SERUM PROTEOMIC ANALYSIS OF NEUROTRANSMISSION-RELATED PROTEINS ASSOCIATED WITH COGNITIVE FUNCTIONS IN A THAI POPULATION

CHEN CHEN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Medical Sciences
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Thesis entitled "Serum proteomic analysis of neurotransmission-related proteins associated with cognitive functions in a Thai population"

By Chen Chen

has been approved by the Graduate School as partial fulfillment of the requirements

for the Doctor of Philosophy in Medical Sciences of Naresuan University

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Keywords	Cognitive function, sex difference, aging, Wisconsin Card Sorting Test (WCST), neurotransmission, serum, proteomics, protein expression profile, glutamate, NMDAR, excitotoxicity

ABSTRACT

Cognitive function refers to higher-order intellectual processes that gather and process information in the human brain. Intact cognitive function is dependent on the precise exchange of information between neurons. Sex differences in cognitive function exist, but they are not stable, undergoing dynamic change during the lifespan. Although previous research indicated that the changes in sex differences in normal neurological processes with age are modulated by complex molecules, with neurotransmission-related proteins being especially important. Our understanding of how sex-related neural information transmission evolves with age is still in its infancy. Therefore, this study was designed to investigate the molecular mechanisms underlying age-related sex difference in cognitive function in a Thai healthy population, as well as to determine the sex-dependent proteomic clocks for predicting cognitive aging. The Wisconsin Card Sorting Test (WCST) was performed to assess cognitive function in 199 Thai healthy subjects (aged 20-70 years). The results showed that males outperformed females in two of the five WCST sub-scores: %Corrects and %Errors, with a higher percentage of total corrects and lower total errors rate. Sex differences in these scores were related to aging, and it became

noticeable in those over 60. Moreover, the label-free proteomics method and bioinformatic analysis were also used to investigate the age-related alternations in the expression profiles of sex-specific neurotransmission-related proteins. According to the findings, differentially expressed proteins between Thai healthy men and women were significantly enriched in the complement cascade, which may correspond to Glu-induced excitotoxicity. Another result of this research revealed that neurotransmission-related proteins were dynamically assembled as protein complexes in a sex-biased manner, relating to age. For example, the elevation of the cytosolic calcium ion concentration complex was only found in the females, and the N-methyl-D-aspartate type glutamate receptors (NMDARs) complex was enriched exclusively in elderly female samples. These results implied that females might be more susceptible to the excitotoxicity induced by NMDAR hyperfunction and this intensified with age, which was consistent with their performance on the WCST. The NMDAR level in serum would be a novel “proteomic clock” for predicting cognitive aging in Thai healthy women.

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Chen Chen

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ABBREVIATIONS

FSHB	=	Follitropin subunit beta
CGA	=	Gonadotropin alpha chain
ETC	=	Electron transport chain
OMM	=	Outer mitochondrial membrane
CHI3L1	=	Chitinase 3-like 1
AD	=	Alzheimer’s disease
VGLUT	=	Vesicular glutamate transporter
DA	=	Dopamine
WCST	=	Wisconsin Card Sorting Test
Glu	=	Glutamate
AMPAR	=	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
NMDAR	=	N-methyl-D-aspartate receptor
WM	=	White matter
CNS	=	Central nervous system
mtDNA	=	Mitochondrial DNA
E2	=	Estradiol
ER	=	Estrogen receptor
GPER1	=	G-protein coupled estrogen receptor 1
AcbC	=	Accumbens core
mEPSPs	=	Miniature excitatory post-synaptic potentials
T	=	Testosterone
DHT	=	Dihydrotestosterone
LTP	=	long-term potentiation
CbN	=	Cerebellar nuclei
Cc	=	Corpus callosum
GKAP	=	Guanylate kinase-associated protein
MBP	=	Myelin basic protein
Rhes	=	Ras homolog enriched in striatum
SYP	=	Synaptophysin
PPI	=	Protein-protein interaction

NRC	=	NMDAR multiprotein complexes
TMHI-55	=	Thai Mental Health Indicator
MMSE	=	Mini-Mental State Examination
AMBIC	=	Ammonium bicarbonate
DTT	=	Dithiothreitol
LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
CID	=	Collision-induced-dissociation
MS	=	Mass spectra
FDR	=	False discovery rate
GLM	=	General linear model
LIMMA	=	Linear Model for Microarray Data
DEPs	=	Differentially expressed proteins
FC	=	Fold change
BF	=	Bayes factor
95%CI	=	95% Credible interval
GO	=	Gene annotation
MF	=	Molecular function
BP	=	Biological process
CC	=	Cellular component
OCD	=	obsessive-compulsive disorder
MDD	=	Major depression disorder
C3	=	Component 3
PKC	=	Protein kinase C
PAFR	=	Platelet-activating factor receptor
Jak2	=	Janus kinase 2
PLC	=	Phospholipase C
MAPK	=	Mitogen-activated protein kina

CHAPTER I

INTRODUCTION

Background and rationale of the study

Cognitive function refers to higher-order intellectual processes that gather and process information in the human brain, and it contains multiple mental abilities such as learning, memory, language, attention, reasoning, problem-solving and decision making, etc. (Rudolph et al., 2019). Because different cognitive domains permeate all aspects of human life, it is a well-known indicator for maintaining human survival and independence. Intact cognition is dependent on the whole-brain interaction as well as the modulation of endogenous neurochemicals particularly differential neurotransmission-related proteins. For example, projections of white matter fiber bundles in the temporal lobe (uncinate fasciculus, cingulum, fornix, superior longitudinal cortex, and inferior longitudinal fasciculus) are associated with a better executive function (Medaglia et al., 2015; Roberts et al., 2013). Furthermore, recent research has shown that language processing incorporates systems in both hemispheres (Muller,, & Meyer, 2014), and a frontoparietal network was active during attention-demanding tasks (Parks,, & Madden, 2013). Neurotransmission-related proteins, on the other hand, such as neurotrophin (Lu et al., 2014), receptors of estrogen (Barth et al., 2015), and syntaxin binding protein 1b (Vercauteren et al., 2007) just to name a few, are essential for healthy cognitive function.

Sex differences in cognitive function have been seen in humans (Choleris et al., 2018). Male and female may differ in terms of cognitive strategies and/or cognitive styles (Abraham, 2016). Different learning style preferences (Wehrwein et al., 2007), decision-making styles (Bakewell, & Mitchell, 2006), and executive function test performances are examples of this (Gaillard et al., 2021). The efficiency of information transmission in neural circuits is directly responsible for cognitive differences between men and women. Men, for example, demonstrated directionally asymmetrical interhemispheric transmission time of verbal information compared to women (Nowicka,, & Fersten, 2001). In the rodent hippocampus, locally synthesized

sex neurosteroid hormones influence synaptic transmission by binding to sex-specific receptors within each sex (Oberlander, & Woolley, 2016). Other animal studies have shown that sex neurosteroids potentiate excitatory synapses and suppress inhibitory synapses in a sex-specific manner (Huang, & Woolley, 2012; Ter Horst et al., 2009). Besides this, neurotransmission-related gene expression differed between sexes, such as *GABRB3*, which encodes the $GABA_A$ receptor β3 subunit (Mercer et al., 2016). According to the aforementioned literature, there is a significant difference in neural wiring and/or circuits between males and females, and the influence of both sex neurosteroid hormones and genetic factors on cognitive sex differences is achieved through their effects on neuronal information transduction efficiency within each sex. However, the efficiency of sexually dimorphic neural information transmission is not permanent and fluctuates dynamically across the lifespan (Adams, & Morrison, 2003; Nicholson et al., 2004; Spritzer, & Galea, 2007).

The changes in sex differences in normal neurological processes with age are modulated by complex molecules, with neurotransmission-related proteins being especially important. Prior proteomics research using human blood samples discovered that over 60% (895 out of 1379) of proteins changed with age were significantly different across the sexes (Lehallier et al., 2019), with the most dramatically changed proteins including follitropin subunit beta (FSHB) and human chorionic gonadotropin alpha chain (CGA). This is consistent with the findings of Tanaka et al. (2018), who discovered that the association between age and eight proteins, half of which were sex hormones, differed between sexes. Unsurprisingly, sex-specific hormones are required for numerous age-related biological differences in males and females. According to the hormonal drive differences theory (Austad, & Fischer, 2016; Sampathkumar et al., 2020), sex hormones induce sex differences in the neuroanatomy and neurochemistry (Hagg, & Jylhava, 2021) and mediate cognitive function and synaptic plasticity throughout (Hara et al., 2015).

In addition, findings from the animal model revealed that electron transport chain (ETC) proteins, outer mitochondrial membrane (OMM) proteins, and β-actin expressed differently in male and female mice in an age-dependent manner (Moschinger et al., 2019). ETC has been implicated in the etiology of various neuropsychiatric disorders, including depression, bipolar disorder, and schizophrenia

(Rezin et al., 2009). Female schizophrenic patients showed a later age of onset, distinct anatomical brain abnormalities and cognitive impairment (Canuso, & Pandina, 2007) compared to their male counterparts, which was consistent with this. Cognitive deficit might predict the onset of schizophrenia (Nuechterlein et al., 2011). On the other hand, β-actin contributes to the morphological normality of mouse brain tissues such as the hippocampus and cerebellum, which correlates with the cognitive function normalization (Cheever et al., 2012). Similarly, Chitinase 3-like 1 (CHI3L1) protein expression levels rose in healthy women versus men during aging and peaked in senior subjects in the hippocampus and cerebellum (Sanfilippo et al., 2019). The highest expression level of CHI3L1 was reported in women with AD in the same study, which is consistent with previous findings indicating females have a higher risk of AD (Niu et al., 2017; Viña, & Lloret, 2010). These findings suggest that β-actin and CHI3L1 may be potential biomarkers for AD, however, the question of whether these proteins act individually or in interaction remains to be answered. Moreover, a larger rise in vesicular glutamate transporter (VGLUT) expression was found in female flies' dopamine (DA) neurons throughout aging, which has a protective role in DA neuron degeneration and might promotes glutamatergic signaling. This finding is shared by flies, rodents, and humans. By preventing VGLUT upregulation in DA neurons, the sex disparities in DA neuron loss with age were eliminated (Buck et al., 2021).

As aforementioned, the protein composition of cells and bodily fluids provides insights into complex biological processes, as proteins are often direct regulators of cellular signaling pathways. Serum, which contains an abundance of proteins from cells and tissues (Ray et al., 2011), is an ideal biological sample for analyzing changes in the proteome between different groups. Quantitative proteomics represents a powerful tool for the comprehensive analysis of a wide range of the real functional proteins expressed in the serum, and it has been used to understand the mechanism underlying sex differences in several mental disorders associated with cognitive impairment (Smirnova et al., 2019; Steeb et al., 2014). However, none of the prior studies employed a quantitative proteomics technology to identify the global protein changes and pathways perturbed in the cognitive sex differences with age in healthy subjects, especially based on a Thai population. To fill the research gap, the

current study used the quantitative proteomics method and bioinformatic analysis to investigate the molecular mechanisms underlying age-related sex differences in cognitive function as measured by the Wisconsin Card Sorting Test (WCST). Lehallier et al. (2019) purposed a sex-independent 'proteomic clock' consisting of 373 plasma proteins to predict normal aging. Due to the differences in protein expressions among ethnicities (Hernandez et al., 2015; McGraw, & Waller, 2012), as well as sex-specific prevalence and onset time of mental disorders (Bebbington, 1998; Canuso, & Pandina, 2007; Diflorio, & Jones, 2010; Riedel et al., 2016), as a result, this study also aimed to determine the sex-dependent proteomic clocks for predicting cognitive aging in Thai healthy males and females.

Research hypotheses

1. A general hypothesis

Sex cognitive differences, as defined by WCST sub-scores, dynamically change with age in Thai healthy subjects. The mechanism underpinning it is the alternations of neurotransmission-related proteins in serum, and there are sex-dependent proteomic clocks that can predict cognitive aging in men and women.

2. Specific hypotheses

2.1 In Thai healthy subjects, the differentially expressed serum proteins between the sexes are mainly involved in signaling pathways.

2.2 The sex-specific serum proteome alters dynamically with age, influencing the age-related cognitive sex difference in Thai healthy subjects.

2.3 Certain neurotransmission-related proteins interact as a cluster to influence cognitive sex differences and change of such differences with age.

2.4 There are sex-dependent proteomic clocks that can predict cognitive aging in Thai healthy men and women.

Research objectives

1. A General experiment objective

This study aimed to investigate the molecular mechanisms underlying age-related sex differences in cognitive function -as assessed by WCST – in a Thai healthy population using a quantitative proteomics method combined with

bioinformatic analysis, as well as to determine the sex-specific proteomic clocks to predict cognitive aging in men and women.

2. Specific research objectives

2.1 To study the categories and pathways of differentially expressed serum proteins between both sexes and different age groups in Thai healthy subjects using bioinformatic analysis.

2.2 To investigate the interactions of certain functional proteins and their effect on the changing of cognitive sex differences with age in Thai healthy subjects using bioinformatic analysis and WCST test.

2.3 To study changes in sex differences in cognitive function with age in Thai healthy subjects using the WCST test.

2.4 To identify the sex-specific proteomic clocks to predict sex cognitive aging in Thai healthy males and females based on the bioinformatic analysis results.

