The role of DHEA in the aetiology of modern chronic disease

by

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DECLARATION

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Abstract

Dehydroepiandrosterone (DHEA) is an androgenic steroid predominantly viewed as the main precursor to androgen and estrogen hormones in the human body. DHEA exists as a sulphated ester known as DHEAS, which is also the most predominant steroid hormone in human circulation. A decline in circulating DHEA concentrations is associated with age, inflammatory disease, as well as neurodegenerative pathologies. It has numerous demonstrated neuroprotective, anti-inflammatory and anti-glucocorticoid effects. The human adrenal glands and gonads are the main sites for DHEA biosynthesis in the periphery, but historical evidence has suggested that central steroid biosynthesis, termed neurosteroidogenesis, is responsible for the presence of DHEA in the brain. The process of neurosteroidogenesis has to date proven to be an elusive process, as only a handful of relatively dated studies have provided evidence for its existence. Furthermore, clear differences are apparent in systemic rodent steroidogenesis and human steroidogenesis, the latter of which most the evidence of neurosteroidogenesis is formulated upon. In order to exploit the numerous reported beneficial effects of DHEA in the brain, it is pertinent that we understand how it is synthesised, how it may exert its effects centrally and whether species differences will affect the function thereof.

Utilising the sensitivity and specificity of Ultra-Performance Convergence Chromatography (UPC^2)-tandem mass spectrometry we comprehensively assessed the ability of primary human astrocytes (pHAs) and primary rat brain *ex vivo* mixed cell cultures (pRBMCs) to synthesise DHEA from a known substrate, pregnenolone, in the presence or absence of steroidogenic modulators. Additionally, we also sought to elucidate the ability of the cells to metabolise DHEA, in either the presence or absence of steroidogenic modulators.

Both pHAs and pRBMCs were unable to synthesise DHEA from pregnenolone as the substrate in the absence or presence of steroidogenic modulators. pHAs and pRBMCs were able to convert pregnenolone in progesterone, demonstrating 3β-HSD activity. Additionally, although both cell populations were unable to demonstrate DHEA biosynthesis, they were able to convert exogenous DHEA into androstenedione and androstenediol, demonstrating not only 3β-HSD, but17β-HSD activity as well. This is the first study demonstrating androstendiol biosynthesis by human glial cells.

The inability of pHAs and pRBMCs to synthesise DHEA from pregnenolone as a substrate contradicts available literature. The metabolism of exogenous DHEA into downstream metabolites suggests that the numerous beneficial effects of DHEA are not due to the steroids itself, but rather its metabolites. This current characterisation of DHEA metabolism both questions our current understanding of DHEA biosynthesis in the brain and holds promise for new therapeutic development in modern chronic disease and specifically neurodegeneration.

Opsomming

Dehydroepiandrosterone (DHEA) is ‘n androgeniese steroïedhormoon wat hoofsaaklik as die belangrikste voorloper vir androgeen en estrogeen hormone in die menslike liggaam beskou word. DHEA bestaan ook as 'n gesulfateerde ester, bekend as DHEAS, wat ook die volopste steroïedhormoon in die menslike bloedsomloop is. 'n Afname in die sirkulerende DHEA konsentrasies word geassosieer met ouderdom, inflammatoriese siektes, sowel as neurodegeneratiewe patologieë. Dit het talle neurobeskermende, anti-inflammatoriese en anti-glukokortikoïede effekte. Die menslike byniere en gonades is die belangrikste organe vir DHEA-biosintese in die periferie, maar historiese bewyse dui aan dat sentrale steroïedbiosintese, wat neurosteroïedgenese genoem word, verantwoordelik is vir die teenwoordigheid van DHEA in die brein is. Die proses van neurosteroïedgenese is tot dusver 'n ontwykende proses, aangesien slegs 'n handjievol relatief verouderde studies bewys lewer dat dit bestaan. Verder is daar duidelike verskille in sistemiese knaagdiersteroïedgenese en menslike steroïedgenese, waarvan laasgenoemde die bewys van neurosteroïedgenese is. Om die talle gerapporteerde voordelige gevolge van DHEA in die brein te benut, is dit pertinent dat ons verstaan hoe dit gesintetiseer word, hoe DHEA sy effekte sentraal kan uitoefen en of spesieverskille die funksie daarvan sal beïnvloed.

Deur gebruik te maak van die sensitiwiteit en spesifisiteit van ultra werkverrigting konvergensie-chromatografie (UPC^2)-tandem-massaspektrometrie, het ons die vermoë van primêre menslike astrosiete (pHA's) en primêre rotbrein *ex vivo* gemengde selkulture (pRBMC's) om DHEA vanaf die bekende substraat pregnenoloon te sintetiseer, in die teenwoordigheid of afwesigheid van moduleerders van steroïedgenese. Daarbenewens het ons ook probeer om die vermoë van hierdie selle om DHEA te metaboliseer, onder dieselfde toestande, toe te lig.

Beide pHA's en pRBMC's kon nie DHEA van pregnenoloon as substraat sintetiseer nie. Die afwesigheid of teenwoordigheid van moduleerders van steroïedgenese het ook nie hierdie bevinding beïnvloed nie. pHA's en pRBMC's het 3β-HSD-aktiwiteit getoon deur pregnenoloon na progesteroon om te skakel. Alhoewel beide selpreparate nie DHEA-biosintese kon demonstreer nie, kon hulle wel eksogene DHEA in androsteendioon en androsteendiol omskakel, wat nie net 3β -HSD, maar ook 17β-HSD aktiwiteit demonstreer. Dit is die eerste studie wat die biosintese van androsteendiol deur menslike glanselle demonstreer.

Die onvermoë van pHA's en pRBMC's om DHEA vanaf pregnenoloon as substraat te sintetiseer stem nie ooreen met die beskikbare literatuur nie. Die metabolisme van eksogene DHEA na stroom-af metaboliete dui daarop dat die talle voordelige effekte van DHEA nie te wyte is aan dié steroïed spesifiek nie, maar eerder die metaboliete daarvan. Hierdie huidige karakterisering van DHEA-metabolisme bevraagteken ons huidige begrip van DHEA-biosintese in die brein, maar hou ook belofte in vir nuwe terapeutiese ontwikkeling in moderne chroniese siektes en spesifiek neurodegenerasie.

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"May I never be complete, may I never be content, may I never be perfect"

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List of abbreviations

µg	Microgram
µL	Microlitre
µM	Micromolar
16OHP4	16α-hydroxyprogesterone
16OHP5	16α-hydroxypregnenolone
17OHP4	17α-hydroxyprogesterone
17OHP5	17α-hydroxypregnenolone
3-NP	3-Nitropropionic acid
3αAdiol	Androstanediol
5αdione	Androstanedione
A4	Androstenedione
A4S	Androstenedione Sulphate
A5	Androstenediol
AD	Alzheimer’s disease
ALLO	epi-Allopregnanolone
AR	Androgen Receptor
AST	Androsterone
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CD11b	complement receptor 3
CNS	Central Nervous System
CS	Corpus Striatum
CSF	Cerebral Spinal Fluid
CYP2D	Cytochrome P450 2D
DHEA	Dehydroepiandrosterone

DHEAS	Dehydroepiandrosterone Sulphate
DHP4	Dihydroprogesterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's medium
E. coli	Escherichia coli
EAE	Experimental autoimmune encephalomyelitis
ER	Estrogen Receptor
ERK ½	Extracellular signal-regulated kinases -1/2
EtOH	Ethanol
FBS	Foetal Bovine Serum
FIV	Feline Immunodeficiency Virus
GABAA R	GABAA Receptor
GABAA	γ-aminobutyric acid type A
GCMS	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptor
H. pomatia	*Helix pomatia*
hCG	human chorionic gonadotropin
HPA	Hypothalamic Pituitary Adrenal axis
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenase
IFN-γ	Interferon gamma
IL	Interleukin
LCMS	Liquid Chromatography Mass Spectrometry
LH	Luteinising Hormone

LPS	Lipopolysaccharide
LTP	Long Term Potentiation
MAP2C	Microtubule Associated Protein isoform 2C
MCP-1	Monocyte Chemoattractant Protein 1
MeOH	Methanol
mg	Milligram
MHC II	Major Histocompatibility Complex II
mL	Millilitre
mM	Millimolar
MOA	Monoamine Oxidase
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
MSMS	Tandem Mass Spectrometry
MTBE	Methyl-tert-Butyl-Ether
NAc	nucleus accumbens
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NGF	Nerve Growth Factor
nL	Nanolitre
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NMDAR	NMDA Receptor
NT-3	Neurotrophin 3
OATP	Organic Anion Transporting Peptide

P4	Progesterone
P450	Cytochrome P450 enzyme
P450scc	Cytochrome P450 side chain cleavage enzyme
P4S	Progesterone Sulphate
P5	Pregnenolone
P5S	Pregnenolone Sulphate
PBMC	Peripheral Blood Mononuclear Cell
PD	Parkinson's disease
PenStrep	Penicillin-Streptomycin
PI3/Akt	Phosphatidylinositol-4,5-bisphosphate 3-kinase/ Protein Kinase B
PPAR	Peroxisome Proliferator-Activated Receptor
PTSD	Post Traumatic Stress Disorder
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
SAM	Sympatho-adrenal-medullary
SFC	Supercritical Fluid Chromatography
Shc	SHC-transforming protein
SNS	Sympathetic Nervous System
SPE	Solid Phase Extraction
StAR	Steroidogenic Acute Regulatory Protein
STS	Steroid Sulphatases
SULT	Steroid sulphotransferase
T	Testosterone
TNF	Tumour Necrosis Factor

Trk	Tropomyosin receptor kinase
UPC^2	Ultra-Performance Convergence Chromatography
σ-1	Sigma 1

Chapter 1

1.1. Introduction

Dehydroepiandrosterone (DHEA) is an androgen steroid hormone that is largely viewed as a precursor to androgens and estrogens, in men and women respectively. It exists as a sulphated ester, dehydroepiandrosterone sulphate (DHEAS), which is the most abundant steroid hormone in human circulation. Although lacking a specific receptor and long thought to exist solely as a precursor hormone, DHEA has been shown to have numerous beneficial effects in its own right, within various contexts. These include neuroprotective, antioxidant and anti-inflammatory capacity (Grimm *et al.*, 2014; Yabuki *et al.*, 2015; Alexaki *et al.*, 2017; Boghozian *et al.*, 2017; Powrie and Smith, 2018).

Traditionally, the biosynthesis of steroid hormones, termed steroidogenesis, was thought to occur only in the gonads and adrenal glands. However, in 1981, Corpéchot and colleagues published data demonstrating the presence of DHEA and DHEAS in the brains of adult male and female Sprague-Dawley rats (Corpéchot *et al.*, 1981). To discount the possibility of systemic delivery, the animals underwent gonadectomies and adrenalectomies, but DHEAS levels remained unchanged. Systemic administration of dexamethasone or corticotropin similarly had no effect on DHEAS concentrations in the brain. The authors coined the term neurosteroid to differentiate the origins of steroids from the central nervous system versus the somatic endocrine system. In further studies of rat brains by the same authors, the presence of pregnenolone (P5) and its sulphated form, pregnenolone sulphate (P5S) was also reported (Corpéchot *et al.*, 1983; Robel and Baulieu, 1985; Robel *et al.*, 1987). Less than a decade later, a study by a different group reported the presence of other steroid hormones, in addition to DHEA, in the human brain – these included progesterone (P4), androstenedione (A4) and 17-hydroxyprogesterone (17OHP4) (Lacroix *et al.*, 1987).

Although these studies demonstrated the presence of steroids in rat and human brain as well as evidence suggesting central biosynthesis, neurosteroidogenesis remained unproven at this point. The first study directly demonstrating neurosteroidogenesis was conducted in isolated mitochondria from rat oligodendrocytes: these cells were shown to be able to convert cholesterol into P5 – the initial and rate limiting step in steroidogenesis (Hu *et al.*, 1987). In years to follow, multiple studies reported data demonstrating the ability of isolated rat neuroglia, namely oligodendrocytes and astrocytes, to synthesise neurosteroids when provided with the relevant substrate (Jung-Testas *et al.*, 1989b, 1989c; Zwain *et al.*, 1997; Zwain and Yen, 1999a, 1999b; Gago *et al.*, 2001).

In addition to data supporting the existence of neurosteroidogenesis, expression of multiple steroid enzymes in the rat brain has also been reported by a multitude of independent laboratories (Martel *et al.*, 1992; Melcangi *et al.*, 1993; Mellon and Deschepper, 1993; Dupont *et al.*, 1994; Normand *et al.*, 1995; Pelletier *et al.*, 1995; Strömstedt and Waterman, 1995; Guennoun *et al.*, 1995; Zwain *et al.*, 1997; Kohchi *et al.*, 1998; Ukena *et al.*, 1998, 1999; Zwain and Yen, 1999a, 1999b; Gago *et al.*, 2001; Kimoto *et al.*, 2001; Benmessahel *et al.*, 2002, 2004; Sanne and Krueger, 2002; Lovelace *et al.*, 2003; Shibuya *et al.*, 2003; Sierra *et al.*, 2003; Griffin *et al.*, 2004; Lavaque *et al.*, 2006; Mukai *et al.*, 2006; Geerling *et al.*, 2006; Karri *et al.*, 2007; Gottfried-Blackmore *et al.*, 2008; Mizoguchi *et al.*, 2014). Most relevant amongst these is cytochrome P450 c17 (CYP17), which is responsible for conversion of P5 into 17-hydroxypregnenolone (17OHP5) as well as 17OHP5 into DHEA.

Although evidence supporting neurosteroidogenesis in rodent brains appears insurmountable, the data available to support the process in human brains remains limited, if not inconsistent.

Only two studies have reported detectable levels of steroid enzyme mRNA, including CYP17, in the human brain (Yu *et al.*, 2002; Brown *et al.*, 2003). Specific parts of the brain shown to express the mRNA were the amygdala, caudate nucleus, cerebellum, corpus callosum, spinal cord, thalamus and frontal cortex. Interestingly, the first study detected CYP17 mRNA in the hippocampus whilst the second did not (Brown *et al.*, 2003). At least one other study has also reported failure to detect CYP17 mRNA in the hippocampus (MacKenzie *et al.*, 2008). Furthermore, contradicting data from others demonstrate that the human temporal lobe and cerebellum also do not express CYP17 mRNA (Steckelbroeck *et al.*, 2004; MacKenzie *et al.*, 2008).

Despite the inconsistencies in the available data on neurosteroidogenesis in the human brain, as previously mentioned, animal studies have suggested that the process appears to preferentially occur in neuroglial cells. In particular, astrocytes – which express CYP17 and are theoretically capable of synthesising DHEA from P5 – seems to be the commonly accepted primary site of DHEA biosynthesis in the rodent brain (Zwain and Yen, 1999a).

In terms of human models, we could find only one group who has investigated the ability of human astrocytes as well as human glioma cell lines to synthesise DHEA (Brown *et al.*, 2000). They reported that normal human astrocytes (as well as the glioma cells) expressed CYP17, but appeared to synthesise DHEA in a manner independent of the steroid pathway, which they theorised to be a ROS (Reactive Oxygen Species)-dependent pathway (Brown *et al.*, 2000). However, no new data have been published to shed light on this theory for almost 20 years, despite the recent focus on redox in the context of neurodegenerative disease. Also, given the methodological limitations at the time these studies were conducted (Powrie and Smith, 2018),

conclusive interpretation from these few studies in only one group is probably premature. Thus, at this point, human astrocytes have not conclusively been shown to directly synthesise DHEA *de novo* or in the presence of a substrate. However, given the many beneficial associations more recently reported for DHEA in the context of oxidative stress, ageing and neuroinflammation, the possibility of intracrine DHEA biosynthesis – and potential therapeutic manipulation thereof – warrants further research.

Since many species differences have already been reported for androgen steroidogenesis, as recently reviewed (Powrie and Smith, 2018), an important first step in this research approach would be to establish similarities or differences between species in the context of intracrine DHEA production. The current study therefore aimed to comprehensively investigate the ability of primary human astrocytes to produce and/or metabolise DHEA *in vitro* in response to different stimuli.

The layout of the dissertation is as follows: Chapter 2 reviews the pertinent literature on the role of DHEA in modern chronic disease with particular focus on neurodegenerative pathologies. In addition, the potential of central DHEA biosynthesis is discussed in detail with special focus on species differences in steroidogenesis. This chapter concludes with the hypothesis, aims and objectives for the study. Chapter 3 details the materials used and methods employed to satisfy the aims of the study. Chapter 4 is a discussion of the results in the context of current literature. Finally, Chapter 5 summarises the conclusions of the dissertation.

Chapter 2

2. Literature Review

A truncated version of this chapter has been published in the Journal of Neuroinflammation (impact factor 5.7).

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2.1. Introduction

From the recent literature, it is evident that the processes of neuroinflammation and neurodegeneration are inextricably linked. Given the sequestered nature of the brain, which complicates research sample collection for obvious reasons, many investigators seem to extrapolate data generated from peripheral samples in attempts to explain central events. However, as also illustrated in the pages to follow, there is often a disconnect between adaptation in the periphery versus those occurring centrally. In our opinion, there are multiple reasons for this. Firstly, the neuroimmune system is structurally distinct from the peripheral system in that most immune functions are mediated by cells specific to the nervous system, such as microglia and astrocytes (Tian *et al.*, 2012). Incidentally, although recent commentaries and research letters pertaining to the identification of a lymphatic system in the dural spaces are suggesting that the brain may be subject to surveillance by immune cells circulating from the periphery (Louveau *et al.*, 2015), not enough data exist with which to evaluate the relative importance of these immune cells relative to those resident in the brain. Secondly, the brain has a preference for glucose as a substrate, as opposed to most peripheral organs, such as the heart, which mainly derive energy through fatty acid β-oxidation, which may affect the outcome of adaptive – or maladaptive - metabolic responses differently in the brain to peripheral compartments. Lastly, although the brain itself is subject to glucocorticoid-mediated metabolic modulation, as in the peripheral compartment, it is also directly affected by – and thus adapts as result of - psychological input.

From this, it is clear that different factors come into play centrally vs. peripherally in terms of adaptation to stimuli. This questions the validity of treating neuroinflammation – especially in the context of chronic disease aetiology – using the same strategies by which peripheral chronic low-grade inflammation is addressed. It further highlights the need for specific, central investigations for the purpose of answering questions pertaining to central physiological adaptation or maladaptation.

We have identified the hormone (also aptly referred to as a neurosteroid) dehydroepiandrosterone (DHEA) as a relatively under-researched hormone in the context of neurophysiology, despite its very clear association with a plethora of clinical disease states. DHEA, which is predominantly synthesized in the human adrenal cortex, also exists as a sulphated ester known as DHEAS, which is formed when DHEA is processed by the enzyme steroid sulphotransferase (SULT) (Maninger *et al.*, 2009; Arbo *et al.*, 2016). Of note, the majority of DHEA in circulation exists as DHEAS due to it having a stronger binding affinity for its carrier protein, albumin (Puche and Nes, 1962). DHEA is the precursor hormone to both androgenic and estrogenic hormones and as such, is also synthesised to a smaller extent in human gonads. In addition, and most relevant to the context of the current topic, it is claimed to be synthesised *de novo* in the human brain as well. In fact, DHEA concentrations in the human brain have been shown to be higher than that in circulation, while DHEAS concentrations are lower (Stárka *et al.*, 2015; Arbo *et al.*, 2016), which not only supports the theory of local biosynthesis, but also testifies to the importance of this hormone centrally.

In the context of human health, DHEA has historically been deemed to have limited importance, until low circulating levels of DHEAS were associated with chronic and age-related diseases such as diabetes, hypertension, arthritis, etc. (Barrett-Connor, 1992; Haffner *et al.*, 1994; Herbert, 1995; Straub *et al.*, 1998; Ravaglia *et al.*, 2002). In addition, it has since drawn much media attention as being an “anti-ageing” supplement after circulating levels were also reported to dramatically and progressively decline with age (Orentreich *et al.*, 1984; Herbert, 1995). Most relevant to the current context, abnormal changes in DHEA levels in serum and cerebral spinal fluid (CSF) have also been reported in the pathophysiology of neurodegenerative diseases such as Alzheimer’s disease (AD), schizophrenia and multiple sclerosis (MS), as well as neurocognitive pathologies such as post-traumatic stress disorder (PTSD) (a summary of these reports is provided in Table 2-1). However, much remains unknown about DHEA’s mode of action in the human body, or the reason for its age-related decline. Therefore, and given the commonly accepted phenomenon of accelerated ageing in many chronic disease states (Petersen and Smith, 2016), understanding the process of ageing and the physiological role of DHEA in this process, may be of benefit to the development of novel strategies in the treatment of these enigmatic pathologies.

With this review, we provide a comprehensive summary of what is known about the role of DHEA in the context of neurophysiology and ageing. This will be followed by a discussion of specific topical issues, such as the effect of sex and species, as well as the sites for DHEA production. More specifically, relevant literature providing arguments for and against central, intracrine DHEA production is discussed in order to make an interpretation on the likelihood of central DHEA biosynthesis taking place, as well as on the feasibility of modulating central

DHEA levels for therapeutic effect in the context of ageing-related neuroinflammation and -degeneration.

Table 2-1: Summary of reports linking DHEA to neurodegenerative diseases and neurocognitive disorders

Disease	Findings	References
Alzheimer's disease	Significant decrease in serum DHEA and DHEAS levels when compared to aged-matched control patients	(Sunderland *et al.*, 1989; Yanase *et al.*, 1996; Bernardi *et al.*, 2000; Hillen *et al.*, 2000; Murialdo *et al.*, 2001; Genedani *et al.*, 2004; Aldred and Mecocci, 2010)
Schizophrenia	Data on both sides of the spectrum associate both abnormally elevated and declining levels of DHEA/DHEAS with the disease. This opposing data could be related to the heterogeneity of the disease itself, as well as other comorbid aetiological factors, which adds complexity to interpretation of this data.	(Tourney and Hatfield, 1972; Harris *et al.*, 2001; Ritsner *et al.*, 2006, 2007; Gallagher *et al.*, 2007; Strous *et al.*, 2009; Babinkostova *et al.*, 2015)
Multiple Sclerosis	Significantly higher CSF DHEA concentrations in relapsed patients relative to control patients with stable neurological disease	(Orefice *et al.*, 2016)
Post-traumatic stress disorder (PTSD)	Increased plasma DHEA and DHEAS levels when compared to unaffected control patients	(Spivak *et al.*, 2000; Söndergaard *et al.*, 2002; Yehuda *et al.*, 2006; Bremner *et al.*, 2007; Gill *et al.*, 2008; Kellner *et al.*, 2010; Jergović *et al.*, 2015)

2.2. **Process of ageing**

Ageing is an inevitable, natural biological process characterised by a progressive loss of physiological integrity which results in impaired function and increased probability of death. It is an extremely complex process which is affected by a number of lifestyle and genetic factors that may accelerate, ameliorate or even slow down the progression of ageing itself (Petersen and Smith, 2016). The process of ageing is commonly considered the main risk factor in the development of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (PD) (Hindle, 2010; Niccoli and Partridge, 2012). Currently, the global population is

living longer as a result of the advances in medicine and consequently such diseases are becoming more prevalent.

Ageing itself is associated with cumulative oxidative stress, chronic low-grade inflammation linked to age-related dysfunction of the immune system (termed immunosenescence) and most notably, a decline in endocrine function (termed endocrinosenescence) (Straub *et al.*, 2000; Petersen and Smith, 2016). The immediate sections to follow will focus primarily on immunosenescence and endocrinosenescence - highlighting their intricately linked aetiologies in the process of ageing itself, as well as in age-related neurodegenerative pathologies.

2.3. **Immune system maladaptation**

Inflammation is an integral part of the innate immune system: it not only acts as the first line of defence against pathogenic attack and injury, but also serves to execute final humoral immune effects (e.g. antibody-mediated pathogen destruction). It is thus a normal physiological process that is essential for the maintenance of homeostasis. An equally normal consequence of the inflammation-facilitated repair of tissues after any insult, is the transient disruption of the local cellular homeostasis of non-infected or uninjured cells. This injury-repair cycle is efficient during youthful years, but is affected by ageing, resulting in a relatively impaired ability of the body to regenerate damaged tissues (Petersen and Smith, 2016). Systemically, immunosenescent changes in the innate immune system are characterised by a loss of phagocytic capacity, reduced efficiency in leukocyte (neutrophil and macrophage) chemotaxis, reduced intracellular, but increased extracellular levels of free radicals and an increase in production of ”early” or acutely responsive pro-inflammatory cytokines, such as interleukins (IL) -1β, -6 and tumour necrosis factor (TNF) -α (Roubenoff *et al.*, 1998; Dobbs *et al.*, 1999; Wenisch *et al.*, 2000; Guayerbas and De La Fuente, 2003), all of which contribute to a more pro-inflammatory phenotype and which increase the burden of tissue damage-recovery cycles. The loss in efficiency of this process, coupled with an increased exposure to antigenic load and modern lifestyle associated chronic stressors, results in the reduced ability of the body to maintain normal immune function. This manifests as an increased risk for malignancy, auto-immune responses, an impaired ability to mount proper immune responses to stimuli and prolonged inflammatory activity during acute infections (Bruunsgaard *et al.*, 1999b, 1999c, 1999a).

2.3.1. Neuroinflammatory changes

As in the periphery, ageing of the brain is associated with increasing inflammation and local oxidative stress – also resulting in repeated tissue damage-recovery cycles - which predisposes older individuals to developing neurodegenerative pathologies. Age-related neuroinflammation partly manifests as an increased reactivity of microglia, the resident innate immune cells. In a healthy brain, microglia are activated upon stimulation, but do not necessarily release these pro-inflammatory mediators. In the aged brain however, microglia appear to not only release pro-inflammatory mediators, but to do so in an exaggerated and prolonged manner (Barrientos *et al.*, 2012). This appears to be linked to the fact that microglia undergo significant immunophenotypic and functional changes with ageing: the activation status of microglia changes from a quiescent to a “primed” state. This manifests as an increased expression of the glial activation markers major histocompatibility complex II (MHC II) and complement receptor 3 (CD11b), which sensitizes microglia to the exaggerated pro-inflammatory responses seen upon stimulation.

