THE ROLE OF DEHYDROEPIANDROSTERONE SULFATE ON LEARNING AND MEMORY IN RAT HIPPOCAMPUS AND ITS LINK TO THE PREFRONTAL CORTEX

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
3α,5α-TH PROG	3α,5α-tetrahydroprogesterone
3β-HSD	Δ5-3β-hydroxysteroid dehydrogenase
5-CSRTT	5-Choice serial reaction time task
ACTH	Adrenocorticotropin hormone
ADHD	attention-deficit/hyperactivity disorder
AF	Alexa Fluor
Allo	Allopregnanolone
ANOVA	Analysis of variance
AP	Antero-posterior
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium
CA1	Cornu Ammonis-1
CA2	Cornu Ammonis-2
CA3	Cornu Ammonis-3
CLE	Confocal laser microscopy
CNS	Central nervous system
CR	Correct response
CRH	Corticotropin-releasing hormone
CRL	Correct response latency
DBS	Deep brain stimulation
DHEAS	Dehydroepiandrosterone sulfate
DHEA	Dehydroepiandrosterone
DOL	Degree of labelling
DT	Distance travelled

DV	Dorso-ventral
EL	Escape latency
FAAT	Foot shock active avoidance training
FI	Fluorescence intensity
FFM	Fibre Fluorescence Microscopy
FSH	Follicle-stimulating hormone
F/P	Ration of fluorophore/protein
$GABA_A$	γ-aminobutyric acid type A
GCMS	Gas chromatography mass spectrometry
GnRH	Gonadotrophin-releasing hormone
H&E	Hematoxylin & Eosin
Hip-PFC	Hippocampus-Prefrontal cortex
HPA	Hypothalamo-pituitary adrenal
HPG	Hypothalamo-pituitary gonadal
HPLC	High performance liquid chromatography
ICR	Incorrect responses
i.c.v.	intracerebroventricular
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
ITC	Isothiocyanates
LCMS	Liquid chromatography mass spectrometry
LH	Luteinizing hormone
LTD	Long-term depression
LTP	Long-term potentiation
ML	Medio-lateral
MMSE	Mini Mental State Examination

MALDI-ToF MS	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MWM	Morris water maze
NaCl	Sodium chloride
NMDA	N-methyl-D-aspartate
OR	Omission responses
PA	Percent accuracy
PBS	Phosphate buffer saline
PFC	Prefrontal cortex
PR	Premature responses
PROG	Progesterone
PREG	Pregnenolone
PREGS	Pregnenolone sulfate
RIA	radioimmunoassay
ROI	Region of interest
SC	Sulfonyl chlorides
SDP	Sulfodichlorophenol esters
SEM	Standard error mean
SE	Succinimidyl esters
SPE	Solid phase extraction
SQ	subcutaneous
SSE	Sulfosuccinimidyl esters
StAR	Steroidogenic acute regulatory
SS	Swimming speed
THDOC	Allotetrahydrodeoxycorticosterone
TFP	Tetrafluorophenyl esters
UV-Vis	Ultraviolet-visible

PERANAN DEHIDROEPIANDROSTERON SULFAT TERHADAP PEMBELAJARAN DAN DAYA INGATAN DALAM HIPOKAMPUS TIKUS DAN KAITANNYA DENGAN KORTEK PRAFRONTAL

ABSTRAK

Dehydroepiandrosterone sulfat (DHEAS) merupakan salah satu neurosteroid penting yang disintesis di dalam otak. Peranan DHEAS adalah penting dalam meningkatkan ingatan dan pembelajaran dan juga fungsi pelaksanaan. Kajian ini bertujuan untuk mengkaji perubahan tahap pendarflour DHEAS berdasarkan kepada prestasi ingatan dan pembelajaran dan prestasi pemerhatian dalam pengimejan secara langsung di kawasan hipokampus *Cornu Ammonis* 1 (CA1). Dalam kajian ini, tikus jantan *Sprague Dawley* dibahagikan kepada dua kumpulan utama bagi peranan tingkah laku yang berbeza iaitu *Morris water maze* (MWM) dan *5-Choice serial reaction time task* (5-CSRTT). Untuk MWM, tikus yang berusia 8 minggu dan mempunyai berat sekitar 250-290 g manakala untuk 5-CSRTT, tikus berumur 10 minggu dengan berat sekitar 300-330 g dibahagikan kepada dua kumpulan yang mengandungi 9 ekor tikus untuk setiap kumpulan kawalan, C (kumpulan I), dan tugasan tingkah laku beserta pembedahan implan dan pengimejan, BP (kumpulan IIa) dan kawalan bersama pengimejan, C_I (kumpulan IIb). Penglabelan pendarflour telah dibina sebagai penanda kepada DHEAS di dalam kawasan hippocampal CA1. Antibodi DHEAS berpendarflour dikonjugasikan bersama *Alexa Fluor 5-sulfodichlorophenol (SDP) ester 488 dye*. Pembedahan implan dan penglabelan pendarflour telah diletakkan di dalam kawasan hippokampal CA1 bagi MWM dan 5-CSRTT. Ujian MWM dilaksanakan untuk prestasi ingatan dan ruangan pembelajaran di mana 5 hari pertama untuk ruangan pembelajaran dan hari yang ke-6 untuk ujian *probe*. Ujian 5-CSRTT

digunakan untuk menentukan prestasi pemerhatian bagi fungsi pelaksanaan. Seterusnya, tikus dipengsankan dengan isofluran dan pada masa yang sama visual dan imej pendarflour DHEAS dalam pengimejan langsung dengan *Fibre Fluorescence Microscopy* (FFM) *probe* bersambungan sistem Cell Visio Lab. Tikus dibedah dan hipokampus dikumpulkan untuk mengesahkan kedudukan *probe* dengan histologi hematoksilin dan eosin (H&E). Konjugasi label-pendarflour yang berjaya pertama kali dilaporkan menunjukkan tahap penglabelan (DOL) di dalam julat yang ditetapkan dan signal ekspresi DHEAS pendarflour diimejkan di FFM bersambungan sistem Cell Visio Lab. Pengoptimuman ke atas pembedahan implan telah dilakukan untuk menentukan koordinat kawasan hipokampal CA1 dengan tepat. Keputusan MWM menunjukkan perubahan yang signifikan bagi kedua-dua kumpulan (IIa dan IIb) terhadap prestasi ruangan ingatan dan pembelajaran seiring dengan peningkatan pendarflour intensiti DHEAS. Keputusan menunjukkan bahawa pendarflour intensiti DHEAS meningkat disebabkan oleh peningkatan prestasi tingkah laku ruangan pembelajaran dan ingatan. Ujian 5-CSRTT menunjukkan tikus belajar dan terarah kepada tindak balas apertur yang bersesuaian. Keputusan menunjukkan prestasi pemerhatian dihasilkan dan integrasi dengan peningkatan pendarflour intensiti DHEAS. Penemuan daripada kajian ini menentukan peranan DHEAS dan hubung kait di antara DHEAS dengan prestasi pembelajaran dan daya ingatan dan juga fungsi eksekutif seterusnya menghubungkan kawasan hipokampus CA1 pada kortek prafrontal.

