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Spatiotemporal expression of androgen receptors in the female rat brain during the oestrous cycle and the impact of exogenous androgen administration: A comparison with gonadally intact males

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ABSTRACT

Little is known about the regulation and cellular distribution of androgen receptors (ARs) in female rodent brains at various stages of the oestrous cycle. This information is critical for further studies of androgen signalling in the regulation of brain function under physiological and pathophysiological conditions. In this report, we show that the distribution of AR immunoreactivity in the female rat brain is consistent with reported *AR* mRNA hybridisation signals in the male brain, except for the dentate gyrus of the hippocampus. Immunohistochemical and Western blot analyses performed herein revealed that the onset of region-specific changes in AR proteins was strongly correlated with circulating and ovarian levels of estradiol and testosterone across the oestrous cycle. During the metestrus and diestrus stages, however, the highest levels of AR expression were abolished by chronic dihydrotestosterone (DHT) treatment. This demonstrates that fluctuations in endogenous androgens are required for the regulation of AR expression in the female rat brain. Colocalisation studies revealed that: (1) anatomical variations in AR protein localisation existed between female and male brains, (2) AR immunoreactivity was both neuronal and non-neuronal, and (3) AR protein expression was lower in female rat brains at all stages of the oestrous cycle compared to age-matched males. Our results indicate the presence of regional sex differences in AR expression and changes in the proportion of AR between different subcellular compartments. Furthermore, DHT was found to down-regulate the level of AR in the subcellular compartment in females in a region-specific manner. As a whole, the present study provides the first step toward understanding the dynamics of AR expression and regulation in the brain during normal physiological conditions and for differences in neuronal androgen effects based on sex.

1. Introduction

Androgens play a pivotal role in the dynamic regulation of reproduction in both males and females (Davison and Bell, 2006; Quigley et al., 1995). Moreover, androgens are potent endogenous neuromodulators that operate in the central nervous system (CNS). They are vital for the regulation of brain development, sexual differentiation and behaviour (Hines, 2008; Wilson and Davies, 2007). The clinical relevance of central androgen signalling for female health is implied by findings that androgen replacement therapy can improve depressive states (Amiaz and Seidman, 2008; Rohr, 2002) and spatial memory in women (Postma et al., 2000). Androgens are also important for the regulation of female sexual behaviour (Van Goozen et al., 1997). In addition, regional brain hypometabolism in women with anorexia nervosa, which has the highest death rate of any psychiatric disorder, can be attenuated by testosterone (T) replacement (Miller et al., 2004, 2005). Androgens are also perturbed in certain neurological pathologies, most notably in Alzheimer's disease (Rosario and Pike, 2008), although

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the precise aetiology of these diseases remains unknown. Therefore, it is important to elucidate the neurobiological mechanism of central androgen signalling for both normal physiology and androgen-related pathophysiology in females. Understanding this mechanism may lead to new approaches that may prevent and/or treat psychiatric and neurological disorders.

Cellular responsiveness or susceptibility to androgens is primarily mediated by the classical intracellular androgen receptor (AR). The AR is a ligand-regulated transcription factor, comprised of a central DNA binding domain, nuclear localisation signals, a ligand-binding domain, and several transactivation function domains (Gao et al., 2005). In the absence of a ligand, the AR is part of a large protein complex that interacts with heat shock proteins (hsp). Following ligand binding, the AR undergoes a conformational change and translocates into the nucleus. In so doing, it communicates with the basal transcriptional machinery, co-regulators, other transcription factors, and DNA, thereby modulating the expression of many target genes, largely in a cell- or tissue-specific context (Patchev et al., 2004). AR-knockout mouse models have been developed to define the physiological effects of androgen signalling through its cognate receptor (Kerkhofs et al., 2009). In male mice, *AR* gene disruption leads to the elimination of male-specific sexual and aggressive behaviour. There is evidence that neuroendocrine response alterations are linked to the disruption of the *AR* gene for female mice as well (Walters et al., 2009). However, developmental compensatory mechanisms in the AR-knockout mouse model may obstruct the distinction between the direct and indirect effects of AR loss in the androgen-target tissues/cells *in vivo*. Recently, mice with a brain-specific AR deletion were generated, providing evidence for a vital and distinctive role for ARs in the CNS (Raskin et al., 2009). Although the relevance of central AR signalling in female mutants has not been reported, studies in brain selective-AR-knockout mice show that mutant males exhibit impaired neuroendocrine dysfunction and behavioural changes. These changes occur in parallel with increased circulating T and its non-aromatisable metabolite, dihydrotestosterone (DHT) (Raskin et al., 2009). While the importance of ARs toward brain function appears clear, our mechanistic understanding of direct androgen actions in the female brain remains insufficient. In part, this is due to the presence of higher androgen levels in males. To date, there is only a small amount of compelling evidence for the cellular localisation and regulation of the AR protein in female rodent brains during various stages in the oestrous cycle. The intracellular distribution of ARs has generally been demonstrated in the past using several brain regions of males and immature females during development (Bakker et al., 1997; Bingham et al., 2006; DonCarlos et al., 2003, 2006; Herbison, 1995; Kritzer, 2004; Kritzer and Creutz, 2008; Leranth et al., 2004; Lorenz et al., 2005; McAbee and DonCarlos, 1999b; Nunez et al., 2003; Ravizza et al., 2002; Tabori et al., 2005; Williamson and Viau, 2007).

A previous report has shown that the T levels correlate significantly with regional brain activation in women with a normal menstrual cycle (Schoning et al., 2007). This enables the speculation that AR expression in different brain regions of rodents would be affected by fluctuations in gonadal steroid hormone levels across the oestrous cycle. Differences in brain function based on sex, as attributed to gonadal steroid hormones, have long been recognised (Patchev et al., 2004). Several lines of evidence suggest that the regulation of AR expression in specific brain regions is sex dependent. For example, regional sex differences in AR expression have been observed in the rat brain, during postnatal development (McAbee and DonCarlos, 1998, 1999b; Nunez et al., 2003; Ravizza et al., 2002), and in the human hypothalamus (Fernandez-Guasti et al., 2000). Although decreases in brain androgen levels have been found for both women and men with Alzheimer's disease (AD) (Rosario et al., in press), the expression of cytoplasmic AR has been shown to significantly decrease only in AD women (Ishunina et al., 2002). Moreover, the milieu of gonadal steroid hormones induces sex-specific forms of behaviour (Dulac and Kimchi, 2007). These may be due to structural differences in the brain between the two sexes (Fernandez-Guasti et al., 2000), as well as to varying levels and secretion patterns between females and males (Davison and Bell, 2006; Quigley et al., 1995). Although the molecular mechanism that mediates the tissue selectivity of AR modulators are still speculative, JNJ-28330835, a nonsteroidal AR modulator, has been shown to enhance the sexual preference for intact male rats over nonsexual orchidectomized males in ovariectomized female rats (Allan et al., 2007). These results further highlight the need to explore sex-specific, AR-dependent function in adult female rodent brains. The purpose of this research was to investigate (1) the variation in AR localisation and protein expression in the female rat brain at different stages of the oestrous cycle; (2) the levels of T in serum and selected tissues (ovary and androgen-target regions of the brain) in female cyclic rats; and (3) the differential distribution of AR protein expression in the brain of adult female and male rats treated for 7 days with DHT. While mammalian brain tissue holds the answers to many interesting biological questions regarding androgen-related effects, our mapping study provides a detailed representation of the *in vivo* pattern of AR expression and its regulation in the brain of both sexes.

2. Materials and methods

2.1. Experimental animals and treatment

All experimental procedures and protocols were approved by the ethics committee at Gothenburg University. Adult (13–14 weeks) female (body weight = 239 ± 17 g) and male (body weight = 284 ± 18 g) Sprague–Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were housed individually in polycarbonate plastic cages with wood chip bedding in a room under defined conditions (i.e., temperature = 21 ± 2 °C, relative humidity = 45–55%, 12-h light/dark illumination schedule) in the pathogen-free animal facility of the Department of Experimental Biomedicine. All animals had free access to tap water and standard laboratory chow *ad libitum* (Teklad global 16% protein rodent diet, 2016, Harland). The chow did not contain alfalfa or soybean meal, thus minimising access to natural phytoestrogens. To examine androgenic effects on AR expression in the brain, female rats (n = 6–9 per group) received either a subcutaneous injection of dihydrotestosterone (DHT, daily dose = 1.66 mg/kg, Sigma, St. Louis, MO, USA), or vehicle (sesame oil) for 7 days. DHT was dissolved in sesame oil and injected intraperitoneally (200 μl/rat). All rats were injected at the diestrus stage to exclude variations from the oestrous cycle. In addition, male rats (n = 6 per group) were handled in a manner similar to that of females during vaginal smear determination. Male rats were injected s.c. with DHT using the same concentration as for the female group. The pharmacological dose of DHT selected was previously found to increase spine synapse density in the female rat hippocampus (Leranth et al., 2004). DHT, a major metabolite of T, is regarded as a more potent androgen than T because of its higher affinity to AR *in vivo* (McLachlan et al., 1996) and its lack of aromatisation into oestrogen (Davison and Bell, 2006). Animals were monitored daily during the experiment for continued cyclicity. In rats, DHT suppressed the incidence of regular oestrous cyclicity during the treatment period. Rats were killed 24 h after the last injection.

