Presence of oestrogen receptor type β in human retina

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Abstract

***Background/aims*—Recent studies have demonstrated the existence of two oestrogen receptor subtypes α (ORα) and β (ORβ) with significant differences of expression among organs. Since important pathologies of human eye could be linked to hormonal status, the expression of ORβ in ocular posterior segment was sought.**

***Methods*—Immunohistochemical localisation of ORβ and ORα protein and detection of OR mRNAs by reverse transcription-polymerase chain reaction (RT-PCR) were performed in macular and extramacular regions of the retina and in the choroid on male and female donors eyes.**

***Results*—ORβ protein was localised in the ganglion cell layer and in the choroid. At the transcriptional level, mRNA for ORβ and for ORα were both present. Local differences in the expression level were observed, however, suggesting the possibility of variation in the ratio of ORα *v* ORβ.**

***Conclusions*—The coexistence of two oestrogen receptor subtypes in the human ocular posterior segment raises acute questions about their potential physiological role, but offers a perspective for preferential targeting of a specific receptor subtype.**

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Oestrogens are steroid hormones traditionally connected with either reproductive system or with bone tissue and cardiovascular system. Their receptors are members of the nuclear receptor superfamily (including among others, receptors for steroids, vitamin D, and retinoic acid) and they mediate most of the known effects of oestrogens. These intracellular proteins function as ligand activated transcription factors regulating gene transcription.

A new type of oestrogen receptor, ORβ has recently been cloned[1] and subsequently its mRNA was found in various organs of male and in different areas of the brain, while ORα is found mainly in most female organs. The finding of a second receptor having different tissue distribution and molecular characteristics is obviously raising a question about the interpretation of oestrogens actions in different organs.

The roles of oestrogens in ophthalmic pathology have been mainly evaluated in the anterior segment. Epidemiological and experimental data suggest a protective effect of oestrogens in age related cataractogenesis.[2–4] Tear function is influenced by sex hormones, and the onset of dry eye is very common during the menopause.[5] The situation in the retina is more controversial. Different epidemiological studies reported a protective effect of oestrogen replacement therapy on the development of neovascular age related macular degeneration (AMD)[6 7] while others failed to demonstrate a significant sex difference in the frequency of the disease.[8] The reasons for these discordant findings are unknown, but could partly be explained by the considerable differences among clinically used oestrogens for their binding affinities on their receptor subtypes.[9] Kobayashi *et al* convincingly demonstrated the presence of an oestrogen receptor in the rat and bovine retina, without differentiating ORα and ORβ.[10] In the human eye, a recent report suggested the presence of ORα in the young female retina.[11]

As the retina obviously shares common biological features with the brain, we hypothesised that ORβ could also be expressed in the retina and/or in the choroid. We report here that the β type oestrogen receptor is present in human male and female ocular posterior segments, with some regional differences in the level of mRNA expression.

Materials and methods

TISSUE COLLECTION

Human male and female donor eyes (eight males and five females) with a limited postmortem enucleation time (1–15 hours) were collected from the Cornea Bank, University of Liège, Belgium. Mean age was 65 years (range 46–82). After removal of the anterior segment structures, 5 mm diameter punches were made in the macular region and in the peripheral retina. Neural retina was then separated from the retinal pigment epithelium and from the choroid (RPE-choroid complex) and tissues were stored at −80°C. Alternatively, posterior segments were fixed in 4% formalin, dehydrated, and embedded in paraffin.

The mean blood testosterone level in males (measured post mortem by radioimmunoassay, Immunotech) was 0.84 μg/l (range 0.11–2.38) and the mean oestrogen level (Immunotech) in females was 148 ng/l (range 32–303).

IMMUNOCYTOCHEMICAL LOCALISATION OF ORβ

Frozen sections of 5 μm were fixed 5 minutes in acetone at room temperature, air dried, and covered for 1 hour with 3% normal goat serum. Then, rabbit anti-human oestrogen α or β receptor (Santa Cruz Biotechnology, CA, USA) diluted 1/25 was applied for 1 hour, sections were rinsed in TRIS/HCl pH 7.4 saline, and covered with one drop of EnVision (Dako, ready to use goat anti-rabbit peroxidase conjugated antibody) for 30 minutes. After rinsing in TRIS/HCl, one drop of AEC+ (Dako, 3-amino-9-ethylcarbazole) was added. Sections were washed in water, counterstained for 1 minute in haematoxylin, and mounted in Aquamount. Deparaffinised sections were similarly treated excepting for a preliminary microwave step (350 W, four times for 5 minutes in 10 mM sodium citrate buffer, pH 6.0) to unmask antigenic sites. Negative controls were obtained by omitting the