THE ROLE OF DEHYDROEPIANDROSTERONE SULFATE ON LEARNING AND MEMORY IN RAT HIPPOCAMPUS AND ITS LINK TO THE PREFRONTAL CORTEX

ABSTRACT

Dehydroepiandrosterone sulfate (DHEAS) is one of the important neurosteroids that are originally synthesised in the brain. The role of DHEAS is important in enhancing learning and memory performance, and executive functions. The aim of this study was to investigate the changes of DHEAS fluorescence level corresponding to the learning and memory task and attentional performance task in real time imaging in the Cornu Ammonis 1 (CA1) area of hippocampus. In this study, adult male Sprague Dawley rats were divided into two major different groups of behavioural tasks which are Morris water maze (MWM) and 5-Choice serial reaction time task (5-CSRTT). For MWM, eight-week-old rats weighing 250-290 g whereas for 5-CSRTT, ten-week-old rats weighing 300-330 g were divided into two groups of 9 animals for control group, C (group I), and behavioural task with surgical implantation and imaging, BP (group IIa) and control with imaging, C_I (group IIb) each. Fluorescence labelling was developed as a tagging to the DHEAS in the CA1 area of hippocampus for both major groups. DHEAS antibodies were fluorescently conjugated with Alexa Fluor 5-SDP ester 488 dye. Surgical implantation and fluorescence labelling were delivered into the CA1 area of hippocampus for each task. The MWM test was performed for spatial learning and memory performance by which 5 days was for the spatial learning test and on the 6^{th} day for probe test. The 5-CSRTT test was used to determine attentional performance of executive function. Following this, the animals were anaesthetized with isoflurane and the DHEAS fluorescence was

simultaneously visualised and imaged in real time imaging with FFM probe connected to Cell Visio Lab system. The animals were dissected and their hippocampi were collected for the histological validation of the probe placement using hematoxylin and eosin (H&E) staining. The conjugation of fluorescent-labelled was the first successful report which demonstrated the degree of labelling (DOL) in the range of desired results and showed the signal of DHEAS expression on the FFM connected to the Cell Visio Lab system. Optimisation on surgical implantation was made to determine the coordinates at the CA1 area of hippocampus precisely and accurately. The results in MWM test indicated that both groups (group IIa and IIb) showed significant changes for spatial learning and memory with enhancement of DHEAS fluorescence intensity. The results revealed that DHEAS fluorescence intensity significantly increased due to spatial learning and memory performance. In the 5-CSRTT test, the results exhibited that the animals learned and tended to respond in an appropriate aperture. The results indicated attentional performance occurred and integrated with increase in the intensity of DHEAS fluorescence. Findings form this study were able to elucidate the role of DHEAS and the association between DHEAS with learning and memory performances as well as executive function thus linking the CA1 area of the hippocampus to the prefrontal cortex.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dehydroepiandrosterone sulphate (DHEAS) is a neurosteroid that is found in greater concentration within the brain rather than in any other body organ (Dong and Zheng, 2011). DHEAS is synthesised from DHEA in the presence of sulfotransferase enzyme and can be converted back to DHEA. Several studies have shown that DHEAS play a role in enhancing both learning and memory (Markowski *et al.*, 2001; Bodensteiner *et al.*, 2008) as well as executive function (Cai *et al.*, 2018). Neurosteroids are endogenous steroids that are synthesised *de novo* in the central nervous system. Several neurosteroids have been identified in both human and animal brains, which include dehydroepiandrosterone (DHEA) and pregnenolone (PREG), their sulfated derivatives DHEAS and PREGS, and progesterone (PROG), deoxycorticosterone, 3α,5α-tetrahydroprogesterone (3α,5α-TH PROG) or allopregnanolone (Allo) and 3α,5α-TH DOC (Charalampopoulos *et al.*, 2008). All neurosteroids are derived from the precursor cholesterol, which is transported into the inner mitochondrial membrane of neurosteroidogenic cells by steroidogenic acute regulatory protein (StAR).

Although neurosteroids are identical to the steroid hormones that produced in the endocrine system, neurosteroids have a unique function and mechanism of action in the nervous system. In the nervous system, neurosteroids function as modulators of neurotransmitter receptors, such as γ-aminobutyric acid type A ($GABA_A$) receptors, *N*-methyl-D-aspartate (NMDA) receptors, and sigma 1 receptors (Mòdol *et al.*, 2011; Khare *et al.*, 2016). Neurosteroidogenesis has been demonstrated in several types of

brain cells including glial cells, hippocampal pyramidal neurons and cerebellar Purkinje neurons (Baulieu and Robel, 1990; Mukai *et al.*, 2006; Tsutsui *et al.*, 2009). DHEAS has been demonstrated to act as an antagonist of $GABA_A$ receptors (Švob Štrac *et al.*, 2012) and also a positive allosteric modulator of NMDA receptors (Svob Strac *et al.*, 2016). Thus, DHEAS plays a crucial role in the enhancement of learning and memory via the activation of NMDA receptors (Huang *et al.*, 2001). Administration of DHEAS has also been shown to enhance NMDA receptors activity as well as improve performance in neurocognitive-related behavioural in the hippocampus, where the important region of learning and memory (Chen *et al.*, 2006). Thus, the hippocampus region is focused in this current study aiming to explore the effect of DHEAS in the modulation of learning and memory in rats.

DHEAS has been hypotesised to modulate synaptic neurotransmission related to learning and memory (Dong and Zheng, 2011). There are many factors that can impact learning and memory, hence the observation showed that neurosteroids are one of the many neurobiological factors involved in learning and memory processes as well as impaired cognitive performance (Vallée *et al.*, 2001). Learning and memory are closely related that mainly involved in the hippocampus, otherwise memory and executive function are fundamental processes that connected each other. Thus, this current study is focusing on the involvement of DHEAS in both learning and memory processes as well as attentional processes of executive function. Behavioural assessment for spatial cognition and memory can be conducted by Morris Water Maze (MWM) and 5-Choice Serial Reaction Time Task (5-CSRTT), the cognitive assessment to determine attentional performance of executive function. In this current study will be able to provide new insights in determining the relationship between neurosteroidogenesis and neurocognitive pathways.