Animal studies have also suggested that chronic and significant elevation of levels of pro-inflammatory cytokines such as IL-1β may be linked to impaired memory formation and long term potentiation (LTP) (Katsuki *et al.*, 1990; Cunningham *et al.*, 1996; Barrientos *et al.*, 2012; Gonzalez *et al.*, 2013). Systemically, IL-1β is released by monocytes upon recognition of a pathogen or site of injury, as well as by any cell sustaining damage (Smith *et al.*, 2008). IL-1β enhances B and T cell lymphocyte proliferation and stimulates IL-6 and TNF-α production in many other immune cells. The systemic elevation of circulating pro-inflammatory cytokines, particularly IL-1β, exerts a significant effect on microglial activation as well. However, in this context, the age-related maladaptation seems to occur at different magnitudes in the brain relative to the circulation. For example, studies in mice have shown that stimulation of the peripheral innate immune system with an intraperitoneal injection of either lipopolysaccharide (LPS) or live *E.coli* bacteria resulted in a more prolonged and exaggerated elevation of IL-1β and IL-6 levels in aged brains relative to those of younger mice (Godbout *et al.*, 2005; Barrientos *et al.*, 2009). Interestingly, the exaggerated and prolonged inflammatory response was restricted to the brain and not paralleled with a similar peripheral response. In addition, it was reported that the hippocampus was more affected when compared to other brain regions, such as the hypothalamus, parietal cortex or prefrontal cortex (Barrientos *et al.*, 2009). The hippocampus plays a critical role in memory formation and consequently it is implicated in many neurodegenerative pathologies, such as Alzheimer’s disease. These data give credibility to the more recent suggestions that a severe inflammatory episode may trigger

development of Alzheimer's disease (Wyss-Coray, 2006; Potgieter *et al.*, 2015). It also identifies the hippocampus specifically as the area of interest for preventative intervention.

However, before one can move on to interventions, it is necessary to more fully understand the cause(s) of this exaggerated neuroinflammatory outcome upon ageing. A complexity is that the exact cause of the overactive response in microglia from aged individuals has in fact not been elucidated convincingly. We believe that the answer might lie in the progressive and chronic elevation of glucocorticoid hormones that is also associated with ageing.

2.4. **Endocrine Dysfunction**

2.4.1. The ageing Hypothalamic-Pituitary-Adrenal Axis (HPA)

Cortisol released into circulation can readily cross the blood-brain barrier (BBB) and therefore can exert its effects both locally (in the brain) and systemically (in the periphery), by binding to either of its two intracellular receptors: mineralocorticoid receptor (MR) or glucocorticoid receptor (GR) (Barrientos *et al.*, 2012). A study in rats showed that MRs have a significantly higher affinity for corticosterone (the most abundant endogenous glucocorticoid in rodents) than GRs; the latter is consequently only bound by corticosterone upon complete saturation of MRs during periods of highly elevated serum corticosterone (Veldhuis *et al.*, 1982). This phenomenon of preferential MR binding by the endogenous glucocorticoid cortisol (the most abundant one in humans) is also well-documented in humans (Frey *et al.*, 2004). Binding of cortisol to its receptors causes it to translocate to the nucleus and decrease gene transcription of pro-inflammatory cytokines – effectively the body's most important anti-inflammatory mechanism. The brain expresses high levels of both MRs and GRs, with highest MR expression mainly in the hippocampus, which has the highest MR:GR ratio in the brain overall (Ferrari and Magri, 2008).

To return to the context of this review, rat hippocampal long term potentiation (LTP), a correlation of synaptic plasticity and strength (and thus capacity for memory formation), has been shown to be suppressed by GR activation (Pavlides *et al.*, 1996), which highlights this pathway as role player in the pathology related to neurodegeneration, such as Alzheimer's disease. The hippocampus is a brain region that is frequently highlighted in neurodegeneration research. Of note, and as mentioned earlier, it is affected more by peripheral inflammation than the rest of the brain. Therefore, should the hippocampus be specifically targeted in the treatment of inflammation-related neurodegeneration? In line with this question, how exactly do glucocorticoids link ageing, inflammation and neurodegeneration? We put forward the following hypothesis: as one ages, the repeated injury-repair cycles require repeated

glucocorticoid mediated anti-inflammatory responses. Initially, the hippocampus is sensitive to glucocorticoids due to a high expression of GR and MR receptors. However, after numerous glucocorticoid responses, the hippocampus downregulates receptor expression – something which is indeed seen in acute and chronic stress (López *et al.*, 1998; Zhe *et al.*, 2008; Smith and van Vuuren, 2014; Füchsl and Reber, 2016). In fact, more than 30 years ago Sapolsky and colleagues reported aged rats to have elevated basal corticosterone levels and an impaired capacity for levels to return to basal after an acute stress response (Sapolsky *et al.*, 1983). This landmark paper proved to be the basis of the glucocorticoid hypothesis, which postulated that increasing age correlated with gradual loss of negative feedback control resulting in the gross accumulation of cortisol/corticosterone not only systemically, but also in the brain (Sapolsky *et al.*, 1986). The loss in negative feedback may be attributed to a decrease in MR and GR expression in the brain. For example, in cases and animal models of neuropsychiatric diseases in which patients present with hypercortisolemia, such as major depressive disorder and PTSD, MR expression has been found to be downregulated specifically in the hippocampus (Zhe *et al.*, 2008; Medina *et al.*, 2013). A final possibility that we propose, is that MR and GR activation may be insensitive to glucocorticoid binding in aged human hippocampi. For example, it has been demonstrated in human peripheral blood mononuclear cells that in cells expressing high levels of GRs, GR affinity for dexamethasone (a synthetic corticosteroid) is significantly reduced when compared to individuals with peripheral blood mononuclear cells (PBMCs) with lower expression levels (Elakovic *et al.*, 2007).

Given the age-related exaggerated and prolonged inflammatory responses discussed above, an exaggerated anti-inflammatory counter in the form of increased glucocorticoid levels is probably to be expected. However, the problem with the pro- and anti-inflammatory system crosstalk lies in that this exaggerated response, which is well-recorded in the ageing literature, is rendered insufficient to counter and resolve the inflammatory response by the endocrine maladaptation discussed here.

2.4.2. The effect of age on the sympatho-adrenal-medullary pathway (SAM pathway)

In parallel to its role in the HPA-axis, the hypothalamus also plays a critical role in the release of catecholamines, such as epinephrine and norepinephrine from the adrenal medulla, facilitated by sympathetic innervation of the medulla (Heffner, 2011). Importantly, in the context of this review, is the fact that catecholamines induce both the systemic and localised upregulation of IL-6 biosynthesis, which can prompt adrenocortical release of glucocorticoids (Päth *et al.*, 1997; Johnson *et al.*, 2005; Blandino *et al.*, 2006, 2009; Kim *et al.*, 2014). In

addition, epinephrine and norepinephrine act synergistically with cortisol by upregulating glucose metabolism and increasing cardiac output during acute periods of stress, i.e. the “fight or flight” response.

Similar to the HPA-axis, ageing also affects the SAM pathway, mainly due to changes in the sympathetic nervous system (SNS). Evidently it appears that overall tonic SNS activity increases with age (Esler *et al.*, 2002). Circulating norepinephrine levels appear to increase whereas epinephrine levels decline with age (Franco-Morselli *et al.*, 1977; Esler *et al.*, 1995, 2002; Kudielka *et al.*, 2000; Amano *et al.*, 2013). Whether these observations are due to impaired clearance or an increased output by the adrenal medulla is still unknown. However, it is also probable that the increasing circulating concentrations of norepinephrine may in part be due to a spill over from synaptic junctions at effector sites – which has already been reported in at least one study (Esler *et al.*, 2002). Although no consensus has been reached with regard to why these changes may occur with age, it is very clear that they do occur.

When compared to the vast knowledge gathered on the effects of age on cortisol and the HPA axis, relatively little is known about the SAM pathway and even less so about its effects on the inflammatory process. However, hormones in the SAM pathway have demonstrated immune modulating behaviour. For example, epinephrine has been shown to potently inhibit LPS-stimulated human monocytes from synthesising pro-inflammatory cytokines, such as TNF-α and IL-12, as well as to stimulate the biosynthesis of the anti-inflammatory cytokine, IL-10 (Elenkov *et al.*, 2008). On the other hand, in the brain, norepinephrine may have a pro-inflammatory effect in the hypothalamus. For example, norepinephrine was shown to play a critical role in foot shock stress-induced hippocampal and splenic production of IL-1β (Blandino *et al.*, 2006). Pre-treatment with the selective beta-adrenergic receptor antagonist, propranolol, abrogated the IL-1β response. Furthermore, when animals were treated with the microglial inhibitor, minocycline, upregulation of IL-1β was completely inhibited in the hypothalamus, but not the spleen (Blandino *et al.*, 2006). These results are extremely relevant in the context of ageing, since it is known that microglia play a critical role in age-related neuroinflammation and the fact that noradrenaline levels increase with age.

2.4.3. The effect of ageing on sex hormones

The HPA- and SAM-pathways are not the only endocrine systems affected by ageing. It is commonly known that the most evident effects of ageing occur through a decline in sex hormone production. With regard to a decline in androgen production and the biosynthesis pathways involved, the female menopause is probably best characterised - in particular, the complete cessation of estrogen production by the ovaries (Labrie, 2015a). Thus, after

menopause DHEA becomes the major androgenic hormone in the female circulation. Interestingly, a recent review suggested that in aged individuals, DHEA – although not able to facilitate ovarian estrogen production - may be able to serve as precursor for tissue-specific estrogen biosynthesis through intracrine mechanisms (Labrie, 2015a). According to this theory, estrogen would be synthesised in low concentrations at tissue level (in any tissue where all the required steroidogenic enzymes are present), where it would act in para- or autocrine fashion, with little or no clearance into circulation. Similarly, in males, extragonadal male androgen production has also been demonstrated in peripheral tissue (Labrie, 2015b). With this in mind, a decline in adrenal DHEA production with advancing age would therefore not only have implications for reproduction, but also potentially for other detrimental effects related to a decrease in extragonadal estrogen or testosterone production at peripheral tissue level.

The decline of DHEA with ageing has been well-established. Peak DHEA levels have been reported to occur slightly earlier in life for females when compared to males (15-19 vs. 20-24 years) (Orentreich *et al.*, 1984), after which it steadily declines, reaching levels of ≈20% of peak levels after age 70 (Rutkowski *et al.*, 2014). Epidemiological studies have correlated DHEA levels to longevity in both humans and non-human models (Gaby, 1996; Roth *et al.*, 2002), while the decline of DHEA has been linked to a number of ageing-associated characteristics, including immunosenescence, cognitive decline, osteopenia and sarcopenia (Rutkowski *et al.*, 2014).

From what has already been discussed, it is clear that ageing is linked to several adaptations/ maladaptations in the endocrine system. What will become equally clear from the literature to follow, is the relative absence of directly relevant data on DHEA, its involvement in these processes and thus the effects its decline might have. From studies conducted in contexts other than ageing, it will become clear that DHEA has a significant role to play. We discuss these potential roles below.

2.5. **DHEA – the magic bullet?**

2.5.1. Pre-clinical evidence suggesting neuroprotective effects of DHEA

Since the turn of the century, several papers suggesting beneficial effects after DHEA supplementation, in the context of neuroprotection, have been published – we have summarised findings illustrating the variety of models used in Table 2-2. Reported benefits include modulation of neurogenesis, neuronal function, metabolism and longevity. However, a significant limitation to available information is that the majority of these effects have only been illustrated in *in vitro* and animal studies.

Table 2-2: Evidence for beneficial effects of administered DHEA/DHEAS or metabolites reported in pre-clinical studies

Demonstrated beneficial effects **General neuroprotective effects demonstrated both *in vitro* and *in vivo* in:**	**Reference**
E18 Sprague–Dawley rat hippocampal cell culture model of *N*-methyl-*D*-aspartate (NMDA) neurotoxicity	(Kimonides *et al.*, 1998)
HT-22 mouse hippocampal cell line model of glutamate and amyloid-β neurotoxicity	(Cardounel *et al.*, 1999)
E18 Sprague–Dawley rat cerebral cortical cell culture anoxia model	(Marx *et al.*, 2000)
Mouse hippocampal neurodegeneration model	(Maurice *et al.*, 2000)
Rats with induced forebrain ischemia model	(Li *et al.*, 2001)
Rat hippocampal slice culture ischemia model	(Pringle *et al.*, 2003)
Primary rat cerebellar granule cell anoxic and glucose deprivation model	(Kaasik *et al.*, 2003)
Mouse spinal cord ischemic injury model	(Fiore *et al.*, 2004)
Rat inflammatory neurodegeneration model	(Dudas *et al.*, 2004)
P19 neuronal NMDA-induced excitotoxicity cell line model	(Xilouri and Papazafiri, 2008)
Aged rat brain model	(Kumar *et al.*, 2008)
Rat Corpus striatum (CS) and the nucleus accumbens (NAc) model of assessing effects of DHEA on monoamine oxidase (MOA) activity	(Pérez-Neri *et al.*, 2009)
Human SH-SY5Y neuroblastoma cell line model assessing the effects of neurosteroids on mitochondrial bioenergetics	(Grimm *et al.*, 2014)
Rat brain 3-Nitropropionic acid (3-NP) induced neurotoxicity model	(Hanna *et al.*, 2015)
Transient brain ischemic mouse model	(Yabuki *et al.*, 2015)
Primary male and female mouse cultured hippocampal neurons, as well as human SH-SY5Y neuroblastoma cell line glucose deprivation model	(Vieira-Marques *et al.*, 2016)
Anti-apoptotic effects demonstrated *in vitro* in:	
Undifferentiated P19 neuronal cell line model of NMDA-induced apoptosis	(Xilouri and Papazafiri, 2006)
PC12 rat pheochromocytoma cell line model of serum deprivation-induced apoptosis in adrenal medulla cells	(Charalampopoulos *et al.*, 2004)
Primary rat embryonic cultured neural precursor cell model investigating the effect of DHEA and DHEAS on Akt phosphorylation	(Zhang *et al.*, 2002)
Primary rat cerebellar granule cell model of hypoxia and glucose deprivation	(Kaasik *et al.*, 2001)
Anti-glucocorticoid effects demonstrated both *in vitro* and *in vivo* in:	
Primary rat embryonic hippocampal neurons exposed to neurotoxic doses of corticosterone	(Kimonides *et al.*, 1999)
HT-22mouse hippocampal cell line model of glutamate and amyloid-β-induced neurotoxicity	(Cardounel *et al.*, 1999)
Mitigating effects on corticosterone-induced suppression of neurogenesis and survival of new neurons in dentate gyri (hippocampi) of Lister Hooded Rats	(Karishma and Herbert, 2002)
Inhibitory effects of DHEA on glucocorticoid amplification in 3T3-L1 adipocyte cell line and C57BL/6J mouse white adipose and liver tissue	(Apostolova *et al.*, 2005)
Inhibition of 11β- Hydroxysteroid Dehydrogenase 1 (11β-HSD1) mediated conversion of cortisol by DHEA metabolites in human skin samples	(Hennebert *et al.*, 2007)
Suppression of 11β-HSD1 mRNA in HEK-293 rat cortical collecting duct cell line, as well as kidneys of C57BL/6J mice and Sprague-Dawley rats	(Balazs *et al.*, 2008)

It is clear that DHEA is neuroprotective within these various disease model contexts, but how is DHEA relevant to inflammation – and in particular to neuroinflammation-related neurodegeneration?

2.6. Direct and indirect evidence for neuroprotective anti-inflammatory effects of DHEA

To the best of the authors' knowledge, very little evidence for direct anti-inflammatory action of DHEA or DHEAS in the brain or central nervous system (CNS) has been elucidated *per se*. We were however able to find at least three studies that have demonstrated the anti-inflammatory effects of DHEA *in vitro* in models relevant to the brain or CNS. The first demonstrated that DHEA inhibits the biosynthesis of TNF-α and IL- 6 by cultured foetal rat astroglia in response to exposure to *Mycoplasma fermetans* (Kipper-Galperin *et al.*, 1999). The second and third studies, which are also the most recent, were performed both *in vitro* and *in vivo* (Alexaki *et al.*, 2017; Boghozian *et al.*, 2017). In the *in vitro* study, DHEA treatment of cultured mouse microglia exposed to LPS showed a significant reduction in TNF-α, IL-6, IL-12 and Monocyte Chemoattractant Protein 1 (MCP-1) mRNA expression when compared to LPS exposed microglia receiving no treatment (Alexaki *et al.*, 2017). The *in vivo* study was an experimental model of autoimmune encephalomyelitis (EAE) in mice, which showed a significant reduction in IL-1β and Interferon gamma (IFN-γ) mRNA expression in spinal cord tissue in animals treated with DHEA-S when compared to untreated EAE affected animals (Boghozian *et al.*, 2017)

Numerous other studies have illustrated the anti-inflammatory action of DHEA and DHEAS in systems and disease models other than those related to the brain or CNS (Table 2-3). Although this does not necessarily prove that these same effects may be seen in the brain under the same or similar circumstances, the possibility cannot be discounted.

Table 2-3: Studies which have demonstrated anti-inflammatory effects of DHEA or DHEAS outside of the CNS

Disease/model	Reference
Reduced regulation of IL-6 production in aged mice	(Daynes *et al.*, 1993)
Reversed effects and decreased production of IL-4 and IL-6 in antigen induced immunosuppression in mice	(Kim *et al.*, 1995)
Reduced production of IL-6 in splenocytes of retrovirus infected mice as well as aged-induced immunocompromised mice	(Araghi-Niknam *et al.*, 1997)
Decreased production of IL-4 and increased production of IL-2 by concanavalin-A-stimulated PBMCs from patients with atopic dermatitis	(Tabata *et al.*, 1997)
Reduction of TNF-α serum concentrations in obese Zucker rat model	(Kimura *et al.*, 1998)
Inhibition of IL-6 production in isolated primary human peripheral blood mononuclear cells (PBMCs)	(Straub *et al.*, 1998)
Reduced production of IL-1, IL-6 and TNF-α in LPS-stimulated murine macrophage cell line	(Padgett and Loria, 1998)
Inhibition of TNF-α-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB)-mediated gene transcription in HuH7 human hepatocyte cell line	(Iwasaki *et al.*, 2004)
Suppression of pro-inflammatory genes (IL-1β, IL-6, TNF-α) in primary human HIV-positive macrophages, as well as feline immunodeficiency virus (FIV)-positive felines	(Maingat *et al.*, 2013)
Inhibits acute LPS-induced microglia-mediated inflammation both *in vivo* and *in vitro* through the activation of TrkA-Akt1/2-CREB-Jmjd3 pathway and reduces IL-6, TNF-α, IL-12 and MCP-1 gene expression	(Alexaki *et al.*, 2017)

From these studies it seems that DHEA exerts its anti-inflammatory effects primarily by modulating either the effects or production of pro-inflammatory cytokines. However, it also appears that the same pro-inflammatory cytokines have the ability to regulate DHEA production in turn. For example, in macrophage-depleted primary murine Leydig cell cultures, both IL-1 and TNF-α was shown to inhibit androgen steroidogenesis by downregulating the expression of cytochrome P450c17 mRNA – which encodes for an enzyme that is critical to androgen formation (Hales, 1992; Li *et al.*, 1995). TNF-α has shown similar inhibitory effects in cultured porcine Leydig cells by reducing the binding of luteinising hormone (LH) or human chorionic gonadotropin (hCG) to the respective receptor – which would stimulate steroidogenesis (Mauduit *et al.*, 1991). These authors further suggested that the primary inhibitory effect of TNF-α was mainly by decreasing availability of cholesterol, the initial substrate required for steroidogenesis (Mauduit *et al.*, 1991).

Regardless of how cytokines may inhibit the biosynthesis of androgens, these studies provide a plausible explanation for the age-related decline of DHEA not only systemically, but centrally as well – given the relatively pro-inflammatory status associated with ageing. Furthermore,

since it has been shown that the inflammatory maladaptation seen with ageing affects the brain to a larger extent (specifically the hippocampus), it is plausible that the age-related decline of DHEA occurs even more rapidly in the brain than it does in the rest of the body.

In contrast to the idea that DHEA is largely beneficial, a minority of studies have suggested that DHEA may have neurotoxicity under specific conditions. In a model of global cerebral ischaemic insult in rats (Li *et al.*, 2009), intraperitoneal administration of a supraphysiological dose of DHEA 3 to 48 hours after ischaemic injury was neuroprotective (e.g. decreased neuronal death in the CA1 hippocampal region). However, DHEA administration 1 hour before or after the insult, exacerbated the effects of the injury. Given the known anti-inflammatory role of DHEA, this may suggest that (at least at this high dose) DHEA inhibited early inflammatory or oxidative responses that were required for cytoprotection. Additionally, decreased cell viability was reported in primary murine neuronal cultures and human neuroblastoma (SK-N-SH) cells after (24-72hr) exposure to a large dose-range of DHEA – a result not seen in mixed neural or glial cultures. Furthermore, the neurotoxic effects of DHEA were abrogated by simultaneous treatment with DHEAS (Gil-ad *et al.*, 2001). From this, it is difficult to formulate an interpretation on potentially toxic effects of DHEA. On the one hand, it is certainly feasible that a high dose of an antioxidant such as DHEA may have damaging effects, as this has been illustrated in the nutraceutical literature (Burkitt, 2001). On the other hand, the second study clearly highlights the limitations of interpretations possible from single cell culture models, although it does provide insight on the complexity of the mechanisms at play. In our opinion, given the relatively larger body of evidence in support of a beneficial effect of DHEA, significant concern is probably not warranted in our context of intracrine DHEA production.

In order to further our understanding of how DHEA may impact on the ageing brain, it is necessary to delve deeper into the potential molecular actions and interactions of DHEA and DHEAS.

2.7. Potential mechanisms for DHEA-mediated neuroprotective effects

There are at least two ways via which DHEA can facilitate its effects centrally. Through following the conventional steroidogenesis pathway, DHEA may be converted to endpoint sex hormones, which would then activate their respective receptors. Reviews by different groups have suggested that DHEA can elicit genomic effects through its conversion into testosterone and dihydrotestosterone (DHT) – thereby activating androgen receptors (AR) – as well as through its conversion into estradiol and the subsequent activation of estrogen receptors (ER) (Stárka *et al.*, 2015; Arbo *et al.*, 2016). In support of this suggestion, It has indeed been demonstrated that isolated neonatal rat cortical and hypothalamic astrocytes can synthesize

both testosterone and estrogen from exogenous DHEA (Zwain and Yen, 1999a). Alternatively, DHEA itself may be able to directly bind to and activate these hormone receptors. Although no cell membrane-bound or nuclear receptor has been described to have an affinity specifically for DHEA or DHEAS, a recent comprehensive review has revealed that DHEA and DHEAS have affinity for a number of membrane and nuclear receptors and binding sites (Prough *et al.*, 2016), allowing DHEA to directly activate receptors in the brain and CNS specifically. DHEA is known to interact with the cell membrane receptors γ-aminobutyric acid type A ($GABA_A$), *N*-methyl-*D*-aspartate (NMDA) and Sigma-1 (σ-1), as well as nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) -α and finally neurotrophin receptors such as Tyrosine receptor kinase (Trk) -A, -B and -C (Lazaridis *et al.*, 2011; Pediaditakis *et al.*, 2015; Prough *et al.*, 2016; Alexaki *et al.*, 2017). These potential mechanisms are briefly discussed in the following paragraphs.

Firstly, $GABA_A$ receptors are ligand-gated chloride and bicarbonate channels that induce hyperpolarisation to inhibit the postsynaptic potentials when activated (Prough *et al.*, 2016). DHEA and DHEAS (to a greater extent) have both been shown to act as non-competitive antagonists of ionotropic $GABA_A$ receptor-mediated activity in isolated neurosynaptosomes from Sprague-Dawley rat brains (Imamura and Prasad, 1998). Similarly, DHEAS was shown to block recombinant $GABA_A$ receptor ($GABA_A$ R) -mediated currents in transfected HEK293 cells (Švob Štrac *et al.*, 2012; Sachidanandan and Bera, 2015). Another study of reversible spinal cord injury in rabbits showed that DHEAS significantly delayed the onset of ischemia-induced paraplegia (Lapchak *et al.*, 2000). However, despite widely reported, the exact mechanisms of how DHEA or DHEAS may elicit neuroprotective effects through $GABA_A$ receptors still remain to be fully elucidated. It is important to point out that DHEA appears to induce multiple effects through GABA receptors – of note and in the context of ischemia injuries it demonstrates antioxidant capacity, which is already accepted in literature and highlights the beneficial pleiotropic effects of DHEA.

Secondly, the NMDA receptor (NMDAR) is a cell membrane bound receptor that is a member of a large family of glutamate receptors. Glutamate receptors bind to neurotransmitters and allosteric effectors which regulate transmembrane ion channels involved in learning and memory, i.e. LTP (Prough *et al.*, 2016). DHEAS has been shown to activate NMDARs in both rat hippocampal slices and cultured mouse embryonic cultured neocortical neurons (Monnet *et al.*, 1995a; Compagnone and Mellon, 1998). These reports suggest a more active function of DHEA in the context of NMDARs.

Thirdly, Sigma-1 (σ-1) receptors are associated with cellular membranes, endoplasmic reticulum, nuclear membranes and mitochondrial membranes of many cells of neural origin,

including astrocytes, oligodendrocytes and microglia (Prough *et al.*, 2016). DHEA activation of sigma-1 receptors improved memory deficits induced in mouse models (Meunier and Maurice, 2004). In addition, the effect that DHEA and DHEAS has on NMDARs appears to be linked to sigma-1 receptor interactions. Evidence has shown that DHEAS can potentiate NMDA evoked release of norepinephrine in rat hippocampal brain slices through its action as a sigma-1 agonist (Monnet *et al.*, 1995b).

Fourthly, peroxisome proliferator-activated receptors or PPARs are nuclear associated transcription factors that are capable of exerting pleiotropic physiological effects, such as regulating lipid metabolism, participating in glucose homeostasis and even having a role in apoptosis (Stahel *et al.*, 2008). DHEA and DHEAS may exert neuroprotective effects at nuclear receptors such as PPARα. Specifically, inhibiting NF-kB binding to PPARα. In a rodent study, the effects of DHEA and DHEAS treatment in aged wild type ($PPAR\alpha^{+/+}$) vs aged PPARα knockout mice ($PPAR\alpha^{-/-}$) were investigated. The aged wild type mice treated with DHEA and DHEAS exhibited reduced tissue lipid peroxidation, reduced NF-kB activation in the spleen and lower pro-inflammatory cytokine production when compared to knockout mice also treated with DHEA and DHEAS (Poynter and Daynes, 1998). It is worth noting though that no specific binding affinity of PPAR for DHEA or DHEAS has been reported (Peters *et al.*, 1996). Despite this fact, PPARα receptors have increasingly been reported to have a role in mediating oxidative stress and inflammation in brain pathologies such as traumatic brain injury (TBI) (Feng *et al.*, 2008; Stahel *et al.*, 2008).

