NEUROPROTECTIVE AND NEUROTHERAPEUTIC EFFECTS OF DIHYDROTESTOSTERONE, 17β-ESTRADIOL, AND *Pueraria mirifica* EXTRACT ON COGNITIVE IMPAIRMENT IN ANDROGEN DEFICIENT RATS

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ฤทธิ์ในการป้องกันและรักษาระบบประสาทของ ไดไฮโดรเทสโทสเทอโรน, 17บีตา-อีสทราไดออล และสารสกัด *Pueraria mirifica* ต่อภาวะรู้คิดบกพร่องในหนูแรทพร่องแอนโดรเจน

นายธราธร ฝายนันทะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษากลไกการออกฤทธิ์ของภาวะพร่องฮอร์โมนเพศชาย ที่มักพบในชายสูงอายุ และผลของไดไฮโดรเทสโทสเทอโรน (DHT) 17 บีตา-อีสทราไดออล (E_2) และสารสกัดกวาวเครือขาว (PME) ต่อการป้องกันและรักษาภาวะรู้คิดบกพร่องในหนูแรทเพศผู้ การทดลองแบ่งออกเป็น 4 ชุด คือ การทดลองที่ 1 เพื่อศึกษากลไกการออกฤทธิ์ของภาวะพร่องฮอร์โมนเพศชายต่อภาวะรู้คิดบกพร่อง นำหนูแรทอายุ 4 เดือน มาตัด (ODX) หรือไม่ตัดอัณฑะ (SH) ทิ้งไว้เป็นเวลา 3 วัน, 2, 4, 6 และ 8 เดือน (กลุ่ม M0, M2, M4, M6 และ M8 ตามลำดับ) ทดสอบความสามารถในการเรียนรู้และจดจำ และวัดการแสดงออกของยีนที่เกี่ยวข้องกับการสื่อประสาท (*Syn*, *GluN1*, *a7-nAChR*, *M_1-mAChR*, and *Bdnf* ในระดับเอ็มอาร์เอ็นเอ และการแสดงออกของ SYN และ PSD95 ในระดับอิมมูโนรีแอกติวิตี) นิวโรไฟบริลลารี แทงเกิล (*Tau3* and *Tau4* ในระดับเอ็มอาร์เอ็นเอ และโปรตีนเทา และโปรตีนเทาที่มีการเติมหมู่ฟอสเฟต) และแผ่นแอมีลอยด์ (*App*, *Bace1*, and *Adam10* ในระดับเอ็มอาร์เอ็นเอ) ในสมองส่วนฮิปโปแคมปัส พบว่าเมื่อหนูมีอายุมากขึ้น ระดับฮอร์โมนเทสโทสเทอโรนค่อย ๆ ลดลงและลดลงอย่างมีนัยสำคัญทางสถิติที่อายุ 12 เดือน (กลุ่ม SH-M8) แต่กลับเริ่มตรวจพบภาวะรู้คิดบกพร่องได้ในหนูอายุ 8 เดือน (กลุ่ม SH-M4) โดยไม่พบการเปลี่ยนแปลงในการแสดงออกของยีนต่าง ๆ ที่สมองส่วนฮิปโปแคมปัส ในขณะที่ภาวะพร่องฮอร์โมนเพศชายอย่างฉับพลันในหนูที่ถูกตัดอัณฑะไปกระตุ้นให้เกิดภาวะรู้คิดบกพร่องได้เร็วขึ้น โดยเริ่มตรวจพบภาวะรู้คิดบกพร่องได้ในหนูที่ตัดอัณฑะไปเพียง 2 เดือนเท่านั้น และภาวะรู้คิดบกพร่องมีความรุนแรงขึ้นภายหลังจากตัดอัณฑะไปนาน 8 เดือน (กลุ่ม ODX-M8) การแสดงออกของยีน ทั้งระดับเอ็มอาร์เอ็นเอและโปรตีน ที่เกี่ยวข้องกับการสื่อประสาทและนิวโรไฟบริลลารี แทงเกิล ในหนูที่ตัดอัณฑะเมื่อเทียบกับหนูที่ไม่ตัดอัณฑะ พบว่าแย่ลงและมีการเปลี่ยนแปลงเพิ่มสูงขึ้นเมื่อหนูอายุได้ 12 เดือน และตัดอัณฑะไปนาน 8 เดือน การทดลองที่ 2 เพื่อตรวจจับการเปลี่ยนแปลงเริ่มแรกในการแสดงออกของยีนที่เกี่ยวกับการสื่อประสาทและนิวโรไฟบริลลารี แทงเกิล นำหนูแรทมาตัดอัณฑะ ทิ้งไว้เป็นเวลา 1 - 9 วัน และวัดการแสดงออกของยีนเช่นเดียวกับการทดลองที่ 1 การเปลี่ยนแปลงในระดับเอ็มอาร์เอ็นเอที่ตรวจพบเป็นลำดับแรก คือ การแสดงออกของยีนที่เกี่ยวกับการทำงานของไซแนปส์โดยพบภายใน 1 วันหลังตัดอัณฑะ จากนั้นเป็นการเปลี่ยนแปลงที่เกี่ยวข้องกับนิวโรไฟบริลลารี แทงเกิล และโครงสร้างของไซแนปส์ในวันที่ 6 และ 9 ตามลำดับ การทดลองที่ 3 เพื่อศึกษาผลต่อการป้องกันเซลล์ประสาทของ DHT E_2 และ PME นำหนูแรทมาตัดอัณฑะ ทิ้งไว้เป็นเวลา 1 วัน และป้อนน้ำกลั่นหรือ PME ในขนาด 100 มก./กก น้ำหนักตัว ทุกวัน หรือฉีด DHT ในขนาด 1มก./กก น้ำหนักตัว หรือ E_2 ในขนาด 80 ไมโครกรัม/กก น้ำหนักตัว เข้าทางใต้ผิวหนัง เป็นเวลา 2 เดือน พบว่า DHT E_2 และ PME สามารถป้องกันภาวะรู้คิดบกพร่องและการเปลี่ยนแปลงการแสดงออกในระดับเอ็มอาร์เอ็นเอของยีนที่เกี่ยวกับโครงสร้างและการทำงานของไซแนปส์ และในระดับเอ็มอาร์เอ็นเอและโปรตีนของยีนที่เกี่ยวกับนิวโรไฟบริลลารี แทงเกิล และเมื่อเปรียบเทียบระหว่างการให้สารทั้ง 3 ตัว พบว่า DHT มีฤทธิ์สูงสุด การทดลองที่ 4 เพื่อศึกษาผลต่อการรักษาเซลล์ประสาทของ DHT E_2 และ PME นำหนูแรทมาตัดอัณฑะ ทิ้งไว้เป็นเวลา 2 เดือน และให้สารเช่นเดียวกับการทดลองที่ 3 นาน 2 เดือน พบว่ามีเพียง DHT ที่สามารถรักษาภาวะรู้คิดบกพร่องได้ ผ่านการกระตุ้นการแสดงออกของยีน *Syn* และ *Bdnf* และลดการแสดงออกของยีน *α7-nAChR* และ *M_1-mAChR* โดยสรุปภาวะพร่องฮอร์โมนเพศชายเป็นปัจจัยหลักที่ไปเร่งให้เกิดและทำให้ภาวะรู้คิดบกพร่องรุนแรงขึ้นเมื่อผู้ชายมีอายุมากขึ้น โดยเกิดจากการสูญเสียโครงสร้างและการทำงานของไซแนปส์และเกิดนิวโรไฟบลิลลารี แทงเกิล ซึ่งสามารถป้องกันได้โดยการให้ DHT E_2 หรือ PME แต่ในการรักษาจะใช้ได้เฉพาะ DHT เท่านั้น จากงานวิจัยนี้จึงกล่าวได้ว่าการป้องกันเป็นวิธีที่เหมาะสมที่สุดต่อภาวะรู้คิดบกพร่องในผู้ชาย ทำได้โดยการให้ DHT เป็นสารหลักและให้ E_2 เป็นสารเสริม นอกจากนี้งานวิจัยนี้ยังสนับสนุนการพัฒนาผลิตภัณฑ์จากธรรมชาติ เช่นกวาวเครือขาวให้เป็นสารทางเลือกสำหรับภาวะรู้คิดบกพร่องในผู้ชายที่อยู่ในภาวะพร่องฮอร์โมนเพศ

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Taratorn Fainanta : NEUROPROTECTIVE AND NEUROTHERAPEUTICEFFECTS OF DIHYDROTESTOSTERONE,17β-ESTRADIOL, AND *Pueraria mirifica* EXTRACT ON COGNITIVE IMPAIRMENT IN ANDROGEN DEFICIENT RATS . Advisor: Prof. Suchinda Malaivijitnond, Ph.D. Co-advisor: Prof. Ishwar S. Parhar, Ph.D.,Asst. Prof. SUKANYA JAROENPORN, Ph.D.

This study aims to understand the mechanism of action of androgen deficiency, which usually occurs in elderly men, and the neuroprotective and neurotherapeutic effects of dihydrotestosterone (DHT), 17β-estradiol (E_2) and *Pueraria mirifica* extract (PME) on cognitive impairment in male rats. Four series of experiments were conducted. Experiment I was to understand the mechanism of action of androgen deficiency on cognitive impairment. Rats at the age of 4 months old were orchidectomized (ODX) or sham-operated (SH), kept for 3 days, 2, 4, 6, and 8 months (M0, M2, M4, M6 and M8, respectively), determined spatial learning behavior and memory capacity, and examined transcriptional and translational levels of genes associated with synaptic plasticity (*Syn*, *GluN1*, *α7-nAChR*, *M_1-mAChR* and *Bdnf* mRNA levels, and SYN and PSD95 immunoreactivity levels), neurofibrillary tangles (*Tau3* and *Tau4* mRNA levels, and total tau and phosphorylated tau protein levels), and amyloid plaque (*App*, *Bace1*, and *Adam10* mRNA levels) in hippocampus. With advancing in age, serum testosterone levels were decreased gradually and significantly in 12-month old SH-M8 rats. A cognitive impairment was first detected in 8-month old SH-M4 rats while it had no changes in any hippocampal marker genes. An abrupt androgen deficiency in the ODX rats accelerated the onset of cognitive impairment as early as 2 months after orchidectomy, and became worsen after 8 months in the ODX-M8 rats. Transcriptional and translational levels of genes associated with synaptic structure and function, and neurofibrillary tangles were deteriorated in comparison with the age-match SH rats, and the degrees of change were exacerbated in 12-month old ODX-M8 rats. Experiment II was to detect the early onset of molecular changes of synaptic plasticity and neurofibrillary tangles. Rats were ODX, kept for 1 - 9 days, and examined mRNA and protein expression levels of genes mentioned in Exp. I. The onset of changes was detected as early as 1 day after orchidectomy for mRNA levels of genes associated with synaptic function, and the chronological changes were detected at 6 and 9 days for neurofibrillary tangle and synaptic structure, respectively. Experiment III was to examine the neuroprotective effects of DHT, E_2, and PME. Rats were ODX, kept recovery for 1 day, and fed daily with distilled water or 100 mg/kg BW of PME, or subcutaneously injected with 1 mg/kg BW of DHT or 80 µg/kg BW of E_2 for 2 months. DHT, E_2 and PME could prevent the cognitive impairment and changes of mRNA levels of genes associated with synaptic structure and function, and mRNA and protein levels of genes associated with neurofibrillary tangle. Among 3 treatments, DHT showed the strongest effects. Experiment IV was to examine the neurotherapeutic effects of DHT, E_2, and PME. Rats were ODX, kept for 2 months, and treated with DHT, E_2, and PME as mentioned in Exp. III for another 2 months. Only DHT could rescue the cognitive impairment by increasing *Syn* and *Bdnf* and decreasing *α7-nAChR* and *M_1-mAChR* mRNA levels. In conclusion, androgen deficiency is a key factor in accelerating and exacerbating the age-associated cognitive impairment in males through a sequential deterioration of synaptic function and structure, and formation of neurofibrillary tangles. It can be prevented by DHT > E_2 ≥ PME, while only DHT can cure the symptoms. Taken together, the preventive approach is suggested for male cognitive impairment, and DHT should be a major treatment, while E_2 is an ancillary agent. The present study also encourages the development of natural product, *P. mirifica*, as an alternative treatment for cognitive impairment in androgen deficient men.

Field of Study:	Biological Sciences	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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3xTg-AD	The triple transgenic mouse model of Alzheimer's disease
AChR	Acetylcholine receptors
AD	Alzheimer's disease
ADAM10	A Disintegrin and metalloproteinase 10
ADT	Androgen deprivation therapy
AICD	APP intracellular domain
AMPA	α-amino-3-hydroxy- 5-methylisoxazole-4-propionic acid
APP	Amyloid precursor protein
AR	Androgen receptor
BACE1	β-site APP cleaving enzyme 1
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis
CaMKII	Calcium calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CREP	Cyclic adenosine monophosphate response element binding protein
DG	Dentate gyrus
DHT	Dihydrotestosterone
E_2	17β-estradiol
EC	Entorhinol cortex
ERK	Extracellular signal-regulated kinase
ERs	Estrogen receptors
USFDA	The United States Food and Drug Administration

fEPSP	Field excitatory postsynaptic potential
FSH	Follicle stimulating hormone
GluN1	N-methyl-D-aspartate receptor subunit 1
GnRH	Gonadotropin-releasing hormone
GPER1	G protein-coupled estrogen receptor 1
GTPase	Guanosine triphosphatase
IHC	Immunohistochemistry
LH	Luteinizing hormone
LOD	Limit of detection
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Metabotropic muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment,
MWM	Morris Water Maze
nAChR	Ionotropic nicotinic acetylcholine receptor
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
ODX	Orchidectomized
PHFs	Paired helical filaments
PI3K	phosphoinositide 3-kinase
PKA	Protein kinase A

PKC	Protein kinase C
PL	Placebo control
PLC	Phospholipase C
PLCγ	Phospholipase Cγ
PME	*Pueraria mirifica* extract
PSD-95	Postsynaptic density protein molecular weight of 95
qrt-RT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
SAMP8	The senescence-accelerated-prone mouse 8
sAPPα	Soluble amyloid precursor protein α fragment
sAPPβ	Soluble amyloid precursor protein β fragment
SD	Sprague-Dawley
SH	Sham operation
SHBG	Sex hormone-binding globulin
SYN	Synaptophysin
Tau3	Three repeat microtubule-binding domain containing tau
Tau4	Four repeat microtubule-binding domain containing tau
TrkB	Tropomyosin receptor kinase B
TS	Testosterone supplementation
α7-nAChR	α7 subunit ionotropic nicotinic acetylcholine receptor

CHAPTER I
GENERAL INTRODUCTION

Cognitive impairment is a stage when a person experiences trouble in remembering, learning new things, concentrating or making a decision that affects their daily life. People more than 1 out of 9, aged 65 years or over, are facing with cognitive impairment. The percentage of cases increases with increasing age: from 5.3% to 13.8% and 34.6% when people are 65 - 74, 75 - 84, and ≥85 years old, respectively (Rajan et al., 2021). Thus, increasing age has been widely considered as a major factor of cognitive impairment. As the world population is shifting in distribution toward the older age, the number of people suffering from cognitive impairment is expected to be increased globally and as high as 152 million cases are expected by 2050 (Alzheimer's Disease International, 2018; World Health Organization, 2019). Once the symptom has been diagnosed, the survival time of the patient becomes shorter in average of 5 years (Joling et al., 2020). Following the increase in life expectancy of the world population, cognitive impairment and related diseases have been accounted as the seventh leading cause of death (World Health Organization, 2020). Mostly, cognitive impaired patients are unable to live alone and require expensive medications, daily assistance with personal care, and, at some point in time, they will lose autonomic functions to be alive. Beside the impact on the diagnosed patient themselves, cognitive impairment also has huge consequences on their relatives and society. The global economic cost of healthcare for cognitive impaired people was estimated to be more than US$ 1 trillion in 2018 and will rise up to $ 2 trillion by 2030 (Alzheimer's Disease International, 2018).

Cognitive impairment is a consequence of insufficient function in several brain regions, especially hippocampus which is responsible for thinking, learning and memory. It progressively loses abilities to communicate between neurons and structural connections termed synapse, and presents the extracellular accumulations of toxic amyloid-β proteins into amyloid plaques and the intracellular accumulations of the hyperphosphorylated tau proteins into neurofibrillary tangles. However, the changes in the brain may begin several decades before the first clinical signs of

cognitive impairment appears, and the cause and the complete sequences of events is currently unclear (Braak et al., 2011b; Villemagne et al., 2013). Although increasing age is a major factor of the cognitive impairment, this is not always detectable. Cognitive impairment may start to be noticeable at any point of age without disturbance on performing individual's daily activity which may or may not progress to a more severe stage (Petersen et al., 2018). In severe stage of cognitive impairment, neurons in parts of the brain, specifically at hippocampus are damaged or destroyed. The initiation of brain changes toward neurodegeneration are unnoticeable. Therefore, it is still questionable when the brain starts to deteriorate and at what age.

Along with the physiological age, reproductive senescence with a decline in sex steroid hormones has been related to and listed as a potential risk factor of cognitive impairment (Gauthierm, 2021). Most of studies revealed that woman has a higher risk of cognitive impairment than man, especially when she enters the menopausal or estrogen-deficient period (Mosconi et al., 2017; Mosconi et al., 2018; Rosario et al., 2011). Similarly, men with lower physiological levels of testosterone have a higher risk of cognitive impairment than those with normal testosterone levels (Ford et al., 2018; Moffat et al., 2004; Yeap et al., 2008). However, a number of studies have argued that the levels of testosterone are not associated with cognitive function in elderly men (Fonda et al., 2005; Martin et al., 2007; Martin et al., 2008; Yonker et al., 2006). Whether a male reproductive senescence or testosterone deficiency can lead to a cognitive impairment in elderly men remains to be elucidated.

Up to now, none of the pharmacological treatments can prevent or cease the cognitive impairment. So far, the U.S. Food and Drug Administration (FDA) has approved only four drugs: rivastigmine, galantamine, donepezil, and memantine for the treatment. The main drugs prescribed to people with early stage of cognitive impairment are donepezil, rivastigmine and galantamine which act as acetylcholinesterase inhibitors. These drugs can promote cholinergic signaling at the synapse by inhibiting acetylcholine degradation and maintain its levels in the extracellular space (Zhu et al., 2013). The shortcoming of these drugs is that they provide only the symptomatic treatment but do not alter the course of the disease, and their efficacy varies between individuals. Although the most ideal strategy is that the treatment should be provided at the early stage of the symptom, the suspected

cognitive impaired patients usually come to consult with the clinician at the later stages: moderate or severe stage, when the neuronal pathology extends to defect the glutamatergic system. A ‘glutamate cycle’ occurring between the presynaptic and postsynaptic neurons was disturbed and led to a state of extracellular glutamate accumulation, thus the excess glutamate receptors, or NMDA (N-methyl-d-aspartate) receptors, were stimulated and later the nerve cells were damaged (so-called excitotoxicity; (Revett et al., 2013). Meanwhile the only drug approved for the clinical use in moderate to severe stage of cognitive impairment is memantine, an uncompetitive NMDA receptor antagonist, which probably counteracts the effects of hyperactive excitatory circuits and prevents the weakening synapse strength by high levels of glutamate (Talantova et al., 2013). Sadly, this drug did not elicit the positive effect in at least half of the patients who take them.

Based on the fact that the cognitive impairment occurs together with reproductive senescence, the use of sex steroid hormones to prevent the symptom had been taken into account. In women, estrogen administration before the onset of menopausal transition, a window of opportunity for ameliorating cognitive impairment, could provide the benefits on cognitive function (Kantarci et al., 2016; Mosconi et al., 2018; Paganini-Hill and Henderson, 1994; Rasgon et al., 2014). In contrast, the testosterone supplementation in men, aged approximately 60 years old and had normal cognitive function, could either enhance (Cherrier et al., 2001; Cherrier et al., 2005a; Janowsky et al., 2000; Janowsky et al., 1994; Wahjoepramono et al., 2016) or not affect the cognitive function (Borst et al., 2014; Emmelot-Vonk et al., 2008; Haren et al., 2005; Huang et al., 2016; Kenny et al., 2002; Maki et al., 2007; Vaughan et al., 2007). This questions the use of testosterone to prevent the cognitive impairment in men. Furthermore, an attempt to investigate the potential benefits of testosterone in elderly men diagnosed with moderate to severe cognitive impairment was also noted. In contrast to an abrupt cessation of estrogen production in menopausal women, the androgen levels are gradually decline in men and a critical point of reduction cannot practically be detected. Therefore, a variation of endogenous testosterone levels among subjects, within and across studies, and timing of treatment are considered as the important confounding factors of an effectiveness of testosterone supplementation on prevention and therapeutics of cognitive impairment in men.

Another interesting factor that possibly intervenes in the beneficial effects of testosterone supplement on cognitive function in men is its mechanisms of action. It is well known that testosterone acts via three major forms: testosterone itself without biotransformation, and after transformed into two active metabolites, dihydrotestosterone (DHT) and 17β-estradiol (E_2). It is currently unknown which form of testosterone acts on cognitive function in men. Besides, both types of sex steroid hormone receptors; androgen (AR) and estrogen (ERs) receptors are expressed in male hippocampus (Gonzalez et al., 2007). Thus, administration of DHT in androgen deficient male mice was reported to stimulate synaptic function and structure and prevent cognitive impairment through AR (George et al., 2013; Kang et al., 2014). Some beneficial effects of E_2 supplementation on several cognitive functions, such as verbal memory and spatial abilities, was also reported in men (Beer et al., 2006; Cherrier et al., 2005a; Wibowo, 2017) and in male rodents (Jacome et al., 2016; Koss et al., 2018; Kritzer et al., 2001; McConnell et al., 2012). Inhibition of transformation of testosterone to E_2 ablated the neuroprotective effect in elderly men. This suggested that E_2 and ERs are important players for sex steroid hormone to act on cognition (Cherrier et al., 2005a). Estrogen could also increase the number of synaptic connections in the hippocampus (Jacome et al., 2016; Zhao et al., 2018), and play an important role in memory consolidation process in male mice (Koss et al., 2018). The neuroprotective effects of testosterone on ameliorating some Alzheimer disease-like neuropathology through estrogen pathways were also reported in male mice (Rosario et al., 2010). Thus, it is of great interest to investigate the neuroprotective and neurotherapeutic effects of E_2 as the best measure on cognitive impairment in men. However, synthetic E_2 might contribute some adverse side effects, e.g., venous thromboembolism, ischemic stroke (Getahun et al., 2018), cardiovascular toxicity (Phillips et al., 2014) and erythema nodosum (Coyle et al., 2015a) when it is a long-term use. Thus, the use of natural products to remedy or ameliorate the cognitive impairment is attracted attention to the modern world people.

Pueraria mirifica, a Thai medicinal herb, which has long been noted for its estrogenic activity in cell lines (Cherdshewasart et al., 2008), rodents (Cherdshewasart et al., 2007b), monkeys (Trisomboon et al., 2006b) and humans (Manonai et al., 2008), contains a variety of phytoestrogenic substances such as

daidzein, daidzin, genistin, genistein, puerain, mirificin, coumestrol, miroestrol, deoxymiroestrol and isomiroestrol (Malaivijitnond, 2012). Recently, it was reported that the crude extract of the *P. mirifica* (PME) could cure the early and late stage of cognitive impairment in estrogen deficient rats (Anukulthanakorn et al., 2016). Several phytoestrogens isolated from *P. mirifica* and used as a single compound were also reported the high potential benefits on brain and cognitive function. For example, puerarin, the major constituent and species-specific phytoestrogen of *Pueraria* plants that shows a weak estrogenic activity, was reported to ameliorate the learning and memory deficit in rodents (Hong et al., 2016; Li et al., 2010; Li et al., 2019; Liu et al., 2015), and attenuate the toxicity of amyloid-β (Li et al., 2010) and tau hyperphosphorylation (Hong et al., 2016) in hippocampus. Miroestrol, a species-specific phytoestrogen of *P. mirifica* and eliciting high estrogenic activity, could ameliorate cognitive deficits in ovariectomized mice. It attenuated the oxidative stress and down-regulated the brain derived neurotrophic factor in the brain (Monthakantirat et al., 2014). Genistein, which was also found in soybean, could protect neuronal apoptosis either in *in vitro* hippocampus (Zeng et al., 2004) and in *in vivo* ovariectomized rats (Huang and Zhang, 2010). Since the PME had no toxic effect on reproductive organs (Jaroenporn et al., 2006; Urasopon et al., 2007), it did not show acute toxicity (Mohamad et al., 2019) but exhibited estrogenic activity in male rats (Malaivijitnond et al., 2004). Therefore, it is interesting to investigate the neuroprotective and neurotherpeutic effects of PME in males.

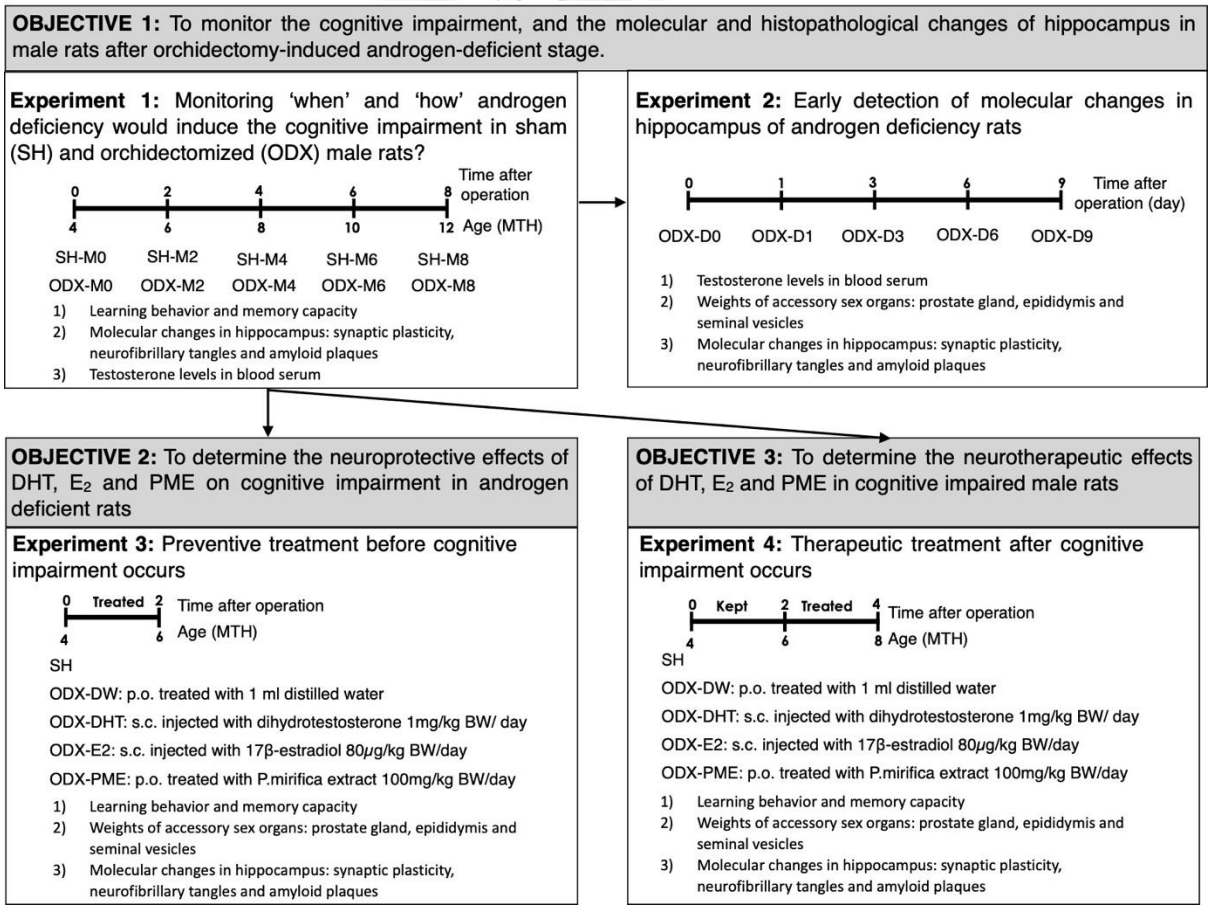
Following all concerns about aging and cognitive impairment in males mentioned above, this study aims to investigate 'when' and 'how' the androgen deficiency induces cognitive impairment in male rats. Male Sprague-Dawley (SD) rats were orchidectomized to induced an androgen deficient stage when the animals were young adults to middle age. The onset of spatial learning and memory impairment was assessed by the Morris Water Maze test. The progressive changes at both transcriptional and translational levels of the complete set of the neuropathological hallmarks, including synaptic plasticity, neurofibrillary tangle and amyloid plaque, in hippocampus were determined. After the mechanism of action of testosterone on cognitive function in males is thoroughly elucidated, the neuroprotective (preventive approach) and neurotherapeutic (therapeutic approach) effects of DHT (a non-

aromatizable androgen), E_2, and PME on cognitive function and neuropathological changes were investigated in orchidectomy-induced androgen-deficient male rats. If PME could sustain the cognitive function in males, it would be a promising phytopharmaceutical product of aging society of Thailand.

OBJECTIVES

1. To monitor the cognitive impairment, and the molecular and histopathological changes of hippocampus in male rats after orchidectomy-induced androgen-deficient stage.
2. To determine the neuroprotective effects of DHT, E_2 and PME on cognitive impairment in androgen deficient rats
3. To determine the neurotherapeutic effects of DHT, E_2 and PME in cognitive impaired male rats

CONCEPTUAL FRAMEWORK



CHAPTER II
LITERATURE REVIEW

1. Aging society and cognitive impairment

According to World Population Prospect 2019, the human life expectancy was an average of 47 years in 1950, and had increased by 25.6 years to an average of 72.6 in 2019 (United Nations, 2019). The improvements of life expectancy during the 20th century has been widely attributed to advances in public health, modern medicine, improved nutrition, healthier lifestyles, and education (Lindsay et al., 2014), which are advantages against the leading causes of illness and death from infectious and parasitic diseases that shortened the mortality of infants and children. However, up to the present time, the healthy longer life is potentially tempered by chronic conditions and several non-communicable diseases that more commonly affect at older ages as biological aging processes (World Health Organization, 2020). In particular, all countries worldwide are experiencing growth in the number and proportion of older persons, aged 65 and older defined by the United Nations, with the number of 727 million (9.3% of global population) in 2020, and projected to reach over 1.5 billion (15.9%) in 2050 (United Nations, 2020). Older persons are a growing demographic group in society, and in 2019 it becomes an unprecedented outnumber over the children aged ≤5 years for the first time ever in human history (United Nations, 2019). Aging process occurs together with the accumulation of wide variety of molecular and cellular damages which lead to a gradual decline in physiological integrity, physical and mental health. Among the top age-related chronic illness in elderly, cognitive impairment is a condition that has huge adverse physical, psychological, social, and economic impacts. It becomes a nationally and globally healthcare problem that affects cognitive impaired patient as well as their relatives, families, and society.

Cognitive impairment is fundamental in the usual consequences of biological aging that affects the ability to learn, remember, think, orientate, comprehend, calculate, language, and judge which varies in the severity and task. In general, getting older is most typically having slower process of speed of cognitive functions or sometimes forgetting names or appointments, but remembering them later, which is not necessarily be a warning sign of neurocognitive disease, frequently termed *age-*

related cognitive impairment (Crook et al., 1986; Levy, 1994). If there is extended cognitive impairment such as becoming forgetful of recent events and people's names or experiencing behavioral changes, including wandering and repeated questioning, which is serious enough to be recognized by an individual, caregiver, or health professional and requires the compensation using tools such as lists, maps, or pill boxes to perform daily activities independently, the individual is said to have some intermediate form of cognitive impairment, clinically termed *mild-cognitive impairment* (*MCI*) (Petersen et al., 2014; Petersen et al., 1999). If memory disturbance is more severed which may include having difficulty to recognize relatives and friends or experiencing behavioral changes and aggression to the point where a person cannot maintain independent function and interfere with daily activities, this level of cognitive impairment is called *dementia*. Dementia is an umbrella term encompassing a range of neurological disorders characterized by cognitive impairment as a result from that the neurons in parts of the brain involving in thinking, learning and memory have been damaged or destroyed, and the most common form is Alzheimer's disease (AD), accounting for almost 80% of cases (Alzheimer's Association, 2016). It is important to note that the initiation and progression of brain changes causing cognitive impairment is mostly unnoticeable to the person affected, and not all individuals eventually develop to dementia (Petersen et al., 2018; Ward et al., 2013). Thus, the major goal of current research is identifying which individuals are more likely to develop MCI or dementia.

Globally, over 55 million people are suffering from cognitive impairment and related diseases, and more than half of cognitive impaired patients are elderly persons, aged ≥65 years (Gauthierm, 2021; Rajan et al., 2021). Counting the possibility, more than one of the nine elderly are in the stage of cognitive impairment. This suggests that age is one of the major factors of cognitive impairment. Along with the increase in global demographics of older persons, the numbers of cognitive impaired patients had been expected to increase up to 152 million by 2050 (Alzheimer's Disease International, 2018). It is important to note that there is currently no treatment available to prevent and cure cognitive impairment. Although numerous new treatments are being investigated in various stages of clinical trials, the medicines and disease-modifying therapies developed to date have limited efficacy. Moreover, the

recent report from Alzheimer's Disease International estimated that up to 75% dementia patients across the globe, equating to 41 million individuals, are undiagnosed, possibly due to several roadblocks for diagnosis such as fear of diagnosis and cost, lack of access to trained clinicians, specialized diagnostic tests and knowledge in making a diagnosis (Gauthierm, 2021). Thus, in addition to an urgent need of drug development to be achieved, one of the principal goals for dementia care is the early diagnosis in order to promote timely and optimal management.

As the progression of neuronal damages extend to affect the brain area involved in movement, the cognitive impaired individuals will experience autonomic dysfunctions such as walking and become bed-bound which leads to disabled and depend on others. In addition to the medical drugs, most of the cost are attributed to social care, e.g., skilled nursing care, home healthcare, hospice care, and informal care (unpaid), e.g., caregiving from family and quality of life that are commonly underestimated and are associated with significant negative societal and personal burden. (Alzheimer's Disease International, 2015; Wong, 2020). This makes cognitive impairment becoming one of the costliest long-term illnesses. In the United States, the cost for taking care of the cognitive impaired patients was about US$52,481 per person per year (Alzheimer's Association, 2021). Globally, the economic cost of healthcare for cognitive impaired people was estimated to be more than US$ 1 trillion in 2018 and will be raised up to US$ 2 trillion by 2030 (Alzheimer's Disease International, 2018). Eventually, the survival time of the patients becomes shorter (Joling et al., 2020). In 2020, the cognitive impairment and related diseases had been accounted to the seventh leading cause of death among global population, and is the sixth rank among Thai's population (World Health Organization, 2020).

2. Cognition and hippocampus

2.1 Cognitive function

Cognitive functions refer to the internal mental processes underlying how people perceive, remember, speak, think, make decisions, and solve problems. In case of age and the cognition impaired, the most prominent cognitive function being interfered is ability to store and recall recent events called *episodic memory* (Park et al., 2002;

Small et al., 1999; Verhaeghen and Salthouse, 1997; Zelinski et al., 1993). Episodic memory refers to the memory system that allows an individual to consciously retrieve a previously experienced events such as the location of placing keys or having dinner with friends last month. Similarly, the earliest symptom in neurodegenerative disease is mostly the difficulty in formation of new episodic memory (Bondi et al., 1999; Collie and Maruff, 2000; Schmitt et al., 2000; Smith et al., 2007). Although the individuals with age-related cognitive impairment may remain stable or just slowly deteriorate or possibly develop MCI and dementia (Hampel and Lista, 2016; Ward et al., 2013), the detection of one's performance referring the episodic memory is the potential early sign of cognitive impairment and related diseases, and is included in memory test for clinical diagnosis (Albert et al., 2011; Dubois et al., 2014).

In parallel with clinical assessment of episodic memory impairment in humans, changes in this cognitive domain has also been reported in laboratory animals, particularly rodents (Webster et al., 2014). Since animals cannot tell what they have experienced, the assessment of episodic paradigm in rodents is the spatial learning and memory which is an ability to navigate using distal cues located outside and at some distance from the organism. One of the most extensively used and accepted assessment tools in detection of the spatial learning and memory in rodents is *Morris Water Maze (MWM)* task. The MWM consists of a large open pool with a hidden (submerged) escape platform located somewhere within the pool. Animals must learn where the platform is, remember the platform's location, and then use spatial cues on subsequent trials to navigate back to the hidden platform. This behavioral task requires the function of a specific brain region: the hippocampus. The lesions, pharmacological inhibition, and loss-of-function genetic mutations of signaling molecules or receptors within the hippocampus induced the deficits in performing the MWM (McNamara and Skelton, 1993; Morris et al., 1986; Morris et al., 1982). It was reported that the transgenic AD animals showed cognitive deficits tested in the MWM (Webster et al., 2013). There are two versions of behavioral task assessing spatial abilities: working and reference, representing the short- and long-term memory, respectively (Cowan, 2008; Olton and Paras, 1979).

2.2 Hippocampus

Hippocampus is a brain region located deep under the cerebral cortex within the medial temporal lobe of the brain, being part of the limbic system. The structural and functional integrity of the hippocampus is crucial for normal learning and memory consolidation, especially episodic memory. Along with the decline in the processing of episodic memory, the hippocampus is also particularly vulnerable to aging (Driscoll et al., 2003; Geinisman et al., 1995; Rosenzweig and Barnes, 2003) and susceptible to being affected by neurodegenerative disease (Mormino et al., 2009). This suggests that the neural circuitry of the hippocampus may be impaired earlier than other brain regions. The hippocampus is subdivided anatomically based on cellular and synaptic composition into three discrete subregions: dentate gyrus (DG), Cornu Ammonis (CA) 1, and CA3 (Figure 2.1) (Kandel et al., 2013). In DG region, the primary constituent is the granule cell type while it was pyramidal neurons in CA1 and CA3 regions. The hippocampus and its associated structures in the medial temporal lobe mediate the neural circuit for learning and memory predominantly via *trisynaptic pathway* (Kandel et al., 2013; Neves et al., 2008). The hippocampus receives the input from the nearby entorhinal cortex (EC) which is composed of six distinct layers. Within the trisynaptic pathway, the axon of neurons from layer II of EC project to the granule cells of DG through the Perforant pathway (see Figure 2.1). Then, the granule cell axons from DG send projections to excite the pyramidal cells in CA3 through Mossyfiber pathway. Finally, CA3 pyramidal neurons relay the excitatory information to CA1 pyramidal neurons through the Schaffer collateral pathway. In addition to the trisynaptic pathway, CA1 also receive the input directly from layer III of EC via Perforant pathway, but this excitation is weak compared to input from the Schaffer collateral pathway. The CA1 completes the circuit by sending the output projection to a number of brain regions, including the neighboring subiculum and neocortex through fornix for subsequent long-term storage. In case of the output from CA1 is strong enough, the signals go back to excite layer V of EC, which in turn send excitatory feedback input to layer II and III. Then, the synaptic loop in subsequent subregions of the hippocampus is repeated (Kandel et al., 2013). Lesion studies indicated that each component in the hippocampal circuit is important,

especially the disruption at CA1 and CA3 impaired the spatial learning and memory in rodents (Daumas et al., 2004; Florian and Roullet, 2004; Lee and Kesner, 2004; Ocampo et al., 2017; Okada and Okaichi, 2009; Tsien et al., 1996). The short-term memory which lasts no longer than a few hours involved with the temporal activation and/or post-translational modification of preexisting molecules whereas the long-term memory which lasts from several hours to days or even longer depends on a crucial phase of gene expression and *de novo* protein synthesis to transform newly learned information into a permanent and stable state (Bozon et al., 2003; McGaugh, 1966; 2000), leading to the strengthening of the specialized contact sites between neurons along the hippocampal circuit, termed *synapse*.

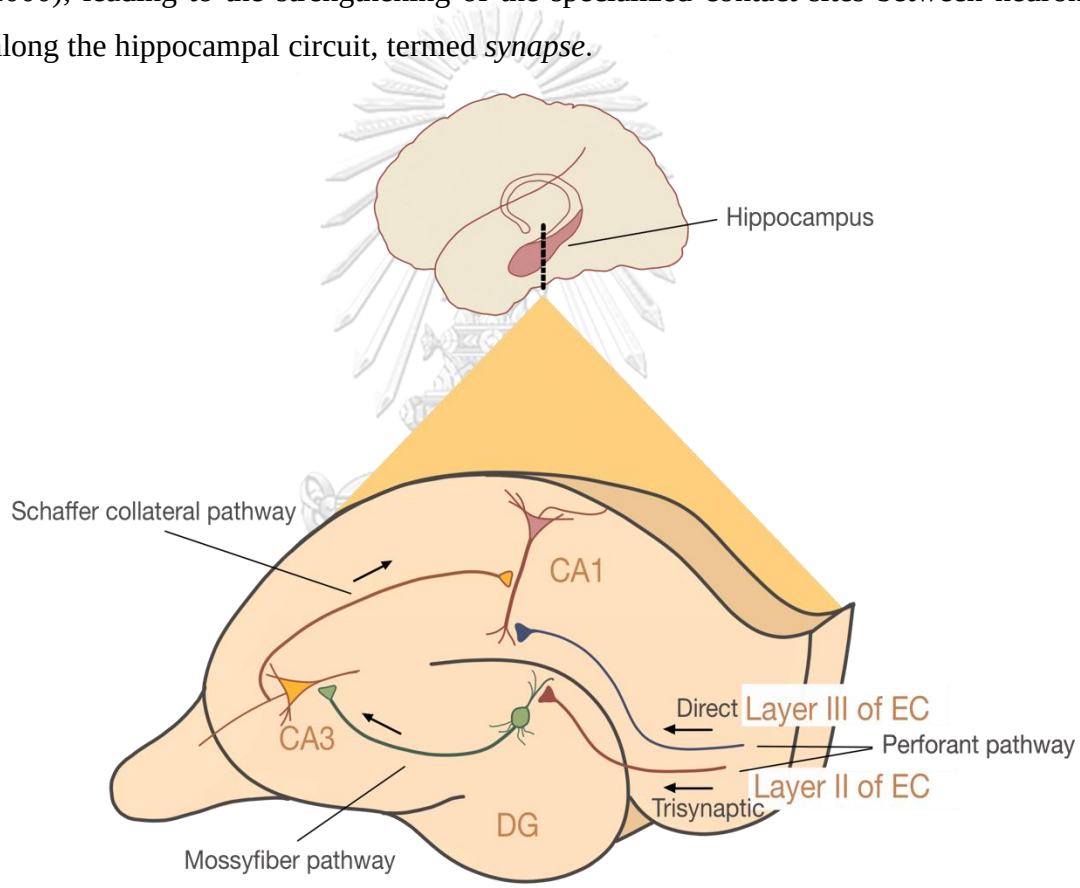


Figure 2.1 Illustration of hippocampus and neural circuits via trisynaptic pathway and a direct input

2.3 Synapse

Synapses are the functional unit of the brain where neurons communicate with each other by transmission of neuronal signals either electrical or chemical of which the latter one is the most common type found in the mammalian central nervous system. A synapse is composed of two essential elements: a presynaptic terminal at the end of an axon where the signal molecules are released in response to electrical impulses or action potential; and a postsynaptic terminal where the receptors are available to receive the chemical signals from the pre-synaptic terminal and convert them back to action potentials. This process is able to be modulated to changes in signaling strength based on inputs from other neurons, termed synaptic plasticity, and underlies important brain functions such as learning and memory (Bailey et al., 2015; Bannerman et al., 2014; Mayford et al., 2012).

2.3.1 Synaptic transmission

Synaptic transmission at chemical synapses involves several steps: (i) arriving of an action potential at the terminal of presynaptic axon causes voltage-gated Ca^{2+} channels at the active zone to open; (ii) opening of Ca^{2+} channels produces a high concentration of intracellular Ca^{2+} near the active zone, causing vesicles containing neurotransmitter to fuse with the presynaptic cell membrane and releasing their contents into the synaptic cleft (a process termed exocytosis); and (iii) binding of the released neurotransmitter molecules to specific receptors on the postsynaptic membrane, causing ion channels to open (or close), thereby changing the membrane conductance and membrane potential of the postsynaptic cell (Jahn and Fasshauer, 2012; Kandel et al., 2013; Südhof, 2004).

The release of neurotransmitters is an important initial step of synaptic transmission between neurons. This process requires several steps starting from translocation of neurotransmitter-filled synaptic vesicles to the release sites, docking to scaffold proteins at active zone terminal, and priming to get ready for fusion to the plasma membrane for rapid exocytosis (Figure 2.2). The Ca^{2+} influx upon arrival of action potential stimulates the rapid exocytosis of synaptic vesicles. Then, the final step is the retrieval of the synaptic vesicle membrane by endocytosis for recycling. Although the molecular sequence of events from vesicle docking to calcium-triggered

membrane fusion is currently uncertain, the number of vesicles available for exocytosis presumably define the efficacy of neurotransmitter releasing. This multistep process requires a series of protein-protein interaction between synaptic vesicles and active sites at the plasma membrane of the presynaptic terminal for trafficking (Figure 2.2). Synaptophysin, the first integral membrane protein and the most abundant, has been identified in the synaptic vesicle (Figure 2.2) (Jahn et al., 1985; Wiedenmann and Franke, 1985), and involved in efficacy of neurotransmission (Adams et al., 2015; Kwon and Chapman, 2011; Raja et al., 2019; Valtorta et al., 2004). Since synaptophysin is exclusively localized to synaptic vesicles, it has been widely used as a marker to study the distribution of synaptic vesicles and presynaptic terminal in the brain (Osimo et al., 2019; Valtorta et al., 2004; Valtschanoff and Weinberg, 2001; Wilhelm et al., 2014). Synaptophysin is depleted in conditions associated with synaptic loss such as AD and other dementias (Sinclair et al., 2015; Sze et al., 2000). Previous study has demonstrated that synaptophysin knockout mice exhibited impaired spatial learning and memory (Schmitt et al., 2009).

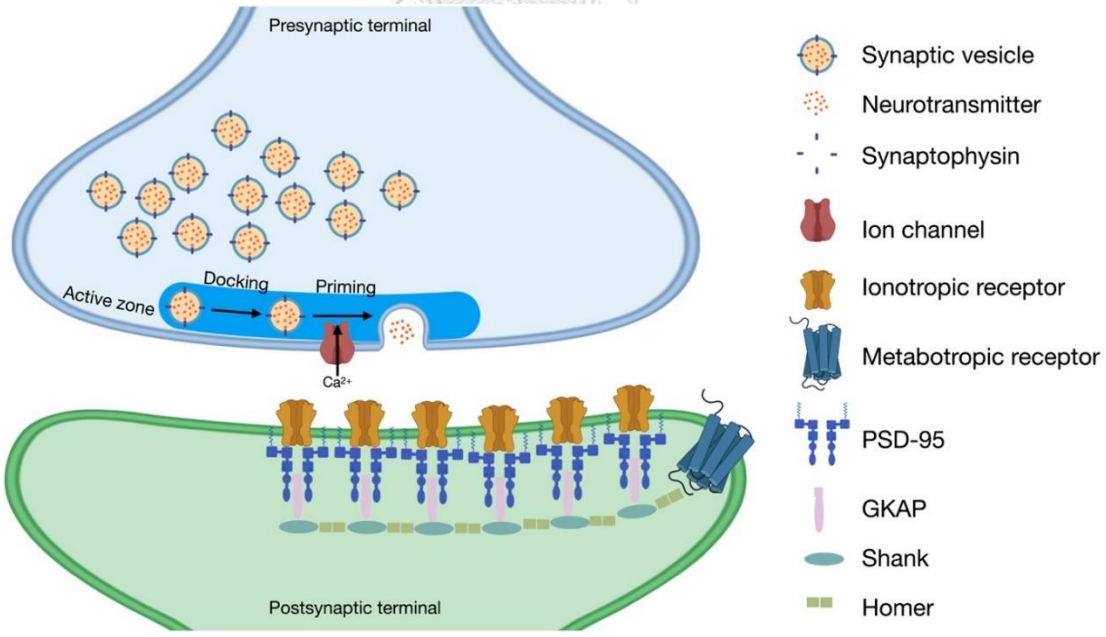


Figure 2.2 Illustration the location of synaptic proteins in the synapse.