Using a motorised stereotaxic apparatus guided by coordinates from a rat brain atlas, the FFM probe will be accurately placed in the CA1 area of the hippocampus in the rat brain to be imaged. Previous studies of changes in neurosteroidogenesis have relied on determination of DHEAS levels in brain tissue or plasma samples by using gas chromatography mass spectrometry or high performance liquid chromatography-mass spectrometry (Liu *et al.*, 2003; Lionetto *et al.*, 2017). In addition, studies of neurosteroid expression in specific areas of the brain such as the hippocampus require cell cultures or histological approaches (Biggio, 2007). Thus, in this study there is a more appropriate approach to determine DHEAS fluorescence level that utilises real-time imaging, *in vivo* monitoring undergoing behavioural assessment of learning and memory as well as executive function to stimulate specific neurocognitive pathways.

1.2 Rationale of the study

In recent years, there has been increasing evidence suggesting that endogenous neurosteroids act as paracrine modulators of neurotransmission and synaptic plasticity (Akk *et al.*, 2007; Dewangan *et al.*, 2018). Additionally, DHEAS influence neurocognitive functions as modulators of learning and memory processes (Wolf and Kirschbaum, 1999). It has been postulated that due to this role, DHEAS level increased in response to the modulation of learning and memory assessment, and attentional performance as well. However, it has yet to be established how neurosteroidogenesis is affected as a response to neurocognitive processes.

Several studies have found a correlation between DHEAS levels with cognitive function where decreased concentrations of DHEAS levels enhanced the development of Alzheimer's disease (Pan *et al.*, 2019) as well as affect patient with Attention-Deficit Hyperactivity Disorder (ADHD) (Strous *et al.*, 2001; Trent *et al.*, 2012). Thus,

this current study helps to provide a deeper understanding of DHEAS in order to explore potential therapeutic role for neurocognitive problems such as Alzheimer's disease and neuropsychiatric disorders.

In this study, changes occurring in the DHEAS fluorescence level specificly, in the CA1 region of the hippocampus in response to stimulation of spatial cognition, memory and executive function pathways of the rat brain were evaluated. To achieve this, novel fluorescence labelling with DHEAS monoclonal antibodies was developed and fluorescently labelled to endogenous DHEAS in the CA1 region of the hippocampus.

Moreover, the lack of knowledge regarding neurosteroidogenesis and the factors affecting neurosteroid production is mostly due to analytical difficulties to detect and measure neurosteroidogenesis in the brain. It was found that DHEAS level was found in low concentrations in the brain and associated steroidogenic proteins (Baulieu and Robel, 1998), low sensitivity of existing techniques and unavailability of suitable techniques for measuring neurosteroids in the nervous system (Baulieu, 2001). Thus, a probe-based FFM is a recently developed technique that enables to visualise neural structures in specific areas of the brain (Eisenstein, 2009). This will probably provide new insights of knowledge and can be used as a basis for future research regarding neurosteroidogenesis and its relationship to neurocognition.

1.3 Aim of the study

General Objective

The main objective of this current study was to investigate the role of dehydroepiandrosterone sulfate (DHEAS) in the CA1 region of the rat hippocampus in relation to the cognitive and executive function.

Specific Objectives

This current study involved *in vivo* imaging in order

1. To develop and optimise the fluorescence labelling method for visualisation of DHEAS monoclonal antibodies in real time imaging.
2. To investigate the effects of surgical procedure on behavioural assessments.
3. To quantify the changes occurring in the levels of dehydroepiandrosterone sulfate (DHEAS) in the CA1 region of the hippocampus rat brain in response to spatial learning, memory and executive function.

CHAPTER 2

LITERATURE REVIEW

2.1 Steroid hormones

Steroid hormones are lipophilic molecules utilised as chemical messengers by organisms, in which they act on a wide range of tissues and biological functions (Albrecht and Pepe, 2003; Chowen *et al.*, 2004). Active steroids are bound to transport proteins that increase their half-life and ensure ubiquitous distribution. The steroids can act by binding to cell surface receptors, binding to cytoplasmic or nucleic receptors and ultimately activating gene transcription (Holst *et al.*, 2004). Steroid hormones play an important role in the development, growth, maturation and differentiation of the brain (Stoffel-Wagner, 2001).

It is a well-established fact that steroid hormones are produced in the gonads, adrenal glands and the placenta which are all derived from cholesterol (Baulieu, 2001; Holst *et al.*, 2004; Baulieu, 2018). The adrenal cortex within adrenal glands secrete mineralocorticoids, glucocorticoids, and androgens. They have three anatomic zones which are the zona glomerulosa, zona fasciculata and zona reticularis. Meanwhile, the steroid hormones produced in the gonads consist mainly of sex steroids (Holst *et al.*, 2004; Capper *et al.*, 2016).

2.1.1 Steroidogenesis

Steroidogenesis is the biosynthesis of steroid hormones and is classified by two neuroendocrine axes which are the hypothalamic-pituitary adrenal axis as a pathway for adrenal steroidogenesis and the hypothalamic-pituitary gonadal axis for gonadal steroidogenesis (Capper *et al.*, 2016) (Figure 2.1).