Research scope

The current study investigated the molecular mechanism underlying change of cognitive sex differences with age in Thai healthy subjects, specifically, the alteration of serum protein expression profile between both sexes with age was analyzed by quantitative proteomics analysis, the categories and interactions of differentially expressed proteins between sexes were investigated by bioinformatic analysis, and cognitive performance of each group was assessed by WCST test.

Keywords

Cognitive function, sex difference, aging, Wisconsin Card Sorting Test (WCST), neurotransmission, serum, proteomics, protein expression profile, glutamate, NMDAR, excitotoxicity.

Research contributions

1. To advance our understanding of the molecular mechanisms behind the change of cognitive sex differenced with age in a Thai healthy population.

2. To provide a better insight into the role of sexes plays in the prevalence and onset time of mental disorders in a Thai healthy population.

3. To propose the potential biomarkers for early detection of cognitive function impairment related diseases in men and women in a Thai healthy population.

Conceptual Framework of this study

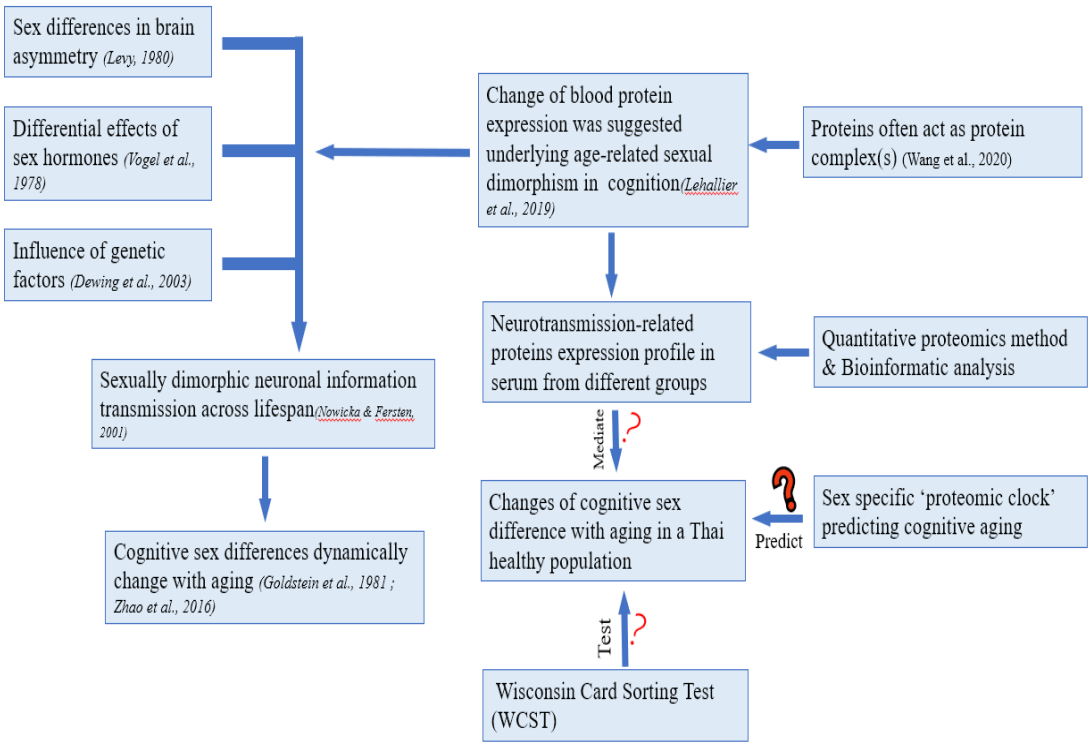

Figure 1 Conceptual Framework of this study

CHAPTER II

LITERATURE REVIEW

To better understand the molecular mechanisms underlying the change of cognitive sex differences with age, as well as to identify the potential proteomic clocks to predict sex-dependent cognitive aging are the central driving forces and integral components of this study, and they provide the overarching connectors and links for this literature review. The literature review is divided into four main sections - theoretical perspectives, the efficiency of information transmission in the neural system may be the real cause of the sex-biased trajectories in cognitive aging, neurotransmission-related proteins modulate age-dependent sex-biased efficiency of information transmission in the neural system, and proteomics methods reveal neurotransmission-related Protein complexes.

Under the influence of genetic factors and/or sex hormones, the endogenous molecules, particularly neurotransmission-related proteins are constantly altering throughout life, resulting in the development of divergent biological processes in the brain between sexes (Bocklandt, & Vilain, 2007; Marrocco, & McEwen, 2016). Because the focal point of the current study is to investigate molecular mechanisms of sex-dependent cognitive aging in a Thai healthy population, it is essential to know what research has been done in this area, what it has shown, and the impact on humans.

Theoretical perspectives

1. Age-related sexual dimorphism in cognition originates from sex differences in brain asymmetry

In the 1970s, it was trendy to believe that cognitive sex differences stem from sex differences in brain asymmetry. Specifically, Levy (1980) proposed that men had more asymmetric brain architecture, with the left hemisphere specialized for verbal processing and the right hemisphere dedicated to spatial processing. In women, the brain is more bilateral, meaning that both hemispheres are responsible for verbal

processing. Subsequent neuroimaging studies found stronger interhemispheric activation in women on a language task, which they excelled at (Shaywitz et al., 1995), and greater focal intrahemispheric activation in men on a spatial ability task, where they excelled (Gur et al., 2000). These findings substantially corroborate Levy's idea. Furthermore, a brain graph theory study reveals that brain functional asymmetries are affected by both intra- and interhemispheric white matter (WM) projection (Ocklenburg et al., 2016). Male brains were shown to have enhanced local, short-range WM projections within the lobe, indicating that they are optimized for intrahemispheric cortical connectivity, whereas female brains are structured to facilitate interhemispheric WM projections (Ingalhalikar et al., 2014), See Figure 2. Because WM is mainly composed of myelinated axons, which are responsible for transferring electrical impulses with speed and stability, there is likely a male-female differential in the efficiency of signal transduction along the axons.

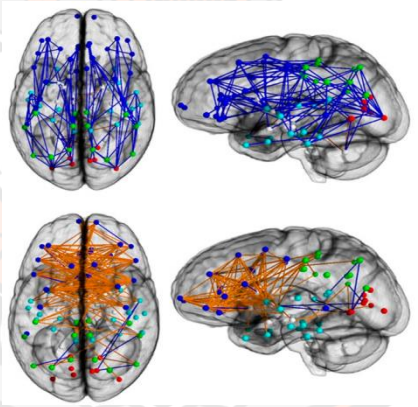

Figure 2 Brain network connectivity in males (upper) and females (lower) Intrahemispheric connections are shown in blue, and interhemispheric connections are shown in orange

Source: Ingalhalikar et al. 2014

However, sex-related hemispheric asymmetries are not stable and fluctuate over time. The differential-aging hypothesis (Goldstein, & Shelly, 1981) stated that aging is associated with greater loss of right-hemisphere functions than left-

hemisphere functions (Ellis, & Oscar-Berman, 1989), but the hypothesis of hemispheric asymmetry reduction in old adults (HAROLD) (Cabeza, 2002) developed the idea that brain activity is less lateralized in older adults compared to their younger counterparts (Bernstein et al., 2002; Grady et al., 2000). Each hypothesis was supported by empirical evidence, the differential-aging hypothesis attempts to explain the changes throughout the entire brain, whereas HAROLD mainly accounts for changes in the prefrontal cortex (Dolcos et al., 2002). In addition, Hausmann et al. (2003) discovered that age-related changes in hemisphere asymmetry depends are sex-dependent. Women's left hemisphere performance fell more dramatically, while the decline in the right hemisphere in men was more pronounced. Although the aforementioned theories create a sketch of brain structures for sex-specific cognitive aging, they failed to specify which endogenous molecules influence or even change such structures in males and females as they age.

2. Effects of sex hormones on age-related changes in cognitive sex differences

Sex-specific hormones are crucial for numerous behavioral and biological distinctions between males and females. Broverman's theory, proposed in 1978, argued that sex disparities in cognitive performance are due to the differential effects of sex hormones (androgen and estrogen) on the activation and inhibition of neural processes (Vogel et al., 1978). Data from animal models and human research have recently indicated that ovarian hormone deficiency can have a negative impact on cognitive functioning, including hippocampus and prefrontal cortex-mediated behaviors (Brinton, 2009). Besides this, only boys showed a relationship between higher levels of testosterone and lower performance in specific domains of executive function, such as monitoring the action process and flexibly shifting between actions (Nguyen et al., 2017). A clinical study, on the other hand, found that testosterone substitution may improve some aspects of cognitive capacity in male patients with Alzheimer's disease (AD) and mild cognitive impairment (Beauchet, 2006).

Concerning why men and women have diverse trajectories of cognitive aging, Samorajski's neurotransmitter hypothesis, developed in 1977, suggested that secretion of pituitary hormone-releasing factors relies on neurotransmitters, particularly catecholamine in the hypothalamus, age-related decrements of these

neurotransmitters could account for multiple age-related alterations in pituitary and periphery endocrine functions (Samorajski, 1977), see Figure 3. The following animal experiments supported this idea, demonstrating that age-related declines in luteinizing hormone secretion are caused by decreased hypothalamic norepinephrine metabolism (Simpkins, & Millard, 1987; Steger et al., 1985). As men age, testosterone levels gradually decline (Fentie et al., 2004), whereas estrogen levels fluctuate throughout women's menstrual cycles and diminish towards menopause (Pollard et al., 2007). As a result, it is intriguing to investigate which sex-specific endogenous factors change in the aging process affect the catecholamine neurotransmitter system in the hypothalamus, which controls gonadal hormone release.

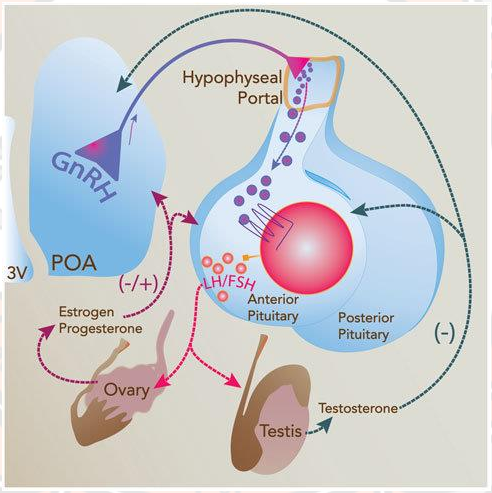

Figure 3 Schematic diagram of the hypothalamic-pituitary-gonadal (HPG) axis and its regulator

Source: Oyola, & Handa, 2017

3. Genetic factors affect sex-biased brain function independently

Another potential contributor to sexual dimorphism in cognitive function is genetic factor. According to the extant literature, 2.6% (448/17051) of the genes analyzed in the central nervous system (CNS) exhibited sex differential expression in at least one brain region, and such expression difference could be observed in almost every region of adult human brain (Trabzuni et al., 2013). In 2003, Dewing and

colleagues provided an alternative hypothesis, which contradicts the classical theory that gonadal hormones act directly to promote sex differences in neural and behavioral development, implying that sex-biased genes (54 genes) expression in embryonic mouse brain occurs before the gonad hormonal effect (Dewing et al., 2003). Another animal study discovered that cultured midbrain cells from rodents evolved into more dopaminergic neurons when the cultures were formed with XY cells rather than XX cells, regardless of the gonadal phenotype of the embryos from which the cells were harvested (Carruth et al., 2002). Autosomes, in addition to sex chromosomes, are implicated in the sex-biased differential expression of genes in the brain (Dewing et al., 2003). These findings indicate that sexually dimorphic brain and behavioral development is unlikely to be completely dependent on gonadal secretions.

The instability of nuclear and mitochondrial genomes has been proposed as a mechanism for the CNS aging (Gaubatz, & Tan, 1994). A prior genomics study found that 1% of genes expressed more severely (more than 1.7-fold change) in the neocortex and cerebellum as mice aged (Lee et al., 2000). In humans, around 4% of the over 11,000 genes studies from frontal cortex samples exhibited substantial changes with aging, with genes involved in synaptic function and plasticity, which underpin learning and memory, being the most affected (Lu et al., 2004). Moreover, recent work has demonstrated significant differences in the trajectory of a collection of AD-related genes change with age in the hippocampus of male and female mice, with females experiencing such changes considerably earlier than males (Zhao et al., 2016). Several lines of evidence established a link between mitochondrial DNA (mtDNA) and nervous system function, demonstrating that mitochondrial genes have a role in neuronal growth and structure, as well as axonal and synaptic activity (Liu et al., 2002; Zenisek, & Matthews, 2000). These effects were mainly caused by interaction with nucleus DNA, a process that becomes more pronounced with age (Roubertoux et al., 2003). Further, mtDNA sequence variants have been linked to dementia risk and disparities in longitudinal changes in cognitive function (Tranah et al., 2012). Because mtDNA is maternally inherited and the variants in mtDNA can only respond to selection acting directly on females (Innocenti et al., 2011), which leads to mutation loads affecting patterns of aging in males but not females (Camus et al., 2012).

The efficiency of information transmission in the neural system may be directly responsible for sex-biased trajectories in cognitive aging

Neurons are the fundamental units of the brain and nervous system. They are wired into circuits in the brain that receive, process, and transmit information. Neurons communicate through a specialized structure known as the synapses, which permit targeted communication between functionally related neurons (Ovsepian, 2017), as shown in Figure 4. At most synapses (chemical synapses), neurons communicate by releasing neurotransmitter packets that activate specific receptors located on the following neuron in a chain. The chemical signals received in the postsynapses then trigger ion channels, which generate action potentials that conduct across the axons.