Almost 90% of the neurons in the brain use glutamate as their neurotransmitter, and thus the major synapse in the brain is glutamatergic (Braitenberg and Schüz, 1998). At the postsynaptic side of the synapse in the hippocampus, there are abundant of glutamate receptors to receive the glutamate neurotransmitter from the presynaptic terminal and transduce it into electrical and biochemical changes. Glutamate receptors can be divided into two broad categories: (i) the ionotropic receptors, which are ligand-gated channels where glutamate binding directly causes opening of the channel, and (ii) metabotropic receptors, which are G protein-coupled receptors that indirectly gate channels through the production of second messengers. There are three major subtypes of ionotropic glutamate receptors: α-amino-3-hydroxy- 5-methylisoxazole-4-propionic acid (AMPA) receptor, kainate receptor, and *N*-methyl-*D*-aspartate (NMDA) receptor. In particular, NMDA receptor is both voltage- and ligand-gated ion channel. Thus, activation of NMDA receptor requires the depolarization of the postsynaptic membrane and glutamate released from an opposed presynaptic terminal, resulting in a strong postsynaptic Ca^{2+} influx. A rise in intracellular Ca^{2+} acts as a second messenger to activate a number of downstream signaling cascades, for example, calcium calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC), protein kinase A (PKA) and mitogen-activated protein kinase (MAPK), which are critical to induce the long-lasting changes in synaptic strength that are thought to underlie higher cognitive functions (Huang et al., 2013; Kerchner and Nicoll, 2008; Lisman et al., 2012; Malenka and Bear, 2004; Traynelis et al., 2010). Blocking of NMDA receptor by antagonist impaired an ability of rats to learn a new spatial location in MWM test (Morris et al., 1986).

The structure of NMDA receptor is tetrameric proteins arranged around a central pore with four subunits of the following different seven subunits. The seven different subunits characterized into three subfamilies: the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D) which are encoded by four different genes, and a pair of GluN3 subunits (GluN3A and GluN3B) arising from two separate genes (Paoletti et al., 2013). Each NMDA receptor structure consists of two GluN1 subunits and two of the different types of the other subunits. Thus, GluN1 subunit is an essential component of all NMDA receptor complexes. The importance of subunit GluN1 on memory consolidation has been reported (Cercato et al., 2016).

The decline of GluN1 expression levels in hippocampus has been associated with the presence of amyloid-β peptide and cognitive impairment in mice (Gao et al., 2018).

In addition to the predominant excitatory glutamatergic synapse, the cholinergic synapse has also been involved in hippocampal-dependent learning and memory (Hasselmo, 2006). The primary cholinergic input to the hippocampus arises from the medial septum and diagonal band of Broca (Amaral and Kurz, 1985; Dutar et al., 1995), and a small population of cholinergic interneurons exist in the hippocampus (Yi et al., 2015). The action of the cholinergic system uses acetylcholine as a neurotransmitter which is mediated via two major types of acetylcholine receptors (AChR): the ionotropic nicotinic receptor (nAChR) and the metabotropic muscarinic receptor (mAChR). These receptors are classified according to their pharmacological response to the molecules other than acetylcholine which is sensitive to be activated by nicotine and muscarine, respectively. Blockade of mAChRs by drugs such as scopolamine impairs the encoding of new memories (Atri et al., 2004; Hasselmo and McGaughy, 2004) while drugs that activate nAChRs enlarge synapses (Oda et al., 2014) and enhance the encoding of new information (Buccafusco et al., 2005; Levin et al., 2006).

The nAChR is ligand-gated ion channel composed of five subunits which are arranged symmetrically into pentameric structures around a central pore. There are 17 subunit types, α1-α10, β1-β4, δ, γ, and ε subunits. The subunit composition is highly variable across different tissues. The α7 subunit (α7-nAChR) is highly expressed in the hippocampus throughout CA1, CA3, and DG (Adams et al., 2002; Alkondon and Albuquerque, 2004; Alkondon and Albuquerque, 2006; Alkondon et al., 1997; Fabian-Fine et al., 2001; Fayuk and Yakel, 2004; Jones et al., 1999; Sudweeks and Yakel, 2000) and unique in existing predominantly in homopentameric structure. The α7-nAChR has a distinctive property on very high permeability to Ca^{2+} which exceeds that of NMDA receptors (Broide and Leslie, 1999; Castro and Albuquerque, 1995; Corringer et al., 1999; Quik et al., 1997). This ability of α7-nAChR channels can effect on several Ca^{2+}-dependent mechanisms, including activation of second messenger pathways contributing to synaptic plasticity (Morley and Happe, 2000; Suzuki et al., 2006) as well as long-term changes mediated by transcriptional regulators via cAMP response element-binding protein (CREB) and extracellular

signal-regulated kinase (ERK)/MAPK signaling cascades on the enhancement of learning and memory (Dajas-Bailador and Wonnacott, 2004). Activation of the α7-nAChR by agonists has been shown to improve hippocampal-dependent learning and memory, especially spatial cognition (Bitner et al., 2007; Blake et al., 2014; Timmermann et al., 2007; Yaguchi et al., 2009), while genetically deletion and pharmacological inhibition of the α7-nAChR impaired those cognitive functions (Fernandes et al., 2006; Levin et al., 2002; Levin et al., 2009; Nott and Levin, 2006; Young et al., 2007). Moreover, it has been reported that the α7-nAChR can be found at both pre-synaptic and post-synaptic regions of the glutamatergic synapse. At the pre-synaptic terminal, α7-nAChR increases a glutamate release (Gray et al., 1996; McGehee et al., 1995; Rousseau et al., 2005). At the post-synaptic compartments (Fabian-Fine et al., 2001), it has been involved in altering the membrane permeability of Ca^{2+} (Frazier et al., 1998) and modulation of the efficacy of glutamate transmission in the hippocampus (Ge, 2005; Ji et al., 2001; Li et al., 2013b; Zappettini et al., 2010).

In contrast to nAChR, the mAChRs are seven-transmembrane G-protein-coupled receptors. The binding of acetylcholine to mAChR activates other ion channels via second messenger cascade, e.g., inositol 1,4,5 triphosphate and diacylglycerol which are responsible for the mobilization of intracellular Ca^{2+} and activation of PKC and subsequently MAPK. There are five subtypes of mAChR; M_1, M_2, M_3, M_4, and M_5 (Caulfield and Birdsall, 1998). The M_1 subtype is the predominant form of the mAChR in the brain including the cerebral cortex, hippocampus, striatum and thalamus (Gerber et al., 2001; Hamilton et al., 1997; Kubo et al., 1986; Miyakawa et al., 2001), and involved in several domains of cognitive function, especially hippocampus-dependent learning and memory, and synaptic transmission in the hippocampus (Anagnostaras et al., 2003; Miyakawa et al., 2001; Shinoe et al., 2005; Wess, 2004). Chemically blockade of M_1-mAChR by its antagonist impaired the recall of memory in rats (Soma et al., 2014), and genetically deletion of *M_1-mAChR* gene severely impaired working memory and memory consolidation process in mice (Anagnostaras et al., 2003). Moreover, the M_1-AChR also expressed in glutamatergic pyramidal neurons and has been thought to involve with the cholinergic modulation of glutamatergic neurotransmission (Levey, 1996; Mrzljak et al., 1993).

2.3.2 Synaptic structure

The neurotransmitter receptors are concentrated at the postsynaptic membrane opposing to the presynaptic terminals. In particular, they are embedded in the postsynaptic density, a dense and large number of regulatory protein network, comprised of anchoring and scaffolding molecules, signaling enzymes, cytoskeletal components, and forming a remarkably stable structure. One of the most prominent proteins in the postsynaptic density which is important for the clustering of glutamate receptors is Postsynaptic Density protein molecular weight of 95 kD (PSD-95) localized closely to the postsynaptic membrane (Figure 2.2).

The PSD-95 is crucial for anchoring glutamate and acetylcholine receptors (Chen et al., 2015; Neff et al., 2009), synaptic transmission, and stabilization of synaptic contact (Ehrlich et al., 2007; Taft and Turrigiano, 2014). PSD-95 contains three repeated regions: PDZ domains which are important for protein-protein interactions, and two other protein-interacting motifs: SH3 domain and guanylate kinase (GK) domain. For example, in NMDA receptor, certain PDZ domains of PSD-95 bind to the carboxyl terminus of GluN2 subunit of NMDA receptor whereas AMPA receptor interacts with PSD-95 indirectly through transmembrane AMPA receptor regulatory protein (TRAP) family as an auxiliary subunit. PSD-95 also acts as a scaffold for various cytoplasmic proteins by binding to the guanylate-kinase-associated protein (GKAP), which interacts with Shank, a large protein that associates into a meshwork linking the various components of the postsynaptic density. The metabotropic glutamate receptor is localized on the periphery of the synapse by interacting with the protein Homer, which in turn binds to Shank. Furthermore, PSD-95 also interacts with the cytoplasmic region of postsynaptic membrane protein, neuroligin, which forms an extracellular contact in the synaptic cleft with the presynaptic membrane protein, neurexin, and interaction important for synapse development. Accordingly, it has been reported that synaptic transmission is maintained by corresponding distribution of presynaptic vesicle fusion sites to the position of receptors in the postsynaptic density (Tang et al., 2016). The localization of PSD-95-immunopositive at postsynaptic terminal adjacent to synaptophysin-positive nerve terminals has been reported (Fukaya and Watanabe, 2000). Therefore, the analysis of immunoreactivity

of a presynaptic synaptophysin and a postsynaptic PSD-95 has been utilized to indicate the changes of synaptic structure (Li et al., 2015; Yu et al., 2011).

The intense synaptic activity leads to more long-lasting changes in synaptic structure or strengthening although the pathway is not fully understood (Bao et al., 2004). One of the important pathways is that a high influx of Ca^{2+} which in turn transduces through a protein signaling cascades; ERK, CaMKII/IV, phosphoinositide 3-kinase (PI3K), and phospholipase C (PLC), resulting in increased cAMP response element binding (CREB) protein that can stimulate an expression of brain-derived neurotrophic factor (BDNF) (Hong et al., 2008; Tao et al., 1998; Zheng and Wang, 2009).

The BDNF is a member of the neurotrophic family of growth factors, helping to support the survival of existing neurons, encouraging the growth and differentiation of new neurons, dendritic growth and arborization, and the formation of synapses (Acheson et al., 1995; Huang and Reichardt, 2001), and exhibiting via mainly tropomyosin receptor kinase B (TrkB) receptor (Patapoutian and Reichardt, 2001). The action of BDNF, especially in the hippocampus, is vital for learning and memory, and higher thinking (Bekinschtein et al., 2008; Yamada and Nabeshima, 2003). BDNF enhances the synaptic structure via several pathways. For example, BDNF upregulates the expression of glutamate receptor in hippocampus (Caldeira et al., 2007; Wu et al., 2004), promotes a formation of new synapse, termed synaptogenesis (Wilson Horch et al., 1999), and an enlargement of the postsynaptic structure or synaptic spine via enhancing the PSD-95 trafficking to the terminal (Yoshii and Constantine-Paton, 2007). This action is regulated through its receptor TrkB triggering three downstream signaling pathways; PI3K, phospholipase Cγ (PLCγ) and MAPK/ERK (Yoshii and Constantine-Paton, 2010; Yoshii and Constantine-Paton, 2014). BDNF can also control the dendritic growth by activation of the Rho GTPase (Cohen-Cory et al., 2010; Penzes et al., 2003; Takemoto-Kimura et al., 2007) which later regulates microtubule dynamics (Jaffe and Hall, 2005), an important cytoskeleton component for the development and stability of the dendrite and axon. The genetically deletion of BDNF receptor, TrkB, in mice resulted in reduction of dendritic spines and reduction of synaptic spines of the excitatory synapses on CA1 and DG in hippocampus (De Vincenti et al., 2019).

In addition to its main effects on enhancing the synaptic structure, BDNF also exhibits the transient effects on enhancing the synaptic transmission regulating through both presynaptic and postsynaptic terminals. The binding of BDNF and TrkB receptor at postsynaptic terminal transduced the protein phosphorylation cascades via ERK and PKC, resulting in enhance the synaptic transmission through NMDA receptor (Slack et al., 2004) and α7-nAChR (Fernandes et al., 2008). While the autocrine regulation of BDNF by binding to TrkB receptor at the presynaptic terminal increased neurotransmitter release by several potential mechanisms. This rapid actions of BDNF played important roles on synaptic transmission and plasticity in the brain and has been shown to be important for spatial memory in the hippocampus (Mizuno, 2003). The bidirectional communication between the presynaptic releasing glutamate on regulating postsynaptic releasing of BDNF which in turn enhanced glutamate receptor response to neurotransmitter resulted in synapse stabilization and improved efficacy of neurotransmission.

2.4 **Neuropathological hallmarks of cognitive impairment**

At the gross anatomical level, the neurodegenerative brain especially in hippocampus was characterized by atrophy associated with loss of synapses and neurons. The brain of an AD patient displayed marked shrinkage gyri, widened sulci, reduced brain weight, and enlarged ventricles in comparison to age-matched normal brains.

At the microscopic level, the neurodegenerative brain is characterized by three dramatic abnormalities: the loss of synaptic transmission and structure; the deposition of neurofibrillary tangles located within neurons; and the deposition of amyloid plaques in the extracellular space. Although the alterations does not occur uniformly throughout the brain, the hippocampus is especially vulnerable and the structural underpinnings of problems with declarative memory are the first symptoms of AD.

2.4.1 Loss of synaptic transmission and structure

The earliest susceptible sites for being disturbed which implicated in neurodegenerative disease is the release of neurotransmitter at the presynaptic terminal (Gray et al., 2009; Milnerwood and Raymond, 2010; Scheff et al., 2007; Waites and Garner, 2011). The reduced expression of synaptophysin is associated with a decrease in learning and memory abilities (Davies et al., 2003; Wang et al.,

2007). The alterations in synaptic transmission of both glutamatergic and cholinergic synapses have been involved with the early stage of neurodegeneration. The overloaded of Ca^{2+} influx in the postsynaptic terminal as a result of excessively high concentrations of glutamate can be toxic to neurons, and is thought to activate calcium-dependent proteases and phospholipases leading to the production of free radicals that is toxic to the cell. This action is called glutamate excitotoxicity (Choi, 1994; Doble, 1999; Dong et al., 2009). Glutamate-induced excitotoxicity, particularly in the hippocampus, has been linked to a decreased neuronal regeneration and dendritic branching, leading to impaired spatial learning (Cortese and Phan, 2005). The aberrant glutamate hyperexcitability has been thus reported as an early event of neurodegeneration (Hijazi et al., 2019; Noebels, 2011; Vossel et al., 2017). The importance of cholinergic transmission has also been involved in pathological changes in neurodegeneration (Contestabile, 2011). In transgenic mice, the increase in the level of α7-nAChR correlated with learning and memory deficits (Dineley et al., 2001; Dineley et al., 2002). The upregulation of α7-nAChR and M_1-mAChR was previously proposed to serve as a marker for the progression of AD (Harrison et al., 1991; Hellstrom-Lindahl et al., 1999; Overk et al., 2010; Teaktong et al., 2004). Thus, both α7-nAChRs and M_1-mAChR were under consideration as drug targets for treating cognitive decline in AD (Jiang et al., 2014; Kruse et al., 2014; Pohanka, 2012; Wallace and Porter, 2011). Since the formation of new synapses was activity-dependent processes that provided a basis for learning ability and memory formation, a loss or alteration of these structures has been described in patients with neurodegenerative disorders. Such alteration was thought to be responsible for cognitive deficits long before or even in the absence of neuronal loss, but the underlying mechanisms are poorly understood. Accordingly, post-mortem analysis has shown lowered level of BDNF in the brain tissues of people with AD (Mattson, 2008), suggesting that the low level of BDNF could indicate the degeneration of the neuronal structure.

2.4.2 Neurofibrillary tangles

Neurofibrillary tangles are filamentous inclusions of abnormal hyperphosphorylated tau protein in cell bodies and proximal dendrites causing destruction of neuronal structure. Tau protein is encoded by the microtubule-associated protein tau (*MAPT*) gene, comprising of 16 exons. With alternative splicing of exon 2, 3 and 10, the six tau isoforms are generated (Figure 2.3) (Chu and Liu, 2019). The absence or presence of exon 10 results in three or four amino acid repeat sequences in the microtubule-binding domain, respectively, so-called 3R-tau (Tau3) or 4R-tau (Tau4) (Goedert et al., 1989; Himmler et al., 1989). In the adult human brain, levels of the 3R and 4R forms are roughly equal (Goedert and Jakes, 1990), and the aggregation of tau in AD is a mixture overexpression of Tau3 and Tau4 isoforms (Arima, 2006; Goedert et al., 1989; Noble et al., 2013).

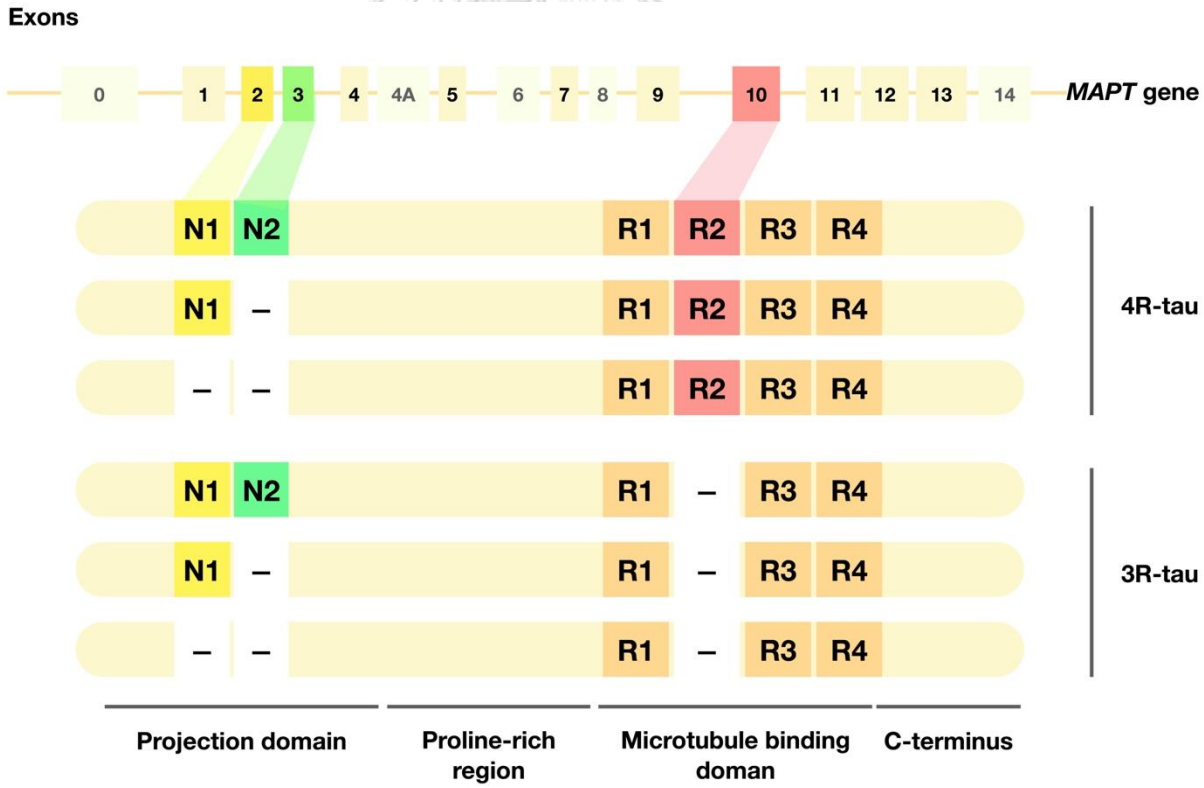


Figure 2.3 MAPT, the gene encoding tau protein, and six isoforms of tau.

Under normal conditions, tau provides microtubule assembly and stabilization facilitating the neuronal structure and supporting synaptic integrity and contribute to the regulation of intracellular trafficking (Dixit et al., 2008; Vershinin et al., 2007). Tau bound at the interface between tubulin heterodimers through its microtubule-binding repeats thereby stabilizing microtubules (Kadavath et al., 2015). Tau4 isoforms has a greater affinity than Tau3 isoforms and are more efficient at promoting microtubule assembly (Goedert and Jakes, 1990). The ability of tau protein on binding to microtubule is regulated by its phosphorylation (Lindwall and Cole, 1984). However, the abnormal post-translation of tau or hyperphosphorylation, as a result of the imbalance between tau kinase and phosphatase activities (Cavazzin et al., 2004; Chen et al., 2017; Jin et al., 2015; Liu et al., 2005; Martin et al., 2013), interfered with tau–microtubule binding and promoted tau misfolding. Normal brain tau contained 2–3 mol of phosphate per mole of the protein, while the AD brain contained two- to three-fold hyperphosphorylated (Kopke et al., 1993), or approximately 6–8 mol of phosphate per mole of the protein (Ksiezak-Reding et al., 1992a). The multiple phosphorylation sites on tau protein that seemed to be associated with tau pathology were reported, i.e., Ser199, Ser202/205, Thr231, Ser262 and Ser^{396} (Augustinack et al., 2002; Luna-Munoz et al., 2007). Among those phosphorylation sites, Ser^{396} was more prominent in severe neuronal dysfunction (Augustinack et al., 2002; Kimura et al., 1996), and was reported as one of the earliest events in AD (Mondragón-Rodríguez et al., 2014). The hyperphosphorylation of tau decreased the binding affinity leading to microtubule destabilization, and thus elevated the soluble tau monomers in the cytoplasm.

Moreover, the hyperphosphorylated tau undergo protein conformational changes which is an exposure of the microtubule-binding domain (Mandelkow et al., 2007), leading to increase the potential for tau-tau interactions, polymerization, and aggregation. The phosphorylated tau monomers dimerize into oligomeric tau, aggregate and constituted into subunits of filaments, so-called protomers. Protomers, in turn, assemble in paired helical filaments. The final step is an aggregation of the paired helical filaments into neurofibrillary tangles (NFTs). In humans, the progressive aggregation of hyperphosphorylated tau and the presence of NFTs correlates closely with cognitive impairment and neuronal loss (Andorfer et al., 2003;

de Calignon et al., 2012; Nelson et al., 2007; Polydoro et al., 2009). The presence of hyperphosphorylated tau can be seen in the brain before the onset of cognitive impairment, while neurofibrillary tangles appear at a later stage (Braak et al., 2006; Braak et al., 2011a; Wharton et al., 2016), and the deposition of tau aggregates always begins in the hippocampus before spreading to other regions (Braak and Braak, 1991; 1997). The study in mice showed that tau phosphorylation in the hippocampus was sufficient to induce neurodegeneration and cognitive impairment (Di et al., 2016).

Since tau-microtubule association is essential for cytoskeleton and intracellular transportation, the disability of tau basically led to dysfunction of neuronal structure and function. In AD, neurons show a reduction in the number and length of microtubules (Cash et al., 2003), reduced levels of acetylated tubulin which is a marker of stable microtubules (Hempen and Brion, 1996; Zhang et al., 2015), and axonal swellings containing vesicles and organelles (Stokin, 2005). Induction of tau phosphorylation decreases synaptic structure and function in the hippocampus of mice (Di et al., 2016). Furthermore, an aberrant tau influences the movement of kinesin and dynamin along the axon (Dixit et al., 2008; Vershinin et al., 2007) and inhibits the transport of organelles (Mandelkow et al., 2002). Neurons containing tangles also have fewer synapses and reduced levels of synaptophysin mRNA compared with tangle-free neurons (Callahan et al., 1999; Ginsberg et al., 2000).

Along with each stage in the development of tau pathology, from the expression of tau itself to post-translational modifications and aggregation, the interventions attempting to reduce tau pathology has been explored such as reducing tau expression, phosphatase modifier (Rueli et al., 2016), kinase inhibitor (Nunes et al., 2013), and tau aggregation inhibitors (Panza et al., 2016). However, numerous preclinical researches for tauopathy-modifying strategies in animal models has failed to translate the results to benefits humans. Therefore, the development of therapies that are effective once symptoms have progressed is being needed, and the continuation of basic research to elucidate the underlying mechanisms of tau dysfunction is essential to identify new targets for intervention (Congdon and Sigurdsson, 2018).

2.4.3 Amyloid plaques

Amyloid plaques are composed of fibrils formed by amyloid-β peptide, which is derived from the sequential cleavage of an integral membrane glycoprotein called "amyloid precursor protein", encoded by the *APP* gene. APP is highly expressed in the brain and kidneys; however, the precise function is not completely understood (Prox et al., 2012). APP can be processed via two alternative pathways: amyloidogenic and non-amyloidogenic. In the non-amyloidogenic pathway (Figure 2.4A), APP is cleaved by α-secretase, a complex that contains A Disintegrin and Metalloproteinase 10 (ADAM10), resulting in a soluble extracellular sAPPα fragment and the C-terminal fragment (CTFα) (Canter et al., 2016). The subsequent cleavage of CTFα by γ-secretase produces a soluble extracellular p3 peptide and APP intracellular domain (AICD). The amyloidogenic pathway (Figure 2.4B), APP is cleaved by β-secretase (encoded by β-site APP cleaving enzyme 1; BACE1), which provides the soluble ectodomain sAPPβ and CTFβ, and cleavage of CTFβ by γ-secretase results in amyloid-β peptide. According to the amyloid cascade hypothesis, an imbalance between these two pathways, particularly a higher β-secretase activity, leads to an over production of amyloid-β peptide (Rockenstein et al., 2005). Accumulating evidence suggested that the declined synaptic function and structure, and progressive formation of neurofibrillary tangles were supposed to be the key triggers of an aberrant amyloid-β (Ghosal et al., 2016; Ittner et al., 2010; Mufson et al., 2008; Roberson et al., 2007). The accumulation of amyloid-β into amyloid plaques plays an important part in the pathophysiology of AD although the physiological functions of amyloid-β remain unknown. The build-up of amyloid-β in the brain parenchyma probably contributes to the loss of synapses, neurodegeneration, and alterations in neuronal activity. Each of these changes disrupts neural circuits, which can lead to widespread network dysfunction and cognitive decline.

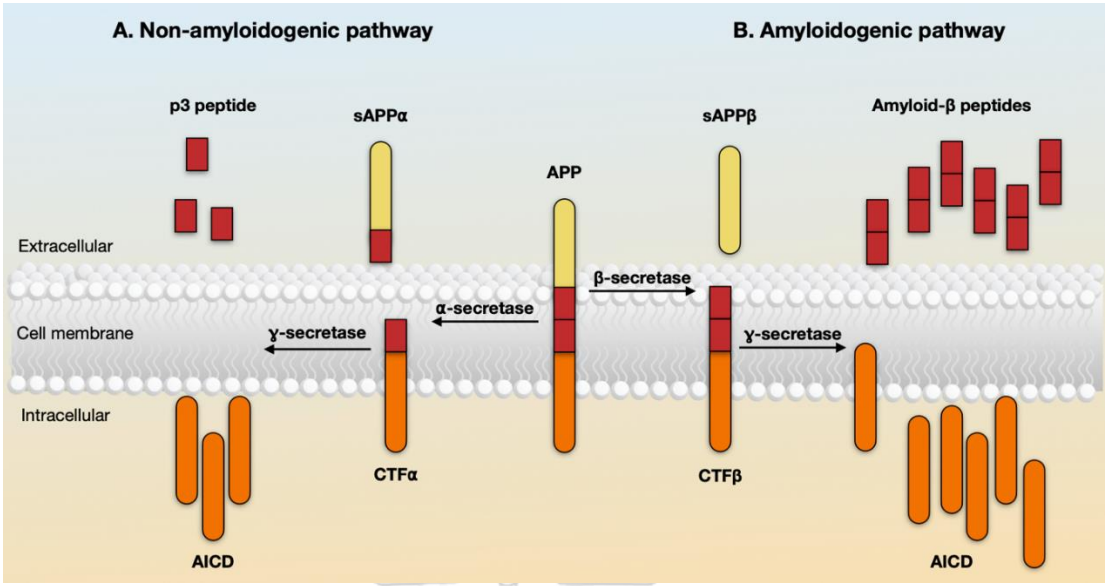


Figure 2.4 The proteolytic pathways of amyloid precursor protein which are (A) non-amyloidogenic pathway and (B) amyloidogenic pathway.

An aberrant production of amyloid-β peptide has been evidenced in association with advancing age in animals. The increased *Bace1* and decreased *Adam10* mRNA and protein expression levels in the hippocampus have been reported in aged (22 months old) male Sprague Dawley rats (Hou et al., 2011) and ICR mice (Mukda et al., 2016) , and it was associated with the impairment in spatial learning and memory (Hou et al., 2011; Mukda et al., 2016). Moreover, the investigation in the 3xTg-AD mice, the transgenic model of AD harboring three human mutant genes (APP, TAU, and PS1), has been reported that amyloid plaques in the hippocampus increased with advancing in age (Belfiore et al., 2019).

3. Androgen deficiency and reproductive senescence in males

Along with the physiological age, a reproductive senescence with a decline of sex steroid hormones has been related to and listed as a potential risk factor of cognitive impairment (Gauthierm, 2021). In male, testes are the major source of sex steroid hormone production, and androgens are predominant.

3.1 Androgens in males

Androgens are a group of steroid hormones which have important roles for male phenotype including male sexual differentiation during development, maintenance of secondary male characteristics, and function of male genital organs and spermatogenesis. Androgens are also involved in many physiological processes such as stimulating muscle and hair growth, bone development, erythropoiesis as well as controlling male psychosocial behaviors. The major androgen in male is testosterone synthesized mostly by Leydig cells in the testes, accounted for more than 95% of androgen in the blood circulation. In addition to testes, the adrenal glands also contribute to testosterone synthesis but in only small amount. The production of testosterone relies on the interplay of hypothalamic-pituitary-testicular axis (Figure 2.5). The hypothalamus releases gonadotropin-releasing hormone (GnRH) in pulsatile manner, about every 90 to 120 minutes, travels to the anterior pituitary gland, and binds to GnRH receptors. This interaction promotes the release of luteinizing hormone (LH) from anterior pituitary gland into the systemic blood circulation downward to testes where LH receptors present. Binding of LH to its receptor presented on the Leydig cells induces testosterone synthesis. Then, testosterone exerts the negative feedback control on GnRH secretion through androgen receptor (AR) on the hypothalamus and on LH secretion in the pituitary gland (Figure 2.5).

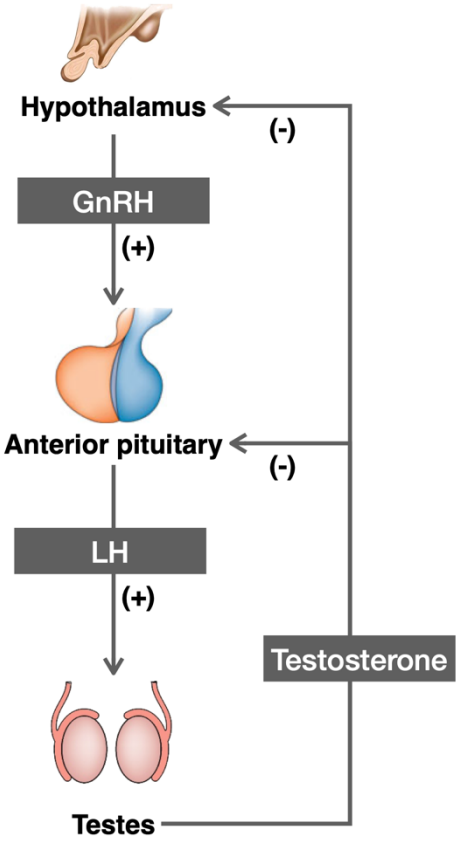


Figure 2.5 The hypothalamic-pituitary-testicular axis.

3.1.1 Biosynthesis of testosterone

Testosterone is synthesized from the cholesterol precursor through steroidogenesis in the Leydig cells. This process can be broadly divided into five major steps.

1) Cholesterol is acquired from the systemic circulation by receptor-mediated endocytosis and *de novo* synthesis endogenously by endoplasmic reticulum in Leydig cells and stored in the form of cholesterol esters in cytoplasmic lipid droplets.

2) LH regulates Leydig cells to stimulate the mobilization of cholesterol from lipid droplet stored CEs to mitochondria.

3) Cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane by a steroidogenic acute regulatory protein (StAR).

4) The cytochrome P450 side chain cleavage enzyme (P450scc) localized at the inner mitochondrial membrane converts cholesterol to pregnenolone, the common precursor for the synthesis of all of the other steroid hormones. The transport of cholesterol to inner mitochondria membrane and conversion into pregnenolone is the rate-limiting step in testosterone biosynthesis.

5) Pregnenolone then diffuses out to the smooth endoplasmic reticulum where remaining steps of testosterone biosynthesis are carried out by several membrane-bound enzymes including 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase (CYP17α) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Along the enzymatic cascades, the intermediate androgens are generated including dehydroepiandrosterone, androstenedione, and androstenediol.

3.1.2 Transportation of testosterone

Testosterone released from the Leydig cells mediates the action at target organs in three different routes: autocrine effects on the Leydig cells themselves; paracrine effects on myoid cells and Sertoli cells to regulate the spermatogenesis; and endocrine effects on other target organs via systemic blood circulation. Testosterone transported in the bloodstream mainly binds to carrier proteins (for approximately 98%) while the remaining (only 2%) is in free form or unbound. Thus, total testosterone refers to the sum of the concentrations of protein-bound and unbound testosterone in circulation. About 40% of testosterone is tightly bound to sex hormone-binding globulin (SHBG), and therefore considered unavailable to target cells. The remaining testosterone (about 58%) is weakly bound to albumin protein, and can be easily dissociated in the tissue capillaries and effectively be available for biological activity. Along with free testosterone, albumin-bound testosterone is also considered to be bioavailable testosterone (Goldman et al., 2017).

3.1.3 Androgenic activity of testosterone

Testosterone mediates androgenic activity after binding with AR that functions as a ligand-activated transcription factor, involving a multistep mechanism. The AR signaling pathway initiates when androgen crosses the plasma membrane, enters the cytoplasm and binds to the AR (at c-terminal ligand-binding domain of AR) which dissociates the chaperone proteins, simultaneously accompanies by conformational changes of the receptor protein into a transformation complex, and the complex is translocated to the nucleus. Then, the complex dimerizes, and the DNA-binding domain binds to DNA at androgen response element to begin transcription and to regulate the expression of androgen-regulated genes. Thus, this genomic dependent signaling pathway has become known as the classical androgen pathway, which can take several hours to days until the responses can be observed.

Additionally, androgens can activate signaling pathways via non-DNA binding-dependent actions, or “non-genomic” effects, through interactions with ARs embedded in the cell membrane that involves a rapid induction of secondary messengers to initiate cellular events, i.e., protein phosphorylation. Functionally, the effect is not removed by inhibition of transcription or translation, and is often activated by membrane-impermeant steroid conjugates. This non-genomic signaling pathway requires neither AR nuclear translocation nor AR-DNA binding. Thus, this rapid steroid effect is activated within seconds or minutes. However, in some case, a rapid pathways of androgen action might ultimately act to modulate transcriptional activity of AR or other transcription factors, for example the activation of kinase signaling cascades (PI3K/Akt) which in turn activates MAPK/ERK, leading to cell proliferation (Liao et al., 2013). Therefore, the term of non-classical, rather than non-genomic, has been often used (Rahman and Christian, 2007).

3.1.4 Metabolites of testosterone

The androgenic activity of testosterone could be either via its highly potent metabolite, DHT, or converted to E_2 by aromatization. Because E_2 mainly interacts with discrete receptors, estrogen receptors (ERs), it completely differs in biological activities comparing to androgens.

Dihydrotestosterone

DHT is reduced from only 5% to 10% of testosterone in bloodstream by 5α-reductase enzyme, which mostly occurs in target organs including prostate gland, seminal vesicles, epididymis, skin, hair follicles, liver, and brain (Gao et al., 2005). DHT is presents only in small amount in the circulating system relative to its precursor testosterone (Swerdloff et al., 2017). While testosterone and DHT act through the same AR, the interaction of both androgens with AR is different. DHT is the most potent agonist of AR with 4-fold higher binding affinity than that of testosterone (Gao et al., 2005), and the dissociation rate of DHT from the receptor is 5-fold slower than that of testosterone (Grino et al., 1990). The metabolic clearance of DHT is slower than testosterone, indicating a modestly longer residence time for DHT (Swerdloff et al., 2017). Unlike testosterone, DHT cannot be converted by the aromatase enzyme into E_2. Therefore, it is frequently used in research settings to distinguish between the effects of testosterone caused by binding to the AR and those

caused by testosterone's conversion to E_2 and subsequent binding to and activation of ERs.

17β-estradiol

Although estrogen is considered as a female sex steroid hormone, the predominant form of estrogen, E_2, also plays the essential roles in males, in both reproductive functions and non-reproductive functions. In adult males, E_2 is derived from about 0.2% of circulating testosterone by the aromatase enzyme at peripheral organs such as testis, adipose tissue, male reproductive tract, bone, and brain (Gao et al., 2005). Estrogen mediates the action via three types of ERs, including ERα, ERβ and G protein-coupled estrogen receptor 1 (GPER1). Since ERα and ERβ belong to the steroid hormone superfamily of nuclear receptors, like AR, the structural architecture composes of three major functional domains, including N-terminal domain, DNA-binding domain, and ligand-binding domain. Thus, the biological actions of estrogen through ERs are mostly similar to AR via both genomic and non-genomic effects. On the other hand, GPER1 (membrane-bound) is mostly responsible for the rapid non-genomic actions with the activation of various protein-kinase cascades. The expression of ERs in males has been reported in many organs such as testis, cardiovascular system, adipose tissue, bone and various regions of brain. Thus, estrogen is involved in the regulation of, for example, the remodeling mechanisms of bone resorption and formation (Falahati-Nini et al., 2000) and prevention of bone loss (Finkelstein et al., 2016). Besides, in the hypothalamic-pituitary-testicular axis, the aromatization of circulating testosterone to E_2 is required for negative feedback of LH secretion at the pituitary gland (Hayes et al., 2000; Rochira et al., 2006).

3.2 Levels of testosterone in circulatory system

Measurement of testosterone levels from circulatory system could represent biological condition of male reproductive system and biological activity of the hormone throughout the body. However, blood testosterone levels fluctuate in a circadian pattern: highest in the morning and lowest in the late afternoon. Health conditions, e.g., obesity, medication and chronic diseases, also greatly influence on individual's testosterone levels (Travison et al., 2017; Wang et al., 2011; Wu et al., 2008). Exposure to endocrine disrupting chemicals from environment also interferes biosynthesis of testosterone (Jeng, 2014; Knez, 2013). Therefore, a considerable

interindividual variability in testosterone levels was common. The clinical practice guidelines from the American Association of Clinical Endocrinologists stated that physiological levels of serum testosterone in adult men range from 280 to 800 ng/dL (Petak et al., 2002). Further, the recent study from 9,054 men across four cohorts had established the reference range of total testosterone levels of 2 6 4 - 1 1 8 2 ng/dL (Travison et al., 2017).

3.3 Age-related androgen deficiency

Like in women that estrogen levels decrease when they enter into the menopausal period, testosterone levels in men also decline with advancing in age. However, in contrast to the abrupt cessation of estrogen production found in menopausal women, the age-related decline in testosterone levels in men, after reaching a peak level in the second and third decade of life, is a slow transition process. The majority of studies have reported that men exhibited a gradually progressive decline in the circulating testosterone at the third to fourth decade of life (Travison et al., 2017). The European Male Aging Study (EMAS) found a decline of total testosterone levels in men between 40 and 79 years of age to be 0.40% per year (Wu et al., 2008). Although a rate of decline of total testosterone levels was low, the decrease in free testosterone level was greater by 1.3% per year. This is explained by the age-related increase in SHBG which increases the bound form and decreases the free form of testosterone. Camacho et al. (2013) reported that total testosterone level and free testosterone level were decreased by 0.59% and 1.41% per year, while the SHBG level was increased by 1.42% per year. With the advancing in age, , the rate of decline in circulating total testosterone is greater; 1.26% per year at 71 to 86 years of age (Lapauw et al., 2008) and 2% per year at 75 and 84 years of age (Yeap et al., 2018).

Although androgens gradually decline with advancing age, elderly men do not normally show an abrupt or drastic cessation of gonadal function; only mild to moderate decline of gamete quality (e.g., semen volume, total sperm count per ejaculation, percentage motility, and normal morphology) and fertility were detected (Humm and Sakkas, 2013; Johnson et al., 2015). Together with the absence of an identifiable inflection point at which testosterone levels begin to decline abruptly or more rapidly, there is no such a phenomenon as ‘andropause’ or ‘male climacteric’. On the other hand, the failure to produce physiological concentrations of testosterone

has been associated with the clinical symptoms and signs such as decreased libido, erectile dysfunction, frailty, and fatigue which are frequently termed as late-onset hypogonadism and required testosterone replacement therapy (Tsametis and Isidori, 2018).

The late-onset hypogonadism, which is diagnosed by the decline of circulating testosterone level below the normal range and defined as androgen deficiency, is a symptom that affects the quality of life (Bhasin et al., 2018; Bhasin et al., 2010; Lunenfeld et al., 2015; Mulhall et al., 2018; Wang et al., 2008). Currently, there is no consensus among the guidelines on what is considered androgen deficiency, and cutoff values of total testosterone levels. For example, the Endocrine Society mentioned that men with total serum testosterone levels below 300 ng/dL are diagnosed as hypogonadism (Bhasin et al., 2010). Meanwhile, a consensus statement from representatives of leading societies concerned with the problem of hypogonadism in men: the International Society of Andrology (ISA), International Society for Study of the Aging Male (ISSAM), European Association of Urology (EAU), European Association of Andrology (EAA), and American Society of Andrology (ASA) recommended that total serum testosterone levels below 230 ng/dL is considered as androgen deficiency, while the levels between 230 and 350 ng/dL required additional result of free testosterone levels for diagnosis (Wang et al., 2008). In 2015, Lunenfeld et al. (2015) recommended that total testosterone levels below 350 ng/d was diagnosed as hypogonadism. Recently, European Society of Endocrinology considered the levels lower than 264 ng/dL (Bhasin et al., 2018) and that of the American Urological Association Clinicians is 300 ng/dL (Mulhall et al., 2018). Although there is the lack of a universally accepted definition for late-onset hypogonadism, several studies have a similar trend of estimation of the prevalence. For example, the Baltimore Longitudinal Study of Aging reported that 12%, 19%, 28%, and 49% of men in their 50s, 60s, 70s, and 80s were diagnosed as hypogonadism (Harman et al., 2001). The 9-year followed up study of Massachusetts Male Aging Study revealed that the prevalence of androgen deficiency in the 40- to 69-year-old men significantly increased up to 12.3% from the baseline (Araujo et al., 2004). Moreover, the overall prevalence of late-onset hypogonadism in the European Male Aging Study, which included 3,369 men aged 40 to 79 years, was 2.1%. The

number was significantly higher for the age group 60 to 69 years (3.2%) and 70 to 79 (5.1%) (Wu et al., 2010). Thus, together with the increasing in life expectancy globally, the number of androgen deficient men is estimated to be increased.

3.3.1 Natural occurrence of androgen deficiency in males

The fundamental mechanisms causing the late-onset hypogonadism remain unknown and its source of origin are difficult to determine. An androgen deficiency in older males reflects multisite failure at the hypothalamic-pituitary-testicular axis which is a loop feedback regulation with a compensatory mechanism. However, recent investigations have disclosed multiple alterations in the hypothalamic-pituitary-testicular axis in older males which may primarily originate from either the testicular failure or *primary hypogonadism* or the dysfunction of the hypothalamic-pituitary unit or *secondary hypogonadism*.

Primary hypogonadism

The failure of testicular function in elderly primarily causes the reduction in testosterone synthesis which is relied on a decrease in the steroidogenesis capacity of Leydig cells (Chen et al., 1994; Chen et al., 2002; Grzywacz et al., 1998; Zirkin et al., 1993). In this case, administration of LH could not restore testosterone synthesis as seen in the adults, suggesting the reduction in sensitivity to LH of Leydig cells. The deterioration of Leydig cells has been reported to associate with the intracellularly increased reactive oxygen species (Chen et al., 2001) and the declined antioxidant capacity (Cao et al., 2004; Luo et al., 2006). The increase in oxidative stress probably led to DNA, protein and/or lipid damage. In responding to the low testosterone level, a negative feedback signal was reduced and the hypothalamic-pituitary unit generally attempted to increase LH secretion. Moreover, the increase in GnRH stimulating signals: kisspeptin and neurokinin B, has been reported in hypothalamus of elderly men (Molnár et al., 2012). The primary hypogonadism has been diagnosed in about 2% out of elderly men aged 40-79 years, and the proportion of primary hypogonadism increased with age (Tajar et al., 2010).

Secondary hypogonadism

For secondary hypogonadism, the dysfunction at hypothalamic-pituitary levels was arisen which was characterized by low testosterone levels and low or inappropriately normal LH levels. In this case, the hypothalamus reduced a GnRH

outflow which led to irregular pulsatile of LH secretion, with lower amplitude and higher frequency, and the sensitivity to the negative feedback from steroid hormones is disturbed (Keenan and Veldhuis, 2001; 2009; 2016; Liu et al., 2009; Mulligan et al., 1997; Pincus et al., 1996). The irregular LH pulses secretion from pituitary mostly reflected the diminished hypothalamic GnRH secretion rather than deterioration on pituitary itself. Indeed, the function of pituitary on secreting LH was basically unaffected by aging, and it exhibited a normal response to GnRH agonist in elderly men (Abbara et al., 2018; Iranmanesh et al., 2010; Kaufman et al., 1991). Decreased GnRH mRNA and protein expression levels have been demonstrated in aged male rats as compared to younger rats (Gruenewald et al., 2000). At the upstream control of GnRH secretion, kisspeptin, neurokinin B, and dynorphin secreted from KNDy neurons, has been reported to decreased in association with a decline in LH levels in aged male rats (Kunimura et al., 2017). However, the underlying mechanisms of the chronological aging contributing to the central axis failure has not been thoroughly addressed.

3.3.2 Medical practice and occurrence of androgen deprivation in males

In addition to a progressively natural decline in testosterone levels in elderly men, some medical practices as seen in prostate cancer patients require suppression of testosterone synthesis in men (Siegel et al., 2020; Sung et al., 2021). Since the malignant prostate cancer cells are androgen responsive for growth and proliferation, the androgen deprivation therapy (ADT) is an essential medical practice. The goal of ADT is to reduce serum testosterone to castrated levels < 20 ng/dL (Cornford et al., 2021). Currently, the injectable LH-releasing hormone agonist, which constantly stimulate the anterior pituitary gland, and in turn decrease LH and testosterone production, is a recommended chemical castration (Cornford et al., 2021). ADT can effectively improve the survival from prostate cancer (Mason et al., 2015). However, several adverse side effects of androgen deficiency due to ADT have been reported such as skeletal-related events (osteopenia, osteoporosis, and fracture of bones), an increased risk of cardiovascular diseases, weight gain, metabolic syndrome, a reduced quality of life (due to hot flushes, sexual dysfunction, fatigue and mental depression), and cognitive impairment (Nead et al., 2017; Nguyen et al., 2015). Thus, the

increasing numbers of prostate cancer patients who are treated with ADT are therefore magnified the numbers of men suffering from androgen deficiency.

4. Androgen deficiency and cognitive impairment in males

The preclinical experiments supporting the important role of testosterone on cognitive function in males have been widely conducted in laboratory rodents. An orchidectomy-induced androgen deficiency by surgically removing testes affected on several domains of behaviors in either non-spatial (Benice and Raber, 2009; Frye et al., 2008; Kritzer et al., 2007) or spatial learning and memory (Daniel et al., 2003; Gibbs and Johnson, 2008; Kritzer et al., 2001; Lagunas et al., 2011). While the crucial effects of testosterone on the spatial working memory have been consistently reported (Bimonte-Nelson et al., 2003; Gibbs and Johnson, 2008; Spritzer et al., 2008), the findings on spatial reference memory were inconsistency. Some studies had reported that orchidectomy-induced androgen deficiency impaired the spatial reference memory assessed by MWM task (Fang et al., 2017; Frye et al., 2010; Moghadami et al., 2016; Pintana et al., 2016). However, some reported an absence of the deleterious effects of orchidectomy on spatial reference performance (Hodosy et al., 2012; Mohammadi-Farani et al., 2015; Pintana et al., 2016; Sandstrom et al., 2006; Spritzer et al., 2008; Zhao et al., 2018). These variations might be because of the difference in age and strain of animals engaged in those studies and the duration of orchidectomy-induced androgen deficiency. Moghadami et al. (2016) reported that orchidectomy in adult male Wistar rats for 2 weeks significantly impaired the spatial performances in MWM test. Additionally, the orchidectomy in 6-week old male Wistar rats for 2 and 3 months, but not at 1 month, significantly impaired their spatial learning and memory (Pintana et al., 2016). In contrast, the orchidectomy in 2-month old rats, for 4 months, had no significant effect on spatial learning and memory compared to age-matched intact rats (Spritzer et al., 2008). Although there is an inconsistency in effects of orchidectomy-induced androgen deficiency on spatial learning and memory in male rodents, the studies experimented in AD animal models have supported the importance of endogenous androgen on cognitive function. They reported that orchidectomy could accelerate the spatial deficit in 3xTg-AD (Rosario et al., 2006;

Rosario et al., 2012) and the senescence-accelerated-prone mouse 8 (SAMP8; a naturally derived animal model for AD) (Kang et al., 2014).