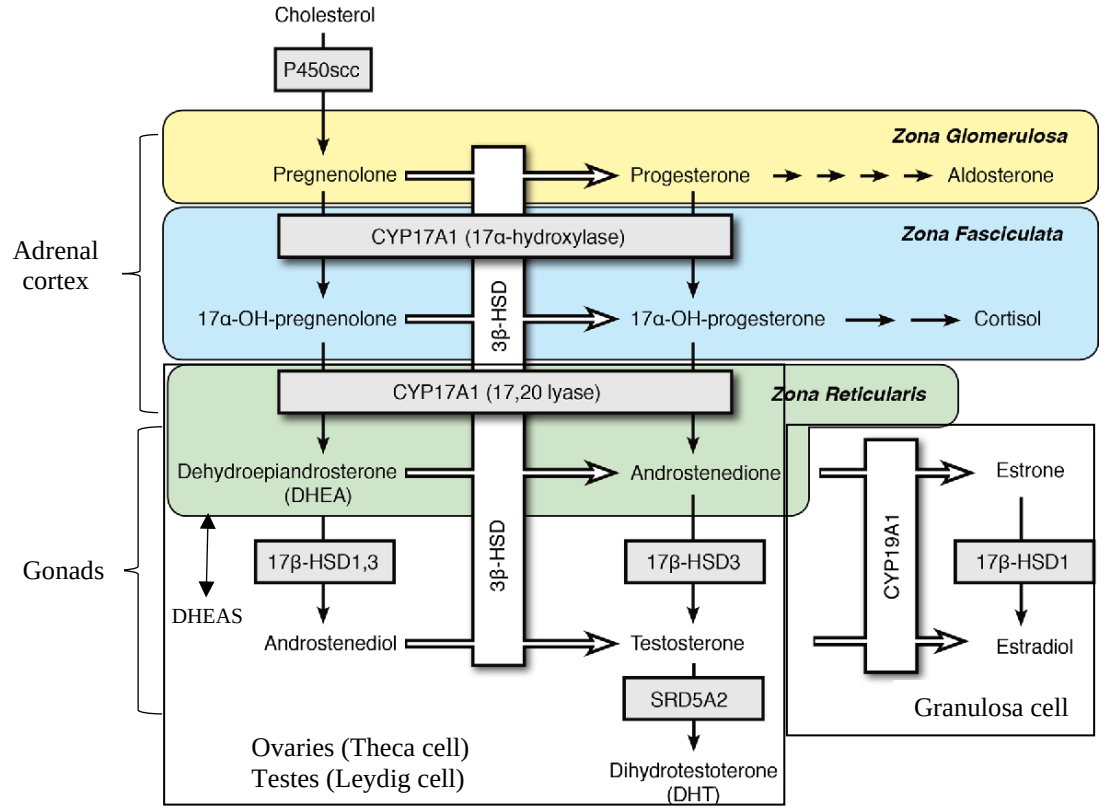

Figure 2.1: Biosynthesis of steroid hormones in adrenal glands and gonads. Adapted from Capper *et al.* (2016).

Cholesterol is the precursor for all steroid synthesis. Steroid synthesis begins with the translocation of cholesterol in the presence of steroidogenic acute regulatory (StAR) protein from the outer mitochondrial membrane to the inner mitochondrial membrane (Holst *et al.*, 2004; Capper *et al.*, 2016).

The steroidogenesis begins with the conversion of cholesterol to pregnenolone (PREG) by side chain cleavage enzyme (P450scc, CYP11A1). Pregnenolone is then converted to progesterone in the presence of enzyme 3βHSD. Subsequently, CYP17A1 acts as a catalyst of PREG and progesterone to form 17OH-pregnenolone and 17OH-progesterone, respectively. These 17-hydroxy products are substrates for other enzymes in their metabolism to cortisol. Then, 17OH-pregnenolone is converted to dehydroepiandrosterone (DHEA) and 17OH-progesterone to androstenedione in the presence of enzyme CYP17A1 (17,20-lyase) (Capper *et al.*, 2016).

Meanwhile, gonadal steroidogenesis is primarily related to the synthesis of sex steroids where the ovarian theca cells are responsible for the conversion of androstenedione to testosterone. Testosterone is then converted to estrogen and estrone in the presence of aromatase (P450aro, CYP19A1) enzyme. This is followed by further secretion of aromatizing androgen to produce estradiol in the granulosa cells by 17βHSD type 1. On the other hand, the testicular Leydig cells in the male gonads express CYP17A1 to produce androgen precursors and are converted to testosterone by expression of the androgenic 17βHSD3 (Capper *et al.*, 2016).

In the hypothalamo-pituitary adrenal (HPA) axis, neurons in the hypothalamus release corticotropin-releasing hormone (CRH) and stimulate adrenocorticotropin (ACTH) secretion in the anterior pituitary. Stimulation of cortisol and androgen precursors synthesis when the ACTH binds to the adrenal cortex extracellular receptor

on cells. Cortisol exerts negative feedback on CRH and ACTH production for homeostasis whereas aldosterone is primarily under the control of the renin-angiotensin-aldosterone system (Capper *et al.*, 2016). Meanwhile, in the hypothalamo-pituitary gonadal (HPG) axis, the gonadotropin-releasing hormone (GnRH) is secreted in the neurons of the hypothalamus. The GnRH enters the portal circulation and stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the gonadotropes in the anterior pituitary. In male, the LH acts on the testicular Leydig cells whereas FSH acts on Sertoli cells to facilitate spermatogenesis. In female, LH acts on the ovarian theca cells and the FSH induced the expression of the enzyme to convert androstenedione and testosterone from the theca cells to the estrogens and estradiol in granulosa cells of the ovary. Consequently, the androgens (androstenedione and testosterone) and estrogens exert negative feedback mechanisms on GnRH, LH and FSH production (Santen, 1981; Capper *et al.*, 2016) (Figure 2.2).