In line with these mechanisms, DHEA has been shown to activate the pro-survival Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein Kinase B (PI3/Akt) pathway. In fact, a study investigating the effects of NMDA-induced excitotoxicity in culture mouse brain cells, illustrated that DHEA was able to mitigate the detrimental effects by activating the PI3/Akt pathway through a calcium dependent mechanism (Xilouri and Papazafiri, 2008). Similarly, DHEA supplementation in aged Wistar rats was able to increase the expression of phosphorylated-Akt in liver tissue (Jacob *et al.*, 2011).

In terms of neurotrophin receptors, DHEA has been shown to bind with high affinity to the Nerve Growth Factor (NGF) receptor TrkA in HEK293 cells transfected with TrkA plasmid cDNA (Lazaridis *et al.*, 2011). Binding of DHEA to TrkA led to phosphorylation of the receptor and activation of downstream pathways such as the Akt, extracellular signal-regulated kinases (ERK) -1/2 and SHC-transforming protein (Shc) signalling cascades (Lazaridis *et al.*, 2011). In a similar study, also conducted in transgenic HEK293 cells, DHEA was shown to also bind to TrkB, which is mainly activated by brain derived neurotrophin factor (BDNF), and TrkC, which is preferentially activated by neurotrophin-3 (NT-3) (Pediaditakis *et al.*, 2015). However,

this study showed that DHEA binding only led to the phosphorylation and activation of TrkC, but not TrkB (Pediaditakis *et al.*, 2015). It was noted that DHEA bound these receptors by two orders magnitude lower than that of the inherent neurotrophins.

Finally, DHEA may also have a modulatory role in cytoskeletal dynamics. In this context, DHEA has been found to bind to the N-terminal of the dendritic localised microtubule associated protein type 2C (MAP2C). MAPs bind to and facilitate the dynamic microtubulin polymerisation and DHEA was reported to increase the length of dendrites expressing MAP2C (Laurine *et al.*, 2003). This finding is of particular relevance in the context of neurodegenerative pathologies such as AD where a pathological change in neuritic morphology is associated with disease (Nixon *et al.*, 2005).

Taken together, these studies indicate a significant modulatory role of DHEA/DHEAS via its binding to a wide variety of receptors. Of particular relevance to the current topic, DHEA-linked anti-inflammatory – albeit mainly demonstrated in circulation – and neuroprotective outcomes indirectly suggest that several benefits may come from prevention of DHEA decline. Supplementation is one clear avenue through which this may be achieved.

2.8. Efficacy of therapeutic DHEA administration

DHEA supplementation has historically been used in the treatment of reproductive-related diseases, particularly in women, where it has been shown to alleviate menopause-related pathologies such as vaginal atrophy (Labrie *et al.*, 2009). In support of our interpretation of benefit from maintaining DHEA availability, in diseases not related to reproduction, DHEA supplementation has been shown to be beneficial to both sexes in the treatment of numerous other pathologies or conditions (see Table 2-4).

Table 2-4: Illustrating the variety of studies reporting favourable effects of DHEA/DHEAS supplementation in clinical pathologies

Disease State	References
Autoimmune disease	
Systemic Lupus Erythematosus	(van Vollenhoven *et al.*, 1994, 1995, 1998, 1999; Barry *et al.*, 1998; Chang *et al.*, 2002; Petri *et al.*, 2002, 2004; Sánchez-Guerrero *et al.*, 2008)
Metabolic disease	
Hypercholesterolemia	(Felt and Stárka, 1966)
Metabolic Syndrome	(Villareal and Holloszy, 2004)
Genetic diseases	
Hereditary Angioedema	(Koó *et al.*, 1983)
Ageing-associated	
Advanced age	(Morales *et al.*, 1994, 1998)
Osteoporosis	(Sun *et al.*, 2002; von Mühlen *et al.*, 2008)
Endocrine disorders	
Hypopituitarism	(Johannsson *et al.*, 2002; Bilger *et al.*, 2005; Brooke *et al.*, 2006a, 2006b)
Adrenal Insufficiency/Addison's disease	(Arlt *et al.*, 1999; Gebre-Medhin *et al.*, 2000; Hunt *et al.*, 2000; Callies *et al.*, 2001; Libè *et al.*, 2004; Dhatariya *et al.*, 2005; van Thiel *et al.*, 2005; Gurnell *et al.*, 2008)

Despite these many clinical studies involving DHEA supplementation, to the best of the authors' knowledge, the effects of exogenous DHEA supplementation has not been directly assessed in clinical manifestations of neurodegeneration or any related pathologies. Similarly, there are also currently no clinical studies assessing the effects of DHEA or DHEAS supplementation as an adjuvant therapy for neurodegenerative diseases in either the European or American clinical trial database.

In addition, it should be noted that other studies have reported oral DHEA supplementation to show little to no benefit in the treatment of other ageing-related pathologies such as cognitive performance, lipid metabolism, glucose metabolism, bone health and muscle function (Wolf *et al.*, 1997; Percheron *et al.*, 2003; Corona *et al.*, 2013).

However, the available clinical studies collectively do provide some insight. The conditions included in our summary (Table 4), which all suggests benefit from DHEA administration, are linked by the fact that inflammation is a characteristic feature in all. Similarly, neuroinflammation is a critical hallmark of the generalised pathophysiology of neurodegeneration and its occurrence appears to have a causative role in this degenerative condition. Thus, given the already discussed anti-inflammatory effect of DHEA (refer to Table 3), as well as the anti-inflammatory therapeutic effect suggested by the results just presented, theoretically, if one can increase DHEA production at cellular level in the central compartment, one should be able to alleviate or prevent neuroinflammation and thus neurodegeneration.

2.9. Is it possible to stimulate extragonadal biosynthesis of DHEA or DHEAS?

Since exogenous DHEA supplementation has yielded varying benefit, an obvious alternative therapeutic – or preventative – strategy to consider is whether it might be naturally or artificially possible to increase endogenous DHEA production.

From the sport and exercise literature, it is known that exercise in older males can significantly increase serum DHEA and DHEAS concentrations (Boudou *et al.*, 2000; Ravaglia *et al.*, 2001; Tissandier *et al.*, 2001; Tremblay *et al.*, 2004; Arai *et al.*, 2006; Cadore *et al.*, 2008; Heaney *et al.*, 2013). These results do not provide proof of extragonadal androgen biosynthesis though, as moderate exercise is known to increase gonadal testosterone production. However, interestingly, similar effects of exercise has been reported for DHEA in older, post-menopausal females (Copeland *et al.*, 2002; Giannopoulou *et al.*, 2003; Kemmler *et al.*, 2003). In this population, where gonadal androgen production is less possible, it is possible that extragonadal DHEA production may occur in response to exercise.

Interestingly, a study conducted in Sprague-Dawley rats has illustrated skeletal muscle to have endogenous steroidogenic capacity and that acute bouts of aerobic exercise can significantly

enhance the localised production of DHEA in both male and female animals (Aizawa *et al.*, 2010). This adds substantial support for our theory that extragonadal DHEA biosynthesis may be elicited through intervention.

Furthermore, higher DHEAS levels have been reported in long-term practitioners of alternative therapies such as transcendental meditation and the ancient martial art of tai chi, when compared to age-matched controls (Glaser *et al.*, 1992; Lai *et al.*, 2017). Although these studies do not prove that DHEA production is stimulated by these practises, the relatively higher levels suggest at least an effect to sustain long-term production rate. In line with these results obtained in psychology-based practices, environmental enrichment in caged rats was linked to increases in steroidogenic enzyme expression associated with DHEA production, relative to unstimulated rats of the same age (Rossetti *et al.*, 2015). Indeed, enrichment therapy is already being employed to treat patients with dementia and Alzheimer's disease (da Cruz *et al.*, 2015; Matias-Guiu *et al.*, 2016; Quintana-Hernandez *et al.*, 2016; Sanchez *et al.*, 2016; Garcia-Casal *et al.*, 2017), although the authors could not find data on the effect of this strategy on DHEA biosynthesis.

Considering this evidence, perhaps it is necessary to first understand which tissues may possess the ability for DHEA biosynthesis, before considering the feasibility of this approach in the context of neurodegeneration.

2.10. **DHEA Biosynthesis**

DHEA is produced during steroidogenesis, in a manner dependent on mainly two classes of steroidogenic enzymes; namely the P450 enzymes and the hydroxysteroid dehydrogenases (Miller *et al.*, 2007). Also, DHEA can be sulphated into DHEAS via enzymes called steroid sulphotransferases (SULT), while conversion of DHEAS back into DHEA is mediated via steroid sulphatases (STS). DHEAS levels continue to increase after puberty, peaking in the mid-20s, after which levels begin to progressively decline with age in both men and women (Orentreich *et al.*, 1984; Maninger *et al.*, 2009). Serum DHEA and DHEAS levels are thought to be almost exclusively maintained by their biosynthesis in the adrenal zona reticularis (Miller *et al.*, 2007). However, in theory any tissue that expresses the steroid enzymes can be classified as steroidogenic. In fact, in the early 80s Fernand Labrie, a well-known endocrinologist, was the first to note that prostate glands of patients with prostate cancer still had high levels of dihydrotestosterone (DHT) even after castration (Labrie, 2010). He illustrated that some peripheral tissues, such as the prostate, possesses the steroidogenic enzymes needed to transform DHEA and DHEAS into its androgenic and estrogenic metabolites, thereby capable of exerting a relatively more localised effect. The study of this unique ability of peripheral tissues was termed "intracrinology" and has subsequently gained

some momentum (Re, 2014). Given the importance of intracrine derived hormone production in terms of female reproductive health, it is clear that investigations into intracrinological mechanisms in other contexts are warranted, such as in neurodegeneration and ageing.

Although it is known that intracrinology takes place in peripheral tissues, all tissue types do not express the same isoforms of the steroidogenic enzymes, nor are they expressed in the same quantity or constitutively expressed as we age. For example, a recent study in rats demonstrated that the expression of several steroidogenic enzymes decline significantly in the brain with age (Rossetti *et al.*, 2015). It is important to have an understanding of where the capacity for steroidogenesis exist, as well as to consider sex and species differences, as these aspects significantly impact on choices for experimental models, as well as on the relevance and applicability of research data gained in any particular model. It is not possible to address steroidogenesis in its totality within the scope of this review. Rather, in the next few sections we describe some of these intricacies in the context of DHEA biosynthesis specifically.

2.11. **Production sites of DHEA**

In terms of substrate delivery for steroidogenesis, the Steroidogenic Acute Regulatory Protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc) are required for the transport of cholesterol to the mitochondria and conversion into pregnenolone (P5), respectively. Interestingly, these enzymes are expressed at significantly lower levels in the Leydig cells of aged rat testes relative to those of younger rats (Luo *et al.*, 2001), which - since Leydig cells are the primary cells for testosterone production in the male body - provides a plausible explanation for the age-related decline of the hormone in both rodents and humans. Interestingly, in contrast, P450scc protein and mRNA expression in the human adrenal glands appears to be relatively stable with ageing (Suzuki *et al.*, 2000; Yu *et al.*, 2002).

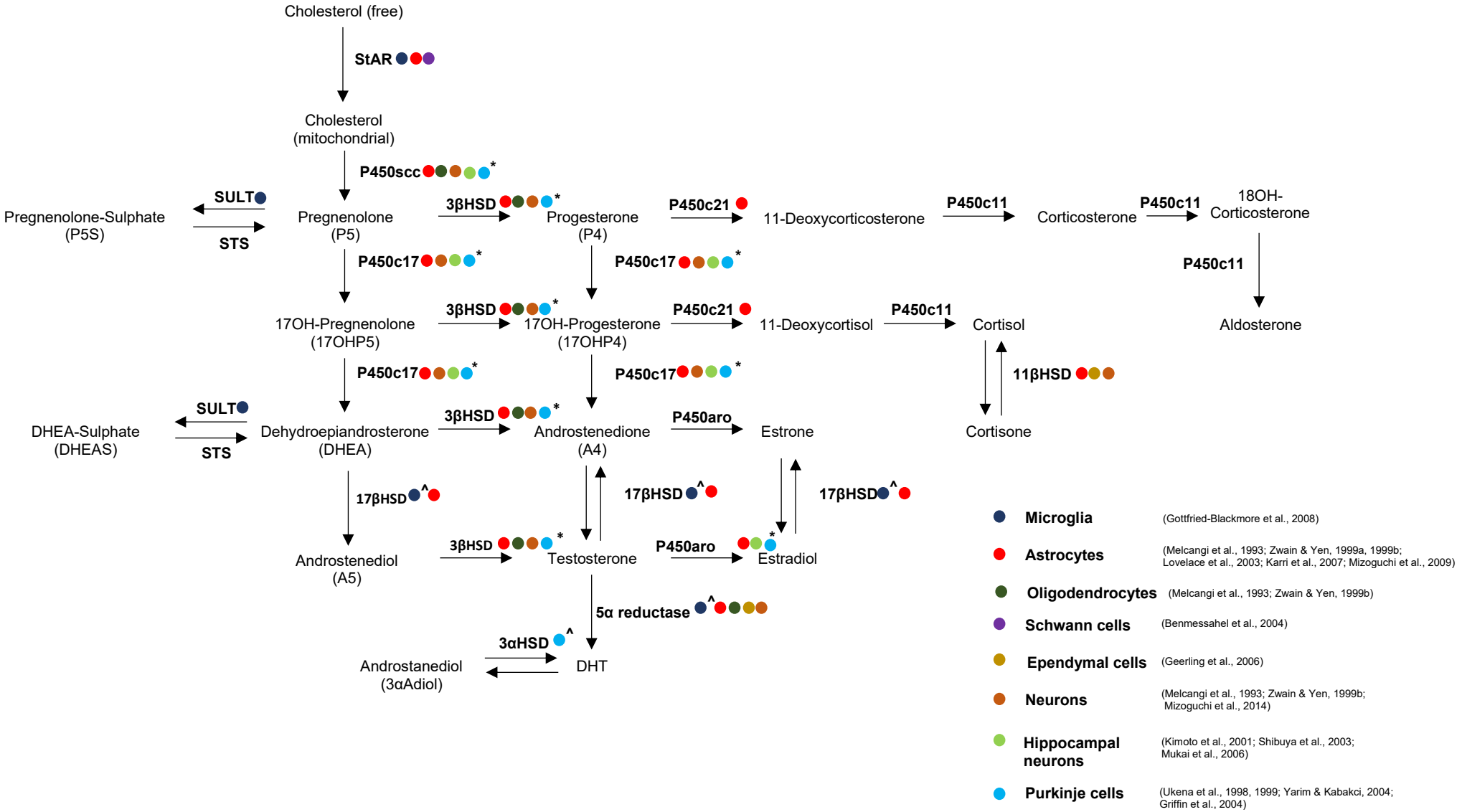

Figure 2-1: **Representative diagram of reported steroidogenic enzyme expression reported in different brain cell types**. Species employed were rat, unless otherwise additionally indicated (^mouse or *canine).

In the context of central intracrine production of DHEA, P450scc mRNA has been detected in cultured human astrocytes and oligodendrocytes (but not neurons) in at least one study (Brown *et al.*, 2000). Many other studies have detected steroidogenic enzyme expression in both neurons and neuroglial cells in vertebrates other than humans (Fig. 2-1). We tabulated these studies for brevity, but it is important to note that although steroidogenic enzyme mRNA was detectable in these different animal brain cell types, it is not clearly indicated to what extent the level of gene expression may differ from one cell type to another. Our interpretation of the data presented in most of these cited studies, is that neuroglial cells appear to express higher levels of steroidogenic enzyme mRNA relative to neurons, where levels were barely detectable in some cases. This suggests that mainly neuroglial cells are capable of significant intracrine biosynthesis, as already reported for peripheral tissue other than gonads and adrenals (Labrie, 2015a, 2015b). In contrast to the P450scc findings, StAR mRNA expression in rat brains has been shown to indeed decline significantly with age (Sierra *et al.*, 2003), suggesting a relative decline in substrate delivery similar to that reported for the testis. Whether this occurs in human brain tissue could not conclusively be answered from the literature, but it certainly cannot be ruled out. Nevertheless, the presence of both StAR and P450scc in astrocytes and oligodendrocytes argues in favour of sufficient substrate availability to indeed allow for localised production of DHEA in these cells.

Another protein of potential relevance in the context of cholesterol transport to the mitochondria has been described. Translocator protein (TSPO), also known as peripheral-type benzodiazepine receptor (PBR), a protein mainly bound to the outer mitochondrial membrane, has also been suggested to play a role in the facilitation of cholesterol transport via StAR (Lacapère and Papadopoulos, 2003). Whether the presence of TSPO is critical in the transport of cholesterol is a matter of controversy within literature. It was shown in mice, with a specific TSPO gene knockout in their Leydig cells, that testosterone could still be produced despite the absence of what thought to be a critical protein for the process (Morohaku *et al.*, 2014). Despite the uncertainty surrounding its role in steroidogenesis, TSPO has recently been identified as a potential biomarker of neurodegeneration, as its expression increases with inflammation and neurodegeneration associated with Alzheimer's disease, HIV encephalitis and MS (Cosenza-Nashat *et al.*, 2009; Kreisl *et al.*, 2013).

More specifically in terms of its function centrally, *in vitro* experiments in primary human astrocytes have demonstrated that various synthetic TSPO ligands of the N,N-dialkyl-2-phenylindol-3-ylglyoxylamide class (PIGAs) are capable of activating TSPO activity and stimulating neurosteroidogenesis (Da Pozzo *et al.*, 2016). In fact, PIGAs have shown to be neuroprotective in L-buthionine-(S,R)-sulfoximine (BOS) induced cytotoxicity of C6 rat glioma cells (Santoro *et al.*, 2016). The mechanism of neuroprotection was associated with a

reduction in lipid peroxidation and inflammation, which was abrogated in presence of the P450scc and StAR inhibitor aminoglutethimide and was therefore thought to occur due to steroidogenesis. Given the known anti-inflammatory and antioxidant properties of DHEA, TSPO may be a role player in the maintenance of DHEA levels.

Another fact in favour of local DHEA production in the brain, is the relatively increased demand for DHEA in brain relative to the periphery. Firstly, steroid sulphotransferase isoform 2A1 (SULT2A1) – which increases circulating DHEAS by sulphating DHEA, rendering it bio-inactive (Suzuki *et al.*, 2000) – seems to not be present in brain tissue at all, while SULT2B1 expression has only been reported for the prefrontal cortex, hippocampus and cerebellum, but not for the cerebral subcortex or neocortex (Steckelbroeck *et al.*, 2004; Lin *et al.*, 2014). Secondly, sulphatase (STS) expression – which is responsible for conversion of DHEAS into the bioactive DHEA - is generally very high in the CNS (Stárka *et al.*, 2015). Together, this suggests a relatively higher demand for DHEA in the brain when compared to the periphery. This scenario would also increase the likelihood of mechanisms for local production to be in place.

The idea of *de novo* biosynthesis of DHEA and DHEAS in the brain itself remains a highly debated topic in the field. As mentioned, the human adrenal glands were initially thought to be the only site of endogenous steroidogenesis, apart from the gonads (i.e. testes, ovaries) which produce DHEA and DHEAS to a lesser extent. However, the brain has recently and controversially been highlighted as a possible alternative site for production. Given the neuroprotective and central anti-inflammatory effects of DHEA already discussed, this possibility for intracrine DHEA production has far-reaching implications for both neurodegenerative disease and ageing – not only to increase our understanding of the disease processes, but also in terms of development of preventative and/or therapeutic strategies.

The first suggestion of DHEA biosynthesis in the brain was already published in the 1980s – when DHEA and DHEAS concentrations were reported to be higher in rat brains than in circulation (Corpéchot *et al.*, 1981). A few years later, similar circumstantial evidence for DHEA biosynthesis in the human brain emerged (Lacroix *et al.*, 1987). DHEA was subsequently commonly referred to as a “neurosteroid”, based on its relatively increased central localisation. Although DHEA can be detected in both animal and human brain tissue, it remains unclear whether *de novo* biosynthesis indeed occurs. For evident reasons, it is practically difficult to accurately assess actual DHEA production in the human brain, so that most data are generated from rodent models. To add complexity to this topic, subsequent evidence may suggest that a species-difference in terms of central DHEA levels may exist. In terms of criticism of analytical techniques, it has for example been suggested that DHEA levels in rat

brain tissue reported in earlier studies may have been overestimated due to a lack of sensitivity of the radio-immunoassays (RIA) technique used at the time, which relied on antibody specificity to detect the steroid hormones (Liere *et al.*, 2000). A newer method encompassing solid phase extraction (SPE), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) was developed to generate more accurate data (Liere *et al.*, 2000). Ironically, nearly a decade later it was reported by the same authors that this technique may indeed also be flawed when it came to the detection of sulphated steroids (Liere *et al.*, 2009). They showed in rodent samples that the levels of DHEA and P5 were higher in the lipoidal fraction of the SPE. It was suggested that cholesterol contamination may have contributed to these findings through cholesterol autoxidation, generating DHEA and P5, and that actual levels of DHEA and DHEAS are likely extremely low or essentially non-existent in rat brain tissue (Liere *et al.*, 2009). Interestingly, in the same paper, the authors were able to confirm the presence of significant amounts of both DHEA and P5 in human brain tissue samples using this improved methodology. The exact mechanisms of how cholesterol autoxidation leads to DHEA and P5 generation remains to be elucidated. Nevertheless, these results highlight the necessity of considering species in the design of experimental models. The fact that DHEA cannot be detected in rodent brains may be a function of generally lower levels when compared to humans. Thus, although rodents can still be feasible models, the parameters to be assessed, might have to differ depending on analytical sensitivity.

Given these methodological limitations in the measurement of hormone levels directly, many indirect methods have been employed to investigate the possibility of DHEA production centrally. However, results have again posed more questions than answers. For example, on one hand, P450c17 mRNA – which transcribes an enzyme that is crucial in the formation of DHEA – has been detected in cultured human glial cells (Brown *et al.*, 2000). Subsequently, P450c17 mRNA expression has also been demonstrated in the amygdala, caudate nucleus, cerebellum, corpus callosum, hippocampus and thalamus of the human brain (Yu *et al.*, 2002). In addition and similarly to circulating DHEA levels, the enzyme expression also declines with age in the rat brain, which argue in favour of the possibility for local DHEA biosynthesis (Munetomo *et al.*, 2015). Interestingly, the age-related decline in P450c17 mRNA was reported to be more pronounced in the cerebral cortex and cerebellum, but not the hypothalamus (Munetomo *et al.*, 2015) – this fact should be further explored in order to determine its relevance.

However, the expression of P450c17 in the human brain has been a topic of debate – at least two other studies could not detect any P450c17 mRNA in the human cerebellum, hippocampus or temporal lobe and reported no P450c17 activity in the temporal lobe (Steckelbroeck *et al.*, 2004; MacKenzie *et al.*, 2008). There are a variety of possible

explanations for these different results. Firstly, it is known that age has a significant effect on steroidogenic enzyme expression, so it is possible that this may have affected the results. Indeed, in the study where the P450c17 mRNA was detected, samples were pooled from patients between the ages of 10 to 78 years. On the other hand, in at least one of the studies failing to detect enzyme activity, samples were collected from a much older patient cohort (80 years and older) (Yu *et al.*, 2002; MacKenzie *et al.*, 2008). Secondly, in terms of the failure of any group to report activity of the enzymes which catalyse the reaction in the brain (Stárka *et al.*, 2015), there is currently no way to determine at which rate potential local DHEA biosynthesis would occur. An important consideration here is that P450c17 expression reported for the CNS is substantially lower than for the testes, which exceeds it by nearly 200-fold (Stárka *et al.*, 2015). It is therefore likely that the conventional methods previously employed in studies with negative outcome, may not have been sufficiently sensitive to detect these possibly very low levels of activity. Thus, the issue remains to be elucidated across the ageing spectrum, to eliminate the potential for a false negative outcome by using aged individuals.

Additionally, it is possible that localised DHEA biosynthetic mechanism in the brain may differ from that at other sites of local DHEA production. One group has indeed suggested that DHEA and DHEAS can be synthesised in a manner independent of P450c17. Their evidence suggests that cultured human astrocytes and oligodendrocytes, but not neurons, could synthesize DHEA in the presence of a P450c17 and 3β-HSD inhibitor. It is suggested that this alternative pathway may be dependent on reactive oxygen species and produce DHEA from an unknown “precursor” molecule other than P5 (Brown *et al.*, 2000). Although this study provided interesting data, there is a limitation to the scientific rationale, as the authors admitted that the P450c17 inhibitor used could not block endogenous DHEA production in the glial cells. At least one other lab has also speculated on the possibility for an alternative pathway of DHEA production linked to free radicals (Maayan *et al.*, 2005). This is not impossible, since a precedent in this regard exists: an alternative steroid production mechanisms in the brain was clearly demonstrated in the case of 21-hydroxylation, which appears to be mediated through CYP2D instead of CYPC21 (Kishimoto *et al.*, 2004).