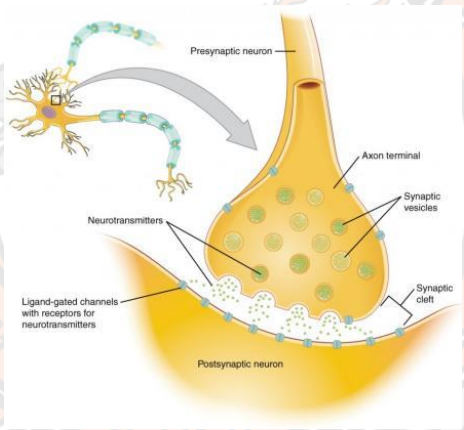

Figure 4 The synapse

Source: https://philschatz.com/chemistry-book/contents/m51011.html

There are latent male-female differences in the information synaptic transmission (Nowicka, & Fersten, 2001), and these differences are multifactorial and change with age. The first and foremost factor is the sex steroids, extant literature supports the idea that the brain, a target for sex steroids, is also a site for the synthesis of such steroids, neurosteroids (Fester, & Rune, 2021; Hojo et al., 2009). Neurosteroids have a different physiological role than gonadal steroid hormones. The neurosteroid estradiol (E2) in rodents' hippocampus has been reported to promote

both presynaptic glutamate (Glu) release probability and postsynaptic sensitivity to Glu in both sexes, but through distinct processes that function largely at discrete synapses within each sex (see Figure 5). In female animals, E2 promotes presynaptic Glu release probability via the estrogen receptor β (ERβ) and increases postsynaptic sensitivity to Glu via the G protein-coupled estrogen receptor 1 (GPER1). In males, on the other hand, E2 acts through estrogen receptor α (Erα) to enhance presynaptic Glu release probability and via ERβ to boost postsynaptic sensitivity to Glu (Oberlander, & Woolley, 2016). Besides this, subsequent research revealed sex differences in molecular signaling that underpins excitatory synaptic potentiation in the same brain region. Jain and colleagues found that, while E2 potentiates synapses to the same extent in both sexes, cAMP-activated protein kinase (PKA) is required to initiate potentiation in female rats but not males, and both L-type calcium channels and calcium release from internal stores are required for E2-induced potentiation in females. In contrast, in males, either L-type calcium channel activation or calcium release from internal stores is sufficient to permit potentiation in males (Jain et al., 2019). Parallel signaling may exist in the hippocampus of male rats, allowing one calcium source to compensate for the other, or it may be produced by changes in calcium source regulation. Furthermore, male rats might have higher baseline cAMP levels and PKA activity, it is likely that the basal state of males includes PKA-dependent phosphorylation of the inositol triphosphate receptor to elevate calcium level (Wagner et al., 2008).

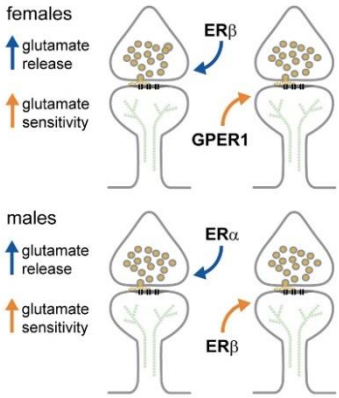

Figure 5 A latent sex difference in the mechanisms of E2-induced synaptic potentiation in the rodent hippocampus

Source: Oberlander and Woolley, 2016

Consistently, E2 modulates excitatory synapse properties in a sex-specific manner in other brain regions of rats, including the nucleus accumbens core (AcbC) (Krentzel et al., 2019). The frequency of miniature excitatory post-synaptic potentials (mEPSPs) falls dramatically in response to the E2 in female medium spiny neurons of AcbC, but not of males, thus E2 may have a bidirectional influence on glutamatergic signaling in the striatum (Krentzel et al., 2019). Aside from its effects on excitatory synaptic transmission, E2 has been shown to rapidly suppress GABAergic synaptic transmission in female rat hippocampus by mobilizing endocannabinoids (CB), which reduces the likelihood of GABA release at a subset of CB type 1 receptor (CB1R)-containing presynaptic inputs (Huang, & Woolley, 2012).

According to the literature, E2 affects glutamatergic synaptic signaling by binding to specific ERs, and one of the proposed mechanisms explaining synaptic alterations induced by E2 in the hippocampus is such alternation was mediated by interactions between ERα and NMDA receptors, the excitatory ionotropic glutamate receptors (Adams, & Morrison, 2003; McEwen, 2002). However, such interaction might be uncoupled with aging. When compared to young rats, the old rats had a dramatic decrease in the number of spines/synapses containing Erα, indicating a reduced capacity to form new synapses (see Figure 6). Furthermore, in the absence of

estrogen, the aged synapses had fewer NMDA NR1 (NMDAR1) subunits per synapse (Adams, & Morrison, 2003).

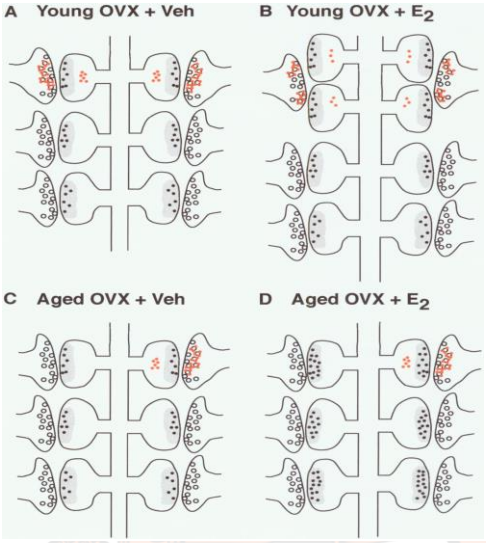

Figure 6 Schematic diagram illustrating the potential sites of synaptic alterations induced by estrogen in the hippocampus of young and old female rats. Small black dots represent NMDAR1 associated with postsynaptic, small red dots represent ER-α, open circles represent synaptic vesicles, and the gray zones represent the postsynaptic density OVX = ovariectomized; Veh = vehicle-treated; E_2 = estrogen-replaced

Source: Adams, & Morrison, 2003

The effects of E2 on synaptic efficiency have attracted the most attention, but does another sex steroid alter synaptic transmission in males? Brandt and colleagues found that testosterone (T) and its metabolite dihydrotestosterone (DHT) control synaptic density in the hippocampus of male mice but not females. Inhibiting DHT local synthesis impairs long-term potentiation (LTP) only in adult male rats' hippocampal slices (Brandt et al., 2020). Further, Ter Horst et al. (2009) demonstrated that the expression of signal transduction proteins, as well as the phosphorylation of extracellular-regulated protein kinase 1/2 (ERK1/2) and cAMP responsive element binding protein (CREB), differed between males and females. The ERK1/2-CREB signaling pathway is required for T-induced rapid spinogenesis in cultured rat

hippocampal neurons (Guo et al., 2020). Consistent with previous research linking the ERK1/2-CREB signaling pathway to formations of mushroom-type spins and glutamatergic excitatory synapses (Haque et al., 2018). Moreover, a senescence-accelerate mouse model revealed that T promotes neuronal survival and changes in synaptic plasticity in aging animals by boosting the protein expression level of synaptic NMDA receptors and promoting Ca^{2+} flux into the cell (Jian-xin et al., 2015). As with estrogen, the effect of testosterone on cognitive capabilities is mediated through the stimulation and interaction of specific androgen receptors (Spritzer, & Galea, 2007).

Apart from neurosteroids, recent literature has extensively suggested that genetic factors also contribute to the divergent synaptic transmission mechanisms across sexes. The epigenetic study discovered 248 genes and loci with a significant sex difference in histone-3 lysine-4 trimethylation (H3K4me3) in the bed nucleus of the stria terminalis and preoptic area, and 71% of them had larger H3K4me3 peaks in females. These genes and loci with elevated H3K4me3 in female animals are involved in synaptic functions (Shen et al., 2015). Another clinical research investigated sex-differentially methylated regions (DMRs) related to human psychiatric disorders and observed a high enrichment of 2080 sex-biased DMR genes in synapse-related pathways and signaling pathways, including glutamatergic, dopaminergic, serotonergic, and GABAergic synapse (Xia et al., 2021).

Furthermore, Mercer et al. (2016) uncovered that deleting one copy of the gene *GABRB3,* which encodes the $GABA_A$ receptor β3 subunit, in the mouse cerebellum. Consequently, male cerebellar nuclei (CbN) neurons upregulate their group 1 metabotropic glutamate receptors (mGluR1/5) that respond to synaptic stimulation, whereas females do not. With normal aging, the expression of a greater number of synaptic genes decreases significantly in a region-specific pattern. Specifically, synaptic gene expression changed substantially in neocortical regions but was relatively mild in limbic regions (Berchtold et al., 2013). This is consistent with prior literature, which indicated that age-related cognitive decline is more likely due to changes in synaptic connectivity and function rather than neuron loss (Nicholson et al., 2004).

Whereas synapses receive information, axons send it in the form of electrical signals with precision and stability, which together determine how efficiently the information is transmitted through the neural network. The male-female difference in axon types partitioning has been observed in rats' corpus callosum (Cc), a region connecting left and right cerebral hemispheres, with females having more unmyelinated axons than males in both genu and splenium regions of Cc (Juraska, & Kopcik, 1988; Mack et al., 1995), whereas males have a thicker myelin sheath and a higher proportion of glia (Juraska, & Kopcik, 1988). There was no significant sex difference was observed in the number of myelinated axons (Juraska, & Kopcik, 1988). A recent ultrastructural analysis further indicated that female axons had consistently smaller diameters with fewer microtubules than males (Alexander et al., 2010; Perrin et al., 2009). This implies that there is a significant difference in the efficiency of axon signal transduction between the sexes, however, studies on how such difference varies dynamically over time are rare.

Based on the theories related to the study, the current section further indicates that the efficiency of information transmission within neural circuits is directly responsible for sex-specific trajectories in cognitive aging. For instance, sex differences in brain asymmetries are primarily caused by divergent white matter wiring and projections. Both sex neurosteroids, and sex-specific genetic factors directly influence synaptic information transmission through effects on a wide range of endogenous and exogenous molecules, resulting in sex-biased information neuronal transmission throughout life.

Neurotransmission-related proteins modulate the sex-biased age-dependent efficiency of information transmission in the neural system

Synapses, as the key structure for signal transmission and plasticity in the CNS, are thought to comprise thousands of different proteins (Dieterich, & Kreutz, 2016; Kahne et al., 2016). A recent study investigated the sex-specific synaptic proteome from divergent brain regions of adult mice, and the highest divergence has been observed in the hippocampus, with 71 proteins deferentially expressed between the sexes. In the cerebellum, 28 proteins were found to have different expression levels in male and female mice, with most of them associated with neuron projection,

synaptic transmission, and RNA binding and processing. While only little male-female differences were seen in the striatal and the cortical synaptic proteome, 7 and 8 proteins differed dramatically in their abundance respectively (Distler et al., 2020). This is consistent with the findings of Block et al. (2015), who explored sex differences in protein expression for a selected panel of 100 proteins involved in learning, memory, and synaptic plasticity in the cortex, hippocampus, and cerebellum of normal male and female mice and their trisomic littermates. The hippocampus was found to have the largest difference, with 40% of those protein levels were remarkably higher in female controls, followed by the cerebellum. Furthermore, another quantitative proteomic study discovered that several neurotransmitters and nervous system-related proteins expressed differentially in rat organotypic hippocampus slice culture, such as guanylate kinase-associated protein (GKAP), the scaffold proteins in postsynaptic density-HOMER1, and high-affinity calcium sensor synaptotagmin-7, which is involved in exocytosis, were upregulated more severely in males. Proteins associated with axon guidance process were also upregulated in male animals, including ROBO1, the semaphorin receptor plexin A1-PLXNA1, the receptors tyrosine kinase Ephrin type-A receptor 4 and 7 (EPH4 and EPHA7, respectively), and the Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase calcineurin (PPP3R1, PPP3CA, PPP3CB, and PPP3CC subunits). In females, however, the most upregulated protein was myelin basic protein (MBP), which is essential for the formation of neuron myeline (Weis et al., 2021).