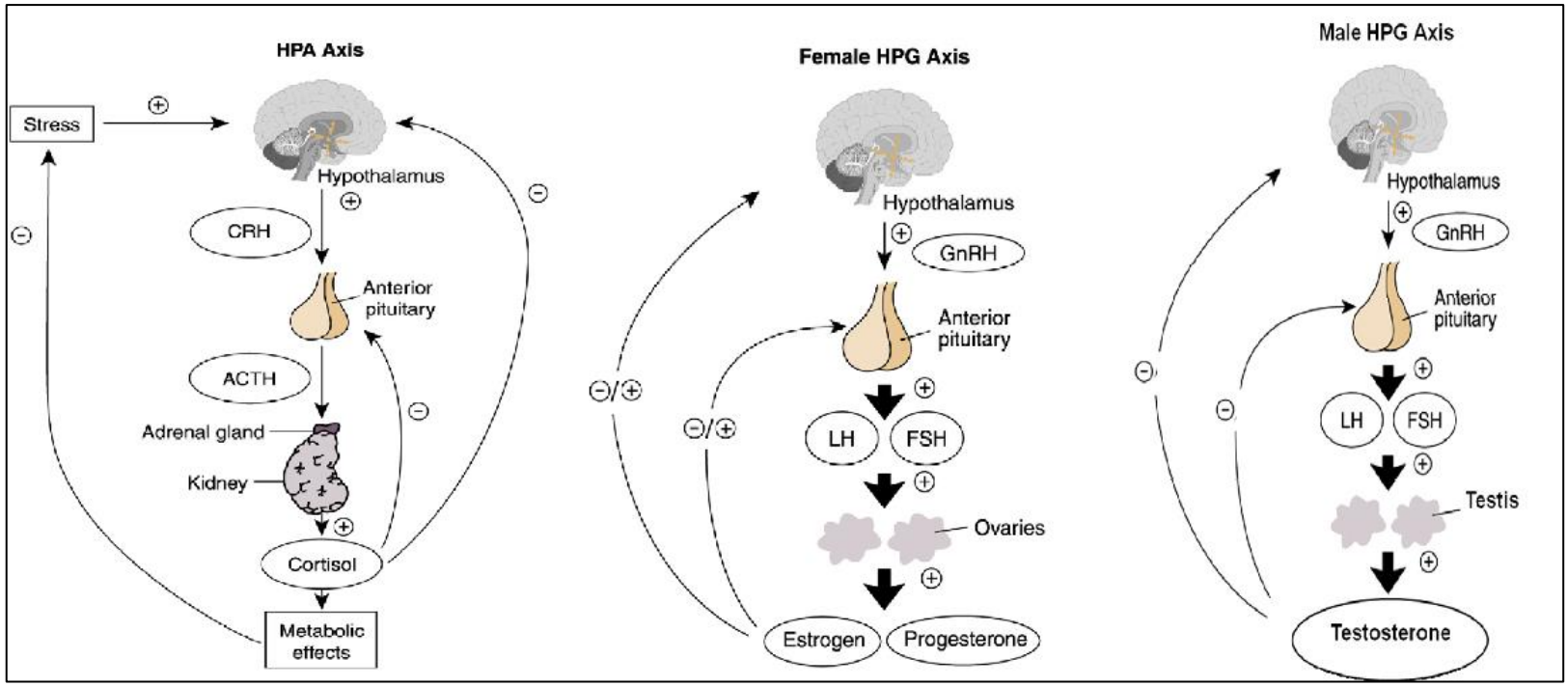

Figure 2.2: Schematic diagram of proposed feedback actions and mechanisms of hypothalamo-pituitary adrenal (HPA) axis and hypothalamo-pituitary gonadal (HPG) axis in male and female. Abbreviations: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin; GnRH, gonadotrophin-releasing hormone; LH, luteinsing hormone; FSH, follicle-stimulating hormone. Adapted from Löfgren (2009).

2.2 Neurosteroids

While it is an established fact that steroid hormones are produced in the gonads or adrenal glands and reach the brain via the blood circulation (Wingfield *et al.*, 2018), nevertheless Baulieu (1981) put forward the idea that steroids can accumulate in the brain in the absence of steroidogenic glands and can be synthesised in the brain from endogenous precursors (Wingfield *et al.*, 2018). These steroids are independently produced in the brain and thus are known as "neurosteroids". The neurosteroids that are synthesised in the brain include dehydroepiandrosterone (DHEA) and pregnenolone (PREG), their sulfated derivatives DHEAS and PREGS, and progesterone (PROG), deoxycorticosterone, 3α,5α-tetrahydroprogesterone (3α,5α-TH PROG) or allopregnanolone (Allo) and 3α,5α-TH DOC (Charalampopoulos *et al.*, 2008; Tuem and Atey, 2017). Local endogenous neurosteroid synthesis occurs in the hippocampus and other brain structures where their function is to modulate synaptic plasticity (Mukai *et al.*, 2006; Hojo and Kawato, 2018). Initially, observation by measuring the concentrations of DHEAS levels showed that greater amounts in the brain matter rather than in the serum (Corpéchot *et al.*, 1981; Charalampopoulos *et al.*, 2008). Neurosteroids can be categorised into three components (1) pregnane neurosteroids, such as allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), (2) androstane neurosteroids, such as androstanediol and etiocholanone, and (3) sulfated neurosteroids, such as PREGS and DHEAS corresponding to structural features (Reddy, 2010).

Within the central and the peripheral nervous system, neurosteroids are also produced from cholesterol, in glial cells, and in neurons. The emerging evidence

indicates that these neurosteroids have numerous functions within the nervous system. They can affect neurotransmission systems in both an excitatory or inhibitory way (Maurice *et al.*, 1999; Mellon, 2007; Dewangan *et al.*, 2018). They can also act as modulators of neurotransmitter receptors, such as $GABA_A$ receptors, NMDA receptors, and sigma 1 receptors (Mòdol *et al.*, 2011; Khare *et al.*, 2016). The $GABA_A$ receptor is a member of the ligand-gated ion channel family which is modulated by several effector sites which bind benzodiazepines, barbiturates and ethanol. On the other hand, NMDA receptors are involved in long-term potentiation (LTP), memory and learning, hypoxic/ischemic neuronal damage and epilepsy (Kawato *et al.*, 2003) while other neurosteroids activate important glial functions, such as myelination (Baulieu and Schumacher, 2000). Thus, it can be seen that these neurosteroids have a neuromodulatory function with the potential to be used as therapeutic agents for various conditions such as neuropsychiatric disorders, Alzheimer's disease, anxiety disorders, stress sensitive conditions and epilepsy (Herbert, 2001). Thus, this current study helps to provide a deeper understanding of neurosteroidal function as a step towards exploring various opportunities for therapeutic development (Reddy, 2010).

While neurosteroids have many functions in the brain, their concentrations can vary according to environmental and behavioural circumstances, such as stress, sex recognition, aggressiveness, depression, anxiety, sleep, epilepsy, and memory formation (Maurice *et al.*, 1999; Tuem and Atey, 2017). Neurosteroids have also been proposed as one of the neurobiological factors involved in the disruption of cognitive processes in aging (Beason-held *et al.*, 2005; Powrie and Smith, 2018). Research shows that the alteration of neurosteroids with age is linked to disruption of both learning and memory processes. This has led to the hypothesis that varying concentrations of steroids may affect both physical and cognitive aging (Vallée *et al.*,

2001). To this end, animal studies are necessary in order to examine various neurosteroids levels under physiological, pathological and clinical conditions. Thus, in this current study, animal studies were conducted to determine the location of the neurosteroid dehydroepiandrosterone sulfate and to develop a method for the analysis of neurosteroid levels in the brain.

It is not only the concentration of neurosteroids that affect the brain. The different metabolites have either inhibitory or excitatory effects in the brain. Allopregnanolone is one of the metabolites of progesterone (PROG) which has shown an ability to inhibit neural activity through the modulation of $GABA_A$ receptors (Majewska *et al.*, 1986; Wang, 2011). It has also been shown that allopregnanolone can inhibit learning in animals undergoing a Morris water maze test (Johansson *et al.*, 2002). On the other hand, numerous studies have shown that pregnenolone sulfate (PREGS) is one of the neurosteroids that is a negative modulator of $GABA_A$ receptor as well as a positive modulator of NMDA (Smith *et al.*, 2014). Administration of PREGS to rats undergoing the Morris water maze and Y-maze tasks showed that their performance in learning and spatial memory was enhanced (Vallée *et al.*, 1997; Darnaudéry *et al.*, 2000).

To add further to the complexity of the effect of neurosteroids in the brain, it has been shown that the sex steroids can cross the blood brain barrier and influence neuronal functions in the brain (Baulieu and Schumacher, 2000). Previous reported studies showed that receptors of androgens increase the number of synaptic contacts in a group of spinal motor neurons (Matsumoto *et al.*, 1988; Verhovshek and Sengelaub, 2013), oestrogens increase the density of dendritic spines of hypothalamic and hippocampal neurons (Frankfurt and Luine, 2015), and progesterone prevent death of facial motor neurons after nerve transection (Yu, 1989). Indeed, Adhya and co-

researchers (2017) have also shown that sex steroids are able to act as therapeutic agents in neuronal injury.