Most of the studies have also examined and reported a positive association between the decline in endogenous testosterone levels and several cognitive domains in men (Table 2.1). The older men with low levels of testosterone in the blood circulation performed poorer in measures of cognitive function such as global cognition, verbal memory, visuospatial functioning, spatial ability, executive function, and attention (Barrett-Connor et al., 1999; Driscoll et al., 2005; Matousek and Sherwin, 2010b; Muller et al., 2005; Thilers et al., 2006; Yeap et al., 2008). One of the largest cross-sectional studies determined the cognitive function in elderly men (70 – 89 years old) using Standard Mini-Mental State Examination (SMMSE) test encompassing a range of cognitive domains; orientation to time and place, immediate memory and recall, visuospatial ability and language and revealed that the cognitive scores were positively correlated with the serum testosterone levels (Yeap et al., 2008). For further analysis, this study had grouped the participants based on the SMMSE scores and showed that the lower-score groups had significantly lower testosterone levels than those in the higher scores (Yeap et al., 2008). These evidences supported the important role of endogenous testosterone on cognitive function. However, the results were not aligned between studies (Fonda et al., 2005; Martin et al., 2007; Martin et al., 2008; Yonker et al., 2006). The interference of the effect of age to confound the effects of testosterone's decline was suspected. Most of the findings yet supported the significantly positive association between testosterone levels and cognitive function when the factor of age was excluded from the analyses. This suggested that the association between testosterone levels and cognitive function is age-independence (Barrett-Connor et al., 1999; Driscoll et al., 2005; Matousek and Sherwin, 2010b; Muller et al., 2005; Yeap et al., 2008). However, in some studies, the significant effect of androgen deficient was mitigated (Yaffe et al., 2002) or even disappeared (Fonda et al., 2005) after adjusting the age factor. Possibly, the advancing in age has amplified the positive association between testosterone levels and cognitive function (Thilers et al., 2006). Although the association between testosterone decline and cognitive function in men was inconclusive, a number of studies have reported that men diagnosed with AD have a significantly lower testosterone level (Chu et al.,

2010; Hogervorst et al., 2001; Moffat et al., 2004; Okun et al., 2004; Paoletti et al., 2004).

With longitudinal analyses tracking the cognitive changes over time supported the positive association between decreased testosterone levels and cognitive impairment as mentioned previously in cross-sectional studies. For example, in the Baltimore Longitudinal Study of Aging, 407 men aged 50–91 years were followed for an average of 10 years and found that the cognitive performances including: verbal memory, visual memory, visuospatial skills, and visuomotor scanning were declined in comparison with the baseline level and the decline was significantly associated with the decreased testosterone levels (Moffat et al., 2002). Likewise, the 10.5-year longitudinal study in a larger cohort of 4069 old men, aged 71–88 years has revealed the positive association between a testosterone decline and cognitive impairment (Ford et al., 2018). Moreover, men with a lower testosterone level at the baseline had a higher risk of developing cognitive impairment compared to those who had a higher level. Moffat et al. (2002) have reported their 19.1-year data collection that the decrease in testosterone levels was significantly associated with an increase in incidence of men diagnosed as AD, and this statistical significance remained evident after eliciting the effects of aging (Moffat et al., 2004). From these longitudinal findings, it suggested that the testosterone depletion is possibly an early component of cognitive impairment which is apparent prior to the clinical diagnosis of AD. These evidences have also strengthened the hypothesis that the decline of testosterone is a risk factor of a cognitive impairment in elderly men. However, one important question which has yet to be elucidated is " When does the cognitive impairment occurr in elderly men?"

Several longitudinal studies attempted to predict the cognitive changes over time in association with testosterone levels measured at the baseline levels (Table 2.1). In almost all studies, the baseline endogenous testosterone levels were varied among individuals and statistically characterized as low, middle and high. With the 2 - 6 year following-up, the overall cognitive function declined compared to baseline values (Geerlings et al., 2006; Hogervorst et al., 2010; Hsu et al., 2015; LeBlanc et al., 2010; Muller et al., 2009; Yaffe et al., 2007); but no significant association with baseline testosterone levels was detected. These findings suggested that baseline testosterone

levels could not predict the cognitive changes. Regarding the fact that testosterone levels basically decreased in approximately 1% to 3 % per year in men, this possibly contributes to the cognitive decline. Hsu et al. (2015) reported that testosterone levels significantly decreased after 5-year follow up and significantly associated with cognitive impairment. All in all, although it clearly depicts that a decrease in testosterone level is a major factor for cognitive impairment, it is very difficult to catch a critical point of a significant reduction of testosterone level and the utmost confounding effect is interindividual variability in testosterone levels.

Some evidences have corroborated that the cognitive decline in men was a consequence of lowered testosterone below the physiological levels which was marked in ADT in prostate cancer patients. Several domains of impaired cognitive function such as verbal memory, spatial abilities, and attention have been observed after the ADT for 6 - 12 months (Cherrier et al., 2009; Green et al., 2002; Salminen et al., 2004). The recent longitudinal investigation, up to 4 years, have supported a strong impact of ADT on cognitive dysfunction (Cherrier and Higano, 2019; Jhan et al., 2017). Moreover, men receiving ADT had a higher incidence of AD than a non-treated group (Nead et al., 2016; Nead et al., 2017). From these evidences, it raises the next question "How long does it take androgen deficient men to develop conditions of AD?".

Thus, in order to further elucidate 'when' and 'how' the testosterone decline would contribute to a cognitive impairment, the timing of a significant decrease in testosterone level and the initiation of cognitive impairment should be carefully determined and clearly defined, and the chronological advancing age which would possibly confound the outcome must be controlled. Furthermore, the early detection of changes in the corresponding brain regions which lead to cognitive impairment should be considered. With knowing the time point of a significant decline in testosterone level and how this decline contributes to a cognitive dysfunction, the preventive and therapeutic measured could be developed.

Table 2.1 Evidence of the association between cognitive function and androgen decline in elderly men

References	Age (mean±SD)	Number of participants	Study design	Major findings
Barrett-Connor et al., 1999	55 - 89 (70.2±8.3)	547	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The verbal memory and global cognitive function test scores were significantly worsened with older age. - Positive association between testosterone levels with all cognitive function was independent from factor of age.
Yaffe et al., 2002	(73.0±7.1)	310	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The global cognition, memory, and attention test scores were significantly worsened with older age. - Positive association between testosterone levels and all cognitive function was marginally lessened after adjusting a factor of age.
Driscoll et al., 2005	20 - 60 (51.9)	33	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The spatial ability significantly declined with older age. - Positive association between total testosterone levels and spatial ability was independent from factor of age.
Fonda et al., 2005	48 – 80 (62.7±8.2)	981	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The working memory, attention and spatial abilities declined with older age. - Positive association between testosterone levels and working memory, attention, spatial ability was absent when the effects of age was elicited.
Muller et al., 2005	40 – 80 (60.2±11.3)	400	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - No association of verbal memory, attention, and executive function and advancing in age has been reported. - Positive association between testosterone levels and cognitive function was independent from factor of age.
Thilers et al., 2006	35 – 90 (62.2±13.5)	1107	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The episodic recall, episodic word recognition, visuospatial performance declined with older age. - Positive association of free testosterone and episodic recall, episodic word recognition, visuospatial performance was amplified with age.

References	Age (mean±SD)	Number of participants	Study design	Major findings
Yonker et al., 2006	35 – 80 (54.4±12.8)	450	Cross-sectional study	- The association of testosterone levels and age has not been reported. - The spatial visualization, episodic memory, semantic memory, problem solving, and verbal fluency declined with older age. - No significant association between testosterone levels and cognitive function has been reported.
Martin et al., 2007	35 – 80 (54.3±11.3)	1046	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The verbal memory significantly declined with older age. - Negative association between total and free testosterone and verbal memory.
Yeap et al., 2008	70 - 89	2932	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - An increasing in age was associated with decline in global cognitive function. - Positive association between testosterone levels and global cognitive function was independent from factor of age.
Matousek and Sherwin, 2010b	61 - 77 (68.6±4.39)	54	Cross-sectional study	- The levels of testosterone were significantly lower in older men. - The working memory and spatial abilities declined with older age. - Positive association between testosterone levels and working memory, which was independent of age. - No significant association between total testosterone or free testosterone and spatial abilities.
Moffat et al., 2002	50 – 90 (64.07±9.40)	407	Longitudinal study (9.7 years)	- The levels of testosterone significantly decreased over time of investigation. - The verbal memory, visuospatial functioning, and visuomotor scanning test scores declined significantly with advancing in age. - Positive association between testosterone levels and verbal memory, visuospatial functioning, visuomotor scanning, this significant association was independent from factor of age
Geerlings et al., 2006	71 – 93 (77.4±4.3)	2974	Longitudinal study (6 years)	- The global cognitive function declined with advancing in age determined over 6-year follow-up. - No association between baseline testosterone levels and global cognition at endpoint. - Note: testosterone levels at endpoint was not reported.
Yaffe et al., 2007	70 - 79 (75.4±2.7)	439	Longitudinal study (2 years)	- The global cognitive function, verbal memory, and executive function declined with advancing in age, assessed over 4-year follow-up. - No significant association between baseline testosterone levels and any cognitive function at

References	Age (mean±SD)	Number of participants	Study design	Major findings
				endpoint. - Note: testosterone levels at endpoint was not reported.
Muller et al., 2009	>70 (77.4±3.2)	242	Longitudinal study (4 years)	- The global cognitive function significantly declined with advancing in age, assessed over 4-year follow-up. - No significant association between baseline testosterone levels and global cognition over 4-year follow-up. - Note: testosterone levels at endpoint was not reported.
Hogervorst et al., 2010	64 - 94 (74.4±5.8)	257	Longitudinal study (2 years)	- The global cognitive function and verbal memory declined with advancing in age, determined at 2-year follow-up compared to baseline. - No significant association between testosterone levels at baseline and cognitive functions at endpoint. - Note: testosterone levels at endpoint was not reported.
LeBlanc et al., 2010	65 - 79 (73.6)	1397	Longitudinal study (4.5 years)	- No significant association between testosterone levels at baseline and global cognition and executive function. - Note: testosterone levels at endpoint was not reported.
Hsu et al., 2015	>70 (76.9±5.5)	958	Longitudinal study (5 years)	- Testosterone levels and global cognitive function decline with advancing in age determined over time of 5-year follow-up in comparison to baseline. - No significant association between testosterone levels at baseline and cognitive function at endpoint. - Positive association between the decline of testosterone levels and cognitive function, which is still existed after adjusting the effects of age.
Ford et al., 2018	71 - 88 (66.3±10.3)	4069	Longitudinal study (10.5 years)	- Testosterone levels and cognitive function decline over time of 10.5-year follow-up in comparison to baseline. - Positive association between testosterone levels and global cognition, which is still existed after adjusting the effects of age. - Men with lower testosterone levels has a higher risk of cognitive impairment.

5. Hormonal treatment and its effects on cognitive function in males

In addition to the varying results in preclinical and clinical studies mentioned above, one important factor that should be noted for the effects of testosterone on cognition is the metabolisms of testosterone contributing to the complexity of its action. Testosterone can act via three major pathways: i) directly as testosterone without biotransformation, ii) converted by 5α-reductase to a highly potent androgen, DHT, and 3) converted by aromatase to E_2. Testosterone and DHT mediate their actions via the AR, while E_2 mediates its actions via ERs. Furthermore, both AR and ERs are expressed in male hippocampus (Gonzalez et al., 2007; Hojo and Kawato, 2018), brain region involving in learning and memory process.

5.1 Testosterone supplementation

With multiple reports denoted the association between endogenous testosterone levels and cognitive function in elderly men, a growing number of clinical trials on the effects of testosterone supplementation and cognitive function have been reported. Testosterone supplementation significantly enhanced spatial performances, working memory and verbal memory in cognitively normal older men (Table 2.2) (Cherrier et al., 2001; Cherrier et al., 2005a; Janowsky et al., 2000; Janowsky et al., 1994; Wahjoepramono et al., 2016). The testosterone supplementation on verbal memory and spatial abilities in elderly men who had low levels of testosterone and suffering from MCI and AD have been also observed (Cherrier et al., 2015; Cherrier et al., 2005b). However, many other studies failed to replicate those effects in elderly men who had normal cognition or had been diagnosed with MCI or AD (Borst et al., 2014; Emmelot-Vonk et al., 2008; Haren et al., 2005; Huang et al., 2016; Kenny et al., 2002; Kenny et al., 2004; Lu et al., 2006; Maki et al., 2007; Vaughan et al., 2007). Additionally, the largest randomized control trial, examined in 492 older men with low testosterone levels, over a period of one year, has also reported that testosterone supplementation failed to provide the beneficial effects on verbal episodic memory and visuospatial performances (Resnick et al., 2017). With meta-analysis using pooled previously published random data of nineteen randomized placebo control trials has shown non-significant effects of testosterone supplementation on executive function, verbal fluency and memory, visuomotor ability, visuospatial ability, attention and working memory in elderly men (Corona et al., 2020). One available

report noted that the weak efficacy of exogenous testosterone supplementation on cognitive functioning in elderly men might be affected by the variation of dosing, timing, and the route of administration (Nelson et al., 2008).

Table 2.2 Evidence of the effects of testosterone supplementation on cognitive function in elderly men

References	Age (mean ± SD)	Number of participants	Cognitive status	Duration of treatment (mth)	Major findings
Janowsky et al., 1994	60-75 (67.40)	TS = 27 PL = 29	Normal	3	- TS significantly increased endogenous testosterone levels. - TS significantly enhanced spatial cognition, but not for executive function, verbal memory, and visuomotor function.
Janowsky et al., 2000	61-75 (67.5)	TS = 10 PL = 9	Normal	1	- TS significantly increased endogenous testosterone levels. - TS significantly enhanced working memory.
Cherrier et al., 2001	50-80 (67.4)	TS = 13 PL = 12	Normal	1.5	- TS significantly increased endogenous testosterone levels. - TS significantly improved spatial performances and, verbal memory, but not for executive function, visuomotor function, attention, and working memory.
Kenny et al., 2002	65-87 (76.0)	TS = 24 PL = 20	Normal	12	- TS significantly increased endogenous testosterone levels. - No significant effects on attention and working memory was observed.
Kenny et al., 2004	73-87 (80.0)	TS = 6 PL = 5	MCI	3	- TS significantly increased endogenous testosterone levels. - No significant effects on executive function, visuomotor function, attention and working memory was observed.
Cherrier et al., 2005a	50-85 (65.0)	TS = 20 PL = 21	Normal	1.5	- TS significantly increased endogenous testosterone levels. - TS significantly enhanced spatial memory and verbal memory.
Cherrier et al., 2005b	63-85 (76.0)	TS = 19 PL = 13	AD	1.5	- TS significantly increased endogenous testosterone levels. - TS significantly improved spatial memory and verbal memory, but not for attention and language.
Haren et al., 2005	60-86 (68.5)	TS = 39 PL = 37	Normal	12	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on visuomotor function, attention, and working memory.
Lu et al., 2006	(69.8 ± 8.7)	TS = 7 PL = 9	AD	6	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on verbal memory, visuomotor function, and visuospatial function.

References	Age (mean ± SD)	Number of participants	Cognitive status	Duration of treatment (mth)	Major findings
Maki et al., 2007	66-86 (73.9)	TS = 9 PL = 7	Normal	9	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on verbal memory, visuospatial learning, attention and working memory.
Vaughan et al., 2007	65-83 (70.8)	TS = 17 PL = 15	Normal	36	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on executive function, verbal memory, visuospatial function, attention, and working memory.
Emmelot-Vonk et al., 2008	60-80 (67.3)	TS = 113 PL = 110	Normal	6	- Levels of endogenous testosterone was not reported at endpoint. - No significant effects of TS on executive function, verbal memory, and visuospatial function.
Borst et al., 2014	(70.0 ± 8.9)	TS = 14 PL = 16	Normal	12	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on executive function, verbal learning and memory, visuospatial learning and memory, attention and working memory.
Cherrier et al., 2015	60-88 (70.5)	TS = 10 PL = 12	MCI	6	- TS significantly increased endogenous testosterone levels. - TS significantly improved verbal learning and memory, but not for executive function, visuospatial learning and memory.
Huang et al., 2016	60-75 (67.5 ± 5.1)	TS = 140 PL = 140	Normal	36	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on executive function, verbal memory, visuospatial learning and memory.
Wahjoepramono et al., 2016	(61.1 ± 7.7)	TS = 22 PL = 22	Normal	6	- TS significantly increased endogenous testosterone levels. - TS significantly improved global cognitive function, but not for verbal memory.
Resnick et al., 2017	(72.3 ± 5.8)	TS = 247 PL = 245	MCI	12	- TS significantly increased endogenous testosterone levels. - No significant effects of TS on executive function, verbal memory, and visuospatial learning.

TS = Testosterone supplementation group, PL = Placebo control group

A few studies have reported that orchidectomy-induced androgen deficiency impaired the spatial reference memory in male rats assessed by MWM task and testosterone replacement could reverse this deficit (Fang et al., 2017; Frye et al., 2010; Moghadami et al., 2016; Pintana et al., 2016). Although the deleterious effects of orchidectomy on spatial reference performance were absent in most studies (Hodosy et al., 2012; Mohammadi-Farani et al., 2015; Pintana et al., 2016; Sandstrom et al., 2006; Spritzer et al., 2008; Zhao et al., 2018), the exogenous testosterone administration could enhance this behavior paradigm (Hodosy et al., 2012). Testosterone administration could also reverse the orchidectomy-accelerated spatial deficit in 3xTg-AD (Rosario et al., 2006; Rosario et al., 2012) and the SAMP8 mice.

5.2 Estrogen supplementation

The possible beneficial role of E_2 on cognitive function in men has been noted. A higher level of endogenous E_2 was associated with a better visual memory in young healthy men (Kampen and Sherwin, 1996), a visuospatial and verbal memory in elderly (Hogervorst et al., 2004; Yaffe et al., 2007; Zimmerman et al., 2011) and in prostate cancer men receiving ADT (Salminen et al., 2005). Moreover, the randomized double-blind control study in healthy elderly men aged 50 to 90 years have revealed that the conversion of testosterone to E_2 was important for the enhanced verbal memory (Cherrier et al., 2005a). Supplementation of E_2 in men who received ADT significantly improved verbal memory (Beer et al., 2006), executive function, attention and working memory (Taxel et al., 2004), but its beneficial effects were absent for visuospatial abilities and language (Beer et al., 2006; Matousek and Sherwin, 2010a; Taxel et al., 2004). The possible reason for this inconsistent result could be the difference in the dose and the duration of time between the initiation of ADT and the onset of E_2 treatment. In Beer et al. (2006)'s study, the patient had been androgen-deprived for approximately 5 years before the onset of E_2 treatment, while in Taxel et al. (2004)'s study the ADT was shorter; 3 weeks to 2 years. The beneficial effects of E_2 administration on spatial memory has also been reported in orchidectomized (ODX) rodents (Hodosy et al., 2009; Koss et al., 2018; Lagunas et al., 2011; Locklear et al., 2015; Locklear and Kritzer, 2014). Given aromatase inhibitor to inhibit endogenous estrogen synthesis in male rodents also impaired cognitive functions (Koss and Frick, 2019; Marbouti et al., 2020; Zhao et al., 2018).

5.3 **Actions of testosterone, dihydrotestosterone and estrogen in male brain**

Although the exact effects of testosterone on cognitive function in males are still under debated, its neuroprotective roles such as delaying neuronal apoptosis (Hammond et al., 2001; Spritzer and Galea, 2007), accelerating the rate of nerve regeneration (Pike et al., 2009), modulating neuronal damage (Ahlbom et al., 2001), exerting anti-inflammatory action (Wise et al., 2010), and reducing amyloid-β peptide (Wahjoepramono et al., 2008) have been widely reported. As mentioned previously that testosterone possibly exerts its roles after binding with either AR (via testosterone or DHT) or ERs (via E_2), to investigate the discrete mechanisms of testosterone, the independent administration of DHT and E_2 were conducted by several researches. Following are the reports on the effects of testosterone and its metabolites on three hallmark of neurodegenerations: synaptic plasticity, neurofibrillary tangle and amyloid plaque.

For synaptic plasticity, administration of testosterone could reverse the orchidectomy-decreased synaptic spine density in hippocampus of male transgenic mice via an elevation of BDNF and PSD-95 (Li et al., 2012). The significantly decreased numbers of neurons, synaptic spines and expression of synaptophysin after orchidectomy has been reversed specifically in DHT treated male SAMP8 mice (Kang et al., 2014; Li et al., 2013a). Androgens had been involved with the expression of PSD-95 in hippocampal HT22 cells (Zhang et al., 2019), and enhanced synaptic spine density in hippocampal slices of male rats through AR and several kinase signaling pathways; PKC, PKA, and ERK/MAPK (Hatanaka et al., 2015). Apart from testosterone and DHT, E_2 also mediated changes of synaptic structure in males. E_2 increased the dendritic spines in CA1 hippocampal slices of male rats (Mukai et al., 2007; Murakami et al., 2006; Soma et al., 2018) via ERs and multiple kinases; Src tyrosine kinase, MAPK, and PI3K (Mukai et al., 2010; Soma et al., 2018; Znamensky et al., 2003). E_2 also significantly increased synaptic spine density in the hippocampus of ODX rats (Jacome et al., 2016). Estrogen is likely involved with synaptic transmission in male hippocampus. E_2 enhanced the transmission of ion at the postsynaptic terminal through NMDA receptor and facilitated the long-term potentiation recording from CA1 pyramidal cells of male rats (Di Mauro et al., 2015; Foy et al., 1999; Grassi et al., 2011; Hasegawa et al., 2015; Ooishi et al., 2012).

Furthermore, E_2 exhibited a beneficial role in memory consolidation process via enhancing the phosphorylation of the transcription factor CREB in hippocampus of male mice (Koss et al., 2018).

There are several evidences pointing to the action of sex steroid hormones on tau pathology in hippocampus. Testosterone treatment can prevent or ameliorate the hyperphosphorylation of tau protein in ODX Sprague Dawley rats (Papasozomenos, 1997) and the 3xTg-AD mice (Rosario et al., 2010). The possible action of testosterone was on an alteration in testosterone regulating GSK-3β activity (Papasozomenos and Shanavas, 2002). E_2 could inhibit PKA overactivation and PKA-induced tau hyperphosphorylation in human embryonic kidney 293 (HEK293) cells (Liu et al., 2008).

The increased amyloid-β peptide levels significantly correlated with decreased testosterone levels in mild cognitive impaired men (Rosario et al., 2011). Testosterone and DHT, but not E_2 treatment, reversed (Ramsden et al., 2003) orchidectomy-increased amyloid-β levels in the brain of Sprague Dawley male rats (Ramsden et al., 2003), and the accumulation of amyloid plaques in hippocampus of 3xTg-AD male mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). This was supported by the study in the genetically knock-out of gene encoding aromatase enzyme in male AD model mice which significantly decreased endogenous E_2, but increased testosterone levels, that these animals exhibited significant reduction of amyloid-β accumulation (McAllister et al., 2010). They suggested that endogenous testosterone basically prevented the accumulation of amyloid plaques via mainly androgen pathway and was E_2-independent.

6. Drug development for cognitive impairment

Up to now, there are no pharmacologic treatments or drugs that can prevent or cure the damage and destruction of neurons that cause cognitive impairment, particularly AD. The current available treatments approved by the U.S. Food and Drug Administration (FDA) for cognitive impairment are only four drugs: rivastigmine, galantamine, donepezil, and memantine. The main expected effects of these drugs are to ameliorate symptoms and to improve quality of life enabling patients to maintain daily functions a little longer than they would without the

medication. However, these drugs are not disease-modifying drugs targeting the underlying causes of a disease, thus brain degeneration still progresses. Galantamine (Razadyne®), rivastigmine (Exelon®), and donepezil (Aricept®) are acetylcholinesterase inhibitors, temporarily increase the amount of acetylcholine in the brain aiming to support communication between neurons. These drugs are primarily prescribed for mild to moderate cognitive impairment, aiming to reduce or stabilize some cognitive and behavioral symptoms, which could provide an individual's independency for a longer period of time and assist their caregivers. As the brain deteriorated over time, acetylcholine is produced less and less. Thus, these medicines may eventually lose their efficacy. Although these acetylcholinesterase inhibitors work in a similar way, the effectiveness of each drug may vary among patients. Memantine (Namenda®) competitively blocks NMDA receptor (antagonist) in the brain from excess glutamatergic transmission that can damage the neurons. This drug is primarily prescribed for moderate-to-severe AD patients and is used alone or in combination with donepezil, in order to improve memory, attention, reason, language, and ability to perform simple tasks. However, these drugs may cause adverse side effects to the patients including headache, vomiting, loss of appetite, increased frequency of bowel movements, constipation, confusion and dizziness.

Since then, there have been many attempts to develop an effective disease-modifying drugs, such as Aβ-targeting agents, β-secretase inhibitors, tau-targeting drugs; however, most of them had failed in phase 3 clinical trials (Bachurin et al., 2017). Those failures were mostly a test in patients with severe cognitive impairment. Thus, it is presently believed that clinical trial of AD-modifying drugs should be shifted to investigate in much earlier stages of cognitive impairment. Most recently, FDA has approved the use of Aducanumab (Aduhelm™), the first therapy targeting the amyloid plaque lessening, for specifically MCI people. Aducanumab was a human IgG monoclonal antibody binds selectively to aggregated fibrils and soluble oligomers of Aβ (Schneider, 2020). Aducanumab was granted on June 07, 2021, through the FDA's Accelerated Approval Program, but it is currently available only in the United States. The further clinical trial phase 4 for aducanumab is expected to be available by early 2030. The most common side effects of aducanumab included headache, dizziness, nausea, confusion and vision changes. Once a person is on

aducanumab, their doctor or specialist may require routine brain imaging to monitor the side effects such as brain swelling or bleeding. Aducanumab is administered intravenously via a 45- to 60-minute infusion every 4 weeks, and it cost approximately $56,000 per year for each person. Notably, the use and the cost of aducanumab are not practical for low- and middle-income countries, other local or endemic medicinal herbs that could be used to ameliorate the cognitive impairment should be alternative choices for them.

7. *Pueraria mirifica* herb and its potential use on cognitive impairment

Pueraria mirifica is a phytoestrogens containing plant that belongs to the family Leguminosae, subfamily Papilionoideae (Malaivijitnond, 2012). It has long been used as a rejuvenating agent in Thai folklore medicine including for the memory loss (Malaivijitnond, 2012; Suntara, 1931). *P. mirifica* is well-known for its estrogenic activity which has been widely tested in cells, laboratory animals and humans. *P. mirifica* contains at least 17 phytoestrogens which can be categorized into three main classes: isoflavonoids, comprised of daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puermiricarpene; coumestans comprised of coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol; and chromenes comprised of miroestrol, deoxymiroestrol and isomiroestrol (Malaivijitnond, 2012). Isoflavionoids have been reported in large amounts in *P. mirifica*, and puerarin is the major constituent and species-specific phytoestrogen of *Pueraria* plants (Cherdshewasart et al., 2007b; Urasopon et al., 2008a). Miroestrol, a species-specific phytoestrogen of *P. mirifica*, elicits high estrogenic activity comparable to that of E_2 (Malaivijitnond, 2012). *P. mirifica* extract (PME) could enhance the proliferation of primary baboon osteoblasts (Tiyasatkulkovit et al., 2014) and prevent bone loss in ovariectomy-induced estrogen deficient rats (Urasopon et al., 2008b). *P. mirifica* increased uterine weight in female rats and attenuated the gonadectomy-induced LH and FSH elevation in both female and male rats (Cherdshewasart et al., 2007a; Malaivijitnond et al., 2004) and post-menopausal elevation of LH level in cynomolgus monkeys (Jaroenporn et al., 2014; Kittivanichkul et al., 2016; Trisomboon et al., 2006a; Trisomboon et al., 2006b). *P. mirifica* disturbed an ovarian function and menstrual cycle in cyclic female

cynomolgus monkeys (Trisomboon et al., 2004). It also improved vaginal atrophy and alleviated cortical bone loss in naturally menopausal monkeys (Jaroenporn et al., 2014; Kittivanichkul et al., 2016; Trisomboon et al., 2006a; Trisomboon et al., 2006b). Based on its estrogenic activity, *P. mirifica* has been consumed in postmenopausal women in order to relieve the climacteric symptoms such as vasomotor symptoms (hot flushes and night sweats), reproductive symptoms (loss of libido, urogenital disorders), depression, and musculoskeletal pain (Kongkaew et al., 2018; Muangman and Cherdshewasart, 2001b).

Although the clinical research of using *P. mirifica* in men has been scarce, its effects have been evidenced in male animals. Oral administrations of *P. mirifica* at doses of 10, 100, 1000 mg/kg BW/day for 3 months could prevent bone loss dose-dependently in ODX rats (Urasopon et al., 2007). Dosing of PME at doses of 10, 100, 1000 mg/kg BW/day for 30 days dose-dependently alleviated of prostate enlargement in male rats (Masrudin and Mohamad, 2015). *P. mirifica* at a dose of 1000 mg/kg BW/ day treated in male rats for 14 days decreased LH levels and increased epididymis weight (Malaivijitnond et al., 2004). Treatment of *P. mirifica* at lower doses of 10-100 mg/kg BW/day for 8 weeks in adult male ICR mice and for 14 days in adult male rats had no adverse effect on reproductive organs, serum LH, FSH, and testosterone levels, and fertility (Jaroenporn et al., 2006). Thus, *P. mirifica* appears to be safe for male reproductive organs, hypothalamic-pituitary-testicular axis, and fertility at the lower doses. In addition, treatment of *P. mirifica* had no toxic effect on kidney and liver in male rodents (Malaivijitnond et al., 2004; Mohamad et al., 2019). Moreover, the investigation of acute toxicity of PME, up to 2000 mg/kg, in male rats according to OECD guidelines reported that PME was safe as no mortality was observed (Mohamad et al., 2019).

The neuroprotective and neurotherapeutic effects of *P. mirifica* and its constituent phytoestrogens were tested both *in vitro* and *in vivo*. *P. mirifica* mitigated the ovariectomy-induced cognitive impairment in rats (Anukulthanakorn et al., 2016). It also stimulated the synaptic function in hippocampal neurons (Chindewa et al., 2008). The neuroprotective and neurotherapeutic effects of puerarin such as ameliorating the learning and memory deficit in rodents (Hong et al., 2016; Li et al., 2010; Li et al., 2019; Liu et al., 2015), attenuating the toxic of amyloid-β (Li et al., 2010) and tau

hyperphosphorylation (Hong et al., 2016), and suppressing the overexpression of *Tau4*, *App*, and *Bace1*mRNA levels in hippocampus of female rats (Anukulthanakorn et al., 2016) had been extensively reported. Miroestrol could ameliorate cognitive deficits in ovariectomized mice via attenuation of oxidative stress and down-regulation of BDNF and CREB (Monthakantirat et al., 2014). In addition, genistein could protect the amyloid-β peptide-induced neuronal apoptosis in the hippocampus (Zeng et al., 2004), and the ovariectomy-induced neuronal apoptosis in rats (Huang and Zhang, 2010). The beneficial effects of genistein on preventing the learning and memory impairment against amyloid-β was also reported in rats (Bagheri et al., 2011).

CHAPTER III
ANDROGEN DEFICIENCY ACCELERATES AND WORSENS THE AGE-RELATED COGNITIVE IMPAIRMENT IN MALE RATS

Introduction

Cognitive impairment is an important age-related change in elderly. In particular, the episodic memories in association with spatial and temporal contexts are mostly deteriorated (Park et al., 2002; Small et al., 1999). The prevalence of cognitive impairment increases with function of age, especially in persons older than 65 years (Rajan et al., 2021). As the world is facing a shift in the distribution of population towards the older ages, the number of elderly has been estimated to increase more than double from 2020 and might be over 1.5 billion people in 2050 (United Nations, 2019). About 10% of those elderly are expected to suffer from cognitive impairment or up to 152 million people by 2050 (Alzheimer's Disease International, 2018). Although four licensed drugs, rivastigmine, galantamine, donepezil, and memantine, are available in the pharmaceutical markets for the cognitive impaired patients, those drugs cannot prevent or cure the symptom, they can only slow the process. Although advancing age is counted as the most common factor of cognitive impairment, it does not always occur in all elderly people.

Potentially, an androgen deficiency, a reduction of circulating testosterone below physiological levels, has been proposed as a key factor leading to cognitive impairment in men. Elderly men who have lower testosterone levels in the blood circulation (or reproductive senescence) show poorer cognitive performances than those men with higher testosterone levels (Barrett-Connor et al., 1999; Driscoll et al., 2005; Matousek and Sherwin, 2010b; Muller et al., 2005; Thilers et al., 2006; Yeap et al., 2008). Additional longitudinal analysis, followed up to 10 years, revealed a positive correlation between testosterone levels and cognitive functions in elderly men (Ford et al., 2018; Moffat et al., 2002). However, some other studies could not detect the association between testosterone levels and male cognitive impairment, for example, cross-sectional studies (Fonda et al., 2005; Martin et al., 2007; Martin et al., 2008; Yonker et al., 2006) and several shorter longitudinal (2-5 years) studies (Geerlings et al., 2006; Hogervorst et al., 2010; LeBlanc et al., 2010; Muller et al.,

2009; Yaffe et al., 2007). Whether the cognitive impairment in elderly men is a result of androgen deficiency is currently under investigation. It is noteworthy that androgen levels in men gradually decline, at approximately 1-2 % each year from middle age, without a practically noticeable landmark of reduction, this leads to the difficulty to seek for the clue of androgen deficiency on cogitation in aged men comparing to those seen in menopausal women (Camacho et al., 2013; Travison et al., 2017; Yeap et al., 2018). Besides the baseline physiological levels of testosterone varied between individuals.

Apart from those studies in humans, the effects of androgen deficiency on cognitive function have been extensively experimented in laboratory animals, mostly rodents. Male animals were induced an androgen deficient condition by castration and their cognitive function, i.e., performances on spatial reference version of learning and memory, was assessed by MWM task (Fang et al., 2017; Frye et al., 2010; Jia et al., 2013; Kang et al., 2014; Moghadami et al., 2016; Pintana et al., 2015; Pintana et al., 2016). However, the results were still inconsistent (Benice and Raber, 2009; Hodosy et al., 2012; Pintana et al., 2016; Sandstrom et al., 2006; Smith et al., 2020; Spritzer et al., 2008; Zhao et al., 2018). The discrepancy between studies might be because of the difference in age of animals recruited into the studies, the ages of animals when they were initiated the castration, and duration of time that the animals were held in androgen deficient condition. For example, Frey et al. (2010) reported that male rats, which were orchidectomized (ODX) at the age of 4 months old and kept for the androgen deficient condition for 2 weeks, performed a poorer spatial function than age-matched intact rats. This behavioral deficit was also presence in rats which were ODX at the age of 2 months old and kept longer after orchidectomy for 2 and 3 months (Pintana et al., 2016). However, if the animals were recruited at the older age, for example at 13 and 24 months old and kept under androgen deficient condition for 2 weeks (Frey et al., 2010), or kept in a shorter time of androgen deficient condition, for example 2 months of age and kept for only a month after castration (Pintana et al., 2016), the behavioral deficit was not detected. Generally, the deficit of spatial learning and memory was emerged in male testes-intact rodents as early as they entered into the middle age, ranging from 12-14 months old (Bizon et al., 2009; Francia et al., 2006; Frye et al., 2010; Guidi et al., 2014; Smith et al., 2020).

To be able to observe the significant changes, however, the comparison of data between those animals before and after they enter the middle age should be done

While the association between androgen deficiency and cognitive impairment in aging males remains inconclusive, the functional significances of androgens (testosterone and DHT) on the brain, particularly hippocampus, a region known to play an important role in learning and memory, have been reported (Atwi et al., 2016; Murakami et al., 2018). For example, androgens have been involved to modulate the synaptic transmission between neurons in hippocampal circuits of male rats (Di Mauro et al., 2015; Pettorossi et al., 2013; Skucas et al., 2013). The application of androgens on hippocampal slices could enhance the synaptic structure by increasing and strengthening the synaptic connecting sites (Hatanaka et al., 2015; Hatanaka et al., 2009; Soma et al., 2018). Administration of DHT to male SAMP8 mice could rescue a decrease in numbers of neuron, synaptic spines and expression of synaptophysin induced by orchidectomy (Kang et al., 2014; Li et al., 2013a). Testosterone treatment can prevent or ameliorate the hyperphosphorylation of tau protein in ODX rats (Papasozomenos, 1997) and 3xTg-AD mice (Rosario et al., 2010). Testosterone and DHT reversed orchidectomy-increased amyloid-β levels in the brain of male rats (Ramsden et al., 2003), and the accumulation of amyloid plaques in hippocampus of 3xTg-AD male mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). Taken together, an underlying mechanism of the cognitive impairment in association with age and androgen levels is dubious and more information is still needed. The final goal of the research is to seek for the interventions that revert or at least prevent the development of cognitive impairment for the era of the dawn of an aging society.

Thus, the present study aimed to investigate ‘when’ and ‘how’ the androgen deficiency induced the cognitive impairment in males. Male Sprague-Dawley (SD) rats at the age of young adults (4 months old) were recruited for this study. They were induced an androgen deficient condition by orchidectomy and kept for 8 months when they reached the middle age (12 months old). Their cognitive abilities were assessed through the spatial learning behavior and memory capacity by using MWM task. To ease the effects of chronological aging which might confound the effect of androgen deficiency, a series of age-matched intact rats has been included in this study. Aligned

with an assessment of the cognitive function, a completed set of the transcriptional and translational expression levels of three neurological hallmarks of neurodegeneration, including loss of synaptic plasticity, neurofibrillary tangles and amyloid plaque in the hippocampus were determined.

Materials and methods

Animal subjects and ethic note

All animal experimental protocols followed the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes issued by the National Research Council of Thailand and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Sciences, Chulalongkorn University (Protocol review number: 1573004). Male Sprague-Dawley rats, at the age of 2 months old, were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were housed in an individually ventilated cage system (22 ± 1 ˚C) with a 12: 12 h light-dark cycle (lights on at 0600 am) at Chulalongkorn University Laboratory Animal Center (CULAC), Thailand. They were provided with standard rat diet (Teklad Global Diets®: ENVIGO Harlans Laboratories, USA) and sterile water *ad libitum*. Basically, male rats reach sexual maturity from 1 month of age whereas the brain is fully developed at approximately 4 months of age (Bandeira et al., 2009; Fu et al., 2013; Mengler et al., 2014; Mortera and Herculano-Houzel, 2012). Therefore, the rats were reared until they were 4 months old and then used in this study

Experimental design

Rats were randomly divided into two main groups: sham (SH) and orchidectomy (ODX). ODX rats were given a surgical castration by removing the bilateral testes to induce an androgen deficient condition, while SH rats were given the same castration surgery except that their testes were kept intact. In each SH and ODX group, rats were subdivided further into five groups (M0, M2, M4, M6, and M8; n = 18-19 rats/group).

Rats were assessed for their learning behavior and memory capacity using the MWM test for six consecutive days from 3 days, and 2, 4, 6, and 8 months after surgery for M0, M2, M4, M6, and M8 groups, respectively. After the learning behavior and memory capacity test, rats were euthanized by decapitation under deep anesthesia with carbon dioxide at a flow rate of 3 L/min for 60 s. The trunk blood was

collected for serum testosterone determination. The whole brain from each rat was rapidly collected from the skull, immediately frozen with dry ice, and then kept at -80 °C until use. Eight to nine specimens from each experimental group were randomly selected for analyzing the transcriptional expression levels of genes associated with synaptic plasticity (function; *Syn*, *GluN1*, *α7-nAChR, M_1-mAChR* and structure; *Bdnf*) neurofibrillary tangles (*Tau3* and *Tau4*), and amyloid plaques (*App*, *Adam10* and Bace1) using two-step quantitative real-time reverse transcription (qrt-RT)-PCR, and the translational expression levels of genes associated with neurofibrillary tangles (total tau and phosphorylated tau Ser^{396}) using western blotting. Five specimens from the M6 and M8 groups of SH and ODX rats were collected for immunoreactivity levels of synaptic function and structure by immunohistochemistry (IHC).

Serum testosterone levels in androgen deficient and aged male rats

The trunk blood of SH and ODX rats was collected in a chilled tube at 4 °C, centrifuged (600×g, 4 °C, for 20 min), and the serum was harvested and kept frozen at -20 °C until use. Serum testosterone level was measured using a Testosterone ELISA Kit (Cat no. ab-108666, lot no. 20160402, Abcam, MA, USA) following the manufacturer's instruction. Inter- and intra-assay coefficients of variation were 6.52% and 3.57%, respectively. The limit of detection (LOD) of the assay was 0.2 ng/ml.

Assessment of learning behavior and memory capacity performance in androgen deficient and aged male rats using MWM test

The MWM, the gold standard assessment for evaluating hippocampal learning and memory in rodents, was used to detect cognition deficits in SH and ODX rats. The test was conducted in a circular pool making from composites resin (180 cm in diameter and 50 cm in height) filled with water (22-25°C). The pool was divided geographically into four quadrants (NE, NW, SE and SW), and a transparent circular platform making from acrylic (10 cm in diameter) was hidden submerged 1 cm below the surface of the water at the center of the target quadrant (NW). Four distinctive stable external cues were place around the pool. The ambient light was set at 50 lux on the water surface. The video camera was mounted above the center of maze and linked to a computer to record the swimming path data using a video tracking system (Smart JUNIOR®, Panlab-Harvard Apparatus, Barcelona, Spain). The learning

behavior test was performed consecutively on day-1 to day-5, and the memory capacity test was performed on day-6 as described previously (Vorhees and Williams, 2006). Each day of the learning behavior test consisted of four trials (90 s each) with a 30 min interval between trials. If any rat failed to locate the platform within 90 s, it was gently guided to the platform and rested for 30 s, and a time of 90 s was recorded. A video tracking system was used to record (i) the latency to find the hidden platform (or escape latency), (ii) the distance to arrive at the hidden platform (or travel distance), and (iii) swimming patterns (Line, Taxis, Random, and Circle) based on the report of Anukulthanakorn et al. (2013). After the rats completed the learning behavior test on day-5, they were continuously assessed the memory capacity test using the spatial probe test on day-6. For the spatial probe test, the platform was removed from the pool to check the memory of the animal for the platform location. The rat was released to the pool at the quadrant opposite to the previously existing platform location and allowed to swim for 30 s. During those 30 s, the number of crossings to the previously existing platform location, and the time and distance spent in the targeted quadrant, where the platform used to be located, were recorded using the video tracking system.

Determination of transcriptional levels of genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques using two-step qrt-RT-PCR

Left hippocampus was dissected, and total RNA was extracted using TRIzol® Reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The total mRNA concentration and purity was determined by spectrometry based on optical density, and a 260/280 ratio of 2.0 ± 0.1 was accepted for mRNA purity. The transcriptional expression levels of the genes associated with: (i) synaptic function and structure, including *Syn*, *GluN1*, *α7-nAChR*, *M_1-mAChR*, and *Bdnf*; (ii) neurofibrillary tangles (*Tau3* and *Tau4*); and (iii) amyloid plaques, including *App*, *Bace1*, and *Adam10* were examined using two-step qrt-RT-PCR.

In the first step RT-PCR, the extracted RNA (5 μg) was reverse transcribed to cDNA in a total volume of 20 μl, containing 5 μl of RNA, 4 μl of RT buffer, 1 μl of dNTP mix (10 mM), 1 μl of Oligo $(dT)_{18}$ Primer Mix, 1 μl of Random Hexamer Primer Mix, 1 μl of RNase Inhibitor (10 U/μl), 1 μl of Reverse Transcriptase (200

U/μl), and 6 μl of diethylpyrocarbonate (DEPC)-treated water using the Tetro cDNA Synthesis kit (Bioline Reagent Ltd., London, UK). The samples were incubated for 10 min at 25 °C, 30 min at 45 °C, and finally 5 min at 4 °C. The obtained cDNA was then diluted in five volumes of DEPC-treated water prior to use in the second step qrtPCR.

The second-step qrtPCR utilized the gene fragment specific primer pairs shown in Table 3.1, where the 28S rRNA gene was used as a reference house-keeping gene in this study. The qrt-PCR was performed using a SensiFAST^{TM} SYBR$^{®}$ kit (Bioline Reagent Ltd., UK) according to the manufacturer's instructions. Each qrt-PCR reaction was performed in a final volume of 20 μl with 10 μl of SensiFast SYBR$^{®}$ Hi-ROX mix, 0.8 μl each of forward and reverse primer (10 μM), 5 μl of cDNA, and 3.4 μl of DEPC-treated water. The thermal cycling consisted of 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 10 s, and then the melting curve analysis (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s before finally cooling at 25 °C for 5 min). Real-time detection of SYBR Green fluorescence intensity, indicating the amount of PCR product formed, was measure at the end of each extension phase. Amplification products were quantified using the StepOnePlus^{TM} qrt-PCR system. To confirm the amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. The amount of the PCR product of each target gene was normalized against the housekeeping 28S ribosomal RNA (28S RNA) gene in the corresponding samples and analyzed by the $2^{-\Delta\Delta CT}$ method.

Table 3.1 Primers used in the second stage qrt-PCR analyses

Target gene	Accession No.	Forward/reverse primer (5'-3')	Product size (bp)
α7-nAChR	NM_012832	TCATGCCAGCAACATCTGATTC AGAGAGGCCCACGATGATCAT	77
Adam10	XM_001054737	GGCCCTCTAGCTAGGCTGAAA ATCAGGGCAATTCCCATAAGC	113
App	X07648	CACACCCACATCGTGATTCCT GTCCATCCGCTCCTGGTGTA	105
Bace1	AF190727	TTGCCATGTGCACGATGAG GCCGTGACAAACGGACCTT	57
Bdnf	NM_001270630	CAAGGCAACTTGGCCTACCC GAGCATCACCCGGGAAGTGT	210
GluN1	NM_001270602	GCAGCCTTTTCAGAGCACACT ATCTTCCTCCTCCTCCTCACTGT	133
M_1-mAChR	NM_080773	TACCACGTACCTGCTCATGG GCTAGGCCAATCATCAGAGC	201
S28RNA	V01270	GGCCGAAACGATCTCAACCT GCCACCGTCCTGCTGTCTAT	217
Syn	NM_012664	TCTTCCTGCAGAACAAGTACC CCTTGCATGTGTTCCCTGTCTG	198
Tau3	ENSRNOT00000045127	GAGGCGGCAAGGTGCAAATAGTCT CCACCTCCTGGCTTGTGATGGAT	100
Tau4	ENSRNOT00000042984	GATCTTAGCAACGTCCAGTCCAA TCCCTAAGGAACCACACTTGGAG	133

Determination of translational levels of genes associated with neurofibrillary tangles by western blotting

The right hemisphere of the hippocampus was isolated and homogenized on ice using RIPA buffer (ThermoScientific, MA, USA) mixed with Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoScientific, MA, USA). The homogenate was centrifuged (10,000×g, 4 °C, for 15 min), and the supernatant was collected. The supernatant protein concentration was determined by the Bradford assay (Sigma-Aldrich, MO, USA). The immunoreactivity of total tau and phosphorylated tau at the Ser^{396} site was quantified by western blotting. Electrophoresis of lysate protein (30 µg) was performed on one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (w/v) acrylamide resolving gel.

Separated proteins were transferred onto PVDF membranes and nonspecific binding sites were blocked by incubation with 5% (w/v) bovine serum albumin (BSA fraction V, Sigma-Aldrich, MO, USA) dissolved in phosphate-buffered saline (PBS)

pH 7.4 at room temperature for 1 h. The blots were then probed overnight at 4 °C with the respective primary antibody (dilution 1:1000): mouse monoclonal antibody Tau-5 (#ab80579, Abcam, MA, USA) , rabbit monoclonal Tau-phospho Ser^{396} (#ab109390, Abcam, MA, USA) , and rabbit monoclonal β-actin (#13E5, Cell Signaling Technology, MA, USA). After washing three times with PBS containing 0.1% (v/v) Tween-20 (Sigma-Aldrich, MO, USA), the blots were incubated at room temperature for 2 h in the relevant specific secondary antibody (dilution 1:2,500): anti-mouse IgG HRP-conjugated antibody or goat anti-rabbit IgG HRP-linked antibody (#7076 for Tau-5, and #7074 for tau-phospho Ser^{396} and β-actin; Cell Signaling Technology, MA, USA).The resultant immunoreactivity was visualized by chemiluminescence reactions using the Clarity™ Western ECL Substrate Kit (#170-5060 Bio-Rad, CA, USA) and exposure to the GelDoc-It® Imaging System together with VisionWork® software (UVP Bioimaging, Cambridge, UK). The intensity of each band was determined using an image-analyzing system (ImageStudio software, NE, USA).