Apart from the debate on the capacity for intracrine DHEA biosynthesis in the CNS, several theories have been formulated in attempts to explain the relatively higher DHEA and DHEAS concentrations in the central compartment. For example, it has been suggested that DHEA in the brain may be produced from DHEAS delivered from circulation, through conversion by steroid sulphatase (STS). Indeed, it has been shown that the human temporal lobe expresses high amounts of STS mRNA as well as activity in the cerebral cortex relative to subcortical white matter (Steckelbroeck *et al.*, 2004). However, there are two arguments against this

hypothesis. Firstly, sulphated hydroxysteroids such as DHEAS are hydrophilic and do not readily cross the blood-brain barrier (Kishimoto and Hoshi, 1972). Although sulphated steroids such as DHEAS and P5 sulphate may enter the brain through circulation via organic anion transporting peptides (OATP) transporting them both ways, it would appear that OATP transport of sulphated steroids favours movement out of the CNS, not in (Asaba *et al.*, 2000). However, a recent study investigating the transport of DHEAS and P5S through the blood brain barrier (BBB) of rats found that although P5S entered the blood brain barrier more readily, DHEA was rapidly metabolised into androstenedione (A4) and androstenediol (Qaiser *et al.*, 2017). Further investigation found that the enzymes responsible for the rapid desulphatasion were mainly localised in the BBB capillaries as opposed to the brain parenchyma (Qaiser *et al.*, 2017). This argues that the DHEA in brain is likely from peripheral sources as opposed to *de novo* biosynthesis. However, it is important to note that the overall efflux of DHEAS through the BBB may still not be rapid enough to account for the differences in concentration in the brain versus the periphery.

Secondly, as already mentioned, DHEA and DHEAS concentrations have been reported to be markedly higher in the human brain than in circulation (Lacroix *et al.*, 1987). In this study, DHEA concentrations were determined in the brains of 10 subjects (9 females and 1 male, ages ranging from 80 – 93). Nevertheless, in rat brains DHEA concentrations were higher relative to circulation and remained so even 15 days after an adrenalectomy and/or orchiectomy (Corpéchot *et al.*, 1981). This argues against the idea of systemic delivery. However, as mentioned earlier the techniques that were used to determine the concentration of DHEA in these studies may reassessed due to possible cholesterol contamination.

Taken together there still exists a strong body of evidence to support that *de novo* biosynthesis occurs in the brain. Although it is near impossible to prove this specifically in humans, as one will be faced with clear practical and ethical hindrances, it is the opinion of the authors that the human brain does indeed produce DHEA centrally. In order to understand how DHEA may be produced in the brain, the use of animal models may be the only way to elucidate key pathways. This, however, does not come without its challenges.

2.12. **Practical challenges in steroidogenesis research**

Several practical challenges have already been alluded to in this review, such as methodological issues pertaining to mRNA and protein determination, as well as gender-specificity. A remaining consideration which will be touched on in this section, is the importance of species selection for investigations of this nature.

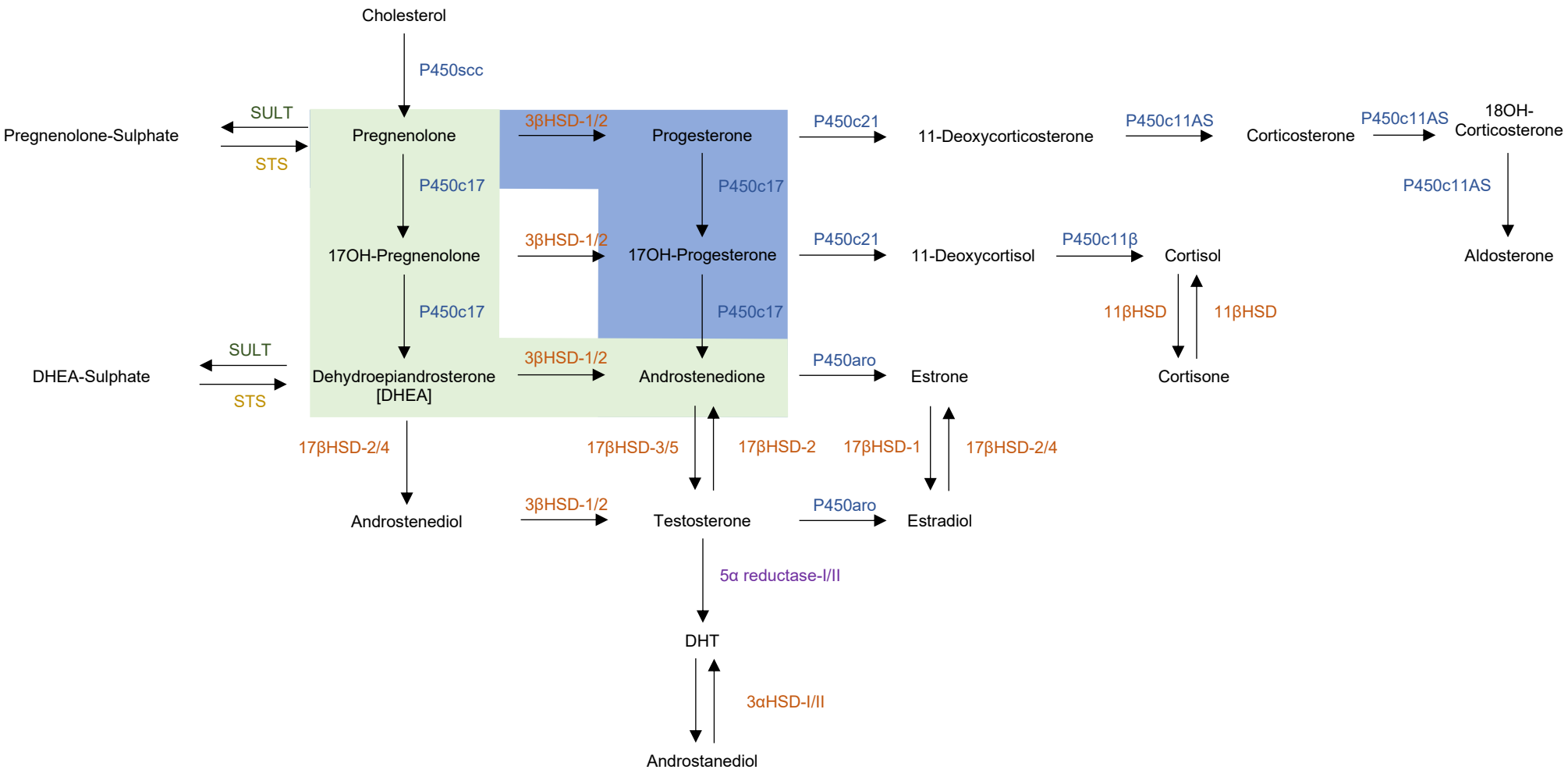

Figure 2-2: **The process of steroidogenesis in rodents and humans**. Cytochrome P450 enzymes (blue font), Hydroxysteroid dehydrogenases (orange font), Reductases (purple font), and Steroid Sulphatases (yellow font) and Sulphotransferases (green font). Part of the pathway highlighted in green denotes the Δ5 pathway, which is favoured in human steroidogenesis, and the blue denotes the Δ4, which is favoured in rodent steroidogenesis.

For example, it is important to consider that systemic steroidogenesis in humans, higher primates and bovines differ from rodents in two significant ways. Firstly, humans primarily convert pregnenolone (P5) to androstenedione (A4) through the Δ^5 pathway in the following manner: P5 » 17OH-P5 » DHEA » A4. Rodent steroidogenesis on the other hand favours the Δ^4 pathway; P5 » progesterone (P4) » 17OH-progesterone (17OHP4) » A4 (Fig. 2-2). This is speculated to be the reason why humans have high levels of circulating DHEAS, while in rodents levels are significantly lower when compared to humans (van Weerden *et al.*, 1992; Pluchino *et al.*, 2015).

Secondly, in humans the adrenal gland is the main source of DHEA production, but in rodents the gonads are the main source, as the adrenal glands of most species mice and rats appear to lack P450c17 enzyme activity (Le Goascogne *et al.*, 1991; van Weerden *et al.*, 1992). Although human gonads do produce androgens such as DHEA, the reason for adrenal preference of androgen production is not clear. The use of rodent models in studying the systemic role DHEA has thus garnered concern due the significant differences between humans and rodents. However, the fact that DHEAS has been found in both human and rodent brain tissue suggests that potential for *de novo* DHEA production exists in the brain in both species. Therefore, rodents could still serve as reliable model systems in studying DHEA and its role in the brain.

Of specific interest in this context, it was recently discovered that the precocial species of mouse, *Acomys cahirinus* or Spiny mouse, possess adrenal glands that express P450c17 and are in fact capable of producing DHEA (Quinn *et al.*, 2013, 2014). This unique mouse has a longer gestational period (± 39 days) when compared to other mice and rats and unlike other rodents, has a brain growth pattern that is comparable with humans at the time of birth (Brunjes *et al.*, 1989; Quinn *et al.*, 2016b). It has also been shown in spiny mice that cortisol is the main glucocorticoid in the blood, unlike in rats where corticosterone is the main circulating glucocorticoid (Quinn *et al.*, 2013, 2014). Most importantly, explanted foetal, neonate and adult spiny mouse brain tissue in culture have been shown capable of producing DHEA in the presence of P5 (Quinn *et al.*, 2016a). In addition, spiny mouse also exhibit an age-related decline in DHEA biosynthesis (Quinn *et al.*, 2016b). These mice may prove to be a particularly reliable and relevant model for studying the mechanisms of DHEA in many neurodegenerative disease states.

2.13. Conclusions

Based on the successes reported in experimental model systems, DHEA has been proven to be beneficial to limit progression of various inflammation-associated neurodegenerative diseases. From our review of the relevant literature, we conclude that *central*, intracrine biosynthesis of DHEA is highly probable and we have highlighted several mechanisms by which locally synthesised DHEA may affect preventative and/or therapeutic benefit in the context of neurodegeneration. Current literature is limited by controversies regarding methodological accuracy and the suitability of basic neuronal cell culture models.

2.14. Problem Statement

Although substantial evidence exists demonstrating the beneficial effects of DHEA in various disease contexts – especially neurodegeneration and neuroinflammation – a paucity exists in literature on the exact site and/or mechanism of central DHEA biosynthesis. It is first necessary to elucidate the potential mechanisms of neurosteroidogenesis before we can exploit central DHEA availability for therapeutic benefit.

2.15. Hypothesis

In the context of chronic disease, astrocytes may play a neuroprotective role via DHEA biosynthesis or metabolism. We hypothesise that astrocytes are the main central site of intracrine DHEA biosynthesis and/or metabolism.

2.16. Aims

Given the known limitations of earlier techniques employed to study DHEA-related steroidogenesis, we aimed to elucidate biosynthesis and/or metabolism of DHEA in astrocytes using modern, accurate and sensitive methodology.

A second aim was to elucidate potential species differences in this context.

2.17. Objectives

The following specific objectives were formulated:

I. Investigate the ability of primary human astrocytes and rodent mixed brain culture to produce DHEA when supplied with appropriate substrate
II. Investigate the ability of primary human astrocytes and rodent mixed brain culture to metabolise exogenous DHEA

Chapter 3

3. Materials and Methods

3.1. Materials

Primary cortical human astrocytes (cat # 1800) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM), gamma-irradiated Foetal Bovine Serum (FBS), Penicillin-Streptomycin (100X), Hanks Balanced Salt Solution (HBSS), 10x Phosphate Buffered Saline (PBS), Alexa Fluor® 488 donkey anti-mouse secondary antibody, Alexa Fluor® 594 donkey anti-rabbit secondary antibody and 0.25% Trypsin-EDTA solution were purchased from ThermoFisher Scientific (Glasgow, UK). Cell culture plasticware (T75 and 6-chamber dishes) was purchased from Nest® (Jiangsu, China). Mouse anti-Iba1 monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-GFAP monoclonal antibody was purchased from Cell Signalling Technologies, Inc. (CST) (Beverly, MA, USA). Donkey serum was purchased from Celtic Diagnostics (Cape Town, ZA).

Acetic acid, sodium acetate, forskolin (from *C. forskohlii*), type H-1 sulphatase enzyme (from *H. pomatia*), Trilostane, Dimethyl Sulfoxide (DMSO), Methyl-*tert*-Butyl-Ether (MTBE), Methanol (MeOH), formic acid, Nunc® 8-chamber glass slides, Poly-L-lysine (PLL) and steroids (Pregnenolone (P5), Dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T), androstanedione (5a-dione), androstanediol (3a-Adiol), androsterone (AST), dihydrotestosterone (DHT), progesterone (P4), 17-hydroxyprogesterone (17OHP4), 5α-pregnan-17α-ol-3,20-dione (Pdione) were purchased from Sigma-Aldrich (now incorporated into Merck)(St. Louis, MO, USA). Dihydroprogesterone (DHP4), 5α-pregnan-3α,17α-diol-20-one (Pdiol), epi-allopregnanolone (ALLO), androstenediol (A5), 17-hydroxypregnenolone (17OHP5) and pregnanetriol were purchased from Steraloids (Wilton, USA). 16-hydroxyprogesterone (16OHP4) was purchased from BDH Chemical Ltd (Poole, England, UK). Deuterated steroid reference standards were purchased from Cambridge isotopes (Andover, MA, USA), these include; D2-testosterone (1, 2-D2, 98%) (D2-T), D9-progesterone (2, 2, 4, 6, 6 , 17A, 21, 21-D9, 98%) (D9-P4), D8-17-hydroxyprogesterone (2, 2, 4, 6, 6, 21, 21-D8, 98%) (D8-17OHP4), D7-androstenedione (D7-A4), D6-dihydroprogesterone (D6-DHP4), D2-androsterone (D2-AST), D4-dihydrotestosterone (D4-DHT), D6-dehydroepiandrosterone (D6-DHEA) and D3-androstanediol (D3-3a-Adiol).FOODFRESH CO_2 was purchased from Afrox (Cape Town, ZA). The ACQUITY UPC$^{2®}$ Viridis ethylene-bridged hybrid (BEH) 2-ethylpyridine (2-EP) column (3mm×100 mm, 1.7 µm) was purchased from Waters (Milford, USA).

3.2. **Methods**

3.2.1. Primary human astrocyte (pHA) cultures

Primary human astrocytes (pHAs) were grown and maintained in high-glucose DMEM (25 mM D-glucose, 1 mM sodium pyruvate, 4mM L-glutamine) supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin (PenStrep) (10,000 U/mL and 10 mg/mL), in a humidified incubator at 37°C under 5% CO_2 atmosphere.

3.2.2. Primary rat brain *ex vivo* mixed cell cultures (pRBMCs)

3.2.2.1. Animals: ethical considerations

For the rodent mixed brain cultures, brain tissue had to be harvested from young, weaned male Wistar rats. Ethical exemption for tissue collection was granted by the Animal Research Ethics Committee of Stellenbosch University (ACU-2019-11454), on the grounds that tissue was harvested from animals that were culled as part of the normal husbandry procedures in the small experimental animal unit. The animals were maintained in a South African Veterinary Council (SAVC) approved facility under strict standard laboratory conditions in 12:12 light-dark cycle. Animals were fed standard rat chow and water was provided *ad libitum*.

3.2.2.2. Tissue isolation and culture technique

The animals were first anaesthetised with halothane gas and subsequently euthanised through CO_2 asphyxiation. Whole brains were rapidly excised and placed in 15 mL ice cold 1X HBSS supplemented with 1% (v/v) Penicillin-Streptomycin. Cerebellums were removed and the cerebral and midbrain tissue minced with a scalpel, after which they were placed in 5 mL warm (37°C) 1X HBSS supplemented with 0.25% trypsin. The tissue was left to digest in a shaking incubator at 37°C for 30 minutes, with agitation every 10 minutes. After digestion the tissue suspensions were centrifuged for 5 minutes at 300 xg. The supernatants were aspirated and discarded, and the pellets were resuspended and triturated in high-glucose DMEM supplemented with 10% (v/v) FBS and 1% PenStrep (v/v). The tissue suspension was then placed through 70 µm cell strainer to generate a single cell suspension. The strained cell suspensions were then seeded into 12-well culture dishes previously coated with poly-L-lysine (50 µg/mL) for 1 hour at 37°C. The cell suspensions were left to grow in a humidified incubator at 37°C under 5% CO_2 atmosphere. The culture media was refreshed 2 days after the initial plating of the suspensions and then every 3 days thereafter until the cultures became confluent.

3.2.2.3. Characterisation of primary brain cell culture

Scanning laser confocal microscopy was utilised to visually confirm the presence of glial cells, particularly astrocytes, in isolated rat brain cell cultures (pRBMCs).

Sample preparation:

Described similarly as before, after extractions, cell suspensions were seeded on Nunc® 8-chamber glass slides previously coated with poly-L-lysine (50 µg/mL). Once cell monolayers reached 80% confluency, they were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed 3x for 5 min each in 1X PBS, permeabilised with 0.1% Triton-X in 1x PBS for 2 min, then subsequently washed again 3x for 5 min with 1x PBS. Cells were then blocked in 5% donkey serum in 1x PBS for 60 min at room temperature. After blocking, cells were incubated overnight with primary mouse anti-Iba1 (1:100) and rabbit anti-GFAP (1:200) antibodies in 1x PBS containing 5% donkey serum. The following day, cells were again washed 3x 5 min after which they were incubated in 1x PBS containing Alexa Fluor® 488-conjugated donkey anti-mouse (1:200) and Alexa Fluor® 594-conjugated donkey anti-rabbit (1:200) antibodies for 60 min. After 60 min a Hoechst 33342 dye was added to the cells and left to incubate for a further 10 min. Cells were then washed 3x 5 min and then immersed in ≈50 µL of Dako® Fluorescent mounting medium and then frozen at -20 °C until imaging.

Confocal Microscopy

Confocal microscopy was performed on a Zeiss LSM 780 system (Carl Zeiss Microimaging, Germany). Data were collected by acquiring z-stacks, each with 8-12 image frames, and increments of ≈2 µm step width. An LCI Plan-Apochromat 63x/1.4 Oil DIC M27 objective equipped with a Diode 405nm CW/PS (pulsed), 488 nm laser, 561 nm laser and GaAsP detector (32+2 PMT), was utilised. Maximum intensity projections of z-stacks were processed and generated in the Zeiss Zen Black Software (2012) and utilised for subsequent experimental analysis.

3.2.3. Intervention treatments

Once primary human astrocyte cell cultures reached ≈80% confluence, they were seeded into 12-well dishes at a density of $1x10^5$ cells per well with 2 mL of complete culture medium. Cells were allowed to grow for 24 hours after which the media was replaced with experimental culture medium.

In the case of the *ex vivo* mixed rat brain cell culture, cells were seeded directly into PLL coated 12-well dishes, as previously described. Once the cells reached a density of ≈80% confluence, the steroidogenic stimulation experiment proceeded.

Experimental medium was defined as high-glucose DMEM, as previously described, but with a significantly reduced serum content (0.1%). Serum was reduced, due to the presence of endogenous steroids in FBS, which would have been a potential confounding factor.

The EM was then divided into 8 groups supplemented with different treatments to stimulate or inhibit parts of the steroidogenesis pathway, the groups are described as follows: a basal (control) condition containing no treatment; Forskolin (10 µM); Pregnenolone (1 µM); Forskolin (10 µM) and Pregnenolone (1 µM); Forskolin (10 µM), Pregnenolone (1 µM) and Trilostane (10 µM); DHEA (1 µM); and lastly Forskolin (10 µM), Pregnenolone (1 µM) and DHEA (1 µM) (See Table 3-1).

The rationale for these treatments is as follows: Forskolin stimulates steroidogenesis by upregulating adenylate cyclase activity, thereby increasing intracellular cyclic adenosine monophosphate (cAMP) – a secondary messenger known to stimulate steroidogenic enzyme activity (Seamon *et al.*, 1981; Moriwaki *et al.*, 1982). Forskolin was used to assess steroidogenic capacity in the presence or absence of P5 as well as DHEA.

Trilostane is a known inhibitor of 3β-HSD, an enzyme critical in the conversion of P4, 17OHP4, A4 and T to P5, 17OHP5, DHEA and A5 respectively (Komanicky *et al.*, 1978). Trilostane was used in order to distinguish whether or not the potentially produced DHEA may have been metabolised before it could reach detectable levels in our method.

Cells were stimulated under these various conditions for 48 hours, after which 0.5 mL of media was collected and frozen at -80 °C until steroid extraction procedure.

Table 3-1: Different experimental groups containing different stimulants and inhibitors of steroidogenesis

	1 B	**2** F	**3** P	**4** FP	**5** FPT	**6** D	**7** FD	**8** FDT
Forskolin (10 µM)		X		X	X		X	X
Pregnenolone (1 µM)			X	X	X			
Trilostane (10 µM)					X			X
DHEA (10 µM)						X	X	X

3.2.4. Steroid Extraction

Steroids were extracted from cell culture media using a liquid-liquid extraction technique utilising MTBE as a solvent. Briefly, 50 µL of deuterated reference standard mix were added to 0.5 mL of cell culture media in a 10 mL borosilicate screw cap vial, after which 3 mL of MTBE were added. Samples were then placed on an automated vortexer (SI-600, MRC Lab, Beijing, China) for 15 min at 1300 RPM to allow phases to interact. Once samples completed the shaking cycle, they were placed in the -80 °C freezer for 20 min. At this temperature, the aqueous phase separates, settles and freezes at the bottom of the vial while the organic phase, that now contains the extracted steroids, remains unfrozen on top of the aqueous phase. The organic phase was then decanted into a clean borosilicate glass vial and dried under N_2 gas at 40 °C. Steroid residues were then resuspended in 150 µL of 50% HPLC-grade MeOH after which they were decanted into LCMS vials for analysis on the UPC^2-MSMS.

Table 3-2: Master mix of deuterated internal standards. Each 50 µL of the master mix contained the amount of respective standard below (ng):

Internal Standard	**Concentration (ng/sample)**
D6-DHP4	5
D9-P4	10
D7-A4	10
D2-AST	15
D4-DHT	5
D6-DHEA	25
D8-17OHP4	10
D2-T	10
D3-3αAdiol	25

3.2.5. Deconjugation assay

Conjugated steroids such as DHEAS, P5S and DHT glucuronide (DHTG) are not extracted in the MTBE liquid-liquid extraction technique due to the polarity of the molecules and the non-polar nature of MTBE. One way in which to extract conjugated steroids is by enzymatically deconjugating an equal volume of sample and calculating the conjugated steroid content by the difference between the untreated and the enzyme treated samples.

A commercially available sulphatase enzyme (STS) from the gastric juices of *H. pomatia* (Wittstock *et al.*, 2000) was used to deconjugate potentially sulphated steroids in samples. This extract also possesses potent glucoronidase activity and therefore also deconjugates steroids such as DHTG.

The assay is described briefly as follows; 66 units of sulfatase (≈20 µL of 2 mg/mL in ddH_2O) was added to each 500 µL of sample. The pH of the sample was lowered to ≈ pH 5 (the optima for the sulphatase enzyme) by the addition of a sodium acetate buffer – to a final concentration of ± 200mM (≈110 µL of buffer). Samples were then incubated for 3 hours at 55 °C in a shaking incubator. Samples were then removed from the incubator to cool, internal standards were then added and steroids were extracted as previously described.

For every assay performed, positive controls were also deconjugated concurrently to calculate assay efficiency for both sulphatase and glucoronidase enzymes. Positive controls included were 1 µM of DHEA, DHEAS, DHT and DHTG that were extracted with or without treatment with sulphatase enzyme.

3.2.6. Ultra-Performance Convergence Chromatography (UPC^2) – tandem mass spectrometry

Steroids were separated and quantified on the ACQUITY UPC^2-MS/MS (Waters, Milford, USA) utilising an ACQUITY UPC^2 Viridis BEH 2-EP column operating in multiple reaction monitoring (MRM) mode (settings can be seen in table 3-3) using a positive electrospray ionisation (ESI+) mode. Sample injection volume was 2 µL with a method run time of 6 minutes. The validation for this method forms part of a different PhD dissertation (Ms Desmare van Rooyen) and thus is not presented here for examination.

All steroid quantifications were referenced to a standard range (0.0001–2 ng/µL) prepared in experimental culture media and extracted as previously described. Individual steroid quantifications were also referenced to a deuterated internal standard to normalise any potential errors in sample preparation (See Table 3-3). Internal standards that eluted closest RT of a given steroid analyte were selected for referenced quantification. Data were collected and analysed in Waters Masslynx 4.1 (Waters®).

Limits of detection (LOD) and limits of quantification (LOQ) for each analyte are indicated in Table 3-4. LODs were defined as the lowest concentration detectable with a signal-to-noise ratio (S/N) of >3 for the quantifier ion. LOQs were defined as the lowest concentration detectable with a S/N of >10 for the quantifier ion and a S/N of >3 for the first (1°) qualifier ion.

