Universidade da Beira Interior – Centro de Investigação em Ciências da Saúde

Androgen receptor is expressed in murine choroid plexuses and downregulated by 5α-dihydrotestosterone in male and female mice

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Androgen receptor is expressed in murine choroid plexuses and downregulated by 5α-dihydrotestosterone in male and female mice.

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Abbreviations Index

- **Aβ** - β-amyloid peptide
- **AD** - Alzheimer's disease
- **AF1** - Activation function 1
- **AF2** - Activation function 2
- **AGE** - Advanced glycation products
- **APP** - Aβ precursor protein
- **AR** - Androgen receptor
- **BDNF** - Brain derived nerve factor
- **b2mgb** - β-2 microglobulin
- **CNS** - Central nervous system
- **CPs** - Choroid plexuses
- **CSF** - Cerebrospinal fluid
- **DBD** - DNA-binding domain
- **DHEA** - Dehydroepiandrosterone
- **DHT** - 5α-dihydrotestosterone
- **ELISA -** Enzyme-linked immunoadsorbent assay
- **ERβ** - Estrogen receptor β
- **ERK** - Extracellular signal regulated kinase
- **ERT** - Estrogen replacement therapy
- **GDNF** - Glial cell line-derived neurotrophic factor
- **GDX** - Gonadectomy
- **Hsp** - Heat shock protein
- **ISH** - *In situ* hybridization
- **IHC** - Immunohistochemistry
- **LBD** - Ligand binding domain
- **MAPK** - Mitogen activated protein kinase
- **MCI** - Mild cognitive impairment
- **NGF** - Nerve growth factor
- **NFT** - Neurofibrillary tangles
- **NLS** - Nuclear localization signal
- **NTD** - N-terminal domain
- **PBS** - Phosphate buffered saline
- **PCR** - Polymerase chain reaction
- **PFA** - Paraformaldehyde
- **PNS** - Peripherical nervous system
- **PR** - Progesterone receptor
- **PREG** - Pregnenolone
- **PROG** - Progesterone
- **PVDF** - Polyvinylidene diflouride
- **P450c11β** - P450scc and 11 β -hydroxylase
- **sAPPα** - Soluble APP-α
- **SARMS** - Selective androgen receptor modulators
- **SP** - Senile plaques
- **SSC** - Saline sodium citrate
- **TBS** - Tris buffered saline

- **TGF-β** - Transforming growth factor β
- **TTR** - Transthyretin
- **17β-HSD** - 17β-hydroxysteroid dehydrogenase
- **3β-HSD** - 3β-hydroxysteroid dehydrogenase-isomerase

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I. Resumo

A doença de Alzheimer (AD) é um dos maiores problemas de saúde pública, nos países desenvolvidos. É caracterizada por uma deteorização progressiva da memória e das funções corticais superiores, culminando numa degradação total das actividades intelectuais e mentais. As características neuropatológicas da doença são: a deposição extracelular de placas de amilóide, constituídas por fibrilhas do péptido amilóide β (Aβ), a formação de agregados neurofibrilares, e a perda de neurónios. Estudos recentes comprovam que alterações morfológicas e funcionais nos plexos coróideu (CPs) estão associadas à AD. Os CPs formam uma barreira única entre o sangue periférico e o líquido cefalorraquidiano (CSF). Vários péptidos envolvidos no processo de neuroprotecção, tais como, o factor de crescimento neural (NGF), o factor β de crescimento e transformação (TGF-β), o factor neural derivado do cérebro (BDNF), a transtirretina (TTR), e o factor neurotrófico derivado de uma linha de células da glia (GDNF), são secretados pelos CPs, e regulados pelos androgénios noutros tecidos, porém os mecanismos de regulação destes, nos CPs, continuam por elucidar. Importa salientar que os androgénios podem actuar a nível cognitivo, e como neuroprotectores. Sabe-se que, a testosterona tem um papel inibidor sobre o stress oxidativo, a apoptose, e a toxicidade da Aβ, sendo todos estes efeitos mediados pelo receptor de androgénios (AR). O AR foi identificado em várias zonas do sistema nervoso central (CNS): no hipotálamo, na amígdala, no hipocampo e no córtex, porém a sua presença nunca foi descrita no CP.

O AR nunca foi identificado no CP do cérebro, nem a hipótese deste ser um órgão responsivo aos androgénios foi averiguada. Para esclarecer estas questões, investigamos e caracterizamos a distribuição e expressão do AR nos CPs de ratos, machos e fêmeas, e analisamos a expressão do receptor em resposta ao tratamento com 5 α-dihidrotestosterona (DHT), em ratinhos castrados, machos e fêmeas.

Os nossos resultados mostram que o AR é expresso nas células epiteliais dos CPs de rato, e parece ser mais abundante nas fêmeas do que nos machos. Além disso, demonstrámos que o receptor é regulado negativamente pela DHT, sendo este efeito mais proeminente nas fêmeas.

Palavras chave: Doença de Alzheimer, 5α-dihidrotestosterona, receptor de andogénios, plexos coróideu.

I. Abstract

Alzheimer's disease (AD) is one of the major health problems in the economically developed countries. It is characterised by the progressive deterioration of memory and higher cortical functions, that ultimately result in total degradation of intellectual and mental activities. The key neuropathological characteristics of AD are: senile plaques (SP), which are associated with β-amyloid peptide (Aβ), neurofibrillary tangles (NFT), and the loss of neurons.

Several studies showed that morphological and functional alterations in the choroid plexuses (CPs) are related to AD. The CPs of the brain form a unique interface between the peripheral blood and the cerebrospinal fluid (CSF). Peptides involved in neuroprotection, like nerve growth factor (NGF), transforming growth factor β (TGF-β), brain derived nerve factor (BDNF), transthyretin (TTR), and glial cell line-derived neurotrophic factor (GDNF), are secreted by CPs, and are regulated by androgens in other tissues, but the mechanisms underlying the regulation of these peptides in CPs remain unknown. Moreover, there are several experimental evidences showing that androgens enhance cognition and act as potential protective factors against degenerative diseases. It has been shown that testosterone exerts neuroprotective actions against oxidative stress, apoptosis, and against the toxicity of Aβ, all via androgen receptor (AR). AR has been identified in several regions of the central nervous system (CNS): hypothalamus, amygdala, hippocampus and the cortex, but not in the CPs.

The presence of AR in CPs has never been investigated; neither the CPs have been considered a potential androgen responsive tissue. In order to fulfil this gap, we investigated and characterised AR distribution and expression in male and female rat CPs, and analysed its response to 5α-dihydrotestosterone (DHT), in castrated male and female mice, subjected to DHT replacement. We show that AR is expressed in rat CPs epithelial cells, and seems to be more abundant in female CPs than in males'. Moreover, we demonstrate that AR is down-regulated by DHT in mice CPs, an effect more prominent in females than in males.

Key words: Alzheimer's disease, 5α-dihydrotestosterone, androgen receptor, choroid plexus.

II. Introduction

1) Alzheimer's Disease

AD is one of the major health problems in the economically developed countries along with cardiovascular disorders and cancer (Bachurin, 2003). It is characterised by progressive deterioration of memory and higher cortical functions, that ultimately result in total degradation of intellectual and mental activities (Cocabelos, 1994; Ebly et al., 1994). AD is the most common form of dementia (Ebly et al., 1994). It affects up to 10% of people over the age of 65 and 30 to 35% or more of those over 85 years (Sykes et al., 2001). Currently, AD affects approximately 20 million people all over the world, and imposes an annual economic burden of about US $ 100 billion (Cocabelos, 1994).

The key neuropathological characteristics of AD are: SP, which are associated with Aβ, NFT, and the loss of neurons in the hippocampus and nucleus basalis of Meynart (Morrison et al., 1998; Selkoe, 1991). Alterations in the morphology and in the functions of CPs have been linked to this disease. In the next chapter we explain the structure and function of this organ. Moreover, we also made a literature revision about ageing of CPs, and the cross-talk, between the CPs and AD.

2) Choroid Plexus

2.1) Structure

The CPs are located throughout the ventricles of the brain. Within the lateral ventricles, they projects from the choroidal fissure and extends from the interventricular foramen to the end of the temporal horn, and projects into the third and fourth ventricles from the ventricular roof (Emerich et al., 2005). Choroid tissues are composed of villi covered by an unistratified epithelium with a central vascular axis. Epithelial cells are cuboid with a rounded central or basal nucleus (Dohrmann, 1970). At the apical pole numerous microvilli of uniform diameter, enmeshed with each other, and a few cilia (Serot et al., 2003). Mitochondria are more numerous at the basal and apical poles, occupying 15 % of the cytoplasm in primates (Cornford et al., 1997). The Golgi apparatus contains columns of cisternae and smooth endoplasmic reticulum and clear vesicles are distributed throughout the apical cytoplasm (Emerich et al., 2005). The epithelial cells lie on an epithelial basement membrane surrounding a thin stroma with

numerous collagen fibbers, scarce dendritic cells, macrophages, fibroblasts and large capillaries with a fenestrated endothelium (Dohrmann, 1970). The basolateral membrane contains numerous interdigitations (Serot et al., 2003). CPs are richly innervated, receiving adrenergic, cholinergic, peptidergic and serotoninergic fibers. The distribution of nervous fibers varies widely according to species (Nilsson et al., 1990). Figure 1 represents the structure of CPs.

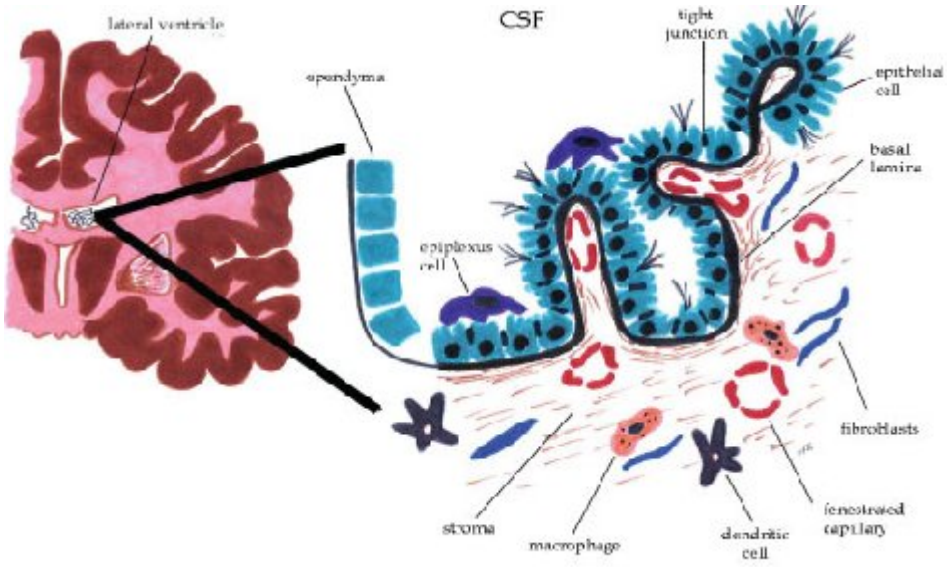

Figure 1: Morphological illustration of CPs within the lateral ventricle. The CPs extends from the ependymal cell layer of the ventricular wall forming a continuous strand of cuboidal epithelial cells resting upon a basal lamina and inner core of connective and highly vascularized tissue. The apical membrane of the epithelial cells faces the CSF where the cells contain numerous infoldings and scattered villi. Adjacent epithelial cells are bound together forming tight junctions and globular macrophages, dendritic cells, and fibroblasts are found throughout the stroma. Dendritic cells are also found in between the epithelial cells while epiplexus cells are located on the apical surface of the epithelial cells (modified from (Emerich et al., 2005)).

2.2) Function

The best-recognized function of the CPs is CSF production (Serot et al., 2003). CSF is produced mainly by active secretion with water entering the CSF from the blood along an osmotic gradient or by specific water channels such as aquaporins (Oshio et al., 2003). The epithelial cells replenish the CSF by moving Na^{2+}, Cl^- and HCO^{3-} from the blood to the ventricles to create the osmotic gradient that drives the secretion of H_2O (Emerich et al., 2005). The CSF is a clear, slightly viscous liquid with few cells and little protein (Rall, 1964). CSF composition is different from plasma (Segal, 2000), but similar to brain interstitial fluid (Felgenhauer, 1986). The CSF pH is slightly acid. Compared to plasma, the levels of Na^+, K^+, Ca^{2+}, HCO^{3-}, proteins and glucose are lower, but Cl^- and Mg^{2+} levels are higher (Segal, 2000). Folate levels are 2 to 3 times

higher in CSF than in plasma (Spector, 1977). TTR represents 25 % of proteins synthesized by CPs and 5 % of CSF proteins (Serot et al., 2003).

Lying within the central ventricular system, the CPs are in an ideal position to monitor the CSF for the presence of noxious compounds, or potentially damaging cellular invasion (Emerich et al., 2005). The CPs protect the brain against acute neurotoxic insults by using a complex, multilayered detoxification system (Engelhardt et al., 2001; Gao, 2007). The CPs aid or impede the overall bio-distribution of drugs and toxic compounds by being the source of a full range of metabolizing enzymes including Phase I–III enzymes for functionalization, conjugation and transport of drugs (Emerich et al., 2005). A second example of the monitoring and modulating role of the CPs come from a recent understanding of its function within the neuroimmune system (Gao and Meier, 2001). Traditionally, the CNS has been considered an immunologically privileged site with no inherent need for immunosurveillance. The first indication that the CPs mediated interactions and/or signalling between the peripheral immune system and the brain came from demonstrations that the CPs contain inducible lymphoid cells (Emerich et al., 2005).

The CPs possesses numerous specific transport systems, contains a broad array of receptors, and also serves as a major source of biologically active compounds (see Table 1). These capabilities allow the CPs to monitor and respond to the biochemistry of the brain by manipulating and maintaining baseline levels of the extracellular milieu throughout the CNS (Chodobski and Szmydynger-Chodobska, 2001; Stopa et al., 2001).