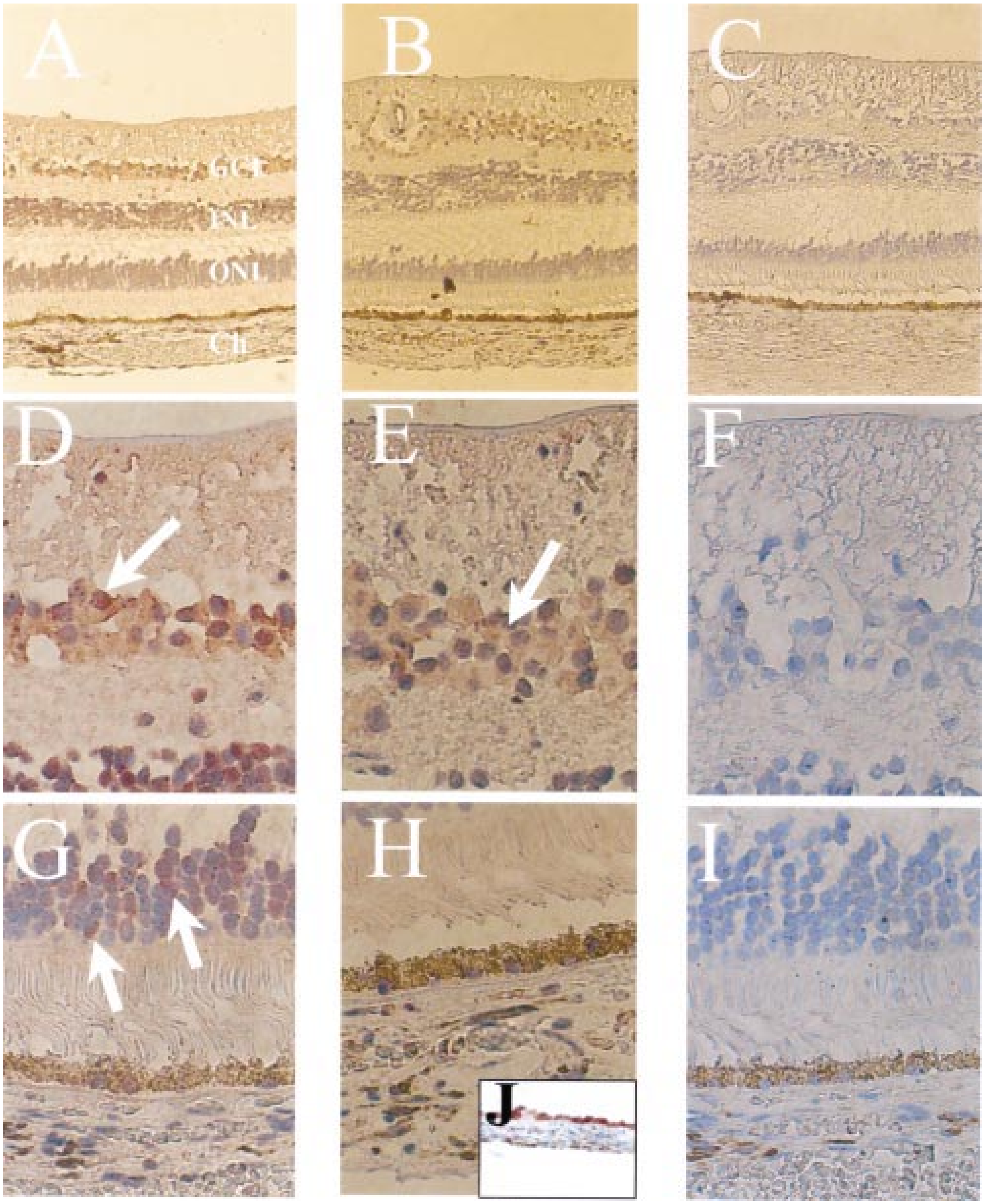

Figure 1 Immunolocalisation of ORα (A, D, G) and ORβ (B, E, H) and negative controls (C, F, I) on paraffin sections of ocular posterior segment at low magnification (×100, A, B, C) or higher magnification (×400) focused either on ganglion cell layer (D, E, F), on the choroid-RPE complex (G, H, I), or selectively on the RPE (J). Note the staining of ORα (arrows) in all nuclear layers (A) and the concentration of ORβ (arrows) in ganglion cell layer (E) and in choroidal structures (H). Ch = choroid; ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer.