The Ras homolog enriched in striatum (Rhes) is a small GTP-binding protein. It has been reported that Rhes affects striatal cAMP-PKA dependent signaling in a sex-sensitive manner. For example, in male mice, Rehs deletion mainly perturbed basal cAMP/PAK activity, whereas in females, Rhes deficiency selectively increased phasic activation of this pathway (Ghiglieri et al., 2015). Outside of the striatum, rhes mRNA was highly expressed in other brain regions such as the hippocampus (Harrison et al., 2008). As aforementioned, cAMP/PAK is required to initiate potentiation in female rats' hippocampus only (Jain et al., 2019). Thus, Rhes could be a potential mediator for the sex-biased molecular signaling that underlies excitatory potentiation in the striatum and hippocampus. Furthermore, Salzberg and colleagues found that local ubiquitin-mediated protein degradation in selected synapses of one

sex leads to dimorphic circuits upon sexual maturation in *C. elegans*. Specifically, the E3 ligase SEL-10/FBW7 degrades the netrin receptor UNC-40 in the sensory phasmid neuro PHB in hermaphrodites only, resulting in synapse pruning while leaving males unaffected (Salzberg et al., 2020). Data from another animal study elucidated that GPER1 agonist G1 strongly increased the density of maturing spines in CA1 stratum lacunosum-molecular cultures from female mice exclusively, and G1 also upregulated protein expression of PSD 95 and decreased the p-/n-coffin ratio only in cultures from the female animals (Li et al., 2021). PSD 95 is a scaffolding protein found in the excitatory synapses that is involved in the stabilization, recruitment, and trafficking of NMDA and AMPA glutamate receptors (Chen et al., 2000). It plays an essential role in the glutamatergic transmission and synaptic plasticity (Funke et al., 2005; Gilman et al., 2011). These data show that PSD 95 may function as a mediator for E2 acts through GPER1 to increase postsynaptic sensitivity to Glu in female animals (Oberlander, & Woolley, 2016). The following study found that PSD 95 levels in old female zebrafish were much higher than in their younger counterparts (Karoglu et al., 2017). Moreover, there is a male-female difference in the expression of dopamine (DA) neuron vesicular glutamate transporter (VGLUT), with DA neurons expressing remarkably higher levels of VGULT in females than males, a finding that is consistent across species from flies to rodents to humans. In addition, the expression of DA neuron VGLUT increases dynamically with aging, and inhibiting such age-related upregulation of VGULT in DA neurons eliminates sex difference in DA neuron degradation with aging (Buck et al., 2021). Besides this, synaptophysin (SYP), a presynaptic vesicular protein, has been reported to be drastically downregulated with aging in male zebrafish (Karoglu et al., 2017), which is consistent with another study using a mouse model that showed a similar result (Benice et al., 2006).

Proteomics methods reveal neurotransmission-related Protein complexes

At the molecular level, the core functions of the brain require psychical interactions among a range of cell surface and intracellular proteins (Basu et al., 2021). For example, synaptic transmission is dependent on transient and stable protein-protein interactions (PPI) between the hundreds of components that form the pre- and post-synaptic compartments. Neurotransmission requires the psychical

interaction of a network of scaffold proteins, calcium sensors, and SNARE proteins at the presynapse; as well as interacted protein 90/PSD 95- associated proteins construct a structural and functional framework at the postsynapse (Frank et al., 2017). Thus, understanding the mechanisms of synaptic transmission efficiency entails investigating the spatial and temporal interactions of multi-protein complexes that shape synapse function. In recent years, proteomic methods have been used to construct a draft of a synapse protein interactome. The first proteomic study of a synapse protein complex was published in 2000, when researchers isolated NMDAR multiprotein complexes (NRC) from the mouse brain, which contains 77 proteins. The structure of such NRC implies that subsets of neurotransmitter receptors, second messengers, cell-adhesion proteins, adapters, and cytoskeletal proteins are organized together into a physical unit containing signaling pathways, and the NRC features provide a better understanding of the physiological context of NMDAR-dependent synaptic plasticity (Husi et al., 2000). Subsequent research uncovered even more neurotransmission-related protein complexes. Abul-Husn and colleagues (2009) applied graph theory-based algorithms to analyze proteomic datasets and found a protein complex containing 17 proteins in rodent presynaptic nerve terminals, as well as predicted 92 additional components. In another publication, a stable and specific complex of 118 proteins related to PSD 95, containing a series of essential synaptic receptors, channels, and signaling molecules, was isolated from the postsynaptic proteome of mice (Fernandez et al., 2009).

The majority of the existing literature on neurotransmission-related protein complexes, however, is based on animal brain tissue proteome studies, which does not work well in human experiments, particularly those involving healthy participants. Because blood contains proteins from different cells and tissues (Ray et al., 2011), and it is convenient to access. Making it an ideal biological sample for investigating neurotransmission-related protein complexes linked to changes in sex-biased cognitive function with normal aging.

CHAPTER III

RESEARCH METHODOLOGY

The purpose of this study was to investigate at how sex differences in cognitive performance change with aging as assessed by Wisconsin Card Sorting Test sub-scale scores. In addition, serum proteomics and bioinformatic analysis were employed in the current work to examine how sex-biased neurotransmission-related protein expression evolves with aging.

Human subjects

The current study used samples from a prior research program that investigated the relationship between dopamine neurotransmission and the executive function (Khanthiyong et al., 2019). The subjects were 199 adult Thai healthy volunteers (male and female). Traditional concepts define healthy old as people aged 60 and above (Baltes, & Smith, 2003). Moreover, a previous longitudinal study revealed that a gradual decline in whole brain volume starts at age 35 (0.2% per year) and accelerates to 0.5% per year at age 60 (Hedman et al., 2012). To explore sex differences in the cognitive performance evolving with aging, all human subjects were divided into three age groups: (1) young adult group, age range from 20-34 years (n=70), (2) middle-aged adult group, age range from 35-59 years (n=59), (3) elderly group, age range from 60 years and above (n=70).

Human subjects with abnormal mental health as evaluated by the Thai Mental Health Indicator (TMHI-55) were excluded from this study. The Mini-Mental State Examination (MMSE) was also applied to exclude subjects with dementia. The MMSE is a 30-point questionnaire that is used extensively in clinical and research settings to screen dementia, and it examines cognitive functions including language, recall, attention and calculation, orientation, and ability to follow simple commands (Pangman et al., 2000). An MMSE score of 24 or higher (out of 30) indicates normal cognition, and all research subjects scored higher than that.

All subjects are of Thai ethnicity, to reduce the possibility of confounding by the population stratification (Freedman et al., 2004).

The Naresuan University Human Ethics Committee had previously approved the recruiting (IRB No.553/2017). Every human subject was provided informed consent before participation.

However, all the experimental protocols involved in the current study were approved by the Human Ethics Committee of Naresuan University (IRB No. 0262/2022).

Blood sample collection

A 3ml cubital vein blood sample was collected from each enrolled subject immediately after completing the WCST test. The blood sample was centrifuged at 3,000 rpm for 5 minutes. The serum was then transferred into a 1.5ml microcentrifuge tube and stored at -80℃ in a refrigerator for future use. All samples were coded to ensure anonymity (Khanthiyong et al., 2019).

Quantitative proteomics analysis

The analytic processes were performed by the National Centre for Genetic Engineering and Biotechnology, Pathum Thani, Thailand, including protein digestion, Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis, protein identification, and protein quantitation.

1. Sample preparation

The protein concentration of all serum samples was determined by the Lowry assay using BSA as a standard protein (Lowry et al., 1951). Five micrograms of protein samples were subjected to in-solution digestion. Samples were completely dissolved in 10 mM ammonium bicarbonate (AMBIC), disulfide bonds were reduced using 5 mM dithiothreitol (DTT) in 10 mM AMBIC at 60 °C for 1 hour, and sulfhydryl groups were alkylated at room temperature for 45 mins in the dark using 15 mM *Iodoacetamide* (IAA) in 10 mM AMBIC. The protein samples were digested for 16 hours at 37°C with sequencing grade porcine trypsin (1:20 ratio). The tryptic peptides were dried using a speed vacuum concentrator and resuspended in 0.1%

formic acid for nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis.

2. LC-MS/MS analysis

The prepared tryptic peptide samples were injected into an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics) equipped with a Nano-captive spray ion source. In brief, one microlitre of peptide digests was enriched on a µ-Precolumn 300 µm i.d. X 5 mm C18 Pepmap 100, 5 µm, 100 A (Thermo Scientific, UK), and separated on a 75 µm I.D. x 15 cm and packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper (Thermo Scientific, UK). The C18 column was placed inside a thermostated column oven that was set to 60 ℃. Solvent A and B containing 0.1% formic acid in water and 0.1 % formic acid in 80% acetonitrile respectively were supplied on the analytical column. A gradient of 5–55% solvent B was used to elute the peptides at a constant flow rate of 0.30 µl/min for 30 min. Electrospray ionization was carried out at 1.6kV using the CaptiveSpray. Nitrogen was used as a drying gas (flow rate about 50 l/h). Collision-induced-dissociation (CID) product ion mass spectra were obtained using nitrogen gas as the collision gas. Mass spectra (MS) and MS/MS spectra were obtained in the positive-ion mode at *2 Hz* over the range (m/z) 150–2200. The collision energy was adjusted to 10 eV as a function of the *m*/*z* value. The LC-MS analysis of each sample had been done in triplicate.

3. Protein quantitation and identification

MaxQuant 1.6.6.0 was utilized to quantify the proteins in individual samples using the Andromeda search engine to correlate MS/MS spectra to the Uniprot *Homo sapiens* database (Tyanova, Temu,, & Cox, 2016). Label-free quantitation with MaxQuant's standard settings was performed: a maximum of two miss cleavages, a mass tolerance of 0.6 Dalton for the main search, trypsin as digesting enzyme, carbamidomethylation of cystein as a fixed modification, and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. Only peptides with a minimum of 7 amino acids, as well as at least one unique peptide, were required for protein identification. Only proteins with at least two peptides, and at least one unique peptide, were considered as being identified and used for further data analysis. Both peptide and protein false discovery rates (FDR)

were set to 10%. The maximum number of modifications per peptide was set at 5. As a search FASTA file, the proteins present in the *Homo sapiens* proteome were downloaded from Uniport. Potential contaminants are present in the contaminants_. fasta file that comes with MaxQuant were automatically added to the search space by the software.

After loading the MaxQuant ProteinGroups.txt file into Perseus version 1.6.6.0 (Tyanova, Temu, Sinitcyn, et al., 2016), all potential contaminants that did not correspond to any UPS1 protein were removed from the data set. Max intensities were $\log_2$ transformed. Missing values were also imputed in Perseus using a constant value (zero).

4. Bioinformatics analysis

Prior to analysis, data cleansing and preprocessing were performed by Perseus (version 1.6.15.0) software. Venn diagrams were utilized to show common proteins shared by different groups (Bardou et al., 2014). PANTHER database (version 16.0) was employed for the functional annotation and enrichment analysis (Mi et al., 2021). Pathway analysis was performed using the DAVIDA database (version 6.8) and Pathway Studio. Neurotransmission-related protein complexes were studied by COMPLEAT (Vinayagam et al., 2013). The false discovery rate (FDR) was set at 1%.

Wisconsin Card Sorting Test (WCST)

The cognitive performance of the subjects in this study was measured by computer-based Wisconsin Card Sorting Test (Inquisit 3.0.6.0). The WCST employs 4 stimulus cards and 128 response cards. The response cards comprise 3 different dimensions including color (red, green, blue, and yellow), number (1, 2, 3, and 4 items), and form (circles, stars, squares, and crosses) (Singh et al., 2020). The WCST test rules are as follows: The participants were shown 4 response cards and asked to choose one of them to match the stimulus card with the sorting rules based on color, number, or form. Participants must select the correct card that corresponds to the rule and maintain 4 consecutive correct responses. Following that, the test abruptly shifted to the new sorting rule. The program only gives feedback as correct or incorrect and records the scores for each WCST subscale. The test was terminated after completing

6 categories of 3 different sorting rules or reaching 128 trails (see Figure 7). The WCST subscale reflects the distinct aspect of cognitive function outlined below:

- The percentage of total corrects (%Corrects): the total number of correct response cards multiplied by 100 and divided by the total cards, reflecting initial conceptualization and attention.
- The percentage of total errors (%Errors): the total number of incorrect response cards multiply 100 and divided by the total cards, reflecting nonspecific cognitive impairment.
- The number of categories completed (Category completed): determined by using the score range from 1 to 6, reflecting cognitive set-shifting.
- The perseverative errors (PE): the score was used to measure the inability to correct the response due to ignorance of relevant stimuli, reflecting cognitive inflexibility.
- Trails to complete the first category (1st Category): the score ranges between 0 and 128, which is the number to complete the initial category of the task.

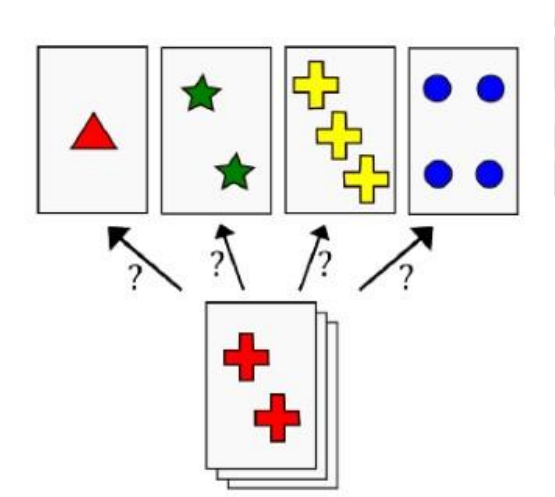

Figure 7 Illustration of Wisconsin Card Sorting Test (WCST)

Source: Khanthiyong et al., 2019

Statistical analysis

Using R-programming ver. 4.1.2, a General Linear Model (GLM) approach paired with Bayesian statistics was applied to analyze the age difference between both sexes, the relationship between education level and sex, the difference in educational level among three age groups, sex differences in WCST sub-scores (covarying for age and educational level), and the difference of WCST scores between males and females from each age group (covarying for educational level) (Fife, 2020), because age and education are associated with cognitive performance (Deary et al., 2009; Murman, 2015). Bayes factor was offered to illustrate the likelihood of supporting the alternative hypothesis (see Table 1).

Linear Model for Microarray Data (LIMMA) approach within R-programming ver. 4.1.2 was employed to find differentially expressed proteins (DEPs) between men and women (Smyth, 2005), with FDR set at 1%. The fold changes (FC) of DEPs were calculated and displayed as $\log_2^{(FC)}$, and the volcano plot was constructed by R-programming ver. 4.1.2 for the two groups. In this study, $P \leq 0.05$ was considered significant.