Table 3-3: List of steroid standards with retention times (RT); molecular ion species and MRM mass transitions; and reference internal standards

Steroid	RT (min)	Mass Transitions			Reference Internal Standard
		Quantifier	Qualifier 1	Qualifier 2	
5aDione	1.39	289.20 > 253.10	289.20 > 271.20	289.20 > 97.20	D6-DHP4
DHP4	1.42	317.00 > 95.00	317.00 > 105.20	317.00 > 175.20	D6-DHP4
P4	1.85	315.20 > 79.15	315.20 > 123.04	315.20 > 297.00	D9-P4
A4	1.88	287.20 > 123.00	287.20 > 173.00	287.20 > 211.10	D7-A4 or D9-P4
AST	2.89	273.20 > 185.10	273.00 > 55.14	273.00 > 69.07	D2-AST
Pdione	2.97	333.40 > 137.26	333.40 > 288.76	333.40 > 70.82	D2-AST
DHT	3.08	291.00 > 255.29	291.00 > 159.00	291.00 > 104.90	D4-DHT or D2-AST
DHEA	3.32	271.20 > 253.20	271.20 > 91.05	271.20 > 213.00	D6-DHEA
ALLO	3.38	319.00 > 189.20	319.00 > 80.90	319.00 > 283.15	D6-DHEA
P5	3.46	317.24 > 80.97	317.24 > 131.00	317.24 > 158.90	D6-DHEA
17OHP4	3.65	331.10 > 122.90	331.10 > 312.80	331.10 > 295.00	D8-17OHP4
T	3.71	289.20 > 123.00	289.20 > 186.80	289.20 > 271.18	D2-T
16OHP4	3.94	331.20 > 97.01	331.20 > 109.10	331.20 > 313.10	D8-17OHP4
3αAdiol	4.13	275.20 > 257.25	275.20 > 81.10	275.20 > 175.00	D3-3αAdiol
Pdiol	4.18	317.40 > 111.00	317.40 > 299.00	317.40 > 93.15	D3-3αAdiol
A5	4.27	273.09 > 201.17	273.00 > 129.14	273.00 > 185.28	D3-3αAdiol
17OHP5	4.32	297.20 > 159.12	297.20 > 133.10	297.20 > 147.30	D3-3αAdiol
Pregnanetriol	4.42	301.20 > 283.20	301.20 > 135.01	301.20 > 187.00	D3-3αAdiol
D6-DHP4	1.42	323.17 > 85.00	323.17 > 287.07	323.17 > 305.00	
D9-P4	1.85	324.20 > 107.15	324.20 > 134.95	324.20 > 194.24	
D7-A4	1.88	294.30 > 234.00	294.30 > 118.90	294.30 > 121.15	
D2-AST	3.82	275.20 > 67.00	275.20 > 54.95	275.20 > 215.10	
D4-AST	3.04	295.20 > 105.05	295.20 > 163.19	295.20 > 215.30	
D6-DHEA	3.31	277.30 > 219.20	277.30 > 97.20	277.30 > 202.10	
D8-17OHP4	3.65	340.10 > 322.00	340.10 > 147.20	340.10 > 168.20	
D2-T	3.72	291.00 > 201.30	291.00 > 105.19	291.00 > 164.84	
D3-3αAdiol	4.15	260.20 > 178.10	260.20 > 109.10	260.20 > 135.10	

Table 3-4: Limits of Detection (LOD) and Limits of Quantifications (LOQ) for different steroid analytes

		Q – S/N > 3		Q – S/N > 10; 1° > 3		Range
	Hormone	**LOD (ng/µL)**	**LOD (µM)**	**LOQ (ng/µL)**	**LOQ (µM)**	
1	5αdione	0,001	0,003	0,001	0,003	0,001-2
2	DHP4	0,002	0,006	0,002	0,006	0,002-2
3	PROG	0,001	0,003	0,001	0,003	0,001-2
4	A4	0,001	0,003	0,001	0,003	0,001-2
5	AST	0,020	0,069	0,020	0,069	0,020-1
6	PDIONE	0,020	0,060	0,020	0,060	0,020-1
7	DHT	0,001	0,003	0,001	0,003	0,001-2
8	DHEA	0,010	0,032	0,010	0,035	0,010-2
9	ALLO	0,002	0,006	0,002	0,006	0,002-0,2
10	PREG	0,010	0,032	0,010	0,032	0,010-0,2
11	17OHP4	0,001	0,003	0,010	0,032	0,001-2
12	T	0,001	0,003	0,001	0,003	0,002-0,2
13	16OHPROG	0,0002	0,0006	0,0001	0,0003	0,0002-0,2
14	3αAdiol	0,010	0,032	0,010	0,032	0,01-0,2
15	PDIOL	0,010	0,032	0,010	0,032	0,01-2
16	A5	0,010	0,032	0,010	0,034	0,01-2
17	17OHP5	0,010	0,032	0,010	0,032	0,01-2
18	Ptriol	0,020	0,064	0,020	0,064	0,0-2

Q – Quantifier Ion; 1° – Qualifier Ion; S/N – Signal-to-noise ratio
LOD – Limit of detection; LOQ – Limit of quantification

3.2.7. Statistical Analysis

Statistical analysis of data was performed in GraphPad Prism® 5. Data were tested for normalcy of distribution after which the appropriate statistical test were applied. These included one-way ANOVA, two-way ANOVA, as well unpaired student-t tests. For normally distributed data, the Bonferroni post hoc test was employed, while Dunn's test was used for non-parametric data. A p-value of $p<0.05$ was accepted as statistically significant.

Chapter 4

4. Results

4.1. Steroidogenesis characterisation of primary human astrocytes

Despite the use of high sensitivity methods, no steroids were detected in cell culture media from cells under basal conditions, nor from cells treated with 10 μM forskolin in the absence of P5 and/or trilostane, and DHEA.

P5 was detectable only in treatment conditions containing ≈1μM P5 as a substrate; namely groups P (Fig. 4-1A), FP (Fig. 4-1B) and FPT (Fig. 4-1C). P5 concentrations were detected independent of forskolin and/or trilostane treatment.

DHEA and A5 were detectable only in groups that received ≈1 μM DHEA as a substrate; namely groups D (Fig. 4-2A), FD (Fig. 4-2B) and FDT (Fig. 4-2C).

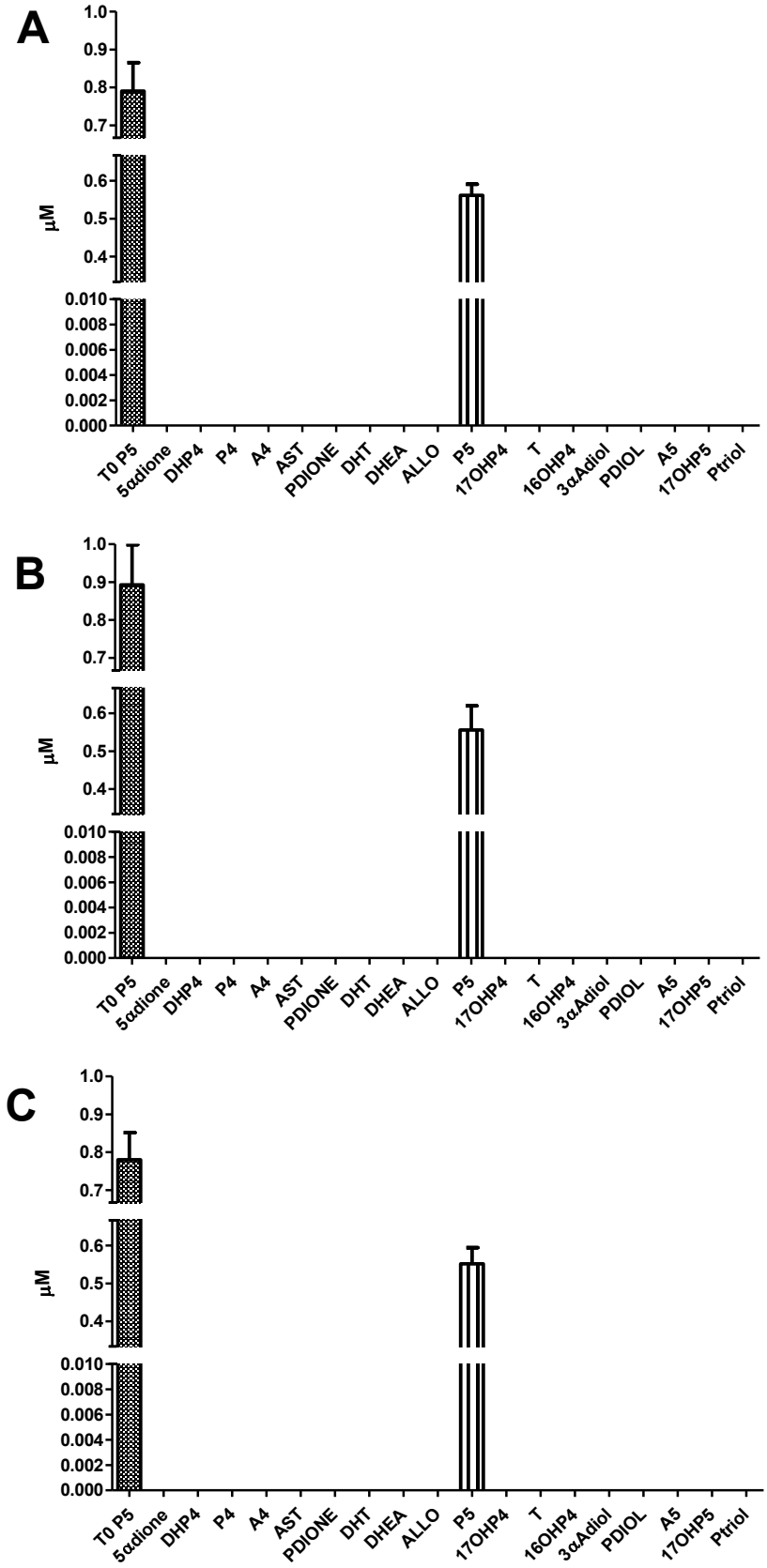

Figure 4-1**: Detectable steroids produced by pHAs in experimental conditions containing P5 as a substrate with or without steroidogenic modulators**: **(A**) P – 1 µM P5; (**B)** FP – 10 µM Forskolin & 1 µM P5; **(C)** FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane**.** T0 P5 indicates initial P5 concentration of treatment. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate.

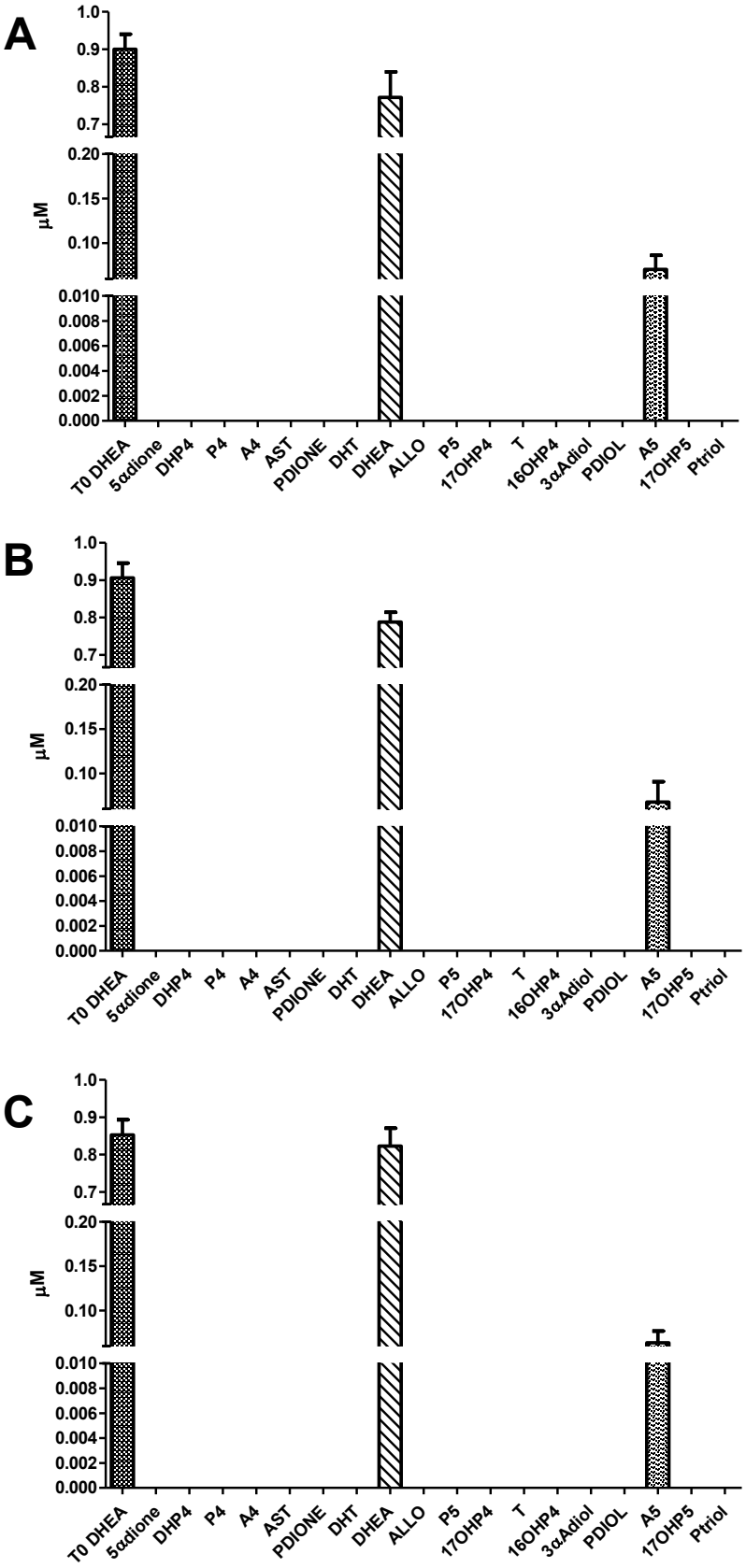

Figure 4-2 **Detectable steroids produced by pHAs in experimental conditions containing DHEA as a substrate with or without steroidogenic modulators**: **(A**) D – 1 µM DHEA; (**B)** FD – 10 µM Forskolin & 1 µM DHEA; **(C)** FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane**.** T0 DHEA indicates initial DHEA concentration of treatment. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate.

4.1.1. Summary of substrate metabolism and analytes produced by pHAs

Taken together, these results suggest that primary human astrocytes in isolation are able to convert DHEA into A5 (Figs 4-2A, B and C)

Initial P5 concentrations were significantly reduced when compared to P5 recovered in groups P, FP and FPT after treatment of cells (Fig 4-3).

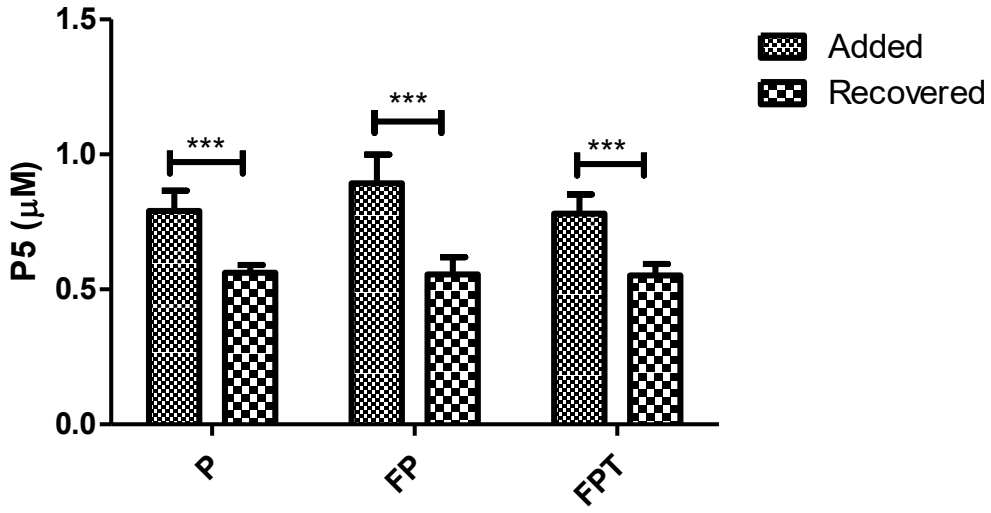

Figure 4-3: **Comparison between initial P5 concentration and recovered concentration after treatment of pHAs**. P – 1 µM P5; FP – 10 µM Forskolin & 1 µM P5; FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. **(p<0.001); ***(p<0.0001)

A significant reduction in DHEA concentration was observed when compared to the initial concentration in groups D and FD. Although a decrease was too observed in group FDT, it was not significant (Fig. 4-4A).

A5 was synthesised in groups D, FD and FDT. A5 concentrations in group FD was significantly higher when compared to both group D and FDT (Fig. 4-4B).

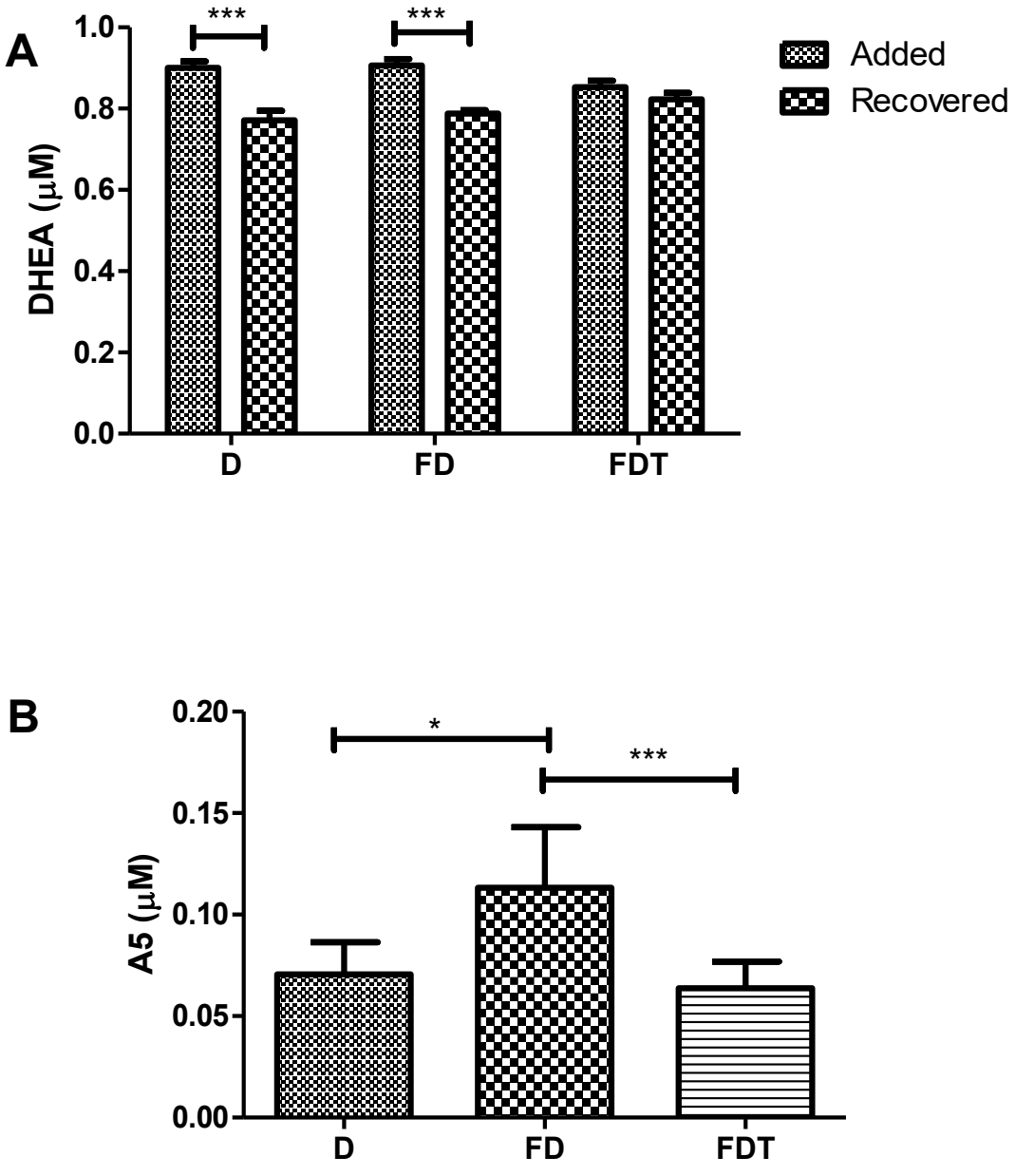

Figure 4-4: **Comparison between initial DHEA concentration and recovered concentration after treatment of pHAs (A); A5 concentrations in different conditions (B).** D – 1 µM DHEA; FD – 10 µM Forskolin & 1 µM DHEA; FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); **($p<0.001$); ***($p<0.0001$)

4.2. Light microscopy morphological characterisation of primary rat brain *ex vivo* mixed cell culture

After 7 days of plating primary rat brain ex vivo mixed cell culture, two distinct cell populations with different morphologies were observed (Fig. 4-5). The first (indicated by white arrows) had an elongated epithelial cell-like morphology and appeared to grow in aggregated colonies. The second cell population (indicated by green arrows) had distinctly small cell bodies, were ovoid in shape and lacked processes.

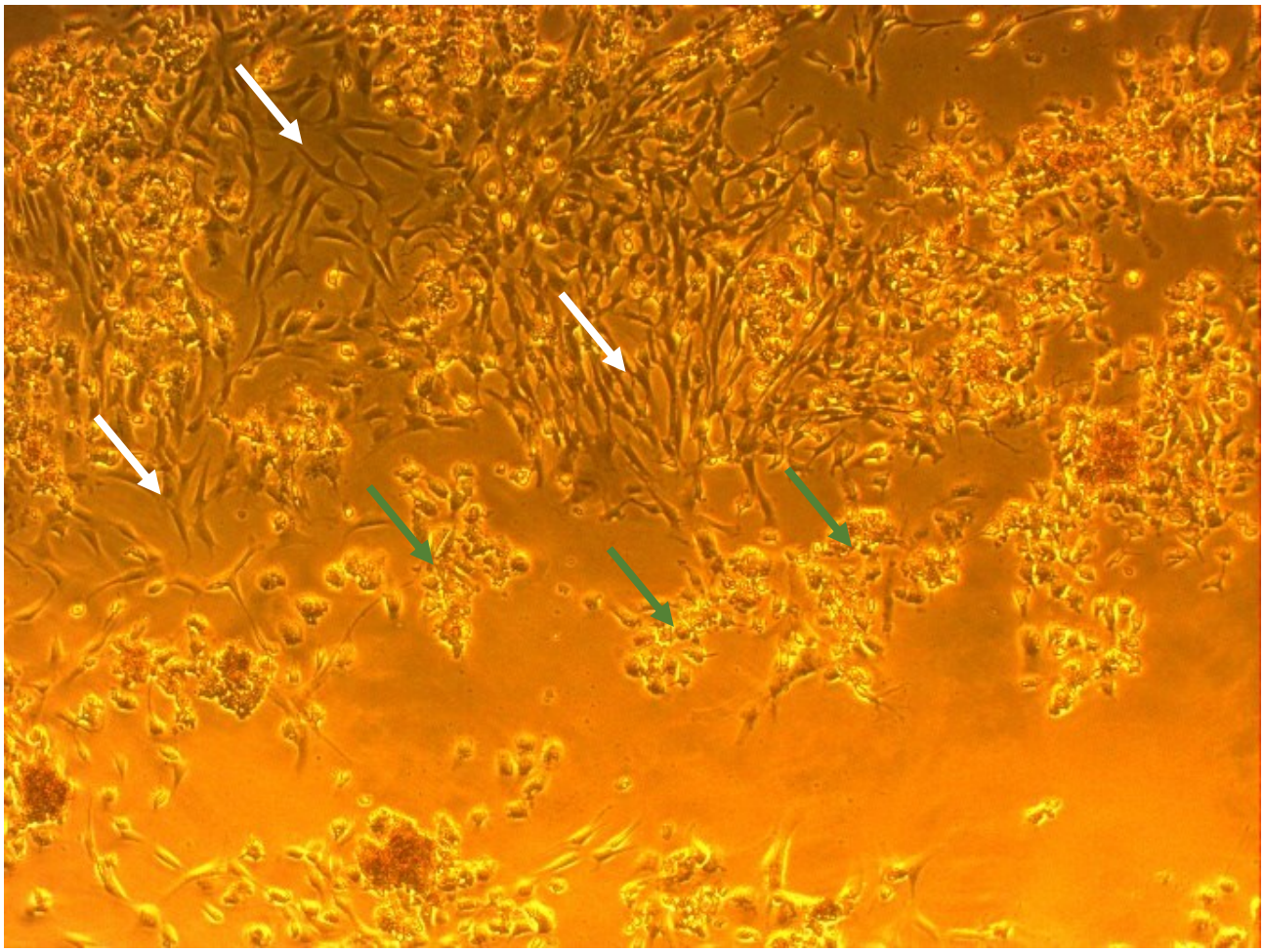

Figure 4-5: **Light micrograph of primary rat brain *ex vivo* mixed cell culture after 7 days of culture.** 10x magnification. White arrows indicate cell population with epithelial like morphology. Green arrows indicate cell population with small cell bodies lacking processes.

4.3. Confocal analysis of primary rat brain *ex vivo* mixed cell culture confirmed the presence of astrocytes and microglia

Confocal microscopy analysis revealed a mixed population containing both cells that were positive for the astrocyte (GFAP) marker and cells positive for the microglial (Iba-1) marker (Figure 4-6). Microglia appeared to occur in a larger proportion to astrocytes and also appeared to grow on top or at least in close proximity of the astrocytes. This is deduced due the nature in which data is acquired in the z-plane of the microscope. Like a light microscope, the objective (and therefore laser source) is inverted, so the detectors resolve the fluorescent pixels from closest to furthest from the objective.

Two populations of Iba-1 positive cells with distinctly different morphologies were observed. One population was characterised as relatively smaller cells with diffuse Iba-1 staining, while the other consisted of larger cells with Iba-1 expression (green fluorescent signal) that formed structural patterns appearing cytoskeletal in nature (white arrows). These cells also seemed to possess larger nuclei (yellow arrows).

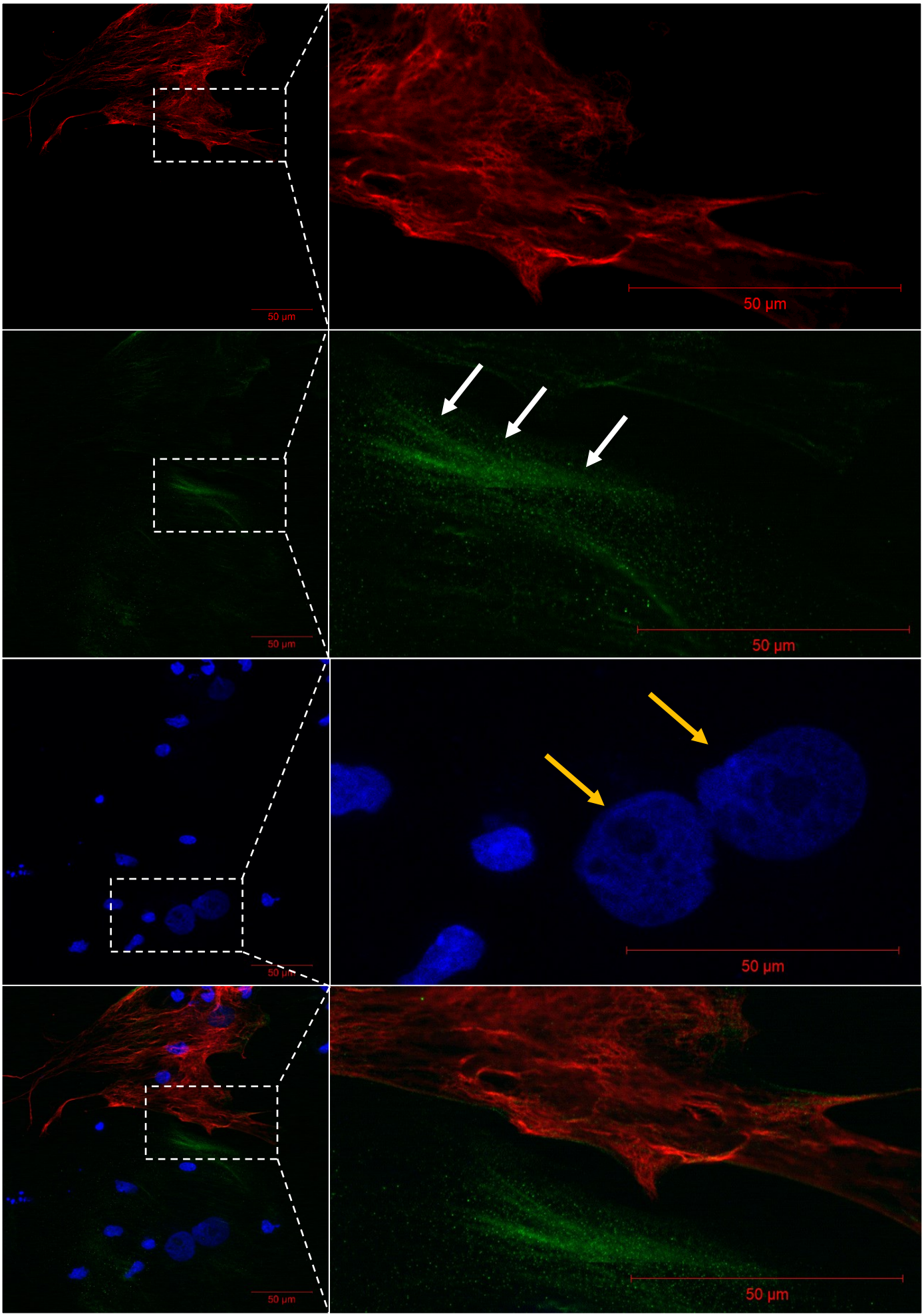

Figure 4-6: **Confocal analysis of primary rat brain *ex vivo* mixed cell culture**. GFAP (Red) labelled astrocytes and Iba-1 (green) labelled microglia in mixed cell population. Hoechst nuclei stain is seen in blue. 60x magnification with 2x2 tile scan (1024x1024 pixels per tile). Scale bar = 50 µM

4.4. Steroidogenesis characterisation of primary rat brain *ex vivo* mixed cell cultures

Similar to pHA samples, no steroid analytes were detected in culture media from cells under basal conditions nor by cells stimulated by 10 µM forskolin in the absence of P5, DHEA or trilostane (data not shown).