The modulation of neuronal activity involves the synaptic release of neurochemicals where it regulates the amplitude or frequency of postsynaptic membrane potentials (Schlinger and Remage-Healey, 2011). This can be seen in terms of synaptic plasticity, where estradiol synthesis can modulate spine density, long-term potentiation (LTP), long-term depression (LTD), and learning and memory in the neurons of the hippocampus (Hojo and Kawato, 2018) (Figure 2.3).

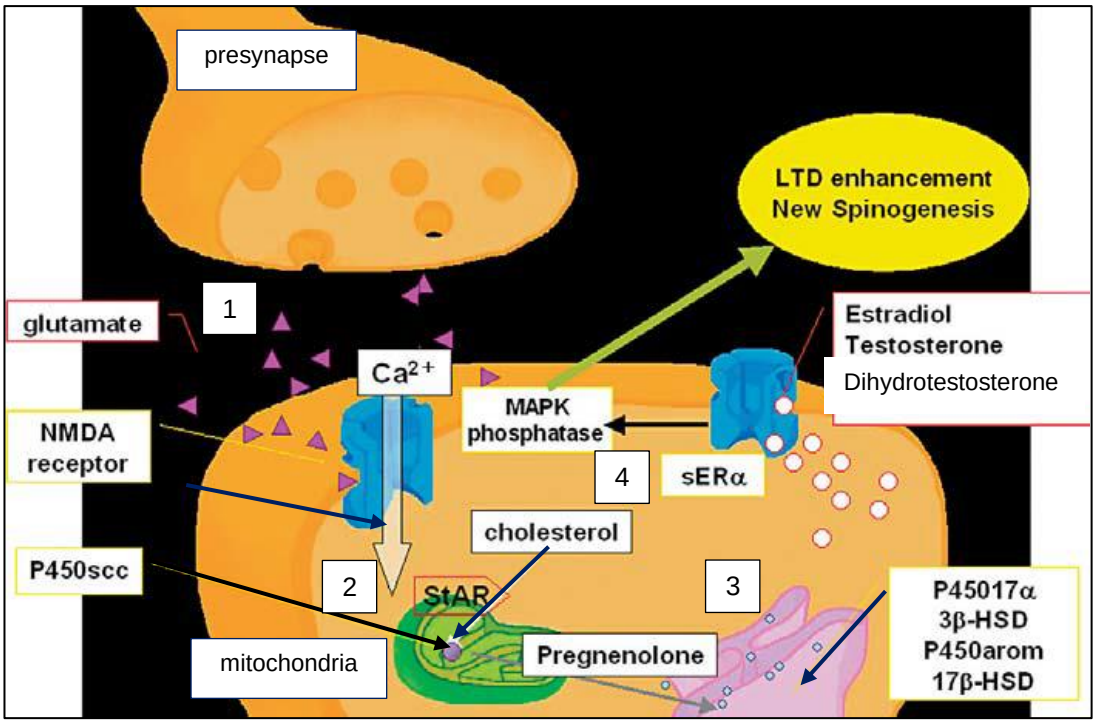

Figure 2.3: Schematic illustration of the neurosteroids modulation of synaptic plasticity. 1) Glutamate release from the presynapse and binds to NMDA receptors and induces Ca^{2+} influx; 2) The Ca^{2+} influx drives StAR as a cholesterol transportation into the mitochondria, where the conversion from cholesterol to pregnenolone by P450scc; 3) The conversion from PREG to DHEA to androstenediol to testosterone to estradiol or testosterone to dihydrotestosterone in the presence of P45017α, 3β-HSD, P450arom, 17β-HSD which are located in the synaptic membrane; and 4) Estradiol binds to synaptic ERα and activation of MAP kinase phosphatase pathway induced the modulation of long-term depression (LTD) and new spine appeared. Adapted from Mukai *et al.* (2006).

2.2.1 Neuroactive neurosteroids

Dehydroepiandrosterone (DHEA), unconjugated steroid and its sulphate ester (DHEAS) are the most abundant circulating hormones in humans (Baulieu and Robel, 1998; Maninger *et al.*, 2009; Rutkowski *et al.*, 2014) and has been shown to have multiple effects in animals (Baulieu and Robel, 1998; Maninger *et al.*, 2009; Sujkovic *et al.*, 2011; Chen *et al.*, 2014). Dehydroepiandrosterone and DHEAS are steroids which can exist as circulating hormones or produced within the brain from cholesterol. In particular, several studies reported that the existence of DHEA and DHEAS at low amounts in the rat's brain (Liere *et al.*, 2009). Dehydroepiandrosterone and DHEAS were the first neurosteroids to be identified in large concentrations in the rat's brain (Corpéchot *et al.*, 1981; Charalampopoulos *et al.*, 2008). An examination of whole rat brains have found 6.47 ng/g DHEAS and 0.54 ng/g DHEA, which are greater than that found in the plasma (Corpéchot *et al.*, 1981). It has been shown that both the DHEA and DHEAS are synthesised in the brain from cholesterol (Baulieu and Robel, 1998; Dong and Zheng, 2011; Stárka *et al.*, 2015).

Dehydroepiandrosterone and DHEAS may influence the cognitive function. Flood and co-researchers (1988) were the first to report that DHEA and DHEAS improved learning and memory in a variety of learning and memory tasks in animals. For example, the effects of DHEA and DHEAS enhanced neural and glial cell survival and/or differentiation of dissociated cell cultures in 14-day-old mice (Roberts *et al.*, 1987). Some studies have demonstrated DHEA and DHEAS enhanced the performance in various cognitive tasks such as Morris water maze, Y-maze and passive avoidance tasks (Frye and Lacey, 1999). Indeed, the evidence that DHEA and DHEAS is associated with learning and memory enhancement is further strengthened

by other supporting studies. DHEA and DHEAS have been shown to alter cognitive performance in rats (Frye and Lacey, 1999) while several other studies have demonstrated that these compounds improved memory retention in passive and active footshock avoidance (Reddy and Kulkarni, 1998) and T-maze tasks (Melchior and Ritzmann, 1996) in male mice. Thus, the evidence seems to indicate that both DHEA and DHEAS have with similar complementary effects on memory enhancement but on different behavioural tasks. Administration of DHEA (3.0 mg/kg) and DHEAS showed that DHEA can affect the cognitive performance on water maze task while DHEAS did not showed any effect on water maze task. On the other hand, DHEA did not show any significant differences on Y-maze task but DHEAS enhanced the cognitive performance on Y-maze task in a dose-dependent manner (0 mg/kg, 3.0 mg/kg and 7.5 mg/kg) (Frye and Lacey, 1999). These complementary effects of both DHEA and DHEAS on memory enhancement and cognitive performance in rats suggest that there is a link to their neurotransmitter receptors (Wolf and Kirschbaum, 1999). Moazedi *et al.* (2008) has investigated this idea and found that the NMDA receptor is indeed involved in the mechanisms related to learning and memory impairment.