2.2. Oestrous cycle evaluation

Stages of the oestrous cycle (diestrus, proestrus, oestrus and metestrus) in female rats (Marcondes et al., 2002) were determined using daily vaginal smears collected between 9:00 a.m. and 10:00 a.m. Cycles were followed for at least 2 weeks before animal treatment and tissue collection. Sequences of oestrous cytology interrupted by at least 2 days of metestrus and diestrus were recorded as a single cycle. Cycles were considered prolonged if the rats remained in one stage for >3 days and acyclic if rats remained in one stage for >15 days. Only rats with at least two consecutive 4- or 5-day oestrous cycles (n = 8–12 per stage) were considered to be regulatory cycling and were used in the present study. Female rats were killed after detecting the occurrence of an oestrous cycle. The vaginal smears were stained with hematoxylin and eosin (H&E) using standard protocols and staged by light microscopy. Care was taken to avoid mechanical stimulation of the cervix during this procedure to prevent pseudopregnancy. Ovarian and uterine tissues, as well as serum were collected for measurement of the respective steroid hormones or for histological analysis.

2.3. Antibodies and reagents

The primary antibodies that were used for either immunohistochemistry (IHC) or Western blot (WB) assays included: rabbit anti-AR (sc-816, 1:200 for IHC, 1:250 for WB, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), mouse anti-α-tubulin (B-5-1-2, 1:1000 for WB, Sigma), rabbit anti-Histone H3 (9701, 1:100 for WB, Cell Signalling Technology, Danvers, MA, USA), and Alexa 488 (green light emitter) conjugated mouse anti-neuronal nuclei (NeuN, MAB377X, 1:100 for WB, Millipore, Billerica, MA, USA). The optimal working dilution was based on different preliminary experiments at the various dilutions reported herein. IHC and WB are critically dependent upon the choice and careful characterisation of the antibody to be used. The antibody used to detect AR in this study was raised against human AR and recognised its ligand-binding domain (aa: MEVQLGLGRVYPRPPSKTYRG) in rodent tissues including the rat brain (Menard and Harlan, 1993). Competition with a specific antigen (usually a synthetic peptide) is the typical approach used to establish the specificity of an antibody. Therefore, the AR antibody used here was evaluated in an antigen adsorption experiment with its respective blocking peptide (sc-816p, Santa Cruz) (Shao et al., 2007a) and in AR-knockout mouse tissues using IHC and WB analyses *in vivo* (De Gendt et al., 2005; Raskin et al., 2009; Sato et al., 2004; Shiina et al., 2006). The same antibody was previously utilised for the detection of AR in human (Adachi et al., 2005; Puy et al., 1995), rat (Creutz and Kritzer, 2004; DonCarlos et al., 2003; Kritzer, 2004; Lorenz et al., 2005; Milner et al., 2007; Williamson and Viau, 2007) and mouse (Loyd and Murphy, 2008; Meyer et al., 2006; Sheng et al., 2004) brains. Alexa 594 (red light emitter) donkey anti-rabbit IgG (Molecular Probes Inc., OR) was used for immunofluorescence assays. Alkaline phosphatase conjugated goat-anti-mouse IgG (A-1682, Sigma) and alkaline phosphatase conjugated goat-anti-rabbit IgG (AC31RL, Tropix, MA, USA) were used as secondary antibodies for WB analyses. Other reagents were purchased from Sigma or Merck AG (Darmstadt, Germany) and were of the highest purity grade available.

2.4. Tissue preparation for IHC analysis

Rats were deeply anesthetised with thiobutabarbital sodium (130 mg/kg i.p.; Inactin, Sigma) and perfused transcardially with 200 ml of cold physiological saline, followed by 100 ml of Histofix (Histolab, Sweden). The brains were dissected out and post-fixed in Histofix containing 20% sucrose for 48 h at 4 °C. Subsequently, this was performed in 0.1 M PBS containing 30% sucrose for at least 24 h at 4 °C. Finally, the brains were sectioned into frontal slices (20 μm) by means of a vibratome (LEICA CM3050S, Leica Microsystems, Nussloch GmbH, Germany). Frontal sections were serially sectioned and stored in tissue culture wells containing 30% sucrose and 30% ethylene glycol in 0.1 M PBS (pH 7.4) at −20 °C, until the assay was performed. In this study, we used perfusion fixation, rather than postmortem immersion, to rapidly immobilize neuronal proteins.

2.5. IHC analysis

Both immunoperoxidase and dual-immunofluorescence labelling was essentially carried out as described previously (Feng et al., 2009; Shao et al., 2007b). For a single IHC assay, free-floating brain sections (20 μm) were washed three times at 5 min each in Tris-buffered saline (TBS; 50 mM Tris, 0.9% NaCl, pH 7.5). Endogenous peroxidases and nonspecific binding were removed by pre-incubation with 3% H_2O_2 for 30 min, 0.5% Triton X-100 for 10 min at room temperature, and 10% normal horse serum for 1 h at 37 °C. Sections were then incubated with primary antibody for 1 h at 37 °C, followed by overnight incubation at 4 °C. After washing, the sections were incubated with the appropriate biotinylated secondary antibody for 1 h at 37 °C. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for the avidin–biotin peroxidase complex (ABC) detection system, according to the manufacturer's instructions. Antigens were visualised by reaction with the chromogen 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Sigma) and hydrogen peroxidase for 1 min. Sections were imaged on an Olympus DP50 microscope (Japan) under bright-field optics and photomicrographed using Image-Pro plus software (version 5.0, Media Cybernetics Inc., MD, USA). Because the brain tissue that expressed AR contained a diverse population of cells, the dual-fluorescence IHC of AR and NeuN was used to determine if neurons actually expressed AR. Sections were washed with TBS and incubated in goat normal serum for 2 h at 37 °C to minimise nonspecific binding. Tissues were then incubated with two different primary antibodies in 1× TBS supplemented with 0.05% Triton X-100 (TBST) for 1 h at 37 °C, followed by overnight incubation at 4 °C. After washing in TBST for 5× 10 min, sections were incubated with secondary antibody for 1 h at 37 °C and washed in TBST for 5× 10 min. Sections were mounted using Vectashield mounting medium, which contains 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories), and covered by coverslips. Sections were viewed either on an Olympus DP50 microscope using Image-Pro plus software or on an Axiovert 200 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss), and all sections were photomicrographed. Rat testis tissue served as a positive control for AR. Additional control studies were performed to further validate results. To this end, all IHC and immunofluorescence assays included the following: (1) substitution of primary antibodies with nonimmune serum; (2) comparison of primary antibodies with multiple dilution strategies to obtain optimal staining in the brain sections; (3) substitution of secondary antibodies alone; and (4) comparison between two secondary antibody detection systems. Prolonged incubation in DAB did not reveal AR translocation in brain sections. Changes in detection methods did not affect the cellular distribution pattern. To enable the comparative evaluation of immunostaining, brain sections from animals at different stages of the oestrous cycle, or in the same experimental setting for different treatments, were processed concurrently. As a large number of brain tissues were processed, the animals were divided into test groups containing at least one animal from each cycle stage. Two of the groups included sections from intact female and male rats treated with or without DHT. For each run, incubation times at every instance were strictly controlled. The cellular localisation and a qualitative estimate of the relative number of AR-positive cells were evaluated by conventional light microscopy. The assigned grades were: – (absent), + (1–10 positive cells), ++ (11–100 positive cells), or +++ (>100 positive cells). Variability between IHC replications was controlled by having an equal number of animals for each group (three sections/region) in each IHC run. Care was taken to ensure that the area of regions selected for comparison did not differ between sections and subjects. For the determination of AR-density, the maximal background intensity observed in control sections (no primary antibody) was set prior to the morphological analysis. Only sections immunostained in the same experiment, which provided comparable time of DAB exposure, were evaluated to avoid interexperimental variability. The rat brain atlas in stereotaxic coordinates (Second Edition by George Paxinos and Charles Watson) was used as a guide to identify different brain regions. Two different observers, who were blinded to the hormonal state of the animals, performed the quantification.