Table 1: Polypeptides synthesized in CPs (Chodobski and Szmydynger-Chodobska, 2001)

Polypeptide	**Is the polypeptide secreted by the CPs?**	**Are the cognate receptors expressed in CPs?**
Adrenomedullin	Yes	No
Apolipoprotein	No	Yes
APP	No	No
Basic fibroblast growth factor	No	Yes
BDNF	No	Yes
Cystatin C	No	No
Endothelin-1	Yes	Yes
Hepatocyte growth factor	No	No
Insulin-like growth factor-II	Yes	Yes
Insulin-like growth factor binding protein 2–6	Yes	No
Interleukin-1β	No	Yes
Interleukin 6	No	No
NGF	No	Yes
Neurotrophin-3 and 4	No	Yes
Prostaglandin D synthase	Yes	No
TGF-β (isoforms b1, b2, b3)	No	Yes
Transferrin	Yes	Yes
TTR	Yes	No
Tumor necrosis factor-α	No	No
Vascular endothelial growth factor	No	Yes
Vasopressin	Yes	Yes

2.3) Ageing of the choroid plexus

Most of our knowledge about the morphology and function of human CPs during aging comes from control tissues in studies investigating changes in AD (Emerich et al., 2005). Physiological events in the ageing CPs include epithelial atrophy, weight increase (Wilson et al., 1999), and slightly different modifications according to species (Wilson et al., 1999). In humans, the height of CPs epithelial cells decreases about 10–11% during life (Serot et al., 2000), and in elderly rats epithelial cells loose 15 % of their normal height (Serot et al., 2001b). The aged epithelial cell cytoplasm becomes rich with Biondi Ring Tangles and lipofuchsin deposits (Serot et al., 2000) and the nuclei appear irregular and flattened while the basement membrane thickens (Wen et al., 1999). The stroma also thickens and contains collagen fibers, hyaline bodies, calcifications and psammoma bodies, and the infiltrating arteries become thicker and fragmented (Serot et al., 2003; Shuangshoti and Netsky, 1970).

The functions of the CPs are energy-dependant, and the ageing CPs cannot maintain its normal energy output (Emerich et al., 2005). Synthesis of enzymes needed for anaerobic respiration and oxidative phosphorylation declines in aging rats (e.g. lactate dehydrogenase and succinate-dehydrogenase decrease 9% and 26%, respectively) (Ferrante and Amenta, 1987). There are age-dependent increases in the number of epithelial cells deficient in cytochrome C oxidase, altering the respiratory mitochondrial chain with a concomitant decrease in cellular production of ATP (Miklossy et al., 1998). Reductions in Na^+K^+-ATPase and in the Na^+K^+-$2Cl^-$ co-transporter also occur (Cottrell et al., 2001). The anatomic and enzymatic modifications of CPs related to ageing are probably responsible for the drastic diminution of CSF secretion (Serot et al., 2003). In animal models, CSF secretion decreases as much as 45 % during ageing. In rats it has been evaluated that 1.2 mL/min of CSF are secreted at 3 months of age, and only 0.65 mL/min are secreted in 30 months old animals (Preston, 2001). In man, the volume of CSF secreted diminishes with age, from 0.41 mL/min at 28 years of age to 0.19 mL/min at 77 years (May et al., 1990). Due to the decreasing secretion and the simultaneously brain atrophy the CSF turnover takes longer (Preston, 2001) in elderly rats (7.9 h) than in young rats (2.2 h) (May et al., 1990). In man, the turnover of CSF is estimated to occur 6 times a day in young adults compared to 1.7 times in elderly subjects (Tanna et al., 1991).

The effects of these cumulative changes on brain functioning caused by alterations in the CPs, and CSF, lead to inadequate distribution of nutritive substances, additional cellular stress, and reduced clearance of toxic compounds that may play a role in age-related cognitive decline, and in the development of specific neurological disorders.

2.4) Implication in Alzheimer's disease

Accumulating evidence supports the idea that the progressive decrease in CPs function during ageing exacerbates AD (Johanson et al., 2005). As part of a new paradigm to explain brain interstitium deterioration in age-related dementias, more attention is being given to the role of the compromised blood-CSF (Johanson et al., 2005), and blood-brain (Huber et al., 2001) barriers. Structural alterations and functional failure in CPs, as well as in brain capillary systems adversely affect fluid dynamics and composition (Rubenstein, 1998; Silverberg et al., 2001).

Abnormalities similar to those observed in ageing CPs are greatly enhanced, in AD. Epithelial cell atrophy is greater, with cell height decreasing up to 22% in relation to age-matched controls. These cells also contain numerous lipofuchsin vacuoles (Serot et al., 2000). The percentage of epithelial cells containing Biondi bodies is significantly increased in AD patients (Wen et al., 1999). Epithelial basement membranes are very irregular and thickened; their thickness increases by 28% compared to controls of the same age (Serot et al., 2000). Stroma of villi is irregularly fibrotic; its thickness can reach several tenths of a μm (Jellinger, 1976; Serot et al., 2000). The CPs contains vessels with thickened walls, hyaline bodies, calcifications, and psammomas mainly at the glomus level. Immunohistology reveals many linear deposits of IgG, IgM and C1q along the epithelial basement membrane suggesting intervention of immunological processes (Serot et al., 1994). The pathological changes in CPs are resumed in Table 2.

Table 2: Pathological changes of CPs in AD (in (Johanson et al., 2004))

Biondi bodies ↑
Basement membrane thickening ↑ (3-fold)
Stromal fibrosis↑
Epithelial atrophy ↑ (cell size decreases by 1/3)
Lipofuchsin vacuoles
IgG and IgM depositions

The increased atrophy of the choroidal epithelial cells in AD is associated with pronounced decreases in secretory activity and transport functions (Emerich et al., 2005). Indeed, during isotopic scinticisternographies, there is a major delay of reabsorption as well as a transitory ventricular contamination phenomena, which promotes a decrease in CSF turnover (Brusa et al., 1990; Coblentz et al., 1973) and epuration capacities. CSF production has been measured by using an intraventricular catheter in patients with AD (Silverberg et al., 2001). The mean CSF production rate decreased significantly in AD patients. Due to cerebral atrophy, and the consequent ventricular dilatation, the CSF turnover is about 36 h in AD patients compared to 14 h in age matched controls (Serot et al., 2003). The decrease of CSF production could favour the glycation of proteins and the formation of Aβ oligomers.

Advanced glycation end products (AGE) are a result of a diverse class of post translational modifications, generated by the non-enzymatic reaction of a sugar ketone or aldehyde group with the free amino groups of a protein or amino acid, specifically lysine, arginine and possibly histidine. In the first step of protein glycation, a labile Schiff base is formed, which subsequently rearranges into a stable Amadori product. Finally, through a complex cascade of dehydration, fragmentation, oxidation and cyclization reactions, AGE are formed as a mixture of protein-bound nitrogen-andoxygen-containing heterocyclic compounds (Harrington and Colaco, 1994). AD brain contains 3 times more advanced glycation products than controls (Vitek et al., 1994). Diffuse deposits of Aβ, SP and NFT are known to contain AGE (Smith et al., 1996). Glycation of proteins promotes their aggregation, the polymerisation of tau proteins with formation of filaments, and Aβ fibril transformation (Troncoso et al., 1993; Vitek et al., 1994). *In vitro*, fibril transformation is accelerated in the presence of AGE, probably through initiation of a nucleation phenomena (Munch et al., 1998). In ageing, levels of CSF proteins, glucose and Amadori bodies remain stable, the reduction in CSF turnover is insufficient to increase protein glycation. On the contrary, in AD the Amadori bodies increase significantly, while CSF protein and glucose levels remain stable (Klein et al., 2001).

Aβ clearance, which is significantly reduced in elderly animals (Preston, 2001), is still unknown in AD patients (Serot et al., 2003). In rats, clearance of intraventricularly injected Aβ decreases from 10.4 mL/minute at 3 months of age to 0.71 mL/minute at 30 months. Consequently, the brain content of Aβ increases from 7%

at the end of CSF perfusion in young rats to 49% in old animals (Preston, 2001). The brain from AD patients contains 12 times as many oligomers as controls (Kuo et al., 1996). AD severity appears to be more closely correlated to Aβ oligomers levels than to SP number (Klein et al., 2001).

Levels of TTR, a CPs-synthesized molecule that associates with Aβ to form complexes, are more than 10% lower in AD (Serot et al., 1997). Ascorbic acid and α-tocopherol levels, the two major scavengers of free radicals of CSF, are decreased in AD likely adding to oxidative stress (Schippling et al., 2000; Tohgi et al., 1994). CSF folate and vitamin B12 (important for methylation of numerous molecules) are significantly lower (Ikeda et al., 1990; Selley et al., 2002; Serot et al., 2001a) while homocysteine, which mediates lipid peroxidation and increases the production of toxic (E)-4-hydroxy-2-nonenal, is increased in AD CSF. The impaired capacity of the CPs to clear molecules from the CSF of AD patients has potentially profound implications (Serot et al., 2003). As pointed out by Serot and colleagues (Serot et al., 2003), these changes could lead to an even greater impoverishment of the brain, conducting to methylation problems, increased oxidative stress and lipid peroxidation, decreased amyloid clearance, augmented tau protein polymerization, and amyloid peptide oligomers and fibril formation.

2.5) Neuroprotective molecules produced by CPs and regulated by androgens

Recent studies reinforced the neuroprotective roles of CPs, showing that conditioned media from CPs prevents cultured embryonic neurons from death (Borlongan, 2004a; Borlongan, 2004b; Watanabe et al., 2005). CPs ependymal cell grafts promote the regeneration of damaged spinal cord (Ide, 2001), and enable the reduction of the functional and structural consequences of cerebral ischemia (Borlongan, 2004a; Borlongan, 2004b). Transcripts and/or protein for several neuroprotective molecules, such as nerve NGF, neurotrophin 3 and 4, TGF-β, BDNF, TTR, and GDNF (Dickson et al., 1986; Emerich et al., 2005; Ikeda, 1999; Koo, 2001) have been identified in CPs, as well as some of their cognate receptors.

Some of these peptides involved in neuroprotection, like NGF, GDNF, BDNF and TGF, secreted by CPs, are regulated by androgens in other tissues.

Studies by Katoh-Semba (1994) and his co-workers have showed that the levels of β-NGF protein decrease in the hypothalamus and hypophysis, but not in the

cerebellum and olfactory bulb, in male mice after castration (Katoh-Semba et al., 1994). Another study using nandrolone shows an increase of NGF levels in the hippocampus and septum and a decrease in the hypothalamus (Tirassa et al., 1997). Moreover, testosterone, but not DHT decreased hippocampal NGF protein in aged rats (Bimonte-Nelson et al., 2003). Castration of male mice reduces the submandibular gland NGF levels to those found in control females, and androgen treatment produced an increase in the submandibular NGF mRNA (Black et al., 1992).

Zhen et al, in 2003, have shown that the expression of GDNF mRNA in prostate decreased significantly after castration, in rats (Zheng et al., 2003).

BDNF interacts with testosterone in the maintenance of spinal nucleus of the bulbocavernosus (dendritic arbors), in male rats (Yang et al., 2004). The castration, in rats, induced an increase of both BDNF tissue concentration and mRNA expression, in the musculature of the vesicular gland and in the fibromuscular stromal cells of both dorsal and ventral prostatic lobes (Mirabella et al., 2006). Levels of BDNF protein are increased by testosterone, in the female high vocal center of adult canary (*Serinus canaria*) (Rasika et al., 1999).

In the case of the TGFβ, chronic DHT treatment increased TGFβ receptor binding, and also increased TGFβ induced cell proliferation in female rats fetal lung fibroblast cells (Dammann et al., 2000). In pig immature Leydig and Sertoli cells, TGFβ levels decreased after testosterone treatment (Avallet et al., 1994). Androgens negatively regulate the expression of TGFβ ligands and receptors, in the human prostate, (Chipuk et al., 2002). In ovarian cancer HEY cells treatment with DHT down-regulated the expression of mRNA for TGFβ receptors I and II (Evangelou et al., 2000). Studies performed in rats have demonstrated that after castration TGF-β 1 increases in prostate, seminal vesicle and epididymis after 3 days of treatment, and decreased after 5 days (Desai and Kondaiah, 2000).

We reasoned that some of these peptides could also be regulated by androgens in CPs, should AR or other androgen activated signalling pathways be present in this tissue.

3) Neurosteroids

3.1) Neurosteroids synthesis

Recent evidence suggests that the brain is a steroidogenic organ, with the ability to synthesize steroid hormones from cholesterol. The steroids produced in the brain from cholesterol and other blood-born precursors, which accumulate in the nervous system at a level partially independent of traditional steroidogenic organs (adrenal glands and gonads), are termed neurosteroids (Mellon et al., 2001; Plassart-Schiess and Baulieu, 2001). Many neurosteroids were previously thought to have a passive role as precursors or metabolites of other steroids, however it has been shown, that they have effects in the nervous system, ranging from targeting gene expression to modulating neurotransmission (Bates et al., 2005).

The first evidence that the brain was capable of producing steroids occurred when Corpéchot et al (1981) demonstrated the presence of pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate derivates in the brains of castrated and adrenalectomized rats (Corpechot et al., 1981). Subsequent studies showed that both glial cells and neurons contain the enzymes necessary for steroid synthesis (Bates et al., 2005).