P5 was detected groups P (Fig. 4-7A), FP (Fig. 4-7B) and FPT (Fig. 4-7C). DHEA and A5 were detectable only in groups that received 1 µM DHEA as a substrate; namely groups D (Fig. 4-8A), FD (Fig. 4-8B) and FDT (Fig. 4-8C).

No other steroid analytes were detected in any of the other treatment groups.

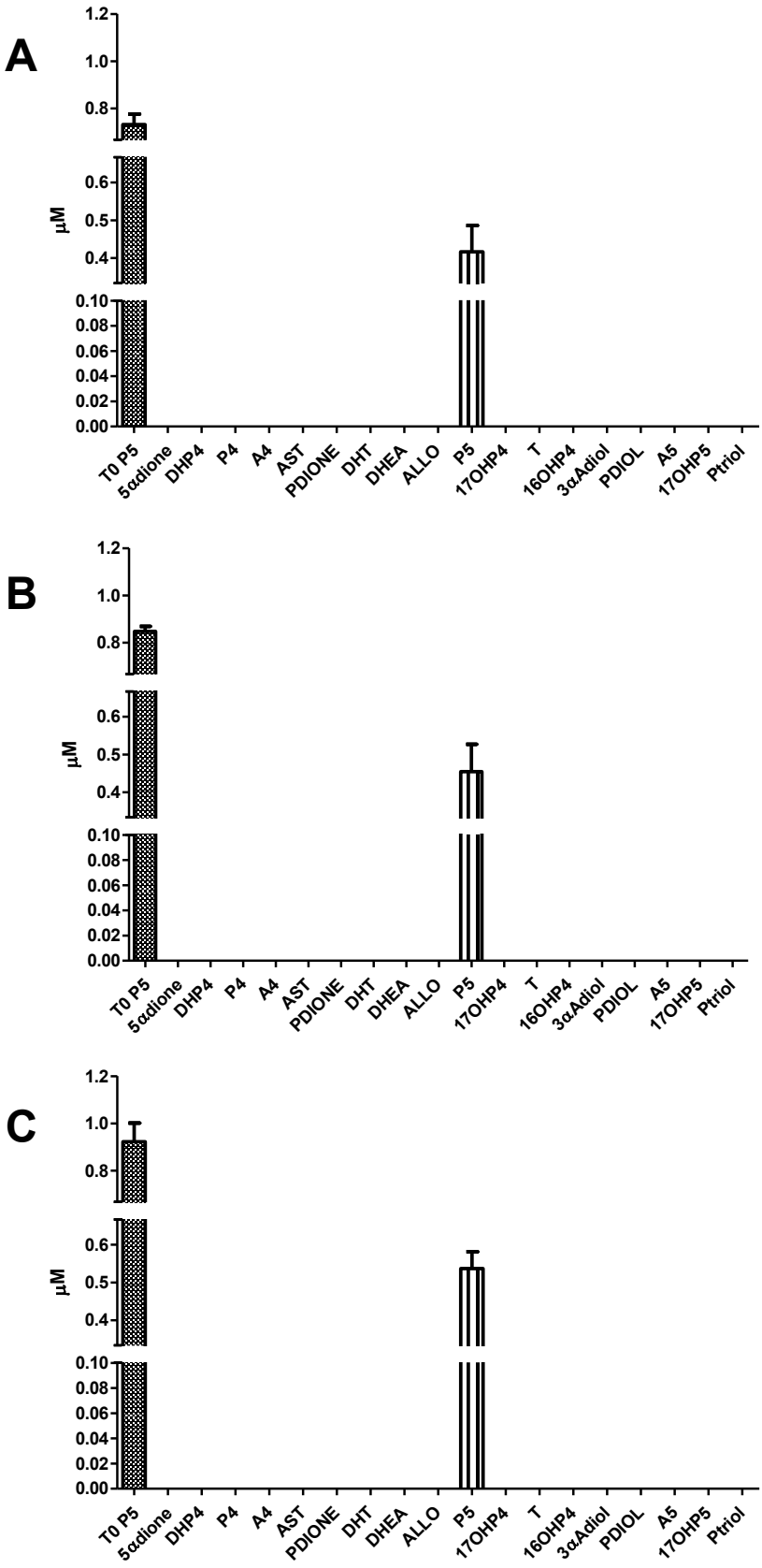

Figure 4-7: **Detectable steroids produced by pRBMCs in experimental conditions containing P5 as a substrate with or without steroidogenic modulators**: **(A**) P – 1 µM P5; (**B)** FP – 10 µM Forskolin & 1 µM P5; **(C)** FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane**.** T0 P5 indicates initial P5 concentration of treatment. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate.

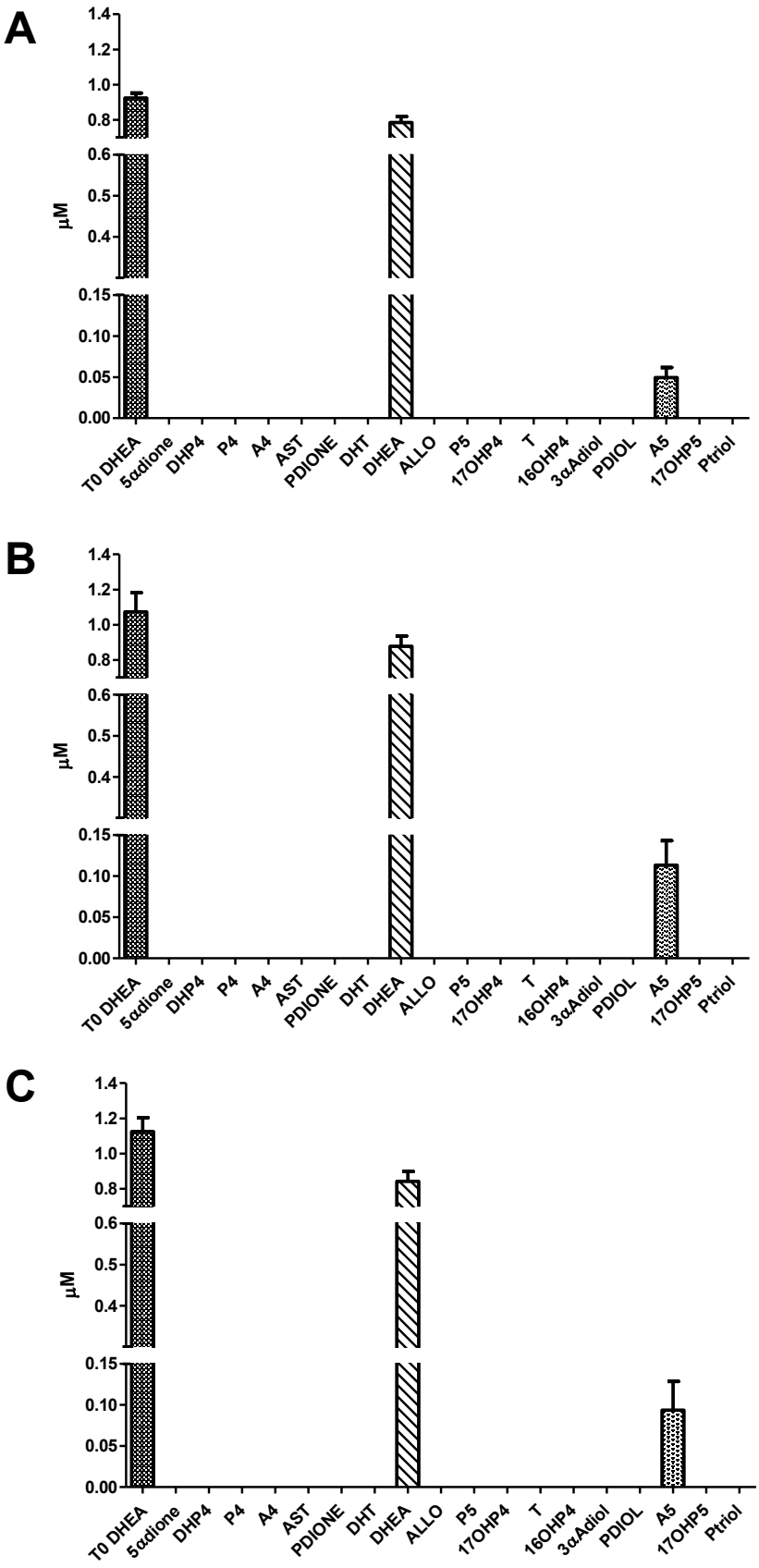

Figure 4-8 **Detectable steroids produced by pRBMCs in experimental conditions containing DHEA as a substrate with or without steroidogenic modulators**: **(A**) D – 1 µM DHEA; (**B)** FD – 10 µM Forskolin & 1 µM DHEA; **(C)** FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane**.** T0 DHEA indicates initial DHEA concentration of treatment. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate.

4.4.1. Summary of substrate metabolism and analytes produced by pRBMCs

Concentrations of P5 decreased significantly when compared to initial (T0) concentration of P5 in all groups after treatment of cells; namely groups P, FP and FPT (Fig. 4-9).

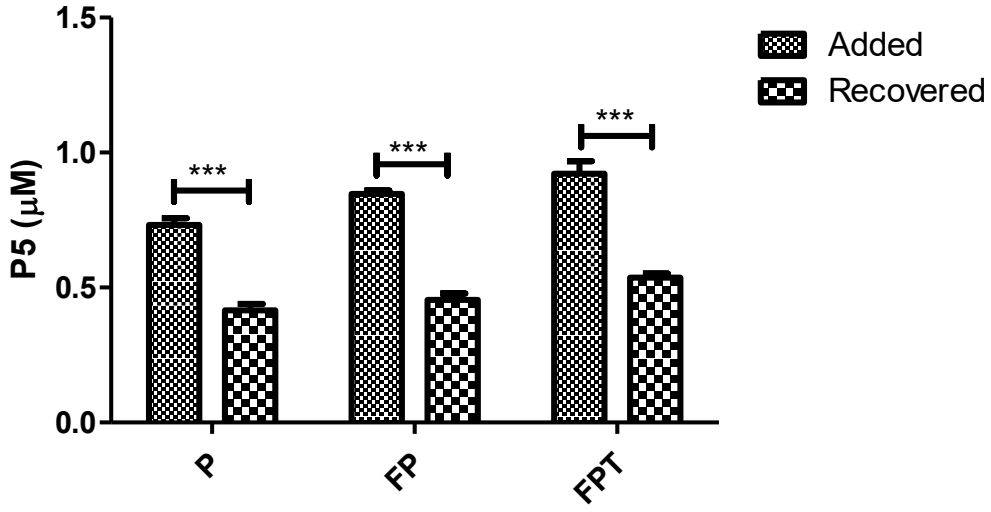

Figure 4-9: **Comparison between initial P5 concentration and recovered concentration after treatment of pRBMCs**. P – 1 µM P5; FP – 10 µM Forskolin & 1 µM P5; FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); ***($p<0.0001$)

Concentrations of DHEA decreased significantly when compared to initial concentration in treatment groups D, FD and FDT (Fig. 4-10A).

A5 was synthesised in groups D, FD and FDT. A5 concentrations were significantly higher in groups FD and FDT when compared to group D, respectively (Fig. 4-10B).

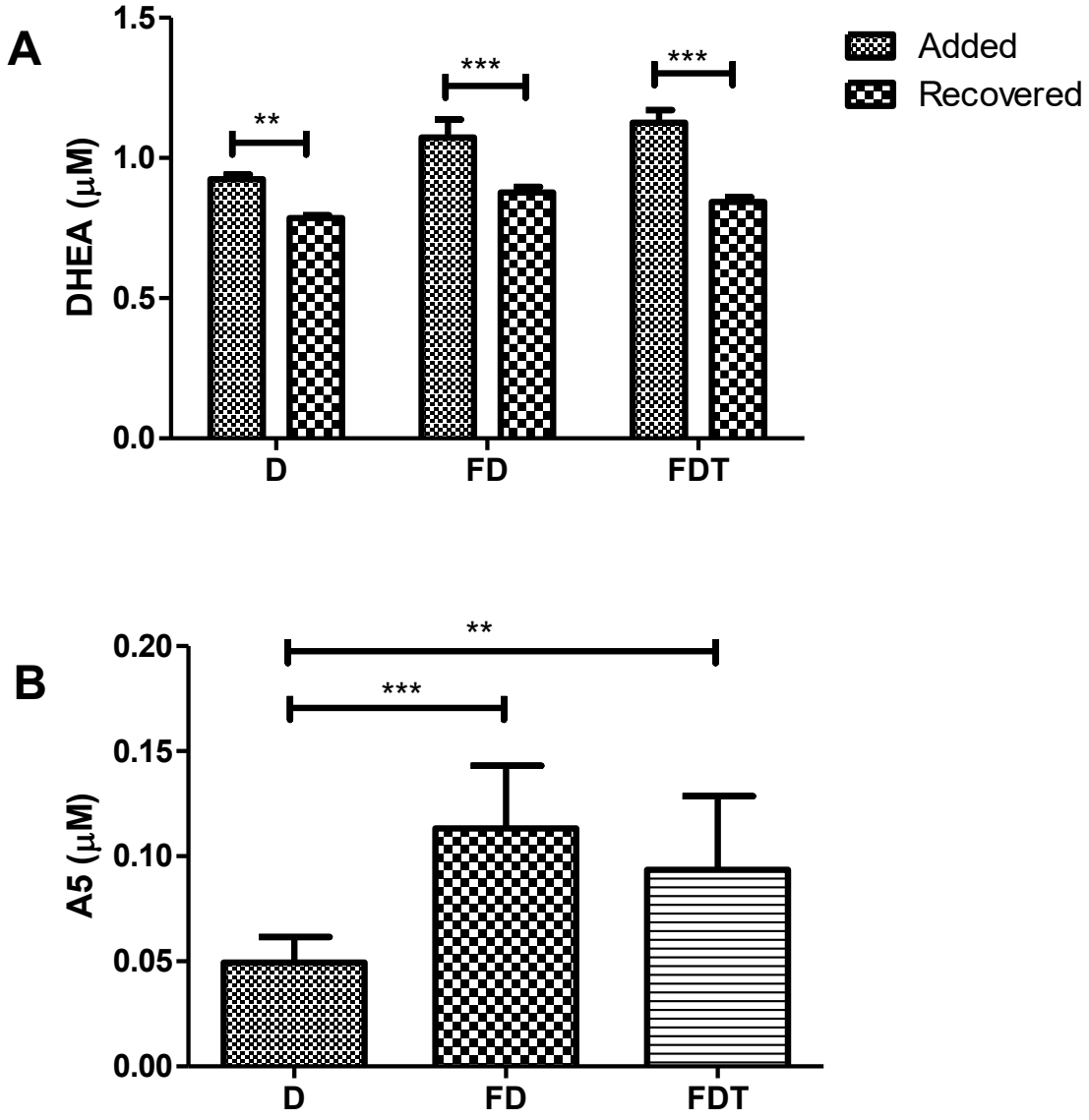

Figure 4-10: **Comparison between initial DHEA concentration and recovered concentration after treatment of pHAs (A); A4 concentrations in different conditions (B).** D – 1 µM DHEA; **(B)** FD – 10 µM Forskolin & 1 µM DHEA; **(C)** FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); **($p<0.001$); ***($p<0.0001$).

4.5. Comparison of synthesised analyte concentrations between pHAs and pRBMCs (species differences)

A5 concentrations were significantly higher in pHAs compared to pRBMCs in group D. Concentrations remained the same between cell populations in group FD but were significantly higher in pRBMCs when compared to pHAs in groups FDT (Fig. 4-11C).

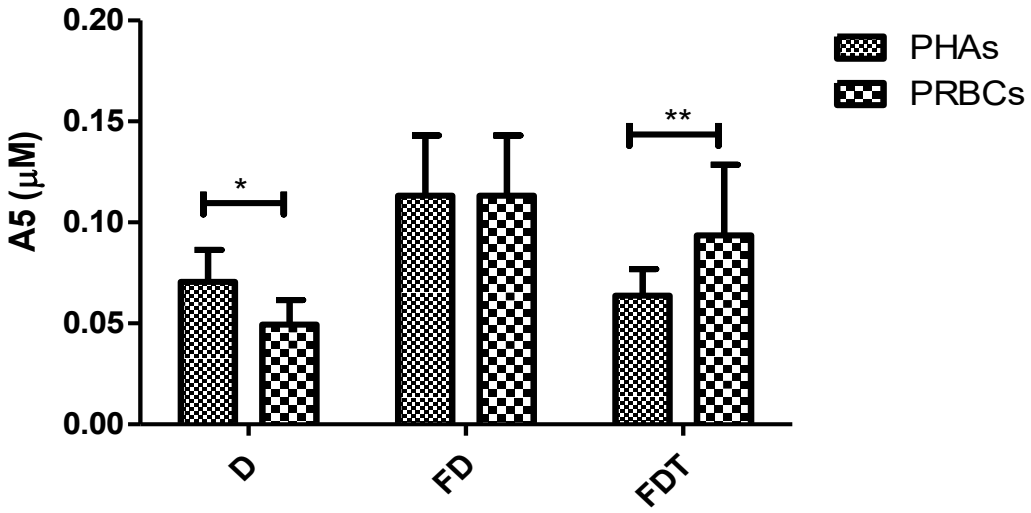

Figure 4-11: **Comparison between pHA and pRBMC synthesised A5 concentrations of different treatment groups**. D – 1 µM DHEA; FD – 10 µM Forskolin & 1 µM DHEA; FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); **($p<0.01$); ***($p<0.0001$).

4.6. **Deconjugation assay**

4.6.1. Efficiency of sulphatase and glucoronidase enzymes

As detailed in the methods description, conjugated steroids such as DHEAS and DHTG are capable of being extracted by MTBE due to their polar nature. To quantify conjugated steroids, samples were treated with a commercial sulphatase (STS) preparation and compared to an untreated (enzyme) counterpart to calculate the fraction that may exist as a conjugated steroid.

Significantly less DHEA was detected in STS treated 1 µM DHEAS positive control when compared to the 1 µM DHEA positive control (Fig. 4-12A). This is calculated as desulphatasion efficiency of ≈48%.

No significant difference in DHT concentration was observed when comparing 1 µM DHT to STS-treated 1 µM DHTG (Fig. 4-12B). This was calculated as deglucuronidation efficiency of ≈100%.

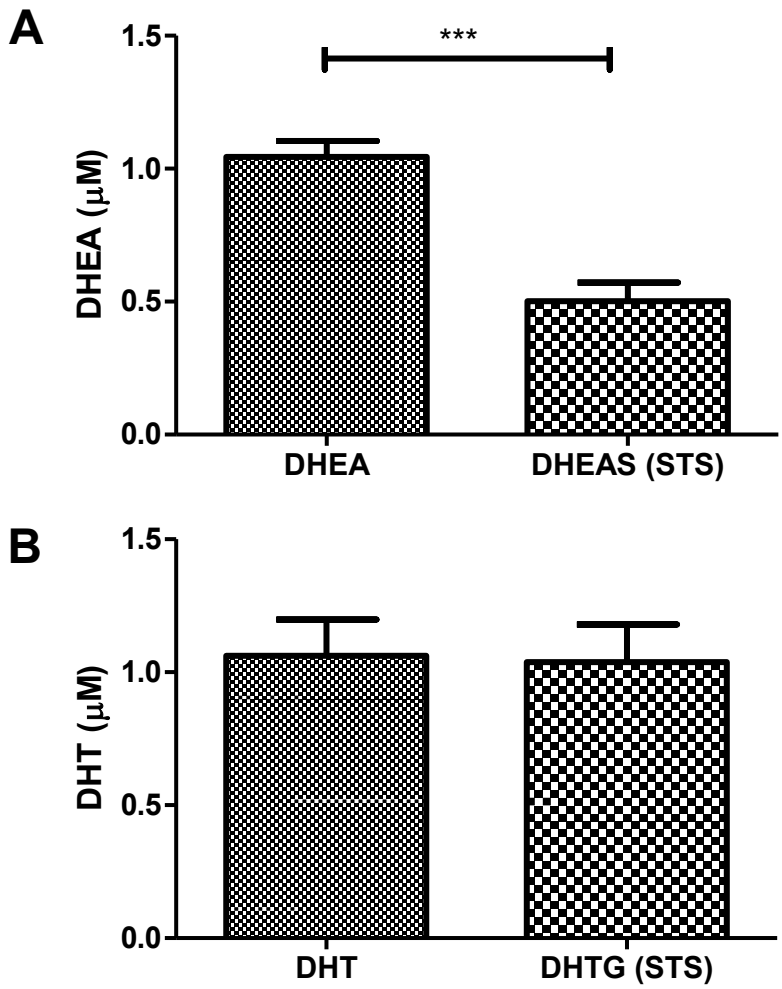

Figure 4-12: **Efficiency of sulphatase (A) and glucoronidase (B) enzymatic activity**. DHT – Dihydrotestosterone; DHTG (STS) – Sulphatase treated DHTG. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. ***($p<0.001$)

4.6.2. Commercial sulphatase enzyme from *H. pomatia* demonstrates endogenous 3β-HSD activity

A4 was detected in STS treated positive controls for both 1 µM DHEA and DHEAS. However, significantly less A4 was present in the DHEAS STS-treated vs DHEA STS-treated samples (Fig. 4-13).

This data suggests endogenous 3β-HSD activity in the commercially available enzyme. The presence of this enzyme activity is a confounding variable as some analytes detected in non-STS-treated samples are substrates of 3β-HSD. The implications of the presence of this enzyme will be discussed in context later in the discussion chapter.

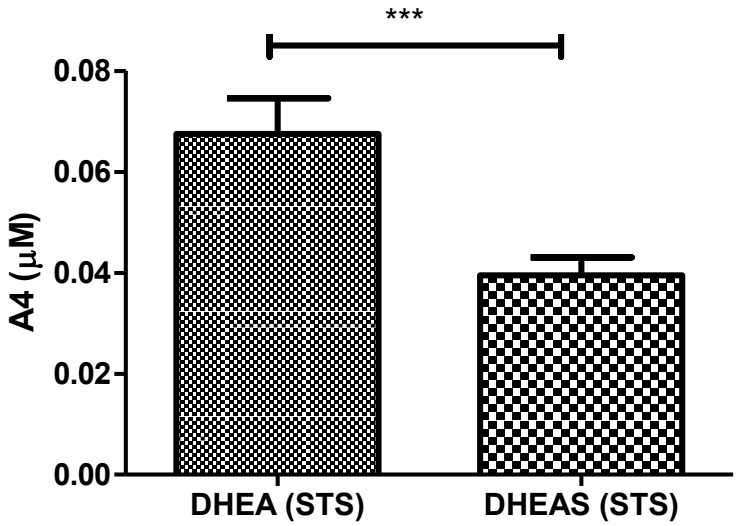

Figure 4-13: **Biosynthesis of A4 in STS treated DHEA and DHEAS positive controls**. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. ***(p<0.0001)

4.7. Detectable steroids in sulphatase enzyme treated pHA and pRBMC samples

Steroid analytes detected in STS treated samples from both pRBMCs and pHAs were identical to untreated samples (i.e. samples not treated with STS).

Comparisons of all detected analytes in both pHA and pRBMC STS-treated samples are described further in the subsections to follow.

4.7.1. Effect of STS enzyme on concentrations of P5 and P4 in pHA samples

Significantly less P5 was present in STS-treated samples when compared to non-STS-treated samples in groups P, FP and FPT (Fig. 4-14A).