2.6. Tissue/cell fractionation

Subcellular fractionation of tissue was performed using a previously described protocol, with minor modification (Shao et al., 2004). Briefly, frozen tissues ($n = 5–7$ per stage) were lysed in ice-cold buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 0.5 $MgCl_2$, 1% Nonidet P-40 (vol/vol), 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor mixture according to the manufacturer's instructions (Roche Diagnostics). The homogenate was left on ice for 5 min and then centrifuged at 800 × *g* for 10 min. The supernatant contained cytosolic and membrane proteins, and the pellet contained nuclear proteins. The supernatant was centrifuged at 165,000 × *g* for 1 h to obtain the cytosolic fraction with minimal contamination from membrane proteins. Concurrently, the pellet was completely solubilised in an ice-cold nuclear isolation buffer containing 10 mM Tris–HCl (pH 7.8), 1% Nonidet P-40 (vol/vol), 0.5 mM PMSF, 5 mM NaF, 10 mM beta-mercaptoethanol, and a protease inhibitor mixture. Nuclear proteins were recovered by centrifugation at 13,000 × *g* for 1 h at 4 °C and washed once using the nuclear isolation buffer described above. Protein content in tissue lysates and subcellular fractions was determined using a Spectramax Plus fluorometer (Molecular Devices, Sunnyvale, CA, USA) with BSA as the standard. All homogenate samples were stored at −80 °C.

2.7. Electrophoresis and quantitative WB analysis

Total tissue/cell lysates and subcellular fractions (cytosolic and nuclear) were analysed for AR protein expression by WB analysis as described previously (Shao et al., 2004, 2007a). Equal amounts of protein were directly electrophoresed on 4–12% one-dimensional Bis–Tris gels (Novex, San Diego, CA, USA). When ARs were assessed, the gel was cut into two pieces along a line corresponding to approximately 61 kDa. The section containing higher molecular weights was exposed to anti-AR antibody, while the lower molecular weight section was exposed to anti-α-tubulin antibody, as a loading control. In parallel, the gels were stained with Coomassie blue to confirm equal loading per lane. To further check the purity of cytosolic and nuclear preparations, the expression of histone 3, a marker specific for cell nuclei, was analysed by WB analysis under reducing conditions. The immunosignal-CDP-Star substrate for alkaline phosphatase systems (Tropix, Bedford, MA, USA) was used to visualise the protein bands. Immunoblotted signals were visualised using a LAS 1000-cooled charge-coupled device camera (Fujifilm, Tokyo, Japan) and ECL-film (Amersham Int). Individual bands were quantified directly from membranes by densitometry using the ImageQuant (version 5.0) software program (Molecular Dynamics, Sunnyvale, CA, USA). Tubulin was used for normalisation between samples.

2.8. Assessment of ovarian and circulating hormones

Circulating, intraovarian and brain tissue estradiol (E2), progesterone (P4), and T levels were determined with radioimmunoassay kits according to protocols provided by the manufacturer (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland).

2.9. Statistics

Grouped results from at least five different animals were expressed as the mean ± SEM. Data were analysed with SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). Results were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. Significance was accepted at the 0.05 level of probability.

3. Results

3.1. Determination of cyclicity

Uterine tissue collected from each stage of the rat estrous cycle was examined by H&E staining. Representative sections are shown in the upper panel of Fig. 1A. Oestrous cycle stages for intact females were verified by examining the cytology of vaginal smears (Becker et al., 2005) and representative sections showing polymorphonuclear leukocytes, nucleated vaginal epithelial cells, and keratinized epithelial cells, which indicate the four stages of the cycle (Fig. 1A, lower panel). As expected, the levels of ovarian and circulating estradiol, progesterone, and testosterone in female rats exhibited clear periodic changes across the oestrous cycle (Fig. 1B and C). After careful assessment, rats with organised oestrous cycles were grouped according to cyclic stage for the following experiments.

3.2. Changes in AR expression: comparison between stages of the oestrous cycle

To investigate the expression of ARs in the female rat brain during the estrous cycle, adult rats were divided into four groups according to anatomically matched sections of the uteri and the cytology of vaginal smears. The distribution patterns of AR in the brain across different stages of the oestrous cycle are summarised in Table 1. Notably, in the retrosplenial agranular (RSA) and retrosplenial granular cortex (RSG) (Fig. 2A), paraventricular (PV; Fig. 2B), medial preoptic area (MPO; Fig. 2C), and nucleus of the solitary tract (Sol; Fig. 2D), both the numbers of AR-positive cells and the density of AR immunoreactivity varied across the oestrous cycle. In general, the lowest immunoreactivity of AR was found in the oestrus stage, whereas the highest immunoreactivity of AR was seen in the diestrus (Fig. 2A and A1) and metestrus (Fig. 2B–D) stages in a region-dependent manner. In the nuclear trapezoid body (TZ), the density of AR immunoreactivity changed across the oestrous cycle (Fig. 2E). Prolonged DAB reaction did not result in enhancement of specific staining, but instead increased nonspecific background staining. In addition, there was no detectable staining when AR antibodies were replaced by nonimmune serum or pre-incubated primary antibodies with their corresponding antigen peptide (data not shown).

To confirm the IHC, showing differences of AR immunoreactivity across the oestrous cycle in different regions of the rat brain, Western blot experiments were performed in selected regions of the brain that were collected at different stages of the oestrous cycle (Fig. 3). Immunoblotting experiments using an anti-AR antibody

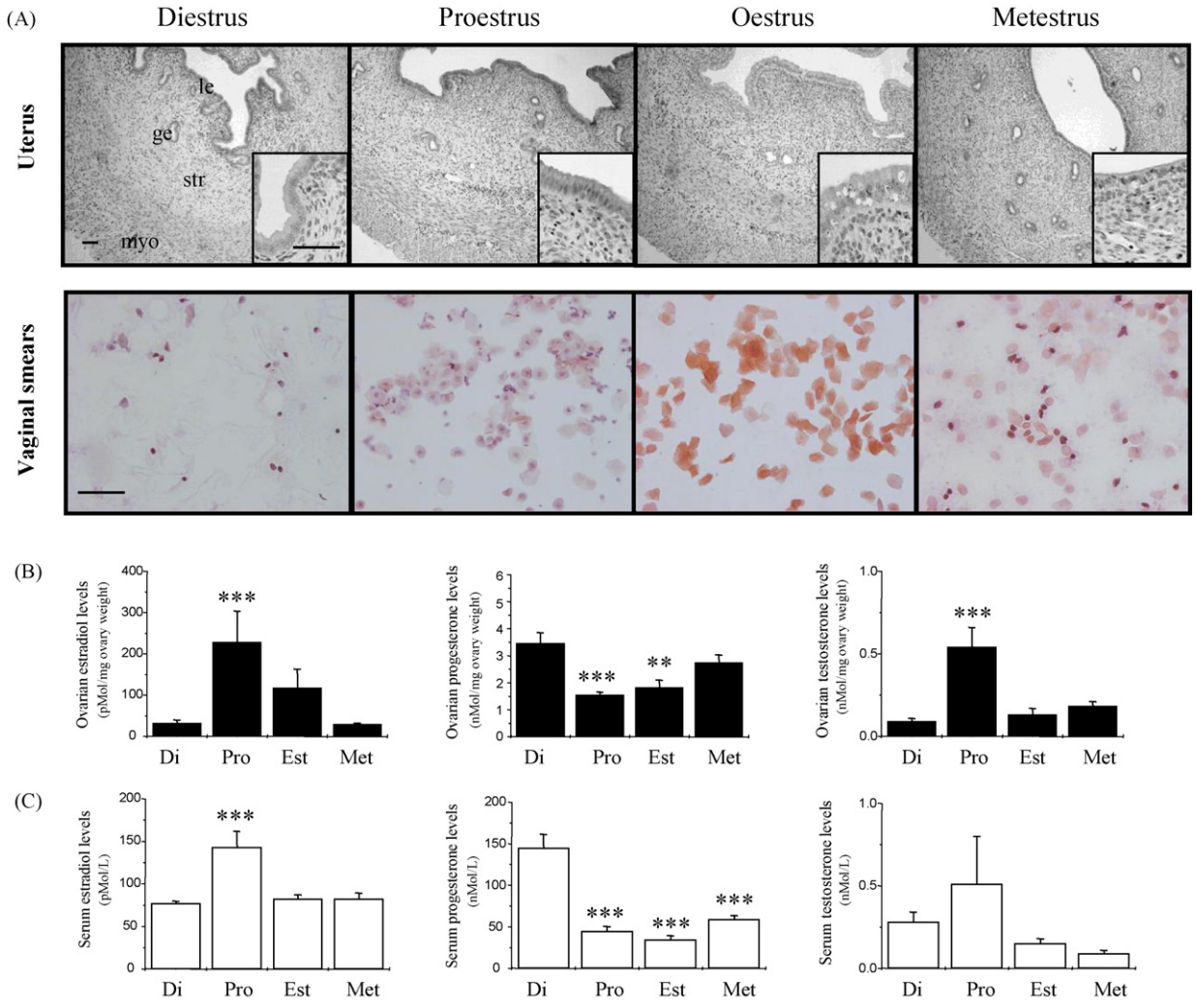

Fig. 1. Histological, cytological, and endocrine correlations of the oestrous cycle for adult female rats. Rats were divided into four groups (Di, diestrus; Pro, proestrus; Est, oestrus; and Met, metestrus), based on uterine morphology and vaginal cytology (A). Insets showing stage-dependent changes in the height of luminal epithelial cell layers during the oestrous cycle. le, luminal epithelial cells; ge, glandular epithelial cells; str, stromal cells; and myo, myometrium. Scale bar = 100 μm. Profiles of ovarian (B) and circulating (C) sex steroid hormones, including estradiol, progesterone, and testosterone, during the oestrous cycle. Data are presented as the mean ± SEM (n = 5–7 per group). Significance of differences relative to hormone levels at diestrus. $^{**}p < 0.01$; $^{***}p < 0.001$.