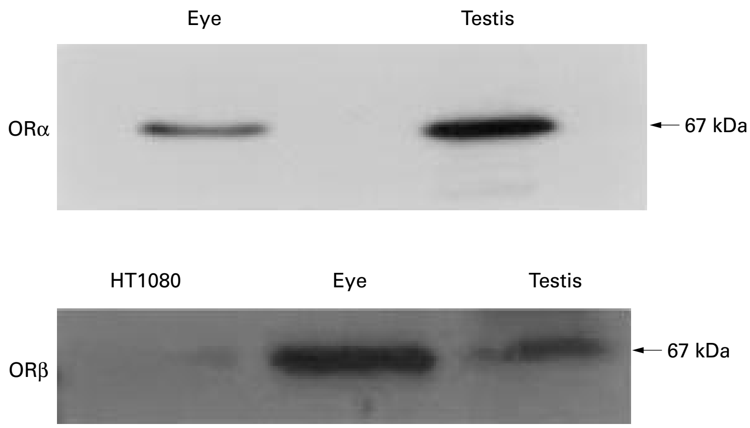

Figure 2 Human retina contained a band of oestrogen receptor α and β (relative molecular mass of 67 kDa). Testis was used as a positive control, and the HT1080 cell line was negative.

primary antibody, while for positive controls, uterine tissue known to express oestrogen β was used (data not shown).

WESTERN BLOT ANALYSIS

Analyses of ORα and ORβ protein expression were performed from 5 mm punches made in the macular region, from testis and HT1080.

Protein extracts were prepared from the cell pellet in RIPA buffer (50 mM TRIS (pH 7.4), 150 mM NaCl, 1% Igepal (v/v), 1% sodium deoxycholate (w/v), 5 mM iodoacetamide, 0.1% SDS (w/v)) containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin).

For HT1080, total extracts were performed by scraping, in RIPA buffer. Protein concentration was determined with the DC protein assay (BioRad, Richmond, CA, USA).

Samples (20 μg for OR-α and OR-β analyses) were mixed with 1/5 sample buffer (0.31 M TRIS (pH 6.8), 10% SDS (w/v), 25% glycerol (v/v), 12.5% β mercaptoethanol (v/v), and 0.125% bromophenol blue (w/v)) and boiled for 5 minutes. They were then separated on 7.5% and 12% SDS-PAGE gels for ORα and ORβ analyses respectively and transferred to a PVDF filter (NEN, Boston, MA, USA). The membranes were then blocked with 5% milk (w/v), 0.1% Tween 20 (w/v) in phosphate buffered saline (PBS) for 2 hours before exposure to the primary antibody overnight at 4°C: rabbit anti-human oestrogen α or β receptor (Santa Cruz Biotechnology, CA, USA). The filters were then incubated either with a horseradish peroxidase conjugated swine anti-rabbit or goat anti-mouse antibody (Dako). Signals were detected with an enhanced chemiluminescence (ECL) kit (NEN, Boston, MA, USA).

DETECTION OF ORα AND ORβ BY RT-PCR

Total RNA from 5 mm punches were extracted using RNeasyMini Kit (Qiagen) as described by the manufacturer. 28S rRNA, ORα and ORβ mRNA were measured in 10 ng aliquots of total RNA using the GeneAmp Thermostable rTth reverse transcriptase RNA-PCR kit (Perkin Elmer) and three pairs of primers (Gibco BRL Life Technologies) (sense: 5'-GTTTCCCCCCACTCAACAGCGT-3' and reverse: 5'-ACTTCCCTTGTCATTGGTACTGGC-3' for ORα mRNA,[12] sense: 5'-TTCCCAGCAATGTCACTAACT-3' and reverse: 5'-CTCTTTGAACCTGGACCAGTA-3' for ORβ mRNA,[13] and sense: 5'-GTTCACCCACTAATAGGGAACGTGA-3' and reverse: 5'-GGATTCTGACTTAGAGGCGTTCAGT-3' for 28S rRNA[14]). Reverse transcription was performed at 70°C for 15 minutes, followed by 2 minutes' incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification started by 15 seconds at 94°C, 20 seconds at 58°C, and 15 seconds at 72°C (35 cycles for ORα and ORβ and 19 cycles for 28S) and terminated by 2 minutes at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analysed using a Fluor-S MultiImager (BioRad) after staining with Gelstar dye (FMC BioProducts).