The initial step in steroidogenesis is the conversion of cholesterol to PREG on the inner mitochondrial membrane by the enzyme cytochrome P450 side chain cleavage (P450scc). PREG can then be converted to progesterone (PROG) by the enzyme 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) in the endoplasmic reticulum, or to DHEA by cytochrome P450c17 (P450c17, also known as 17α-hydroxylase/c17-20-lyase). The former pathway results in the synthesis of PROG and PROG metabolites, such as 20α-dihydroprogesterone and 5α-dihydroprogesterone. The latter pathway culminates in the formation of testosterone via conversion of androstenedione by 17β-hydroxysteroid dehydrogenase (17β-HSD) (androstenedione is also derived from PROG). Testosterone can in turn be converted into estradiol via the enzyme P450 aromatase (Bates et al., 2005). All these pathways are represented in the figure 2.

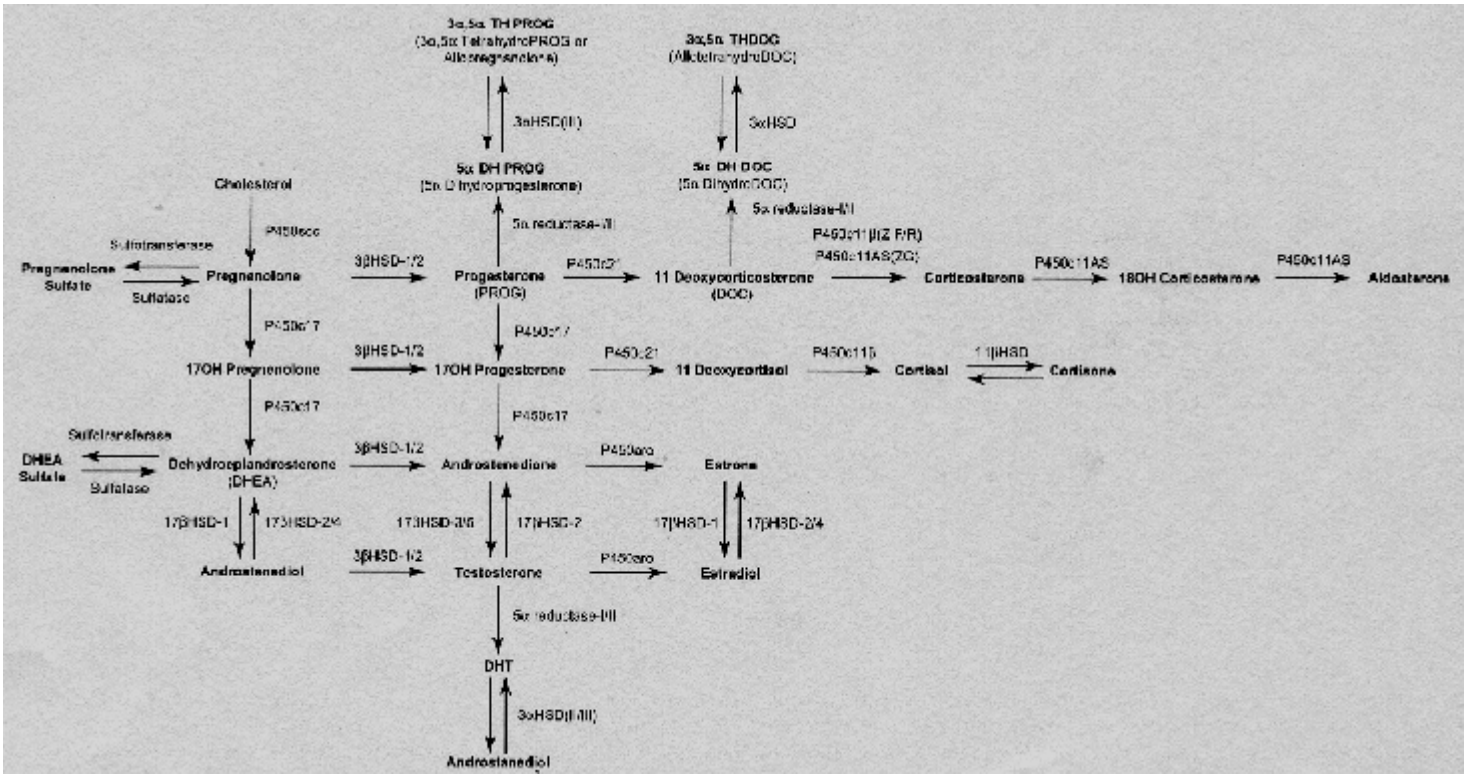

Figure 2: The neurosteroidogenic pathway. The names of the enzymes mediating each reaction are shown above the arrows (in (Mellon and Griffin, 2002))

The expression of the neurosteroidogenic enzymes in the CNS is cell type specific (Figure 3). In vitro analysis of messenger RNA expression and steroid production has revealed, that astrocytes are the most steroidogenic cells in the brain, expressing P450scc, P450c17, 3β-HSD, 17β-HSD, and aromatase (Zwain and Yen, 1999). Astrocytes are therefore, capable of producing PREG, PROG, DHEA, androstenedione, testosterone, estradiol and estrone. Oligodendrocytes, the myelinating cells of the CNS, express P450scc and 3β-HSD, producing PREG, PROG and androstenedione. Neurons express P450scc, P450c17, 3β-HSD and aromatase, and thus produce PREG, DHEA, androstenedione and estrogen (Bates et al., 2005). The relative production capability of these cells can be summarized as follows: astrocytes are the major producers of PROG, DHEA and androgens, oligodendrocytes are the predominant source of PREG, and neurons are the main source of estrogens (Bates et al., 2005). Furthermore, the regulation of some neurosteroidogenic enzymes is sex specific and developmentally regulated. The expression of 3α-hydroxysteroid dehydrogenase (involved in the generation of neurosteroids through ring-A reduction of hormonal precursors progesterone and corticosterone) is high on postnatal day 7, and is

gender specific during puberty in the rat (Mitev et al., 2003). The expression of mRNA for P450scc and 11β-hydroxylase (P450c11β, involved in the synthesis of corticosterone) in the rat is region specific. P450scc mRNA is most abundant in the cortex of both male and female adult animals, and is also found in the amygdala, hippocampus and midbrain, but absent in the cerebellum and hypothalamus (Mellon and Deschepper, 1993). P450c11β mRNA is detected mainly in the amygdala and cortex, but also in the cerebellum and hippocampus of both male and female rats (Mellon and Deschepper, 1993). Interestingly, female rats have higher expression of P450c11β in the hippocampus than male rats. Neurosteroid synthesis and metabolism is thus a complex process. Steroidogenic enzymes are differentially expressed by CNS cells, therefore adding a temporal and spatial dimension to sex steroid synthesis in the CNS (Bates et al., 2005). Neurosteroidogenesis can be envisaged as an autocrine event, with precursors produced by cells, that are required by other cell types to produce the necessary products.

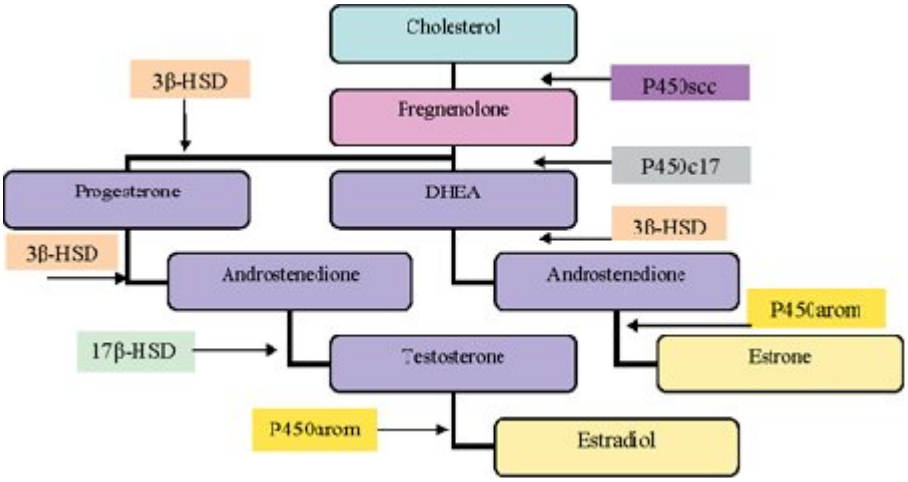

Figure 3: Sex steroid synthesis in the CNS. Major products and enzymes involved are indicated. Astrocyte products are shown in purple, oligodendrocytes products in pink, and neuron products in yellow (in (Bates et al., 2005)).

3.2) Testosterone and ageing

Findings from clinical and basic science studies indicate that testosterone and its androgen metabolites have a wide range of beneficial actions in the CNS (Pike et al., 2007). Androgen actions not only influence the development of the CNS, but also help to maintain its proper function in adulthood. However, as a normal consequence of ageing in men, both circulating (Feldman et al., 2002; Gray et al., 1991) and brain

(Rosario et al., 2004) levels of testosterone exhibit gradual, but eventually functionally significant depletion. The decrease in testosterone production by the testes is progressive, though in men, there is no state analogous to the menopause. Total serum testosterone decreases about 30% and free testosterone by as much as 50% between 25 and 75 years of age (Morley et al., 1997; Vermeulen, 1991). Circulating testosterone not only decreases in ageing men, but also in women, as a consequence of the age-dependent decline in ovarian and adrenal androgen production. The mid-cycle rise in free testosterone and androstenedione seen in younger women (19–37 years old) is consistently absent in older women (43–47 years old) (Mushayandebvu et al., 1996). The 24 h mean plasma concentrations of total and free testosterone also show a steep diminution with ageing in healthy women between the ages of 21 and 51 (Zumoff et al., 1995). This age-related androgen loss has senescent effects in androgen responsive tissues throughout the body, as demonstrated by both impaired function, and increased vulnerability to disease (Morley, 2001). As an androgen-responsive tissue, the brain is also thought to suffer deleterious consequences of age-related androgen depletion. Neural manifestations of androgen deficiency in ageing males include disturbances in mood, cognition, and libido (Gooren and Kruijver, 2002; Morley, 2001; Swerdloff and Wang, 2003). Recent evidence suggests that androgen depletion in men also increases the risk of developing age related neurodegenerative disorders including AD (Pike et al., 2007). How androgen depletion contributes to CNS dysfunction is not clear, but likely includes the diminished activation of androgen signalling pathways that affect behaviour, neuron viability, and regulation of specific pathologies. Further, the cellular and molecular mechanisms that underlie androgen mediated cell signalling pathways remain incompletely defined (Pike et al., 2007).

3.3) Implication of testosterone in neuroprotection

The non-reproductive effects of androgens in the nervous system are considerably less well characterized than those of estrogens and progestins. Testosterone influences neuroplastic changes in different nuclei of the limbic system (De Vries et al., 1994; Johnson et al., 1989; Malsbury and McKay, 1994), and also exerts neuroprotective effects, which can be mediated either directly or indirectly via its aromatization to estradiol (Balthazart and Ball, 1998). Other data suggest that testosterone may also exert neurotrophic actions. For example, Beyer et al., and Lustig

have observed neuronal differentiation and increase in neurite outgrowth after activation of androgen pathways in the cultured neural cells (Beyer et al., 1994; Beyer and Hutchison, 1997; Lustig, 1994). Physiological concentrations of testosterone have also been shown to protect primary cultures of human neurons against apoptosis induced by serum deprivation. The effect of testosterone was directly mediated through androgen receptors, and did not involve its aromatization to estradiol: (1) it could be mimicked by the non-aromatizable androgen mibolerone; (2) it could be blocked by the anti androgen flutamide; and (3) it was not prevented by an aromatase inhibitor (Hammond et al., 2001).

Other experiments in male rodents suggest that testosterone is linked to an increase in neuron size, neuritic growth, plasticity and synaptogenesis in both motoneurons of the spinal nucleus of the bulbocavernosus (Forger et al., 1992; Matsumoto, 1997), and several populations of pelvic autonomic neurons (Keast and Saunders, 1998). Moreover, studies with motoneuron populations, including facial, spinal and pudendal motoneurons, have demonstrated that the administration of testosterone immediately after nerve injury promotes their survival and regeneration through actions mediated by the AR (Jones et al., 2001; Tanzer and Jones, 1997). Ogata et al. have reported that testosterone protected spinal cord neurons against neuronal damage induced by glutamate. The hormone reduced the extent of the spinal cord damage *in vitro* (Ogata et al., 1993).

Testosterone also regulates the production of Aβ by neurons. Treatment of neuroblastoma cells, and of rat primary cerebrocortical neurons with testosterone increases the secretion of the non-amyloidogenic Aβ precursor protein (APP) fragment, and decreases the secretion of amyloidogenic Aβ (Gouras et al., 2000). Testosterone can prevent the hyperphosphorylation of tau, another important factor in AD pathogenesis (Papasozomenos, 1997).

Androgens also play a role in myelination, and have been shown to modulate P0 gene expression in the peripherical nervous system (PNS). As Schwann cells do not express the intracellular AR, different alternative mechanisms by which androgens regulate peripheral myelin gene expression have been proposed: (1) the testosterone metabolite DHT may activate P0 expression by interacting with the progesterone receptor (PR), which is present in Schwann cells; (2) the testosterone metabolite 3α,5α-androstane-diol may interact with GABAA receptors; and (3) testosterone may

influence the myelination process indirectly by acting on the neurons that are myelinated (Magnaghi et al., 1999).

3.4) Testosterone and Alzheimer's disease

Progressive dysfunction and death of neurons characterize neurodegenerative diseases. There are some evidences supporting the hypothesis that testosterone may act protectively in some neurodegenerative disorders: AD, mild cognitive impairment (MCI), and depression (Bialek et al., 2004).