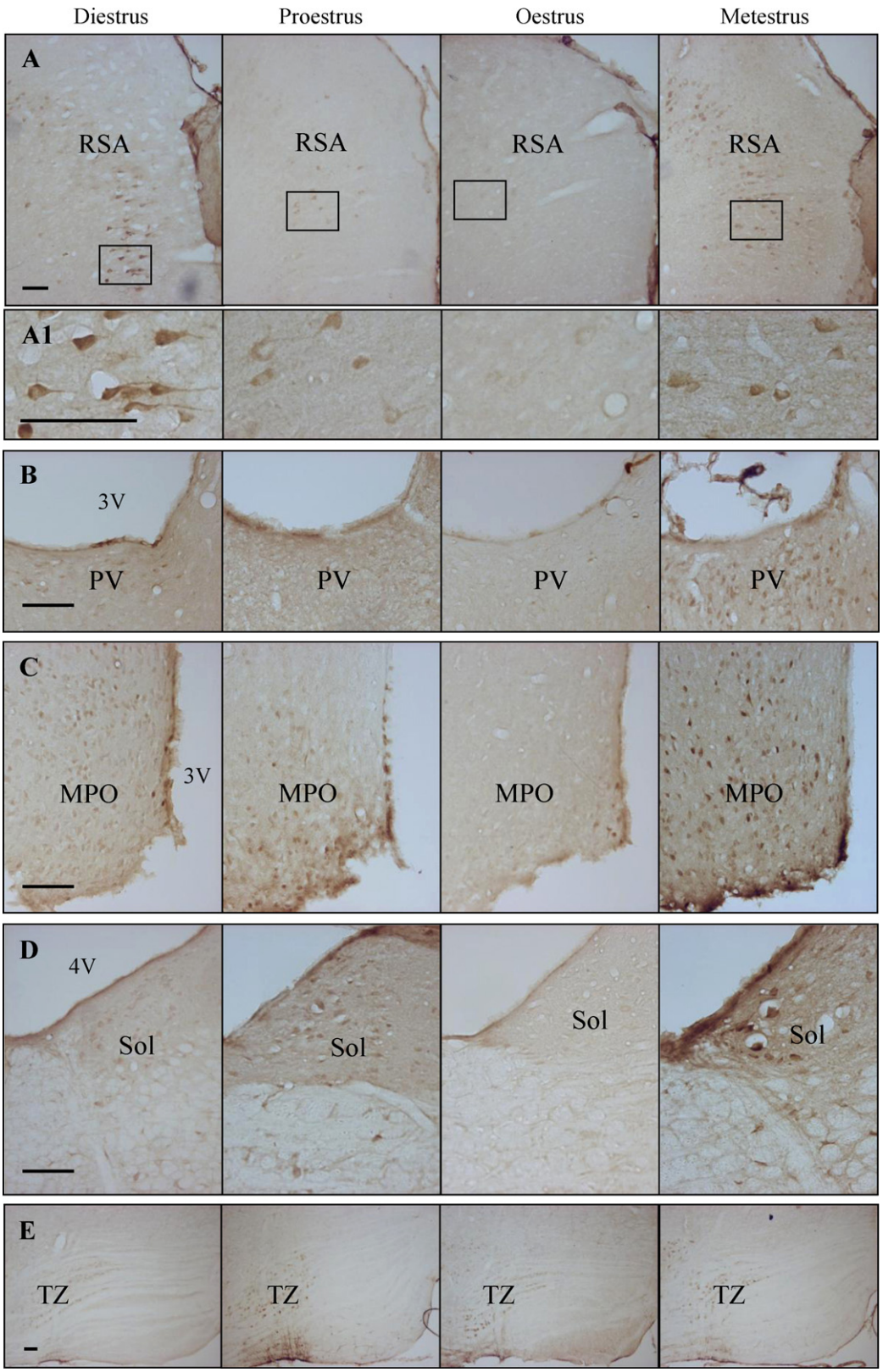

Fig. 2. Immunohistochemical distribution of AR in selected brain regions of female rats during the oestrous cycle. Immunoperoxidase staining of AR was performed on 20-μm thick brain sections showing the retrosplenial agranular (RSA) and retrosplenial granular cortex (RSG), paraventricular (PV), medial preoptic area (MPO), nucleus of the solitary tract (Sol), and nuclear trapezoid body (TZ). AR-positive cells were rarely detected in the RSA/RSG, PV, MPO, and Sol at the oestrus stage, whereas AR immunoreactivity in those regions was strong at the metestrus and diestrus stages. Images in (A1) are at higher magnifications than (A). Scale bar = 100 μm. These experiments were repeated using three rats/group with similar results. 3 V, third ventricle; 4 V, fourth ventricle.

Table 1
Distribution of AR-immunoreactive cell bodies/nuclei in rat central nervous system.

	Female				Male	
	Diestrus	Proestrus	Oestrus	Metestrus	Vehicle	DHT
FOREBRAIN						
Olfactory bulb						
External plexiform layer (EPI)	++	+	++	++	++	++
Anterior olfactory nu, lateral (AOL)	++	+	+/++	++	++	++
Lateral olfactory tract (lo)	++	−/+	+	+	+/++	+
Isocortex						
Cingulate (Cg)	++/+++	++	+	++	+/++	−
Frontal cortex (Fr)	++/+++	++	+/++	++	++	++
Striatum						
Caudate putamen (CPu)	++	++	−	++	++/+++	++
Diagonal band of Broca						
Horiz limb diagonal band (HDB)	++	++	−	++	−	+
Ventral pallidum (VP)	+	−	−	++	++	+
Bed nucleus of stria terminalis (BST)	+	++	−	−	++	−
Amygdala						
Anterior amygdaloid area (AA)	+	−	+	+	+	−
Amygdalohippocampal nu (AHi)	+/++	−	+	+	+	−
Preoptic						
Lateral preoptic area (LPO)	++	+/++	−	++	+++	−
Medial preoptic area (MPO)	++	++	+	+++	+++	+
Hypothalamus						
Arcuate nu (Arc)	++	++	+/++	++	++	++
Paraventricular hypothalamic nu (PVN)	+	+	−	−/+	++	−
Ventromedial hypothalamic (VMH)	++	++	−	−/++	++	−
Supraoptic nu (SO)	+	+	−	++	+	−
Premammaliar nu, ventral part (PMV)	++	++	−	++	++	−/+
Median eminence (ME)	++	++	+	+	++	−
Hippocampus						
CA1	−	−/+	−	+/++	++	−/+
CA2	−	−/+	+	+/++	+	−/+
CA3	+	−/+	+	+	−/+	−/+
Dentate gyrus (DG)	+	−/+	+	+	+/++	+
Thalamus						
Paraventricular (PV)	+	+	−	++	+	+
Zona incerta (ZI)	++	++	++	++	++	+
Midbrain/pons/cerebellum						
Pontine retivilar nu (PnO)	++	−	−	++	++	++
Superior olive						
Dorsal periolivary region (DPO)	+	+	+	+/++	++	+
Suporior paraolivary nu (SPO)	+	+	+	+/++	++	+/++
Lateral superior olive (LSO)	+	+	+	+/++	++	+
Cerebellar lobules (CL)	++	++	+	++	++	++
Nu of the solitary tract (Sol)	++	++	−/+	++	++	+/++
Lateral reticular tract (LRt)	++	++	+	++	++	−/+
Nu trapezoid body (TZ)	++	+/++	+/++	++	++	++

−: absent; +: 1–10 immunoreactivity cells; ++: 11–100 immunoreactivity cells; +++: >100 immunoreactivity cells.
Abbreviation based on *The Rat Brain in Stereotaxic Coordinates (Second Edition)* by George Paxinos & Charles Watson.

detected a specific, ~97 kDa band, which is in agreement with previous data on human and rodent brains (Lu et al., 1998, 1999; Puy et al., 1995). A linear relationship with *R*-values ranging from 0.9 to 0.95 was established between the optical density of immunostained bands for AR protein and brain protein values ranging from 0 to 75 μg (Fig. 3A). The specificity of the AR immunoreactive band in the same brain tissue samples was demonstrated by preabsorbtion (Fig. 3A). Detailed Western blot analysis for the detection of AR in different regions of the female brain confirmed that AR protein was present throughout the cerebral cortex (CC), hippocampus (HIP), hypothalamus (HYP), amygdale (AMY), cerebellar cortex (CeC), and medulla oblongata (MO), which is in agreement with our immunohistochemistry data (Fig. 2 and Table 1). Quantitative Western blot analysis of whole tissue/cell lysates (Fig. 3B) corroborated the immunohistochemical data (Fig. 2, Table 1). This analysis demonstrated that the AR levels mirrored the number and density of AR-positive cells within the same brain region (e.g., CC, HYP, CeC and MO), but not in the HIP, at the different stages of the oestrous cycle (Fig. 3B). In the AMY, AR levels were slightly decreased during the proestrus stage, but this was not a significant effect (Fig. 3B). The expression of AR in the CC, CeC, and MO (Fig. 3B) suggested that the hormonal fluctuations associated with the oestrous cycle did not influence or participate in the regulation of AR expression in these brain regions. Furthermore, HIP and AMY testosterone concentrations during the diestrus and proestrus stages were significantly higher than those during the oestrus and metestrus stages. However, in the HYP, low levels of testosterone were only observed during the oestrus stages compared to all other stages across the