P4 was detected in STS-treated samples when compared to non-STS-treated samples in groups P, FP and FPT (Fig. 4-14B)

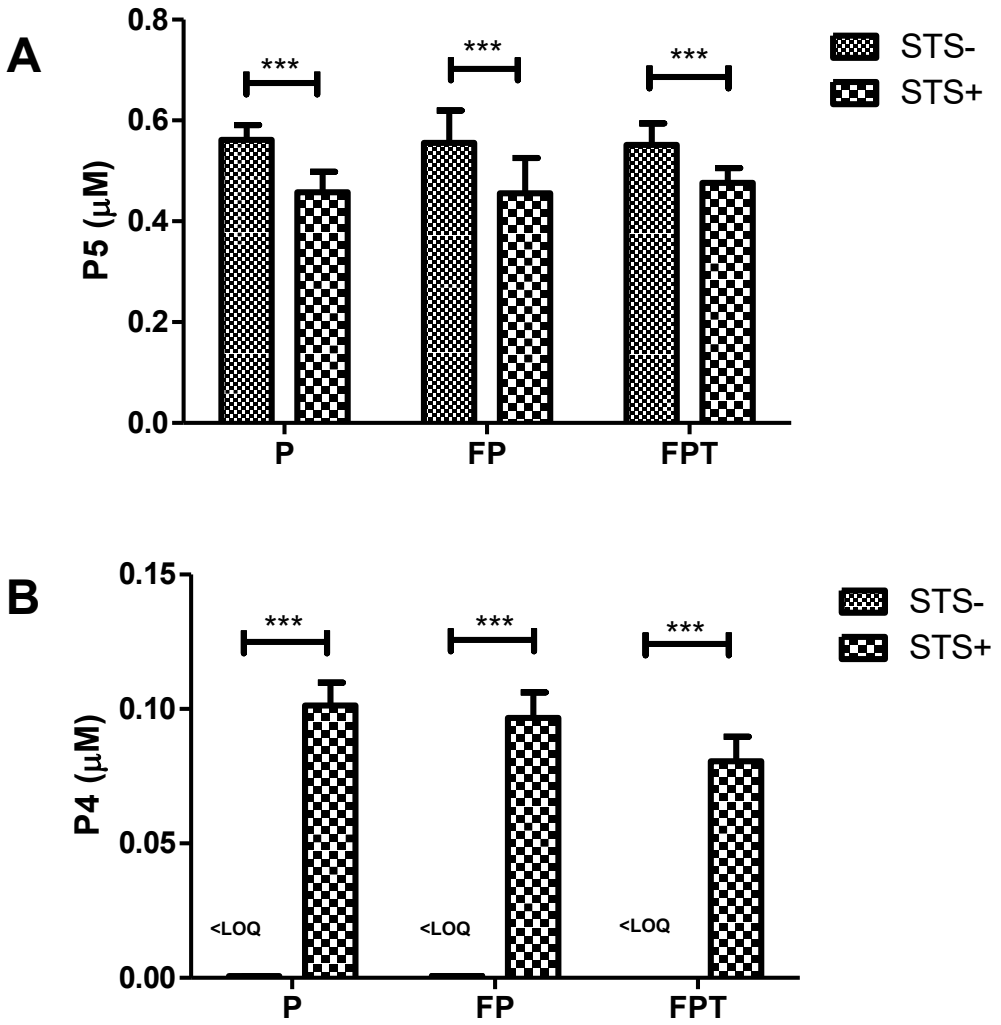

Figure 4-14: **Comparison between P5 (A) and P4 (B) concentrations of samples from pHAs before and after treatment with STS in different treatment groups**. P – 1 µM P5; FP – 10 µM Forskolin & 1 µM P5; FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. ***(p<0.0001)

4.7.2. Effect of STS enzyme on concentrations of DHEA, A4 and A5 in pHA samples

DHEA concentrations remained relatively unchanged in both STS-treated and non-STS-treated samples in groups D, FD and FDT. Although a slight decrease was noted in each group, albeit non-significant (Fig. 4-15A).

A4 were detected in STS-treated compared to non-STS-treated samples in groups D, FD and FDT (Fig. 4-15B).

A5 concentrations were significantly decreased in STS-treated compared to non-STS-treated samples in group D. A decrease was noted in STS-treated vs non-STS-treated samples in groups FD, although these changes were non-significant (Fig. 4-15C).

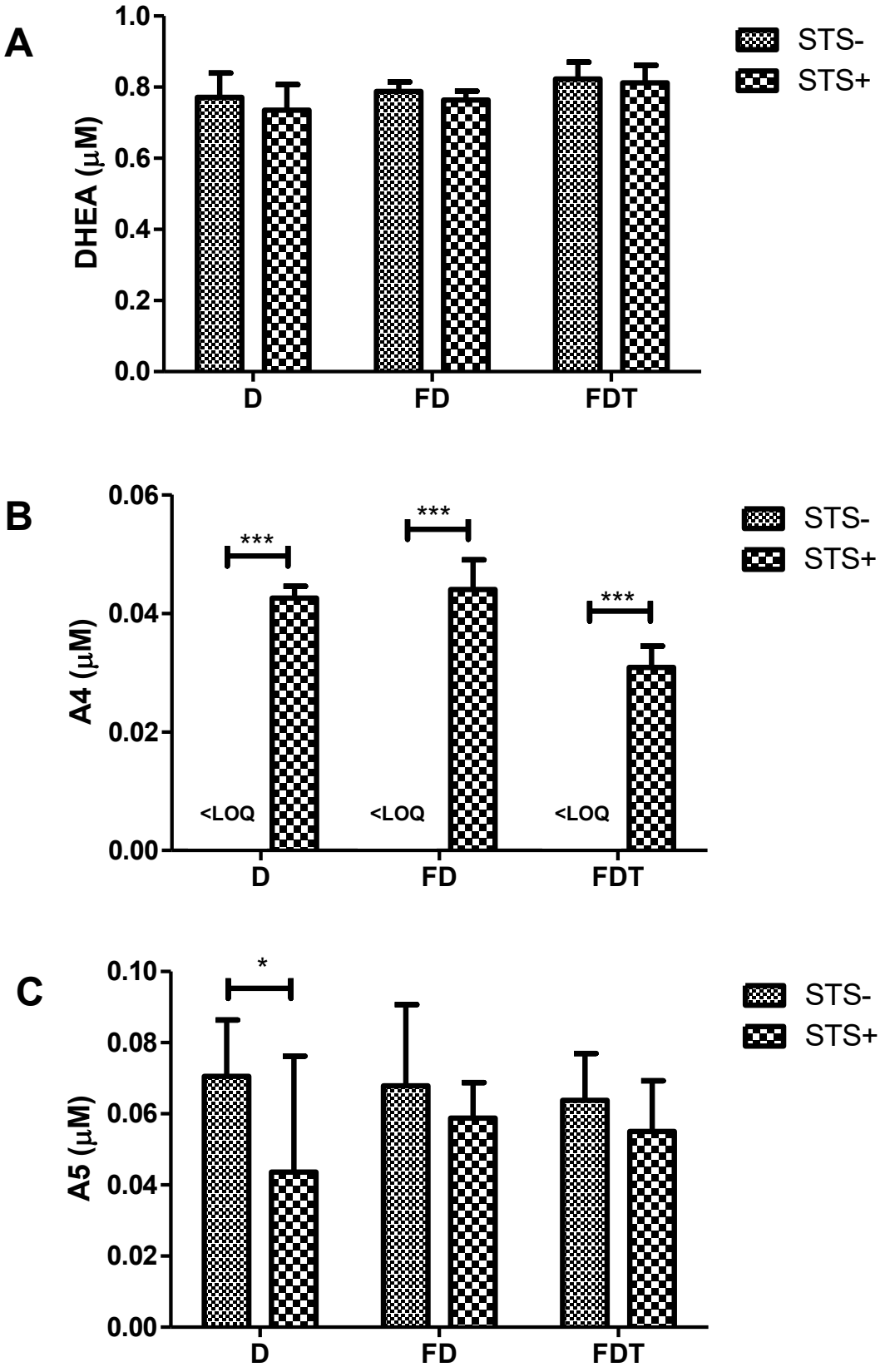

Figure 4-15: **Comparison between DHEA (A), A4 (B) and A5 (C) concentrations of samples from pHAs before and after treatment with STS in different treatment groups**. D – 1 µM DHEA; FD – 10 µM Forskolin & 1 µM DHEA; FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); **($p<0.0001$). LOQ – Limit of quantification

4.7.3. Effect of STS enzyme on concentrations of P5 and P4 in pRBMC samples

P5 concentrations remained relatively unchanged in both STS-treated and non-STS-treated samples in groups P, FP and FPT. Although a slight decrease was noted in group P and FP, albeit non-significant. Surprisingly a slight increase was noted in group FPT (Fig. 4-16A).

P4 was detected in STS-treated samples when compared to non-STS-treated samples in groups P, FP and FPT (Fig. 4-16B). However, P4 was likely present due to the exogenous 3β-HSD enzyme in the STS preparation.

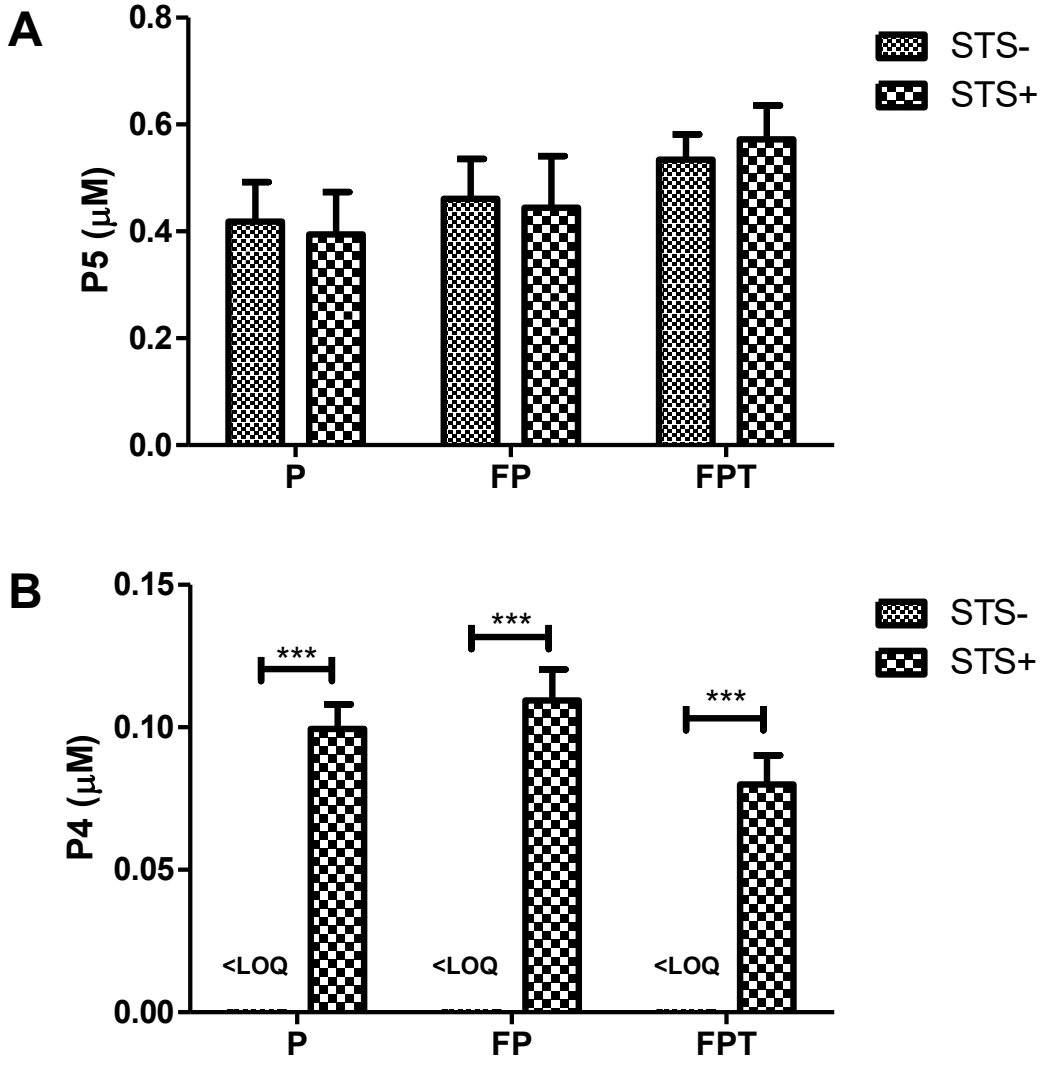

Figure 4-16: **Comparison between P4 (A) and P4 (B) concentrations of samples from pRBMCs before and after treatment with STS in different treatment groups**. P – 1 µM P5; FP – 10 µM Forskolin & 1 µM P5; FPT – 10 µM Forskolin, 1 µM P5 & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. ***(p<0.0001). LOQ – Limit of quantification

4.7.4. Effect of sulphatase enzyme on concentrations of DHEA, A4 and A5 in pRBMC samples

DHEA concentrations remained relatively unchanged between STS-treated and non-STS-treated samples in groups D and FDT – with non-significant decreases noted. However, a significant increase was observed in group FD (Fig. 4-17A).

A4 was detected in STS-treated compared to non-STS-treated samples in groups D, FD and FDT (Fig. 4-17B).

A5 concentrations were significantly decreased in STS-treated compared to non-STS-treated samples in group FD and FDT. A decrease was noted in STS-treated vs non-STS-treated samples in groups D, but this change was non-significant (Fig. 4-17C).

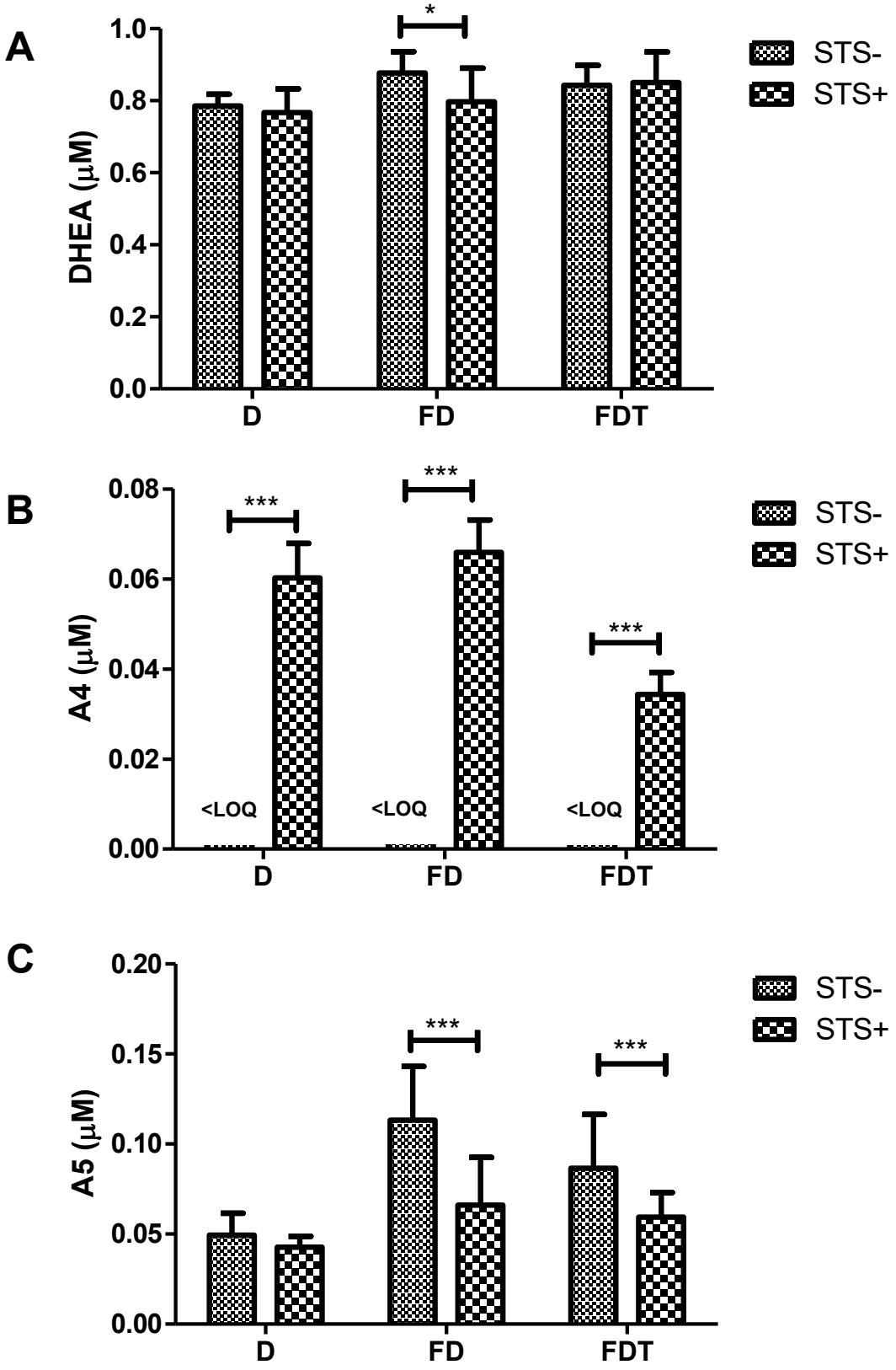

Figure 4-17: **Comparison between DHEA (A), A4 (B) and A5 (C) concentrations of samples from pRBMCs before and after treatment with STS in different treatment groups**. D – 1 µM DHEA; FD – 10 µM Forskolin & 1 µM DHEA; FDT – 10 µM Forskolin, 1 µM DHEA & 10 µM Trilostane. Data are presented as absolute steroid concentrations in µM (mean ± SD). N=3 independent experiments in triplicate. *($p<0.05$); ***($p<0.0001$). LOQ – Limit of quantification

Chapter 5

5. Discussion

5.1. Main findings

The topic of this thesis was to elucidate the pathways of DHEA-related neurosteroidogenesis in primary human astrocyte monocultures and primary rat brain mixed cell cultures, in order to allow an interpretation on the potential role that this neurosteroid may play in modern chronic disease.

We report as main findings that, despite using the high sensitivity and specificity of UPC^2-tandem mass spectrometry, we were unable to detect *de novo* DHEA biosynthesis in primary human astrocytes and rat brain mixed glial cultures. In our opinion, this result cannot be ascribed to limitations in our methodology.

Firstly, we included a condition of experimental upregulation of flux through the steroidogenic system. Forskolin treatment stimulates steroidogenesis by activating the adenylyl cyclase enzyme and raising intracellular cAMP levels (Purdy *et al.*, 1991). P450scc is then activated by cAMP-dependent protein complexes (Manna *et al.*, 2002) – thereby upregulating the rate-limiting step in the steroid pathway (conversion of cholesterol to P5). Interestingly, one study has demonstrated the ability of forskolin to selectively upregulate the androgen biosynthesis branch of the steroidogenic pathway in the steroid hormone producing H295R human adrenocorticocarcinoma cell line (Cobb *et al.*, 1996). However, the addition of forskolin could not bring about steroidogenesis resulting in detectable levels of DHEA either.

Secondly, substrate utilisation was not a limitation. The inability of *de novo* steroidogenesis by astrocytes, i.e. conversion of cholesterol into P5, is a shared consensus within the available literature (Brown *et al.*, 2000). At least two independent laboratories have demonstrated that oligodendrocytes appear to be the primary cell type involved P5 biosynthesis in the brain (Zwain and Yen, 1999a, 1999b; Brown *et al.*, 2000). This supports our methodology of providing P5 as substrate and not cholesterol.

Thirdly, to discount the possibility that DHEA levels were non-detectable due to its immediate metabolism, trilostane was used as a complimentary treatment to investigate whether DHEA was undergoing conversion into other androgenic metabolites in cells treated with both P5 and DHEA as initial substrate. Trilostane blocks 3β-HSD activity through competitive inhibition (Thomas *et al.*, 2008). 3β-HSD is responsible for conversion of P5 into P4, 17OHP5 into 17OHP4, DHEA into A4 and A5 into testosterone (T) (Figure5-1). As for other treatments, trilostane did not alter the outcome in terms of DHEA detection.

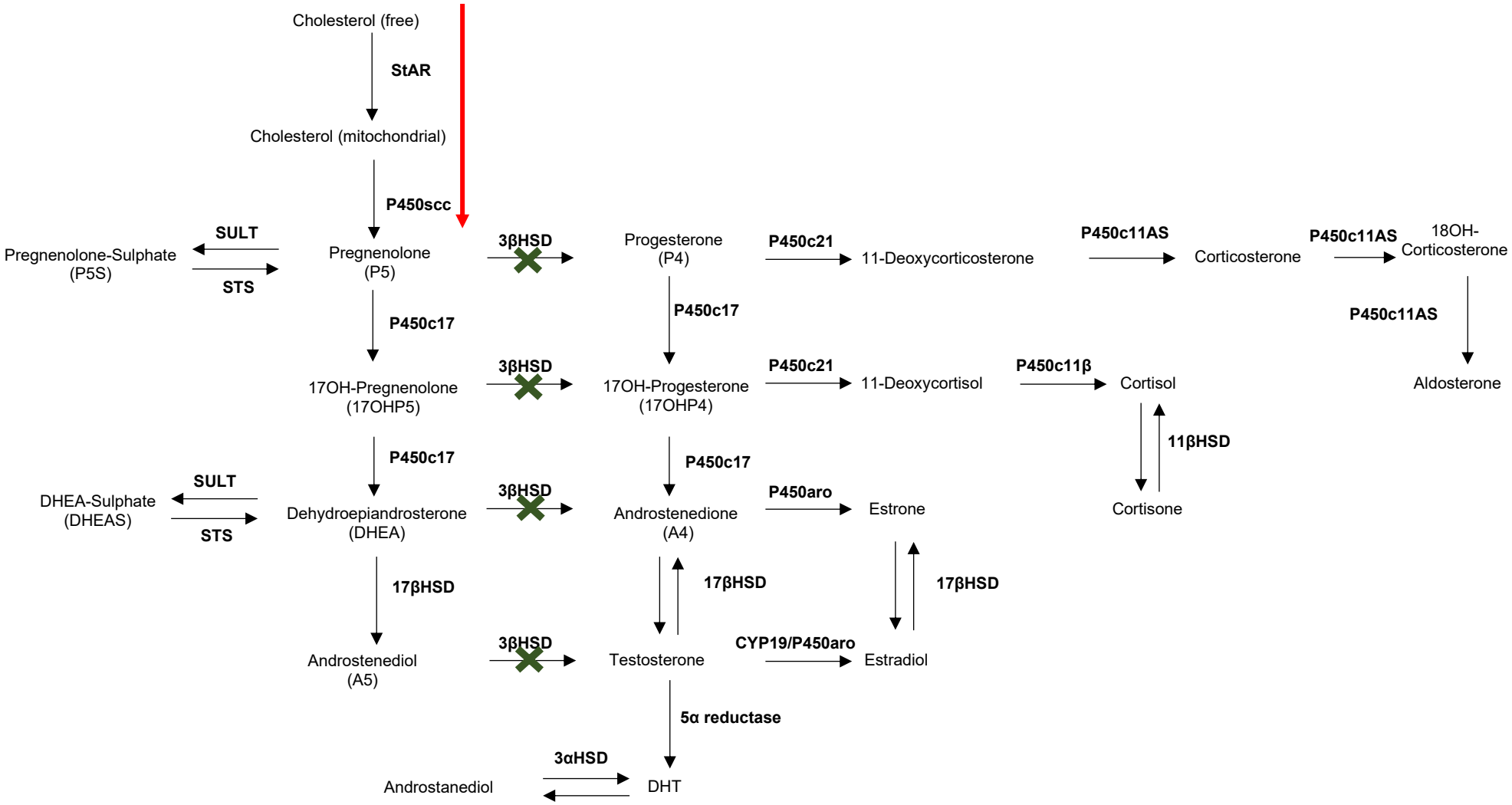

Figure 5-1: **The steroidogenesis pathway revisited**. The red arrow indicates the mechanism in which forskolin increases the biosynthesis of pregnenolone. The green crosses indicate the mechanism of how trilostane inhibits 3β-HSD activity

Finally, a particular challenge was the quantification of conjugated steroids, in particular DHEAS. As detailed in the materials and methods chapter, DHEAS and other conjugated steroids could be extracted in the same manner as their unconjugated counterparts because of the polar nature of their structures. A commercial sulphatase enzyme preparation (STS) isolated from *H. pomatia* was used to deconjugate potentially sulphated and glucoronidated steroids. Unfortunately, as our results have demonstrated, the STS preparation was not pure, as it exhibited exogenous 3β-HSD activity. This complicated the analysis and interpretation of data arising from the assay. Where relevant, analytes potentially affected by the tainted assay will be discussed with the cognisance of exogenous 3β-HSD activity in the assay. Interestingly, we are not the first to report on the artefact of 3β-HSD activity in *H. pomatia* STS preparations (Messeri *et al.*, 1984; Wolthers *et al.*, 1985; Christakoudi *et al.*, 2008; Hauser *et al.*, 2008). In our opinion, the presence of this artefact illustrates how even with highly advanced technologies such as modern chromatography and mass spectrometry, reagent quality may still limit the accurate quantification of conjugated steroids and our understanding of steroidogenesis in the brain. Nevertheless, despite the requirement to be aware of such limitations, we are confident that the method we employed was the best technique available for the purposes of the topic explored.

Taken together, given the sensitive equipment used and the sound methodology employed, and our knowledge and consideration of unavoidable limitations, we are confident in our interpretation that current data does not support a major role for either isolated human astrocytes or rat mixed glial cells to synthesise significant amounts of DHEA. However, when considering potential DHEA metabolism, we have formulated an interpretation in line with both our hypothesis and current data, as well as with data previously reported in the literature (discussed in detail in section 5.2).

However, of further interest, pHAs and pRBMCs were able to convert DHEA into androstenediol (A5), a function that was significantly enhanced by the presence of forskolin but unaffected by addition of trilostane. These data suggest 17β-HSD activity in both pHAs and pRBMCs.

In terms of methodological inclusivity, the analytes selected to quantify were chosen based upon the classical steroidogenic pathway in terms of androgen biosynthesis from pregnenolone (P5) as well as DHEA metabolism. In addition to these, hormone analytes and intermediates that exist in the non-classical or so-called “backdoor pathway”(Auchus, 2004; du Toit *et al.*, 2018) (Fig. 5-2) were also quantified specifically to distinguish between potential biosynthesis of DHT via the classical or backdoor pathways of steroidogenesis.

Given the observation that DHEA was converted into A5 in both human and animal brain cells, it is quite possible that the numerous beneficial effects attributed to DHEA (Kumar *et al.*, 2008; Yabuki *et al.*, 2015; Vieira-Marques *et al.*, 2016) are possibly not a result of this steroid itself, but rather it's metabolites (more on this later).

The layout of the discussion hereon forth will primarily focus on the steroid metabolites that was produced at detectable levels from DHEA, by pHAs and pRBMCs, respectively. Data pertaining to each metabolite will be discussed in context of available literature and the potential physiological relevance of its biosynthesis by glial cells and its presence in human and rodent brains within the context of this dissertation.