Table 1 Bayes factor grades of evidence

BF (H_1:H_0)	Evidence against H_0
0-1	Anecdotal
1-3	Weak
3-10	Substantial
10-30	Strong
30-100	Very strong
＞100	Decisive

Source: Liang, & Xiong, 2013

CHAPTER IV

RESULTS

This chapter reported the findings of a study on neural mechanisms underpinning age-dependent cognitive sex differences. The Wisconsin Card Sorting Test (WCST) and label-free proteomics analysis were used to collect data from 199 Thai healthy subjects for this research. Following data collection, the WCST raw scores were analyzed using the General Linear Model to determine how cognitive sex differences fluctuate with age, and the serum protein expression profiles were analyzed by Bioinformatics. The analysis results are summarized and presented in the following order.

Demographic data

The Subjects were 89 males and 110 females, with a mean age of 45.6±19.3 years (range, 20-70 years). Age differed between men and women (BF=10.7, 95%CI= [2.74, 13.2]), and although a sex difference in education level was discovered (BF=4.9), the evidence was moderate.

In each age group, differences in age and education level between men and women were not adequately supported. However, there were significant differences in education level among the three age groups (see Table 2).

Table 2 Demographic data of subjects

	Male	Female	Education level	Age	95%CI
Young adult	22 (31.4%)	48 (68.6%)	BF=0.26		
	21.2±1.50	21.1±3.06		BF=0.2	[-1.40, 1.44]
Middle-aged adult	32 (54.2%)	27 (45.8%)	BF=4		
	53.2±6.36	49.4±7.93		BF=1.42	[0.05, 7.33]
Elderly	35 (50%)	35 (50%)	BF=0.81		
	65.0±3.59	65.2±3.01		BF=0.18	[-1.55, 1.58]
Inter-groups			BF>100		

Data was presented as mean±SD by General Linear Model. BF=Bayes Factor.
95%CI=95% Credible Interval of difference male against female

Sex differences in cognitive performance

As shown in Figure 8, males performed better in %Corrects and had fewer %Errors, with strong evidence to reject the null hypothesis. Weak evidence suggests the presence of sex differences in the other three scores: Category Completed, 1st Category, and PE. Table 3 shows the parameter estimation of sex disparities in those WCST sub-scores.

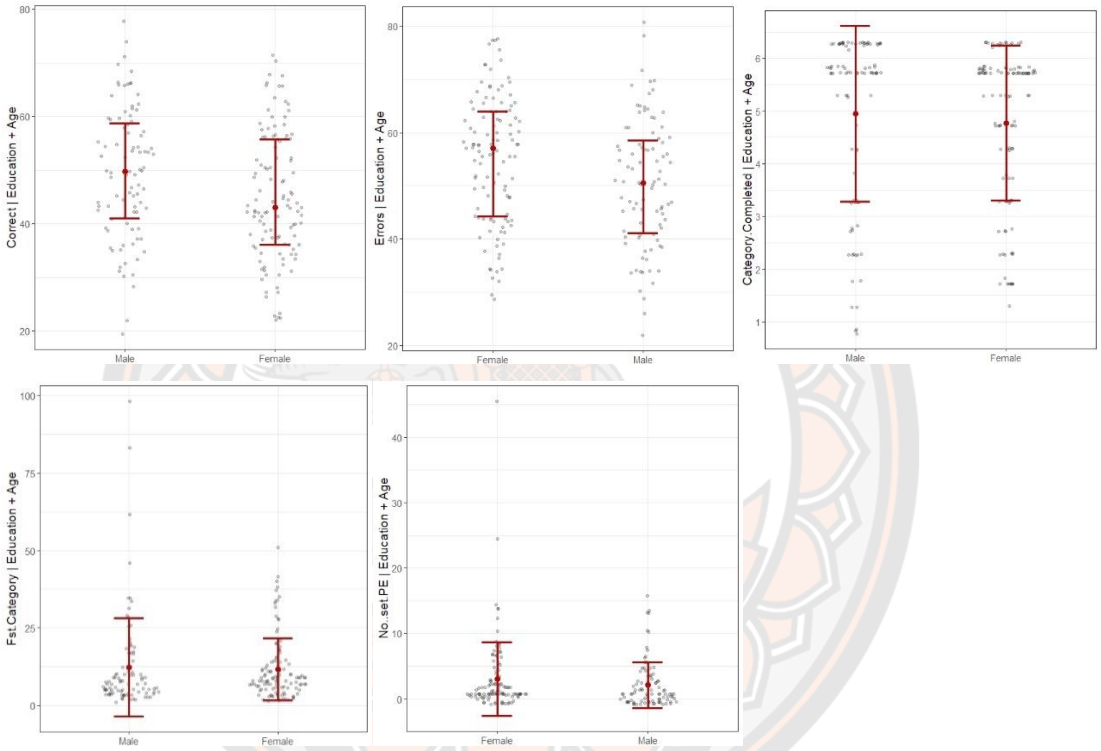

Figure 8 Sex differences in WCST sub-scores after controlling age and education level. Correct=%Corrects, Errors=%Errors, Fst Category=1st Category, No..set PE=PE

Table 3 The parameter estimation of sex differences in WCST sub-scores

	Male	Female	BF	95% CI	Cohen's d
%Corrects	48.8±12.3	45.5±12.6	193	[1.40, 8.23]	0.39
%Errors	51.1±12.2	54.5±12.6	193	[-8.31, -1.30]	0.39
Category completed	4.89±1.68	4.81±1.51	1.54	[-0.27, 0.63]	0.12
PE	2.25±3.55	2.94±5.68	2.48	[-2.32, 0.38]	0.20
1st Category	12.8±16.1	11.3±10.1	1.86	[-3.05, 4.31]	0.05

Data was presented as mean±SD by General Linear Model.
BF=Bayes Factor 95% CI= 95% Credible interval of difference male against female

Change of sex differences in cognitive function with age

Age-related sex differences were found in two of the five WCST scores: %Corrects and %Errors. As shown in Figure 9, males scored better in %Corrects (BF=7.84, 95%CI = [-0.35, 12.6]) and had fewer total errors (BF=7.58, 95%CI= [-12.7, 0.2]) in the young adult group, however, this sex difference reversed in the middle-aged adult group (%Corrects: BF=2.09, 95%CI= [-1.27, 11.1]; %Errors: BF=2.08, 95%CI= [-11.0, 1.54]). Nevertheless, there was insufficient evidence to support sex differences in these two scores in both young and middle-aged adult groups. While male dominance in %Corrects (BF＞100, 95%CI=[4.32, 14.6]) and %Errors (BF＞100, 95%CI=[-14.6, -4.29]) have been found in the elderly group, as in the young adult group, with decisive evidence to support it. In all three age groups, there was weak evidence to support the alternative hypothesis for the other three WCST scores: Category complete, 1st Category and PE (BF＜4).

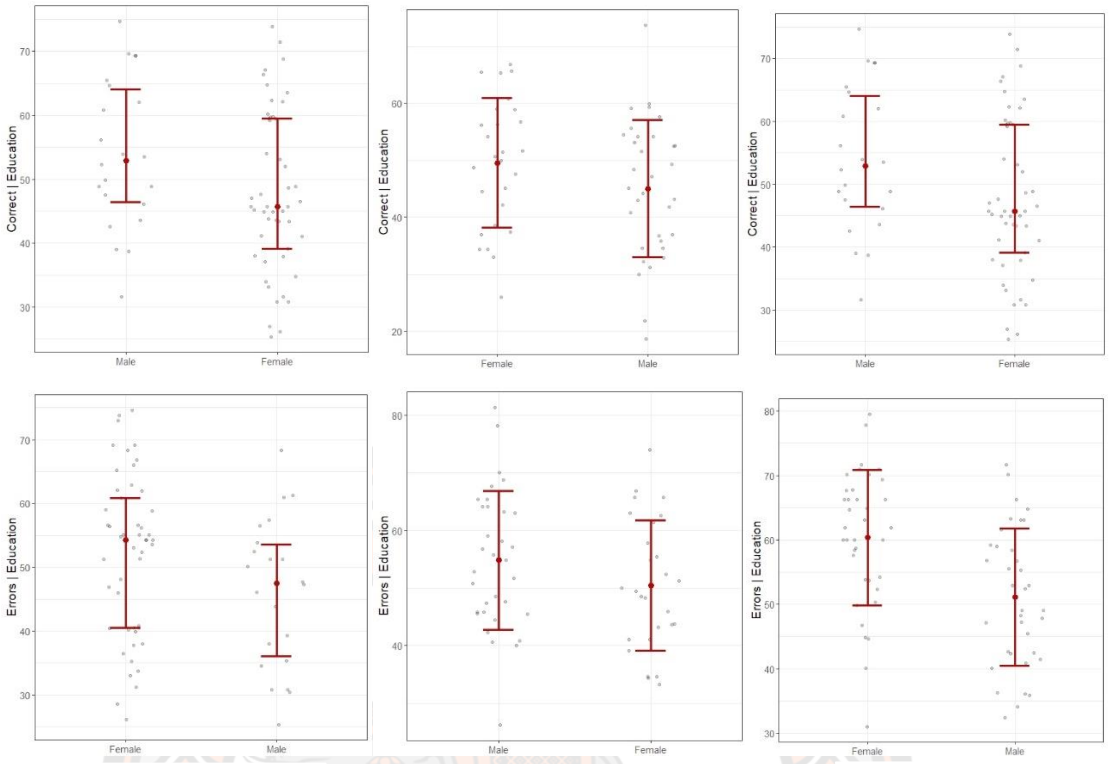

Figure 9 Change of sex differences in WCST scores %Corrects and %Errors with age. Correct=%Corrects, Errors=%Errors

Identification and relative quantification of differentially expressed proteins between males and females

The label-free proteomics detected 19640 proteins in each sample after applying protein FDR=0.1. Then, 886 proteins were further filtered with both protein FDR=10% and Q-value=0.95 in MaxQuant, with 808 of them shared by both men and women (see Fig 10). Of those 808 proteins, we found 52 proteins that were differentially expressed between sexes (FDR<0.01) (see Table 4), with 6 of those 52 DEPs being upregulated in males and the other 46 DEPs downregulated (see Fig 11).

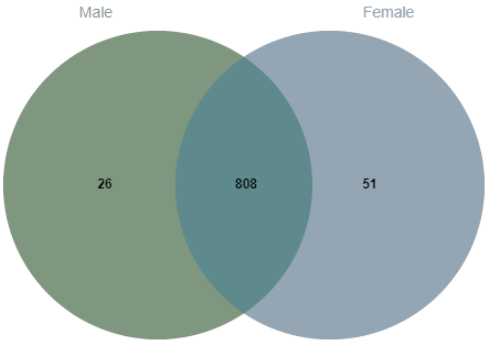

Figure 10 Venn diagram showing 818 proteins shared by both males and females

Table 4 Differentially expressed proteins between males and females detected by LIMMA, FDR=1%

Number	Protein IDs	Gene name	Protein name	Fold change (Male/Female)	FDR
1	A6NGW2	STRCP1	Putative stereocilin-like protein	-9.87378	1.2004E-12
2	P28068	HLA-DMB	HLA class II histocompatibility antigen, DM beta chain	-10.0593	1.9025E-07
3	P0C5Y4	KRTAP1-4	Keratin-associated protein 1-4	5.006562	5.166E-06
4	O00635	TRIM38	E3 ubiquitin-protein ligase TRIM38	-7.38556	9.0655E-06
5	P04731	MT1A	Metallothionein-1A	-5.80523	2.2525E-05
6	P32297	CHRNA3	Neuronal acetylcholine receptor subunit alpha-3	-5.72918	2.3905E-05
7	C9JL84	HHLA1	HERV-H LTR-associating protein 1	-5.64453	2.7304E-05
8	O75084	FZD7	Frizzled-7	-5.46865	2.7304E-05
9	O95221	OR5F1	Olfactory receptor 5F1	-4.76125	3.4929E-05
10	P16298	PPP3CB	Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform	-4.81837	3.4929E-05
11	A0A1B0GV85	REELD1	Reelin domain-containing protein 1	-4.93878	3.4929E-05
12	B4E2M5	ANKRD66	Ankyrin repeat domain-containing protein 66	-6.80293	8.8842E-05
13	P25311	AZGP1	Zinc-alpha-2-glycoprotein	-6.08555	0.00013846
14	P08620	FGF4	Fibroblast growth factor 4	-5.4998	0.00013846
15	P28906	CD34	Hematopoietic progenitor cell antigen CD34	5.718257	0.00016083
16	P02749	APOH	Beta-2-glycoprotein 1	-5.84757	0.00016748
17	P0C0L4	C4A	Complement C4-A	-1.67486	0.00016748
18	O00273	DFFA	DNA fragmentation factor subunit alpha	3.878624	0.0003333
19	P38117	ETFB	Electron transfer flavoprotein subunit beta	-3.96004	0.00036524
20	P14061	HSD17B1	17-beta-hydroxysteroid dehydrogenase type 1	-4.51463	0.00050686
21	P01024	C3	Complement C3	-1.22015	0.00057158