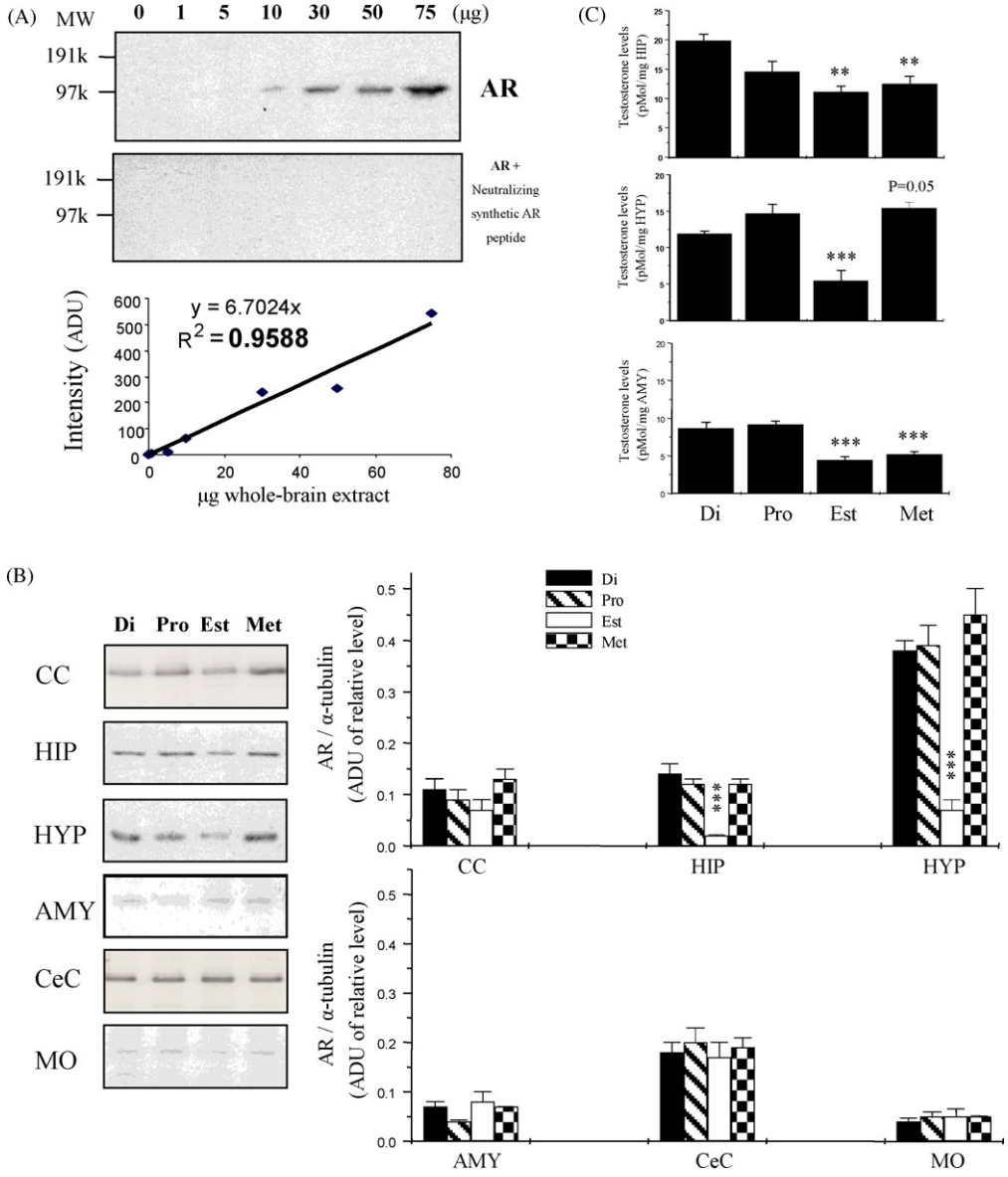

Fig. 3. Quantification of AR protein expression in selected brain regions of female rats by Western blot analysis. (A) Signals from AR immunoblot bands demonstrated that their intensity was linearly proportional to the amount of input tissue proteins from rat brain by a least squares linear regression analysis. Santa Cruz is cited as a source for tissue proteins from the rat brain. (B) Equal amounts of proteins from regional brain lysate were subjected to 4–12% one-dimensional Bis–Tris gels and immunoblotted with antibody against AR (left). The densitometric analysis of levels of AR protein expression is shown on the right. The quantification of AR and α-tubulin (an internal control) band intensities was made directly by densitometry using the ImageQuant software program. The bar graph shows the quantity of AR protein expression calculated by the densitometric value of AR bands divided by the densitometric value of α-tubulin. Data are expressed as arbitrary densitometric units (ADU) and represented as the mean ± SEM (n = 5–7 rats/group). Di, diestrus; Pro, proestrus; Est, oestrus; Met, metestrus; CC, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; AMY, amygdale; CeC, cerebellar cortex; and MO, medulla oblongata. (C) Testosterone levels in the HYP, HIP, and AMY regions of rats across the oestrous cycle. Values are represented as the mean ± SEM (n = 5–7 rats/group). Significance of differences relative to hormone levels at diestrus. $^{**}p < 0.01$; $^{***}p < 0.001$.

oestrous cycle (Fig. 3C). This suggests that the regulation of AR expression is region-specific and independent of T levels only.

3.3. Comparisons of AR expression between females and males

To determine sex differences of AR localisation in rat brains, immunofluorescence staining of AR was performed on both female (the highest level of AR in the metestrus) and male brains. Fig. 4 illustrates dual-fluorescence labelling for AR and NeuN antigens in several regions [dorsolateral outer cortex (DOC), dentate gyrus (DG) and PVN] of both female and male brains. In general, the proportions of AR-positive cells to total cells and AR immunoreactivity were much lower in females (Fig. 4A, C and E) than in males (Fig. 4B, D and F). At higher magnification, AR immunoreactivity was concentrated principally in the cytosol of NeuN-positive cells in female rats (Fig. 4A1), whereas some NeuN-positive cells exhibited cytosolic and/or nuclear labelling of AR in male rats (Fig. 4B1). Colocalisation with NeuN confirmed the AR expression in neuronal cells. No immunofluorescent signal was detected in negative control brain sections from either female or male rats that were processed with both the pre-incubated primary antibody and its corresponding antigen peptide (data not shown).

3.4. Regulation of endogenous AR in different subcellular compartments by DHT

To pinpoint the specific effects of androgens on AR expression in the different regions of brain, we determined if brain AR locali-