One of the initial studies of Hogervorst et al. (2001) reported significantly reduced serum levels of testosterone in men with AD in comparison to age-matched, non-demented men (Hogervorst et al., 2001). Similar findings of low testosterone in men with AD have been reported in several studies (Almeida et al., 2004; Moffat et al., 2004; Paoletti et al., 2004; Rasmuson et al., 2002; Watanabe et al., 2004) but not all (Pennanen et al., 2004). Rosario and her team (2004) investigated the relationship between brain levels of testosterone, and of AD neuropathology, comparing the brain levels of sex steroids in men with and without AD neuropathology. An approximately 50% decrease in brain testosterone in men with AD aged 60–80 years compared to age-matched men lacking any evidence of AD, or other neuropathology was observed (Rosario et al., 2004). They have not founded age-related changes in brain levels of estradiol in these men (Rosario et al., 2004).

Recent findings suggest that testosterone depletion occurs prior to the development of AD, and thus may act as a contributing factor to AD pathogenesis (Pike et al., 2007). Moreover, these relationships may also be affected by apolipoprotein E ε4 status, which is associated with higher salivary testosterone levels in men, but lower salivary testosterone levels in women (Berteau-Pavy et al., 2007).

Interestingly, low testosterone has also been linked to several other neurodegenerative diseases, including Parkinson's disease (Okun et al., 2004), vascular dementia (Watanabe et al., 2004), amyotropic lateral sclerosis (Militello et al., 2002), and Huntington's disease (Markianos et al., 2005).

There are also randomized studies on various human populations that showed the effect of androgen substitution on cognition in men. Janowsky et al. (1994) have demonstrated that testosterone enhances spatial cognition of healthy older men (Janowsky et al., 1994). Some studies have reported that testosterone enanthate

supplementation improves spatial, verbal (Cherrier et al., 2001), and working memory (Janowsky et al., 2000), in healthy older men.

One of the key events in initiating and driving AD pathogenesis is the accumulation of Aβ (Hardy and Selkoe, 2002). Androgens act as endogenous negative regulators of Aβ accumulation. Consequently, the age-related depletion of testosterone likely diminishes the ability of the brain to adequately regulate Aβ, resulting in increased of Aβ accumulation and development of AD. The mechanisms by which androgens regulate Aβ accumulation have yet to be fully determined, but apparently involve at least two pathways (Pike et al., 2007). One mechanism by which androgens may influence Aβ is via aromatization to estradiol and activation of estrogen pathways. Some studies have shown that estrogen can reduce levels of soluble Aβ (Greenfield et al., 2002; Levin-Allerhand et al., 2002; Manthey et al., 2001; Petanceska et al., 2000; Xu et al., 1998; Zheng et al., 2002) by a mechanism that involves regulation of the processing and/or trafficking of APP (Greenfield et al., 2002; Jaffe et al., 1994), the precursor protein of Aβ.

APP is proteolytically cleaved at the amino- and carboxyl-termini of Aβ by β-secretase and γ-secretase, respectively, to generate the 40–42 amino acid Aβ (Sinha and Lieberburg, 1999). However, APP is alternatively processed within the Aβ sequence by α-secretase, which prevents formation of full-length Aβ. This non-amyloidogenic processing of APP results in secretion of soluble APP-α (sAPPα), which can be used as an index of APP metabolism and, indirectly, Aβ production (Pike et al., 2007).

Gouras et al. (2000) evaluated the effect of testosterone on APP metabolism, and Aβ production in cultured cortical neurons, and in a neuroblastoma cell line. They found that, in parallel to the actions of estrogen, prolonged treatment of cultures with testosterone resulted in elevated levels of sAPPα and reduced levels of Aβ (Gouras et al., 2000). Their data clearly demonstrated that testosterone can regulate Aβ levels, but did not indicate whether the mechanism involves AR-dependent pathways, or aromatization to estradiol and activation of established estrogen pathways. Because aromatase is present in neurons and can effectively metabolize testosterone into estradiol (Melcangi et al., 1992; Poletti et al., 1997), an estrogen pathway is certainly reasonable. In fact, a subsequent study, in 2000, indicated that aromatization to estradiol underlies testosterone regulation of APP and, consequently, Aβ (Goodenough et al., 2000). Like Gouras (Gouras et al., 2000), these researchers found that testosterone

increased sAPPα levels, indicating non-amyloidogenic processing of APP (Goodenough et al., 2000). However, the testosterone-mediated increase in sAPPα was blocked by aromatase inhibition, suggesting that testosterone regulation of Aβ occurs at least in part through estrogen pathways. Consistent with these cell culture findings, there are some observations in men with prostate cancer in which treatment with anti androgen therapy reduced circulating levels of testosterone, estradiol, and increased Aβ (Almeida et al., 2004; Gandy et al., 2001). Although experimental evidence demonstrates that testosterone can regulate Aβ by an estrogen-mediated mechanism, the significance of this pathway to AD risk in men is questioned by other findings, that AD in men is associated with brain levels of testosterone, but not estradiol (Rosario et al., 2004), and that soluble Aβ levels in male rats are reduced by DHT, but not estradiol (Ramsden et al., 2003b).

A second mechanism by which androgens regulate Aβ accumulation involves estrogen-independent androgen signalling pathways. To begin distinguishing between androgen versus estrogen actions of testosterone in regulation of Aβ accumulation, Lund et al, used the testosterone metabolite DHT that is not aromatized to estradiol, although it can be metabolized to form 5α-androstan-3β, 17β-diol which has some agonist effects on estrogen receptor β (ER β) (Lund et al., 2006).

Pike and his team evaluated how the brain levels of soluble Aβ in adult male rats were affected by gonadectomy (GDX), and subsequent treatment with vehicle, DHT, or estradiol. If androgens negatively regulate Aβ accumulation, he has predicted that GDX-induced androgen depletion would increase Aβ levels in the brain (Pike et al., 2007). In agreement with this hypothesis, analysis of soluble Aβ in whole brain by a sensitive and specific enzyme-linked immunoadsorbent assay (ELISA) several weeks after GDX showed a significant increase in Aβ in GDX versus sham GDX male rats (Ramsden et al., 2003a). Further, this GDX-induced increase in Aβ was prevented in male rats treated with subcutaneous, slow-release hormone pellets containing DHT. Notably, GDX male rats treated with estradiol pellets did not show any reduction in Aβ (Ramsden et al., 2003a). The finding of Pike et al, that estradiol treatment did not reduce Aβ in GDX male rats (Pike et al., 2007), is in contrast to observations that estradiol can reduce Aβ in GDX female rodents (Carroll et al., 2007; Levin-Allerhand et al., 2002; Petanceska et al., 2000; Zheng et al., 2002).

Together, these findings suggest that brain levels of Aβ in males are regulated by androgens, and that the underlying mechanism involves, at least in part, androgen signalling pathways that are independent of estrogen (Pike et al., 2007). If androgens are in fact endogenous regulators of Aβ, might expect that changes in androgen levels more subtle than GDX-induced depletion may affect Aβ levels. To study this possibility, Pike et al, examined whether inherent differences in circulating DHT levels in gonadally intact, adult male rats predicted brain levels of soluble Aβ. They observed that male rats with relatively high DHT levels showed lower levels of the two primary forms of Aβ protein, Aβ1-40 and Aβ1-42, than male rats with relatively low DHT levels (Pike et al., 2007). This finding predicts that, even the gradual age-related loss of testosterone associated with normal male aging may promote Aβ accumulation (Pike et al., 2007).

Moreover, Pike et al, also have found that in men lacking AD, but characterized by mild neuropathological changes, brain levels of testosterone are inversely correlated with brain levels of soluble Aβ. Further, in male brown Norway rats, they have found that age-related decreases in testosterone and DHT are associated with increased brain levels of Aβ (Pike et al., 2007). One interesting aspect of the data obtained in aging rats is that the observed changes in androgens and Aβ occurred prior to significant increases in the levels of luteinizing hormone, a variable linked to testosterone loss which some have argued that also contributes to regulation of Aβ (Casadesus et al., 2005). Considering the testosterone and Aβ literature, one obvious and clinically important prediction is that low testosterone may promote the development of AD neuropathology, Rosario et al, evaluated how androgen status affects the development of AD-like neuropathology in the 3xTg-AD triple transgenic mouse model of AD. They observed that depletion of endogenous androgens by GDX in male 3xTg-AD mice at 3 months of age resulted in a significant increase in accumulation of Aβ in subiculum, hippocampus CA1, and amygdala at 6 months of age (Rosario et al., 2006). This GDX-induced increase in Aβ was associated with a significant worsening in performance in spontaneous alternation behaviour, a hippocampal dependent task of working memory. However, continuous treatment with DHT beginning at the time of GDX prevented the increase in Aβ accumulation, and worsening in behavioural performance (Rosario et al., 2006). These data confirm in another context that androgen pathways regulate Aβ accumulation, and predict a protective role against AD. Although research findings have established that, androgens can regulate Aβ accumulation through estrogen-

independent, androgen pathways, the signalling mechanism(s) underlying this action remain to be elucidated (Pike et al., 2007).

Tau protein, a microtubular binding protein, predominantly axonal, which stabilizes the neuronal cytoskeleton, is another neuropathological hallmark of AD (Bialek et al., 2004). Papasozomenos et al, has demonstrated that heat shock-induced hyperphosphorylation of tau protein in the brain of orchidectomized male rats can be reduced by testosterone (Papasozomenos, 1997).

Correlations between two markers of AD and level of neurosteroids have been founded. Aβ peptide levels were negatively correlated with PREGS levels in the striatum and cerebellum. Phosphorylated tau protein levels were negatively correlated with DHEAS concentrations in the hypothalamus. Other data demonstrated that administration of progestins reversed the age-dependent myelin abnormalities whereas administration of androgens was without effect (Azcoitia et al., 2003). Ibanez et al, have revealed that PROG administration slows remyelination of axons by oligodendrocytes after toxin-induced demyelination in old male rats (Ibanez et al., 2004). Morales et al, have reported that DHEA increases physical and psychological well-being in woman and men, increases lean body mass and muscle strength in men (Militello et al., 2002; Morales et al., 1994). However, most human trials demonstrating DHEA effect on cognitive performance have failed (Wolkowitz et al., 1995).

Some of the numerous effects of androgens, in neuroprotection, and in AD, previously described are mediated by the androgen receptor. The structure, function and its relation with AD are described below.

4) Androgen receptor

4.1) Gene and protein structure

AR is a member of the steroid and nuclear receptor superfamily (Freedman, 1998), which is composed of over 100 members, and continues to grow. Among this large family of proteins, only five vertebrate steroid receptors, estrogen, progesterone, androgen, glucocorticoid, and mineralocorticoid receptors are known. Like other steroid receptors, AR is a soluble protein, that functions as an intracellular transcriptional factor. AR function is regulated by the binding of androgens, which initiates sequential conformational changes of the receptor that affect receptor protein interactions and

receptor-DNA interactions (Gao et al., 2005). AR-regulated gene expression is responsible for male sexual differentiation and male pubertal changes (Gao et al., 2005).

In 1981, Migeon et al, first localized the AR gene to the human X chromosome (Migeon et al., 1981). In 1998, Lubahn et al, cloned human AR genomic DNA from a human X chromosome library using a consensus nucleotide sequence from the DNA-binding domain of the nuclear receptor family (Lubahn et al., 1988). In the same year, several groups cloned human AR cDNAs (Chang et al., 1988; Colvard et al., 1989; Lubahn et al., 1988; Trapman et al., 1988). To date, only one AR gene has been identified in humans. The AR gene is more than 90 kb long, and codes for a protein of 919 amino acids, that has three major functional domains, as illustrated in figure 4, (Gao et al., 2005).

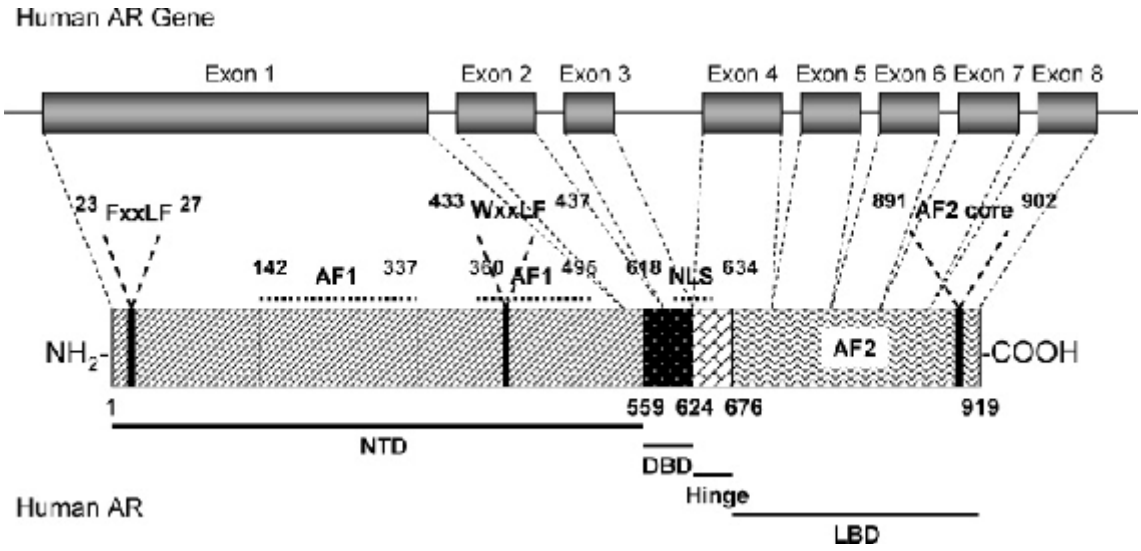

Figure 4: Structural organization of the human AR gene and protein. (from (Gao et al., 2005))

The N-terminal domain (NTD), which serves a modulatory function, is encoded by exon 1 (1586 bp). The DNA-binding domain (DBD) is encoded by exons 2 and 3 (152 and 117 bp, respectively) (McEwan, 2004). The ligand binding domain (LBD) is encoded by five exons, which vary from 131 to 288 bp in size. There is also a small hinge region between the DNA-binding domain and ligand-binding domain. Two transactivation functions have been identified. The N-terminal activation function 1 (AF1) is constitutively active in the truncated receptor, that does not contain the ligand-binding domain, and its sequence is not conserved compared to other steroid receptors, whereas the C-terminal activation function 2 (AF2) functions in a ligand-dependent

manner, and is relatively more conserved compared to other steroid hormone receptors, particularly in the charged-clamp residues (Chawnshang, 2002). A nuclear localization signal (NLS) spans the region between the DNA-binding domain and the hinge region. The human and rat AR amino acid sequence is very similar with identical sequences in the DNA- and ligand-binding domains, and an overall sequence identity of 85% (Lubahn et al., 1988).