Number	Protein IDs	Gene name	Protein name	Fold change (Male/Female)	FDR
22	P04275	VWF	von Willebrand factor	-3.89944	0.00060659
23	P04004	VTN	Vitronectin	-4.00878	0.0006814
24	P02766	TTR	Transthyretin	-4.64038	0.0006814
25	P20338	RAB4A	Ras-related protein Rab-4A	-5.6758	0.0006814
26	P05155	SERPING1	Plasma protease C1 inhibitor	-4.44992	0.0006814
27	O43353	RIPK2	Receptor-interacting serine/threonine-protein kinase 2	-4.3834	0.0006814
28	P0DMS9	TMIGD3	Transmembrane domain-containing protein TMIGD3	-4.81477	0.00069802
29	O75096	LRP4	Low-density lipoprotein receptor-related protein 4	-3.74001	0.00090466
30	P13861	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	-4.93194	0.00093903
31	P26022	PTX3	Pentraxin-related protein PTX3	3.44284	0.00158262
32	P0CG40	SP9	Transcription factor Sp9	3.946251	0.00284037
33	O15397	IPO8	Importin-8	-4.82519	0.00305309
34	G5E9R7	KRTAP4-16	Putative keratin-associated protein 4-16	4.263715	0.00313433
35	O95674	CDS2	Phosphatidate cytidylyltransferase 2	-4.39524	0.00343441
36	O75881	CYP7B1	Cytochrome P450 7B1	-4.64784	0.00349086
37	A0A075B6I0	IGLV8-61	Immunoglobulin lambda variable 8-61	-2.62925	0.00349086
38	P28331	NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	-4.06335	0.00375275
39	P01042	KNG1	Kininogen-1	-5.17065	0.00387168
40	O75891	ALDH1L1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	-3.43624	0.00418803
41	P01008	SERPINC1	Antithrombin-III	-4.40148	0.00418803
42	P36955	SERPINF1	Pigment epithelium-derived factor	-4.6215	0.00424301
43	P14652	HOXB2	Homeobox protein Hox-B2	-2.88034	0.00549476
44	P10412	H1-4	Histone H1.4	-2.86669	0.00590983
45	O75427	LRCH4	Leucine-rich repeat and calponin homology domain-containing protein 4	-4.00204	0.00614704
46	P05783	KRT18	Keratin, type I cytoskeletal 18	-4.18557	0.00756386
47	P08603	CFH	Complement factor H	-3.11493	0.00756386
48	P04264	KRT1	Keratin, type II cytoskeletal 1	-1.29099	0.00756386
49	O14980	XPO1	Exportin-1	-4.08967	0.00849615
50	P0DOX7		Immunoglobulin kappa light chain	-1.35981	0.00863139
51	O15382	BCAT2	Branched-chain-amino-acid aminotransferase, mitochondrial	-3.24202	0.00881399
52	P22897	MRC1	Macrophage mannose receptor 1	-3.98199	0.00972859

The red and green color are corresponding to the highlighted proteins in Figure 11

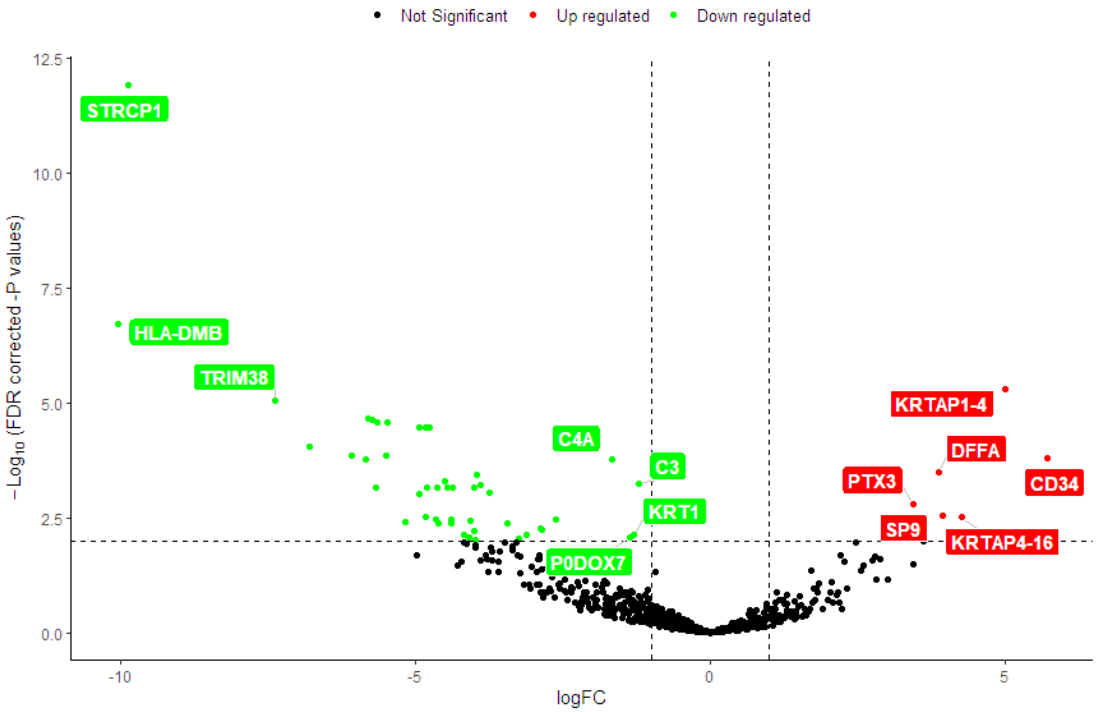

Figure 11 Profile of differentially expressed proteins between males and females. Green color represents protein downregulated in males; red color represents protein upregulated in males. logFC=log^{2}FC (Male/Female)

Overrepresentation analysis of DEPs

To gain insight into the biological changes that occur following a WCST test in both sexes. PANTHER overrepresentation analysis was employed to determine if such DEPs were enriched in certain groups based on the following three Gene annotation (GO) classes: molecular function (MF), biological process (BP), and cellular component (CC). The results showed that in the MF class, the DEPs involved in heparin binding (GO:0008201) were the most significantly enriched (Fold enrichment=14, FDR=0.025), see Figure 12 and Table 5, and in the BP class, the DEPs involved in the regulation of complement activation (GO:0030449) were the most significantly overrepresented (Fold enrichment=55.1, FDR=0.016). The DEPs associated with blood microparticle (GO:0072562) were the most significantly enriched in the CC class (Fold enrichment=22.4, FDR=7.14E-6).

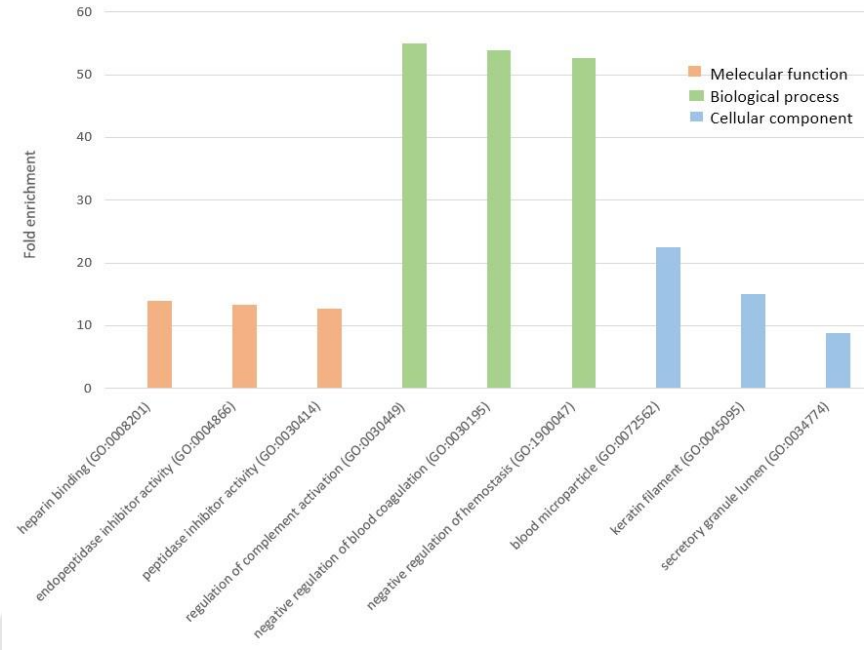

Figure 12 The top three significantly enriched DEPs in each GO class

Table 5 The top three significantly enriched DEPs in each GO class

GO class	Proteins	Fold enrichment	FDR
Molecular function			
heparin binding (GO:0008201)	6	14	0.025
endopeptidase inhibitor activity (GO:0004866)	6	13.24	0.00854
peptidase inhibitor activity (GO:0030414)	6	12.75	0.00841
Biological process			
regulation of complement activation (GO:0030449)	3	55.05	0.0159
negative regulation of blood coagulation (GO:0030195)	6	53.83	1.09E-05
negative regulation of hemostasis (GO:1900047)	6	52.66	9.83E-06
Cellular component			
blood microparticle (GO:0072562)	8	22.43	7.14E-06
keratin filament (GO:0045095)	4	15.09	0.0238
secretory granule lumen (GO:0034774)	7	8.83	0.00339

Pathway enrichment analysis

The KEGG pathway analysis revealed two significantly enriched pathways: complement and coagulation cascades (has:04610) and Staphylococcus aureus infection (has:05150), as shown in Table 6.

Table 6 KEEG pathways the differentially expressed proteins enriched

Number	Pathway ID	Pathway	Mapped DEPs	Fold Enrichment	FDR
1	hsa04610	Complement and coagulation cascades	P04004, P0C0L4, P01042, P08603, P01008, P05155, P04275, P01024	23.1	3.62E-6
2	hsa05150	Staphylococcus aureus infection	P0C0L4, P28068, P08603, P05783, P01024	12.8	0.032

Sex-dependent protein complexes analysis

To investigate the dynamic assembly of protein complexes stimulated by the WCST test, 19,640 previously identified proteins were submitted to the COMPLEAT database. Protein complexes that behaved consistently across both sexes (common complexes), as well as others that showed dynamic changes in either male or female subjects (dynamic complexes) were found.

As demonstrated in Figure 14, analysis of male versus female data sets revealed 54 common complexes and 109 dynamic complexes, with 48 complexes enriched solely in females and the remaining 61 complexes concentrated only in males. All the complexes with $P \leq 0.01$.

In terms of neurotransmission-related protein complexes, the elevation of cytosolic calcium ion concentration complex (P=4.45E-04), activation of protein kinase activity complex (P=6.99E-04), transmembrane receptor protein tyrosine kinase signaling pathway complex (4.38E-03), Multicomponent signaling complex (6.90E-03), positive regulation of kinase activity complex (P=7.63E-03), JAK2-PAFR-TYK2 complex (7.82E-03), and receptor-mediated endocytosis complex (9.41E-03) were exclusively enriched in female samples. In males, mucin type O-glycan biosynthesis complex (P=6.52E-03), activation of phospholipase C activity complex (P=3.98E-03), ULK1-ATG13-FIP200 complex (P=1.31E-03), receptor-

mediated endocytosis (P=8.68E-03), NOTCH-core complex (P=9.29E-03), and protein sumoylation complex (P=9.06E-03) were selectively presence, see Figure 13.

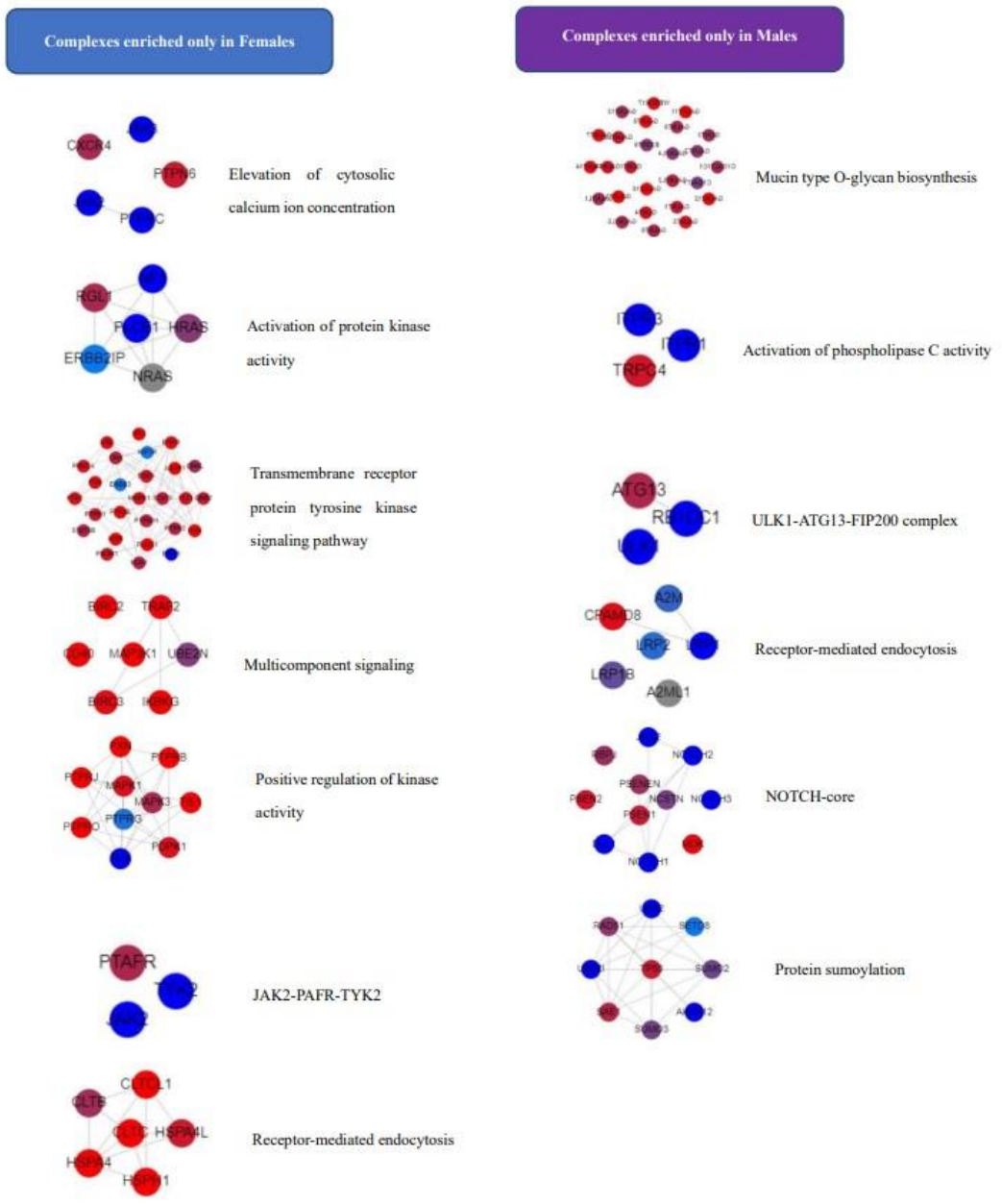

Figure 13 Neurotransmission-related protein complexes only enriched in males or females. Color gradient varies from red (max input score) to blue (min input score). Dashed lines indicate interactions.