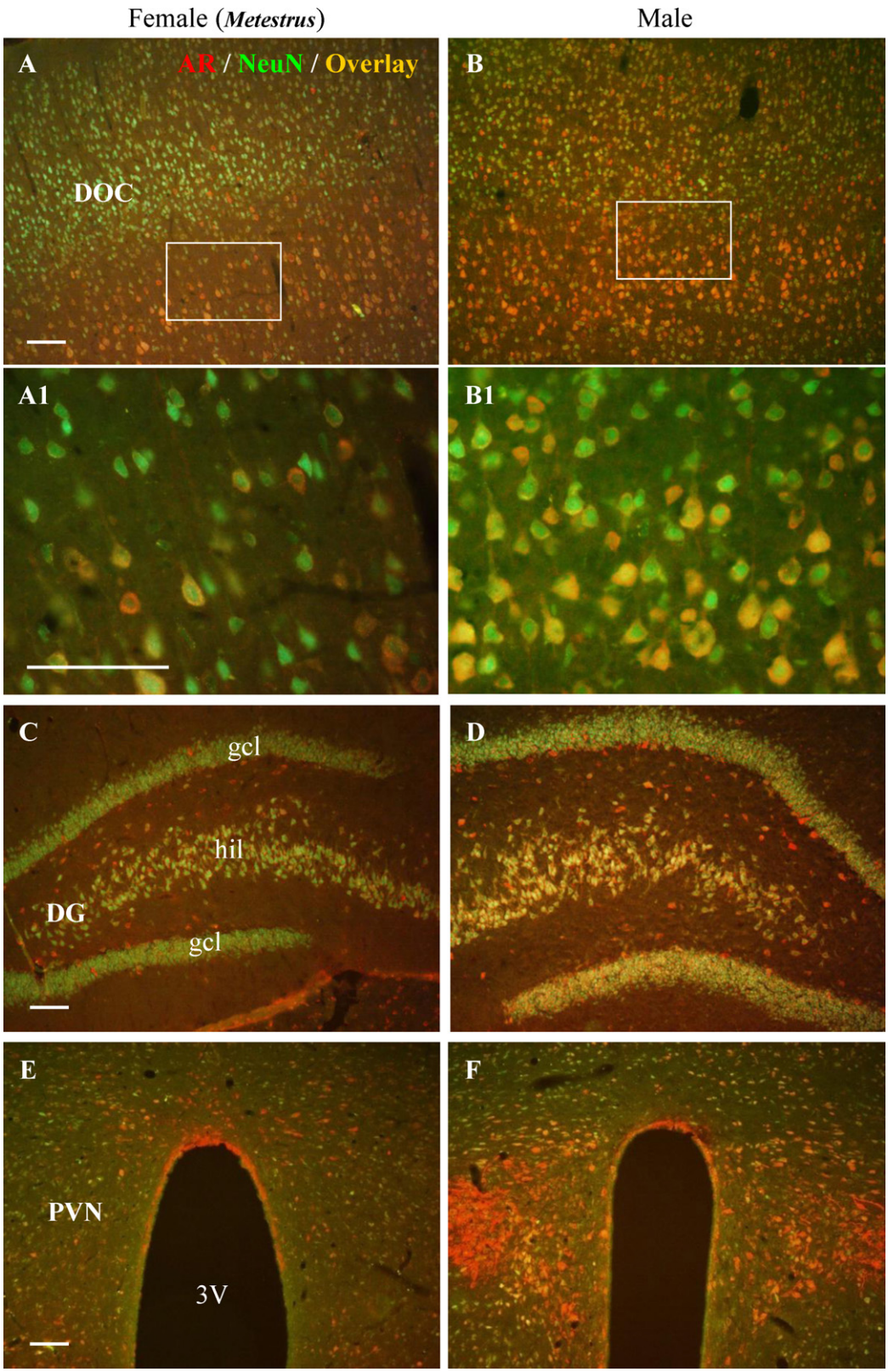

Fig. 4. Comparison of AR expression in female and male brains by immunofluorescence assay. Dual-immunofluorescence labelling of AR (red) and NeuN (green) was performed on 20-μm thick female (metestrus, left) and male (right) rat brain sections showing the dorsolateral outer cortex [DOC; (A) and (B)], the dentate gyrus [DG; (C) and (D)] and the paraventricular hypothalamic nucleus [PVN; (E) and (F)]. The proportions of AR-positive cells and AR immunoreactivity were much lower in female brains compared to male brains. Note that both cytoplasmic and nuclear AR immunofluorescence signals were detected. Images in (A1) are higher magnifications of (A). Scattered interneurons located within the infragranular regions of the hilus contained AR associated with their cell nuclei [(C) and (D)]. Scale bar = 100 μm. These experiments were repeated using three rats/group with similar results. 3 V, third ventricle; gcl, granule cell layer; hil, hilus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

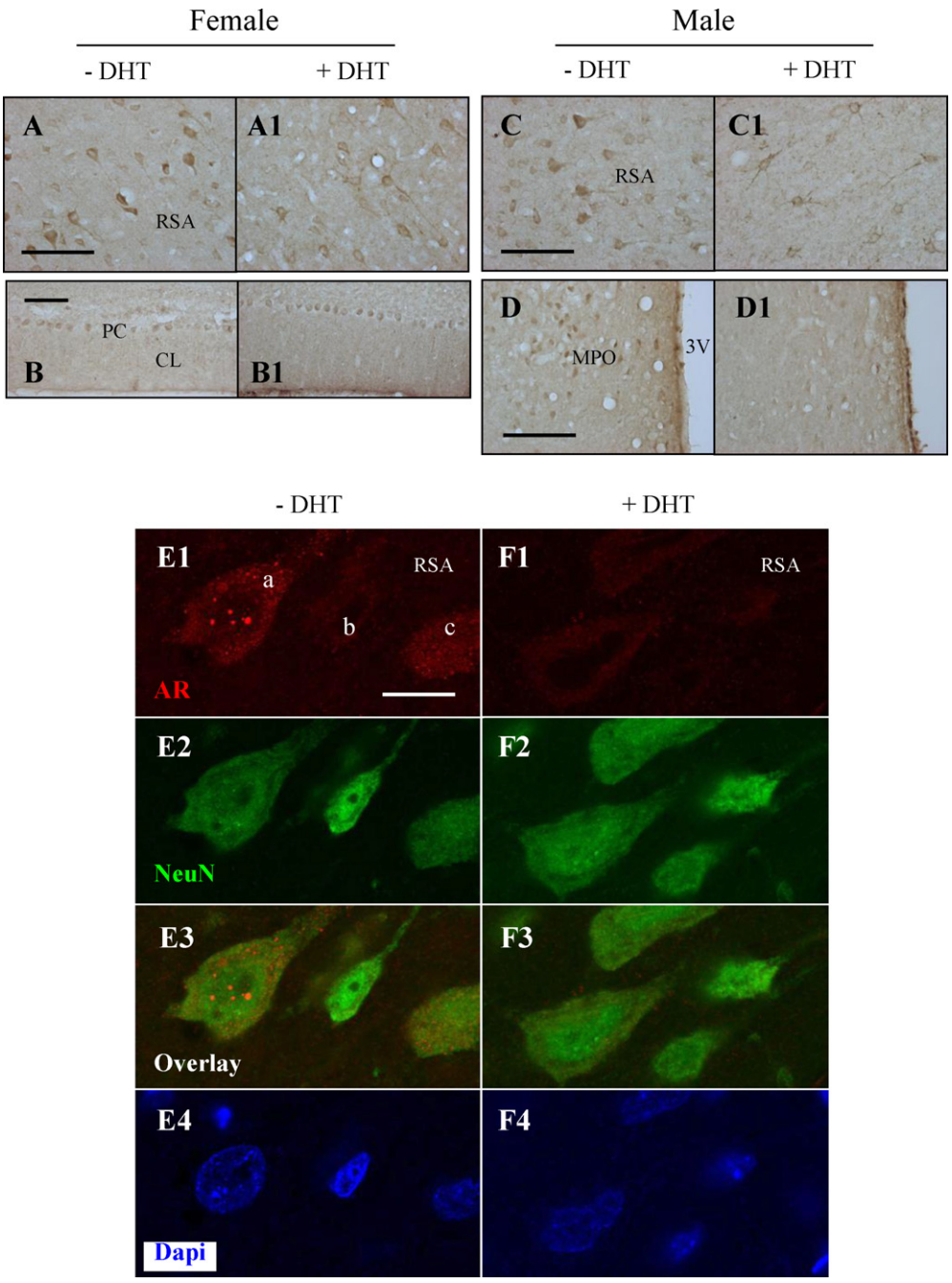

Fig. 5. Effect of DHT on AR localisation in female and male brains by immunohistochemical and immunofluorescence assays. Immunoperoxidase and immunofluorescence labelling of AR was performed on 20-μm thick rat brain sections showing the retrosplenial agranular (RSA), cerebellar lobules (CL), and medial preoptic areas (MPO). While the density of AR immunoperoxidase activity was significantly decreased in the RSA in both sexes after a 7-day DHT exposure, compared to control brains, both the number of AR-positive cells and the density of AR immunoreactivity were significantly decreased in the MPO [(A)–(D) and (A1)–(D1)]. The cerebellum showed prominent immunostaining of AR in the Purkinje cells (PC), identified by their large nuclei [(B) and (B1)]. In addition, the subcellular localisation of AR was further examined by immunofluorescence experiments, with subsequent analysis by confocal microscopy [(E1)–(E4) and (F1)–(F4)]. Four to five randomly selected sections from individual female rat brains per group were performed. In the RSA, AR (red) was localised in the cytosol, or to both cytosol and nuclei, in NeuN (green)-positive neurons (E1). DHT treatment abolished nuclear AR expression and decreased cytosolic AR expression in NeuN (green)-positive neurons (F1). The blue staining represents DAPI-stained nuclei. Scale bar = 100 μm. These experiments were repeated using three to six rats/group with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sation and expression could be regulated by treatment with DHT, which is a non-aromatisable AR agonist, in both female (metestrus and diestrus) and male rats. A clear difference in the patterns of AR immunostaining in brains between DHT-treated and -untreated male rats was detected (Table 1). DHT treatment gave identical results in both males and females at the metestrus and diestrus stages (data not shown). Notably, AR-positive cell bodies with "ring-like" appearance (Fig. 5A1 and C1) were observed in several regions, such as the RSA in both female and male brains after a 7-day DHT exposure. On the other hand, weak AR immunoreactivity remained in the RSA axons after DHT treatment. Moreover, the density of AR immunoreactivity was significantly decreased in the RSA of both female and male brains after a 7-day DHT exposure compared to control animal brains (Fig. 5A and C). However, the treatment of both female and male rats with DHT failed to change AR expression in other regions, such as the cerebellar lobules; CL