4.2) Function and physiologic roles

AR is mainly expressed in androgen target tissues, such as the prostate, skeletal muscle, liver, and CNS. In the brain from monkeys, AR mRNA was found in the medial basal hypothalamus, the bed nucleus of the stria terminalis, the medial preoptic area, anterior hypothalamus, and in the lateral dorsomedial hypothalamus, septum and amygdale, in the hippocampus, cingulate cortex, parietal cortex, cerebellum, anterior pituitary (Abdelgadir et al., 1999), with the highest expression level observed in the prostate, adrenal gland, and epididymis as determined by real-time polymerase chain reaction (PCR) (Keller et al., 1996). AR can be activated by the binding of endogenous androgens, including testosterone and DHT (Gao et al., 2005). Physiologically, functional AR is responsible for male sexual differentiation in utero, and for male pubertal changes. In adult males, androgens are mainly responsible for maintaining libido, spermatogenesis, muscle mass and strength, bone mineral density, and erythropoisis (Goodman, 2001; Johansen, 2004). The actions of androgen in the reproductive tissues, including prostate, seminal vesicle, testis, and accessory structures, are known as the androgenic effects, while the nitrogen retaining effects of androgen in muscle and bone, are known as the anabolic effects (Gao et al., 2005). Numerous and varied site mutations in AR have been identified (The Androgen Receptor Gene Mutations Database World Wide Web Server, http://www.androgendb.mcgill.ca/) (Gao et al., 2005). The majority of these mutations are associated with diseases (Gao et al., 2005). Besides the site mutations documented, AR gene polymorphism, have also been identified, particularly, the poly-Q (CAG)*n* at exon I. The polymorphic (CAG)10-35 triplet repeat sequence, starting from codon 58, codes for polyglutamine. The length of the repeat is inversely correlated with the transactivation activity of AR (Oettel, 2003).

4.3) Implication in neurodisease / Alzheimer's disease

The AR is present in the brain, including regions that can be severely affected in AD such as the cerebral cortex and hippocampus (Simerly et al., 1990). AR are bound to heat shock protein 90 (Hsp90) in the cytosol, stabilising the apoAR in a high affinity ligand binding conformation, and protecting them from degradation (Solit et al., 2003). AR change in structure and dimerize upon binding to androgens, following which, the androgen–receptor complexes enter the nucleus and bind to DNA modulating gene transcription (Fuller et al., 2007). Downstream effects are still being investigated, however the antioxidant enzyme catalase (Ahlbom et al., 2001), and hsp70 (Magrane et al., 2004; Zhang et al., 2004) appear to be induced. Increases in Hsp70 levels may be particularly relevant to AD, as this protein has been shown to be part of a neuroprotective response, against Aβ-induced toxicity (Magrane et al., 2004; Zhang et al., 2004). AR-dependent activation of a mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway, eventually inactivating the pro-apoptotic protein Bad, has also been shown to promote neuroprotection in hippocampal cultures (Nguyen et al., 2005). AR may affect nongenotropic changes as well, for example androgen receptor activation can promote a Src/Shc/ERK signalling pathway, attenuating apoptosis in cell culture models (Kousteni et al., 2001). In other tissues, AR-driven transcription controls muscle growth, bone growth spermatogenesis, and the development of secondary sexual characteristics (Mooradian et al., 1987). The AR gene contains a polymorphic trinucleotide CAG-repeat in exon 1, which encodes a functional polyglutamine tract of variable length (Fuller et al., 2007). The normal CAG-repeat length is within the range 5–35; several studies have attempted to establish an association between variation in AR CAG-repeat length and serum steroid levels, with limited success (for a review see (Kaufman and Vermeulen, 2005)). However, one study has found that short AR CAG-repeat lengths combined with lower than average testosterone levels appears to increase the risk of AD in men (Leder et al., 2004). Few studies of age-related changes in brain expression of AR have been conducted. One study has shown that AR synthesis declines with age in both male and female mice (Tan and Pu, 2001), although AR phosphorylation, thought to be necessary to make the AR transcriptionally active (Wang et al., 1999), has been found to be higher in old mice of either sex compared to younger adult mice. In this study, testosterone supplementation caused a remarkable increase in AR phosphorylation (Tan and Pu, 2001), whereas in

gonadectomised mice, testosterone supplementation caused a drop in AR synthesis. High levels of AR mRNA have been found in the human hippocampus CA1 region (Beyenburg et al., 2000), and hippocampal AR mRNA has been shown to decrease significantly with age (Tohgi et al., 1995). In another study of post-mortem human brains, the vertical limb of the diagonal band of Broca and the nucleus basalis of Meynert, major cholinergic nuclei of the basal forebrain, nuclear AR expression was also found to decrease with aging (Ishunina et al., 2002). In contrast, a study in rat hippocampus showed that AR mRNA levels were significantly higher in old (22 month) than in young adult (5 month) male rats (Kerr et al., 1995). Discrepancies in these results may be due to differences in post-mortem delay, as well as species differences, and further studies are needed to clarify this issue. In the brain, methylation of the AR promotor in mouse brain cortex is induced following testosterone treatment in gonadectomised mice, and results in a decrease in AR mRNA expression (Kumar, 2004). AR, responds to circulating androgen levels to alter AR expression (Kerr et al., 1995). Studies of AR CAG-repeats have shown that shorter repeat lengths are associated with higher expression of AR (Krithivas et al., 1999), and as mentioned above, shorter repeat lengths have also been associated with AD risk (Lehmann et al., 2004), particularly in subjects with low testosterone levels. Alteration of testosterone levels appears to have a significant effect on AR levels, AR phosphorylation and AR promotor methylation, suggesting a complex feedback mechanism that will need much further research before we can predict the outcomes of testosterone supplementation, or other hormonal treatment in putative AD-preventive treatment of elderly males (Patchev et al., 2004). Individual variations in AR CAG-repeats may also cause individual variations in responses to androgen treatments, providing another area that requires further research (Fuller et al., 2007). Nevertheless, AR-targeting therapies have been suggested for AD, including non-steroidal small molecule compounds (selective androgen receptor modulators - SARMS) that have specifically high affinity for brain androgen receptors (Chen et al., 2002). Such compounds could potentially signal through brain receptors and activate only essential pathways, e.g. anabolic pathways in the brain. This may have therapeutic advantages in AD and related disorders in men (Kumar et al., 1999).

III. Objectives

The presence of AR in CPs has never been investigated. Otherwise, the information provided by this literature revision, gives us enough information to raise the hypothesis that should CPs be an androgen responsive tissue, this property may have implications in AD onset and progression. Therefore the present study proposes the following objectives:

- investigate and characterised AR distribution and expression in male and female rats CPs;

- analysed the response of AR to DHT, in castrated male and female mice, subjected to DHT replacement.

IV. Material and methods

1) Animals

All animals were handled in compliance with the NIH guidelines and the National and European Union rules for the care and handling of laboratory animals (Directive 86/609/EEC). Male (n=5) and female (n=5) Wistar rats (3 months old), and mice (see DHT stimulation experiment for details) were housed in appropriate cages at constant temperature in a 12h light /12h dark photoperiod and given standard laboratory chow, and water *ad libitum*. Tissue sampling was carried out in animals euthanized under anaesthesia (Clorketam 1000, Vétoquinol, Lure, France). CPs (dissected from the lateral and fourth ventricles of brains), liver, and prostate were collected from rats and frozen at -80ºC for protein, or RNA extraction. Brains including CPs, were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for *in situ* hybridization (ISH), and immunohistochemistry (IHC). Rat CPs samples were used for Western blotting, ISH, and IHC as larger amounts of tissue were required for these experiments.

2) DHT stimulation experiment

An experiment aiming to determine the response of AR to DHT treatment in CPs was carried out in mice. Female and male mice (129S1/Sv strain, 5 months±2 weeks) were either ovariectomised (n=17) or orchidectomised (n=20) under anaesthesia (Clorketam1000), following standard procedures. Five weeks after surgery, castrated animals were implanted with Alzet mini-osmotic pumps (Charles River Laboratories, Model 1007D, Durect^{TM}, Barcelona, Spain) delivering 419µg/Kg/day of DHT (Fluka, Seelze,Germany) (7 females and 10 males), or vehicle only (placebos, 10 females and 10 males) (0.5% ethanol:99.5% polypropyleneglicol, Aldrich, Saint Louis, USA). Implants were placed in the subscapular region under anaesthesia. Sham operated animals, not implanted (5 females and 5 males), were also included in the experiment.

After one week, mice were euthanized under anaesthesia, and CPs were dissected, and frozen at -80ºC until RNA extraction.

3) AR mRNA detection

3.1) RNA isolation

Total RNA was extracted from CPs dissected from rat and mice brains, upon homogenization in TRI Reagent (Ambion, Applied Biosystems, Austin USA) according to the manufacturers' instructions. RNA was quantified by UV spectrophotometry at 260nm (Pharmacia Biotech, Ultrospec 3000, Denmark), and its quality was assessed by agarose gel electrophoresis containing ethidium bromide (Sigma, St. Louis, USA).

3.2) RT- PCR

Total RNA (60ng) from rat and mice CPs, was reverse transcribed for 1h at 37ºC in a 20µL reaction containing First Strand-5X buffer (50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$) (Invitrogen, Karlsruhe LMA, Germany), 10mM DTT, 0.5mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Amersham, Uppsala, Sweden), 20U of RNAse Out (Invitrogen), 25ρmol of random hexamer primers (Invitrogen), and 200U of M-MLV reverse transcriptase (Invitrogen). PCRs were carried out using 1µL of rat or mice CPs cDNA, in a 25µL reaction containing 20ρmol of forward and reverse primers, 0.2mM of dNTPs, 1.5mM $MgCl_2$ (Promega, Madinson, USA), 1Xbuffer (10mM Tris-HCl, 50mM KCl and 0.1%Triton® X-100) (Promega), and 1.25U of Taq DNA polymerase (Promega). Amplifications were carried out over 35 cycles of 1min denaturation at 94ºC, 1min annealing at 57ºC, and 30s extension at 72ºC. Primer sequences for amplification of rat and mice AR were the following: sense- 5´ GCC AGT GCG TGA GGA TGA 3´ and anti-sense- 5´ GGT GAG CTG GTA GAA GCG C 3´(rat); and sense-5´ GGC GGT CCT TCA CTA ATG TCA CTC 3´ and anti-sense -5´GAG ACT TGT GCA TGC GGT ACT CAT 3´ (Waters et al., 2001). Aliquots of PCR products were resolved by agarose gel electrophoresis containing ethidium bromide (Sigma), and visualized with the Molecular Imager FX Pro Plus MultiImager system (Biorad, Hercules, USA). PCR products were cloned in pGEM-T easy vector (Promega) and sequenced (Stabvida, Oeiras, Portugal) to confirm the identity of the amplicons.

3.3) ISH

Digoxigenin-labeled AR (sense and anti-sense) cRNAs were generated by *in vitro* transcription using the DIG RNA Labeling Kit, according to the manufacturers' instructions (Roche, Basel, Switzerland), from a pGEM-T easy vector (Promega) containing a 237bp fragment of rat AR cDNA, which had been obtained by RT-PCR as described above. Paraffin embedded male and female rat CPs sections (3µm) were hydrated, permeabilised with RNAse free proteinase K (20µg/mL) in TE buffer (100mM Tris, 50mM EDTA, pH 8.0) for 30min at 37ºC, post-fixed in 4% PFA for 10 min, and pre-hybridized for 2h at 45ºC in a buffer containing 50% formamide, 5X saline sodium citrate (SSC), and 40µg/mL of denatured fish sperm DNA (Sigma). Hybridization was carried out at 45ºC for 18h in pre-hybridization buffer containing the synthesized riboprobe (~2.5 µg/mL). Slides were washed at room temperature for 10min in 2XSSC, twice at 65ºC for 15min in 1XSSC, twice at 65ºC for 15min in 0,5XSSC, and blocked with Tris buffered saline (TBS) containing 0.1%Triton X-100, and 2% normal sheep serum, for 30min at room temperature. Sections were then incubated with alkaline phosphatise coupled anti-digoxigenin antibody (Roche) for 4h at room temperature, and subsequently stained using 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrate (NBT/BCIP, Roche), for 16h at room temperature. As negative controls, sections were hybridised with the sense probe, or without any probe, and reactions allowed proceeding during the same time, as with the antisense probe. For further details see point 1 in the annexes.

4) AR protein analysis

4.1) Antibodies

Anti-AR antibodies raised in rabbit against the AR C-terminal (C-19:SC-815), and the AR N-terminal (N20:SC-816), and the corresponding blocking peptides (SC-815P and SC-816P) were purchased from Santa Cruz Biotechnology (Santa Cruz). Preabsorption of the antibodies was carried out by incubation with a five fold (by weight) excess of the corresponding blocking peptide in PBS, overnight at 4ºC.