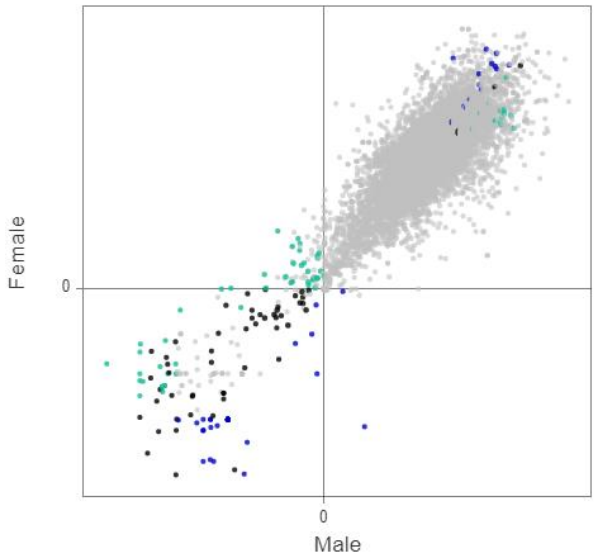

Figure 14 Comparison of complex enrichment scores from males' and females' datasets. Black points correspond to enriched complexes in both datasets. Cyan points correspond to significant complexes in males only, and blue points correspond to female-specific complexes. All the complexes with P≤0.01

The dynamic assembly of sex-dependent protein complexes with age

As shown in Figure 15, analysis of male versus female data sets in the young adult samples revealed 33 common complexes and 44 dynamic complexes, with 17 complexes enriched solely in females and the remaining 27 complexes concentrated only in males. There were 40 common complexes detected in the middle-aged group, as well as 50 complexes enriched exclusively in females and 61 complexes enriched only in males. In the elderly, 40 common complexes were discovered, as well as 38 complexes were concentrated only in females and 56 complexes that were solely enriched males.

In terms of neurotransmission-related protein complexes, we found that the N-methyl-D-aspartate type glutamate receptor (NMDAR) complex (P=0.005), regulation of protein kinase activity complex (P=0.009), and G-protein coupled receptor signaling pathway complex (P=0.008) were solely concentrated in elderly females. There were four neurotransmission-related complexes that were only enriched in elderly men: protein ubiquitination complex (P=0.004), generation of

neurons complex (P=0.002), regulation of JUN kinases activity complex (P=0.008), and negative regulation of apoptotic process complex (P=0.007) (see Figure 16). Besides, the elevation of cytosolic calcium ion concentration complex ($P < 0.005$) was only found in females across all three age groups. The regulation of neuron differentiation complex (P=0.003), NOTCH-Core complex (P=0.0007), and activation of protein kinase activity complex (P=0.0009) were found to be enriched only in females in the elderly samples, however, these complexes existed as common complexes in the middle-aged group and were not seen in young adult samples. While the axon guidance complex was present as common a complex in the young adult group, it was concentrated only in females in both middle-aged (P=0.0007) and elderly (P=0.002) groups. Furthermore, the nervous system development complex was found to be enriched only in males in both middle-aged (P=0.009) and elderly (P=0.008) groups, but not in young adults. The histone H3-K4 methylation complex was not present in the young adult group, was common in the middle-aged group, and was enriched only in males in the elderly group (P=0.007).

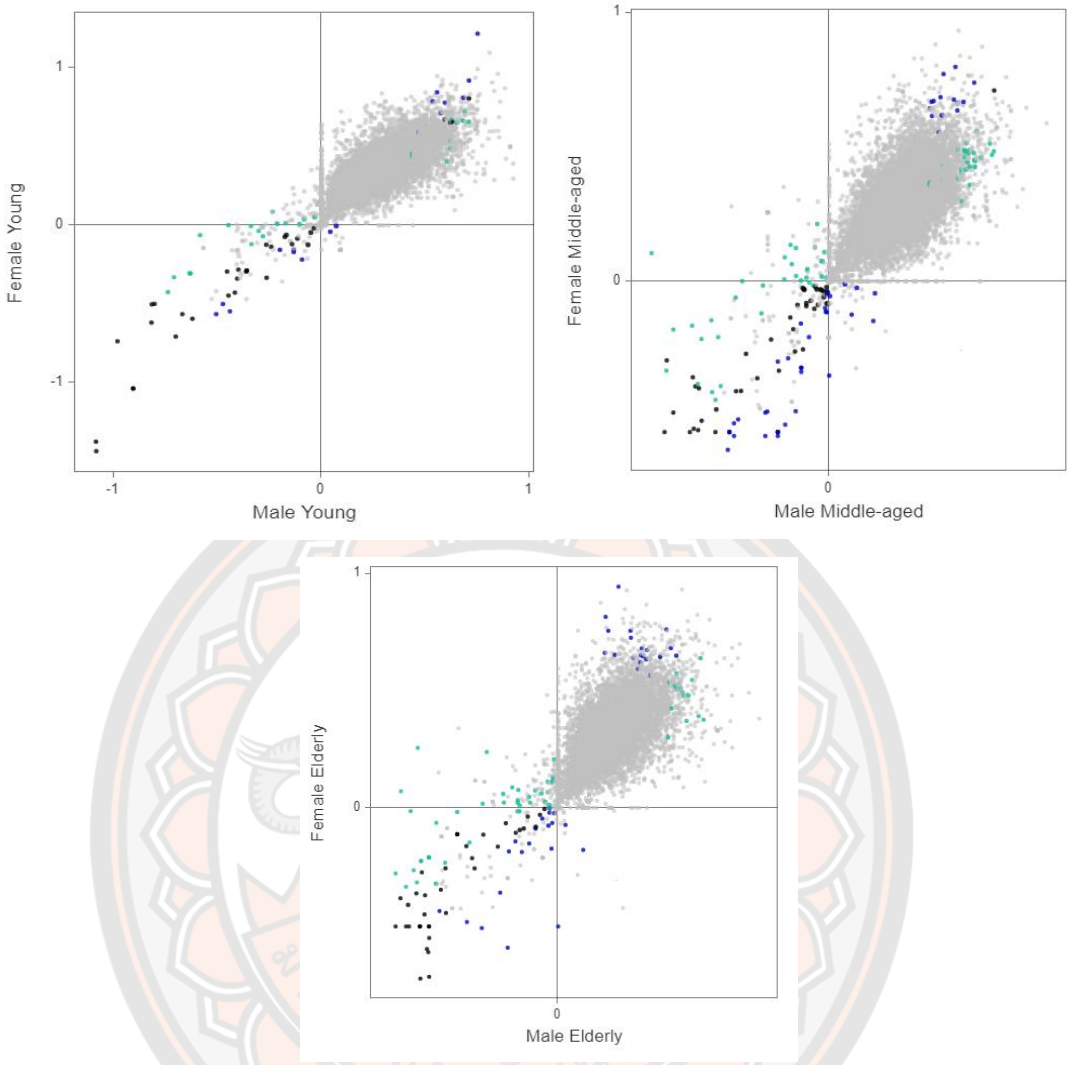

Figure 15 Comparison of complex enrichment scores from males and females' datasets in each age group. Black points correspond to enriched complexes in both datasets. Cyan points correspond to significant complexes in males only, and blue points correspond to female-specific complexes. All the complexes with P≤0.01

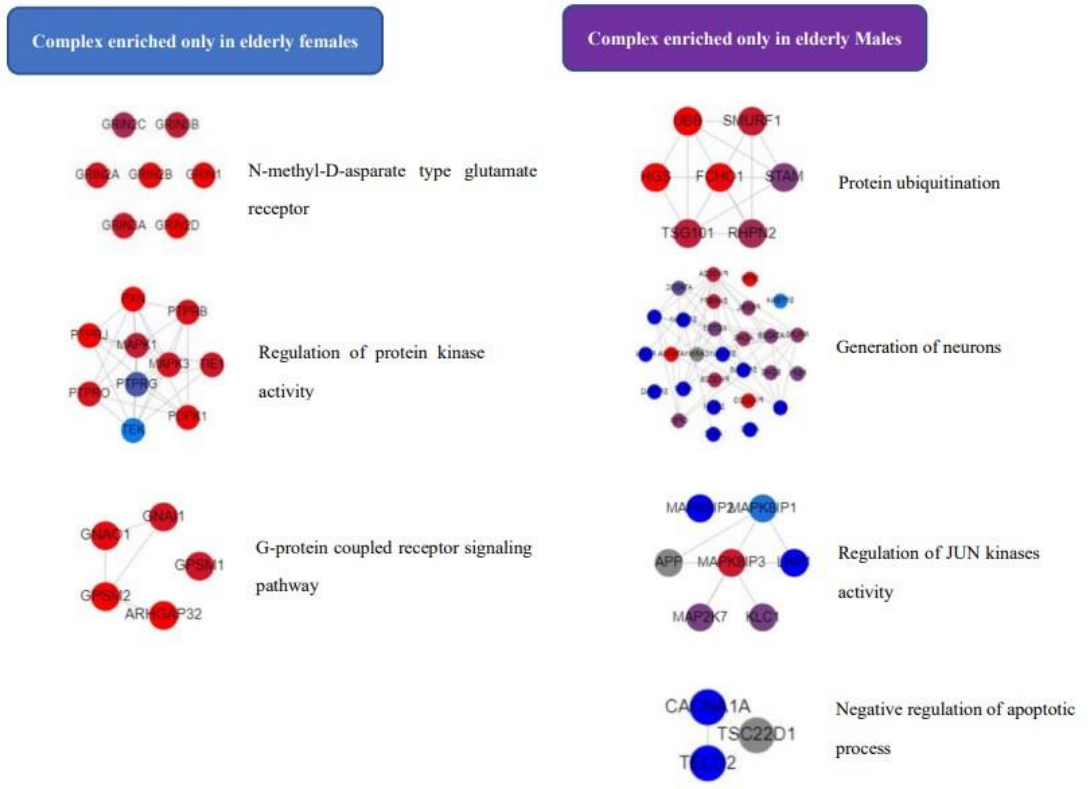

Figure 16 Neurotransmission-related protein complexes enriched only in elderly males or females. Color gradient varies from red (max input score) to blue (min input score). Dashed lines indicate interactions

CHAPTER V

DISCUSSION

This chapter summarizes and discusses the findings of this study and addresses how the age-dependent dynamic assembly of sex-bias neurotransmission-related proteins influences cognitive sex differences. Because the current study included four research objectives and hypotheses, there is a discussion for each objective and subsequent hypothesis. This chapter also discusses the limitations and the practical applications of this study, as well as makes recommendations for future research.

Changes of sex differences in cognitive function with age as shown by the WCST scores in Thai healthy subjects

In the current study, to gain a better understanding of how sex differences in cognitive function evolve with age in Thai healthy subjects, cognitive performance was assessed by using WCST. Males outperformed females in two of the five WCST sub-scores: %Corrects and %Errors, with a higher percentage of total corrects and lower total errors rate.

The WCST is a test of cognitive flexibility, that is the ability to adjust behavioral response mode in the face of changing conditions (Nagahama et al., 1997; Nyhus, & Barcelo, 2009). Its sub-scores %Corrects and %Errors show specific domains of cognitive function, which correspond to the activity of certain brain regions. For example, %Corrects reflects conceptualization and attention, and previous study showed that multiple areas of the PFC may participate in information processing during attentional shift in the WCST (Nagahama et al., 1998). While %Errors reflecting non-specific cognitive impairment and frontal and temporal lobes lesions have been linked to a large number of WCST total errors (Horner et al., 1996; Nelson, 1976).