Immunoreactivity levels of proteins reflecting the synaptic plasticity

Five rats from each of the M6 and M8 groups of the SH and ODX groups were randomly selected, deeply anesthetized with sodium pentobarbital (150 mg/ kg bodyweight, i.p.), and sequentially perfused through the left ventricle with 0.1 M PBS and 4% (w/v) paraformaldehyde in 0.1 M PBS pH 7.4. The brains were removed from the skulls, post-fixed in the same fixative overnight, and cryoprotected in 30% (w/v) sucrose in 0.1 M PBS at 4 °C until it sunk. The brains were then cut on a freezing microtome at 50-μm thickness in the coronal plan. The sections were kept in 0.05 M tris-buffered saline (TBS) pH 7.6. The immunoreactivity of SYN and PSD-95 were quantified by IHC.

To detect the immunoreactivity of SYN and PSD-95, the rabbit monoclonal antibody to SYN (#ab32127, Abcam, MA, USA) and goat polyclonal antibody to PSD-95 (#ab12093, Abcam, MA, USA), of which each antibody recognized a single band by western blot, were used. A serial dilution of each antibody was prepared, and a linear function of antibody concentration against immunoreactivity was obtained. The dilution that produced slightly less than half-maximal labeling was chosen, in

order to optimize the detection of intensity variations.

To provide an identical IHC labeling condition, sections from each individual brain were processed in the same batch. Briefly, sections were rinsed in 0.05 M TBS with 0.5% (v/v) Triton-X-100 (TBST) to remove the cryoprotectant, incubated in a solution containing 0.3% hydrogen peroxide (H_2O_2) to deactivate endogenous peroxidases, and washed three times (5 min each) with 0.05 M TBS. Then, sections were incubated sequentially in (i) 5% BSA in 0.05 M TBS to block nonspecific antibody binding for 24 h at 4 °C, (ii) primary antiserum in 5% (w/v) BSA in TBST (rabbit monoclonal antibody to SYN, 1:8000; goat polyclonal antibody to PSD-95, 1:1000) for 48 h at 4 °C, (iii) biotinylated goat anti-rabbit IgG (for SYN) or biotinylated rabbit anti-goat IgG (for PSD-95; both from Vector Laboratories, CA, USA) in TBST for 60 min, (iv) peroxidase-avidin complex solution (Vector Laboratories, CA, USA) for 60 min; and (v) 0.04% 3,3-diaminobenzidine (DAB; Sigma-Aldrich, MO, USA) and 0.03% H_2O_2 in TBS for 8 min. All incubations were separated by washes in TBS except for after 5% (w/v) BSA. Finally, all sections were mounted onto gelatin-coated glass slides, dried overnight, dehydrated in a series of graded ethanol, cleared in xylene, and cover-slipped with Mount-Quick Mounting Media (Daido Sangyo Co., Ltd, Japan).

Quantitative light microscopic localization of immunoreactivity for SYN and PSD-95 was performed as previously described (Waters et al., 2009). The sections from Bregma -3.90 to -4.20 of each animal that had no ice-crystal in the tissues determined under the light microscope were included in the analysis. Images of regions-of-interest were captured using an all-in-one fluorescence microscope BZ-X710 (KEYENCE, Osaka, Japan) and BZ-X Viewer software (KEYENCE, Osaka, Japan). The consistency of immunoreactivity staining was indicated by the analysis of intensity in the corpus callosum, which was presumed to only contain non-specific staining (Waters et al., 2009), and this revealed a coefficient of variation of 0.16. The optical density in the hippocampal CA1 and CA3 region were determined using the ImageJ software (ImageJ, MD, USA). To compensate for background labeling and control for variations in the overall illumination levels between images, the average optical density for three regions in the corpus callosum was determined and subtracted from all density measurements made on particular regions-of-interest.

Statistical analysis

The effects of (i) an advancing age with a gradual decline in serum testosterone levels in SH rats by comparing the SH-M2, -M4, -M6, and -M8 groups with the SH-M0 group, (ii) an ODX-induced abrupt decline in serum testosterone levels in the ODX rats by comparing the ODX-M0, -M2, -M4, -M6, and -M8 rats with the corresponding age-matched SH rats, and (iii) a combination of an ODX-induced abrupt decline in serum testosterone levels and an advancing in age by comparing the ODX-M2, -M4, -M6, and -M8 rats with the ODX-M0 rats, on the spatial learning behavior and memory capacity, and the biological changes in hippocampus were statistically analyzed. The results are expressed as mean ± standard error of mean (SEM).

Residuals were examined for assumption of normality and heteroscedasticity. For the learning behavioral trials, the significant differences of escape latency, and travel distance for searching the hidden platform were determined using two-way analysis of variance (ANOVA) with repeated measures and a pair-wise comparison between groups was analyzed by using Bonferroni test. The significant differences of swimming patterns between groups were determined by the chi-square test. The one-way ANOVA was used to determine the differences in the mean serum testosterone levels, mRNA and protein expression levels, and the memory capacity test within SH rats or ODX rats. If a significant ANOVA was observed, a pair-wise comparison was performed using the least significant difference (LSD) test. An independent student's t-test was used to determine the difference in the mean mRNA and protein expression levels, and the memory capacity between the ODX group and the age-matched SH group. The level of significance was set as $p < 0.05$ for all statistical tests. Statistical analyses were performed using the SPSS software program version 22 (International Business Machines Corp., NY, USA).

Results

Serum testosterone levels in androgen deficient and aged male rats

Serum testosterone level in the 4-month-old SH rats (SH-M0 group) was 2.375 ± 0.607 ng/ml, and gradually and continuously decreased with advancing age ($F = 2.550$, $p < 0.05$; Table 3.2). The significant reduction was detected (0.660 ± 0.195 ng/ml; $p < 0.05$) when the rats were 12 months old (SH-M8), which was approximately 72% lower than that of the 4-month-old SH rats.

On the other hand, serum testosterone levels in all five groups of ODX rats (ODX-M0, -M2, -M4, -M6, and -M8) were below the LOD (< 0.2 ng/µl). This confirmed their androgen deficient condition (Table 3.2). Noting that, in the ODX-M0 group, serum testosterone levels were measured in rats after 9 days of orchidectomy, because they were kept for recovery from bilateral testes castration for 3 days and the MWM test were conducted for 6 days.

Table 3.2 Testosterone levels of sham (SH) and orchidectomized (ODX) rats

Group	Age (months)	Testosterone levels (ng/ml)	
		SH	ODX
M0	4	2.375±0.607	< 0.2
M2	6	2.050±0.625	< 0.2
M4	8	1.282±0.306	< 0.2
M6	10	1.043±0.310	< 0.2
M8	12	0.660±0.195*	< 0.2

Means ± SEM (n=18-19) *represent $p < 0.05$ compared with M0 group

Effect of increasing in age and androgen deficiency on learning and memory performance in male rats

All five groups (4, 6, 8, 10 and 12 months old) of intact (SH) rats showed progressive decreases in escape latency ($F = 139.844$, $p < 0.001$) and travel distance ($F = 251.394$, $p < 0.001$) over the five consecutive days of the spatial learning trials (hidden platform) in the MWM task, indicating that the rats learned the location of the platform (Figure 3.1A and B). The repeated measures ANOVA across the five days of learning trials revealed the main effect of age on the escape latency ($F = 16.886$, $p < 0.001$) and travel distance ($F = 10.509$, $p < 0.001$) (Figure 3.1A and B). Post-hoc analysis showed that the 8-, 10-, and 12-month-old SH rats (SH-M4, -M6, and -M8 groups, respectively) spent longer time in the escape latency and longer travel distance than those of the 4-month-old SH rats (M0 group). Increasing in age also significantly affected the proportion of strategies for searching the hidden platform on day-5 among SH groups ($\chi^2 = 86.724$, $p < 0.001$). The strategies performed by the 4- and 6-month-old SH rats (SH-M0 and -M2 groups) were only line and taxis, whereas those performed by the 8-, 10-, and 12-month-old SH rats (SH-M4, -M6, and- M8 groups) included a random search pattern (Figure 3.1C).

For the memory capacity tested on day-6 (or probe trial), advancing age significantly reduced the crossing numbers ($F = 9.636$, $p < 0.001$; Figure 3.2A), time in the target area ($F = 16.991$, $p < 0.001$; Figure 3.2B), and distance in target area ($F = 12.406$, $p < 0.001$; Figure 3.2C) in the 8-, 10-, and 12-month-old SH rats (SH-M4, -M6, and -M8 groups, respectively) compared to the the 4-month-old SH rats (M0 group). These results indicated that the SH male rats showed an age-associated deficit in spatial learning and memory initiating from 8 months old onwards.

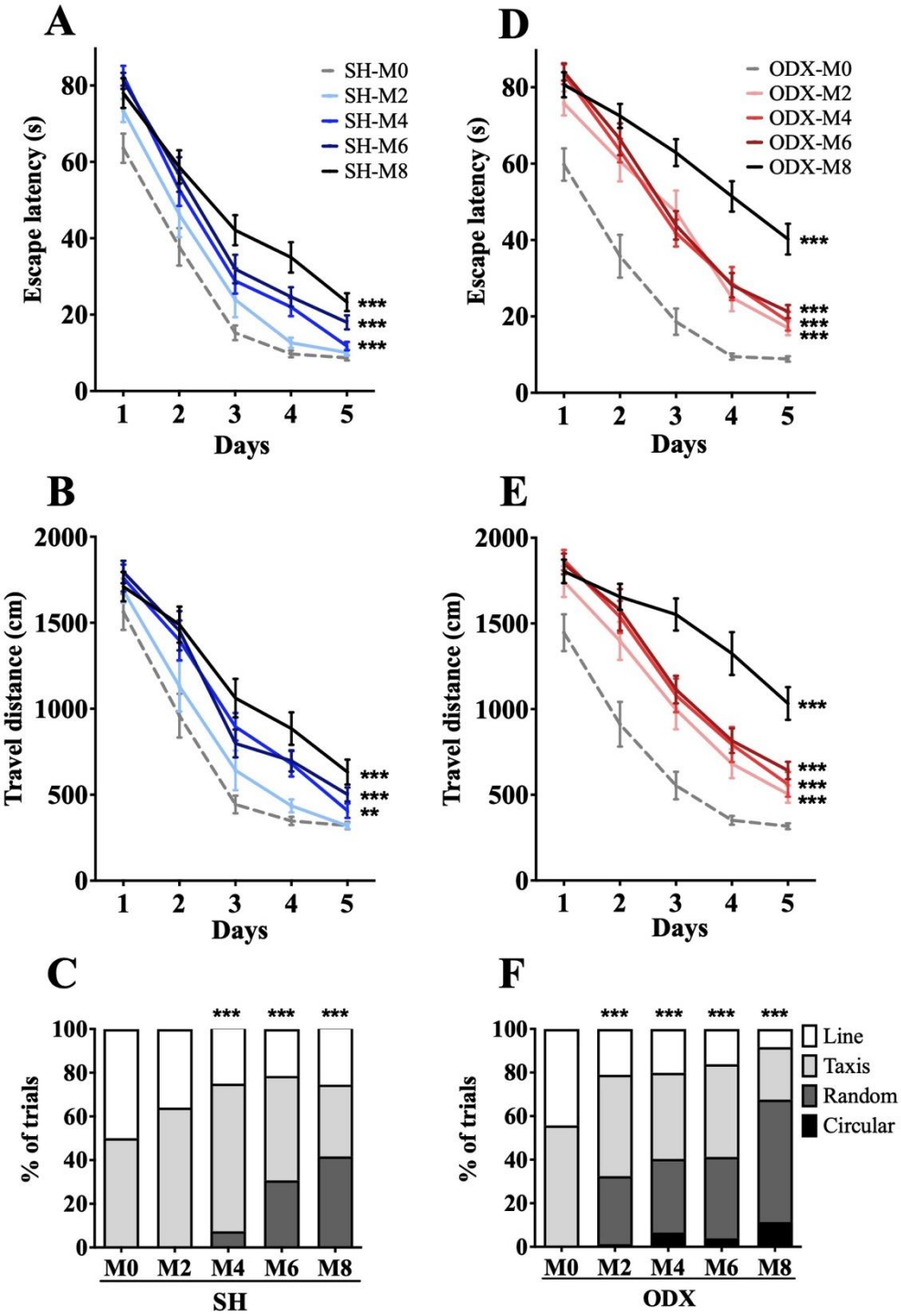


Figure 3.1 The learning behavior assessed on day-1 to -5 of Morris Water Maze task (hidden platform) in sham (SH) and orchidectomized (ODX) rats after testicular surgery for 3 days (M0), 2 (M2), 4 (M4), 6 (M6), and 8 (M8) months.The escape latency and travel distance of the SH and ODX rats are shown in A-B and D-E, respectively. The data are shown in means ± SEM (n = 18-19). The proportion of swimming strategies used on day-5 of the SH and ODX rats are shown in C and F. ** and *** represent $p < 0.01$ and 0.001 compared with the M0 group.

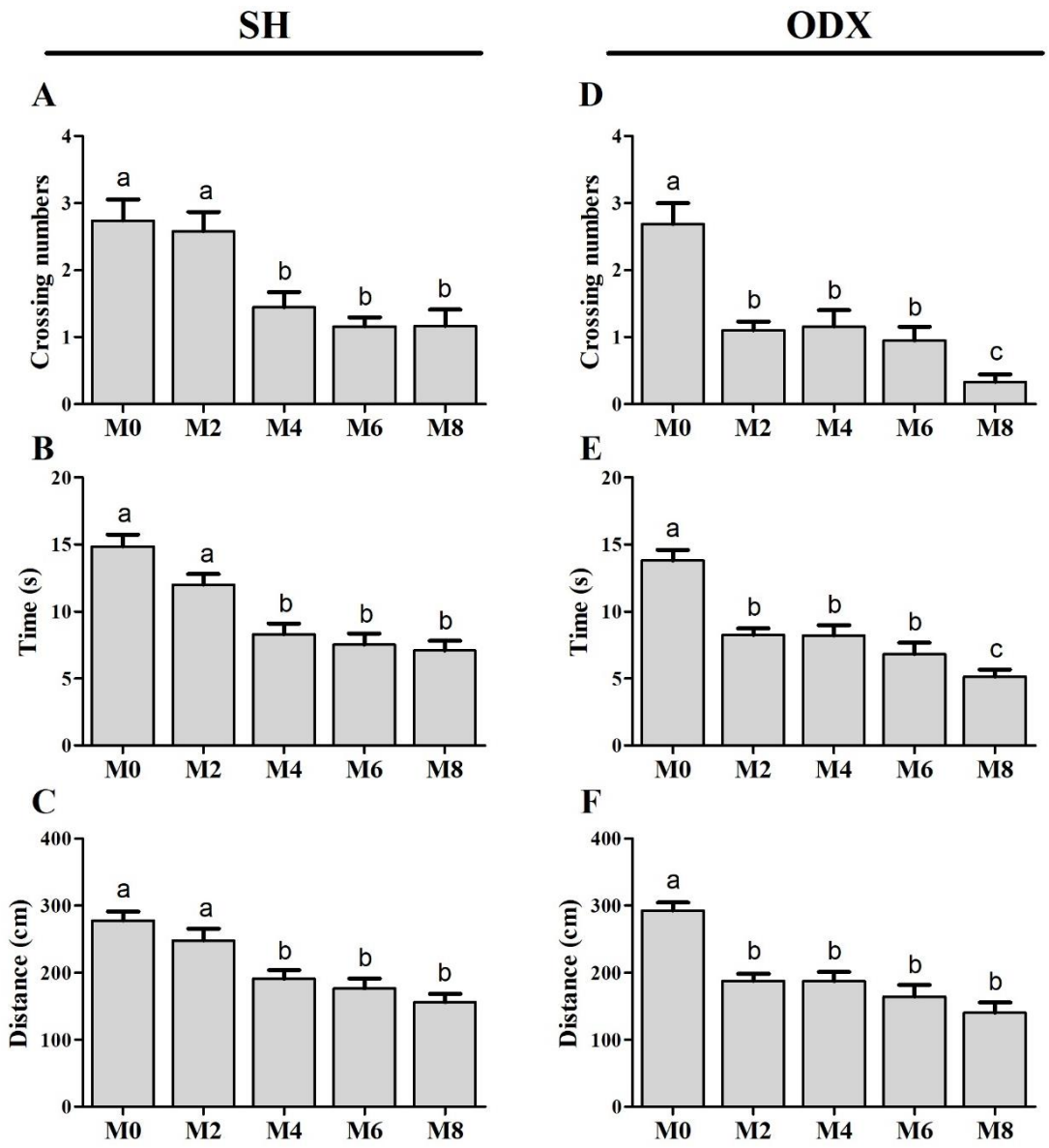


Figure 3.2 The memory capacity assessed on day-6 of Morris Water Maze task (probe trial) in sham (SH) and orchidectomized (ODX) rats after surgery for 3 days (M0), 2 (M2), 4 (M4), 6 (M6), and 8 (M8) months.The number of crossing the location where the platform was previously existed, time and distance spent in targeted quadrant of SH (A-C) and ODX (D-F) rats are shown. Vertical bars represent means ± SEM (n = 18-19). A different letter indicates significant differences between groups of SH or ODX rats ($p < 0.05$).

All five groups (4, 6, 8, 10 and 12 months old) of ODX rats showed progressive decreases in escape latency ($F = 267.312$, $p < 0.001$) and travel distance ($F = 180.812$, $p < 0.001$) over the five consecutive days of the hidden platform trials, indicating that the ODX rats could learn the location of the platform. Post-hoc analysis between each day of learning trials showed that the escape latency and travel distance of day-1 and day-2 were not significanly different in ODX-M2, -M4, -M6 amd -M8 rats indicating that the rate of learning process in ODX-M2, -M4, -M6 and -M8 were markedly slower. Considering the combination of the effects of androgen deficiency and advancing age in the ODX rats, the repeated measures ANOVA across the five days of learning trials revealed the main effect of increasing age in ODX rats on the escape latencies ($F = 31.919$, $p < 0.001$) and travel distances ($F = 24.345$, $p < 0.001$). Post-hoc analysis showed that the 6-, 8-, 10-, and 12-month-old ODX rats (ODX-M2, -M4, -M6, and -M8 groups) spent significantly longer time in the escape latency and longer travel distance than those of the 4-month-old ODX rats (ODX-M0 group). Additionally, these parameters performed by the 12-month-old ODX rats (ODX-M8 group) were significantly longer than those of the 6-, 8- and 10-month-old ODX rats (ODX-M2, -M4, and -M6 groups) (Figure 3.1D and E). Also, the proportion of strategies for searching for a hidden platform was significantly affected ($\chi^2 = 83.073$, $p < 0.001$) by androgen deficiency and increasing age. Together with a random pattern, a circular pattern was used and more frequent in the older rats (ODX-M8> M6 = M4 > M2) (Figure 3.1F).

The effects of androgen deficiency and advancing age also significantly affected the performances in the probe trial (the crossing number, $F = 15.840$, $p < 0.001$; time spent in target area, $F = 20.681$, $p < 0.001$; and distance spent in target quadrant, $F = 17.534$, $p < 0.001$). These three parameters in the 6-, 8-, 10-, and 12-month-old ODX rats (ODX-2, -M4, -M6, and -M8 groups, respectively) were significantly lower than those of the 4-month-old ODX rats (ODX-M0 group) (Figure 3.2). These results indicated that androgen deficiency accelerated the age-associated cognitive impairment observed in the SH rats. That is the cognitive impairment was initiated at 2 months after orchidectomy (ODX-M2 group) when the rats were at a young age of 6 months old, and it could severely exacerbate the cognitive impairment at 8 months

after orchidectomy (ODX-M8 group) when animals entered middle age of 12 months old.

To analyze the effect of orchidectomy-induced androgen deficiency, not associated with increasing in age, on cognitive impairment, comparison between the ODX rats and the age-matched SH rats was performed. The repeated measure ANOVA over five consecutive day of learning trials revealed that escape latencies between the ODX-M2 and SH-M2 groups ($F = 9.993$, $p < 0.01$) and between the ODX-M8 and SH-M8 groups ($F = 24.563$, $p < 0.001$) as well as the travel distances ($F = 6.817$, $p < 0.05$ and $F = 12.845$, $p < 0.01$, respectively) showed significant differences: those of the ODX rats were significantly longer than those of the age-matched SH rats (Figure 3.3). There were no significant differences between the ODX and SH rats at 0, 4, and 6 months (M0, M4, and M6 groups) after orchidectomy (Figure 3.3). While the ODX-M0 group used only line and taxis patterns, which were not significantly different from that of the SH-M0 group ($\chi^2 = 0.864$, $p = 0.353$), the ODX-M2, -M4, -M6, and -M8 rats used random and circular searching more often than those of the age-matched SH rats ($p < 0.01$; Figure 3.1F). In agreement with the learning behavior, the performance in the probe trial of the ODX-M2 and ODX-M8 rats, including crossing number, time, and distance in target quadrant (except the distance in targeted area for the ODX-M8 rats) were significantly lower than those of the age-matched SH rats (Figure 3.4).

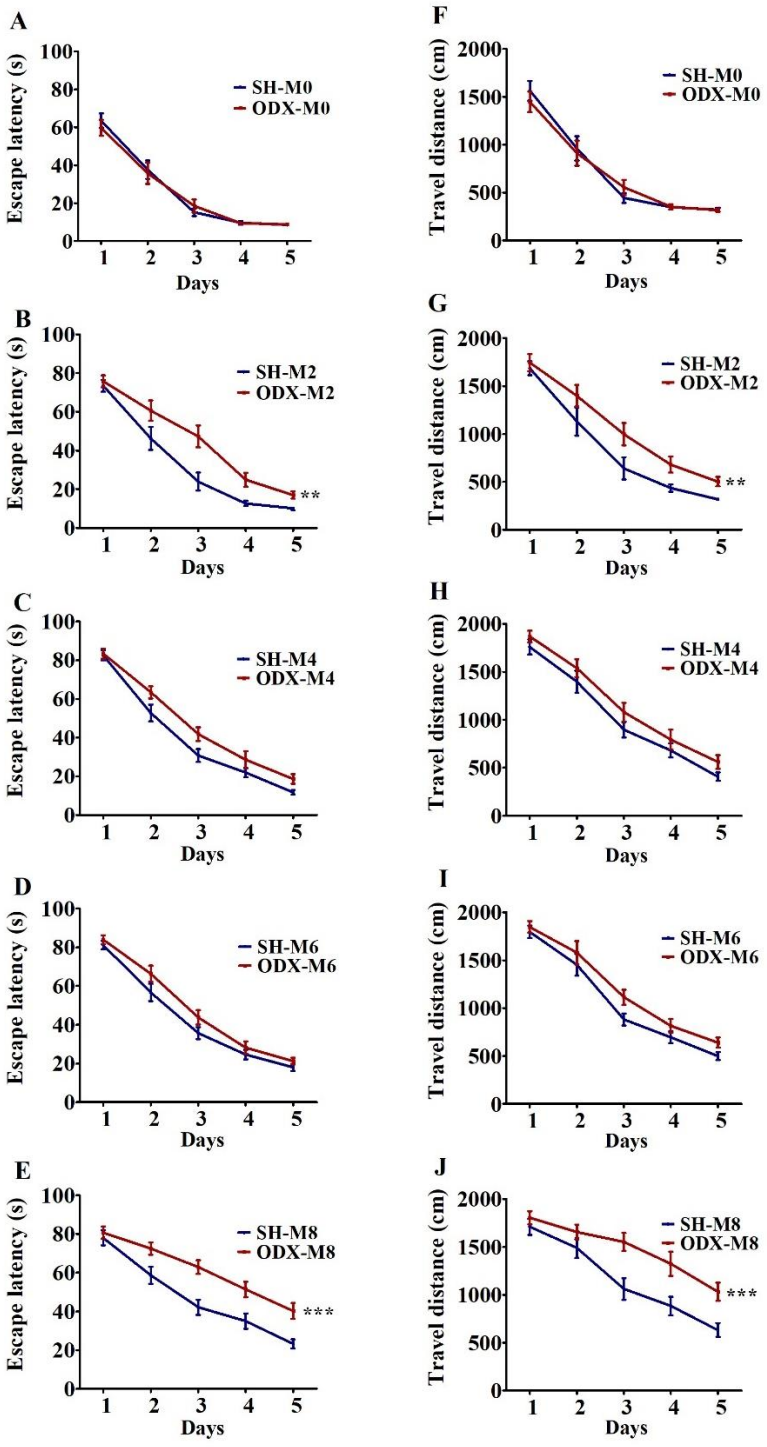


Figure 3.3 The learning behavior assessed on day-1 to -5 of Morris Water Maze task (hidden platform), the escape latency (A-E) and travel distance (F-J) of the ODX-M0, -M2, -M4, -M6 and -M8 rats compared with the SH-M0, -M2, -M4, -M6 and -M8 rats.The data are shown in means ± SEM (n = 18-19). ** and *** represent $p < 0.01$ and 0.001 compared with the age-matched SH rats.

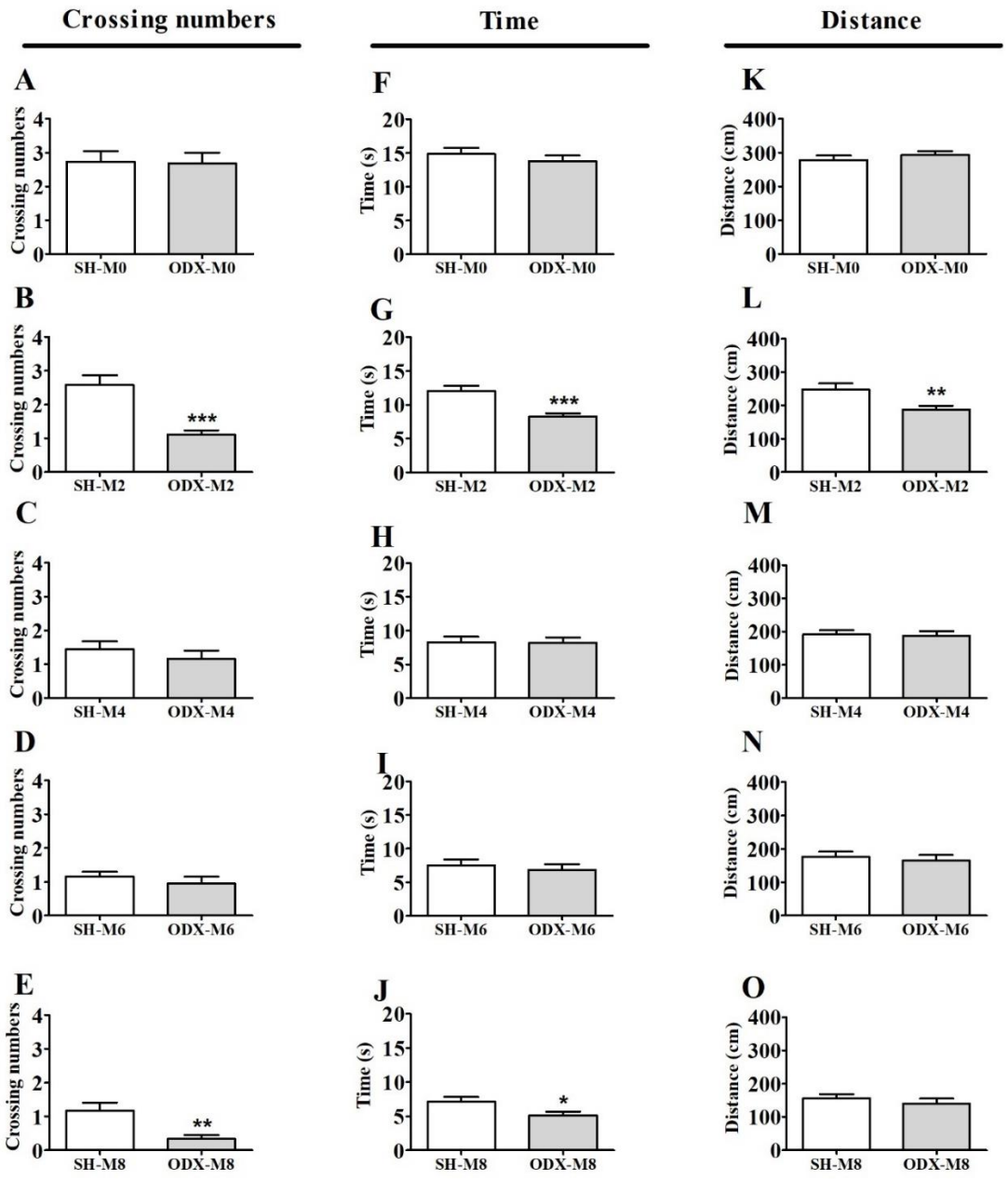


Figure 3.4 The memory capacity assessed on day-6 of Morris Water Maze task (probe trial) in orchidectomized (ODX) rats compared with the age-matched SH rats after operation for 9 days (M0), 2 (M2), 4 (M4), 6 (M6) and 8 (M8) months.The numbers of crossing the previously existing platform (A-E), time (F-J) and distance (K-O) spent in targeted quadrant are shown. Vertical bars represent means ± SEM (n = 18-19). *, ** and *** represent $p < 0.05$, 0.01 and 0.001 compared with age-matched SH rats.

Transcriptional levels of genes associated with synaptic plasticity

In the hippocampus of the intact SH rats, an increasing age significantly affected the transcript levels of *Bdnf* ($F = 3.008$, $p < 0.05$) and *M_1-mAChR* ($F = 0.016$, $p < 0.05$). Post-hoc analysis showed that *Bdnf* level was significantly decreased, while *M_1-mAChR* level was significantly increased in the 12-month-old, SH-M8 rats compared with the 4-month-old, SH-M0 rats (Figure 3.5A and E). However, no significant alterations were detected in the transcript levels of α*7-nAChR*, *GluN1*, and *Syn* from 4- to 12-month-old (SH-M0 to SH-M8) rats (Figure 3.5B-D).

Considering the combined effects of androgen deficiency and advancing age in the ODX rats, the *Bdnf*, α*7-nAChR*, and *GluN1* transcript levels were significantly altered ($F = 7.731$, $p < 0.001$; $F = 6.036$, $p < 0.05$; and $F = 5.427$, $p < 0.05$, respectively). Post-hoc analysis showed that the *Bdnf* and *GluN1* transcript levels were significantly increased in the 10-month-old, ODX-M6 rats (Figure 3.5A and C), while the α*7-nAChR* mRNA level was significantly decreased in the 12-month-old, ODX-M8 rats in comparison with the 4-month-old, 9-day-post-orchidectomy, ODX-M0 rats (Figure 3.5B).

To analyze the specific effect of orchidectomy-induced androgen deficiency on synaptic plasticity, comparisons of mRNA levels between the ODX rats and the age-matched SH rats was performed. The *Bdnf*, *GluN1*, and *Syn* mRNA levels in the ODX rats remained constantly lower than in the age-matched SH rats (Figure 3.5A, C and D), while the α*7-nAChR* and *M_1-mAChR* mRNA levels in the ODX rats were significantly higher than those in the age-matched SH rats (Figure 3.5B and E). The significant differences between the SH and ODX groups were detected from 9 days (M0 group) up to 8 months (M8 group) after orchidectomy, except for *Bdnf* levels in the M4 and M6 group, *GluN1* level in the M6 group, and α*7-nAChR* and *M_1-mAChR* levels in the M8 group (Fig 3.5A-E).

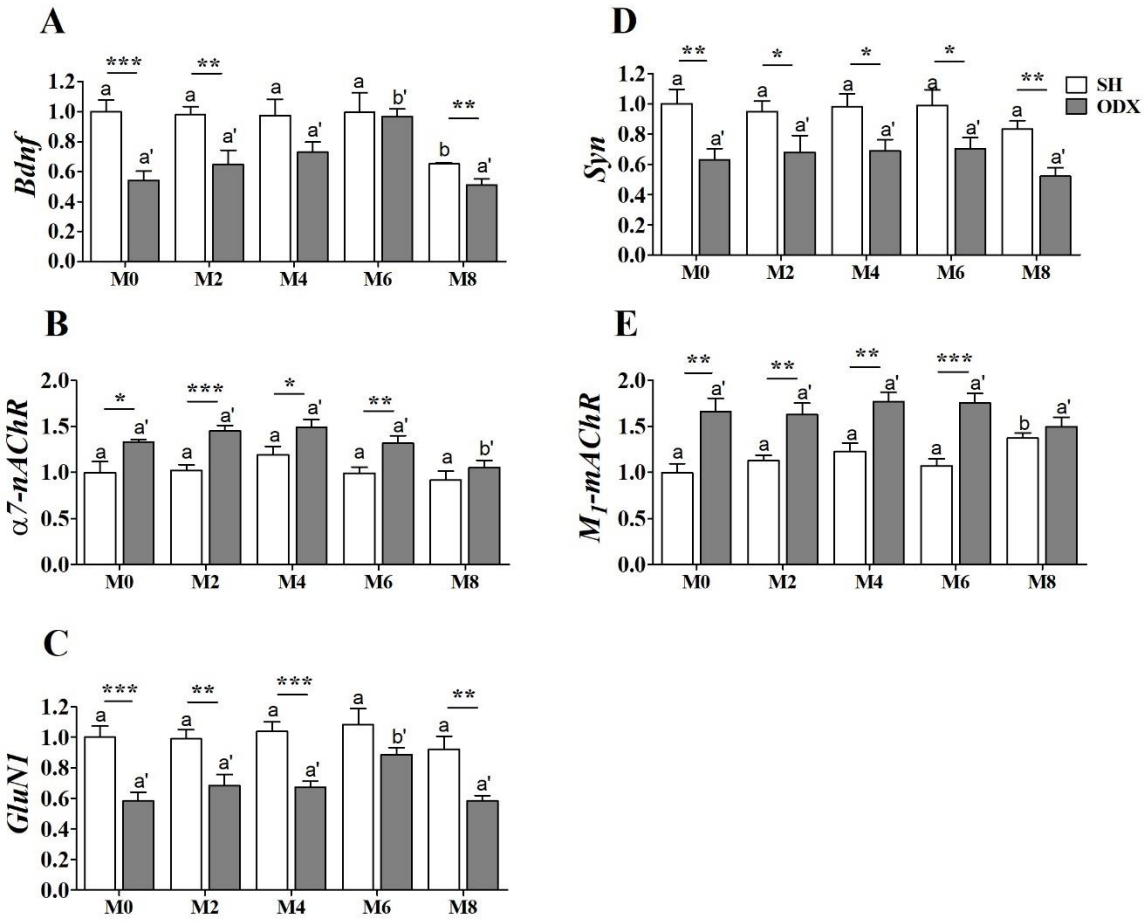


Figure 3.5 The mRNA expression levels of genes associated with synaptic plasticity; *Bdnf* (A), *α7-nAChR* (B), *GluN1* (C), *Syn* (D) and *M_1-mAChR* (E) in male rats after sham (SH) or orchidectomy (ODX) operation for 9 days (M0), 2 (M2), 4 (M4), 6 (M6), and 8 (M8) months.All data show the fold change in gene expression relative to the mean of SH-M0 rats. Vertical bars represent means ± SEM (n = 8-9). A different letter indicates significant differences between groups of SH (white column) or ODX (grey column) rats ($p < 0.01$). *, ** and *** represent $p < 0.05$, 0.01 and 0.001 compared to the age-matched SH rats.

Transcriptional levels of genes associated with neurofibrillary tangles

There was no significant effect of increasing age on mRNA expression levels of *Tau3* ($F = 0.291$, $p = 0.882$) and *Tau4* ($F = 0.701$, $p = 0.534$) in the hippocampus of the intact SH male rats over the age of 4-12 months (SH-M0 to SH-M8; Figure 3.6). On the other hand, orchidectomy and aging significantly and continuously decreased mRNA expression levels of *Tau3* ($F = 7.511$, $p < 0.001$) and *Tau4* ($F = 8.908$, $p < 0.001$) starting from 6-month-old rats (ODX-M2, -M4, -M6, and -M8 groups) in comparison with the 4-month-old rats (ODX-M0 group).

Comparison between the SH and ODX rats revealed that *Tau3* and *Tau4* mRNA levels were significantly higher in the ODX-M0 and -M2 rats than those of the age-matched intact SH rats (Figure 3.6). Interestingly, no significant differences were detected between SH and ODX rats of the M4, M6, and M8 groups. These results indicated that the *Tau3* and *Tau4* mRNA expression levels were abruptly increased by orchidectomy-induced androgen deficiency, and were subsequently reversed back to the base-line levels with advancing age.

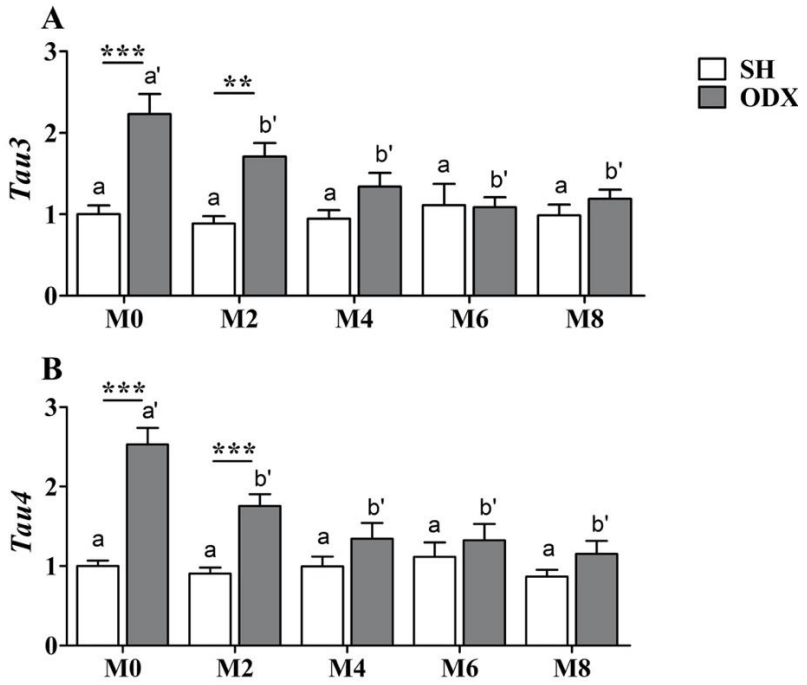


Figure 3.6 The mRNA expression levels of genes associated with neurofibrillary tangles; *Tau3* (A) and *Tau4* (B)in male rats after sham (SH) or orchidectomy (ODX) operation for 9 days, (M0), 2 (M2), 4 (M4), 6 (M6), and 8 (M8) months.All data show the fold change in gene expression relative to the mean of SH-M0 rats. Vertical bars represent means ± SEM (n = 8-9). A different letter indicates significant differences between groups of SH (white column) or ODX (grey column) rats ($p < 0.05$). **and *** represent $p < 0.01$ and 0.001 compared to age-matched SH rats.

Transcriptional levels of genes associated with amyloid plaques

There was no significant effect of advancing age on the mRNA expression levels of *App* ($F = 0.803$, $p = 0.530$), *Bace1* ($F = 1.341$, $p = 0.272$), and *Adam10* ($F = 0.798$, $p = 0.534$) in the hippocampus of intact SH male rats across the age of 4-12 months (SH-M0 to SH-M8; Figure 3.7). Likewise, the effect of androgen deficiency and advancing age had no effect on the *App* ($F = 1.382$, $p = 0.258$) and *Adam10* ($F = 0.604$, $p = 0.662$) mRNA levels, except that the *Bace1* mRNA level was significantly increased in the 10-month old, ODX-M6 rats compared with the 4-month old, ODX-M0 rats ($F = 3.044$, $p < 0.05$). Asides the no differences in the *App* and *Adam10* mRNA levels and the increased *Bace1* mRNA level of the ODX-M6 rats, only the *Bace1* mRNA level of the ODX-M6 rats was significantly higher than that of the age-matched SH rats ($p < 0.001$) (Figure 3.7).

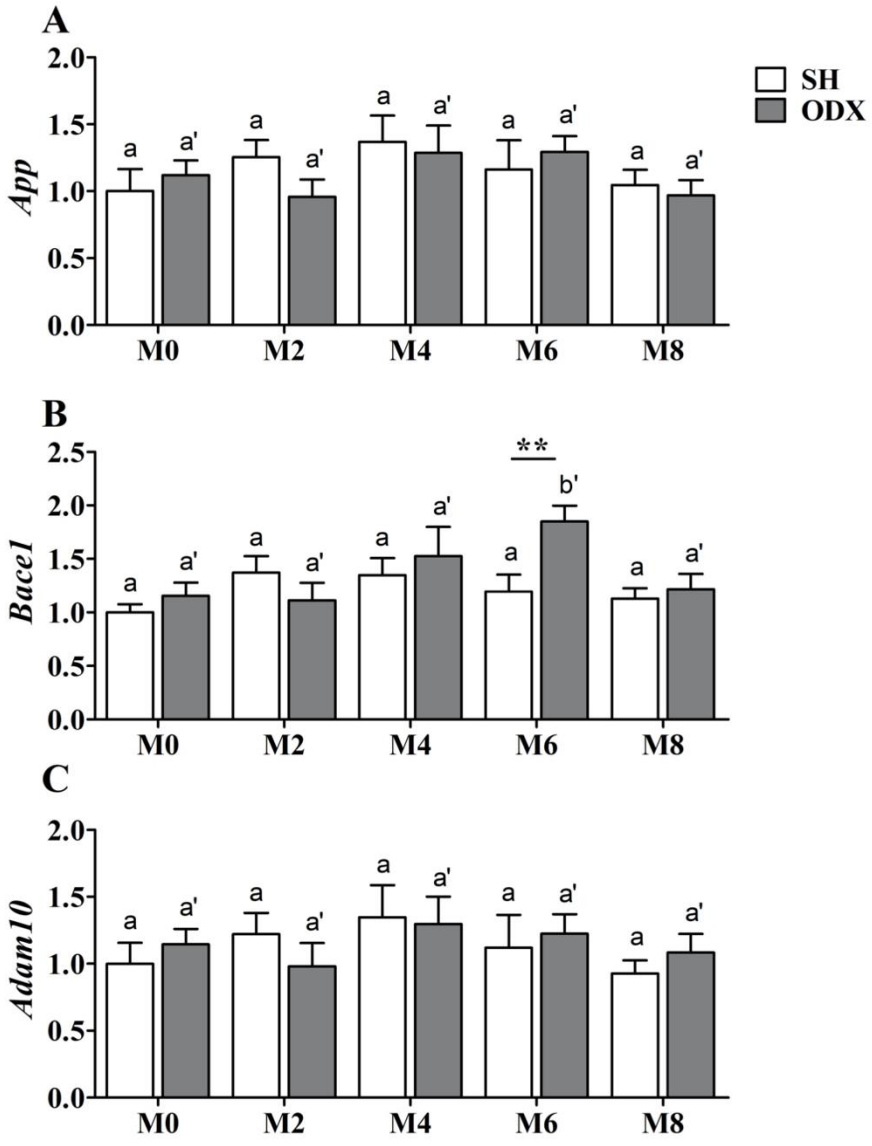


Figure 3.7 The mRNA expression levels of genes associated with amyloid plaques; *App* (A), *Bace1* (B), and *Adam10* (C) in male rats after sham (SH) or orchidectomy (ODX) operation for 9 days, (M0), 2 (M2), 4 (M4), 6 (M6), and 8 (M8) months. All data show the fold change in gene expression relative to the mean of SH-M0 rats. Vertical bars represent means ± SEM (n = 8-9). A different letter indicates significant differences between groups of SH (white column) or ODX (grey column) rats ($p < 0.01$). ** represents $p < 0.01$ compared to age-matched SH rats.

Translational levels of genes associated with the tau protein

To confirm if the remarkable changes in *Tau3* and *Tau4* mRNA levels was carried on to the protein levels, the immunoreactivity of total tau protein (Tau-5) and phosphorylated tau protein at Ser^{396} (p-Tau Ser396) was determined by Western blotting. The patterns of change in the protein levels matched those of the mRNA levels. There was no effect of increasing age, from 4-12 months old (SH-M0 to SH-M8), on Tau-5 and p-Tau Ser^{396} protein levels in intact SH male rats ($F = 0.399$, $p = 0.808$ for Tau-5 and $F = 0.422$, $p = 0.792$ for p-Tau Ser^{396} protein) (Figure 3.8). Androgen deficiency and an increasing age in the ODX rats significantly and continuously decreased the transcriptional levels of Tau-5 ($F = 5.219$, $p < 0.01$) and p-Tau Ser^{396} ($F = 16.760$, $p < 0.001$) in comparison with the ODX-M0 group. In comparison between the ODX rats and the SH rats, the Tau-5 and p-Tau Ser^{396} protein levels in ODX-M0 and -M2 rats were significantly higher than those in the age-matched SH rats ($P < 0.01$ and 0.001). In confirmation with that of the *Tau3* and *Tau4* mRNA levels, Tau-5 and p-Tau Ser^{396} protein levels were abruptly increased by orchidectomy-induced androgen deficiency, and were subsequently reversed back to the base-line levels by advancing age.

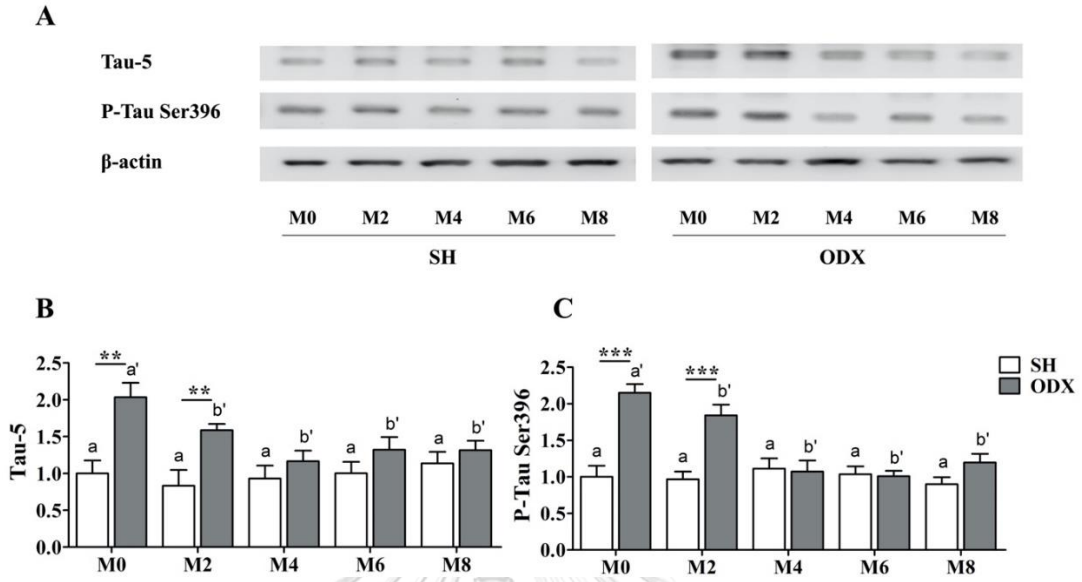


Figure 3.8 The protein expression levels of total tau (Tau-5) and phosphorylated tau (p-Tau Ser^{396}) in male rats after sham (SH) or orchidectomy (ODX) operation for 9 days, 2, 4, 6 and 8 months (M0, M2, M4, M6 and M8, respectively). Representative western blots of Tau-5, p-Tau Ser^{396} and β-actin are shown in (A). The relative quantitation of the western blot signals of Tau-5 and p-Tau Ser^{396} are shown in (B) and (C). All data show the fold change in protein expression levels relative to the mean of SH-M0 rats. Vertical bars represent means ± SEM (n = 8-9). A different letter indicates significant differences between groups of SH (white column) or ODX (grey column) rats ($p < 0.05$). **, *** represent $p < 0.01$ and 0.001 compared to age-matched SH rats.