4.2) Western blot

Total protein was extracted from rat CPs and liver, through homogenization in a buffer (2mL/g of tissue) containing 0.1M Tris (Riedel-de-Haen), 10mM EDTA (Sigma), and 0.05mM PMSF (Sigma). Homogenates were centrifuged at 1500g, for 30min at 4ºC, and the supernatant was collected and centrifuged at 9300g for 15min at 4ºC. Total protein content in samples was measured using the Biorad Protein assay (Biorad, Hercules, USA) using bovine serum albumin (BSA, Sigma) as a standard. Total protein extracts (20μg) from rat CPs, and liver, were boiled in loading buffer (10mM Tris-HCl, pH 6.8, 3% SDS, 5% β-mercaptoethanol, 20% glycerol and 0.6% bromophenol blue) for 5min and resolved on a 12% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to a polyvinylidene diflouride (PVDF) membrane (Amersham, Hybond-P) for 105min. Membranes were blocked with 5% skimmed milk powder (Regilait, France) in TBS containing 0.1% Tween 20 (Applichem, Darmstadt, Germany) (TBST) for 1h, and incubated for 16h with the anti-AR polyclonal antibody (C-19:SC-815) diluted to 1:500 in TBST, at 4ºC, or with the preabsorbed antibody, also diluted to 1:500, as a negative control. After washing with TBST, membranes were incubated with goat anti-rabbit IgG-H+L (1:10000; ECF Western Blotting Reagent Packs, Amersham) for 1h at room temperature. Membranes were washed again with TBST. Reactions were developed using the ECF substrate (ECF Western Blotting Reagent Packs, Amersham) according to the manufacturer's instructions. These procedures were repeated using tissue samples from four different male and female rats. Images of blots were captured with the Molecular Imager FX Pro Plus MultiImager system. For further details see point 2 in the annexes.

4.3) IHC

IHC was carried out in CPs paraffin sections (3 μm) from male and female rats, with the antibodies C-19:SC-815 and N20:SC-816, diluted to 1:100, and to 1:50, respectively, in PBS with 1% BSA. Incubations with the primary antibodies were carried out for 1h, at room temperature. Sections were then incubated with the secondary antibody (biotinylated goat anti-rabbit IgG) diluted to 1:20 in PBS, for 1h at room temperature, and after, with extravidin peroxidase conjugate (ExtrAvidin Peroxidase staining kit rabbit, Sigma) diluted to 1:20 in PBS for 30min, at room temperature. Colour reactions were developed with diaminobenzidine (DAB, Sigma),

and sections were counterstained with hematoxylin. Incubation and developing time exposures used with the primary antibody were the same, as in negative controls. For further details see point 3 in the annexes.

5) Analysis of the AR transcription response to DHT treatment by Real Time PCR

AR expression in CPs of placebos was compared to AR expression in CPs of castrated male and female mice subjected to DHT replacement, and to sham animals, by Real Time PCR. cDNA from mice CPs was obtained as described previously. Beta-2 microglobulin (b2mgb) was used as an endogenous control. B2mgb primers were designed using the Beacon designer v7.0 software (forward primer: 5-CGG TGA CCC TGG TCT TTC TG-3'; reverse primer: 5'-ACT TGA ATT TGA GGG GTT TTC TGG-3'), and AR primers were the same as mentioned above, for RT-PCR. Real Time PCR reactions were carried out in the iCycler iQ™ system (Biorad) using 1µL of cDNA synthesized from mice CPs in a 20µL reaction containing 10µL of syber green supermix (Biorad), and 0.1 or 0.2ρmol of each primer (b2mgb and AR, respectively). Amplification conditions were: 95°C for 3min and 40 cycles of 95°C for 15s, 60°C for 30s, and 72ºC for 30s. Amplified PCR fragments were checked by melting curves: reactions were heated from 55ºC to 95ºC with 10s holds at each temperature (0.05 ºC/s). Primers were validated previously by quantitative PCR reactions with incremented cDNA concentrations, and efficiencies were calculated. Triplicates were run for each sample. Data collected from Real Time PCR experiments were analysed with the Relative Expression Software Tool (REST©) 2005 version 1.9.12 (Pfaffl et al., 2002), which allowed determination of statistically significant differences between placebos and treated animals, and between placebos and sham animals, taking into account reaction efficiency, and reference gene normalization. For each experimental group comparison, 50000 iterations were carried out. Moreover, comparisons of AR expression between males and females in each experimental group (placebos, DHT-treated, and sham) were also carried out, using the same analytical procedure. Statistically significant differences were considered when $p<0.05$.

V. Results

1) AR mRNA and protein are present in CPs epithelial cells

In a preliminary approach to determine if AR was expressed in CPs, RT-PCR with specific primers for AR amplifications were carried out in rat and mice CPs total RNA. Those PCR products were 100% identical to the rat and mice AR sequences available in Genebank (NM_012502 and NT_039706.7, respectively) (data not shown). Moreover, ISH was conducted to identify the AR expressing cells in rat CPs (Figure 5).

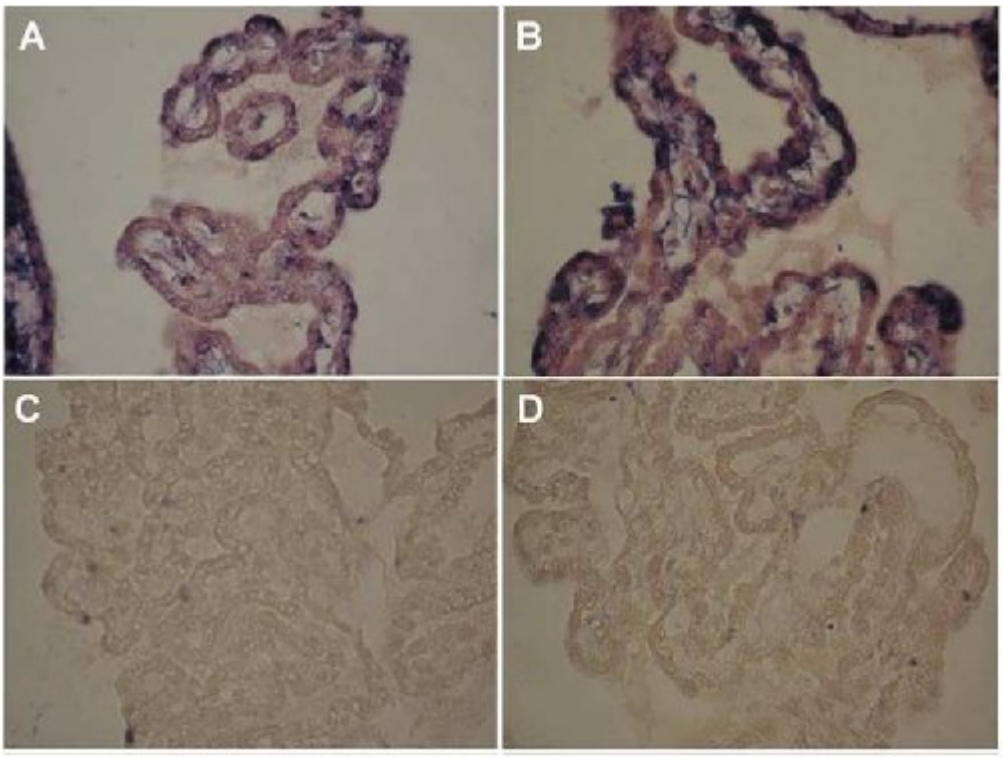

Figure 5: ISH using a specific rat AR cRNA riboprobe. High levels of AR expression were detected in the cytoplasm of male and female rat CPs epithelial cells. Positive staining is shown in dark blue in males (panel A) and females (panel B) (400X; anti-sense cRNA probe). Panels C and D– negative controls for male and female CPs, respectively (400X; sense cRNA probe).

Strong staining was observed in epithelial cells, as dark blue staining in males (Figure 5A) and in females (Figure 5B), but staining was generally more intense in females. No staining was observed when sections were hybridized with the sense probe in male and female tissue (Figure 5C and D, respectively), or without any probe (data not shown), demonstrating the reaction specificity.

The presence of AR protein in rat CPs was detected by Western blot and IHC. Western blot analysis (Figure 6), using an antibody against the AR C-terminal region (C-19), showed the presence of two principal bands with approximately 110 and 87K in CPs (lane 1-males; lane 2-females), and in females liver (lane 3). Similar electrophoretic profiles were detected in males and females, except for the presence of a 35K immunoreactive protein found in females, but staining seemed to be more intense in females CPs than in males'. To analyse the specificity of these bands, duplicates of these blots were incubated with the AR antibody preabsorbed with the AR peptide used to produce the antibody (Figure 6, lanes 4, 5 and 6). Only the third band from top (*) detected in female CP and liver (lanes 2 and 3) did not seem to be specific, as they were also detected when blots were incubated with the preabsorbed antibody (*; lanes 5 and 6).

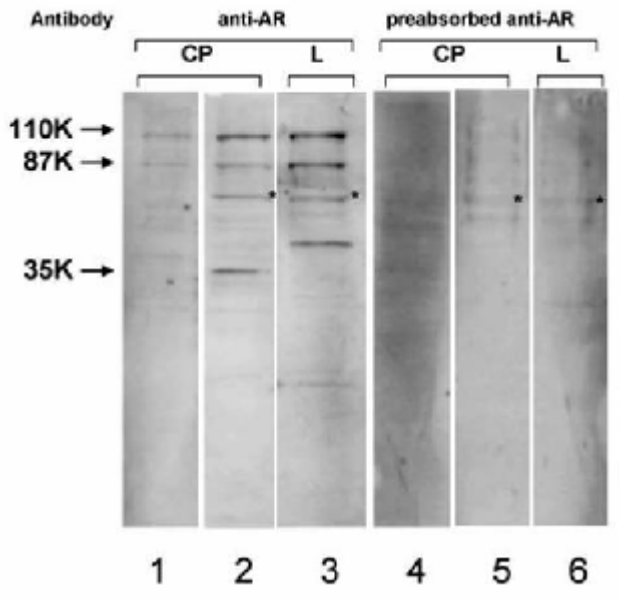

Figure 6: Western blot analysis of 20µg of total protein extracted from male and female rat CPs, and female liver (L) using an antibody against the C-terminal region (C-19) of AR. 1-Male CPs, 2-female CPs, 3-female liver. Lanes 4 to 6 contain the same samples as in 1 to 3, incubated with the preabsorbed AR antibody. The proteins of 110K, 87K and 35K are indicated by arrows. Non-specific band are indicated (*).

IHC enabled the identification of AR cellular localization in rat CPs. Two different anti-AR antibodies (C-19 and N-20) were used for this purpose yielding very similar results, therefore only the results obtained with the C-19 antibody are presented (Figure 7). Alike AR transcripts, AR protein is also present in CPs epithelial cells. Dark brown staining, showing variable degrees of immunoreactivity, was found in the nuclear compartment of these cells in males (Figure 7B), and in females (Figure 7E). In

agreement with Western blot data, again AR seemed to be less abundant in males CPs than in females', in which some cytoplasmatic staining was also detected. No staining was detected when the preabsorbed antibody was used in male and female tissue (panels C and F, respectively), nor when the primary antibody was omitted in the reactions (panels A and D).

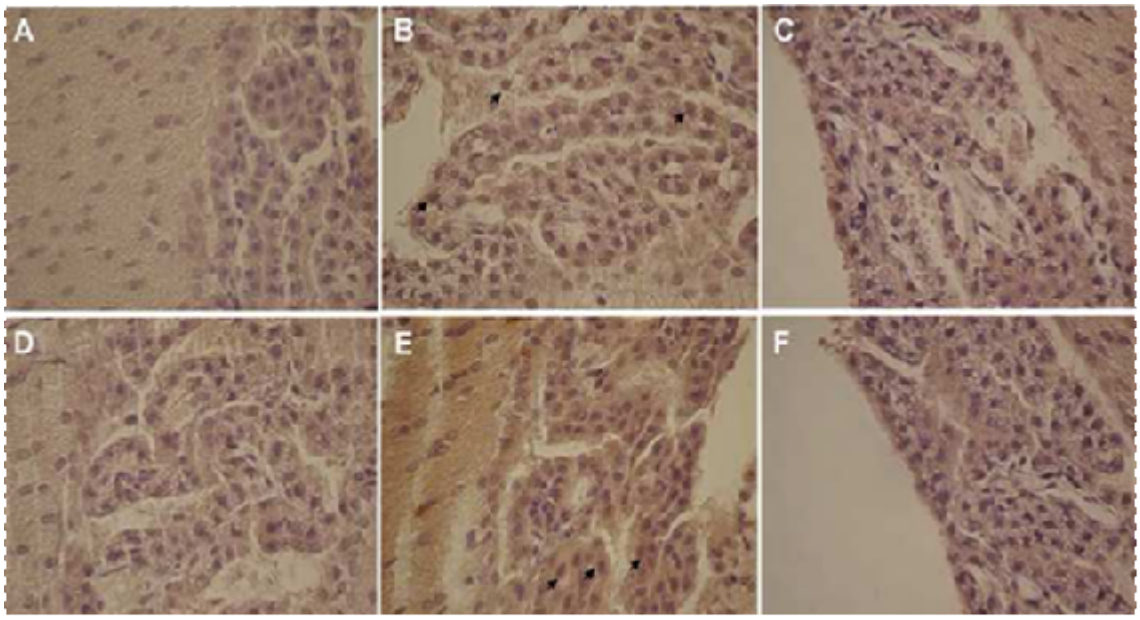

Figure 7: Immunostaining of rat CPs using an antibody against the AR C-terminal region (C-19). A-C male CPs; D-F female CPs. CPs sections in panels B and E were incubated with the C-19 primary antibody. CPs sections in panels C and F were incubated with the preabsorbed C-19 primary antibody, and in panels A and D the primary antibody was omitted in the reactions (negative controls). Positive staining is shown by brownish staining in both cytosolic and nuclear compartments of the epithelial cells of CPs (indicated by arrows) (400X magnification).