Moreover, there is evidence that neurosteroids regulate neuronal function by binding to intracellular receptors with a concurrent influence on neuronal excitability and gene expression (Rupprecht and Holsboer, 1999; Rupprecht *et al.*, 2001; Rupprecht, 2003; Balthazart *et al.*, 2018). Thus, the genomic (classical intracellular steroid receptors) and non-genomic (ion channels and membrane receptors) effects of steroids in the brain may contribute to the mechanism of steroids action on neuronal function and plasticity (Rupprecht, 2003; Tuem and Atey, 2017; Balthazart *et al.*, 2018) (Figure 2.4).

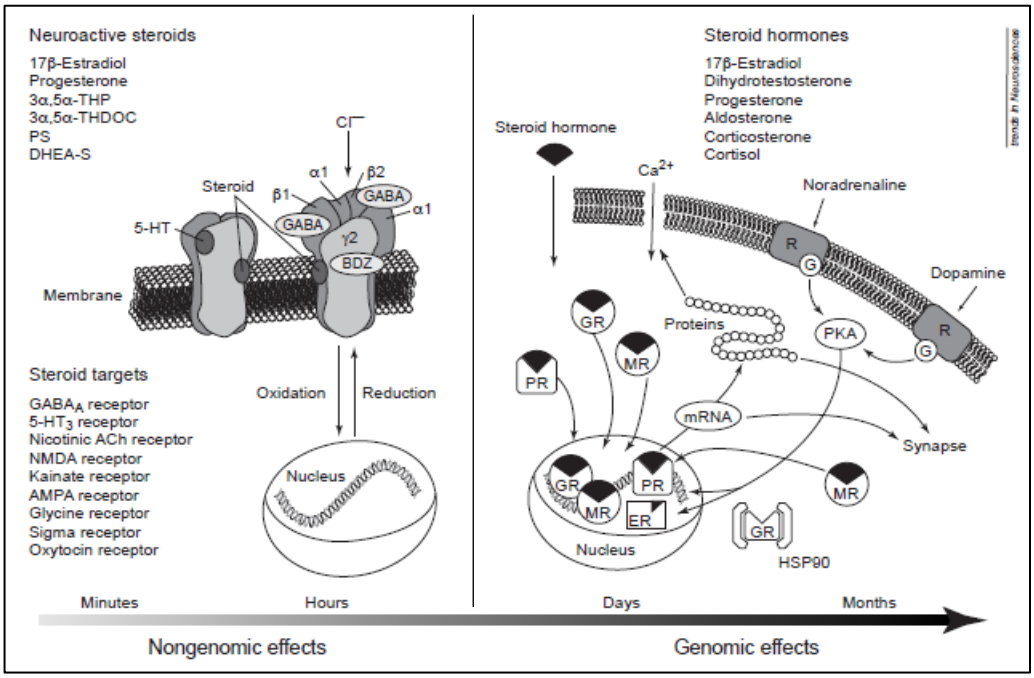

Figure 2.4: Schematic illustrations of non-genomic and genomic effects of neuroactive steroids (DHEAS). Abbreviations: BDZ, benzodiazepines; R, receptor; G, G-protein; PKA, protein kinase A; HSP 90, heat shock protein 90; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; ER, estrogen receptor. Adapted from Rupprecht (2003).

Steroid hormones act via the classical genomic pathway, whereas neuroactive steroids do not bind to steroid receptors. However, they act as modulators of ligand-gated ion channels. Steroid hormone actions via intracellular steroid receptors are usually slow and prolonged duration, whereas the physiolgical effects of neuroactive steroids occur within hours or minutes. On the other hand, steroid hormones may influence neuronal excitability by interfering with the expression patterns of neurotransmitter-receptors and voltage-gated ion channels, and through modulation of ligand-gated ion channels or G-protein-coupled receptors by alteration of the intracellular kinases activity. Furthermore, steroids in the brain are highly complex and do not exhibit high-affinity interaction with nuclear steroid hormone receptors (Rupprecht *et al.*, 1996; Rebas *et al.*, 2017).

Neuroactive steroids have been demonstrated to act as a direct modulator to ligand-gated ion channels and most notably steroid-binding site exists at the $GABA_A$ receptors (Lambert *et al.*, 2003; Wang, 2011). The effects of progesterone at $GABA_A$ receptors might exert sedative, anesthetic, anxiolytic, sleep modulating and anticonvulsant activity in animals and humans (Rupprecht and Holsboer, 1999; Lambert *et al.*, 2003; Rupprecht, 2003; Belelli *et al.*, 2009), whereas classical steroid receptor is not required for that activity of progesterone and related neurosteroids (Reddy *et al.*, 2005). Animal studies have shown that negative allosteric modulators of $GABA_A$ receptors and positive NMDA receptors may produce neuroprotective properties and memory enhancement (Wolf and Kirschbaum, 1999). These actions of neuroactive steroids provide various opportunities and potential for the development of various therapeutic approaches.

2.2.2 Neurosteroidogenesis

Neurosteroidogenesis is defined as the biosynthesis of endogenous steroids in the brain (Dong and Zheng, 2011). Neurosteroidogenesis has an important role in the modulation of neurophysiological and neuropsychological function. It is also involved in the regulation of synaptic plasticity and contributes to the effects of neurotransmission, dendritic spine formation, long-term potentiation or depression, as well as learning and memory (Frankfurt and Luine, 2015; Porcu *et al.*, 2016; Bazzari and Parri, 2019).

The presence of the steroidogenic acute regulatory protein (StAR) plays an essential role in neurosteroidogenesis as cholesterol is transported into the mitochondria in the presence of steroidogenic acute regulating protein (StAR) (Sierra, 2004; Porcu *et al.*, 2016).

Meanwhile, in the mitochondria, neurosteroidogenesis begins with the conversion of pregnenolone (PREG) from cholesterol in the presence of cytochrome P450scc (CYP11A1) (Sierra, 2004; MacKenzie and Maguire, 2013). Pregnenolone is then converted to progesterone in the presence of Δ5-3β-hyroxysteroid dehydrogenase isomerase (3β-HSD). The progesterone is then converted to 5α-dihydroprogesterone by 5α-reductase and subsequently to 3α,5α-tetrahydroprogesterone (allopregnanolone) by 3α-hydroxysteroid oxidoreductase (Maurice *et al.*, 1999; Kawato *et al.*, 2003; Charalampopoulos *et al.*, 2008; Maninger *et al.*, 2009; Dong and Zheng, 2011;) (Figure 2.5).