Immunoreactivity levels of proteins reflecting the synaptic plasticity

In view of the significant changes in the mRNA levels of the *Bdnf* (M6), α*7-nAChR* (M8), and *GluN1* (M6) in the ODX groups, the *Bdnf* (M8) and M_1*-mAChR* (M8) levels in the SH group, and the *Bdnf*, *GluN1*, and *Syn* levels between the ODX-M8 and SH-M8 rats, the immunoreactivity levels of SYN and PSD-95 protein in both the CA1 and CA3 areas of the hippocampus were determined and compared between the M6 and M8 groups of the ODX and SH rats. There were no significant differences in SYN and PSD-95 immunoreactivity levels at either the CA1 or CA3 areas between the SH-M6 and SH-M8 rats (Figure 3.9). On the other hand, both SYN and PSD-95 immunoreactivities in the CA1 and CA3 areas in the ODX-M8 rats were significantly lower than those of the ODX-M6 rats. Comparing between the SH and ODX rats, the immunoreactivity levels of SYN in the M6 and M8 groups, and PSD-95 in the M8 groups in both hippocampal areas were significantly lower than those of the age-matched SH rats (Figure 3.9).

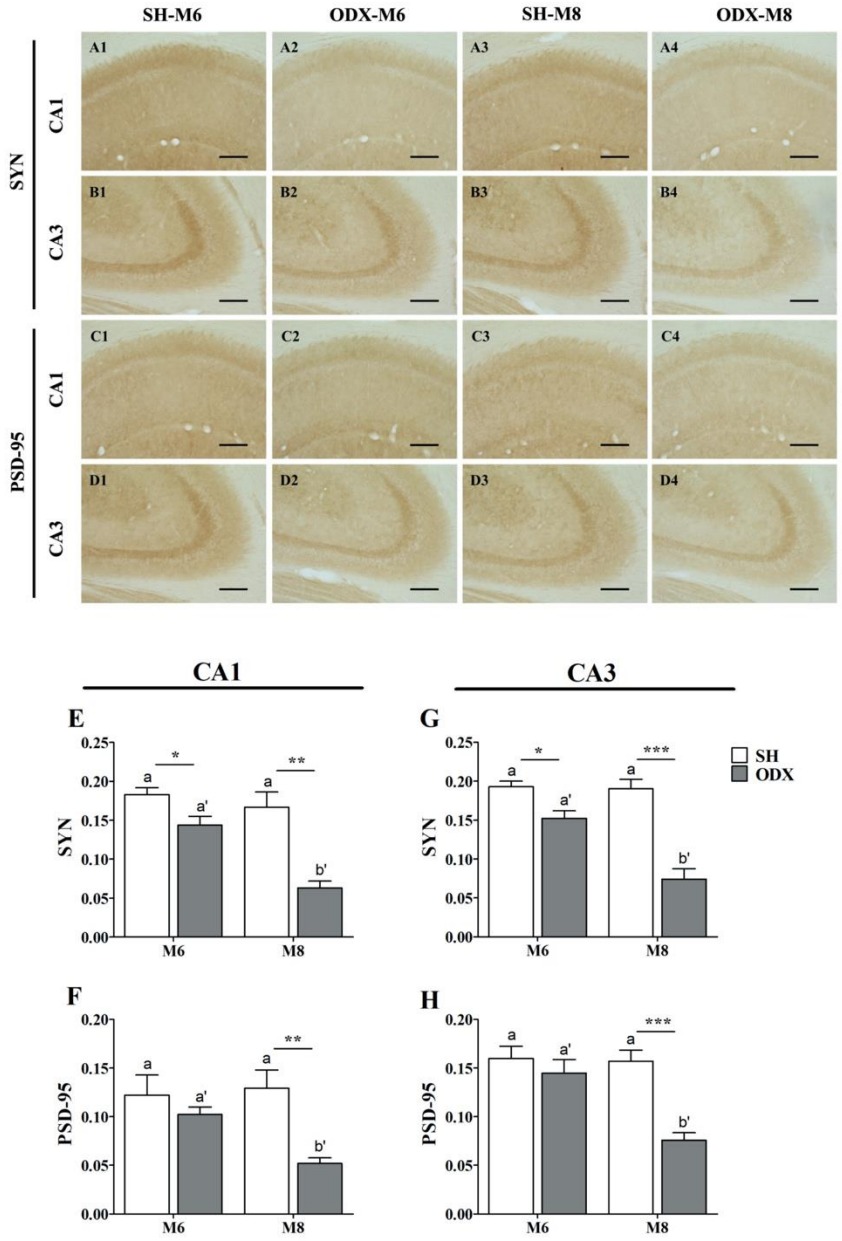


Figure 3.9 Representative photomicrographs of immunoreactivity of synaptophysin (SYN) in CA1 (A1-A4) and CA3 (B1-B4) region and postsynaptic density (PSD)-95 in CA1 (C1-C4) and CA3 (D1-D4) region in hippocampus of male rats after sham (SH) or orchidectomy (ODX) operation for 6 (M6) and 8 (M8) months.The positive immunohistochemical staining for SYN and PSD-95 were brownish yellow particles. Scale bar = 300 μm. The optical density of immunoreactivity signals of SYN in CA1 (E) and CA3 (G), and PSD-95 in CA1 (F) and CA3 (H) were quantified. Vertical bars represent means ± SEM (n = 5). A different letter indicates significant differences between groups of SH (white column) or ODX (grey column) rats ($p < 0.01$). ** and *** represent $p < 0.01$ and 0.001 compared to the age-matched SH rats.

Discussion

The effects of androgen deficiency on the cognitive impairment in both men and male laboratory animals were extensively reported and the results were inconsistent (Ford et al., 2018; Frye et al., 2010; Moffat et al., 2002; Pintana et al., 2016; Yeap et al., 2008) which was speculated that it was confounded by the effects of aging. In this study, the effects of an advancing in age, an androgen deficiency, and a combination between the two factors were assessed step-by-step. The cognitive function in male SH rats was sequentially assessed from young adult (4 months old) to middle age (12 months old) which aimed to answer if a cognitive impairment is the results of an advancing in age. Determination in the M0 to M8 groups of the ODX rats expected give a clue of the association between an abrupt androgen deficiency and aging on cognitive function. While the comparison between ODX rats and their age-match SH-rats should unveil the sole effect of androgen deficiency on cognitive function.

Searching the database of the previous reports, no longitudinal study tracking the course of decline in cognitive function before the middle age in male Sprague-Dawley rats were found. With the finer timescale tracking every 2 months in this study, from young adult to middle-aged rats, the impairment in spatial learning and memory could be detected as early as 8 months old (SH-M4) or at the early middle age. This finding partially aligned with the previous studies showing that the deficit in spatial learning and memory on MWM task emerged at the middle age, approximately 12-14 months old (Bizon et al., 2009; Francia et al., 2006; Frick et al., 1995; Frye et al., 2010; Guidi et al., 2014; Smith et al., 2020; Wang et al., 2020). In parallel with the increasing in age and the impairment in spatial learning and memory, male rats had a gradual and marginal decline in serum testosterone levels starting from 8 months old and a significant reduction was detected at 12 months old (or middle age). On one hand, the initiation of cognitive decline detected in the early-middle aged, 8 months old rats might suggest a hypersensitivity and vulnerability of their cognitive functions to a merely marginal decline in testosterone levels. On the other hand, it should be noted that the cognitive performance of middle age, 12 months old (SH-M8) rats was not worsened compared to that of the early-middle aged, 8 months old (SH-M4) rats though their testosterone levels were significantly lowered. Thus, the cognitive impairment initiated in the early-middle aged male rats and kept until they reached the

middle age, was seemingly independent of a significant decline in testosterone level. But this event might be occurred coincidentally between advancing in age and testosterone reduction and suggested that the cognitive impairment was likely and largely depended on the effects of advancing in age, so-called *age-associated cognitive impairment*.

In contrast to the naturally and gradually decline in serum testosterone levels in SH rats, the ODX-M0 rats had an abrupt decline in serum testosterone levels (below the LOD of <0.2 ng/ml). Noting, serum testosterone level was first measured at 9 days after orchidectomy. Previously, it was reported that the orchidectomy in male rats decreased serum testosterone to undetectable levels as fast as 6 h after surgery (Kashiwagi et al., 2005), and induced an atrophy of androgen responsive organs within 24 h (Fainanta et al., 2019).

There were two steps in the orchidectomy-induced androgen deficiency that impaired the spatial learning and memory in male rats in this study: short and long-term. The short-term effect was observed in ODX-M2 rats (Figure 3.2D, E and F), and the symptom was kept continued to ODX-M4 and ODX-M6 rats where it had no significant differences between ODX-M2, M4 and M6 groups. The long-term effect was observed in the ODX-M8, middle-aged rats that showed a significantly worsened cognitive impairment comparing to the ODX-M2 rats. Bearing in mind that the cognitive impairment in these groups of ODX rats might stem from two factors, aging and androgen deficiency, the comparison between the ODX rats and the age-matched SH rats should resolve the effects of aging. It was found that only the cognitive function of the ODX-M2 and ODX-M8 rats were significantly worsened than the age-matched (SH-M2 and SH-M8) rats. On one hand, the absence of the effects of an androgen deficiency-induced cognitive deficit in ODX-M4 and ODX-M6 rats comparing to the SH-M4 (8 months old) and SH-M6 (10 months old) rats might be concealed by the effects of an age-related cognitive decline in the early-middle aged, 8 months old (SH-M4) rats mentioned above. As seen in the previous report, no cognitive deficits were detected in male rodents after castration at the age of 2 months old and kept for 3-4 months comparing to the age-matched intact animals (Benice and Raber, 2009; Spritzer et al., 2008). Nonetheless, the cognitive deficit was exacerbated (long-term effect) in ODX-M8, middle-aged rats. This suggests that the effect of

orchidectomy-induced cognitive deficit was not straightforwardly and additionally magnified by the effect of advancing in age as a cumulative time-course effect. On the flip side, the previous studies reported that once an age-related cognitive decline occurred in the middle age rats (12-13 months old) it was not exacerbated by androgen deficient condition though the animals were ODX and kept for another 1-3 months (Frye et al., 2010; Smith et al., 2020). It implies that orchidectomy-induced androgen deficiency has no additive effect to age-related cognitive impairment in middle-aged male rats. Taken together, the present findings denoted the crucial role of androgen on cognitive function, especially if the androgen-deficient condition could occur in the young age (ODX-M0, 4 months old rats in this study) because the effects can be cumulative and exacerbated when the males reach the middle age. Because the different time points of cognitive impairment could be observed in the SH rats (initiated in SH-M4, early middle-aged rats) and the ODX rats (occurred in two steps: in ODX-M2, early young aged rats and ODX-M8, middle-aged rats), it possibly suggested a different underlying mechanism on cognitive impairment between age and orchidectomy-induced androgen deficiency which need further investigation.

Interestingly, no significant changes of any hippocampal markers designed for this study could be detected in an age-related cognitive impaired (8 months old) SH-M4 rats. Similarly, there were unchanged in expression of GluN1 protein (Zhao et al., 2009), *nAChR* mRNA and protein (Rogers et al., 1998; Tribollet et al., 2004), *mAChR* mRNA (Nieves-Martinez et al., 2012), *Bdnf* mRNA and protein (Silhol et al., 2005), SYN protein (Nicolle et al., 1999; Smith et al., 2000; Wang et al., 2007), *Tau3* and *Tau4* mRNA and total tau and phosphorylated tau protein (Jung et al., 2011), and genes associated with amyloid plaque formation (Che et al., 2014; Mukda et al., 2016) in the hippocampus of young to early middle-age rodents. Although rodents were reported to experience a cognitive decline with advancing in age, most of cognitive impaired animals were not normally subjected to neurodegenerative diseases. It has been reported that the numbers of principal neuronal cells (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Woodruff-Pak et al., 2010), dendritic arborization (Flood, 1993; Pyapali and Turner, 1996; Turner and Deupree, 1991), numbers and density of synaptic spines (Curcio and Hinds, 1983; Geinisman et al., 2004; Markham et al., 2005) in hippocampus of middle-aged rodents were remarkably preserved. Rather, an

age-related cognitive impairment in early middle-aged rats was thought to correlate with more subtle changes in the functional connectivity of the hippocampus which can be inferred from various electrophysiological measures, i.e., a decline in amplitude of field excitatory postsynaptic potential (fEPSP) (Barnes and McNaughton, 1980; Barnes et al., 1992; Barnes et al., 2000; Deupree et al., 1993; Landfield et al., 1986; Tombaugh et al., 2002), an impairment of induction and maintenance of long-term potentiation (LTP; a synaptic strengthening process) (Barnes et al., 1996; Deupree et al., 1993; Deupree et al., 1991; Moore et al., 1993), and a more susceptible to long-term depression (LTD; a synaptic weakening process) (Kumar and Foster, 2005; Norris et al., 1996). An impairment of electrophysiological properties of synaptic transmission in hippocampal circuits was characterized in early middle-aged rats. The 8 months old male rats required a higher electrical stimulation to induce a population spike in CA1 hippocampus (Papatheodoropoulos and Kostopoulos, 1996), and the magnitude of LTP induction and maintenance in hippocampus were declined (Amani et al., 2021; Fouquet et al., 2011; Monfort and Felipo, 2007; Rex et al., 2005; Schreurs et al., 2017). Taken the results in the present study together with the previous reports, it indicates that an early impairment of synaptic transmission, especially at electrophysiological levels, should be factors for age-related cognitive impairment initiated in early-middle aged SH-M4 rats. In contrast to no changes detected in the early middle-aged (8 to 10 months old, SH-M4 and M6) rats, the mRNA expression levels of *Bdnf* decreased, while *M_1-mAChR* increased, in hippocampus of the middle-aged (12 months old) SH-M8 rats, which coincided with a marked decrease in serum testosterone levels. Thus, circulating testosterone levels might play an important role in regulating the expression of *Bdnf* and *M_1-mAChR* in rat's hippocampus.

In contrast to the SH rats, the biological changes in hippocampus of ODX rats happened as fast as 9 days after operation (or in ODX-M0 group), before the emergence of cognitive impairment. The decrease in *Syn* mRNA expression levels could imply the probability of a decrease in neurotransmitter releasing (Huo et al., 2016; Lau et al., 2014; Li et al., 2013a). The increase in *M_1-mAChR* and *α7-nAChR* mRNA expression levels suggested the compensatory response of cholinergic transmission in hippocampus of androgen deficient rats. Although how androgens are

involved in cholinergic transmission has been rarely investigated, an increase of cholinergic receptors is thought to involve with early signs of neurodegeneration and cognitive impairment (Maurer and Williams, 2017). In contrast to cholinergic system, the decreased expression mRNA levels of glutamatergic NMDA receptor subunit *GluN1* after orchidectomy indicated the different response of glutamatergic transmission and along with the decreasing of *Syn*, it might indicate the perturbation of glutamatergic transmission. It has been reported that orchidectomy could increase EPSP of glutamatergic synaptic transmission measured at CA1 and CA3 regions of hippocampus (Harley et al., 2000; Skucas et al., 2013). Moreover, testosterone also regulated inhibitory GABA neurons which basically inhibited the neuronal excitability (Reddy and Jian, 2010). Thus, an androgen deficient condition is postulated to provide hyperexcitability in hippocampal glutamatergic neurons (Harley et al., 2000; Reddy and Jian, 2010; Skucas et al., 2013), which leads to over influx of Ca^{2+} and consequently contributes to cytotoxic damage and neuronal cell death (Caruso et al., 2004; Piña-Crespo et al., 2014). Therefore, androgens are thought to play an important role on counterbalance the excitation of glutamatergic synapse (Harley et al., 2000; Reddy and Jian, 2010; Skucas et al., 2013). There are 2 major types of glutamate receptors present at postsynaptic terminal of excitatory glutamatergic synapse: NMDA receptor and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. While AMPA receptor could be dynamically up-regulated or down-regulated in response to rapid changes in activity of synaptic transmission, the presence of NMDA receptor on the surface of postsynaptic terminal is more stable and thought to be very important for controlling synaptic plasticity and mediating learning and memory functions (Cercato et al., 2016; Li and Tsien, 2009). Therefore, the increase in glutamatergic transmission as mentioned by previously published studies (Harley et al., 2000; Skucas et al., 2013) might have relied on AMPA receptor. However, the decreased NMDA receptor subunit *GluN1* as reported in this study should indicate the consequent of excitotoxic damage leading to the perturbation of glutamatergic transmission.

Apart from affecting the synaptic transmissions, androgen deficiency also worsened the synaptic structure. The present study showed that *Bdnf* mRNA expression level was decreased in the ODX-M0 rats comparing to the SH-M0 rats. It

has been reported that orchidectomy decreased synaptic spine density in hippocampus of male rats (Leranth et al., 2003) through a reduction in BDNF expression (Li et al., 2012). Thus, the decreased *Bdnf* mRNA expression in the present study could probably indicate that the synaptic structure was likely to be decreased. However, there was a marked increase in *Tau3* and *Tau4* and total tau protein; a microtubule-associated protein facilitates the normal morphology of neurons and supports a synaptic integrity, in the ODX-M0 and M2 rats. The beneficial role of an increased tau level in medial preoptic area of the ODX mice was reported to enhance synaptic structure (Bharadwaj et al., 2013). Therefore, an early elevated tau in the present study migth play a beneficial role to compensate the decline in BDNF in order to maintain the synaptic structure.

It is important to note that a post-translational modification of tau proteins could adversely affect its function on microtubule assembly. The expression levels of the phosphorylated tau Ser^{396} protein, a pathological form, were concordantly increased. Although the exact mechanism for formation of neurofibrillary tangles is currently unknown, the increased expression of hyperphosphorylated tau protein has been proposed to trigger a progressive accumulation of phosphorylated tau which later aggregated into neurofibrillary tangles (de Calignon et al., 2012; Mondragon-Rodriguez et al., 2014). The study in mouse model for tau overexpression showed the chronological changes of phosphorylated tau protein expression levels (Delobel et al., 2008). They reported that, after phosphorylated tau expression reaching the peak level, it decreased abruptly, and then the phosphorylated tau filaments increased instead (Delobel et al., 2008). Similarly, in the present study, the over-expression of total tau and phosphorylated tau at 9-day to 2-month after orchidectomy was markedly decreased when the ODX rats reached 8 months old. It could be implicated that the hyperphosphorylated tau incorporated into filaments although the presence of neurofibrillary tangles needs to be confirmed. Combining changes of all molecular markers determined in this study, it can conclude that an abrupt androgen deficiency in the ODX rats perturbated the presynaptic terminal, and later both cholinergic and glutamatergic postsynaptic terminals attempted to maintain the homeostasis of synaptic functions. It seems to be that hippocampus tried to maintain its function, as a compensatory response of synaptic transmission, in cognitive performances.

As the two steps (short and long-term effects) of cognitive impairment were detected in ODX rats comparing to the SH rats: in the ODX-M2 and ODX-M8 rats, together with the determination of the three neurological hallmarks, changes of synaptic function and structure were proposed to be major players to trigger this event. Changes of mRNA levels of *Bdnf*, *α7-nAChR*, *GluN1*, *Syn*, *M_1-mAChR*, *Tau3*, and *Tau4* were initially detected in ODX-M0 rats before the cognitive impairment was detected in ODX-M2 rats. The deterioration in mRNA expression of gene associated with amyloid plaque in the hippocampus occurred progressively in this study in the ODX-M6 rats: *Bace1* mRNA level was significantly increased implying that the catalytic processing of amyloid precursor protein was likely shifted toward amyloidogenesis pathway, following with the cognitive impairment detected in ODX-M8 rats. It is widely accepted that once amyloid-β has formed in the brain it can induce a higher severity of synaptic impairment (Liu et al., 2010). Together with the increased *Bace1* mRNA levels in the ODX-M6 rats, the significant decreases in SYN immunoreactivity of the ODX-M6 rats and in SYN and PSD-95 immunoreactivity of the ODX-M8 rats in the CA1 and CA3 areas were detected. These probably indicated that the function of the presynaptic terminals in the release of neurotransmitters was likely decreased. Since BDNF is thought to be a regulator for PSD-95 transportation to postsynaptic terminal (Yoshii and Constantine-Paton, 2007), the decline of *Bdnf* transcripts in the ODX-M8 rats comparing to the SH-M8 rats should weaken the synaptic strength. Taken together, a cumulative effect of a long-term androgen deficiency in the ODX-M8 rats can exacerbate a cognitive impairment by progressively deteriorating the synaptic function and structure and the shift to an amyloidogenesis pathway. Aligned with these evidence, the *Bace1* gene should be the first candidate marker to be detected for the pathway of amyloid plaque formation.

In summary, the present study demonstrates that an advancing in age drives an age-associated cognitive impairment in male rats to be occurred starting from early middle age (8 months old) while testosterone levels are non-significantly reduced. However, a significant reduction of serum testosterone level when rats enter a middle aged, 12 months old could worsen the effects of aging. A major defect detected for age-related cognitive impairment is a deteriorateion of the synaptic function and structure. An abrupt decrease in serum testosterone level induced by orchidectomy

could accelerate the age-associated cognitive impairment in young ODX-M2 (6 months old) rats. The process of cognitive impairment was progressed via cumulative disturbances in synaptic function and structure, together with a plausible formation of neurofibrillary tangles in the hippocampus. However, if the two factors of aging and androgen deficiency are combined, as seen in the ODX-M8 rats, the cognitive impairment could be exacerbated via an imbalance of amyloid-β producing pathways which later severely deteriorated the synaptic function and structure. Taken together, the present study unveiled that an advancing in age plays an important role in driving the normal age-associated cognitive impairment in males, and androgen deficiency is a crucial additional risk factor for the progressive cognitive impairment in elderly males.

CHAPTER IV
CHRONOLOGICAL MOLECULAR CHANGES IN NEURONAL COMMUNICATION IN ANDROGEN-DEFICIENT RATS

Introduction

Neurodegenerative diseases are increasing in prevalence with the increased life-expectancy and aging population of humans across the globe. It is estimated that more than 55 million people live with dementia nowadays, and this is expected to increase to more than 152 million patients by 2050 (Alzheimer's Disease International, 2018; World Health Organization, 2019). Dementia is a clinical syndrome characterized by severe cognitive impairment that ultimately affects the individual's ability to live independently. What drives the variation in age-related cognitive impairment and dementia is not completely understood, but a reproductive senescence when a decline or deprivation of sex steroid hormones – estrogens in women (Burger et al., 2002; Butler and Santoro, 2011) and androgens in men (Harman et al., 2001; Leifke et al., 2000) – occurs in the elderly and is viewed as a major cause (Barron and Pike, 2012; Moffat et al., 2004). Although there are a number of studies on the effects of estrogen deficiency in female animals or menopause in women on cognitive impairment, there are very few studies for androgens and the male gender (Li and Singh, 2014). It has been reported that elderly men with low levels of circulating testosterone have a high prevalence of Alzheimer's disease (Barrett-Connor et al., 1999; Moffat et al., 2002; Yaffe et al., 2002). Testosterone can bind with ARs in regions of the brain related to cognitive function (Pike et al., 2009), especially at the hippocampus and cortical regions, which have a high density of ARs (Kerr et al., 1995; Sarkey et al., 2008; Simerly et al., 1990). As a matter of fact, a decline in androgen levels may negatively impact upon the neuronal function and cognitive abilities in males.

Analyses of the brains of severe neurodegenerative patients suggest that the presence of synaptic loss, intracellular inclusions of neurofibrillary tangles rich in hyperphosphorylated tau protein and extracellular aggregation of amyloid-β peptides into amyloid plaques are all pathological hallmarks of dementia disease (Bertoni-Freddari et al., 2003; Gomez-Isla et al., 1997; Ikonomovic et al., 2003; Scheff and Price, 2003; Scheff et al., 2006). Androgens have profound effects on synaptic (e.g., glutamatergic and cholinergic) transmission (Nakamura et al., 2002; Romeo et al.,

2005b) and neuronal structure, such as the induction of spines synapse; (Leranth et al., 2003; Smith et al., 2002). Dysfunction of neurotransmission and neuronal structure and function leads to the impairment of learning and memory performance (Auld et al., 2002; Mufson et al., 2003; Shankar et al., 2007; Wallace and Porter, 2011). Androgens also prevent the accumulation of abnormal protein neurofibrillary tangles and amyloid plaques. Supplementation with androgens can reverse the hyperphosphorylation of tau in ovariectomized rats (Papasozomenos and Shanavas, 2002) and ODX 3xTg-AD transgenic mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). Orchidectomy accelerated amyloid plaque formations in 3xTg-AD (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012) and SAMP8 mice (Kang et al., 2014), and these effects were lessened after subsequent testosterone treatment (Kang et al., 2014; Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). In addition, testosterone also exerted protective effects on amyloid-β induced neurotoxicity rats (Huo et al., 2016).

The above data supports an important role for androgens in preventing neurodegeneration. However, most of these studies were conducted in animals in which the neurodegeneration had already occurred or in transgenic animals that might not reflect the condition of aged, androgen deficient animals or humans. Moreover, knowledge of the molecular changes that occur before neurodegeneration in an androgen-deficient condition is scarce. There is currently no prescription or treatment for neurodegeneration, and so prevention before the disease occurs is likely to be the best measure. Furthermore, the findings from CHAPTER III revealed that changes of molecular markers of synaptic plasticity and neurofibrillary tangle in hippocampus of androgen deficient rats have been detected as early as 9 days after orchidectomy (in ODX-M0 group). Therefore, this study aimed to determine if the onset of molecular changes in the hippocampus of orchidectomy-induced androgen-deficient rats could be occurred earlier than 9 days. The mRNA expression levels of genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques and the protein expression levels of total tau and phosphorylated tau proteins in rats at 1 - 9 days after orchidectomy in comparison to age-matched sham control rats were determined.

Materials and Methods

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University, Thailand (Protocol review number: 1723007). Male Sprague-Dawley rats, at an age of 3.5 months old, were purchased from the National Laboratory Animal Center, Mahidol University, Thailand and housed in the Laboratory Animal Center of Faculty of Science, Chulalongkorn University under a controlled temperature (22 ± 1 °C) and light cycle (12:12 h light: dark). The rats were provided with a rat chow diet (Perfect Companion Group Co. Ltd, Thailand) and water *ad libitum*. They were reared until they reached 4 months old and were then used in this study as reported previously for female rats (Anukulthanakorn et al., 2013).

Experimental design

Experiments were divided into two sets. For Experiment I, to detect the early onset of molecular changes in hippocampus after androgen deficiency, 45 rats were ODX under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, and euthanized by carbon dioxide exposure at D_0 (control), D_1, D_3, D_6 and D_9 (9 animals for each) after orchidectomy. The whole brains of rats were immediately collected and kept frozen at -80 °C until the hippocampus was isolated, and the determination of mRNA expression levels of the genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques was performed. In order to verify the efficacy of orchidectomy-induced androgen deficiency, blood serum was collected from each rat and serum testosterone levels were determined by ELISA, and the prostate gland, seminal vesicles and epididymis were dissected, trimmed free of fat remnants and weighed.

For Experiment II, to ease the effect of increasing age (9 days) on the parameters determined in Experiment I, 20 age-matched intact rats were sham operated (SH-D_9) or ODX (ODX-D_9), kept for 9 days and euthanized. The determination of mRNA expression levels of the genes in the hippocampus (as in Experiment I) was then performed along with the protein expression levels of total tau and phosphorylated tau.

Quantitative qrtRT-PCR for determination of the mRNA expression levels of the genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques

The mRNA expression levels of the genes associated with synaptic plasticity; *Syn*, subunit *GluN1*, *α7-nAChR*, *M_1-mAChR* and *Bdnf*, neurofibrillary tangles (*Tau3* and *Tau4*), and amyloid plaques; *App*, *Bace1* and *Adam10*, were examined using two-stage qrt-RT-PCR.

Total RNA extraction and first stage cDNA synthesis by RT-PCR

The left hemisphere of hippocampal brain region was isolated from the whole brain and stored at -80 °C until RNA extraction. Total RNA was extracted from tissues using TRIzol® Reagent (Invitrogen, CA, USA) following the manufacturer's instruction. RNA concentration and purity were confirmed by spectrometer based on optical density (OD) measurements at 260 and 280 nm.

In the first RT-PCR stage, the extracted RNA (5 μg) was reverse transcribed to cDNA in a total volume of 20 μl containing 5 μl of RNA, 4 μl of RT buffer, 1 μl of dNTP mix (10 mM), 1 μl of Oligo $(dT)_{18}$ Primer Mix, 1 μl of Random Hexamer Primer Mix, 1 μl of RNase Inhibitor (10 U/μl), 1 μl of Reverse Transcriptase (200 U/μl) and 6 μl of diethylpyrocarbonate (DEPC)-treated water using the Tetro cDNA Synthesis kit (Bioline Reagent Ltd., London, UK). The samples were incubated at 25 °C for 10 min, 45 °C for 30 min and finally 85 °C for 5 min. The obtained cDNA was diluted in five-volumes of DEPC-treated water prior to use.

Second stage qrt-PCR detection of specific genes

The second stage qrt-PCR amplification of the obtained cDNA was performed using the gene-specific primers as described in Table 3.1, where S28RNA was used as the reference house-keeping gene in this study. The primers were first tested for optimal annealing temperature by conventional PCR. The qrt-PCR was performed using a SensiFAST™ SYBR® kit (Bioline Reagent Ltd., London, UK) according to the manufacturer's instructions.

Each qrt-PCR reaction was performed in a final volume of 20 μl with 10 μl of SensiFast SYBR® Hi-ROX mix, 0.8 μl each of forward and reverse primers (10 μM), 5 μl of cDNA and 3.4 μl of DEPC-treated water. The thermal cycling consisted of 95

°C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s, and then the melting curve analysis (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s before finally cooling at 25 °C for 5 min). Real-time detection of the SYBR Green fluorescence intensity, used to indicate the amount of PCR product, was measured at the end of each extension phase. Amplification products were quantified by the StepOnePlus^{TM} qrt-PCR system. To confirm the amplification specificity, PCR products from each primer pair were subjected to a melting curve analysis. The amounts of PCR product of the target genes were normalized against the housekeeping gene S28RNA in the corresponding samples and analyzed by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis for tau proteins

Determination of tau proteins (total tau and phosphorylated tau at the Ser^{396} site) was performed only in the rats from Experiment II. The right hemisphere of hippocampal brain region was isolated and homogenized on ice using RIPA buffer (ThermoScientific, MA, USA) mixed with Halt^{TM} Protease and Phosphatase Inhibitor Cocktail (ThermoScientific, MA, USA). The homogenate was centrifuged at 10,000×g, 4 °C for 15 min, and the supernatant was collected. The supernatant protein concentrations were determined by the Bradford assay (Sigma-Aldrich, MO, USA). Electrophoresis of lysate protein (30 µg) was performed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (w/v) acrylamide resolving gel. Separated proteins were transferred onto PVDF membranes and nonspecific binding sites were blocked by incubation with 5% (w/v) bovine serum albumin (BSA fraction V, Sigma-Aldrich, MO, USA) dissolved in phosphate-buffered saline (PBS), pH 7.4 at room temperature for 1 h. The blots were then probed overnight at 4 °C with primary antibody (dilution 1:1000): mouse monoclonal antibody Tau-5 (#ab80579, Abcam, MA, USA), rabbit monoclonal Tau-phospho S^{396} (#ab109390, Abcam, MA, USA) and rabbit monoclonal β-actin (#13E5, Cell Signaling Technology, MA, USA). After washing three times with PBS containing 0.1% (v/v) Tween-20, pH 7.4 (Sigma-Aldrich, MO, USA), the blots were incubated at room temperature for 2 h in the relevant specific secondary antibody (dilution 1: 2,500): anti-mouse IgG HRP-conjugated antibody or goat anti-rabbit IgG HRP-linked antibody (#7076 for Tau-5, and #7074 for tau-phospho S^{396} and β-actin; Cell

Signaling Technology, MA, USA). The resultant immunoreactivity was visualized by chemiluminescence reactions using the Clarity™ Western ECL Substrate Kit (#170-5060 Bio-Rad, CA, USA) and exposure to the GelDoc-It® Imaging System together with VisionWork® software (UVP Bioimaging, Cambridge, UK). The intensity of the bands was determined using an image-analyzing system (ImageStudio software, NE, USA).

Testosterone assay

After the blood samples had been collected, sera were separated by centrifugation at 1000×g for 20 min at 4 °C, and kept frozen at -20 °C until assayed. Serum testosterone levels were measured using a Testosterone ELISA Kit (Cat no. ab-108666, lot no. 20170501, Abcam, MA, USA) following the manufacturer' s instruction. Inter- and intra-assay coefficients of variation were 6.52% and 3.57% , respectively. The limit of detection (LOD) of the assay was 0.2 ng/ml.

Statistical analysis

The results are expressed as the mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was used to determine the significance of differences in the means of body weights, accessory sex organ weights and mRNA expression levels between D_0 and D_1, D_3, D_6 and D_9 in Experiment I. The observed significance was then confirmed by the Bonferroni test. The student t-test was used to determine the significance of differences in the mean mRNA and protein expression levels between the SH-D_9 and ODX-D_9 groups in Experiment II. Significance was accepted at the $p < 0.05$ level in both experiments. Statistical analyses were performed using the SPSS software program version 22 (International Business Machines Corp., NY, USA).

Results

Serum testosterone levels at 1–9 days after orchidectomy

The serum testosterone levels of male rats in the control group (D_0) were 7.434 ± 1.074 ng/ml. No detectable serum testosterone levels (< 0.2 ng/ml) were observed within 1 day after orchidectomy (D_1), and then remained at undetectable levels throughout the 9-day period (Table 4.1).

Accessory sex organs and body weights at 1–9 days after orchidectomy

The onset of a significant reduction in the prostate gland weights was detected at D_1 in comparison with D_0, and at D_3 for seminal vesicle and epididymis weights. A significant reduction in the body weight could also be detected as early as D_1 (Table 4.1).

Table 4.1 Serum testosterone levels, relative weights of the accessory sex organs (prostate gland, seminal vesicle and epididymis) and body weights of male rats at 0, 1, 3, 6 and 9 days after orchidectomy (D_0, D_1, D_3, D_6 and D_9, respectively).

Group	Testosterone concentration (ng/ml)	Accessory organ weights (g)			Body weight (g)
		Prostate gland	Seminal vesicle	Epididymis	
D_0	7.434± 1.074	0.417 ± 0.014^a	1.341 ± 0.056^a	1.365 ± 0.028^a	341.56 ± 7.10^a
D_1	< 0.2	0.357 ± 0.021^b	1.290 ± 0.013^a	1.282 ± 0.071^a	307.78 ± 5.39^b
D_3	< 0.2	0.261 ± 0.011^c	0.579 ± 0.027^b	1.014 ± 0.027^b	288.89 ± 8.48^b
D_6	< 0.2	0.190 ± 0.011^d	0.397 ± 0.015^c	0.718 ± 0.023^c	286.44 ± 3.80^b
D_9	< 0.2	0.115 ± 0.010^e	0.299 ± 0.010^c	0.637 ± 0.023^c	283.33 ± 6.67^b

All data are presented as the mean ± SEM (n = 9). Means within a column followed by a different letter superscript are significantly different ($p < 0.05$)

Changes in mRNA expression levels of genes associated with synaptic plasticity at 1–9 days after orchidectomy

The earliest change in the mRNA expression levels of genes associated with synaptic plasticity was observed with the *Syn* gene, where a significant decrease was detected at D_1 after orchidectomy and then remained at this reduced level up to D_9. Subsequently, there was a significant increase in the *GluN1* mRNA levels from D_3 and increasing up to D_9, a significant increase in *M_1-mAChR* and *α7-nAChR* mRNA levels from D_6 and a further increase at D_9, and a decrease in *Bdnf* mRNA levels at D_9, (Figure 4.1).

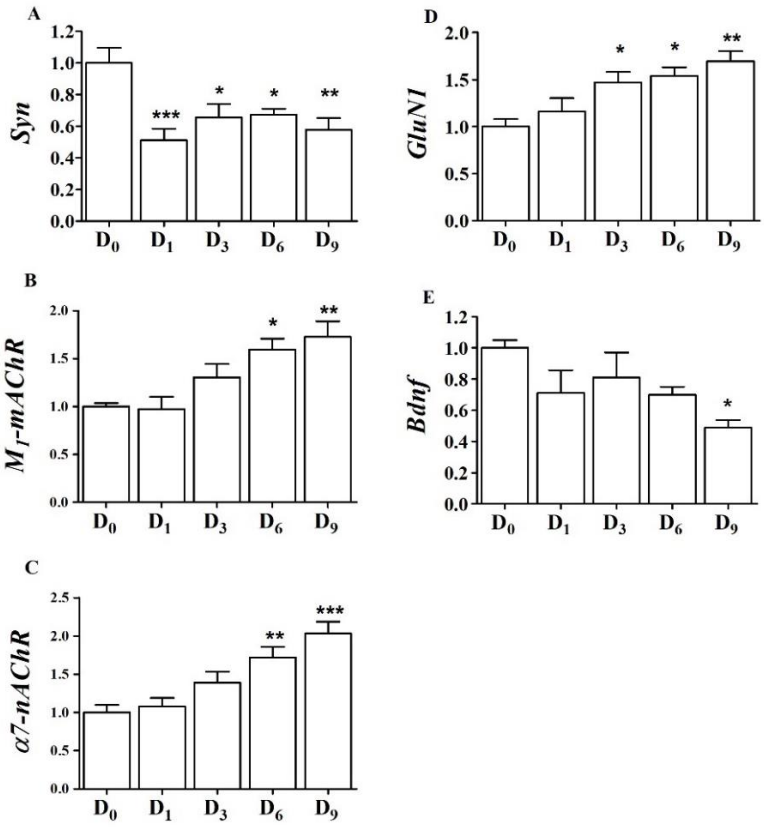


Figure 4.1 The mRNA expression levels of genes associated with synaptic plasticity; (A) *Syn*, (B) *M_1-mAChR*, (C) *α7-nAChR*, (D) *GluN1* and (E) *Bdnf* in the hippocampus of male rats at 0, 1, 3, 6 and 9 days after orchidectomy (D_0, D_1, D_3, D_6 and D_9, respectively). All data are presented as mean ± SEM (n = 9) of the fold changes in gene expression relative to the mean of D_0 group. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared to the D_0 group.

Changes in mRNA expression levels of genes associated with neurofibrillary tangles at 1–9 days after orchidectomy

The mRNA expression levels were significantly increased at D_6 after orchidectomy for the *Tau4* gene and at D_9 for *Tau3* (Figure 4.2).

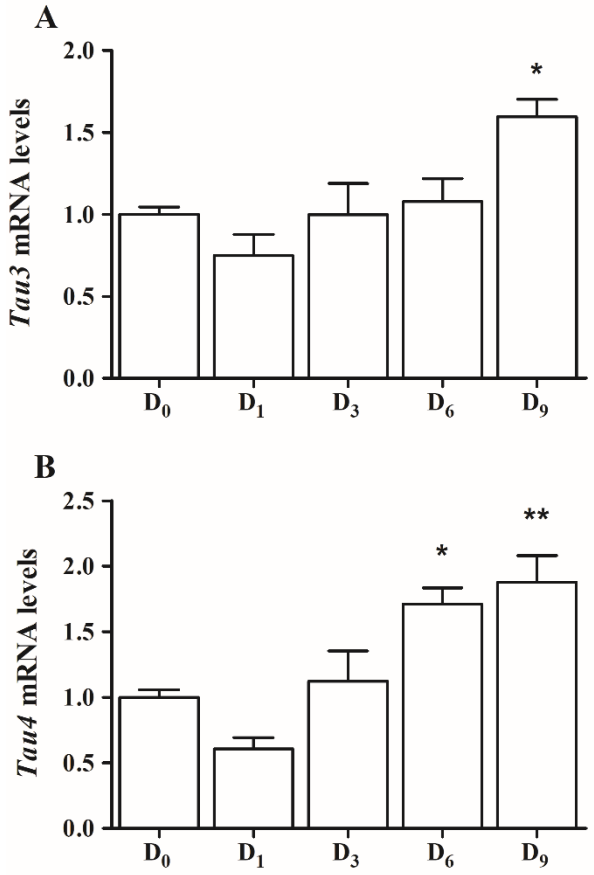


Figure 4.2 The mRNA expression levels of genes associated with neurofibrillary tangles; (A) Tau3 and (B) Tau4 in the hippocampus of male rats at 0, 1, 3, 6 and 9 days after orchidectomy (D_0, D_1, D_3, D_6 and D_9, respectively). All data are presented as mean ± SEM (n = 9) of the fold changes in gene expression relative to the mean of D_0 group. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the D_0 group.

Changes in mRNA expression levels of genes associated with amyloid plaques at 1–9 days after orchidectomy

No significant changes in the mRNA expression levels of *App, Bace1* and *Adam10* were detected in the 9-day period after orchidectomy (Figure 4.3).

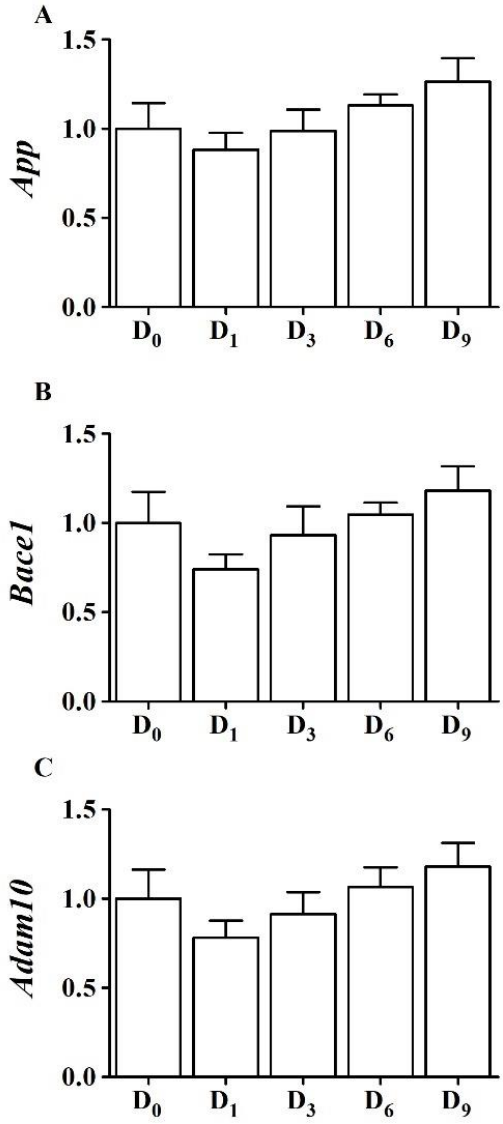


Figure 4.3 The mRNA expression levels of genes associated with amyloid plaques; (A) App, (B) Bace1 and (C) Adam10 in the hippocampus of male rats at 0, 1, 3, 6 and 9 days after orchidectomy (D_0, D_1, D_3, D_6 and D_9, respectively).All data are presented as mean ± SEM (n = 9) of the fold changes in gene expression relative to the mean of D_0 group.

Comparison of mRNA expression levels of genes associated with neurodegeneration between SH-D_9 and ODX-D_9 rats

The mRNA expression levels of genes associated with synaptic plasticity (*Syn*, *GluN1* and *Bdnf*) were highly significantly decreased in the ODX-D_9 rats compared to the SH-D_9 rats, while the *α7-nAChR* and *M_1-mAChR* mRNA levels were significantly increased. In the same line with those of the *α7-nAChR* and *M_1-mAChR* mRNA levels, the mRNA expression levels of the gene associated with neurofibrillary tangles (*Tau4* and *Tau3*) were significantly increased in the ODX-D_9 rats compared to the SH-D_9 rats. As expected, no significant differences in the mRNA expression levels of the genes associated with amyloid plaques (*App*, *Bace1* and *Adam10*) were detected between the ODX-D_9 and SH-D_9 rats (Table 4.2).

Table 4. 2 The mRNA expression levels of genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques in the hippocampus of male rats.The expression levels of age-matched intact ($SH\text{-}D_9$) rats was set as 1 and the expression levels of ODX ($ODX\text{-}D_9$) is given as the fold change compared to $SH\text{-}D_9$.

Gene		Group		p-value
		$SH\text{-}D_9$	$ODX\text{-}D_9$	
Synaptic plasticity				
	Syn	1.000 ± 0.096	0.631 ± 0.071	0.007
	α7-nAChR	1.000 ± 0.119	1.367 ± 0.055	0.013
	M_1-mAChR	1.000 ± 0.097	1.661 ± 0.141	0.001
	GluN1	1.000 ± 0.073	0.585 ± 0.055	< 0.001
	Bdnf	1.000 ± 0.078	0.542 ± 0.062	< 0.001
Neurofibrillary tangles				
	Tau3	1.000 ± 0.109	2.230 ± 0.244	< 0.001
	Tau4	1.000 ± 0.070	2.532 ± 0.207	< 0.001
Amyloid plaques				
	App	1.000 ± 0.165	1.121 ± 0.109	0.561
	Adam10	1.000 ± 0.158	1.146 ± 0.114	0.465
	Bace1	1.000 ± 0.076	1.154 ± 0.127	0.315

All data are presented as the mean ± SEM (n = 9) fold changes in the gene expression level relative to the mean of $SH\text{-}D_9$ group.

Comparison of the tau and phosphorylated tau protein expression levels in the SH-D_9 and ODX-D_9 rats

To confirm if the increase in *Tau3* and *Tau4* mRNA levels was reflected in the protein expression levels, the protein expression levels of total tau and phosphorylated tau (at Ser^{396}) were determined by western blotting. Both the total tau and phosphorylated tau protein levels were significantly increased in the ODX-D_9 rats compared to the age-matched SH-D_9 rats (Figure 4.4).

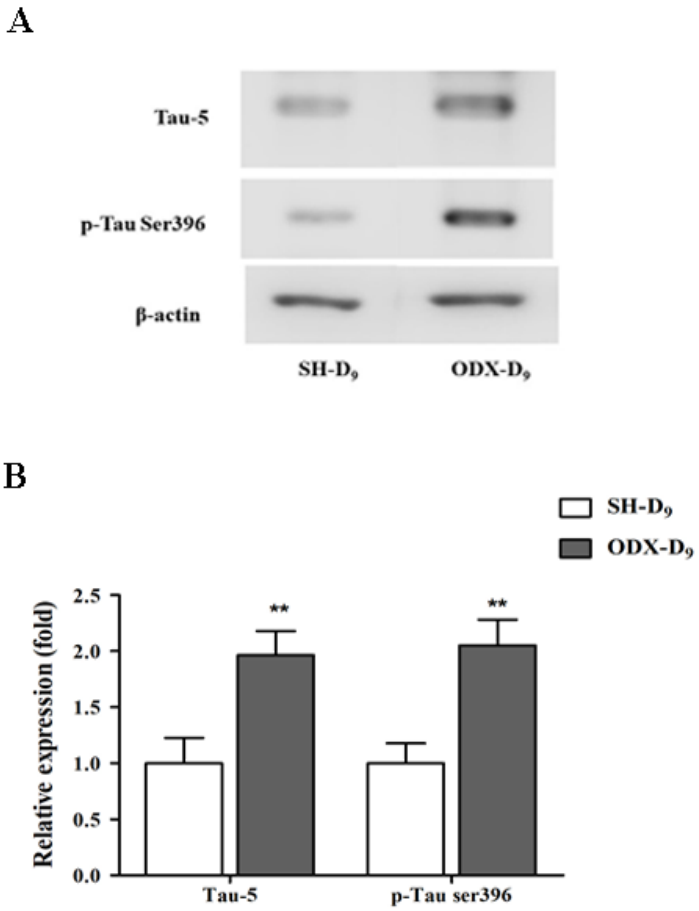


Figure 4.4 Total tau (Tau-5) and phosphorylated tau (at Ser^{396}; p-Tau Ser^{396}) in the hippocampus of ODX rats at 9 days after the operation and age-matched intact (SH) rats. (A) Representative western blots of Tau-5, p-Tau Ser^{396} and β-actin, and (B) after quantitation of the western blot signals. Values are presented as mean ± SEM (n = 9). ** indicates $p < 0.01$ compared with the mean of SH-D_9 rats.

Discussion

Although androgen deficiency has been associated with a high incidence of neurodegenerative disease, the early molecular events of neurodegeneration after the onset of androgen deficiency are not well verified. The earliest detection that has been reported so far was the remarkable upregulation of genes associated with signal transduction, such as gamma aminobutyric acid (GABA) , glutamate receptor, potassium ion channel and cadherin 9, after some two weeks after orchidectomy (Quintela et al., 2015). Since there is currently no medicine or treatment for neurodegeneration, knowledge of the early genetic changes that lead to neurodegeneration might enable prevention of the symptoms. Combining the report of Quintela et al. (2015) mentioned above with the reductions in serum testosterone levels and accessory sex organ weights within 3 days after orchidectomy determined in this study, the influence of androgen deficiency on the mRNA expression levels of various genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques in the hippocampus of male rats at 1, 3, 6 and 9 days after orchidectomy were investigated.