2) DHT down-regulates AR expression in CPs of male and female mice

Once the presence of AR mRNA in CPs was confirmed in rat and mice by RT-PCR, and further confirmed in rat by ISH, the response of AR transcription to DHT treatment in male and female mice was analysed by Real Time PCR. The levels of AR in placebos were considered 100% (by default), and expression data in DHT-treated, and in sham animals are represented as a percentage of AR expression in placebos (Figure 8A). In DHT-treated males, AR transcription was 35% of that of placebos (p=0.037), whereas in females, AR expression decreased to 9.1% of placebo levels in response to DHT treatment (p=0.027). In sham males, AR transcripts were 9.4% of that of placebos, indicating that castration increased AR transcription. In opposition, sham

females had higher levels (267.6%) of AR expression than placebos, though without statistical significance (p=0.908). Remarkably, down-regulation of AR transcription in response to DHT was higher in females than in males. Moreover, the levels of AR transcripts in males were compared to those in females in each experimental group (Figure 8B). In this case expression levels in females were considered 100% (by default), and AR expression levels in males in each experimental group, are represented as a percentage of the AR expression in females. In placebo males, AR levels were 70% lower than in females (p=0.033), and in sham males, AR levels were 0.8% of those in females (p=0.065). No statistically significant differences in AR transcription were observed between males and females in the DHT-treated group.

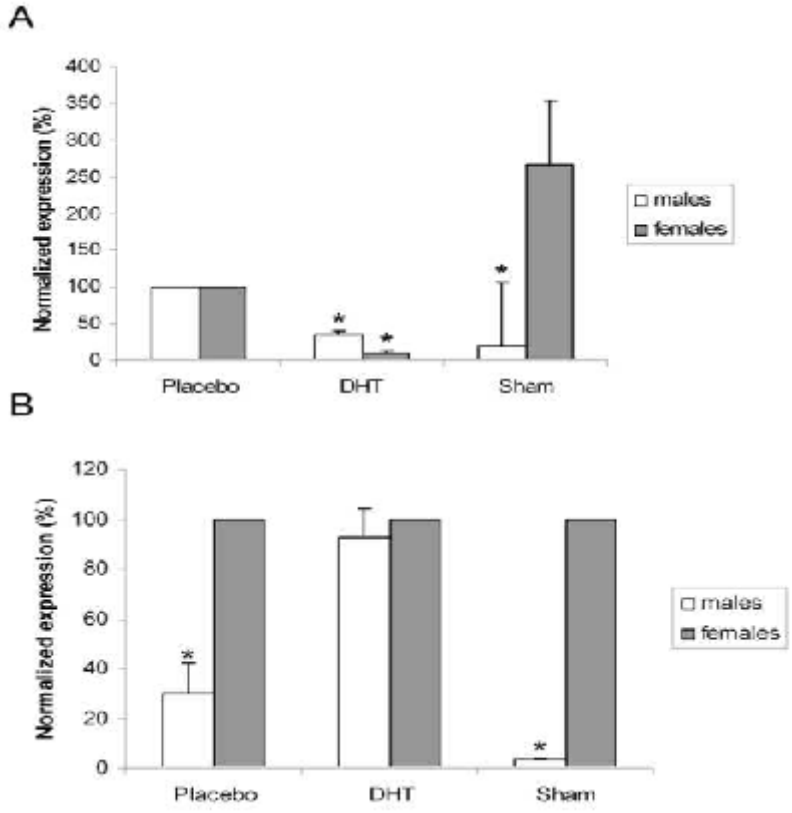

Figure 8: Comparison of AR expression in male and female CPs from mice by Real Time PCR. A- AR expression in male and female mice was compared between placebos (considered 100%) and DHT-treated animals, and between placebos and sham animals (represented as a percentage of placebos expression levels). B- AR expression in males was compared to AR expression in females (considered 100%) among placebos, DHT-treated, and sham animals. Male AR expression levels in each group are presented as a percentage of females expression. Results are presented as normalized expression using b2mgb as an endogenous control, calculated using the Relative Expression Software Tool (REST©) 2005 version 1.9.12. Statistically significant differences among groups (p<0.05) are indicated (*).

VI. Discussion

AR transcripts were initially detected by RT-PCR conducted in rat and mice CPs, yielding amplicons 100% identical to previously AR rat and mice published sequences. To ensure that AR amplification by RT-PCR was not due to contamination of CPs samples with tissue from other brain adjacent regions, which are known to express AR (Kerr et al., 1995). ISH was carried out in male and female rats. AR mRNA transcripts were found in CP epithelial cells, and female samples seemed to contain more AR transcripts than males. The presence of AR protein in CPs was analysed by Western blot and IHC. Western blot analysis showed the presence of two major bands with approximately 110 and 87K in CPs, and in liver. Bentvelsen et al (1996) reported the presence of an AR immunoreactive protein in rat prostate, testis and epididymis with 110K (Bentvelsen, 1996), the same molecular weight herein described for the higher molecular weight band detected. In humans, an additional N-terminal truncated form of the receptor of 87K, has also been reported. Although it was initially thought that this 87K AR was a result of internal translational initiation at Met-188 in the AR open reading frame (Wilson and McPhaul, 1996; Wilson, 1994), Gregory et al (2001) demonstrated that the 87K form of AR most likely results from *in vitro* proteolysis of full-length AR by caspase 3 (Gregory, 2001). This possibility is not to exclude for the 87K detected in rat male and female CPs. No references to smaller AR isoforms have been reported in rats, and the 35K band detected in females CPs, remains to be identified. IHC results showed that AR protein is predominantly located in the nuclei of CPs epithelial cells in males, whereas in females cytoplasmatic staining was also detected. In the absence of ligand, AR has been found in the perinuclear region (Wilson, 1991), and ligand binding (Carson-Jurica, 1990) seems to promote AR translocation to the nucleus. So, it is likely that the AR detected in the cytoplasm may correspond to non-ligand bound receptor, and therefore the more abundant cytoplasmatic AR in females, which in normal conditions have lower levels of circulating androgens than males. Interestingly, AR was down-regulated by DHT in both sexes, but this effect was more pronounced in females than in males. Females seem to have natural occurring higher levels of AR than males, and DHT treatment brings AR levels to similar values in both sexes. Therefore, the steeper reduction of AR levels observed in females suggests that similar exposure to DHT, originate similar levels of AR. In fact when AR mRNA levels where compared between males and females in each experimental group,

it was found that AR transcription was higher in placebo and sham females than in males, and about the same in animals treated with DHT. The major sources of androgens in non-primate mammalians are the gonads (Belanger, 1989). Therefore, after castration, ablation of endogenous androgens production is expected; thus the observed down-regulation of AR transcription in hormone replaced animals must be due to the exogenous administration of DHT, and to endogenous androgens in sham animals. DHT is a non aromatizable androgen, which rules out an effect due to estrogens in DHT treated animals. However, DHT can be converted to the metabolite 5alpha-androstan-3beta, 17beta-diol (3beta-diol) by the enzyme 17β-HSD (Handa et al., 2007; Lund et al., 2006). This metabolite binds ERβ, eliciting ERβ mediated cellular responses. ERβ is present in human CPs (Hong-Goka and Chang, 2004), and recent studies in our laboratory indicate that both ERβ and 17β-HSD transcripts are present in rat and mice CPs. So, an ERβ mediated response to DHT is not to exclude in the regulation of AR in mice CPs. Still, down-regulation of AR by one of its highest affinity cognate ligands is suggestive of a negative feed-back regulation mechanism. This last hypothesis, is reinforced by the lower levels of AR transcripts found in DHT-treated and sham males compared to placebos, and further sustained with the remarkable and consistent indication that AR is more expressed in females than in males CPs.

Several studies on the regulation of ARs by their cognate ligands have shown that it differs between tissues. The levels of AR are diminished by androgens in the rat penis before puberty (Takane, 1990), while in Sertoli cells and in genital skin fibroblasts AR expression is induced by androgens (Gad, 1988; Verhoeven, 1988). In the murine brain, regulation of AR by androgens is age-related and seems to vary from region to region. In the hippocampus, testosterone enhances AR transcription in young animals, but a decrease in testosterone levels in aged rats is concomitant with enhanced AR transcription (Kerr et al., 1995). In the mouse brain cortex, testosterone down-regulates AR levels (Kumar, 2004; Thakur et al., 2000), similarly to our findings in CPs. Our experiments were not conducted to determine if the response of AR to DHT is directly mediated by the AR itself, which would make sense, as a negative feed-back mechanism appears to exist in this tissue, regarding AR regulation. However, androgens can modulate gene transcription through several other mechanisms (Foradori et al., 2007; Heinlein, 2002), which may also have accounted for the results obtained in mice CPs.

Several studies have associated the androgen depletion in men with an increased risk of developing age related neurodegenerative disorders including AD (Pike et al., 2007). It has been shown that testosterone exerts neuroprotective actions against oxidative stress, apoptosis, and against the toxicity of Aβ, all via AR. The presence of AR in CPs enhances the hypothesis that androgens can be important in the regulation of neuroprotective molecules produced in CPs, and in its release to the CSF. It´s, also important not forget that morphologic and function alterations of CPs are related with aging and with AD (Emerich et al., 2005), and some of these, may be associated with the decline in the androgens levels and with their cognate receptor.

The concomitant detection of AR mRNA by RT-PCR and by ISH, with the detection of AR protein by Western blot and IHC in murine CPs, in addition to its down-regulation by DHT, support the hypothesis that this tissue is a potential target for androgen actions, and this response may be important in the treatment and prevention of AD.

VII. Conclusions and Future Perspectives

After analysing and discussing the results we can say that all the objectives of the present study were successfully accomplished. We clearly proved that the AR mRNA and protein are expressed in the murine CPs of both sexes, and that DHT treatment decreases the expression of the AR mRNA, in castrated mice. Moreover the results obtained suggest that AR is more abundant in females than in males. The further step is to elucidate the pathways behind the regulation of AR, using *in vitro* models, and, to evaluate the regulation by androgens of the neuroprotective molecules, produced in CPs, by androgens.

Therefore, this study brings about novel avenues for understanding the regulation of CPs protein synthesis, and release to the CSF, and to further understand the involvement of these peptides in AD.

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IX. Annexes - Protocols

1) In situ Hybridization Protocol (Paraffin sections)

Note: There are almost as many methods for carrying out in situ hybridization as there are tissues that have been probed…So more important than to follow a protocol is to consider the type of probe to use and the best way to label it, the permeabilization steps, the level of stringency etc…

1. Tissue Fixation:

- Fresh tissue should be removed and immediately fixed o/n in 4%PFA in 1XPBS
- Do not prolong fixation beyond 24h
- Follow standard laboratory techniques for dehydrating and embedding tissue in paraffin
- For long term storage paraffin-embedded tissue is best-kept at 4°C in a dry environment
- For in situ hyb. 3-7µm sections should be cut and mounted onto poly-L-lysine coated slides (or equivalent)

2. Dig- labelled riboprobe synthesis:

2.1. *Plasmid linearization and purification*

- Purify plasmid using the Promega Wizard Purification Kit (or equivalent)
- Linearize plasmid with the appropriate restriction enzyme and corresponding buffer, in a final volume of 50µL
- Check template linearization, amount and quality of DNA on an agarose gel
- Purify using a DNA purification kit (e.g. Promega Wizard SV gel and PCR Clean-up system) and elute in H_2O DEPC
- Confirm purification results by electrophoresis

2.2. *RNA Dig-labelling reaction*

- Add reagents to a sterile, RNase-free microfuge tube (on ice) in the following order:

Reagent	Volume	Final concentration
Purified DNA Template	Variable (1µg)	0.05µg/µL
DEPC-treated H2O	Up to 20µL	_
10X NTP labelling mixture	2µL	1X
10X transcription buffer	2µL	1X
Rnase inhibitor	1µL	2U/µL
RNA polymerase (SP6 or T7)	2µL	2U/µL
Final volume	20µL	

- Mix gently and centrifuge briefly
- Incubate 2h at 37°C
- Add 2µL DNase I and incubate 15min at 37°C
- Stop the reaction by adding 2µL 0.2M EDTA pH 8.0

2.3. *Purification and analysis*

- Precipitate riboprobes by adding: 2.5µL LiCl 4M and 75µL 100%ET-OH and incubating at least 30min at -80°C
- Centrifuge 13000g for 15min at 4°C and discard supernatant
- Wash the pellet with 50µL ET-OH 70%
- Centrifuge 13000g for 15min at 4°C and discard supernatant
- Let the pellet dry, ressuspended in 100µL H_2O DEPC
- Analyse the RNA transcripts quality by agarose gel electrophoresis and estimate the concentration.
- aliquot and store at -80°C

NOTE: probes are stable for up to one year.

3. Tissue Preparation:

3.1. *Dewaxing*

- remove slides from 4°C storage and allow to warm to RT
- dewax sections by 2x5min washes using fresh xylene each time

3.2. *Rehydration*

- Rehydrate by performing the following steps:

100% ETOH 2x2min
95% ETOH 1x5min
70% ETOH 1x5min
50% ETOH 1x5min
2 quick washes in DEPC-H_2O
2 x 5min DEPC-PBS

IMPORTANT: once tissue sections are rehydrated do not let sections dry out at any step in the entire in-situ hybridization procedure

3.3. *Permeabilization*

- Permeabilize sections for 30min at 37°C with DEPC-treated TE buffer containing 20µg/mL RNase-free proteinase K.
- Wash for 30s to 1min in PBS+2mg/mL glycine

NOTE: It is desirable when carrying out the first experiments on a new tissue to try different alternatives of permeabilization (exposure time, temperature, concentration of proteinase K) to determine what works best with your particular situation/tissue/fixation method.