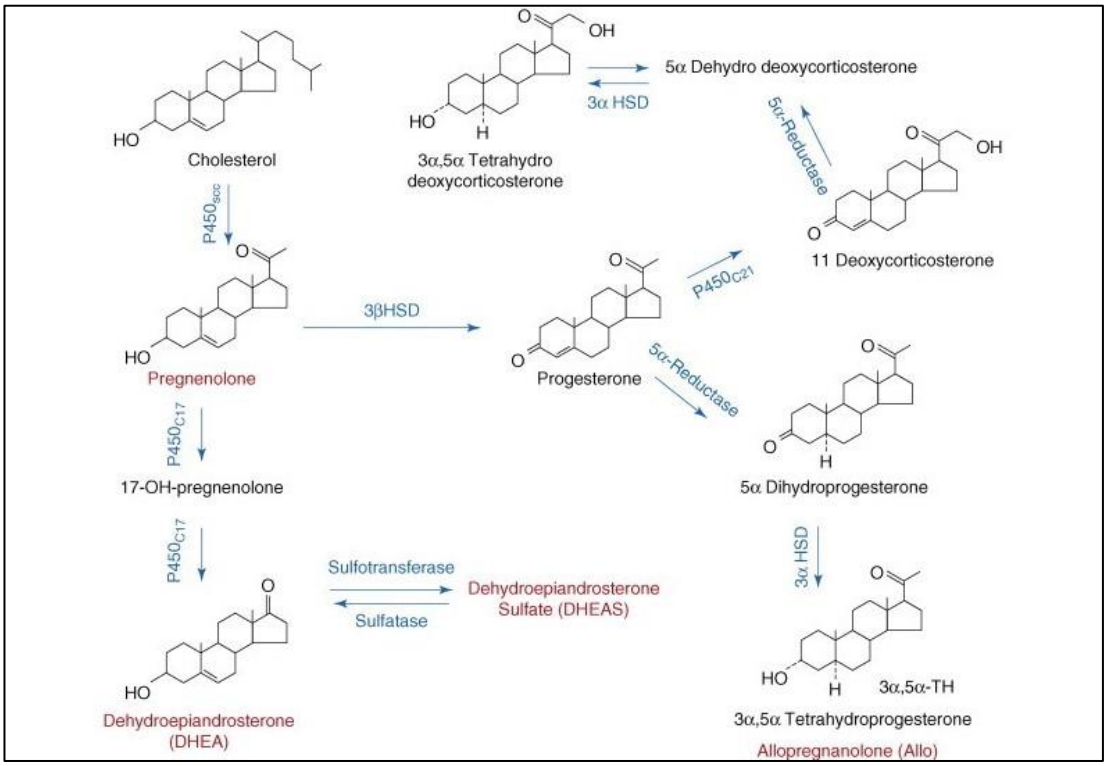

Figure 2.5: Biosynthesis pathways of neurosteroids in the hippocampus. Adapted from Charalampopoulos *et al.*, 2008).

Pregnenolone is also a precursor for dehydroepiandrosterone (DHEA). Dehydroepiandrosterone is a 19-carbon steroid hormone classified as an adrenal androgen. It is derived from the conversion of pregnenolone to DHEA by cytochrome P450c17 (Charalampopoulos *et al.*, 2008; Maninger *et al.*, 2009; Dong and Zheng, 2011; Diotel *et al.*, 2018). In addition, P450c17 is also expressed in the rat hippocampus (Compagnone *et al.*, 1995; Kawato *et al.*, 2003; Hojo *et al.*, 2004). Finally, the conversion of DHEA into its sulphated derivative DHEAS is catalysed by hydroxysteroid sulfotransferase. DHEAS is also known to be metabolically interconvertible to DHEA through hydrolysis by a sulphatase (Maninger et al., 2009; Dong and Zheng, 2011).

Interestingly, cytochrome P450scc, hyroxysteroid sulfotransferase, cytochrome $P450_{17\alpha}$ (CYP17), cytochrome P450arom, steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase/ Δ^5-Δ^4-isomerase (3β-HSD) have all been found to be expressed in the hippocampus and Purkinje cells of adult male rats as seen in various studies involving immunohistochemical staining and confocal laser scanning microscopy, RT-PCR analysis, and enzyme activity assays (Kimoto *et al.*, 2001; Matsunaga *et al.*, 2001; Hojo *et al.*, 2004). Immunohistochemical evidence has also shown presence of P450scc, P45017α, and P450arom within the hippocampus (Kawato *et al.*, 2003). Indeed, Dewangan *et al.*, 2018 also reported that the acute modulation of neurotransmission can occur within the hippocampus with both excitatory and / or inhibitory effects.

Recently, several studies have also shown that hydroxysteroid sulfotransferase activity was present in the rat brain (Aldred and Waring, 1999; Qaiser *et al.*, 2017). It has also been shown that sulfatase activity which has a role in neurosteroidogenesis,

is widely present in the central nervous system and affects memory inhibition (Rhodes *et al.*, 1997; Kříž *et al.*, 2008).

2.2.3 Dehydroepiandrosterone sulfate (DHEAS)

Dehydroepiandrosterone sulfate (DHEAS) is one of the vital neurosteroids which is derived from dehydroepiandrosterone (DHEA) in the presence of sulphotransferase enzymes (Dong and Zheng, 2011). DHEAS is reversibly synthesised from and hydrolysed to the corresponding free steroid DHEA (also secreted by the adrenals), which itself can be partially metabolised into active androgens and estrogens in peripheral tissues (Berr *et al.*, 1996; Stárka *et al.*, 2015). The enzyme hydroxysteroid sulfotransferase is a catalyst which transforms DHEA into the more stable sulfate ester DHEAS which can then be converted back to DHEA by sulfatase enzymes (Maninger *et al.*, 2009) (Figure 2.6).

Several studies have shown that the presence of DHEAS increased the neuronal excitability, enhanced neuronal plasticity, possessed neuroprotective properties, anti-glucocorticoid effects and improved memory and learning (Wolf and Kirschbaum, 1999; Maninger *et al.*, 2009; Höllig *et al.*, 2015). DHEAS has a longer biological half-life (7 – 10 h) compared to DHEA which has a short half-life (30 min or less) (Wolf and Kirschbaum, 1999). Several studies demonstrated that there are relatively high levels of DHEAS in both animals and humans (Corpéchot *et al.*, 1981; Lanthier and Patwardhan, 1986; Lennartsson *et al.*, 2012; Lionetto *et al.*, 2017), with higher concentrations of DHEAS in man (Goldman and Glei, 2007) and comparatively very low concentrations in rats and mice (Fleshner *et al.*, 1997; Liere *et al.*, 2009) which may be related to very low expression of P450c17α enzyme (Baulieu and Robel, 1998;

Dong and Zheng, 2011; Diotel *et al.*, 2018). This is supported by a study in adult male rats which reported that DHEAS levels in the anterior and posterior areas of the brain was in the range of 1.58 ± 0.14 and 4.89 ± 1.06 ng/g, respectively (Corpéchot *et al.*, 1981).