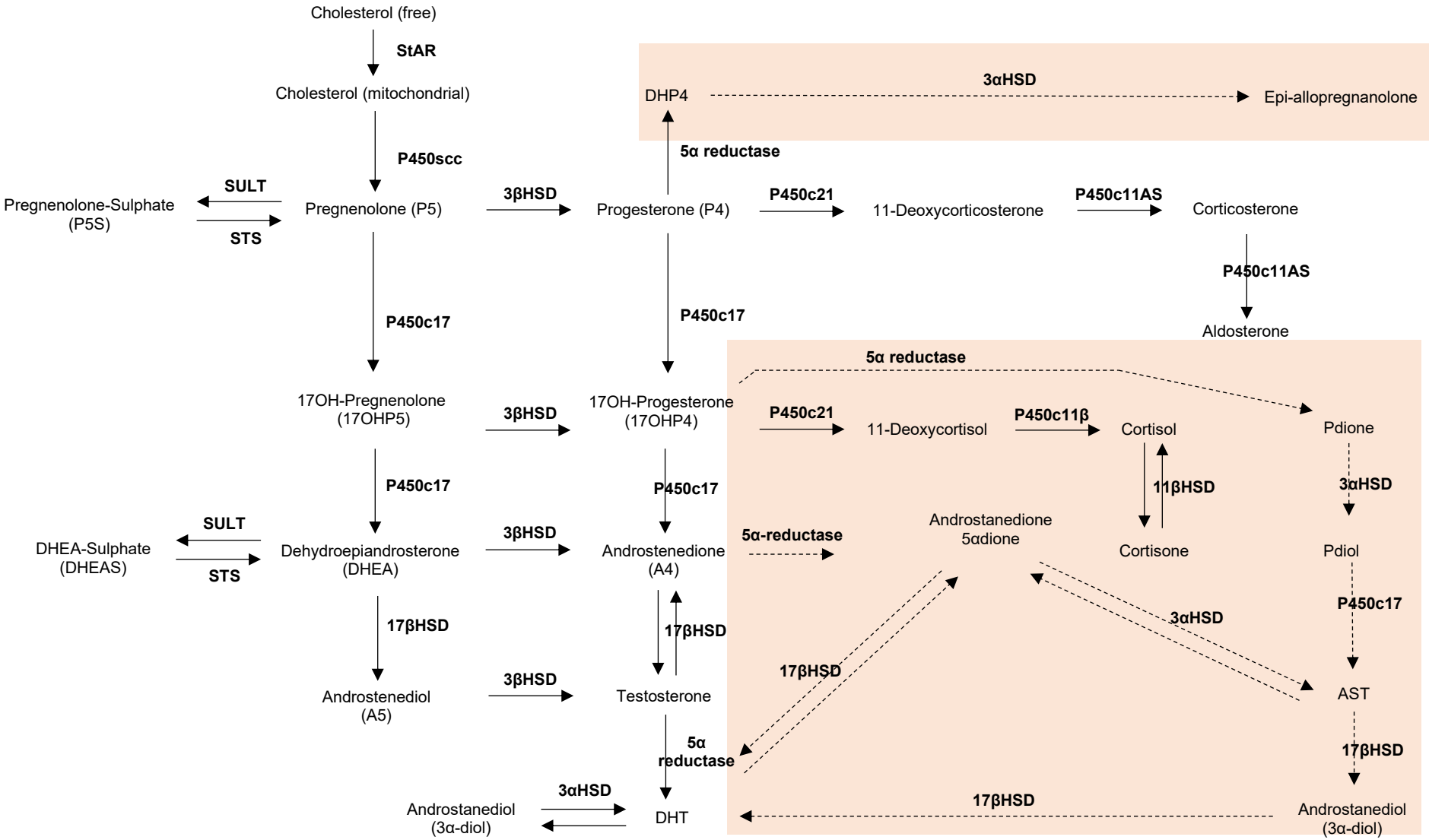

Figure 5-2:**The "hybrid" steroidogenesis pathway**. Indicated in solid lines is the classical androgen branch steroidogenesis. Highlighted in pink background with broken lines, is the alternative or "backdoor" pathway of steroidogenesis culminating in the biosynthesis of dihydrotestosterone (DHT)

5.2. **DHEA**

Since the discovery of so-called neurosteroids in rodent brains by Corpéchot and colleagues in 1981, the process of neurosteroidogenesis has remained somewhat of an enigma in the literature. Although many studies have demonstrated the ability of rodent brain cells or tissue to synthesise steroids when provided with the relevant substrate (Hu *et al.*, 1987; Jung-Testas *et al.*, 1989a, 1989c; Akwa *et al.*, 1991; Zwain *et al.*, 1997; Zwain and Yen, 1999b, 1999a; Brown *et al.*, 2000; Cascio *et al.*, 2000; Gago *et al.*, 2001; Kimoto *et al.*, 2001; Kawato *et al.*, 2002; Shibuya *et al.*, 2003; Hojo *et al.*, 2004; Mukai *et al.*, 2006), the data on DHEA biosynthesis does not appear as comprehensively characterised.

The first study to ever mention DHEA in the rodent brain was in 1972, when it was observed that intracardially administered DHEA and DHEAS accumulated in trace amounts in rat brains (Kishimoto and Hoshi, 1972). As previously mentioned in the literature review, central DHEA biosynthesis was further implied due to the maintained presence of DHEAS in the brains of adult male rats who had undergone adrenalectomies and gonadectomies approximately two weeks prior to measurement (Corpéchot *et al.*, 1981). In this study, DHEAS levels were also significantly higher than that of DHEA in the brain, as well as significantly higher than DHEAS levels in circulation (Corpéchot *et al.*, 1981).

Although many independent laboratories have since reported similar findings illustrating the presence of DHEA and DHEAS in both the human and rodent brain (Corpéchot *et al.*, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Lacroix *et al.*, 1987; Robel *et al.*, 1987; Griffiths *et al.*, 1999; Higashi *et al.*, 2001; Liu *et al.*, 2003; Liere *et al.*, 2004), very few have been able to actually demonstrate biosynthesis in the brain itself or by brain cells.

To our knowledge, the first study demonstrating central DHEA biosynthesis was performed in isolated cells from rat neonatal cerebral cortices (Zwain and Yen, 1999a, 1999b). Utilising reverse transcription polymerase chain reaction (RT-PCR) and radioimmunoassay (RIA) techniques, the authors reported that isolated neonate cortical neurons and astrocytes expressed P450c17 mRNA and were able to synthesise DHEA from P5. Cortical astrocytes were reported to produce DHEA to a larger extent when compared to neurons, while hypothalamic astrocytes was reported to produce approximately 3 times more DHEA compared to cortical astrocytes (Zwain and Yen, 1999a, 1999b). These data were generated using RIA assays.

Another study performed in isolated rat neonatal astrocytes and oligodendrocytes suggested that these glial cells are able to synthesise DHEA in a P450c17-independent pathway (Cascio *et al.*, 2000). In this study, the isolated oligodendrocytes were positive for P450c17 mRNA and

were able to synthesise DHEA from P5. The addition of Fe^{2+} to treatment media increased the biosynthesis of DHEA and this enhanced effect was not affected by the P450c17 inhibitor SU 10603. The isolated astrocytes did not express mRNA or contain P450c17 protein at all, but similarly were able to synthesise DHEA *de novo* when treated with Fe^{2+}. The addition of SU10603 again had no effect on DHEA biosynthesis (Cascio *et al.*, 2000).

There has been only one other study investigating DHEA biosynthesis in primary human astrocytes and/or human brain cells – it may be of interest to note that this study was conducted by the same group as the previously mentioned study. In this study the authors assessed their hypothesised alternative Fe^{2+} dependent pathway in commercial normal human astrocytes (NHA) and human glioma cell lines (KG-1-C, MGM-1 and MGM-3). In addition to these cell lines, also included in the experiment, were NT2 cells, a human teratocarcinoma cell line, and hNT-PF cells, which are NT2 transdifferentiated neuron like cells with retinoic acid (Brown *et al.*, 2000). NHA, KG-1-C, MGM-1, MGM-3, NT2 and hNT-PF all expressed P450c17 mRNA. KG-1-C and MGM-3 cells were able to synthesise P5 *de novo* and synthesise DHEA which was again unaffected by SU10603 (i.e. P450c17 inhibition). All cell lines were able to synthesise DHEA *de novo*, but when cells were exposed to $FeSO_4$, biosynthesis was only enhanced in MGM-1, MGM-3 and NHA cells. The authors theorised that the P450c17 independent mechanism was due to oxidative stress and continued on to show that experimentally increased ROS increased DHEA production in MGM-1 cells – effect ameliorated in the presence of the antioxidant Vitamin E. (Brown *et al.*, 2003). To date, this laboratory and respective collaborators are the first and only that have demonstrated evidence for an P450c17-indpendent pathway for DHEA biosynthesis in the brain. Taken together, these studies seem to present a convincing body of evidence for the presence of DHEA at significant levels in the brain. However, in our opinion, this is highly unlikely for a number of reasons. Firstly, it is commonly known that the majority of DHEA in the body occurs in its sulphated form, DHEAS. Similarly, DHEA may be easily metabolised into a number of metabolites quite similar in structure to DHEA. In addition, the RIA technology employed in these fairly dated studies, lacked the specificity that modern UPC^2-tandem mass spectrometry has. These facts suggest that most likely, the analytes detected and reported as DHEA in these studies, may have been analytes other than DHEA, but sufficiently similar in structure to DHEA, resulting in falsely elevated results for DHEA. Likely examples include 7α-hydroxy- and 7β-hydroxy-DHEA, which will be discussed in detail later. These limitations preclude a firm interpretation on actual steroidogenic events in the brain and highlights a requirement for this to be revisited using modern techniques. This thesis indeed adds to this gap in the literature by confirming that DHEA *per se* is not present in either isolated human or mixed rodent glial cells at significant concentrations.

The possibility that we may not have detected DHEA because it was immediately metabolised into an analyte not listed in our method is a possibility that should be considered. Indeed, in our opinion, given the fact that *in vivo*, DHEA is readily converted into its closely related intermediates in the steroidogenic pathway such as A5, it is likely that if DHEA was produced, it was metabolised into other intermediates which was not specifically included in our assessment panel. A likely possibility is that newly synthesised DHEA may have been metabolised to its hydroxy metabolites 7α-hydroxy-DHEA (7α-DHEA) and 7β-hydroxy-DHEA (7β-DHEA). These hydroxy analytes are synthesised when DHEA is converted by the enzyme CYP7B1 into 7α-DHEA (Rose *et al.*, 1997). 7α-DHEA is a ligand for 11β-HSD1, which interconverts it into 7β-DHEA - the same enzyme responsible for the conversion of cortisol to cortisone. The anti-glucocorticoid activity of DHEA has actually been proposed to be due to 7α-DHEA and 7β-DHEA metabolites (Hennebert *et al.*, 2007). One study has suggested that the accumulation of DHEA in the brains of AD patients may be due to the loss in ability to convert DHEA into its hydroxy metabolites (Kim *et al.*, 2003).

Unfortunately, at the time of the current study, specific reference standards for 7α-DHEA and 7β-DHEA could not be sourced. Thus, given the current data showing non-detectable DHEA levels – in contrast with the earlier literature – it is recommended that this possibility be revisited as soon as appropriate reference standards are available.

Another limitation in terms of reagents - the exogenous 3β-HSD activity in the STS *H. pomatia* preparation - precludes a firm interpretation on whether or not DHEA may have been produced and sulphated to DHEAS by pHA and pRBMCs. However, several indirect results may suffice in the argument that it is unlikely DHEA was sulphated by these cells. For example, in the current study, cells treated with P5 as substrate, DHEA was undetectable, but no androstenedione (A4) (the conversion of which would have been facilitated by STS, from DHEA) could be detected in the STS treated samples either. This suggests that no DHEA was sulphated under the conditions assessed. In addition, SULT – which catalyses the sulphating of DHEA and P5 into DHEAS and P5S respectively – has demonstrated low activity in the rodent brain (Rajkowski *et al.*, 1997) and its mRNA is expressed in very low to non-detectable levels in human brain (Steckelbroeck *et al.*, 2004). In fact, the human brain expresses high levels of STS mRNA – the translation of which would result in enzyme deconjugation of DHEAS and P5S (Steckelbroeck *et al.*, 2004). Furthermore, studies investigating the effect of systemically administered DHEA on brain DHEAS concentrations in rats demonstrated no significant increase in central concentrations of DHEAS (Kishimoto and Hoshi, 1972; Young *et al.*, 1991). These data, albeit dated, suggest that DHEAS concentrations centrally are independent of endogenous SULT activity. Current data corroborates this interpretation.

Taking this into account, the systemically independent central biosynthesis of DHEAS does not preclude it from existing mainly in a precursor capacity.

Lastly, despite the aberration in the sulphatase assay, it is the opinion of the authors that pHAs and pRBMCs are likely not capable of synthesising DHEA in its unmetabolized form, even in the presence of added P5. Although trilostane was used to prevent the conversion of DHEA to A4 and STS used to deconjugate potentially synthesised DHEAS, these are not the only metabolites that DHEA may be converted into, of which the hydroxylated epimers are an example.

In the sections to follow it will become evident that these cell populations/types are anything but devoid of steroidogenic capacity.

5.3. **Androstenediol (A5)**

Both pHAs and pRBMCs demonstrated the ability to convert DHEA into androstenediol (A5), which is in line with the reports of 17β-HSD mRNA (Martel *et al.*, 1992; Normand *et al.*, 1995; Pelletier *et al.*, 1995; Beyenburg *et al.*, 2000; Steckelbroeck *et al.*, 2001, 2003) in both human and murine astrocytes and microglia, as well as 17β-HSD protein (Pelletier *et al.*, 1995) and activity (Zwain and Yen, 1999b) in murine astrocytes. To date, there has only been two reports of A5 presence in rodent brains (Tagawa *et al.*, 2006; Qaiser *et al.*, 2017). The first study assessed and compared the differences of neurosteroid concentration in different strains of mice, and reported A5 in all strains (Tagawa *et al.*, 2006). The strains assessed included DBA/2, ICR, BALB/c, ddY and C57BL/6 mice. Steroid concentrations in all strains were compared to DBA/2 mice as the control and A5 concentrations were significantly lower in BALB/c and ddY mice as a result. The second study, as mentioned already in section 5.3, only demonstrated the ability of rat brains to convert DHEAS into A5 once it crosses the BBB (Qaiser *et al.*, 2017). Murine DHEA conversion to A5 seem to be largely ascribed to microglial cells (Jellinck *et al.*, 2007; Gottfried-Blackmore *et al.*, 2008), but current data suggest a significant role for astrocytes as well. Furthermore, no reports exist on A5 presence in human brains, although the capacity to synthesise A5 and A5 sulphate from DHEA and DHEAS has been demonstrated in human brain tissue homogenates (Kishimoto and Hoshi, 1972; Kishimoto, 1973; Weill-Engerer *et al.*, 2003). Further investigation is required to confirm whether human microglial cells may also contribute to A5 levels in the brain.

The potential for A5 biosynthesis to occur in both astrocytes and microglia in pRBMC culture is a possibility. However, PBCRs only produced significantly more A5 in samples that were treated with DHEA, forskolin and trilostane. pHAs produced significantly more A5 compared to PBCRs when treated with DHEA alone. The fact that A5 concentrations were not higher in

pRBMCs compared to pHAs in all experimental conditions suggests that astrocytes may contribute to A5 biosynthesis to a larger extent when compared to microglia. Interestingly, A5 concentrations were significantly increased with the addition of forskolin relative to DHEA alone in both pHA and pRBMC cultures. Similarly, this has been reported that forskolin also increases 17β-HSD activity in H295R cells (Bird *et al.*, 1996).

Interestingly, systemic administration of LPS in male rats also causes a significant increase in 17β-HSD mRNA expression in brains relative to control, but interestingly, a relative decrease in the testis (Sadasivam *et al.*, 2014). Similarly, LPS treatment of primary mouse microglia also caused a significant increase in 17β-HSD mRNA expression (Gottfried-Blackmore *et al.*, 2008). However, in the latter study the LPS had no significant effect on the conversion of DHEA into A5. The lack of the change in A5 concentrations after LPS treatment could not be attributed to increased conversion of A5 into testosterone, as these concentrations also remained unchanged. Levels of estradiol and estrone, downstream metabolites were not measured, but the isolated primary microglia did not express detectable levels of P450aro mRNA – which encodes for the enzyme responsible for the conversion of T into estradiol (Gottfried-Blackmore *et al.*, 2008). Therefore, the lack in the change of A5 concentrations, despite an increase in 17β-HSD cannot be attributed to increased flux through the pathway.

The data demonstrating the effects that LPS may have on 17β-HSD mRNA expression, further suggest that A5 has a role in limiting neuroinflammation. Indeed, A5 treatment of LPS treated BV2 murine microglia caused a significant reduction in IL-6 mRNA expression relative to control. Similarly, systemic administration of A5 in LPS treated mice caused a significant reduction in IL-6 mRNA expression in the substantia nigra, relative to control and improved clinical outcome measures in a mouse model of experimental autoimmune encephalomyelitis (EAS) (known to mimic multiple sclerosis pathology)(Saijo *et al.*, 2011). Interestingly, this beneficial effect of A5 was abrogated in ERβ knockout mice in this study – suggesting a role for this receptor in A5-mediated anti-inflammatory action. Furthermore, although ERβ had a higher affinity for 17β-estradiol, its commonly excepted ligand, the anti-inflammatory effect was unique to A5 as opposed to 17β-estradiol (Saijo *et al.*, 2011).

Others have also reported A5 to be neuroprotective (Hanna *et al.*, 2015), myelin promoting (Kalakh and Mouihate, 2015, 2017) as well as possessing anti-inflammatory properties (Ben-Nathan *et al.*, 1999; Auci *et al.*, 2005; Suzuki *et al.*, 2006; Szalay *et al.*, 2006; Nicoletti *et al.*, 2010).

5.4. Summary of findings

This study has demonstrated that primary human astrocytes (pHAs) and primary rat brain mixed cell cultures (pRBMC) do not possess the ability to synthesise DHEA when provided with pregnenolone (P5) as a substrate (Fig. 6-1). These findings conflict with previous reports (Zwain and Yen, 1999a, 1999b).

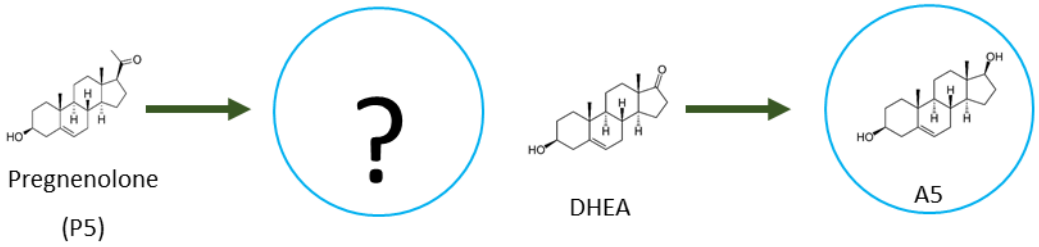

Figure 5-3: **Summary of metabolism of pregnenolone (P5) and dehydroepiandrosterone (DHEA) by primary human astrocytes and primary rat bran mixed cell cultures**.

pHAs and pRBMCs were able to synthesise P4 from P5 as a substrate. Although P4 was detected in both pHA and pRBMC cultures treated with P5, it does not account for the significant loss of P5 after treatment in both cultures. This suggests metabolism of P5 into a steroid(s) that were not in our list of analytes (Fig. 6-1). The P5 may have been metabolised down the mineralocorticoid branch of steroidogenesis, but it is also possible that DHEA was hydroxylated to form 7α- and 7β-hydroxy-DHEA (Rose *et al.*, 1997; Jellinck *et al.*, 2001, 2007; Weill-Engerer *et al.*, 2003). At the time of these experiments, we did not possess the standards for the hydroxylated metabolites of DHEA, but it is certainly something that needs to be investigated in subsequent experiments.

Primary human astrocytes and primary rat mixed glial cells were also able to synthesise androstenediol (A5) from DHEA as a substrate. To the best of the authors knowledge, this is the first study to report the direct biosynthesis of A5 by primary human astrocytes from DHEA as a substrate.

Although we did not measure 17β-HSD enzyme expression, the presence of this enzyme and its activity was clear due to the products of its activity being detected. Additionally, the rationale to measure the expression of other enzymes, such as P450c17, was also decided against since no products could be detected.

Lastly, the lack of detection of some steroid analytes in the method also suggests that pHAs and pRBMCs are not able to biosynthesise DHT through the classical or backdoor steroid pathway. This too is a novel finding.

Chapter 6

6. Conclusion

The published studies supposedly demonstrating the first evidence of DHEA biosynthesis in astrocytes utilised RIA methods, which at the time were probably the best techniques for measuring steroid analytes. Indeed, even presently, immunoassay based techniques are still utilised in clinical environments because of its speed, practicality and cost (Fanelli *et al.*, 2011). However, decades have passed since the publication of these seminal studies and better technologies such as UPC^2-tandem mass spectrometry have emerged. Given the complexity of steroid structures and the multitude of intermediary metabolites that exist in the steroid pathway, it is possible that antibodies used in immunoassays cross-react with other steroids, especially at low concentrations (Fanelli *et al.*, 2011; Krasowski *et al.*, 2014; Stokes *et al.*, 2014), such as is expected at sites of intracrine steroidogenesis.

The demonstrated metabolism of exogenous DHEA provided as substrate suggests a small chance that DHEA potentially produced from P5 may also have been metabolised into other analytes not included in our analysis panel and was therefore not detected in groups treated with P5. However, the fact that more A5 was detectable only in groups that were provided exogenous DHEA, argues against this.

It is therefore our opinion that DHEA is likely not synthesised by astrocytes. Nevertheless, given the importance of astrocyte function in the context of neuroinflammation, as well as the beneficial effects attributed to both DHEA and its metabolites, it is clear that these hormones have a significant role in the limitation of neuroinflammation.

Since current data argues against central biosynthesis of DHEA, the demonstrated significant levels of DHEA present in the brain, can only come from the periphery. Assuming that this is the case, we interpret the ”central DHEA picture” as follows: Firstly, although data suggest that DHEA sulphation is independent of the periphery (Young *et al.*, 1991), a more recent publication reports that DHEAS does not readily cross the BBB (Qaiser *et al.*, 2017) and that when it does, it is desulphated in the process (Qaiser *et al.*, 2017). Systemic administration of DHEA in rats increases central DHEA concentrations, but central DHEAS concentrations remain unchanged (Young *et al.*, 1991). Furthermore, Corpéchot and colleagues showed that DHEA and DHEAS levels in rodent brains remained unchanged after gonadectomies and adrenalectomies (Corpéchot *et al.*, 1981). Similarly, systemic steroidogenic modulators had no effect. However, DHEAS levels still remained higher than those of DHEA and the levels of both remained unchanged after 15 days – this suggested central biosynthesis to the authors. However, we suggest that DHEAS may exists in the brain as a long-term depot for DHEA metabolism. We theorise that DHEA, but not DHEAS, moves freely across the BBB, is

sulphated once inside the brain to keep it there and that it is only desulphated at the appropriate time (i.e. neuroinflammation and/or damage), in order to allow it to be metabolised into anti-inflammatory metabolites.

Secondly, although systemic levels of DHEA decline with age and neurodegeneration, levels in the central compartment are reported to be abnormally elevated in pathologies such as Alzheimer's disease (AD) (Marx *et al.*, 2006) and multiple sclerosis (MS) (Orefice *et al.*, 2016). Generally, the increased levels would be ascribed to increased biosynthesis, but decreased metabolism of DHEA also needs to be considered, as such a decrease in the production of neuroprotective anti-inflammatory metabolites such as A4 and A5, could indeed increase the risk for neurological pathology. Indeed, others have also come to this conclusion (Yau *et al.*, 2003).

Thirdly, our theory of a central DHEA depot also holds true in context of the disproportionate development of neurodegenerative pathologies that affect women more than men, such as AD (Mielke *et al.*, 2014) and MS (Koch-Henriksen and Sørensen, 2010; Kalincik *et al.*, 2013). Serum DHEA and DHEAS levels generally decrease with age in both men and women. After menopause, however, adrenal steroidogenesis becomes the main source of circulating androgens in women. When ovarian and adrenal steroid biosynthesis declines significantly or ceases, due to menopause and age respectively, circulating DHEAS/DHEA levels decline significantly as well. Provided with our theory of the importance of the DHEA metabolites in the brain, if no DHEA from the periphery exists as a precursor this would clearly provide the basis of an explanation for the importance of this hormone in regulating neuroinflammatory conditions. This is also an explanation as to why post-menopausal women are more likely to develop these pathologies compared to men.

Taken together, it remains clear that DHEA has a significant role in the maintenance of homeostasis in the brain and although we cannot provide evidence for its central biosynthesis, we have demonstrated evidence for its metabolism. It is also clear that DHEA metabolism in the brain is significantly different from the periphery.

Finally, in terms of clinical application of this knowledge, although systemic administration of DHEA raises central concentrations of DHEA in mice (Young *et al.*, 1991), it is probably not a viable treatment in humans due to its strong androgenic properties and numerous side effects, such as hirsutism and acne in women (Petri *et al.*, 2004). However, since metabolites downstream of DHEA in the steroidogenic pathway also exert these beneficial effects, should we not attempt to exploit them instead? Further studies should thus move forward by comprehensively characterising any other steroid analytes that may be synthesised from DHEA metabolism, such as 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. The therapeutic benefit

of these metabolites needs to be quantified by ascertaining whether the therapeutic usage may outweigh potential negative systemic side effects and whether these metabolites can cross the BBB after systemic administration.

To test our DHEAS depot hypothesis we propose using a model similar to one utilised by Corpéchot and colleagues (1981). However, after gonadectomies and adrenalectomies, we propose simulating a neuroinflammatory environment through systemic administration of a high dose of LPS. We would then investigate potential changes in central levels of DHEA and DHEAS, as well as metabolite A5, in response to the LPS stimulus. If our theory proves correct, the DHEA and DHEAS concentrations should significantly decline, but won't return to baseline and A5 and other DHEA metabolite concentrations should increase.

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