3.4. *Post-fixation*

- Post fix the tissue sections for 10min in 4%PFA in 1XPB at 4°C
- Wash slides 2x5min with 1XPBS

4. In situ hybridization:

4.1. *Pre-hybridization*

- Get excess buffer solutions off the tissue sections.
- Carefully overlay each section with well mixed pre-heated (45°C) hybridization buffer (~50µL) for a section of 2cmx2cm.
- Incubate your slides in a hybridization chamber for at least 2h at 45°C

4.2. *Hybridization*

- At the end of pre-hybridization drain excess buffer and carefully overlay each section with hybridization buffer containing the denatured probe (5 min at 100ºC)

- Carefully put your slides into the hybridization chamber and incubate overnight at hyb temp (45ºC) for at least ~18h. Hyb time can be extended up to 40h for rare transcripts.

5. Washes

- Make sure SSC solutions are at wash temperature before use.
- At the end of hybridization remove the parafilm from tissue sections by immersing the slides into 2X SSC for 5min.
- Using a shaking water bath at the desired temperature carry out the following washes:

- quick wash	1XSSC	RT
- 2x15min	1XSSC	65°C
- 2x15min	0.5XSSC	65°C
- 1x10min	0.5XSSC	RT

NOTE: time and stringency of washes can be modified. If these give high background alternatively, formamide can be add to the washe solutions (50%)

6. Immunological detection of DIG using anti-DIG antibody conjugated to alkaline phosphatase:

- Small antibody aggregates in the anti-DIG-AP may lead to higher background staining. It is therefore suggested to centrifuge the vial for 5min at 13000 rpm before its first use. Thereafter, it is sufficient to centrifuge for 1 min immediatly before dilution
- Transfer slides to TBS 1X and wash 3x5min
- Cover sections for 30 min with blocking solution (TBS + 0.1% Triton X-100 + 2% normal sheep serum; or 1% blocking reagent (Roche))
- Pour off blocking solution and incubate sections for 4h minimum at RT with anti-DIG antibody diluted 1:200 in TBS + 0.1% Triton X-100. Incubation can alternatively be performed at 4°C o/n

NOTE: the diluted antibody is stable at 2-8°C for 12h. ALWAYS PREPARE FRESH!!

- Wash sections 3x5min with TBS 1X

- Incubate sections 5min with 100mM Tris-HCl (pH9.5), 100mM NaCl, 50mM $MgCl_2$
- Dissolve one NBT/BCPI "ready to use" tablet in 10mL of dH_20 to make 10mL of staining solution
- Incubate sections with approximately 200µl of staining solution in the dark. A blue precipitate will form. The development time will depend on numerous factors but it takes usually from a few minutes for high abundance transcripts to several hours, or even overnight for low level mRNAs
- When colour development is optimal stop the reaction by rinsing slides in TE-buffer pH8
- Finally rinse briefly in dH_2O and mount slides with any water-soluble mounting medium (do not use xylene-based mounting medium).

Solutions

- All glassware, plasticware, pipette tips etc should be autoclaved
- dH_2O must be autoclaved
- DEPC-treated H_2O and buffers:

add 0.2mL DEPC to 100mL of the solution to be treated. Shake vigorously to get the DEPC into solution. Autoclave. Prepare in a fume hood and wear gloves

- Fixative: 4% paraformaldheyde (PFA) in PB

8g PFA dissolved in 100mL of H_2O. Heat to partially dissolve then add 1M NaOH dropwise until solution clears. Add 100mL of 0.2M PB, mix. Filter. Cool to 4°C before use. Store at 4°C. Prolonged storage (> 1 week) requires pH is checked. pH should be around 7.4. DO NOT autoclave.

- Phosphate buffer (PB) 0.2M:

Add 21.8 g of Na_2HPO_4

6.4 g of NaH_2PO_4 to 800 mL. Mix to dissolve, adjust volume to 1000mL and adjust pH to 7.4

Autoclave and store this solution at room temperature.

- Phosphate buffer saline (PBS) 10X:

Add to 800mL DEPC-H20:

80 g	NaCl
80 g	NaCl
2 g	KCl
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4

Adjust pH to 7.4 with HCl and add H_2O to 1L. Autoclave

- Triethanolamine buffer (TEA) (0.1M TEA, pH 8.0)

Add 18.57g triethanolamine.Cl to 900mL dH_2O. Dissolve and adjust pH to 8.0 using NaOH. Adjust to 1L with dH_20. Use the same day

- TE buffer:

Prepare solution of 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0. Autoclave.

- Tris-buffer saline (TBS) 10X (100mM Tris HCl pH 7.5, 150mM NaCl,):

Add to 800mL of dH_20:

88 g	NaCl
121 g	Tris Base

Dissolve and adjust pH to 7.5 with approximately 40mL HCl 0.1N. Add H2O-DEPC to 1L. Autoclave

- Levamisole 1M

Add 2.4g to 10mL sterile dH_2O. Dissolve and store at 4°C for several weeks.

- 20xSSC (3.0M NaCl; 0.3M Sodium citrate):

175.3 g NaCl

88.2 g $C_6H_5O_7Na_3.2H_2O$ (Sodium citrate)

Dissolve in 800 mL of dH_2O. Adjust the pH to 7.0 with NaOH 10N, Add H_2O DEPC to 1L. Autoclave

- Hybridization buffer:

To make 10mL add to a 50mL Falcon tube:

Reagent	Quantity	Final concentration
Formamide (deionised)	5 mL	50%
20xSSC	2.5mL	5X
Denatured fish sperm DNA (10mg/mL)	40µL	40µg/mL

Bring volume to 10mL with H_2O DEPC.

NOTES:

Mix very well before use.

Hybridization buffer can be pre-made and stored at -20°C without fish sperm DNA.

Bring to desired temperature and shortly before use add denatured fish sperm DNA and denatured probe

Denature probe by incubating 5min at 80°C and immediately place on ice. The optimal amount of probe you will need to add will require a bit of trial-and-error, but it is usually within the range of 100-1000ng/mL of hyb. Buffer with 200ng/mL is a good starting concentration. Mix well BY HAND to ensure even probe dispersal, but avoid foaming (bubbles may lead to high background).

- Wash solutions:

0.5X SSC

1XSSC

Dilute 20XSSC to the desired concentration

2) Western blot

1. Electrophoresis

Determinate the appropriated volume to use in the gel, and choose the right plaques 0.75mm (V_{well}=33µL) or 1.5mm (V_{well}=64µL).

- Preparation of the resolving gel (12.5%)
  - 8.3 mL polyacrylamide (40%)
  - 7.5 mL Tris-HCl 1.875M pH 8.8
  - 3.6 mL Water
  - 0.2 mL SDS 10%

Add 250 µL ammonium persulfate (PSA) 10%; 15 µL TEMED

- Preparation of the stacking gel (4.7%)
  - 1.75 mL polyacrylamide (40%)
  - 1.25 mL Tris-HCl 1.25M pH 6.8
  - 6.9 mL Water
  - 1.0 mL SDS 10%

Add 250 µL PSA 10%; 15 µL TEMED

Note: always prepare fresh PSA

Only add TEMED and PSA when the system is all mounted. First add the PSA and then the TEMED.

- Add the resolving gel to the system (up to 1 cm of the top), be careful not to leave any air bubbles.
- Add water and wait until the gel polymerizes
- Prepare the stacking gel
- Remove the water, add the stacking gel and insert the comb
- Let polymerize and remove the comb
- Wash the wells
- Put the gels into the electrophoresis apparatus
- Add the sample buffer to the samples, and boil 5 min at 100 ºC

- Load the samples and the protein standards into the gel
- Run electrophoresis at 110-120 V during ± 90 min at room temperature

2. Electrotransfer

- Activate the PVDF membranes (6x9 cm):
- 5 s in methanol
- 5 min in water
- 5- 15 min in electrotransfer buffer (10 mM CAPS in methanol 10%, pH 11)
- Pick up the gel and remove the stacking gel
- Put the gel in the electrotransfer buffer for 5 min
- Put the filter paper, the gel and the membrane in a sandwich, beware the gel must be placed in the left side of the sandwich
- Remove all the air bubbles
- Do the electrotransfer at 4 ºC, 750 mA, during 90 min

3. Blocking

- After the electrotransfer, pick up the membranes and do the blocking with TBS-T with 5% milk, for at least 1h at room temperature with continuous shaking

4. Incubation with the antibodies

- Incubate the membranes with the primary antibody in TBS-T 1%, overnight at 4 ºC, with continuous shaking
- Quickly wash the membranes with TBS-T 0.5%
- Wash 3X, 10 min, the membranes, in TBS-T 0.5% with shaking
- Incubate the membranes with the secondary antibody in TBS-T 1%, 1h at room temperature, with continuous shaking

Note: the secondary antibodies to the alkaline phosphatase (for ECF) are used in the dilution of **1:10000**

- Wash 3X, during 10 min, the membranes, in TBS-T 0.5% with shaking

5. Revelation

- Use 800 μL of the reagent ECF for membrane, add drop by drop the ECF to a plastic sheet, on top of it put the membrane, and incubate during 5 min

- Capture the images of blots with the Molecular Imager FX Pro Plus MultiImager system, and carry out densitometric analysis of the immuno detected proteins with the software Quantity One™ (Biorad)

Solutions

Sample buffer

100 mM Tris, 100 mM glycine, 4% SDS, 8M urea, 0.01% bromophenol blue

Electrophoresis buffer

25 mM Tris, 192 mM glycine; 0.1% SDS

Electrotransfer buffer (CAPS)

Final solution - CAPS 10 mM in 10% methanol

CAPS (stock 10X)

100 mM CAPS, pH 11- 22.1 g/ L CAPS

TBS 10X, pH 7.6

1L; 24.2g Tris (200 mM); 80 g NaCl (1.37 M)

TBS-T 0.5% (1L); wash solution

100 mL TBS 10X

1 mL Tween (0.1%)

TBS-T 5% (100mL); Blocking solution

5 g of milk

100 mL TBS (0.1%)

100 μL Tween

TBS-T 1% (20 mL/ membrane); Solution for dilution of the antibodies

2 mL TBS

20 uL Tween 20

Sodium azide 0.02%

3) Immunohistochemistry

1. Removal of Paraffin and Rehydration

- Transfer to a xylene bath and perform two changes of xylene for 5 min each
- Shake off excess liquid and rehydrate slides in two changes of fresh absolute ethanol for 3 min each
- Shake off excess liquid and place slides in fresh 90% ethanol for 3 min
- Shake off excess liquid and place slides in fresh 80% ethanol for 3 min
- Rinse the slides gently in running tap water for 30s (avoid a direct jet which may wash off or loosen the section)

2. Antigen Retrieval - Unmasking of Antigen

Note: This step is performed only in cases where weak or no staining occurs, or for antigens requiring "unmasking" according to the primary antibody specifications.

Enzyme retrieval: Apply 0.1% trypsin in PBS or 0.1% protease in PBS for 2-30 min at 37 °C. Extending the incubation time may also enhance specific staining. Rinse in PBS for 10 min.

3. Inactivation of Endogenous Peroxidase

- Place the slides on a flat level surface. Do not allow slides to touch each other. Do not allow the sections to dry out at any time
- Add enough drops of 3% hydrogen peroxide to cover the whole section
- Incubate 5 min at room temperature
- Rinse with PBS from a wash bottle
- Place the slides in PBS wash bath for 2 min

4. Primary Antibody Reaction

Note:

a. Pre-incubation of the sample with 5% BSA for 10 min prior to the primary antibody reaction may decrease background staining. For best results with animal tissues, use 5 to 10% normal serum from the same species as the host of the secondary antibody

b. Optimal dilution and incubation time should be determined for each primary antibody prior to use

- Allow the slides to drain, shake off excess fluid with a brisk motion and carefully wipe each slide around the sections

Dilute the primary antibody or negative control reagent to its optimal dilution in dilution solution (PBS). The dilution solution alone may be used as a negative control, in addition a negative control using the neutralized antibody must be used (Preabsorption of the antibodies was carried out by incubation with a five fold, by weight, excess of the corresponding blocking peptide in PBS, overnight at 4ºC).

- A positive control slide (a tissue known to contain the antigen under study) should also be run.
- Apply 100 µL primary antibody solution to the appropriate slides, covering the tissue sections
- Tilt each slide in two different directions, so the liquid is spread evenly over the slide
- Incubate for at least 60 min at 37 °C in a humidified chamber. Longer incubations are advised for low abundance antigens
- Rinse gently with PBS from a wash bottle. Place the slide in a PBS wash bath for 5 min

5. Secondary Antibody Reaction

Biotin/ExtrAvidin Detection

- Allow the slides to drain, shake off excess fluid and carefully wipe the slide as before
- Dilute the biotinylated secondary antibody in diluent to its optimal concentration
- Apply 100 µL to each slide, covering the tissue sections
- Tilt each slide in two different directions
- Incubate in a humidity chamber for at least 30 min, at room temperature
- Rinse gently with PBS from a wash bottle
- Place the slide in a PBS wash bath for 5 min.

6. Development

- Allow each slide to drain. Shake off excess fluid and carefully wipe the slide as before
- Apply enough drops of freshly prepared substrate mixture to cover the tissue section
- Incubate 5-10 min or until the desired colour is observed, when monitored with a microscope. Terminate the reaction before background staining appears in the negative controls by rinsing gently with distilled water from a wash bottle

7. Counterstaining

Note: When using AEC substrate, do not use alcohol-containing solutions for counter-staining (e.g., Harris' hematoxylin, acid alcohol), since the AEC stain formed by this method is soluble in organic solvents. The slide must not be dehydrated, brought back to toluene (or xylene), or mounted in toluene-containing mountants

- Apply enough Mayer's hematoxylin to cover the section or place the slide in a bath of Mayer's hematoxylin
- Incubate for 0.5-5 min, depending on strength of the hematoxylin used
- Rinse the slide gently with distilled water from a wash bottle
- Rinse the slide under gently running tap water for 5 min (avoid a direct jet which may wash off or loosen the section)
- Mount the sections using aqueous mounting medium such as glycerol or gelatin. Coverslip may be sealed with clear nail polish