It was reported in male rats that have serum testosterone levels fall below the LOD within 6 h after castration (Kashiwagi et al., 2005). In this study, the serum testosterone level was under the LOD (< 0.2 ng/ml) from D_1 (24 h, the earliest time assay point for testosterone determination). Since testosterone is required for normal function and cell proliferation of the accessory sex organs, via activation of the AR, then depletion of testosterone levels would likely lead to accessory sex organ atrophy. Indeed, changes (decreases) in the weights of accessory sex organs are used to indicate the androgenic status in male rats (OECD, 2009; Yamasaki et al., 2001). Among all three accessory sex organs determined in this study, the prostate gland was found to be highly responsive to testosterone deprivation with a significant weight reduction being detected within a day after orchidectomy via a reduction in the epithelial cell numbers (English et al., 1987), while the reduction of the seminal vesicle and epididymis weights were detected afterwards at D_3. Interestingly, a reduction in the rat body weight as early as the reduced prostate gland weight at D_1 after orchidectomy could be detected. Several studies have shown that orchidectomy-induced androgen deficiency can lead to a reduction in body weight by a reduced

daily food intake (Borst and Conover, 2006; Chai et al., 1999; Gentry and Wade, 1976), which was related to the reduced growth hormone output, lowered insulin-like growth factor-1 levels (Inoue et al., 2010) and muscle atrophy (Axell et al., 2006; Borst and Conover, 2006; Gao et al., 2005). Thus, instead of killing the animals to collect and weigh the accessory sex organs, the body weight reduction of male rats could be used as a non-invasive indicator of their androgen deficiency condition.

In the process of neuronal transmission, the neurotransmitter is released from a presynaptic neuron, translocated and bound to specific receptors at postsynaptic neurons. This study revealed that the mRNA levels of synaptophysin (*Syn*), an integral membrane protein of the synaptic vesicle that is involved in neurotransmitter release (Valtorta et al., 2004), decreased immediately within 1 day after orchidectomy, indicating a likely decreased level of neurotransmitter release. Orchidectomy has been reported to also decrease the mRNA and protein expression levels of *Syn* in SAMP8 mice (Li et al., 2013a), which was prevented by DHT treatment. Testosterone also exerted protective effects on presynaptic terminals against amyloid-β-induced neurotoxicity by preserving the protein expression levels of SYN (Huo et al., 2016; Lau et al., 2014). Further, the upregulation of transcripts for genes associated with postsynaptic receptors after orchidectomy at D_3 for *GluN1* and D_6 for *α7-nAChR* and *M_1-mAchR* were found. The upregulation of postsynaptic receptor genes might be a compensatory response in consequence to the reduced amounts of neurotransmitter release (Creese and Sibley, 1981). The upregulation of *α7-nAChR* was previously proposed to serve as a marker for the progression of Alzheimer's disease (Counts et al., 2007; Hellstrom-Lindahl et al., 1999; Teaktong et al., 2004).

Apart from the effects on neuronal transmission, androgens are also involved in the development of neuronal structure, where androgens are implicated in neurite growth, differentiation and neuroprotection. The spine synaptic density was reported to increase after treatment with testosterone or DHT in ODX rats (Leranth et al., 2003; Li et al., 2013a) and in hippocampal slices from adult male rats (Hatanaka et al., 2015). The neurotrophic effect of androgens occurred via the stimulation of mRNA and protein expression of the BDNF (Ottem et al., 2007; Verhovshek et al., 2010). This study found that the mRNA expression levels of *Bdnf* decreased at 9-day

after orchidectomy. Since BDNF is one of the factors that controls dendritic arborization (Horch and Katz, 2002; Tolwani et al., 2002), dendritic spine formation (Bennett and Lagopoulos, 2014) and axonal growth (Liao et al., 2015), the decrease in *Bdnf* mRNA levels should initiate an abnormal neuron structure and function that could finally lead to neurodegeneration. Note that BDNF controls dendritic growth by activation of the Rho GTPase (Cohen-Cory et al., 2010; Penzes et al., 2003; Takemoto-Kimura et al., 2007), which later regulates microtubule dynamics (Jaffe and Hall, 2005). Microtubules are important cytoskeleton components for the development and stability of the dendrite and axon, where tubulin assembly is facilitated by the microtubule-associated tau protein that binds to microtubules.

Alterations in the amount or the structure of tau protein can affect its role on microtubules. Tau protein is encoded by *MAPT*, which has six mRNA isoforms caused by alternative splicing. The isoforms can contain four or three microtubule-binding domains, termed *Tau4* or *Tau3*, respectively. In the present study, the mRNA expression levels of *Tau4* and *Tau3* were found to be increased at 6 and 9 days after orchidectomy, respectively. Previously, it was reported in female rats that *Tau4* mRNA levels was increased within one month after ovariectomy and so this was proposed as a biomarker of the early-onset of neurodegenerative disease in estrogen deficient female rats (Anukulthanakorn et al., 2013).

Indeed, high *Tau4* and *Tau3* mRNA levels could have either a positive or a negative effect on microtubules. On one hand, high Tau3 and Tau4 protein levels should intensify microtubule assembly and stabilization, while on the other hand, if the levels of Tau3 and Tau4 protein are too high, they could undergo modification, mainly through hyperphosphorylation. Phosphorylation normally regulates the binding of tau to microtubules, which is the prominent method of tau function. A healthy human brain has approximately 1.9 moles of phosphate per mole of tau, whereas tau from the abnormal filaments in patients with Alzheimer's disease carries 6–8 moles of phosphate per mole of tau (Ksiezak-Reding et al., 1992b). This hyperphosphorylation makes tau unable to interact with microtubules and so it generates aberrant aggregates that lead to the formation of paired helical filaments and finally become neurofibrillary tangles with the destruction of neurons.

To ascertain if the upregulation of *Tau4* and *Tau3* mRNA levels was beneficial to

the neuronal structure, the total tau and phosphorylated tau protein levels in the ODX-D_9 rats were determined and compared with the SH-D_9 rats. It was found that total tau and phosphorylated tau protein levels were both increased in the ODX-D_9 rats, indicating that the upregulation of *Tau4* and *Tau3* mRNA levels in ODX rats likely deteriorated the microtubule structure. In support of this notion, the *Bdnf* mRNA level, a marker of dendritic branching and axonal growth, was also decreased in the ODX rats, as already mentioned above. In accord with the results of this study, it was previously reported that testosterone treatment can prevent or ameliorate the hyperphosphorylation of tau protein in ODX rats (Papasozomenos, 1997) and ODX 3xTg-AD mice (Rosario et al., 2010).

It has been reported previously that neurofibrillary tangles could induce the formation of amyloid plaques (Ghosal et al., 2016; Ittner et al., 2010; Roberson et al., 2007). Amyloid plaques are composed of amyloid-β peptide, which is derived from the sequential cleavage of APP, encoded by the *APP* gene. APP can be cleaved by α-secretase (encoded by *ADAM10*), which results in a non-toxic, large extracellular APPα fragment, or by β-secretase (encoded by *BACE1*), which provides amyloid-β peptide. In the present study, the mRNA expression levels of *App*, *Bace1* and *Adam10* did not change in the 9-day period after orchidectomy, which indicates that the formation of amyloid plaques likely takes a longer time-course than the dysfunction of synaptic plasticity and formation of neurofibrillary tangles. As seen in previous reports, the amyloid-β peptide levels increased 1 month after orchidectomy in androgen deficient male rats (Ramsden et al., 2003) and at 4 months in ODX 3xTg-AD mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). In estrogen deficient female rats, mRNA expression levels of *App*, *Bace1* and *Adam10* could be detected at 4 months after ovariectomy (Anukulthanakorn et al., 2013).

Determination of gene expression at the mRNA level during the 9-day period after orchidectomy provides the sequences of early molecular changes in the hippocampus. However, those molecular changes could have been influenced by androgen deficiency and/or the advancing age of the animals. In order to ease effect of advancing age, this study determined the mRNA expression levels of those genes in androgen deficient rats 9 days after orchidectomy (ODX-D_9 group) in comparison with age-matched intact rats (SH-D_9 group). All mRNA expression levels of the

ODX-D_9 rats were aligned with the above results for the D_9 rats (of Experiment I), except for the mRNA level of *GluN1* that was significantly decreased in the ODX-D_9 rats but increased in the D_9 rats. This inconsistent result might be due to the expression of the *GluN1* gene is influenced by various other stimuli as well as androgen. Therefore, this study confirmed that androgen deficiency is the major influencing factor, not age, which leads to the early dysfunction of neuronal communication and structure in hippocampus. Accordingly, this finding may help in the setting up of a strategy to protect early neuronal degeneration in elderly people once a low androgen level is detected.

In conclusion, androgen deficiency induces chronological molecular changes in the hippocampus that can be detected as early as 1 day after orchidectomy. Changes started with a likely deterioration in synaptic transmission with a decrease in presynaptic release (*Syn* mRNA expression; D_1) followed by a compensatory of postsynaptic receptor upregulation (mRNA expression of *GluN1* at D_3, *M_1-mAchR* and *α7-nAChR* at D_6 and *Bdnf* at D_9) and the formation of neurofibrillary tangles (*Tau4* and *Tau3* mRNA expression at D_6 and D_9, and total tau and phosphorylated tau protein at D_9). However, the prominent marker of neurodegeneration, the formation of amyloid plaques, takes a longer time (longer than 9 days after orchidectomy) to occur. Thus, these results can hopefully shed some light on the paradigm of neurodegenerative prediction.

CHAPTER V
NEUROPROTECTIVE EFFECTS OF DIHYDROTESTOSTERONE, 17β-ESTRADIOL, *Pueraria mirifica* EXTRACT ON COGNITIVE IMPAIRMENT IN ANDROGEN DEFICIENT RATS

Introduction

Sex steroid hormone deficiency has been accounted as an important risk factor of cognitive impairment (Gauthierm, 2021). The vast majority of evidences revealed that the prevalence of cognitive impairment and the risk of being diagnosed as AD are associated with the estrogen deficiency in postmenopausal women (Mosconi et al., 2017; Mosconi et al., 2018; Rosario et al., 2011), and low levels of circulating testosterone in elderly men (Ford et al., 2018; Moffat et al., 2002; Moffat et al., 2004) and in prostate cancer patients who underwent the androgen deprivation therapy (Cherrier et al., 2009; Green et al., 2002; Salminen et al., 2004). The beneficial effects of estrogen replacement therapy against the cognitive impairment has been widely reported in women during peri-menopausal period (Kantarci et al., 2016; Mosconi et al., 2018; Paganini-Hill and Henderson, 1994; Rasgon et al., 2014), a window of treatment to prevent age-related neurological disease in women (Scheyer et al., 2018). In contrast to women, reports of an advantage of testosterone supplementation on cognitive function are limited in men (Cherrier et al., 2015; Cherrier et al., 2005b), and reports showing these effects are conflicting (Borst et al., 2014; Emmelot-Vonk et al., 2008; Haren et al., 2005; Huang et al., 2016; Kenny et al., 2002; Kenny et al., 2004; Lu et al., 2006; Maki et al., 2007; Vaughan et al., 2007). Unlike the abrupt cessation of sex hormone production in women during menopause, the decline of testosterone synthesis in men is gradual and continuous with increasing in age at approximately 0.4-2% per year, starting from the middle age (Camacho et al., 2013; Lapauw et al., 2008; Yeap et al., 2018). Apart from the noticeable point of a significant decline of testosterone level, each man has his own normal range of physiological testosterone levels (Travison et al., 2017). Thus, these factors potentially forbid the optimal time point for manipulating testosterone supplementation and investigating its effectiveness on cognitive impairment in men.

Testosterone is basically catabolized into either high potent and nonaromatizable DHT by 5α-reductase or E_2 by aromatase. Male brain, especially the brain region that

is responsible for learning and memory as hippocampus (Gonzalez et al., 2007; Hojo and Kawato, 2018), expresses both ARs and ERs. Taken together, all these factors caused complications of if and how the testosterone acts on regulating cognitive function in male brain. The neuroprotective effects of testosterone, DHT and E_2 administration on cognitive functions have been investigated in ODX rodents; however, the results were inconsistent (Frye et al., 2010; Mohammadi-Farani et al., 2015; Pintana et al., 2015; Spritzer et al., 2011). Administration of exogenous testosterone to ODX mice inhibited synaptic transmission from pre- to post-synaptic terminal in hippocampus (Harley et al., 2000; Skucas et al., 2013) whereas DHT treatment exhibited the positive results (George et al., 2013; Kang et al., 2014). Treatment of testosterone could prevent the decay of the synaptic structure, particularly the numbers of dendritic spines, in ODX rats (Kovacs et al., 2003; Leranth et al., 2003). Testosterone and DHT supplementation could decrease β-amyloid peptide, a major component of amyloid plaque, in hippocampus of male SD rats (Ramsden et al., 2003), SAMP8 mice (Kang et al., 2014), and 3xTgAD mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). However, some reports showed that E_2 and ERs were required to regulate several cognitive functions in male rodents (Hodosy et al., 2009; Koss and Frick, 2019; Lagunas et al., 2011; Martin et al., 2003), male common marmoset (Gervais et al., 2019) and men (Beer et al., 2006; Cherrier et al., 2005a; Salminen et al., 2005). E_2 facilitated synaptic transmission and strengthening of synaptic structure in hippocampus (Vierk et al., 2015; Vierk et al., 2012). Treatment of E_2 could decrease the hyperphosphorylation of tau, the major components of neurofibrillary tangles in hippocampus of 3xTgAD mice (Rosario et al., 2010; Rosario et al., 2006; Rosario et al., 2012). As mentioned above that testosterone can act either in the form of DHT or E_2 which binds with ARs or ERs, however, most of experiments have done the test using DHT or E_2 at a time, and no comparison of the results and mechanism of actions of DHT and E_2 on cognitive function in a single study. Therefore, the present study aims to investigate the neuroprotective effects of DHT and E_2 on hippocampal dependent learning and memory in androgen deficient rats, and determine its actions at mRNA expression levels of genes associated with synaptic transmission and structure, neurofibrillary

tangles, and amyloid plaques, and at protein expression levels of total tau and phosphorylated tau in hippocampus

The use of herbal medicines and phytonutrients or nutraceuticals has increased tremendously over the past four decades (Ekor, 2014). Recently, Anukulthanakorn et al. (2016) had reported that oral administration of the crude extract of *P. mirifica* could cure the early- and late-stage of spatial cognitive impairment in ovariectomy-induced estrogen deficient rats. *P. mirifica* contains many phytoestrogenic substances, such as puerarin, miroestrol, deoxymiroestrol, genistein, genistin, daidzin and daidzein (Malaivijitnond, 2012). The beneficial effects of these phytoestrogens on regulating the synaptic plasticity and preventing the formation of neurofibrillary tangles and amyloid plaques in hippocampus have been examined *in vitro*, and in female animals (Anukulthanakorn et al., 2016; Bagheri et al., 2011; Hong et al., 2016; Huang and Zhang, 2010; Li et al., 2010; Li et al., 2019; Liu et al., 2015; Monthakantirat et al., 2014; Zeng et al., 2004). Therefore, it is interesting to expand the knowledge if PME could potentially provide the neuroprotective effects on cognitive function in males, and compare its effect to those of DHT and E_2.

Materials and methods

Animal subjects and ethical note

The animal experiments were conducted under the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes issued by the National Research Council of Thailand and were approved by the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (Protocol review number 17-33-007). Two-month-old male Sprague-Dawley rats were purchased from the Nomura Siam International Co., Ltd., Bangkok, Thailand and were housed (two rats/cage) in a strictly hygienic conventional housing system with controlled temperature of 24±1°C, light (06:00-18:00 h) /dark cycle, and relative humidity of 40-60% at the Laboratory Animal Research Building, Faculty of Pharmaceutical Sciences. The rats were provided access to rat chow diet (Perfect Companion Group Co. Ltd, Thailand) and water *ad libitum.* They were reared until 4 months old, and used in this study as reported previously for female rats (Anukulthanakorn et al., 2013).

Preparation of chemicals

The powder of DHT (#A0462, ≥99.0% purity, Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) and E_2 (#E8875, ≥98% purity, Sigma-Aldrich Co., Ltd., MO, USA) were weighed and dissolved in a small volume of absolute ethanol. The dissolved solution was added with the olive oil and allowed to stand at room temperature to evaporate the ethanol. This stock solution was then diluted with olive oil to give a final concentration of 4 mg/ml DHT and 0.32 mg/ml E_2. A dose of 80 µg/kg/day of E_2 was selected for this study following the previous report in estrogen deficient female rats (Anukulthanakorn et al., 2016; Feng et al., 2004). A dose of 1 mg/kg/day of DHT was used in this study because it was reported to prevent a cognitive impairment in androgen deficient male mice (Kang et al., 2014).

The PME was prepared from *P. mirifica* powder (lot no.141023) kindly provided by Dr. I. Sandford Schwartz, Smith Natural Co. Ltd., Thailand. *P. mirifica* powder was extracted twice in 95% ethanol solvent using a soxhlet apparatus at 80 °C for 8 hours in each time. The extracted solution was filtered through a filter paper (Whatman No. 4) equipped with vacuum suction pump and dried using rotary vacuum evaporator at 40 °C, 70 rpm. The remaining solvent mixed in crude extraction was

evaporated again at 25 °C until the extracted weight was no further changed and provided a 9.95% of yield. The dried PME was kept in a dark bottle and stored at -20°C until used. The stock PME was suspended in distilled water to give a final concentration of 80 mg/ml for the treatment. A dose of 100 mg/kg/day of PME was used in this study because it exhibited the neurotherapeutic effects on cognitive impairment in female rats (Anukulthanakorn et al., 2016).

Phytochemical analysis of PME

The quantitative analysis of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein contents in the PME was performed using liquid chromatography tandem mass spectrometry (LC/MS/MS) at Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The standards of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein were dissolved in DMSO and diluted with methanol. One hundred milligram of PME was dissolved in 1 ml of DMSO, and then diluted 100-fold in methanol. The diluted PME samples were mixed with a 10-fold volume of methanol containing 562.5 ng of glycyrrhetinic acid as an internal standard for the LC/MS/MS analysis, performed using a Nexera UHPLC 100 LC equipped with a Shimadzu 8060 LCMS, controlled by Labsolution software version 5.86 (Kyoto, Japan). The UHPLC system was equipped with a Synergi Fusion-RP C18 column as the stationary phase (Phenomenex Inc, Torrance, CA, USA), while the mobile phase was a methanol: water gradient starting at 10% (vol/vol) methanol for 0.5 min, increased linearly to 90% (vol/vol) methanol at 1.5–3.5min, and then decreased linearly to 10% (vol/vol) methanol at 4–6min. The retention times of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin, daidzein and glycyrrhetinic were 1.515, 1.663, 1.733, 1.650, 1.822, 1.583, 1.762 and 2.206 min, respectively. The MS analysis was performed with negative mode ionization for detection of puerarin (m/z, 415.05/267.00), miroestrol (m/z, 356.90/189.20), deoxymiroestrol (m/z, 341.00/265.15), daidzein (m/z, 253.00/208.05) and glycyrrhetinic acid (m/z, 469.35/409.40), and with positive mode ionization for detection of genistin (m/z, 433.05/271.05), genistein (m/z, 270.95/153.00) and daidzin (m/z, 417.00/255.10). Calibration curves of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein showed good correlation coefficients (R^2>0.996) over the

concentration range of 5-1000 µg/L. The limit of detection of these compounds was estimated to be 1 µg/L with a signal-to-noise ratio of 5. The intra-assay accuracy and precision for analysis of these compounds were within ±10%. The puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein contents were 35.05, 2.29, 0, 19.42, 8.11, 40.16, 14.58 mg, respectively, in 100g PME.

Experimental design

The testes of 4-month old rats were surgically removed under sodium pentobarbital anesthesia (40-50 mg/kg. i.p.), divided into 4 groups (13 rats/group), and orally treated with 1 ml/day of distilled water (ODX-DW group), subcutaneously injected with 1 mg/kg/day of DHT (ODX-DHT group), subcutaneously injected with 80µg/kg/day of E_2 (ODX-E_2 group) and orally treated with 100 mg/kg/day of PME (ODX-PME group) for 2 months. Rats were given those treatments on the next day after surgery based on the reason that the onset of the changes of mRNA expression of genes associated with hippocampal synaptic plasticity were detected as early as 1 day after orchidectomy (see CHAPTER IV). Sham control (SH) rats were surgically operated as did in the ODX rats, but their testes were kept intact.

Rats were assessed for cognitive functions in spatial learning and memory using MWM task for six consecutive days before the termination of the treatment. At the end of the experiment, rats were euthanized and the hippocampus collected for analysis of mRNA expression levels using qrtRT-PCR technique and protein expression levels using the western blot, and the accessory sex organs (prostate gland, epididymis and seminal vesicles) to determine the weight.

Assessment of learning behavior and memory capacity using MWM test

The MWM task was used to assess the hippocampal learning and memory in rats. The test was conducted in a circular pool making from composites resin (180 cm in diameter and 50 cm in height) filled with water at temperature of 22-25°C. The pool was divided geographically into four quadrants (NE, NW, SE and SW), and a transparent circular platform making from acrylic (10 cm in diameter) was hidden submerged 1 cm below the surface of the water at the center of the target quadrant (NW). Four distinctive stable external cues were place around the pool. The ambient light was set at 50 lux on the water surface. The video camera was mounted above the center of maze and linked to a computer to record the swimming path data using a

video tracking system (Smart JUNIOR®, Panlab-Harvard Apparatus, Barcelona, Spain). The learning behavior test was performed consecutively on day-1 to day-5, and the memory capacity test was performed on day-6 as described previously (Vorhees and Williams, 2006). Each day of the learning behavior test consisted of four trials (90 s each) with a 30 min interval between trials. If any rat failed to locate the platform within 90 s, it was gently guided to the platform and rested for 30 s, and a time of 90 s was recorded. A video tracking system was used to record (i) the latency to find the hidden platform (or escape latency), (ii) the distance to arrive at the hidden platform (or travel distance), and (iii) swimming patterns (Line, Taxis, Random, and Circle) based on the report of Anukulthanakorn et al. (2013). After the rats completed the learning behavior test on day-5, they were continuously assessed the memory capacity test using the spatial probe test on day-6. For the spatial probe test, the platform was removed from the pool to check the memory of the animal for the platform location. The rat was released to the pool at the quadrant opposite to the previously existing platform location and allowed to swim for 30 s. During those 30 s, the number of crossings to the previously existing platform location, and the time and distance spent in the targeted quadrant, where the platform used to be located, were recorded using the video tracking system.

qrtRT-PCR for determination of mRNA expression levels of the genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques

The left hemisphere of hippocampal brain region was isolated from the whole brain and stored at − 80 °C until RNA extraction. Total RNA was extracted from tissues using TRIzol® Reagent (Invitrogen, CA, USA) following the manufacturer's instruction. The concentration and purity of the RNA samples were determined by measuring the absorbance at a wavelength of 260 and 280 nm using a Thermo NanoDrop^{TM} One UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA), where a 260/280 ratio of 2.0 ± 0.1 was accepted for mRNA purity. The mRNA expression levels of the genes associated with synaptic plasticity; *Syn*, subunit *GluN1*, *α7-nAChR*, *M_1-mAChR* and *Bdnf*, neurofibrillary tangles; *Tau3* and *Tau4*, and amyloid plaques; *App*, *Bace1* and *Adam10*, were examined using two-stage qrt-RT-PCR.

In the first RT-PCR stage, the extracted RNA (5 μg) was reverse transcribed to cDNA in a total volume of 20 μl containing 5 μl of RNA, 4 μl of RT buffer, 1 μl of dNTP mix (10 mM), 1 μl of oligo (dT)18 primer mix, 1 μl of random hexamer primer mix, 1 μl of RNase inhibitor (10 U/μl), 1 μl of reverse transcriptase (200 U/μl), and 6 μl of diethylpyrocarbonate (DEPC)-treated water using the Tetro cDNA Synthesis kit (Bioline Reagent Ltd., London, UK). The samples were incubated at 25 °C for 10 min, 45 °C for 30 min, and finally 85 °C for 5 min. The obtained cDNA was diluted in five volumes of DEPC-treated water prior to use.

The second-stage qrt-PCR amplification of the obtained cDNA was performed using the gene-specific primers as described in Table 3.1, where S28RNA was used as the reference house-keeping gene in this study. The primers were first tested for optimal annealing temperature by conventional PCR. The qrt-PCR was performed using a SensiFAST™ SYBR® kit (Bioline Reagent Ltd., London, UK) according to the manufacturer's instructions.

Each qrt-PCR reaction was performed in a final volume of 20 μl with 10 μl of SensiFast SYBR® Hi-ROX mix, 0.8 μl each of forward and reverse primers (10 μM), 5 μl of cDNA, and 3.4 μl of DEPC-treated water. The thermal cycling consisted of 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s, and then the melting curve analysis (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s before finally cooling at 25 °C for 5 min). Real-time detection of the SYBR Green fluorescence intensity, which was used to indicate the amount of PCR product, was measured at the end of each extension phase. Amplification products were quantified by the Step One Plus™ Real-Time PCR system. To confirm the amplification specificity, PCR product from each primer pair was subjected to a melting curve analysis. The amounts of PCR product of the target genes were normalized against the housekeeping gene S28RNA in the corresponding samples and analyzed by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis for tau protein expression levels

Determination of the protein expression levels of total tau protein and phosphorylated tau at the Ser^{396} site was performed using the right hemisphere of hippocampal brain region. The total protein was extracted by homogenizing the hippocampal tissue in ice-cold RIPA buffer (#89900, Thermo Scientific, MA, USA)

mixed with Halt^{TM} Protease and Phosphatase Inhibitor Cocktail (#78440, Thermo Scientific, MA, USA). The homogenate was centrifuged at 10,000×g, 4 °C for 15 min, and the supernatant was collected. The protein concentration in the supernatant was quantified by Bradford assay (#B6916, Sigma-Aldrich, MO, USA). Then, the concentration of protein lysates was adjusted to 1 µg/µl with RIPA buffer.

The targeted protein expression levels were determined by a capillary electrophoresis size-based separating using the fully automated ProteinSimple Wes system with the 12-230 kDa Wes Separation Module, 8 x 25 capillary cartridges (#SM-W004; ProteinSimple, CA, USA) following the manufacturer's instructions. Briefly, 6.4 µl of protein lysates were mixed with 1.6 µl of the 5X Fluorescent Master Mix in a ratio 4:1 and then incubated at 95 °C for 5 min.

The prepared samples, the biotinylated ladder, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies, and Luminal-Peroxide Mix were loaded into the independent wells of the plate separation module. The plate was briefly spun, loaded into the Wes instrument (ProteinSimple, CA, USA), and then the electrophoresis separation and immunodetection steps were automatically processed. Firstly, the capillaries were filled with Separation Matrix for 200 s, Stacking Matrix for 15 s and samples for 9 s with vacuum injection. Then, separation was performed at 375 volts for 25 min. After separation, the capillaries were exposed to UV light to attach the separated protein to the capillary wall and then Matrixes were removed. Subsequently, the capillaries were washed with washing buffer for three times and then blocked with blocking reagent for 30 min to prevent non-specific binding.

Targeted proteins were immunoprobed with primary antibodies including mouse monoclonal antibody Tau-5 (1:50; #ab80579, Abcam, MA, USA), rabbit monoclonal antibody Tau-phospho Ser^{396} (1:100; #ab109390, Abcam, MA, USA), and rabbit monoclonal β-actin (1:100; #13E5, Cell Signaling Technology, MA, USA) for 30 min. All primary antibodies were detected by incubated in the HRP-conjugated secondary anti-mouse provided in Anti-Mouse Detection Module Kit (#DM-002 for Tau-5; ProteinSimple, CA, USA) or anti-rabbit antibodies provided in Anti-Rabbit Detection Module Kit (#DM-001for tau-phospho Ser^{396} and β-actin; ProteinSimple, CA, USA) for 30 min, and visualized by chemiluminescence reaction using Luminol-S and peroxide. Images were captured, and band densities of targeted proteins were

analyzed using Compass software (ProteinSimple, CA, USA). The expression levels of targeted proteins were normalized against β-actin and expressed as fold-changed to SH values.

Statistical analysis

The results are expressed as mean ± standard error of mean (SEM). All data sets were performed the criteria of normal distribution and homogeneity of variance. The one-way analysis of variance (ANOVA) was used to determine the differences among treatment groups followed by the least significant difference (LSD) post hoc test. The escape latency and travel distance across five days of the spatial learning were analyzed using two-way repeated measure ANOVA followed by the Bonferroni post hoc test. The significant difference of swimming patterns was tested by the chi-square test. The level of significance was set as $p < 0.05$. Statistical analyses were performed using SPSS software version 22 (International Business Machines Corp., NY, USA).

Results

Androgenic status of the ODX rats after DHT, E_2 and PME treatment

Weights of androgen-dependent accessory sex organs; prostate gland, seminal vesicles, and epididymis, were measured to indicate the androgenic status of the ODX rats and the effects of the treatments. Weights of prostate gland, seminal vesicles, and epididymis in ODX-DW rats were significantly lower than those of the SH rats (Table 5.1), indicating an androgen deficient status of the rats and confirming the complete removal of the testes. Among three treatments of DHT, E_2 and PME in the ODX rats, only the DHT could hamper the reduction of the weights of these accessory sex organs and the weights were comparable to those of the SH rats, except the epididymal weight that was slightly lower than that of the SH rats. On the other hand, the accessory organ weights in the ODX-E_2 and ODX-PME groups were not significant difference from those of the ODX-DW group (Table 5.1). Therefore, these results confirmed that the physiological androgenic activity was found only in the DHT, while E_2 and PME did not elicit the androgenic activity on those organs.

Table 5.1 Weights of androgen-dependent accessory sex organs; prostate gland, seminal vesicles, and epididymis, of intact sham (SH) rats and orchidectomized (ODX) rats treated with distilled water (DW), 1mg/kg/day of dihydrotestosterone (DHT), 80μg/kg/day of 17β-estradiol (E_2), or 100mg/kg/day of *P. mirifica* extract (PME).

Group	Weight (g)		
	Prostate gland	Seminal vesicles	Epididymis
SH	0.692 ± 0.014^a	1.652 ± 0.035^a	1.536 ± 0.047^a
ODX-DW	0.081 ± 0.005^b	0.250 ± 0.020^b	0.481 ± 0.026^b
ODX-DHT	0.726 ± 0.045^a	1.547 ± 0.066^a	0.990 ± 0.032^c
ODX-E_2	0.094 ± 0.006^b	0.209 ± 0.010^b	0.506 ± 0.016^b
ODX-PME	$0.077\pm.011^b$	0.194 ± 0.012^b	0.519 ± 0.020^b

All data are presented as the mean ± SEM. Means within a column followed by a different superscript letter are significantly different ($p < 0.001$)

Effects of DHT, E_2 and PME on spatial learning behavior and memory capacity in the ODX rats

Considering a spatial learning behavior, the escape latency and travel distance for searching the hidden platform significantly decreased in all five groups of rats during 5 consecutive days of the MWM task ($F = 370.679$, $p < 0.001$ for the escape latency; and $F = 3.740$, $p < 0.001$ for the travel distance), indicating that rats could learn where the location of the platform (Figure 5.1A and B). However, a post-hoc comparison showed that the decreased patterns in both escape latency and travel distance of the ODX-DW rats were significantly higher than the SH rats (Figure 5.1A and B). Moreover, the ODX-DW rats showed the bad learning patterns in searching for the hidden platform on day-5 (29% random and 6% circular) which were not detected in the SH rats (only 32% line and 68% taxis patterns were observed; $\chi^2 = 32.732$, $p < 0.001$; Figure 5.1C). These results can be implied that androgen deficiency induced by orchidectomy could worsen the spatial learning behavior in male rats.

Treatment with DHT, E_2, and PME significantly decreased the escape latency and travel distance in the ODX-DHT, ODX-E_2, and ODX-PME rats compared to the ODX-DW rats. The decreased patterns in the ODX-DHT and ODX-E_2 rats were not significantly different from those of the SH rats, while the ODX-PME rats showed the significantly higher values (Figure 5.1A and B). While the escape latency and travel

distance of the ODX-DHT and ODX-E_2 rats were comparable, those of ODX-DHT rats, but not ODX-E_2 rats, were significantly lower than that of ODX-PME rats ($p < 0.05$). The proportion of swimming patterns in the ODX-DHT rats was also resemble to that of the SH rats (only 40% line and 60% taxis patterns were observed; $\chi^2 = 1.050$, $p = 0.305$) which was significantly different from that of the ODX-DW rats ($\chi^2 = 22.359$, $p < 0.001$). Although the swimming pattern strategies used by the ODX-E_2 rats (44% line, 44% taxis, 12% random, and 0% circular) and the ODX-PME rats (27% line, 58% taxis, 15% random, and 0% circular) were significantly improved comparing to the ODX-DW rats (29% line, 36% taxis, 29% random and 6% circular; $\chi^2 = 9.144$; $p < 0.05$ for the ODX-E_2 and $\chi^2 = 8.059$; $p < 0.05$ for the ODX-PME; Figure 1C), the values were remained worsened than the SH rats ($\chi^2 = 13.199$; $p < 0.01$ and $\chi^2 = 11.747$; $p < 0.01$). While ODX-DHT rats used only line and taxis patterns, these pattern were significantly lesser used by ODX-E_2 and ODX-PME rats ($p < 0.05$). These indicted that the improvement of the ODX-induced spatial learning impairment of rats was DHT > E_2 > PME treatment.

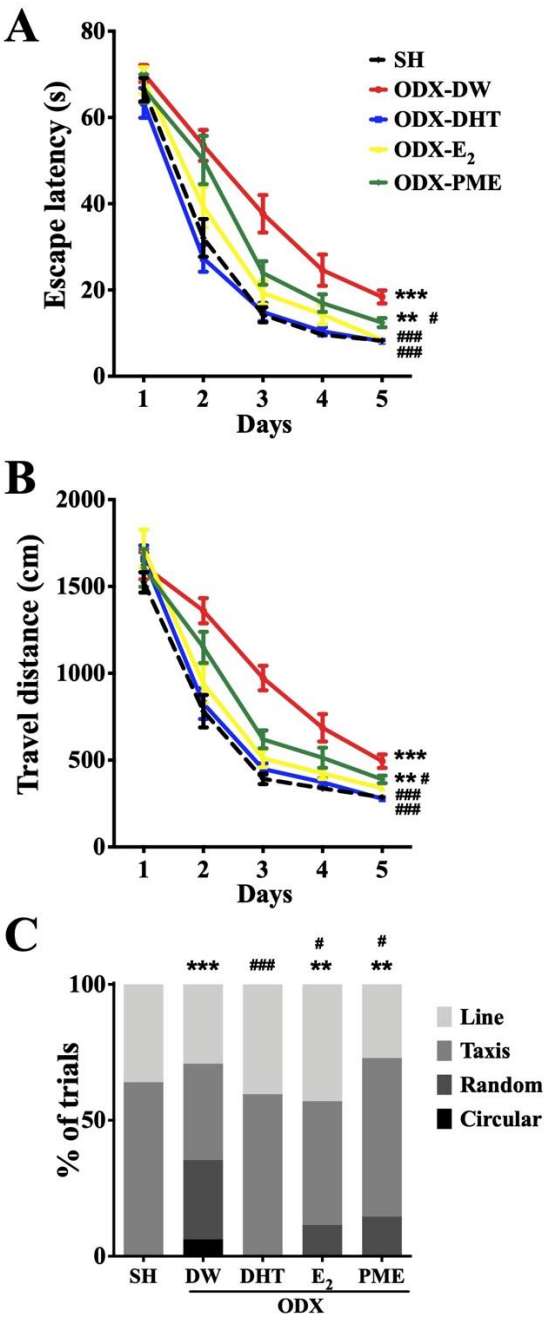


Figure 5. 1 The spatial learning behavior of sham (SH) rats, and orchidectomize (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and P. mirifica extract (PME) for 2 months which were assessed for 5 consecutive days before the termination of the treatments using the Morris Water Maze task. The escape latency, travel distance and strategies of swimming pattern are shown in (A), (B), and (C), respectively. ** and *** represent $p < 0.01$ and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001 compared to the ODX-DW rats.

Rats were determined their memory capacity by probe trial (a hidden platform was taken out of the pool) on day-6. The crossing numbers, time and distance spent in a targeted area in the ODX-DW group were significantly lower than the SH group (Figure 5.2), indicating that orchidectomy-induced androgen deficiency impaired the memory capacity of the ODX rats. While the crossing numbers, time and distance spent in target area of ODX-DHT, ODX-E_2 and ODX-PME rats were not significantly different, treatment with DHT in the ODX-DHT rats could prevent a decrease in crossing numbers, time, and distance spent in a targeted area, and the values were comparable to those of the SH rats (Figure 5.2). Treatments of E_2 and PME to the ODX-E_2 rats and the ODX-PME rats, respectively, could hamper a decrease only in time, and distance spent in a targeted area, while a crossing number was remained significantly lower than the SH rats (Figure 5.2A). These results indicated that the DHT has the higher efficacy on preventing the memory impairment followed by the E_2 and PME (DHT > E_2 > PME), respectively.

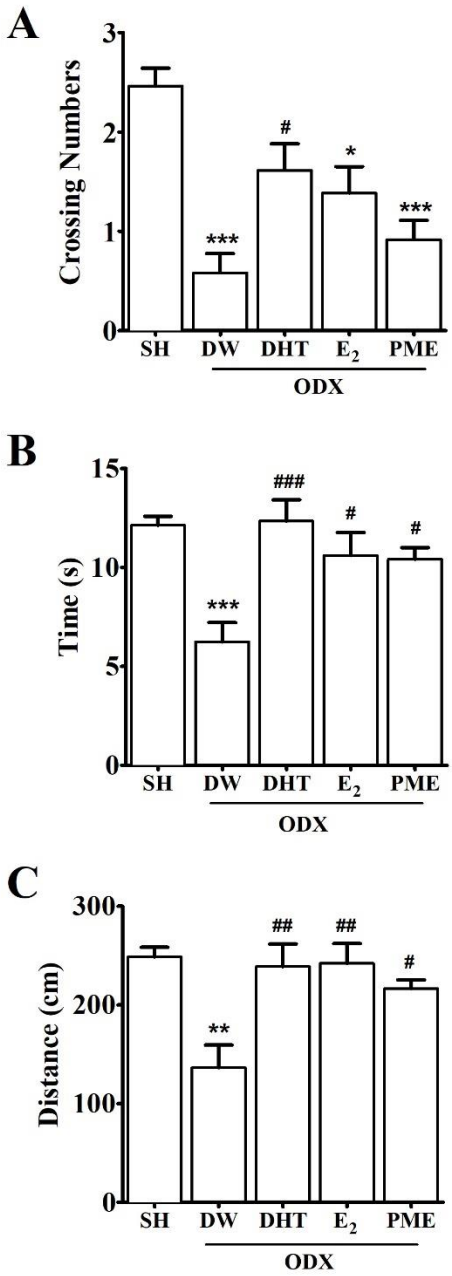


Figure 5.2 The spatial memory capacity of sham (SH) rats, and orchidectomized (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months which were assessed one day before the termination of the treatments (or day-6 of the MWM test program) using the Morris Water Maze task. The crossing numbers, time and distance spent in targeted area are shown in A, B, and C, respectively. *, **, and *** represent $p < 0.05$, 0.01, and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, 0.001, respectively compared to the ODX-DW rats.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with hippocampal synaptic plasticity

Orchidectomy-induced androgen deficiency in the ODX-DW rats significantly decreased mRNA expression levels of *Syn* and *Bdnf*, but increased *M_1-mAChR* and *α7-nAChR* mRNA levels compared to the SH rats (Figure 5.3). Treatment with DHT in the ODX-DHT rats prevented the reduction in mRNA expression levels of *Syn* (p = 0.066) and *Bdnf* ($p < 0.01$) and the increase in *M_1-mAChR* ($p < 0.001$) and *α7-nAChR* ($p < 0.001$) mRNA levels compared to the ODX-DW rats. It is noteworthy that the *M_1-mAChR* and *α7-nAChR* mRNA levels were significantly–lower than the SH rats (Figure 5.3). This indicates that treatment of DHT at a dose of 1mg/kg/day for 2 months elicited the greater effects on synaptic plasticity of hippocampal neurons than the physiological level of the DHT in the SH rats. The treatment of E_2 and PME showed the similar patterns of changes on preventing: a decrease in *Syn* and *Bdnf* mRNA levels and an increase in *M_1-mAChR* and *α7-nAChR* mRNA levels induced by orchidectomy, and all values were comparable to those of the SH rats (Figure 5.3). While DHT exhibit the grater effects on decreasing *M_1-mAChR* ($p < 0.05$) and *α7-nAChR* ($p < 0.01$) than E_2 and PME, it was E_2, but not PME, significantly increased *Syn* over DHT ($p < 0.05$). The ability of preventing the reduction in *Bdnf* of DHT, E_2 and PME were statistically comparable. These results indicated that the recovery of the orchidectomy-induced deterioration of the synaptic plasticity in hippocampus of male rats was greater in DHT followed by E_2 and PME (DHT > $E_2 \geq$ PME). Interestingly, no changes of *GluN1* mRNA levels were detected in any groups of rats.

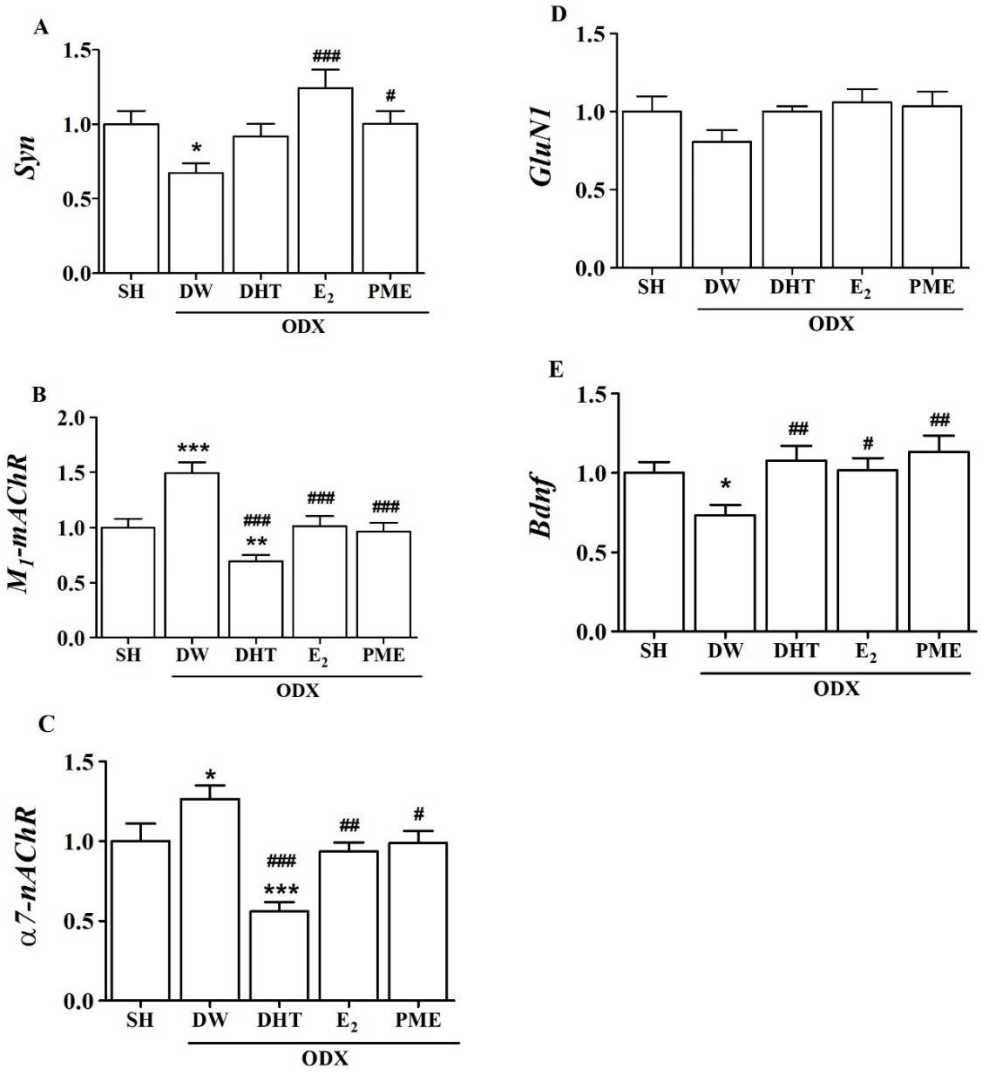


Figure 5.3 The mRNA expression levels of genes associated with synaptic plasticity, *Syn* (A), *M_1-mAChR* (B), *α7-nAChR* (C), *GluN1* (D), and *Bdnf* (E), in hippocampus of sham (SH) and orchidectomized (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data show the fold change in mRNA expression levels relative to the mean of SH rats. *, **, and *** represent $p < 0.05$, 0.01, and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001, compared to the ODX-DW rats.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with neurofibrillary tangles

Orchidectomy-induced androgen deficiency significantly increased the mRNA expression levels of *Tau3* and *Tau4* in ODX-DW rats compared to the SH rats (Figure 5.4). Treatment with DHT, E_2 and PME prevented the significant increases of *Tau3* and *Tau4* in the ODX-DHT, ODX-E_2 and ODX-PME rats induced by the ODX and the levels were not significant difference from those of the SH rats, except that the values in the ODX-E_2 rats remained higher than and showed significant differences from the SH rats (Figure 5.4). While *Tau3* and *Tau4* mRNA levels of ODX-DHT rats and ODX-PME rats were not significantly different, those of ODX-DHT rats, but not ODX-PME rats, were significanty lower than that of ODX-E_2 rats ($p < 0.05$). These results indicated that DHT and PME could mitigate the increases in *Tau3* and *Tau4* mRNA levels better than that the E_2 did (DHT≥PME>E_2).

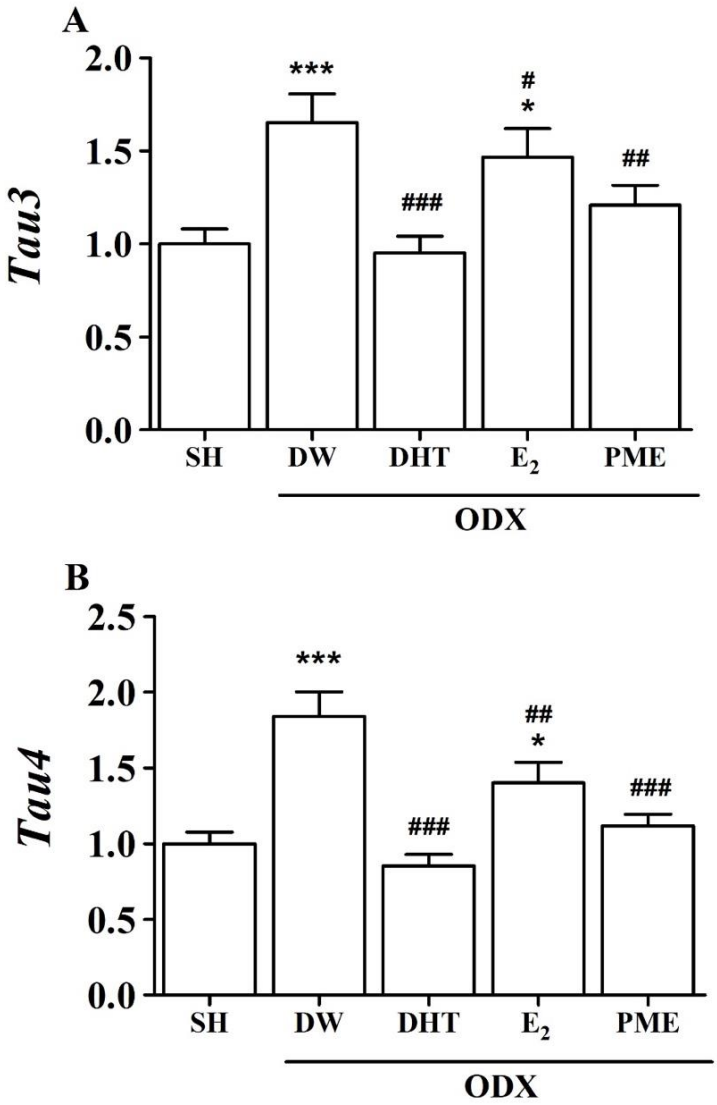


Figure 5.4 The mRNA expression levels of genes associated with neurofibrillary tangles, *Tau3* (A), and *Tau4* (B), in hippocampus of sham (SH) and orchidectomized (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data show the fold change in mRNA expression levels relative to the mean of SH rats. * and *** represent $p < 0.05$ and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001 compared to the ODX-DW rats.