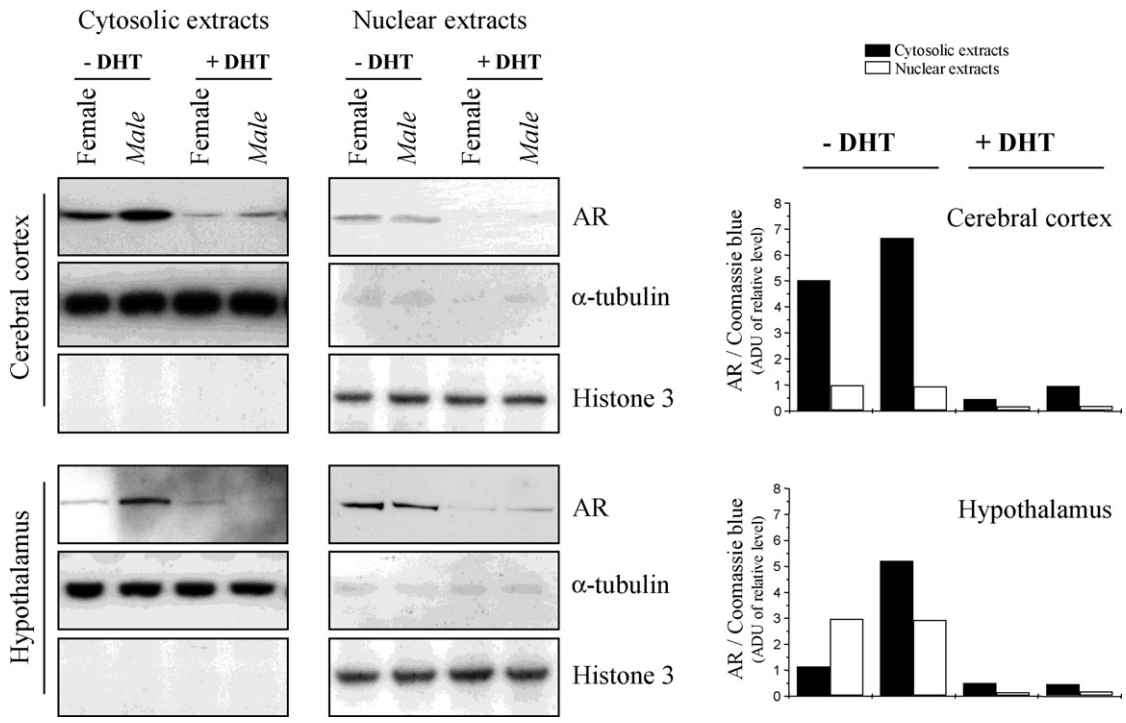

Fig. 6. Effect of DHT on subcellular AR expression in female and male brains by tissue/cell fraction and Western blot analysis. Tissue/cell fractions were prepared by differential centrifugation. Equal amounts of proteins from each subcellular fraction (cerebral cortex and hypothalamus) were subjected to a 4–12% one-dimensional Bis–Tris gels and immunoblotted with antibodies against AR, α-tubulin (cytoplasmic marker), and histone 3 (nuclear marker). Cytosol and nuclear fractions are directly comparable. AR was found in different subcellular compartments in both female and male brains. DHT treatment decreased both cytosolic and nuclear AR expression, while sex differences were observed with the down regulation of cytosolic AR expression in the hypothalamus. Equal sample loading was confirmed by Coomassie blue staining of gel. Relative levels of AR proteins were expressed as a ratio of AR densitometric value to whole proteins in Coomassie blue staining. The results are representative of two independent experiments, each run with independent samples (2 rats/group).

(Table 1), which indicated that Purkinje cells essentially expressed AR (Fig. 5B). While the MPO represents a major source of input to the PVN (Williamson and Viau, 2007), expression of AR in the MPO was not only altered during the oestrous cycle (Fig. 2C) but also by DHT treatment (Fig. 5D1). Furthermore, both the number of AR-positive cells and the density of AR immunoreactivity were significantly decreased in the cingulated isocortex, bed nucleus of stria terminalis (BST), AMY, PVN, ventromedial hypothalamic nucleus (VMH), supraoptic nucleus, premammaliar nucleus (ventral part), median eminence, and the lateral reticular tract (Table 1) following 7-day DHT treatment in both females and males, indicating that AR expression in the cells of these regions is dependent on androgen. In addition, the intensities of AR immunoreactivity were not appreciably different within the same treatment group of animals of the same sex. To increase the resolution of cell structures, we used immunofluorescence confocal microscopy for subcellular AR localisation in the female rat brain. While diffuse AR immunoreactivity was detected in the cytosol, granular immunoreactivity for AR was observed in the nuclei (Fig. 5). Moreover, AR could be localised to the cytosol or to both the cytosol and nucleus in neurons (Fig. 5E1). However, not all NeuN-positive cells contained AR, suggesting that not all mature neurons contain AR within the brain tissue (Lorenz et al., 2005). Stimulation with DHT changed the localisation of AR (Fig. 5F1–4). Expression of AR in the cytosol was rarely detected, and AR immunoreactivity was diminished, in the nuclei of NeuN-positive cells (Fig. 5F1).

After detecting AR in a total rat brain homogenate (Fig. 3), we sought to identify the subcellular compartment that was particularly enriched with AR protein in the different brain regions. As an independent form of evidence to demonstrate the changes in cytosolic and nuclear AR signals by DHT treatment, WB analysis of the tissue/cell fractionation of selected regions of brains was performed. The WB analysis revealed that AR was located mainly in the cytosolic fraction of the cerebral cortex in both sexes (Fig. 6). However, it was located exclusively in the nuclei of the hypothalamus in female rats when compared to males. Although AR was present in the cytosolic and nuclear fractions of the cerebral cortex and hypothalamus, there was only a small amount of AR in the cytosol. In contrast, AR expression was not detected in the nuclei of the cerebral cortex after DHT exposure in either sex, which is supported by the observations from our IHC and immunofluorescence studies (Fig. 5). Significant decreases were also observed in AR expression in the hypothalamus nuclei for both sexes that were treated with DHT. Notably, DHT stimulation did not significantly change AR levels in the hypothalamus cytosol for female rats, in contrast to male rats. This suggests that DHT exposure down-regulates subcellular AR expression in a region-specific manner in the female brain. To further confirm the quality of the tissue/cell fractionation, subcellular fractions were also probed by WB analysis for the cytoplasmic marker α-tubulin, and the nuclear marker histone 3 antibodies. The two marker proteins were mainly found in the expected fractions, although α-tubulin was detected at an extremely low level in the nuclear extracts of both the cerebral cortex and hypothalamus (Fig. 6). This further confirms the subcellular localisation of AR for vehicle and DHT treatment in both female and male brains, as demonstrated by confocal microscopy.

4. Discussion

Androgens modulate brain function and behaviour (Hines, 2008; Wilson and Davies, 2007), yet their contribution to gender-related differences remains enigmatic. Improved mapping of AR in the brain in the physiological context of the intact animal is needed

for a comprehensive understanding of the physiological role of androgens. A better understanding may lead to new insights into the roles of androgens in brain-related disease processes. While ligand-binding studies have indicated oestrous cycle-dependent regulation of AR in rats (Handa et al., 1986), this study examined the relationship between the central AR protein expression, AR cellular localisation and oestrous cycle in adult cycling rats *in vivo*. Our results indicate region-specific differences in AR content across the oestrous cycle. Furthermore, while sex differences in AR expression were observed, we also determined whether or not the distribution of AR in adult female and male rat brains was dependent on the presence of exogenous androgens.