Intact cognitive function is dependent on the precise exchange of information between neurons, which is initiated by the activation of excitatory neurotransmitter pathways (Cheng et al., 2021; Xu et al., 2012), primarily the glutamatergic pathway

since glutamate (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS). However, its excessive activation would result in excitotoxicity, a term initially described in a study by Olney in which acute neuronal cell damage was observed as the consequence of Glu exposure in both infant and adult mice (Olney, 1969). Excitotoxicity is caused by changes in Glu metabolism, dysfunction of Glu transporters, hyperfunction of Glu receptors, particularly N-methyl-D-aspartate receptors (NMDAR) (Lipton, 2008; Vincent, & Mulle, 2009)

Evidence from the clinical field indicated that abnormalities in the prefrontal cortex (PFC) were positively connected to WCST total errors in both obsessive-compulsive disorder (OCD) patients (Gruner, & McKay, 2013) and major depression disorder (MDD) patients (Zhang et al., 2022). Another study conducted by Askari et al. (2022) discovered that after 12 weeks of memantine-a NMDA receptor antagonist-treatment, the WCST total errors decreased in OCD patients. In contrast, N-methyl-D-aspartate (NMDA) treatment increases excitotoxicity markers in the rat frontal cortex (Chang et al., 2008). These data imply that excitotoxicity driven by NMDA receptor overactivation in PFC is linked to the higher number of WCST errors. On the other hand, the PFC plays an essential role in attention control (Bahmani et al., 2019; Kim et al., 2016; Knight et al., 1995), Glu neurotoxicity in this brain region is bound to impaired attention control and results in worsened performance in WCST %Corrects. Furthermore, when comparing females with MDD to their healthy counterparts, increased expression levels of the majority of glutamate receptor genes in the PFC were found, although such differences were not found in male groups (Gray et al., 2015). In healthy individuals, the efficiency of normal cellular uptake mechanisms in removing Glu from the synaptic cleft (Regan, & Choi, 1991; Schousboe, 1981) and the potential neuroprotective effect of estrogen (Burstein et al., 2018; Mendelowitsch et al., 2001; Zhao, & Brinton, 2007) may mask Glu-induced acute neurotoxicity. Thus, WCST errors may be a reflection of excitotoxicity damage in the PFC, males did better because they are less susceptible to such acute and transient glutamate neurotoxicity.

This study also found that the sex difference in %Corrects and %Errors changes with aging, and this difference becomes noticeable in those over 60. This is congruent with the findings of a study by Whitley et al. (2016), which demonstrated

that in the UK residents, men and women subtraction scores and numerical problem-solving ability show different trends with aging beginning at 60. In addition, estrogen levels in women fluctuate throughout their lives and drop after menopause (Wariso et al., 2017). According to the existing literature, Thai women reach menopause at around 50 years of age (Chompootweep et al., 1993), hence the neuroprotective effects of estrogen are likely to be weakened in elderly female participants. This idea is supported by animal model research, which observed that those juvenile female rats were less susceptible to developing Glu-induced excitotoxicity due to the neuroprotective effect of estrogen (Al-Suwailem et al., 2018). As a result, the considerable sex differences in those two scores seen in the elderly group in this work could be attributed to the fact that women had lost the protective effect of estrogen and were thus more vulnerable to the damage caused by Glu-induced excitotoxicity. A neuroimaging study also found that men had larger volumes of the parieto-occipital area than women between the ages 65 to 75 (Coffey et al., 1998), and lesions in this brain region are positively associated with WCST errors (Nyhus, & Barcelo, 2009). This adds to the evidence that supports our findings.

The classifications and pathways involved in differentially expressed serum proteins between both sexes

At the molecular level, the DEPs between sexes were most significantly enriched in complement cascades, additionally, those four proteins with the most complex relationships- C3, APOH, VWF, and KNG1- are either the components of or related to the complement system (Dobó et al., 2011; Feng et al., 2015; Garcia-Arguinzonis et al., 2021; Schrijver et al., 2020). The complement cascade is a major component of the innate immune system (Ricklin et al., 2010). One of the fundamental functions of complement activation in the healthy brain is to protect neurons from potentially harmful toxic stimuli (Ziabska et al., 2021). The complement component 3 (C3), a central component of the complement system, has been found to be expressed in rat hippocampus following acute excitotoxic injury (Hernandez-Encinas et al., 2016). In the current study, complement cascade components such as C3 and C4A, as well as regulators such as CFH, VTN, and SERPING 1 were detected in both males and females, considering the blood drawn from the subjects after the

WCST test, the complement activation is most likely in response to generation of excitotoxicity induced by Glu. Moreover, the aforementioned complement components and regulators are all upregulated in female subjects when compared to males, indicating that females may be more susceptible to Glu-induced excitotoxicity.

Certain neurotransmission-related proteins interact as a cluster to influence sex cognitive differences

To become functional, proteins commonly depend on their interaction with other molecules, which are known to comprise the other proteins, which rapidly interact into protein complexes. Protein complexes are functional units of proteome organization, and they are responsible for the majority of biological processes such as biomedical pathways and signaling cascades in the cell (Acuner Ozbabacan et al., 2011; Basu et al., 2021).

The current study uncovered a number of sex-biased neurotransmission-related protein complexes. The elevation of cytosolic calcium ion concentration complex, which contains 5 proteins, was only found in the females. In classic excitotoxicity, impaired Glu transporter function leads to increased extracellular Glu, which elicits a massive influx of calcium into neurons via NMDARs (Tymianski et al., 1993), whereas elevated calcium contributes ultimately to irreversible excitotoxic injury (Choi, 2020). Protein kinase C (PKC), a family of protein kinase enzymes, has been shown to regulate NMDARs trafficking and gating (Lan et al., 2001), and upregulating cellular PKC activity can exacerbate neurotoxicity mediated by NMDA receptor activation (Wagey et al., 2001). Both activation of protein kinase activity complex and positive regulation of kinase activity complex were selectively seen in females, implying that there would be excitotoxicity induced by NMDAR hyperfunction in women. In addition, platelet-activating factor receptor (PAFR) interacts with Tyk2 to promote Janus kinase 2 (Jak2) activation (Lukashova et al., 2003). Jak is a type of protein tyrosine kinase (Szilveszter et al., 2019), and data from experimental mice and clinical observations have revealed multiple signaling events mediated by Jak in innate and adaptive immunity (Ghoreschi et al., 2009). The enrichment of the JAK2-PAFR-TYK2 complex and transmembrane receptor protein tyrosine kinase signaling pathway complex further indicated the presence of

excitotoxicity induced by Glu. Previous research shows that one function of the multi-component signaling is to mediate environmental stress (Franchini et al., 2000; Janeway, 1992), and its enrichment suggested that aforementioned neurotransmission-related protein complexes are more likely to be assembled transiently in response to the WCST stimulation.

The activation of the phospholipase C (PLC) activity complex was identified only in males. PLC activation is associated with an enhanced NMDAR function (Xiao et al., 2012), whereas PLC inhibition suppressed NMDAR-dependent long-term depression (Horne, & Dell'Acqua, 2007). Another complex that is only found in men is ULK1-ATG13-FIP200 complex, which mediates the mTOR signaling (Ganley et al., 2009; Hosokawa et al., 2009). A previous study has demonstrated that NMDAR activation regulates sociability through its effects on the mTOR signaling pathway (Burket et al., 2015). According to existing research, NOTCH signaling regulates numerous phases of innate immunity (Radtke et al., 2010; Shang et al., 2016). The mucin-type-O-glycans have multiple functions, including acting as receptors for carbohydrate-binding proteins and as an important component of the immune response (Brockhausen, 2006). Furthermore, protein sumoylation, as a post-translational modification, is essential in various biological processes, and an earlier study has shown that global sumoylation level shapes the immune responses (Karhausen et al., 2021). Thus, the selective enrichment of these complexes in males implies that excitotoxicity induced by NMDAR hyperfunction may also occur in males, as males are less susceptible to such Glu-induced neurotoxicity and scored better in WCST test. However, Radiske et al. (2021) revealed that hippocampal NMDARs drive local protein synthesis via mTOR signaling and may control active memory maintenance, and that the enrichment of NOTCH core complex in males may offer a compensating effect on the excitotoxicity-induced cell injury.

There are two receptor-mediated endocytosis complexes selectively enriched in females and males, but with different components, reflecting a sex-biased transient protein assembly in response to the environmental stimuli.

Sex-biased protein complexes dynamically change with age

Protein complexes are not permanent, their dynamic assembly is fundamental to inducing cellular responses to various internal and external stimuli, and the individual protein complexes involved in a signaling pathway assemble in different compartments at different times (Hartwell et al., 1999).

This study uncovered three complexes that were enriched exclusively in elderly females: N-methyl-D-aspartate type glutamate receptors (NMDARs) and its regulators tyrosine kinase (TK) (Salter, & Kalia, 2004; Wang, & Salter, 1994) and mitogen-activated protein kinase (MAPK) (Haddad, 2005), and G-protein coupled receptors (Lutzu, & Castillo, 2021; MacDonald et al., 2007). This suggested a potential NMDAR hyperfunction in the brain of elderly females. Because serum samples were collected shortly after the participants completed the WCST test in this study, aforesaid sex-specific enriched neurotransmission-related protein complexes are more likely to be interacted transiently in response to the external stimuli. According to the hyperfunction theory of aging (Blagosklonny, 2006), aging is not functional decline, but caused by celluar hyperfunction-a function that was not switched off upon its completion-that results in age-related diseases. This theory linked aged-related functional loss to inappropriate activation of signaling pathways. Thus, later in life, higher than the optimal activity of NMDAR directly drives age-related cognitive impairment in women (Newcomer et al., 2000), as indicated in their performance in score %Errors.

Moreover, JUN kinase has been reported to mediate glutamate-induced excitotoxicity (Borsello et al., 2003), with JUN kinase controlling NMDAR-evoked presynaptic glutamate release as a possible mechanism (Nisticò et al., 2015), and NMDAR activation leads to related protein ubiquitination (Xu et al., 2018). The regulation of JUN kinase activity and protein ubiquitination complexes were selectively concentrated in men of the elderly group, suggesting that NMDAR hyperfunction might also be detected in elderly males. However, they had less cognitive impairment than elderly women, as evidenced by the sex difference in both %Corrects and %Errors scores. The neuroprotective efficacy of sex steroids, estrogen (Green, & Simpkins, 2000; Wei et al., 2014) and testosterone (Białek et al., 2004; Moffat, 2005), has been proposed to protect against neuron damage induced by

NMDAR hyperfunction in females and males, respectively. Female estrogen levels dropped after menopause (Wariso et al., 2017), whereas testosterone levels in males gradually decrease with age (Feldman et al., 2002). As a result, disparities in the neuroprotection of sex hormones in older men and women contribute to their differences in cognitive function.

The neurotransmission-related protein complexes identified in middle-aged samples mostly exist as common complexes. Among them, the NOTCH-Core complex associated with NMDAR-induced synaptic plasticity (Lathia et al., 2008), and NMDAR signaling leads to the activation of protein kinase (Giordano et al., 2005; Wang, & Peng, 2016). Only one common complex was found in the young adult samples: the axon guidance complex, which has been suggested to be regulated by NMDAR (Gao et al., 2018; Lee et al., 2005). This indicated that NMDAR hyperfunction may be present as early as the age of 20 in both sexes. In addition, Tymianski et al. (1993) discovered that increasing extracellular glutamate elicits a massive influx of calcium into neurons via NMDARs, and neuronal cell death is related to intracellular calcium ion overload (Zhivotovsky, & Orrenius, 2011). The elevation of cytosolic calcium ion concentration complex was exclusively enriched in women across all three age groups, implying that females may be more susceptible to the harm caused by NMDAR hyperfunction, which could explain the sex difference in cognitive performance. Because NMDAR hyperfunction beyond its optimal value increases with age, the reflection of it is cognitive decline with age, and sex hormone decline at different rates in male and female, which is why we observed different cognitive sex differences in score %Corrects and %Errors, as well as sufficient evidence to support such differences in elderly group only.

Limitation of this study and implication for future research

There are several limitations to our study. First, in an attempt to obtain a sample that is approximately representative of the population, the sample covers a range of subjects with varied age, education levels, socioeconomic statuses, and occupations, these also increase the variability of the sample. Second, except age and education level, the other confounders such as socioeconomic status and occupations were not controlled for in this study, despite the fact that both potentially contribute to

the sex difference in cognitive performance. Third, while some neurochemicals do cross the bran-blood barrier (De Blasio et al., 2019), the majority of them might be locally synthesized in the brain (Veerhuis et al., 2011; Woodruff et al., 2010), and future studies directly comparing protein expression profiles in the brain tissues between males and females is needed to replicate our finding. Last, owing to the moderate sample size, generalizing the findings of this study should be done with caution.

CHAPTER VI

CONCLUSIONS

The purpose of this study was to investigate the neural mechanisms underlying age-related sex differences in cognitive function – as assessed by WCST - in a Thai healthy population using a quantitative proteomics method and bioinformatic analysis, as well as to explore the sex-specific proteomic clocks to predict cognitive aging in men and women.

The first objective was to examine age-dependent sex differences in cognitive function between Thai healthy men and women. There were cognitive sex differences in two of the five WCST scores %Corrects and %Errors. However, when grouped by age, such sex disparities were only sustained by compelling evidence in the elderly group.

The second objective was to investigate the classifications and pathways of the differentially expressed proteins (DEPs) in males and females. They were significantly enriched in the complement cascades, with the majority of such DEPs upregulated in females, showing that females may be more vulnerable to the excitotoxicity induced by NMDAR hyperfunction.

The third objective as to explore the interactions of certain functional proteins and their effect on the change of cognitive sex differences with age. The age-related sex-biased proteins transient assembly in response to WCST stimuli was found in both males and females. This added evidence from the protein global level to the hypothesis that females are more susceptible to Glu-induced excitotoxicity.

The last objective was to identify sex-specific proteomic clocks to predict cognitive aging. The NMDAR complex was only found enriched in elderly female samples, and the NMDAR level in serum would be a novel “proteomic clock” for predicting cognitive aging in Thai healthy women.

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