Effect of DHT, E_2 and PME on protein expression levels of Tau-5 and p-Tau Ser^{396}

Aligning with changes of *Tau3* and *Tau4* mRNA levels, the orchidectomy-induced androgen deficiency significantly increased the protein expression levels of Tau-5 and p-Tau Ser^{396} in the ODX-DW rats compared to the SH rats (Figure 5.5B and C). Treatment with DHT, E_2 and PME prevented the increase in Tau-5 and p-Tau Ser^{396} in the ODX-DHT, ODX-E_2 and ODX-PME rats, and the levels were not significantly different from those of the SH rats, except that the Tau-5 protein level in the ODX-E_2 rats remained higher than and showed a significance from that of the SH rats (Figure 5.5B and C). While Tau-5 and p-Tau Ser^{396} protein levels of ODX-DHT rats and ODX-PME rats were not significantly different, those of both groups were significanty lower than that of ODX-E_2 rats ($p < 0.05$). These protein expression levels confirmed the results of mRNA expression levels mentioned above that the DHT and PME could mitigate the formation of the neurofibrillary tangle better than the E_2 (DHT $\geq$ PME $>E_2$).

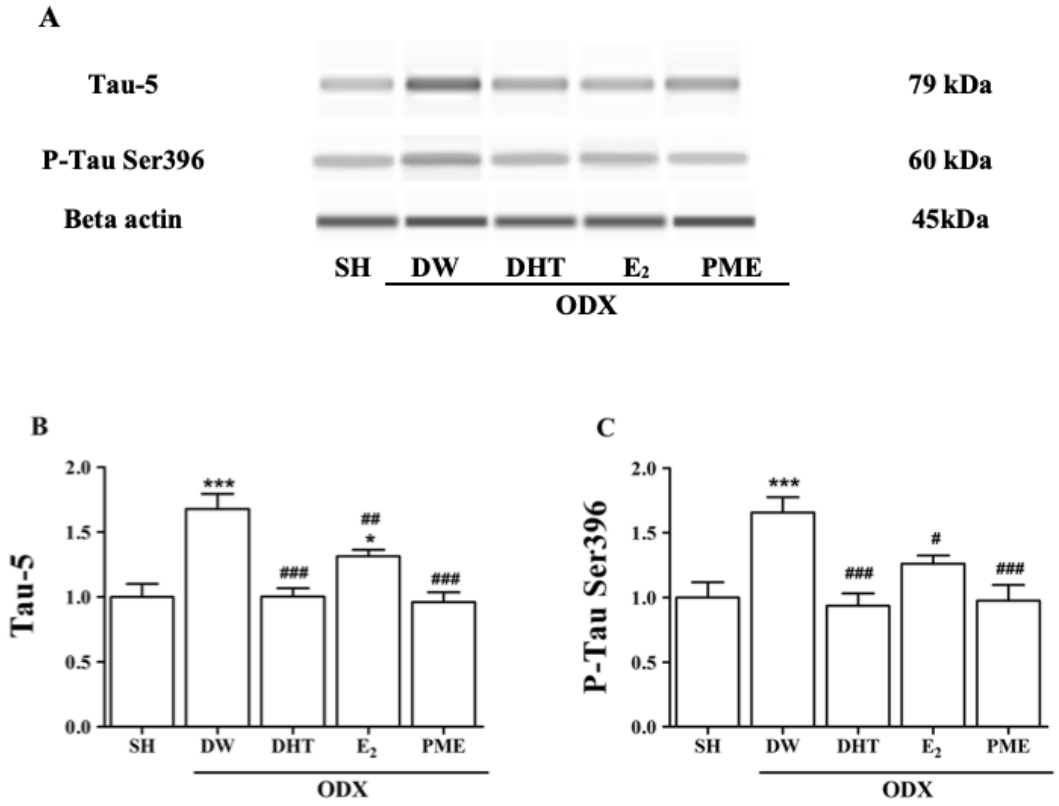


Figure 5.5 The protein expression levels of Tau-5, and p-Tau Ser^{396} in hippocampus of sham (SH) and orchidectomized (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.Representative western blots of Tau-5 and p-Tau Ser^{396} and beta actin are shown in (A). The quantification of the western blot signals of Tau-5 and p-Tau Ser^{396} relative to beta actin are shown in (B) and (C), respectively. All data are the fold changes in protein expression levels relative to the mean of SH rats. * and *** represent $p < 0.05$ and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001 compared to the ODX-DW rats.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with amyloid plaques

The mRNA expression levels of *App*, *Bace1*, and *Adam10* were not significantly different between SH, ODX-DW, ODX-E_2 and ODX-PME groups (Figure 5.6), though they were slightly decreased in the ODX-PME group, indicating that orchidectomy-induced androgen deficiency and treatment of E_2 and PME for 2 months had no effect on expression levels of genes associated with amyloid plaques in hippocampus. Only the treatment of DHT in the ODX-DHT rats could significantly lower the mRNA expression levels of *App* and *Bace1* compared to those of the SH and ODX-DW, and these effects were significantly greater than that of ODX-E_2 rats ($p < 0.01$ for *App*; $p < 0.01$ for *Bace1*), but not for ODX-PME rats ($p = 0.174$ for *App*; $p = 0.186$ for *Bace1*) (Figure 5.6A and B). These results pointed that DHT may ameliorate the formation of the amyloid plaques via suppression of the amyloidogenic pathway (DHT > PME ≥ E_2).

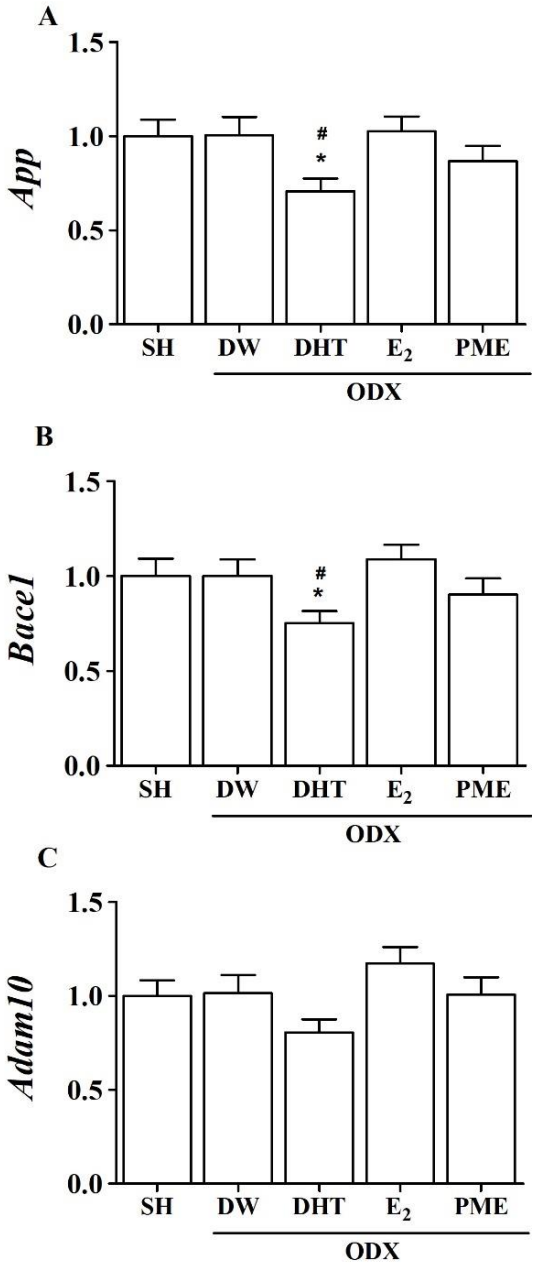


Figure 5.6 The mRNA expression levels of genes associated with amyloid plaques, *App* (A), *Bace1* (B), and *Adam10* (C), in hippocampus of sham (SH) and orchidectomized (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data show the fold change in mRNA expression levels relative to the mean of SH rats.* represent $p < 0.05$ compared to the SH rats. # represent $p < 0.05$ compared to the ODX-DW rats.

Discussion

Androgen deficient condition induced by orchidectomy in the ODX-DW rats was confirmed by weight losses of androgen responsive organs, including prostate gland, seminal vesicles and epididymis, because these organs require androgens to maintain their structure and function. Accordingly, an androgenic activity of hormonal treatments could be tested using this biological assay. Only DHT could prevent the weight loss of these accessory sex organs after orchidectomy. This confirmed that 1 mg/kg BW/day of DHT treatment could exhibit the physiological sufficiency in androgenic activity, while E_2 and PME did not show such an activity. Previously, 80µg/kg BW/day of E_2 and 100mg/kg BW/day of PME were reported their estrogenic activity on regulating an estrogen-dependent accessory sex organ, i.e., uterus, in female rats (Anukulthanakorn et al., 2016).

The neuroprotective effects of testosterone treatment were previously reported. The prevention of the impairment of the spatial learning and memory assessed by MWM test in ODX rats (Frye et al., 2010; Hodosy et al., 2012; Moghadami et al., 2016; Pintana et al., 2015) and mice (Fang et al., 2017) was used as an indicator; however, its effects were absent in some other reports (Mohammadi-Farani et al., 2015; Spritzer et al., 2011). The contradictory results could be due to the age of animals (Frye et al., 2010), administrating routes, doses and durations of testosterone treatment (Hodosy et al., 2012; Moghadami et al., 2016; Spritzer et al., 2011; Spritzer et al., 2013). This study revealed that 2-month-orchidectomy-induced androgen deficiency impaired the spatial learning and memory capacity in adult male rats, and 1 mg/kg BW/day of DHT treatment could rescue the impairment. This finding was consistent with the previous report using a similar dose of DHT, route of administration and duration of treatment in the ODX SAMP8 mice (Kang et al., 2014) and in the ODX 3xTgAD mice (George et al., 2013). Further, it confirms that an immediate administration of DHT after the drop of endogenous androgen levels could effectively prevent the cognitive impairment in males.

Since the action of testosterone can occur through two forms of its metabolites, either DHT or E_2, the possible androgenic and estrogenic pathways were thus investigated in this study. This study disclosed that testosterone acted in both androgenic (via DHT) and estrogenic (via E_2) pathways on prevention of the

cognitive impairment in orchidectomy-induced androgen deficient rats. Previously, the neuroprotective effects of DHT (George et al., 2013; Kang et al., 2014) as well as E_2 (Hodosy et al., 2012) on cognitive impairment in male rodents were also reported. Further, the present study explored the different levels of efficacy of DHT and E_2 on prevention of cognitive impairment determined by MWM test and their different mechanisms of action on expression of genes associated with synaptic plasticity, and formation of neurofibrillary tangle and amyloid plaques in ODX rats. Comparing between DHT and E_2, treatment of DHT had a greater efficacy than the E_2 on spatial learning behavior and memory capacity of the ODX rats, indicating by all behavioral parameters assessed using MWM task. It was reported that an inhibition of endogenous E_2 synthesis by administration of aromatase inhibitor (letrozole) for 7 days did not have any adverse effect on the spatial learning and memory in male mice (Vierk et al., 2015), but if the treatment became longer for 4 weeks, the cognitive function was significantly impaired (Zhao et al., 2018). Taken together, it can conclude that DHT is a major form of testosterone that exerts a crucial role on cognitive function in male rats and E_2 is an ancillary form.

Referring to CHAPTER IV, an investigation of biochemistry in hippocampus of the ODX rats revealed that the onset of the chronological molecular changes (at mRNA levels) of synaptic plasticity and neurofibrillary tangle could be detected within 1 to 9 days after castration as follows; *Syn* (decrease) at day-1, *GluN1* (increase) at day-3, *α7-nAChR*, *M1-mAChR* and *Tau3* (increase) at day-6, *Tau4* (increase) and *Bdnf* (decrease) at day-9, respectively (Fainanta et al., 2019). In the present study, a silimar pattern of changes in those markers were also observed in the ODX-DW rats at 2 months after castration in comparison with the SH intact rats, suggesting that the impairment of spatial learning and memory in the orchidectomy-induced androgen deficient rats has been first involved with the perturbation of synaptic transmission and structure in the hippocampus.

Treatment with DHT showed the greater efficacy than the E_2 in all three neurological hallmarks of cognitive impairment: synaptic plasticity, neurofibrillary tangle and amyloid plaques. Apart from the recovery of the *Syn* and *Bdnf* mRNA levels to resemble those of the SH rats, only DHT could decrease *α7-nAChR* and *M1-mAChR* mRNA levels to be lower than those of the SH rats. This implies that the

release of presynaptic neurotransmitter and the responses of its postsynaptic receptors are simultaneously deceased. In agreement with the present study, it has been reported that a 2-week orchidectomy increased the synaptic transmission, determined by electrophysiological recordings in Mossy fiber pathway (dentate gyrus to CA3), in hippocampal slices isolated from adult male rats which was comparable to that of the SH rats, and the effect was suppressed by DHT treatment (Skucas et al., 2013). Besides, applying of an AR inhibitor (flutamide) could increase synaptic transmission and induce long-term potentiation (LTP) investigated in Schaffer collateral pathway of hippocampal slice isolated from male rats (Pettorossi et al., 2013). Taken together, DHT possibly exhibits a suppressive effect on synaptic transmission in hippocampus of ODX male rats via ARs pathway which has been hypothesized as the mechanisms for preventing a hyperexcitability of male hippocampal neurons (Harley et al., 2000; Skucas et al., 2013). In contrast to the DHT, E_2 treatment could maintain the synaptic transmission in male hippocampus in a comparable manner with the SH rats as indicated by a non-significant difference of mRNA expression levels of *α7-nAChR* and *M_1-mAChR* between ODX-E_2 and SH rats. Several previous reports showed that E_2 could increase the synaptic transmissions and facilitate induction of LTP in hippocampal slices isolated from male rodents (Foy et al., 1999; Kramár et al., 2009; Tanaka and Sokabe, 2013), and the effects were reversed by application of aromatase (Grassi et al., 2011; Tanaka and Sokabe, 2012) and ERs inhibitors (Pettorossi et al., 2013). The action of E_2 on facilitating synaptic transmission in male hippocampus has been proposed to mediate through kinase dependent signaling mechanisms (Hasegawa et al., 2015). The present study showed that the treatment with both DHT and E_2 could prevent a decrease in *Bdnf* expression in the ODX rats, which has been proposed to involve with the strengthening of synaptic contact and increased the synaptic spine density in hippocampus (Hatanaka et al., 2015; Kang et al., 2014; Soma et al., 2018). However, it has been noted that E_2 increases mostly the small-head synaptic spines, implying less strength synaptic connection (Hasegawa et al., 2015), whereas DHT increases mostly the large- and middle-head synaptic spines (Hatanaka et al., 2015).

DHT treatment could completely prevent the overexpression of *Tau3* and *Tau4* mRNA, and total tau and phosphorylated form of tau protein induced by

orchidectomy (see the increase in the ODX-DW rats), and the lowered levels of mRNA and protein in the ODX-DHT rats were comparable to those of the SH rats. Similarly, DHT treatment in the 3xTgAD mice showed the same trend (Mendell et al., 2020; Rosario et al., 2010). Comparing to the DHT, although E_2 could prevent an overexpression of *Tau3* and *Tau4* mRNA and total tau protein, the effects were only partially and the levels were remained higher than the SH rats. It was proposed that E_2 prevented the phosphorylation of tau protein via suppression of activity of the enzymes involved in tau phosphorylation such as protein kinase A (Liu et al., 2008) and glycogen synthase kinase 3β (Goodenough et al., 2005).

Only DHT treatment could decrease mRNA expression levels of *Bace1* and *App* and the levels were even lower than the SH rats, implying that DHT reduces a catalytic process of amyloid peptide. The advantage of androgen on decreasing amyloid peptide levels in hippocampus had been reported in male rats (Ramsden et al., 2003; Yao et al., 2008), 3xTgAD mice (Rosario et al., 2010; Rosario et al., 2006), and SAMP8 mice (Kang et al., 2014). In sum, the neuroprotective effects of DHT exerting via an androgenic pathway were mainly through maintaining a synaptic transmission and structure, and preventing a formation of neurofibrillary tangle and amyloid plaques in male hippocampus, while those of E_2 exerting via an estrogenic pathway were particularly on maintaining a synaptic structure and partly on preventing a formation of neurofibrillary tangle. Thus, it seems to be that DHT should be a primary active agent to use to prevent a cognitive impairment in androgen deficient males, and E_2 should be an ancillary drug.

Although uses of DHT and E_2 to prevent the cognitive impairment for androgen deficient or elderly men are promising, the synthetic hormones can cause many undesirable side effects such as venous thromboembolism, ischemic stroke (Getahun et al., 2018), cardiovascular toxicity (Phillips et al., 2014) and erythema nodosum (Coyle et al., 2015a), and the clinical consequences of prolonged use remained poorly understood (Cox and Crawford, 1995; Coyle et al., 2015b). Thus, the use of natural products to remedy or ameliorate the cognitive impairment has attracted attention of the modern world people. Recently, the neuroprotective effects of PME on spatial learning and memory in ovariectomized rats had been reported (Anukulthanakorn et al., 2016). Since PME has been renowned for its phytoestrogenic constituents and its

high estrogenic activity which has high similarity in chemical structure to E_2, especially a phenolic ring, a prerequisite for ERs binding (Malaivijitnond, 2012; Moreira et al., 2014), its effects on cognitive function was therefore proposed to be resemble those of E_2. Comparing the effects of PME on spatial learning and memory, and expression of genes (at mRNA and protein levels) associated with synaptic plasticity, neurofibrillary tangle and amyloid plaques to those of DHT and E_2, it was found that PME exerts the lowest efficacy on spatial learning and memory, but were in-between DHT and E_2 for other effects.

Treatment of PME to the ODX rats could ameliorate the expression of *Syn*, *α7-nAChR*, and *M1-mAChR* and kept comparable levels to those of the ODX-E_2 rats. This denotes that PME could maintain the synaptic transmission of the hippocampus of the ODX rats. The present findings agreed with the previous report showing that application of PME on primary rat's hippocampal culture increased the expression of synaptophysin, and the effect was blocked by ERs inhibitor (Chindewa et al., 2008). In addition to the synaptic transmission, PME could prevent an overexpression of *Tau3, Tau4* mRNA level and total tau and phosphorylated tau protein levels in a comparable manner with that of the DHT. Although it showed a non-significant decrease in *App* and *Bace1* mRNA level, the effects of PME for prevention of the formation of the amyloid plaques were in the intermediate of DHT and E_2. That is DHT suppressed the mRNA expression of *App* and *Bace1* and no changes were detected for E_2 treatment. PME had also been reported to enhance neuronal morphology, cell viability and cell number, and such effects have been proposed to regulate via the antioxidant ability (Sucontphunt et al., 2011). The major active phytoestrogenic compounds of PME quantified in this study (as indicated in Materials and method) were puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin, and daidzein. It had been reported that puerarin could enhance the synaptic transmission in hippocampus determined by electrophysiological recording (Li et al., 2019), and decreased acetylcholinesterase activity but increased choline acetyltransferase activity (Liu et al., 2015). Furthermore, puerarin (Tantipongpiradet et al., 2019) and miroestrol (Monthakantirat et al., 2014) have been reported to increase *Bdnf* mRNA expression in hippocampus which was related to the increase in neurogenesis ability. Moreover, puerarin could significantly decrease

hyperphosphorylation at Ser^{396} site of tau protein in the rat's hippocampus (Hong et al., 2016). Taken together, it is interesting to promote the development of PME as a phytopharmaceutical product to prevent the cognitive impairment in androgen deficient men in the future.

P. mirifica is a Thai endemic medicinal plant and its consumptoin by local communities has been long recorded over a century for rejuvenating purpose in menopausal women and andropasal men (Kerr, 1932; Suntara, 1931). Based on the precilical research in laboratory animals and clinical studies in women, the amount of consuming *P. mirifica* powder that are effective on alleviating female climacteric symptoms and safe has been suggested at 1-2 mg/kg BW/day or 50-100 mg/day (Chandeying and Lamlertkittikul, 2007; Cherdshewasart, 2003; Chivapat et al., 2005; Manonai et al., 2008; Muangman and Cherdshewasart, 2001a; Saenphet et al., 2005). While the oral administration of *P. mirifica* powder or crude extract at upto dose of 2000 mg/kg BW in male rodents did not show an acute toxicity on mortality (Cherdshewasart, 2003; Mohamad et al., 2019), it has been reported that the long-term treatment of 250 mg/kg BW/day *P. mirifica* for 6 months in male rats reduced hematocrit, red blood cells, hemogloblin and weight of testes (Chivapat et al., 2005). Moreover, treatment of *P. mirifica* at doses of 600-800 mg/kg BW/day for 30 days induced micronucleus cells in bone marrow of male rats (Saenphet et al., 2005), but this effect was absent in rats treated with lower dose at 300 mg/kg BW/day (Cherdshewasart et al., 2009). Thus, the further research for the safety and optimal doses of using *P. mirifica* in men should be studied.

In summary, DHT, E_2 and PME exhibited neuroprotective effects in orchidectomy-induced androgen deficient male rats. DHT showed the strongest efficacy. The mechanism of actions of each treatment, however, was slightly different. The DHT prominently maintained the synaptic transmission and structure, possibly suppressed the formation of neurofibrillary tangles and amyloid plaques in hippocampus. On the other hand, administration of E_2 for 2 months mainly maintained synaptic structure but less ability on suppressing the neurofibrillary tangles and no effect on amyloid plaques. Interestingly, PME could either maintain synaptic function and structure and suppress the neurofibrillary tangles, although its efficacy on preventing the cognitive impairment was slightly lower than both DHT

and E_2. Thus, PME has a high potential as an alternative to hormonal treatment to prevent cognitive impairment in males.

CHAPTER VI
NEUROTHERAPEUTIVE EFFECTS OF DIHYDROTESTOSTERONE, 17β-ESTRADIOL, AND *Pueraria mirifica* EXTRACT IN COGNITIVE IMPAIRED MALE RATS

Introduction

Cognitive impairment is related to progressive brain dysfunction that slowly destroys memory and thinking skills leading to disturbance of independency and mostly affects the elderly people (Rajan et al., 2021). Currently, the available pharmacological treatments for moderate to severe stages of cognitive impairment that have been approved by the US-FDA are acetylcholinesterase inhibitors (i.e., Donepezil, Galantamine, and Rivastigmine) and NMDA receptor antagonist (i.e., Memantine). These drugs are prescribed for counterbalance the disturbance of neurotransmitter. Although these drugs provide the symptomatic benefits, they cannot reverse the process of neurodegeneration in the brain. As the brain deteriorate over time, cognitive impaired patients are eventually inevitable of fatal. Thus, the most ideal strategy is the disease-modifying drugs that are capable to intervene the progression of pathogenesis, e.g., loss of synaptic plasticity, formation of neurofibrillary tangles and amyloid plaques (Cummings et al., 2021; Marasco, 2020). Over the past 20 years, more than 50 drug candidates against cognitive impairment had been tested, but none has passed phase III clinical trial (Bachurin et al., 2017). Up to now, one hundred and twenty-eight agents are being tested in 152 clinical phase trials (Cummings et al., 2021). The possible reason of failures in the late phase clinical trials of drug candidates might be linked to the pathogenesis of cognitive impairment of which the precise molecular mechanisms are not yet fully understood. Moreover, the brain biochemistry changes may begin ambiguously years to decades prior to clinically detectable symptoms (Sperling et al., 2013), and the suspected patients rather come to consult with the clinician when their clinical symptoms are beyond the reversed phase.

Androgen deficiency which commonly occurred along with male reproductive senescence is considered as an important risk factor of cognitive impairment in elderly men (Gauthierm, 2021; Moffat et al., 2004; Yeap et al., 2008). Sex hormone supplementation is therefore counted as practical strategies to cure the symptoms.

However, administration of testosterone to cognitive impaired men who had low testosterone levels was either enhancement (Cherrier et al., 2015; Cherrier et al., 2005b) or no effect on cognitive function (Kenny et al., 2004; Lu et al., 2006; Resnick et al., 2017). The inconsistent findings might be related to the different timing of treatment, the individual variations of endogenous testosterone levels, the degree of cognitive impairment, and the heterogeneous progression of pathologies in the brain. It should be noted that the nature of male reproductive senescence occurred by a gradual decline in testosterone levels which are nearly impossible to detect a critical point of reduction. While the onset of menopausal transition in women has been suggested as a window of opportunity for therapeutic interventions to ameliorate the cognitive impairment (Henderson et al., 1994; Kantarci et al., 2016; Scheyer et al., 2018), the right time for manipulating a hormonal supplementation is more challenging in men.

The important roles of androgens on neuroprotective effects have been previously confirmed in orchidectomy-induced androgen deficient rodents which were treated either with testosterone (Frye et al., 2010; Hodosy et al., 2012; Pintana et al., 2015) or its active metabolized forms: DHT and E_2. Both DHT and E_2 regulated the hippocampal function, which is responsible for learning and memory, after binding with ARs and ERs, respectively. DHT exerted the neuroprotective abilities on maintaining synaptic structure, preventing hyperphosphorylated tau and decreasing amyloidogenic pathway (Kang et al., 2014; Rosario et al., 2010). E_2 could effectively maintain synaptic transmission and mildly prevent hyperphosphorylated tau protein (Rosario et al., 2010; Vierk et al., 2015).

Recently, it was reported that the crude extract of the *P. mirifica* (PME) could cure the early and late stage of cognitive impairment in estrogen deficient female rats (Anukulthanakorn et al., 2016). As reported in CHAPTER V, PME could prevent a cognitive impairment in orchidectomy-induced androgen deficient rats through the maintenance of synaptic plasticity and the decrease in formation of neurofibrillary tangle in hippocampus. With these promising effects of PME on cognitive impairment, the present study aimed to investigate the neurotherapeutic effects of oral administrations of PME in the 2-month-orchidectomy-induced cognitive impaired rats in comparison with the DHT and E_2 injection. Changes in the transcriptional

expression levels of genes associated with synaptic plasticity, neurofibrillary tangles, and amyloid plaques, and protein expression levels of total tau protein and phosphorylated tau at the Ser^{396} site together with spatial learning and memory are determined in this study.

Materials and methods

Animal subjects and ethical note

The animal experiments were conducted under the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes issued by the National Research Council of Thailand and were approved by the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (Protocol review number 17-33-007).

Two-month-old male Sprague-Dawley rats were purchased from the Nomura Siam International Co., Ltd., Bangkok, Thailand. They were housed (two rats/cage) in a strictly hygienic conventional housing system with controlled temperature of 24±1°C, 12-h light /dark cycle (lights on at 06:00-18:00 h), and relative humidity of 40-60% at the Laboratory Animal Research Building, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The rats were provided access to rat chow diet (Perfect Companion Group Co. Ltd, Thailand) and reversed osmosis water *ad libitum*. They were reared until 4 months old, and used in this study as reported previously for female rats (Anukulthanakorn et al., 2013).

Preparation of the chemicals

The powder of DHT (#A0462, ≥99.0% purity, Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) and E_2 (#E8875, ≥98% purity, Sigma-Aldrich Co., Ltd., MO, USA) were weighed and dissolved in a small volume of absolute ethanol. The dissolved solution was added with the olive oil and allowed to stand at room temperature to evaporate the ethanol. This stock solution was then diluted with olive oil to give a final concentration of 4 mg/ml of DHT and 0.32 mg/ml of E_2. A dose of 80 µg/kg/day of E_2 was selected for this study following the previous report in estrogen deficient female rats (Anukulthanakorn et al., 2016; Feng et al., 2004). A dose of 1 mg/kg/day of DHT was used in this study because it was reported to prevent a cognitive impairment in androgen deficient male mice (Kang et al., 2014).

The PME was prepared from *P. mirifica* powder (lot no.141023) kindly provided by Dr. I. Sandford Schwartz, Smith Natural Co. Ltd., Thailand. *P. mirifica* powder was extracted twice by 95% ethanol solvent using a soxhlet apparatus at 80 °C for 8 hours in each time. The extracted solution was filtered through a filter paper (Whatman No. 4) equipped with vacuum suction pump and dried using rotary vacuum evaporator at 40 °C, 70 rpm. The remaining solvent mixed in crude extraction was evaporated again at 25 °C until the extracted weight was no further changed and provided a 9.95% of yield. The dried PME was kept in a dark bottle and stored at -20°C until used. The stock PME was suspended in distilled water to give a final concentration of 80 mg/ml for the treatment. A dose of 100 mg/kg/day of PME was used in this study because it exhibited the neurotherapeutic effects on cognitive impairment in female rats (Anukulthanakorn et al., 2016).

Phytochemical analysis of PME

The quantitative analysis of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein contents in the PME was performed using liquid chromatography tandem mass spectrometry (LC/MS/MS) at Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The standards of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein were dissolved in DMSO and diluted with methanol. One hundred milligram of PME was dissolved in 1 ml of DMSO, and then diluted 100-fold in methanol. The diluted PME samples were mixed with a 10-fold volume of methanol containing 562.5 ng of glycyrrhetinic acid as an internal standard for the LC/MS/MS analysis, performed using a Nexera UHPLC 100 LC equipped with a Shimadzu 8060 LCMS, controlled by Labsolution software version 5.86 (Kyoto, Japan). The UHPLC system was equipped with a Synergi Fusion-RP C18 column as the stationary phase (Phenomenex Inc, Torrance, CA, USA), while the mobile phase was a methanol: water gradient starting at 10% (vol/vol) methanol for 0.5 min, increased linearly to 90% (vol/vol) methanol at 1.5–3.5min, and then decreased linearly to 10% (vol/vol) methanol at 4–6min. The retention times of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin, daidzein and glycyrrhetinic were 1.515, 1.663, 1.733, 1.650, 1.822, 1.583, 1.762 and 2.206 min, respectively. The MS analysis was performed with negative mode ionization for detection of puerarin

(m/z, 415.05/267.00), miroestrol (m/z, 356.90/189.20), deoxymiroestrol (m/z, 341.00/265.15), daidzein (m/z, 253.00/208.05) and glycyrrhetinic acid (m/z, 469.35/409.40), and with positive mode ionization for detection of genistin (m/z, 433.05/271.05), genistein (m/z, 270.95/153.00) and daidzin (m/z, 417.00/255.10). Calibration curves of puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein showed good correlation coefficients (R^2>0.996) over the concentration range of 5-1000 lg/L. The limit of detection of these compounds was estimated to be 1 µg/L with a signal-to-noise ratio of 5. The intra-assay accuracy and precision for analysis of these compounds were within ±10%. The puerarin, miroestrol, deoxymiroestrol, genistin, genistein, daidzin and daidzein contents were 35.05, 2.29, 0, 19.42, 8.11, 40.16, 14.58 mg, respectively, in 100g PME.

Experimental design

The 4-month old rats were surgically removed the testes under sodium pentobarbital anesthesia (40-50 mg/kg. i.p.) and kept for 2 months to induce the cognitive impairment according to the previous report (see CHAPTER V). Then, ODX rats were divided into 4 groups (13 rats/group) and orally treated with 1 ml/day of distilled water (ODX-DW group), subcutaneously injected with 1 mg/kg/day of DHT (ODX-DHT group), subcutaneously injected with 80µg/kg/day of E_2 (ODX-E_2 group) and orally treated with 100 mg/kg/day of PME (ODX-PME group) for 2 months. Sham control (SH) rats were surgically operated as did in the ODX rats, but their testes were kept intact.

Rats were assessed the cognitive function in spatial learning and memory using MWM task for six consecutive days before the end of the treatment. At the end of the experiment, rats were euthanized, the hippocampus was collected for analysis of mRNA expression levels using qrtRT-PCR technique and protein expression levels using western blot technique. The accessory sex organs (prostate gland, epididymis and seminal vesicles) were collected, trimmed off fats, weighed and recorded.

Assessment of learning behavior and memory capacity using MWM test

The MWM task was used to assess the hippocampal learning and memory in rats. The test was conducted in a circular pool making from composites resin (180 cm in diameter and 50 cm in height) filled with water at temperature of 22-25°C. The pool was divided geographically into four quadrants (NE, NW, SE and SW), and a

transparent circular platform making from acrylic (10 cm in diameter) was hidden submerged 1 cm below the surface of the water at the center of the target quadrant (at NW). Four distinctive stable external cues were place around the pool. The ambient light was set at 50 lux on the water surface. The video camera was mounted above the center of maze and linked to a computer to record the swimming path data using a video tracking system (Smart JUNIOR®, Panlab-Harvard Apparatus, Barcelona, Spain). The learning behavior test was performed consecutively on day-1 to day-5, and the memory capacity test was performed on day-6 as described previously (Vorhees and Williams, 2006). Each day of the learning behavior test consisted of four trials (90 s each) with a 30 min interval between trials. If any rat failed to locate the platform within 90 s, it was gently guided to the platform and rested for 30 s, and a time of 90 s was recorded. A video tracking system was used to record (i) the latency to find the hidden platform (or escape latency), (ii) the distance to arrive at the hidden platform (or travel distance), and (iii) swimming patterns (Line, Taxis, Random, and Circle) based on the report of Anukulthanakorn et al. (2013). After the rats completed the learning behavior test on day-5, they were continuously assessed the memory capacity test using the spatial probe test on day-6. For the spatial probe test, the platform was removed from the pool to check the memory of the animal for the platform location. The rat was released to the pool at the quadrant opposite to the previously existing platform location and allowed to swim for 30 s. During those 30 s, the number of crossings to the previously existing platform location, and the time and distance spent in the targeted quadrant, where the platform used to be located, were recorded using the video tracking system.

qrtRT-PCR for determination of the mRNA expression levels of the genes associated with synaptic plasticity, neurofibrillary tangles and amyloid plaques

The left hemisphere of hippocampal brain region was isolated from the whole brain and stored at − 80 °C until RNA extraction. Total RNA was extracted from tissues using TRIzol® Reagent (Invitrogen, CA, USA) following the manufacturer's instruction. The concentration and purity of the RNA samples were determined by measuring the absorbance at a wavelength of 260 and 280 nm using a Thermo NanoDrop^{TM} One UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA), where a 260/280 ratio of 2.0 ± 0.1 was accepted for mRNA purity. The mRNA

expression levels of the genes associated with synaptic plasticity; *Syn,* subunit *GluN1, α7-nAChR, M_1-mAChR* and *Bdnf,* neurofibrillary tangles; *Tau3* and *Tau4,* and amyloid plaques; *App, Bace1* and *Adam10,* were examined using two-stage qrt-RT-PCR.

In the first RT-PCR stage, the extracted RNA (5 μg) was reverse transcribed to cDNA in a total volume of 20 μl containing 5 μl of RNA, 4 μl of RT buffer, 1 μl of dNTP mix (10 mM), 1 μl of oligo (dT)18 primer mix, 1 μl of random hexamer primer mix, 1 μl of RNase inhibitor (10 U/μl), 1 μl of reverse transcriptase (200 U/μl), and 6 μl of diethylpyrocarbonate (DEPC)-treated water using the Tetro cDNA Synthesis kit (Bioline Reagent Ltd., London, UK). The samples were incubated at 25 °C for 10 min, 45 °C for 30 min, and finally 85 °C for 5 min. The obtained cDNA was diluted in five volumes of DEPC-treated water prior to use.

The second-stage qrt-PCR amplification of the obtained cDNA was performed using the gene-specific primers as described in Table 3.1, where S28RNA was used as the reference house-keeping gene in this study. The primers were first tested for optimal annealing temperature by conventional PCR. The qrt-PCR was performed using a SensiFAST™ SYBR® kit (Bioline Reagent Ltd., London, UK) according to the manufacturer's instructions.

Each qrt-PCR reaction was performed in a final volume of 20 μl with 10 μl of SensiFast SYBR® Hi-ROX mix, 0.8 μl each of forward and reverse primers (10 μM), 5 μl of cDNA, and 3.4 μl of DEPC-treated water. The thermal cycling consisted of 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s, and then the melting curve analysis (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s before finally cooling at 25 °C for 5 min). Real-time detection of the SYBR Green fluorescence intensity, which was used to indicate the amount of PCR product, was measured at the end of each extension phase. Amplification products were quantified by the Step One Plus™ Real-Time PCR system. To confirm the amplification specificity, PCR products from each primer pair were subjected to a melting curve analysis. The amounts of PCR product of the target genes were normalized against the housekeeping gene S28RNA in the corresponding samples and analyzed by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis for tau protein expression levels

Determination of the protein expression levels of total tau protein and phosphorylated tau at the Ser^{396} site was performed using the right hemisphere of hippocampal brain region. The total protein was extracted by homogenizing the hippocampal tissue in ice-cold RIPA buffer (#89900, Thermo Scientific, MA, USA) mixed with Halt™ Protease and Phosphatase Inhibitor Cocktail (#78440, Thermo Scientific, MA, USA). The homogenate was centrifuged at 10,000×g, 4 °C for 15 min, and the supernatant was collected. The protein concentration in the supernatant was quantified by Bradford assay (#B6916, Sigma-Aldrich, MO, USA). Then, the concentration of protein lysates was adjusted to 1 µg/µl with RIPA buffer.

The targeted protein expression levels were determined by a capillary electrophoresis size-based separating using the fully automated ProteinSimple Wes system with the 12-230 kDa Wes Separation Module, 8 x 25 capillary cartridges (#SM-W004; ProteinSimple, CA, USA) following the manufacturer's instructions. Briefly, 6.4 µl of protein lysates were mixed with 1.6 µl of the 5X Fluorescent Master Mix in a ratio 4:1 and then incubated at 95 °C for 5 min.

The prepared samples, the biotinylated ladder, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies, and Luminal-Peroxide Mix were loaded into the independent wells of the plate separation module. The plate was briefly spun, loaded into the Wes instrument (ProteinSimple, CA, USA), and then the electrophoresis separation and immunodetection steps were automatically processed. Firstly, the capillaries were filled with Separation Matrix for 200 s, Stacking Matrix for 15 s and samples for 9 s with vacuum injection. Then, separation was performed at 375 volts for 25 min. After separation, the capillaries were exposed to UV light to attach the separated protein to the capillary wall and then Matrixes were removed. Subsequently, the capillaries were washed with washing buffer for three times and then blocked with blocking reagent for 30 min to prevent non-specific binding.

Targeted proteins were immunoprobed with primary antibodies including mouse monoclonal antibody Tau-5 (1:50; #ab80579, Abcam, MA, USA), rabbit monoclonal antibody Tau-phospho Ser^{396} (1:100; #ab109390, Abcam, MA, USA), and rabbit monoclonal β-actin (1:100; #13E5, Cell Signaling Technology, MA, USA) for 30 min. All primary antibodies were detected by incubated in the HRP-conjugated secondary anti-mouse provided in Anti-Mouse Detection Module Kit (#DM-002 for

Tau-5; ProteinSimple, CA, USA) or anti-rabbit antibodies provided in Anti-Rabbit Detection Module Kit (#DM-001for tau-phospho Ser^{396} and β-actin; ProteinSimple, CA, USA) for 30 min, and visualized by chemiluminescence reaction using Luminol-S and peroxide. Images were captured, and band densities of targeted proteins were analyzed using Compass software (ProteinSimple, CA, USA). The expression levels of targeted proteins were normalized against β-actin and expressed as fold-changed to SH values.

Statistical analysis

The results are expressed as mean ± standard error of mean (SEM). All data sets were performed the criteria of normal distribution and homogeneity of variance. The one-way analysis of variance (ANOVA) was used to determine the differences among treatment groups followed by the least significant difference (LSD) post hoc test. The escape latency and travel distance across five days of the spatial learning were analyzed using two-way repeated measure ANOVA followed by the Bonferroni post hoc test. The significant difference of swimming patterns was tested by the chi-square test. The level of significance was set as $p < 0.05$. Statistical analyses were performed using SPSS software version 22 (International Business Machines Corp., NY, USA)

Results

Androgenic status of the ODX rats after DHT, E_2 and PME treatment

Weighing of androgen-dependent accessory sex organs; prostate gland, seminal vesicles and epididymis, were used to indicate the androgenic status of the ODX rats and the androgenic effects of the treatments. Weights of prostate gland, seminal vesicles, and epididymis in the ODX-DW rats were significantly lower than those of the SH rats (Table 6.1), indicating an androgen deficient status of rats and confirming the complete removal of the testes. Weights of the accessory sex organs in the ODX-DHT rats were significantly higher than those of the ODX-DW rats, but they were significantly lower than those of the SH rats (Table 6.1). These results indicated that the 2-month treatment of DHT could exhibit the androgenic effects in androgen-deficient rats, but it could only partially restore the accessory sex organ weights. Following its estrogenic, non-androgenic activity, treatments of E_2 and PME did not significantly change the accessory sex organ weights comparing to those of the ODX-DW rats (Table 6.1).

Table 6.1 Weights of androgen-dependent accessory sex organs; prostate gland, seminal vesicles, and epididymis of sham (SH) rats and orchidectomized (ODX) rats treated with distilled water (DW), 1mg/kg/day of dihydrotestosterone (DHT), 80μg/kg/day of 17β-estradiol (E_2), or 100mg/kg/day of *P. mirifica* extract (PME).

Group	Weights (g)		
	Prostate gland	Seminal vesicles	Epididymis
SH	0.825 ± 0.036^{a}	1.881 ± 0.058^{a}	1.670 ± 0.048^{a}
ODX-DW	0.049 ± 0.004^{b}	0.161 ± 0.011^{b}	0.399 ± 0.020^{b}
ODX-DHT	0.231 ± 0.009^{c}	0.746 ± 0.051^{c}	0.543 ± 0.028^{c}
ODX-E_2	0.063 ± 0.005^{b}	0.212 ± 0.009^{b}	0.462 ± 0.018^{b}
ODX-PME	0.055 ± 0.005^{b}	0.188 ± 0.008^{b}	0.390 ± 0.019^{b}

All data are presented as the mean ± SEM. Means within a column followed by a different superscript letter are significantly different ($p < 0.001$)

Effects of DHT, E_2 and PME on the spatial learning behavior and memory capacity in ODX rats

After 4 months of orchidectomy-induced cognitive impairment, the spatial learning was assessed in ODX rats. The escape latency and travel distance for searching the hidden platform significantly decreased during 5 consecutive days in learning trials of the MWM task ($F = 124.305$, $p < 0.001$; and $F = 181.048$, $p < 0.001$, respectively), indicating that rats could learn the location of the platform (Figure 6.1A and B). Comparing the results of the ODX-DW rats with the rats treated with DHT, E_2 and PME, it showed the significant differences of escape latency ($F = 4.227$, $p < 0.01$) and travel distance ($F = 4.363$, $p < 0.01$) among four groups of rats. A post-hoc comparison between ODX-DW rats and the SH rats showed that the profiles of a decrease in both escape latency ($p = 0.091$) and travel distance ($p = 0.272$) were marginally, non-significantly different (Figure 6.1A and B). However, when the day-5 swimming patters between these two groups of rats were compared, it was found that the ODX-DW rats used random and circular patterns (21% line, 19% taxis 48% random and 12% circular) more frequent than the SH rats (29% line, 60% taxis, 11% random and 0% circular), and it was significantly differed ($\chi^2 = 36.033$, $p < 0.001$; Figure 6.1C). The treatment with DHT significantly decreased the escape latency and travel distance in the ODX-DHT rats compared with the ODX-DW rats (Figure 6.1A and B). The swimming patterns used by the ODX-DHT rats were mainly line and

taxis (48% line, 37% taxis, 15% random, 0% circular), which was significantly different from those of the ODX-DW rats ($\chi^2 = 22.655$, $p < 0.001$), whereas it was comparable to those of the SH rats ($\chi^2 = 5.837$, $p = 0.054$; Figure 6.1C). In contrast, the escape latency and travel distance profiles of the ODX-E_2 and ODX-PME rats were not significantly different from either the ODX-DW rats or the SH rats (Figure 6.1A and B). However, the swimming patterns of line and taxis used by the ODX-E_2 (25% line, 43% taxis, 36% random, 0% circular) and ODX-PME rats (19% line, 46% taxis, 35% random, 0% circular) were significantly higher than the ODX-DW rats ($\chi^2 = 10.727$, $p < 0.05$ for ODX-E_2; $\chi^2 = 12.404$, $p < 0.01$ for ODX-PME), but it was significantly lower than the SH rats ($\chi^2 = 10.617$, $p < 0.01$ for ODX-E_2; $\chi^2 = 10.442$, $p < 0.01$ for ODX-PME; Figure 6.1C). While the escape latency and travel distance of ODX-DHT, ODX-E_2 and ODX-PME rats were not significantly different, ODX-DHT rats used line and taxis swimming patterns more than those of ODX-E_2 rats ($\chi^2 = 9.518$, $p < 0.01$) and ODX-PME rats ($\chi^2 = 10.692$, $p < 0.01$) and the proportion of patterns used by ODX-E_2 and ODX-PME rats were not significantly different ($\chi^2 = 0.076$, $p < 0.963$). This indicated that the spatial learning behavior in cognitive impaired rats could be ameliorated after treatment with DHT followed by E_2 and PME (DHT > $E_2 \geq$ PME).

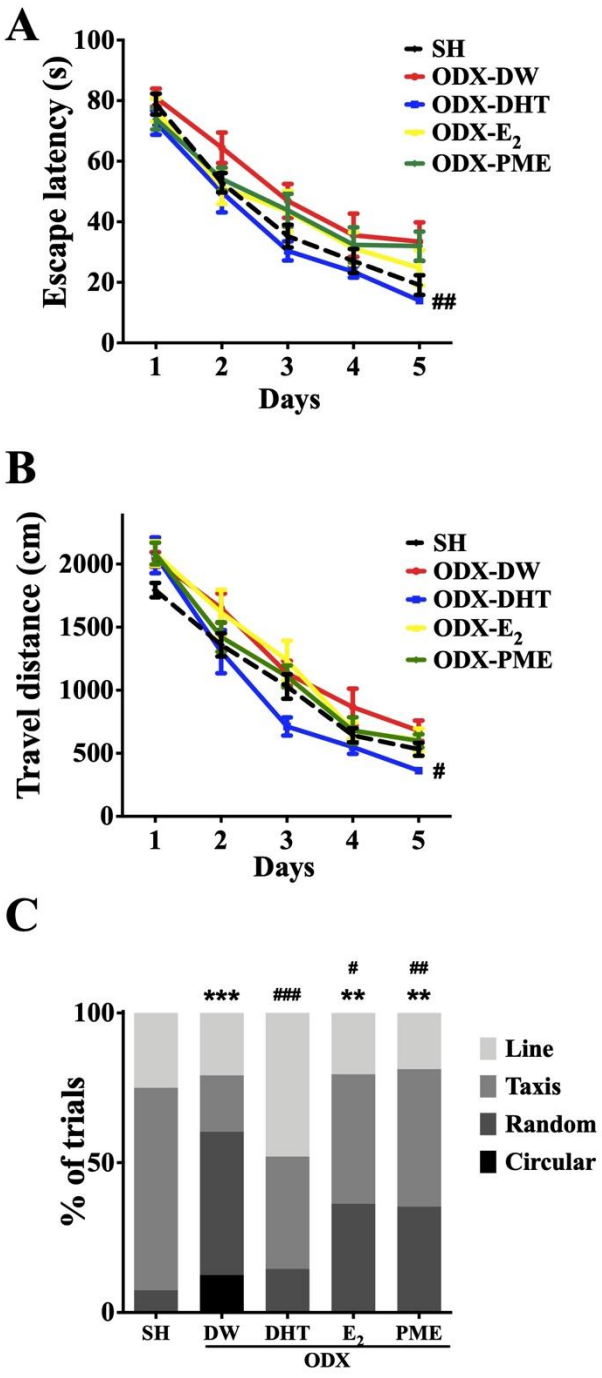


Figure 6.1 The spatial learning behavior of sham (SH) rats, and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months which were assessed using the Morris Water Maze task.The escape latency, travel distance and strategies of swimming pattern are shown in (A), (B), and (C), respectively. ** and *** represent $p < 0.01$ and 0.001 compared to the SH rats. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001 compared to the ODX-DW rats.