4.1. Regulation of AR and its possible physiological implications in females

Although several studies have reported the presence of AR in the female brain (Kumar and Thakur, 2004; Lu et al., 1998; McAbee and DonCarlos, 1998; Xiao and Jordan, 2002), knowledge with regard to how AR action influences brain function in females remains unclear. The major findings of this investigation were that changes in the ovarian steroid hormone environment across the reproductive cycle have a profound impact on the AR protein expression and that this regulation is region-dependent within the female rat brain. The supporting evidence for this hypothesis are: (1) AR expression varied strikingly in distinct anatomical regions of the brain across the oestrous cycle; (2) the low level of AR expression at the proestrus and/or oestrus stages corresponded to high levels of circulating and ovarian estradiol/testosterone, while the high levels of AR expression at the metestrus and diestrus stages correlated with low levels of circulating and ovarian estradiol/testosterone; and (3) while the effect of pharmacological level of DHT used in this study is evidenced by the fact that DHT increased spine synapse density in the female rat hippocampus (Leranth et al., 2004), exogenous DHT treatment of females at metestrus, when central AR levels were high, decreased AR expression in most of the AR expressing brain regions. However, our data also show that low levels of testosterone are associated to the lowest AR levels in the hippocampus and hypothalamus in adult cycling rats at the oestrus stage. A possible interpretation is that changes in central AR expression might be dependent of local levels of DHT rather than testosterone during the oestrous cycle in rats. *In vivo* and *in vitro* studies have provided evidence that the mammalian brain is capable of irreversible converting testosterone into DHT by 5α-reductase (Celotti et al., 1992). In addition, the present results demonstrated that both adult female and male brains have a similar regulatory pattern for AR expression in response to DHT treatment, which indicates a common regulatory mechanism. Expression of AR is regulated by gonadal steroid hormones both during development and adulthood (Kumar and Thakur, 2004; Lorenz et al., 2005; McAbee and DonCarlos, 1998, 1999a,b; Nunez et al., 2003; Ravizza et al., 2002; Wu et al., 2009). However, there is conflicting evidence for how the androgens regulate AR expression in the brain. Previous studies have shown that decreased AR expression runs parallel with the reduction of T production, following gonadectomy in men (Fernandez-Guasti et al., 2000) and male rodents (Apostolinas et al., 1999; Lu et al., 1998, 1999; McAbee and DonCarlos, 1999a,b). Subsequent treatment with T or DHT restores AR expression in rodent brains, reduced by gonadectomy during development (McAbee and DonCarlos, 1999b) and in adulthood (Apostolinas et al., 1999; Lu et al., 1998, 1999; Lynch and Story, 2000; Zhou et al., 1994). In contrast, the results presented here and by others (Kumar and Thakur, 2004; Lorenz et al., 2005) demonstrate that treatment with T or DHT down-regulates AR expression in both adult female and male rodent brains. In support of an inverse relationship between androgen and AR levels, Wu et al. (2009) recently reported a significant increase in brain AR expression that correlates with a gradual decline of circulating T levels in aging rats. Obviously, species, age, or sex differences cannot fully explain these differences in the regulation of central AR expression *in vivo*. In addition to the dosage, route, means of manipulation (via peripheral circulation or via local CNS), and duration of administration, another explanation for these discrepant findings lies in the cross-talk between androgenic and oestrogenic signalling processes. Testosterone can act either directly through binding to AR or indirectly through oestrogen receptors (ER), following its cytochrome P450 aromatase-mediated conversion to estradiol (Raskin et al., 2009). Because the regulation of aromatase is androgen-dependent in the mammalian brain (Roselli, 2007), it is difficult to separate these two modes of androgen action following *in vivo* exposure to testosterone. Moreover, although DHT is considered to be a more potent AR agonist (McLachlan et al., 1996), its metabolite, 5α-androstane-3β, 17β-diol (3β-Adiol), has been shown to function as an endogenous ERβ ligand that could facilitate ER-mediated transcription through classical ER signalling pathways in the neuronal cells (Lund et al., 2006). Therefore, the regulation of AR by androgens is complex and involves different interactions between AR and ERs (ERα and ERβ) in the brain. In accordance with studies showing that oestrogen antagonises some androgen action (Kipp and Ramirez, 2003), several studies have demonstrated that oestrogen and oestrogenic metabolite are able to increase AR in the mouse brain (Kumar and Thakur, 2004; McAbee and DonCarlos, 1999a). Therefore, further study of the specific regulation of central AR expression by different estrogens and androgens in the brain is needed through the combination of ovarectomized animals and an *in vitro* brain-slide culture system. It may provide an easier mean to interpret the outcome of experimental manipulations, or to conceptualize possible roles of AR signalling pathways in the female brain. It is most likely that the relative level of androgens versus oestrogens (i.e., circulating, brain tissue, or both) may be a key regulatory factor for determining functional AR outcomes in the female brain during the oestrous cycle. This possibility merits investigation in the future.

Open questions remain regarding, for example, the significance of the oestrous cycle-dependent regulation of AR expression in specific brain regions in female rats. One vital function modulated across the reproductive cycle in humans and rodents is the neuronal control of ovarian function. This has been related to the convergence of ovarian steroid hormone signalling in the brain, notably in the hypothalamus, that regulates gonadotrophin-releasing hormone (GnRH) secretion (Clarke and Pompolo, 2005). Thus, the information represented by the changes in AR expression in particular brain regions is interesting. It is clear that central AR plays the primary physiological role in the steroid feedback on LH secretion, and ERα is not sufficient to compensate for the absence of AR in brain selective-AR-knockout male mice (Raskin et al., 2009). Thus, we assume that the presence and functionality of the AR is relevant affecting hypothalamic-pituitary-gonadal function in females.

4.2. Regional sex differences in AR expression

The central distribution of AR immunoreactivity in female and male rat brains from this study is generally consistent with a previous study, which showed a widespread distribution of *AR* mRNA in the male rat brain (Simerly et al., 1990). Most of the higher brain regions and phylogenetically old brain regions expressed AR in both sexes (Table 1). In the DG, however, *AR* mRNA expression (Simerly et al., 1990) did not correlate with AR protein expression (Brannvall et al., 2005; Hajszan et al., 2007) (Fig. 4C and D). The reason that AR mRNA is absent in the DG is currently unclear. In the present investigation, IHC and quantitative WB analyses were used to evaluate the number, intensity, and amount of AR protein. Comparison between females at different stages of the oestrous

cycle and age-matched males revealed two kinds of sex differences for AR expression. First, the number of AR-positive cells and the expression levels of AR were higher in the male brain compared to females. In agreement with previous reports on humans and rodents (Bingham et al., 2006; Fernandez-Guasti et al., 2000; Herbison, 1995; Kritzer, 2004; Kritzer and Creutz, 2008; Lu et al., 1998; McAbee and DonCarlos, 1998, 1999a,b; Nunez et al., 2003; Portillo et al., 2006; Xiao and Jordan, 2002), we observed that the CC, HIP, MPO, and PVN contained less AR in females than males, regardless of the stage of the oestrous cycle. Regional sex differences in AR expression may reflect differences in the number of androgen-responsive cells, differences in the amount of AR within individual cells, or both. Our results suggest sex differences in the structure and function of these regions of the brain (Bingham et al., 2006; Ishunina et al., 2002; Leranth et al., 2004; Williamson and Viau, 2007; Xiao and Jordan, 2002) and could also account for the differences in androgen levels between adult females and males (Davison and Bell, 2006; Quigley et al., 1995). Second, region-specific AR expression has been suggested to play a direct role in steroid-dependent sexual differentiation in rodents during development (Lorenz et al., 2005; McAbee and DonCarlos, 1998; Nunez et al., 2003; Ravizza et al., 2002). Clinical observations show that the prevalence for anorexia nervosa is higher in women than men and that T directly regulates the regional brain hypometabolism in anorexic women (Miller et al., 2004, 2005). Given the lack of knowledge with regard to how or what extent AR mediates gender differences, we can only speculate to what extent the regional sex differences in AR expression found here underlie the different susceptibilities of sexes toward androgenic-induced behavioural changes in adults. On the other hand, androgen replacement therapy in women with depression essentially confirms that the female brain responds in a manner similar to men with androgen insufficiency (Amiaz and Seidman, 2008; Rohr, 2002). This suggests that a sex-independent mechanism of androgenic modulation for cognition and mood may exist (Hajszan et al., 2007).

4.3. Intracellular localisation of AR

The AR is a nuclear steroid hormone receptor (Gao et al., 2005) and light and electron microscopy demonstrate that cytosolic AR can be detected in different regions of the human and rat brain (DonCarlos et al., 2003; Fernandez-Guasti et al., 2000; Ishunina et al., 2002; Lorenz et al., 2005; Tabori et al., 2005). This suggests that AR is maintained in the cytoplasm perhaps through a specific cytosolic retention signal. Based upon the results of our subcellular colocalisation and cofractionation experiments, we observed that a constant proportion of AR was present in both the cytoplasm and nucleus. This type of subcellular localisation of AR was dynamic and was influenced by DHT in both adult female and male brains, supporting the notion that androgens are not able to maintain the nuclear localisation of AR in the brain (Wood and Newman, 1993). Although our results cannot distinguish the hsp-free or hsp-complexed forms of AR, the cytosolic versus nuclear compartmentalisation of AR may be associated with the genomic and non-genomic actions of androgen, which potentially represents different signalling processes in the brain (Patchev et al., 2004).

In this study, detailed findings on the spatiotemporal expression of AR in the female brain provide reference data for dissecting the contribution of AR toward androgen-dependent physiological processes ranging from development to adult behaviour. Although the consequences of AR expression changes in the female brain remain to be determined, the functional differences of AR and the diversity of androgen effects in both female and male brains would be defined unequivocally.

Conflict of interest

The authors have declared that no conflict of interest exists.

Contributors

YF IH ESV HB RS: conceived and designed the experiments. YF BW TW EE JFR ESV RS: performed the experiments. YF BW TW EE JFR RS: analyzed the data. YF JFR ESV HB RS: contributed reagents/materials/analysis tools. YF IH HB RS: interpretation of data. YF RS: the final version of the manuscript. All authors contributed to and have approved the final manuscript.

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