For the probe trial tested on day-6 of MWM task (a hidden platform was taken out of the pool), the ANOVA analysis of the crossing numbers, time and distance spent in the targeted area showed the significant differences across five treatment groups ($F = 4.950$, $p < 0.05$; $F = 7.190$, $p < 0.001$; $F = 4.418$, $p < 0.01$, respectively). A post-hoc analysis revealed that the crossing numbers in the ODX-DW rats were marginally, non-significantly lower than the SH rats ($p = 0.084$) while the time and distance spent in targeted area were comparable to those of the SH rats (Figure 6.2). The treatment with DHT significantly increased the crossing numbers, time, and distance spent in the targeted area in the ODX-DHT rats compared with the ODX-DW rats (Figure 6.2). However, this effect could not be observed after 2 months of E_2 and PME treatment, and the crossing numbers, time, and distance spent in the targeted area in the ODX-E_2 and ODX-PME rats were comparable to those of the ODX-DW rats (Figure 6.2). The crossing numbers, time and distance spent in targe area in ODX-DHT rats were significantly higher than those of ODX-E_2 rats and ODX-PME rats ($p < 0.05$) where those of the later groups were not significantly different. This indicated that only DHT could ameliorate the spatial memory capacity in cognitive impaired rats.

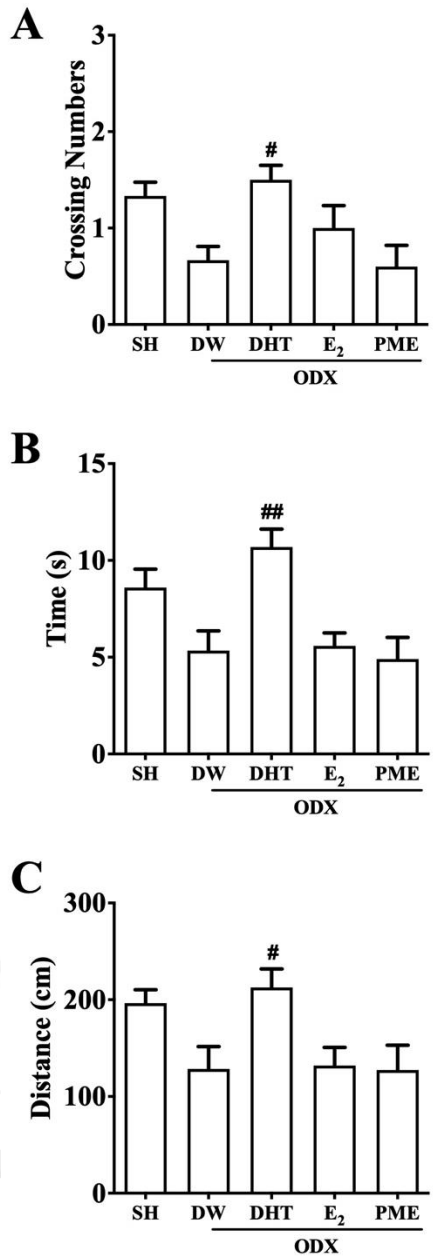


Figure 6.2 The spatial memory capacity of sham (SH) rats, and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months which were assessed using the Morris Water Maze task.The crossing numbers, time and distance spent in targeted area are shown in A, B, and C, respectively. $^{\#}$ and $^{\#\#}$ represent $p < 0.05$ and 0.01compared to the ODX-DW rats.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with hippocampal synaptic plasticity

After the rats were castrated for 2 months and continuously treated with DW for 2 months, or 4 months in the orchidectomy-induced cognitive impairment condition, mRNA expression levels of *Syn* and *Bdnf* were decreased, but *M_1-mAChR* and *α7-nAChR* mRNA levels were increased in the ODX-DW rats compared with the SH rats (Figure 6.3). Treatment with DHT in the ODX-DHT rats significantly increased *Syn* and *Bdnf* mRNA levels and decreased *M_1-mAChR* and *α7-nAChR* mRNA level in comparison with the ODX-DW rats. Noted, the *M_1-mAChR* mRNA level was also lower than that of the SH rats (Figure 6.3B). Treatment with E_2 and PME in the ODX-E_2 and ODX-PME rats resulted in similar patterns of changes in *Bdnf* mRNA levels (non-significant differences from the ODX-DW rats; Figure 6.3E) and *M_1-mAChR* and *α7-nAChR* mRNA levels (lower than the ODX-DW rats; Figure 6.3B and C), except that *Syn* mRNA level was higher in the ODX-E_2 rat, but non-significant difference in the ODX-PME rats compared to the ODX-DW rats (Figure 6.3A). Comparing to the SH rats, *Syn*, *Bdnf*, *M_1-mAChR* and *α7-nAChR* mRNA levels of the ODX-E_2 and ODX-PME rats were non-significant differences from the SH rats, except that *Syn* mRNA level of the ODX-PME rats was lower than the SH rats. The comparison between ODX-DHT, ODX-E_2 and ODX-PME rats revealed that that DHT increased *Syn* and decreased *M_1-mAChR* which were significantly greater than that of E_2 and PME ($p < 0.05$) while the *α7-nAChR* and Bdnf in these three groups of rats were not significantly different. These results indicated that DHT treatment could restore the synaptic transmission and structure in orchidectomy-induced cognitive impaired rats whereas E_2 and PME treatment provided the benefits only on the synaptic transmission (DHT > E_2 > PME). The mRNA expression levels of *GluN1* were not significantly different across all five groups of rats (Figure 6.3D).

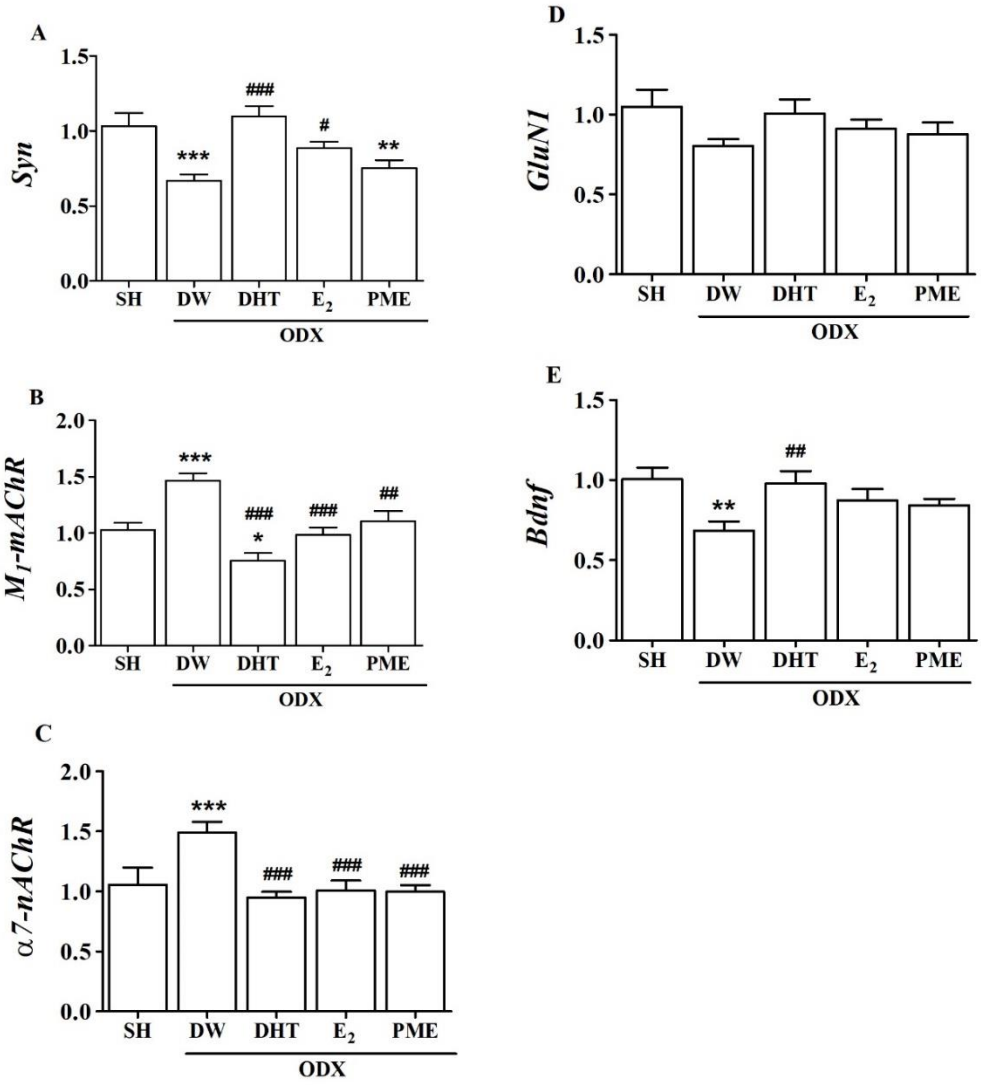


Figure 6. 3 The mRNA expression levels of genes associated with synaptic plasticity, *Syn* (A), *M_1-mAChR* (B), *α7-nAChR* (C), *GluN1* (D), and *Bdnf* (E), in hippocampus of sham (SH) and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data show the fold change in mRNA expression levels relative to the mean of SH rats. *, **, and *** represent $p < 0.05$, 0.01, and 0.001 compared to SH. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ represent $p < 0.05$, 0.01, and 0.001, compared to ODX-DW.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with neurofibrillary tangles

The mRNA expression levels of *Tau3* and *Tau4* were not significantly different across all five groups of rats (F = 1.490, p = 0.219; F = 2.001, p = 0.108, respectively; Figure 6.4). This indicates that 2-month treatment of DHT, E_2 and PME in the 2-month-orchidectomy-induced cognitive impaired (ODX) rats did not affect *Tau3* and *Tau4* mRNA expression in the rat's hippocampus.

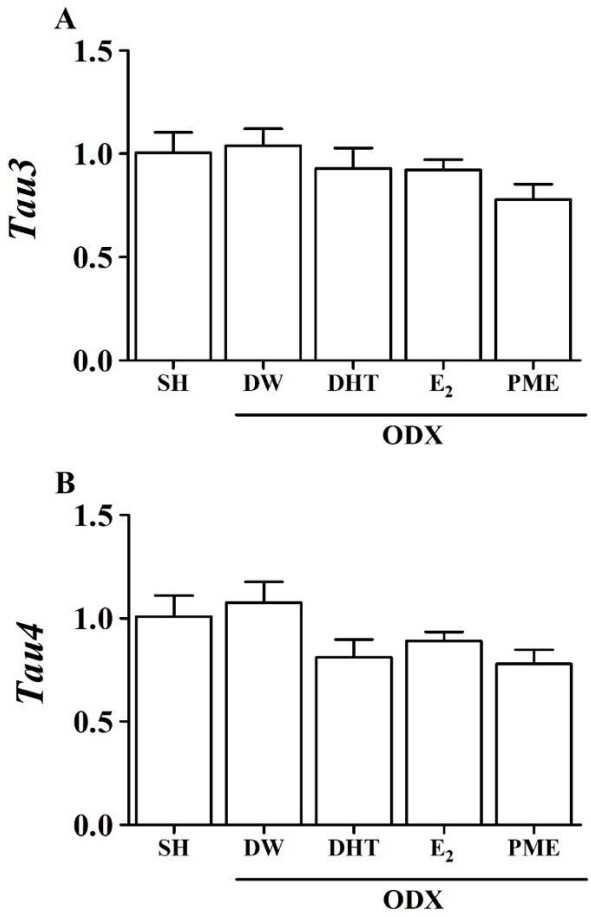


Figure 6.4 The mRNA expression levels of genes associated with neurofibrillary tangles, *Tau3* (A), and *Tau4* (B), in hippocampus of sham (SH) and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data indicate the fold change in mRNA expression levels relative to the mean of SH rats.

Effect of DHT, E_2 and PME on protein expression levels of Tau-5 and p-Tau Ser^{396}

Aligned with the unchanged mRNA levels, the protein expression levels of Tau-5 and p-Tau Ser^{396} were not significantly different across SH, ODX-DW, ODX-E_2 and ODX-PME groups (F = 0.646, p = 0.633; F = 0.902, p = 0.471, respectively; Figure 6.5). This indicates that 2-month treatment of DHT, E_2 and PME in the 2-month-orchidectomy-induced cognitive impaired (ODX) rats did not affect tau-5 and p-Tau Ser^{396} expression in the rat's hippocampus.

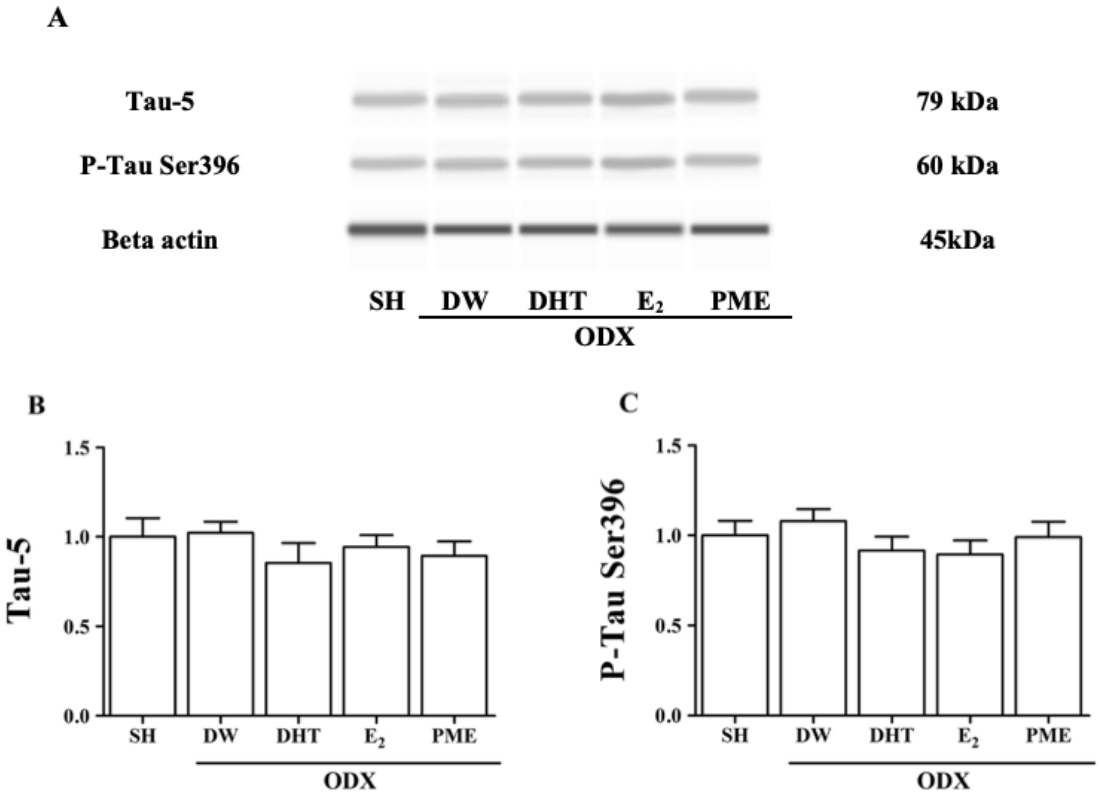


Figure 6.5 The protein expression levels of Tau-5, and p-Tau Ser^{396} in hippocampus of sham (SH) and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.Representative western blots of Tau-5 and p-Tau Ser^{396} and beta actin are shown in (A). The quantifications of the western blot signals of Tau-5 and p-Tau Ser^{396} relative to beta actin are shown (B) and (C), respectively. All data indicate the fold change in protein expression levels relative to the mean of SH rats.

Effects of DHT, E_2 and PME on mRNA expression levels of genes associated with amyloid plaques

The mRNA expression levels of *App, Bace1* and *Adam10* were not significantly different between the SH, ODX-DW, ODX-E_2 and ODX-PME groups (F = 1.860, p = 0.131; F = 2.232, p = 0.078; F = 0.872, p = 0.487, respectively; Figure 6.6). This indicates that 2-month treatment of DHT, E_2 and PME in the 2-month-orchidectomy-induced cognitive impaired (ODX) rats did not affect *App, Bace1* and *Adam10* mRNA expression in rat's hippocampus.

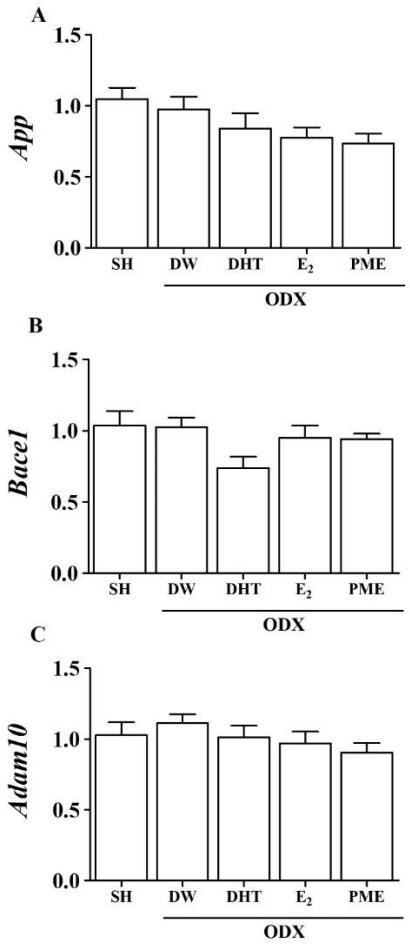


Figure 6.6 The mRNA expression levels of genes associated with amyloid plaques, *App* (A), *Bace1* (B), and *Adam10* (C), in hippocampus of sham (SH) and 2-month-orchidectomy-induced cognitive impaired (ODX) rats treated with distilled water (DW), dihydrotestosterone (DHT), 17β-estradiol (E_2) and *P. mirifica* extract (PME) for 2 months.All data indicate the fold change in mRNA expression levels relative to the mean of SH rats.

Discussion

After it was confirmed in CHAPTER V that the 2-month-orchidectomy-induced androgen deficiency could induce cognitive impairment in male rats and neuroprotective effects after DHT, E_2, and PME treatment, thus the neurotherapeutic effects of DHT, E_2, and PME in the cognitive impaired male rats were investigated further. In the present study, the orchidectomy-induced androgen deficiency and the androgenic activity of the treatments were confirmed by determining the weights of androgen responsive accessory sex organs: prostate gland, seminal vesicles, and epididymis. It indicated that orchidectomy completely induced an androgen-deficient status in rats, and only DHT exhibited an androgenic activity.

Among the controversy in the therapeutic effects of testosterone in cognitive impaired men (Cherrier et al., 2015; Cherrier et al., 2005b; Kenny et al., 2004; Resnick et al., 2017), the variations of an androgenic status, and the time course of treatment in relation to the heterogeneous progression of pathologies in the brain were noted as factors on success of the treatments. The neurotherapeutic effects of testosterone, using spatial learning and memory as indicators, were also reported in male rats. Differing from the present study, the orchidectomy was done in 2 months old rats, kept for 3 months, and injected with 2 mg/Kg BW/ day of testosterone for 1 month (Pintana et al., 2015). It should be noted that the neurotherapeutic effects presented in this study was exerted by subcutaneous injections of DHT, a higher potent metabolized form of testosterone, at the dose only at 1 mg/Kg BW/day. On the other hand, administrations of estrogenic agents, i.e., E_2 – an aromatized form of testosterone and PME – phytoestrogenic form, were unable to rescue the cognitive impairment in male rats, although both E_2 and PME showed the neuroprotective effects on cognitive impairment reported in CHAPTER V. Therefore, this result supports the utilization of DHT as hormonal replacement therapy to remedy the cognitive impairment in males.

Referring to CHAPTER III, the 2-month-orchidectomy-induced cognitive impaired rats presented the perturbation of synaptic transmission and structure, and hyperphosphorylated tau protein, but absence of amyloidogenesis pathway in hippocampus. After an oral administration of DW for 2 months, signs of synaptic perturbation determined at mRNA levels were existed including the synaptic

transmission (decreased *Syn* and increased *M_1-mAChR* and *α7-nAChR* mRNA levels) and synaptic structure (decreased *Bdnf* mRNA level). Interestingly, the perturbation of synaptic transmission was fully rescued by both DHT and E_2 injection, but not PME consumption. This was supported by the study of Pintana et al. (2015) reporting that testosterone replacement in ODX-induced cognitive impaired rats could increase the fEPSP response according to the LTP induction from Schaffer collateral pathway in hippocampus. However, the perturbation of synaptic structure was rescued only by the DHT treatment via a significant increase in *Bdnf* mRNA expression levels in the ODX-DHT rats in comparable level to that of the SH rats. BDNF has been reported to enhance the synaptic structure and increase the synaptic spine density in hippocampus (Hatanaka et al., 2015; Soma et al., 2018; Yoshii and Constantine-Paton, 2007). These results echo the importance of androgenic effect of testosterone on enhancing the synaptic structure via an increase in *Bdnf* expression.

As reported in CHAPTER III and V that 9 days (CHAPTER III) and 2 months (CHAPTER V) after orchidectomy-induced androgen deficiency could induce an overexpression of *Tau3* and *Tau4* mRNA levels and Tau-5 and p-Tau Ser^{396} protein levels which were reverted by DHT, PME, and E_2 treatment, however, the present study showed the non-alterations of tau at both mRNA and protein levels in 4-month ODX rats (ODX-DW group) comparing to the SH rats, and the treatment with DHT, PME, and E_2 had no effects. Following the chronological investigation of the effects of orchidectomy on tau mRNA and protein levels presented in CHAPTER III and IV, it was found that the onset of an increase in *Tau3* and *Tau4* mRNA levels and Tau-5 and p-Tau Ser^{396} protein levels was detected at 9 days after orchiectomy and persisted until 2 months before returned to the basal levels at 4, 6, and 8 months. The lowered overexpression of tau protein has been proposed that tau proteins are aggregated and turned to be tau filament forms (Delobel et al., 2008). However, if these tau filaments will form the neurofibrillary tangles, it needs to be investigated further.

As expected, alterations of an expression of *App*, *Bace1* and *Adam10* mRNA levels were not detected in the 4-month ODX rats, and the treatment with DHT, E_2 and PME had also no effects on these mRNA expressions. The results from the present study indicate that the mild cognitive impairment, induced by the perturbation of synaptic transmission and structure, could be completely rescued by the DHT

treatment, and partially rescued by the E_2 and PME treatment. However, once the formation of neurofibrillary tangle and amyloid plaque occurred as seen in moderate and severe cognitive impaired male patients, even DHT injection could not alleviate the symptoms.

In conclusion, the androgen deficiency-induced mild cognitive impairment in males could be entirely ameliorated by DHT treatment, and partially by E_2 or PME treatment. This result corroborates the high potential use of DHT as a therapeutic agent for the early onset of the cognitive impairment in men. It also suggests that, for the cognitive impairment, the prevention strategy is better than the therapeutic strategy because more choices and more success of the treatments (see CHAPTER V) can be obtained.

CHAPTER VII
GENERAL DISCUSSION AND CONCLUSION

Globally, life expectancy at birth has increased by more than 6 years between 2000 and 2019 – from 67.2 years in 2000 to 73.5 years in 2019 (GBD 2019 Demographics Collaborators, 2020). As people age, the reproductive and cognitive functions decline. About 13% of adults aged ≥ 65 years were found to have age-associated cognitive impairment at moderate to severe stage (Beauchet, 2006). Unlike women that the sex hormone production is abruptly discontinued when they enter menopausal period, men could produce testosterone throughout their life. Though the testosterone level is decreased 1% per year in middle-aged (30 – 50 years old) and older men, this decrease might not be noticeable and a significant change might be implausible to be detected (Camacho et al., 2013; Lapauw et al., 2008; Yeap et al., 2018). Thus, the emergence of cognitive impairment is generally unpredictable in the older men, and a low testosterone level is considered to be a key factor (Ford et al., 2018; Frye et al., 2010; Moffat et al., 2002; Pintana et al., 2016; Yeap et al., 2008). Apart from the unnoticeable decrease in testosterone level in males, the action of testosterone can be occurred through either ARs or ERs because it can be transformed into two active metabolites, DHT and E_2, which attribute to the more complication to unveil its mechanisms of action on cognitive function. This is because both receptor types, ARs and ERs, are expressed in hippocampus; a brain region involves with learning and memory. Thus, the present study has been systematically designed to answer the following related questions: (i) what is the mechanism of action of androgen deficiency on cognitive impairment in association with molecular and histopathological changes at male hippocampal brain region? (ii) can DHT, E_2 and PME elicit the neuroprotective and neurotherapeutic effects on cognitive impairment in androgen deficient males? (iii) if it is, which one shows the greater efficacy? and (iv) do they act differently by examining the chronological changes at transcriptional and translational levels of genes associated with three neuropathological hallmarks of cognitive impairment: synaptic plasticity, neurofibrillary tangle and amyloid plaque. This experimental design was under the assumption that DHT acts via androgenic pathway, while E_2 and PME should act via estrogenic pathways after binding with

their respective receptors in hippocampal neurons. Male SD rats were selected as subjects of this study and the androgen deficient condition was induced by orchidectomy.

To unveil the mechanism of action of androgen deficiency on cognitive impairment and the molecular and histopathological changes at hippocampus, male rats aged 4 months old were ODX, kept for 3 days, 2, 4, 6, and 8 months (M0, M2, M4, M6 and M8 groups, respectively: CHAPTER III). They were determined spatial learning behavior and memory capacity for 6 consecutive days before euthanized and examined transcriptional and translational levels of genes associated with synaptic plasticity (*Syn*, *GluN1*, *α7-nAChR*, *M_1-mAChR* and *Bdnf* mRNA levels, and SYN and PSD95 immunoreactivity levels), neurofibrillary tangles (*Tau3* and *Tau4* mRNA levels, and total tau and phosphorylated tau protein levels), and amyloid plaque (*App*, *Bace1*, and *Adam10* mRNA levels) in hippocampus in comparison with their age-match, testes-intact (SH) rats. The androgen deficient condition was confirmed by a decrease to undetectable level of serum testosterone and a significant decrease in weights of androgen responsive organs including prostate gland, seminal vesicles and epididymis. Rats showed an onset of impairment in spatial learning behavior and memory capacity assessed by MWM task as early as 2 months after orchidectomy (6-month-old ODX-M2 rats), and the impairment remained unchanged until 6 months after orchidectomy (10-month-old ODX-M6 rats) before it became worsened in 12-month-old ODX-M8 rats (Figure 7.1, lower panel). Considering on the side of the SH rats, the cognitive impairment was first observed in 8-month-old SH-M4 rats and the level of impairment was remained stable until the rats were 12-month old (SH-M8) (Figure 7.1, upper panel). Comparing between the ODX rats and the SH rats, the significantly impaired spatial learning behavior and memory capacity were detected in the 6-month-old ODX-M2 and 12-month-old ODX-M8 rats. This can lead to the conclusion that orchidectomy-induced androgen deficiency induces age-associated cognitive impairment in two steps by two mechanisms: short-term (acceleration) and long-term (exacerbation) for M2 and M8 rats, respectively (Figure 7.1, lower panel). The crucial role of androgen on cognitive function is also noted. Later, the mechanisms of action of androgen deficiency at the transcriptional and translational levels were examined and the results between the ODX and SH rats were compared.

The significant differences were detected as early as in the M0 group (or 9 days after orchidectomy) of the ODX rats for both synaptic plasticity (*Syn*, *M_1-mAChR*, *α7-nAChR* and *Bdnf* mRNA levels, and SYN and PSD95 immunoreactivity levels) and neurofibrillary tangles (*Tau3* and *Tau4* mRNA levels, and total tau and phosphorylated tau protein levels). The changes were kept continued up to the M8 group, except that all genetic markers of neurofibrillary tangle examined in this study were not significantly different between the ODX and SH rats starting from M4 groups (see Figure 3.7 and 3.8). Noted, only *Bace1* mRNA level, among three genes analyzed for the amyloid plaque, was significantly increased in the 10-month-old ODX-M6 rats. Thus, this leads to the conclusion that short-term (within 2 months) effect of an abrupt androgen deficiency can induce mild cognitive impairment via the deterioration of synaptic structure and function and the initiative formation of neurofibrillary tangle. While the long-term (within 8 months) effect can induce moderate cognitive impairment via a greater deterioration of synaptic structure and function and the accumulative formation of neurofibrillary tangle, and this unlikely causes by the formation of the amyloid plaque (because of only a single-time increase in *Bace1* mRNA level). The severe deterioration in synaptic transmission and structure of the ODX-M8 rats should have been resulted in the decreases in SYN and PSD-95 immunoreactivity and *Bdnf* transcripts, since BDNF protein is thought to be a regulator for PSD-95 transportation to postsynaptic terminal (Yoshii and Constantine-Paton, 2007). The return to the base-line (SH) levels of *Tau3* and *Tau4* mRNA and total tau and phosphorylated tau protein at 4 to 8 months after orchidectomy (M4 to M8 groups) of the ODX rats suggested that the hyperphosphorylated tau has been aggregated into filament forms prior to the appearance of neurofibrillary tangles (Delobel et al., 2008).

Based on the above-mentioned results that the differences of mRNA and protein levels of genes associated with synaptic plasticity and neurofibrillary tangle between the ODX, and SH rats can be detected as early as 9 days after orchidectomy (or M0 group), it was questioned further when the early onset of changes of the expression of those genes was occurred. Thus, another set of experiment was performed (CHAPTER IV). Male rats were ODX, kept for 1, 3, 6 and 9 days, and examined for mRNA and protein expression levels of genes mentioned in CHAPTER III. The onset

of chronological changes of mRNA expression could be detected as early as 1 day after orchidectomy for genes associated with synaptic function: a reduction in *Syn* mRNA level at 1-day with subsequent increases in *GluN1* (at 3-day), *α7-nAChR*, and *M_1-mAChR* (at 6-day) mRNA levels (Figure 7.1, lower panel), while those of the neurofibrillary tangle and synaptic structure changes afterward (an increase in *Tau4* (at 6-day) and *Tau3* (at 9-day) mRNA levels and total tau and phosphorylated tau (at 9-day) protein levels, and a decrease in *Bdnf* (at 9-day) mRNA level, respectively). These results can be interpreted that the sequential changes have started with a deterioration in synaptic transmission with a decrease in presynaptic release (*Syn* at D_1) followed by a compensatory of postsynaptic receptor upregulation (*GluN1* at D_3, *M_1-mAchR* and *α7-nAChR* at D_6) to the reduced amounts of neurotransmitter release (Creese and Sibley, 1981). After the changes in pre- and post-synaptic communication, a response of neuronal structure and synapses was happened via a decrease in *Bdnf* mRNA expression, and an increase in *Tau4* and *Tau3* mRNA expression and total tau protein. At the same time, the upregulation of phosphorylated tau Ser^{396} protein could adversely affect microtubule assembly and possibly perturbed in components of neuronal structure and synapses.

After the mechanism of actions of androgen deficiency, at the molecular levels, on cognitive impairment in male rats was revealed, the effects of the two active metabolite forms of testosterone, DHT and E_2, to prevent and cure the cognitive impairment in male rats were assessed. Regarding the fact that a long-term use of synthetic DHT and E_2 might cause some adverse side effects, e.g., venous thromboembolism, ischemic stroke (Getahun et al., 2018), cardiovascular toxicity (Phillips et al., 2014) and erythema nodosum (Coyle et al., 2015a), the use of the natural products to remedy or ameliorate the cognitive impairment attracted attention to the modern world people. Recently, it was reported that the PME could cure the early and late stage of cognitive impairment in estrogen deficient rats (Anukulthanakorn et al., 2016). PME did not show an acute toxicity (Mohamad et al., 2019) . PME and its phytoestrogenic constituents have been ratified for their estrogenic activity *in vitro* (Cherdshewasart et al., 2008) and *in vivo* (Cherdshewasart et al., 2007b; Manonai et al., 2008; Trisomboon et al., 2006b), including in male rats (Malaivijitnond et al., 2004). Thus, the PME was included as one of the test agents for

neuroprotective and neurotherapeutic effects on cognitive impairment in the present study.

To determine the neuroprotective effects of DHT, E_2 and PME on hippocampal dependent learning and memory in association with the chronologically molecular changes of three neuropathological hallmarks in androgen deficient rats, male rats at 4 months old were ODX, kept for recovery for 1 day, given 1 mg/kg BW/day of DHT, 80µg/kg BW/day of E_2 or 100 mg/kg BW/day of PME for 2 months, and the results were compared with the age-match, testes-intact SH rats (CHAPTER V). The duration of 2-month treatment was selected for this study in respect to the emergence time course of the mild cognitive impairment observed in CHAPTER III. This study revealed that the treatment with 1 mg/kg BW/day of DHT, 80 µg/kg BW/day of E_2 and 100 mg/kg BW/day of PME for 2 months could prevent the spatial learning and memory impairment in male rats. However, the DHT treatment had a greater efficacy than the E_2 and PME, and the mechanisms of actions were different among three testing agents (Figure 7.2, left panel). The DHT prominently maintained the synaptic transmission and structure through recovery of the *Syn* and *Bdnf* mRNA levels as well as possible suppressive effects of hyperexcitability in hippocampus by a decrease in *α7-nAChR* and *M1-mAChR* mRNA levels (Harley et al., 2000; Skucas et al., 2013). Moreover, DHT possibly suppressed the formation of neurofibrillary tangles by completely prevent the overexpression of *Tau3* and *Tau4* mRNA, and total tau and phosphorylated form of tau protein induced by orchidectomy. On the other hand, an administration of E_2 mainly maintained synaptic transmission and structure in male hippocampus by mitigating the orchidectomy-induced changes of mRNA expression levels of *α7-nAChR*, *M1-mAChR*, and *Bdnf*, while those effects on neurofibrillary tangle associated genes, i.e., suppressing the overexpression of *Tau3* and *Tau4* mRNA and total tau and phosphorylated tau, were less effective than the DHT effects. Supporting this finding, it was reported that an activation of ARs can prevent hyperphosphorylated tau via the inhibition of GSK signaling which is independent of ERs (Papasozomenos and Shanavas, 2002; Papasozomenos, 1997). Noted, the effect of E_2 on presynaptic release (*Syn* mRNA expression level) was greater than that of the DHT. Accordingly, it seems to be that DHT should be a primary active agent used to prevent a cognitive impairment in androgen deficient males, and E_2 should be an

ancillary drug. PME showed the effects in comparable degree to that of the E_2 for synaptic function (*Syn*, *α7-nAChR*, and *M1-mAChR* mRNA expression levels) and structure (*Bdnf* mRNA expression level), but comparable to those of DHT for neurofibrillary tangle (by preventing an overexpression of *Tau3* and *Tau4* mRNA levels and total tau and phosphorylated tau protein levels). Since PME contains several phytoestrogenic substances, particularly a miroestrol that elicits high estrogenic activity comparable to that of E_2 (Malaivijitnond, 2012), thus the effect on synaptic plasticity of PME might act via ERs. On the other hand, its effect on neurofibrillary tangle formation should be on the other pathway that differs from those of the DHT such as antioxidant pathway (Jiang et al., 2016; Nakajima et al., 2013). Based on the LC/MS/MS analysis of phytoestrogen contents in the PME used in this study, puerarin is the second highest content (35.05 mg in 100g PME) next to daidzin (40.16 mg in 100g PME) which is in agreement with the previous reports (Cherdshewasart and Sriwatcharakul, 2007; Cherdshewasart et al., 2007b; Kraithong et al., 2021; Udomsuk et al., 2009). Puerarin is known for its high antioxidant activity (Bebrevska et al., 2010; Cherdshewasart and Sutjit, 2008; Chung et al., 2008). Several studies have reported the inhibition of neurofibrillary tangle formation by antioxidative substances (Jiang et al., 2016). Subcutaneous injection of 7.0 mg/kg BW/day of puerarin for 4 months in the female rats, after they were ovariectomized and kept for 2 months for the induction of cognitive impairment, could decrease the mRNA expression level of *Tau4* in hippocampus (Anukulthanakorn et al., 2016). Thus, PME should be an alternative option to prevent cognitive impairment in males.

While the brain biochemistry changes may begin ambiguously years to decades prior to the clinically detectable symptoms (Sperling et al., 2013), the cognitive impaired patients often come to consult with a doctor at the late stage. Thus, a therapeutic approach to reverse or rescue the cognitive impaired condition is required. With respect to the affirmation of the 2-month-orchidectomy-induced mild cognitive impairment in male rats (in CHAPTER III and Figure 7.1), and the treatments of DHT, E_2, and PME had a promising result on neuroprotection (Figure 7.2, left panel), the neurotherapeutic effects of DHT, E_2, and PME in the mild cognitive impaired male rats were investigated further. Male rats were ODX, kept intact for 2 months for the induction of cognitive impairment, and treated with 1 mg/kg BW/day of DHT,

80µg/kg BW/day of E_2 or 100 mg/kg BW/day of PME for another 2 months (CHAPTER VI). The present study showed that only the DHT treatment can rescue the cognitive function of the mild cognitive impaired ODX rats to resemble that of the testes-intact SH rats, while E_2 and PME did not show the efficacy (Figure 7.2, right panel). The action of DHT is through the restoration of the synaptic transmission (by increasing *Syn* and decreasing *M_1-mAChR* and *α7-nAChR* mRNA levels) and the synaptic structure (by increasing *Bdnf* mRNA level). Although E_2 injection could mitigate an alteration of synaptic transmission (*Syn*, *M_1-mAChR* and *α7-nAChR* mRNA expression levels) in a comparable degree to those of the DHT, it could not restore the synaptic structure (*Bdnf* mRNA expression level). These results corroborate the important of androgenic effects on enhancing the synaptic structure via the *Bdnf* expression. Apart from the action via nuclear ARs, the DHT might exhibit a non-genomic action through the extranuclear ARs localized at the postsynaptic neuron. It was reported that an activation of the extranuclear ARs could enhance the formation of new synaptic spines via MAPK, PKA, and PKC signaling pathways (Hatanaka et al., 2015). It should be noted that neither testosterone nor E_2 had been reported to cure the moderate cognitive impaired patients when the neurofibrillary tangles were formed (Asih et al., 2015; Borst et al., 2014; Rasgon et al., 2014; Uddin et al., 2020). The present study also confirmed this statement for DHT, since the ODX rats injected with DHT and E_2 had no changes in components of tau: *Tau3* and *Tau4* mRNA levels and Tau-5 and P-Tau Ser^{396} protein levels. In contrast to the neuroprotective effects, PME consumption has a slight effect on synaptic transmission (restoring the *M_1-mAChR* and *α7-nAChR* mRNA levels) and no effect on synaptic structure in hippocampus of cognitive impaired rats.

Assessing if the neurotherapeutic effect of E_2 and PME is sex- and time-dependent, the results of the present study were compared with the previous report performed in female rats. They used 4-month-old female SD rats, ovariectomized, kept for 2 months for the induction of cognitive impairment, and treated with 100 mg/kg BW/day of PME (p.o.) or 80 µg/kg BW/day of E_2 (s.c.) for 4 months (Anukulthanakorn et al., 2016). Noted, the duration of treatment is 2 months longer than the present study in male rats. It was found that both E_2 and PME could restore the spatial learning behavior of the ovariectomized rats to resemble the SH rats, via

the reduction of *Tau4*, *App* and *Bace1* mRNA levels. While in the male rats only DHT treatment could decrease *Bace1* mRNA expression level in the ODX rat's hippocampus, not by PME or E_2 treatments. Moreover, in the study of Anukulthanakorn et al. (2016), E_2 and PME could decrease the mRNA expression levels of ERα. Although both ERα and ERβ are distributed throughout hippocampus of male rats, the expression of ERα in females were higher than in the males (Romeo et al., 2005a). This implies that the neurotherapeutic effects and mechanism of action of DHT, E_2 and PME on cognitive impairment are sex- and time-dependent.

In conclusion, the orchidectomy-induced androgen deficiency can induce cognitive impairment in males. The induction was in two steps by two mechanisms: short-term (acceleration) and long-term (exacerbation). The short-term effects, which are caused by the perturbation of the synaptic function and structure and the initiation of neurofibrillary tangle formation, can induce the mild cognitive impairment. The long-term effects, which are caused by a severe perturbation of the synaptic function and structure and an accumulative formation of the neurofibrillary tangle, can induce the moderate cognitive impairment. When it comes to a treatment management, a preventive approach is recommended, and the judgement may need for the treatment intervention. Based on the results in this study, DHT is suggested as a major drug, and E_2 should be ancillary testing agent. Apart from the use of synthetic DHT and E_2, this study encourages the development of PME as a phytopharmaceutical drug for cognitive impairment in males which will be value-added to the Thai medicinal herb because *P. mirifica* products have been sold in food supplement and cosmetic markets since two decades ago.

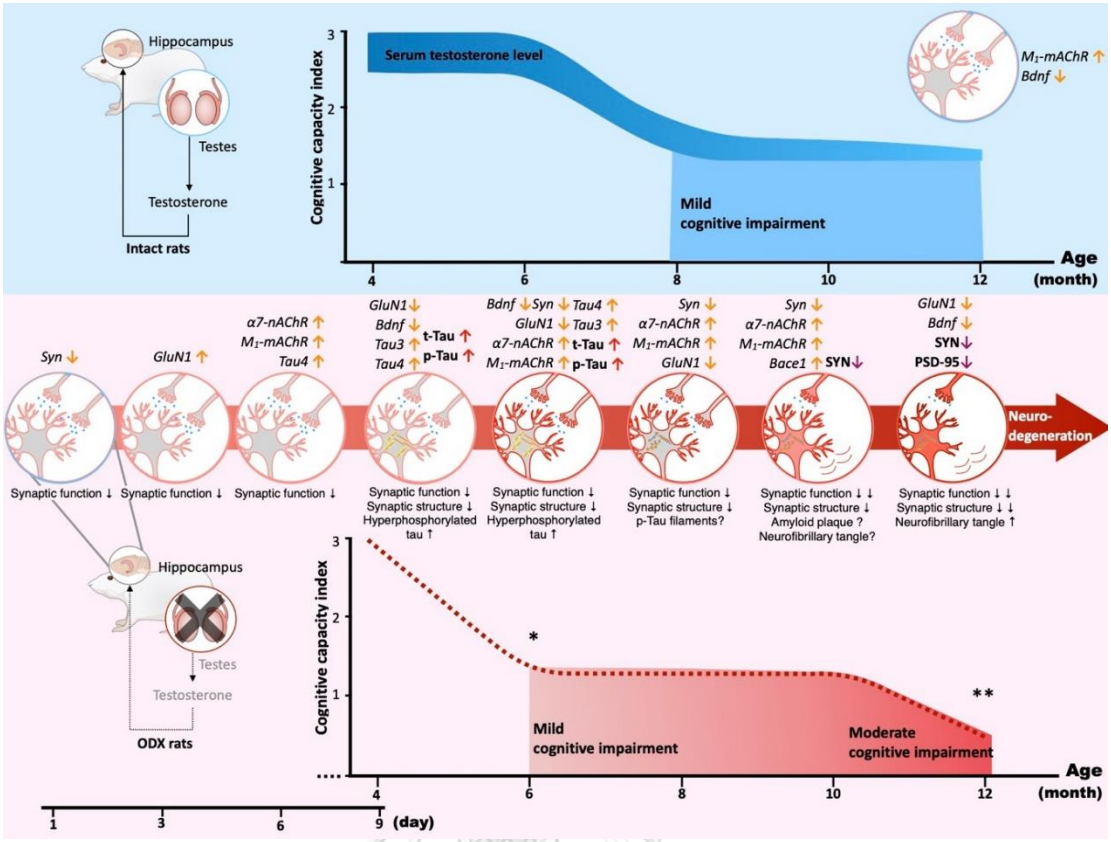


Figure 7.1 Summary diagram of the cognitive capacity index in testes-intact (SH) (upper panel) and orchidectomized (ODX) rats (lower panel) at the age of 4 to 12 months, and the analysis of transcriptional and translational levels of genes associated with synaptic plasticity (*Syn*, *GluN1*, *α7-nAChR*, *M_1-mAChR* and *Bdnf* mRNA levels, and SYN and PSD95 immunoreactivity levels), neurofibrillary tangles (*Tau3* and *Tau4* mRNA levels, and total tau and phosphorylated tau protein levels), and amyloid plaque (*Bace1* mRNA level) in hippocampus. The molecular events shown in circles represent each time point. The cognitive capacity index is calculated from all six parameters of spatial learning and memory and averaged; the degrees of cognitive performances are categorized into mild (3), moderate (2) and severe (1) for each parameter. The thickness of the blue line indicates serum testosterone levels, and the red dash line indicates androgen deficient stage. * and ** indicate two-step of effects of androgen deficiency to accelerate and exacerbate an age-associated cognitive impairment.

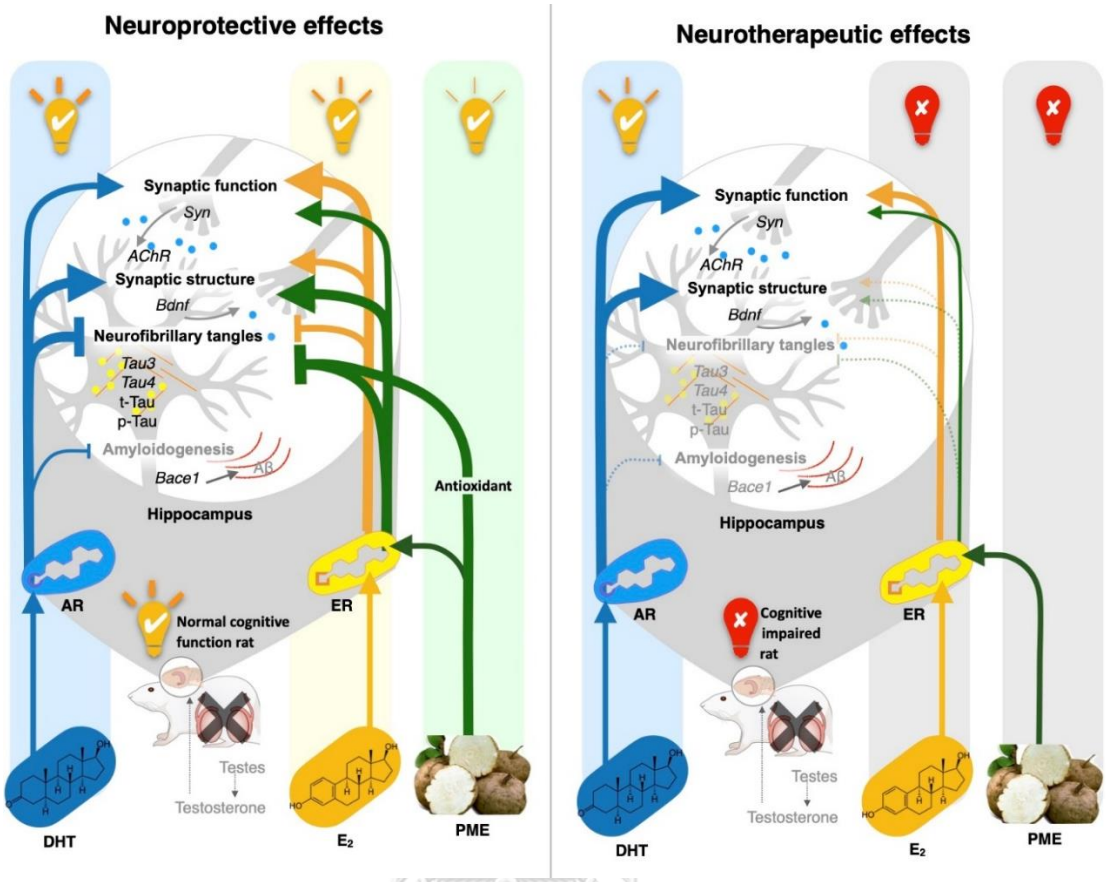


Figure 7.2 A schematic of neuroprotective (left panel) and neurotherapeutic (right panel) effects of dihydrotestosterone (DHT), 17β-estradiol (E_2), and *P. mirifica* extract (PME) on cognitive impairment in androgen deficient rats.A thicker line indicates a higher degree of the efficacy, and a dash line indicates an absence of the effects. An arrowhead line indicates stimulation, while a blunt-head line indicates inhibition. Please refer to the abbreviation index for the meaning of each abbreviation.

Recommendations

1. Since PME is a mixture of many active phytoestrogenic compounds, including puerarin, miroestrol, genistin, genistein, daidzin and daidzein, that have been quantified in this study and the neuroprotective effects on hippocampus and cognitive function in male rats were previously reported, thus, the effects and actions of each compound should be verified if the drug development for the elderly people is planned.

2. Although these experiments were conducted in wild-type rats, not genetically modified rats, which should mimic the neurodegenerative diseases that happen in regular people, an investigation in animal models that have anatomy, physiology, and development of neurodegeneration closely resembled humans such as non-human primates should be considered.

3. Although the present study examined the synaptic function in hippocampus using a molecular technique, it is recommended to determine of synaptic transmission by electrophysiological techniques for intensive and more detailed results.

4. Adding to the knowledge gain in this study on neuroprotective and neurotherapeutic effects of PME, the study of the effects of PME on negative emotions such as fear, anxiety, and depression, which could lead to neurodegeneration or dementia should be interesting.

5. The present study showed that orchidectomized rats lost their body weights, suggesting that decreasing of body weight can be used as a noninvasive indicator of androgen deficiency.

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