Results

OR IMMUNOLOCALISATION ON POSTERIOR SEGMENT SECTIONS

Cytoplasmic and nuclear staining with the antioestrogen receptor β antibody were mainly observed in the ganglion cell layer of the retina (Fig 1B, E, H). Immunolocalisation in the choroid was mild and inconsistent. The staining was similar in tissues of both sexes and its localisation was the same on frozen (not shown) and on paraffin sections. No staining could be observed in the absence of the primary antibody (Fig 1C, F, I). Immunolocalisation of receptor α antibody was more widespread in the neural retina (Fig 1A, D, G) and its localisation correlated with previously performed analysis on human tissue, with more intensity in the ganglion cell layer.[11]

WESTERN BLOT ANALYSIS

The presence of oestrogen receptors β and α in the human retina was confirmed by western blot analysis with the appropriate antioestrogen receptor antibody (Fig 2). Specimens contained a band of oestrogen receptor protein at a relative molecular mass of 67 kDa. The HT1080 cell line was used as a negative control in which no 67 kDa band was observed. A band was present in the testis (positive control).

OR mRNA EXPRESSION IN THE POSTERIOR SEGMENT

The primer specificity was checked using total RNA isolated from human testis and from endometrial Ishikawa cells and breast adenocarcinoma MCF-7 cells (data not shown). ORα and ORβ primers amplified a 234 bp and a 258 bp fragment, respectively. ORα and ORβ mRNA expression was detected in ocular tissues from patients of different age and sex, regardless of the region of the sample (inside or outside the macula) (Fig 3). The expression of ORβ mRNA was relatively constant between different donors, while there was more variation with ORα. This variation was also observed when RT-PCR was performed using total RNA selectively extracted from neural

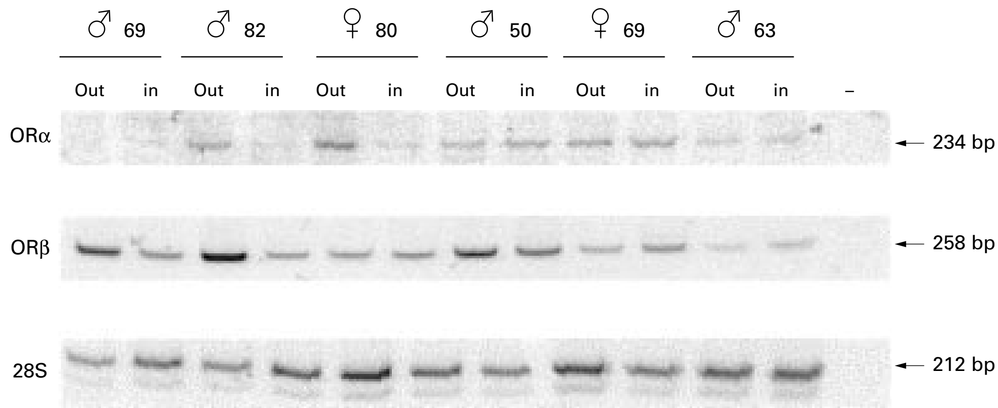

Figure 3 Representative example of ORα and ORβ mRNA expression in the eye of various sex donors (age is given in years with sex symbol). Total RNA (approximately 10 ng) from the macular region (in) or from the peripheral retina (out) were submitted to RT-PCR as described in Materials and methods. The 28S rRNA (lower panel) is used to assess the total amount of RNA loaded.

retina and the choroid region (Fig 4). Both receptor subtypes mRNA were detected in the RPE-choroid complex, but ORα was unequally distributed between the retina and the RPE-choroid (Fig 4).

Discussion

In this report, we demonstrate the presence of ORβ in human male and female ocular posterior segment by immunohistochemistry, western blot analysis, and RT-PCR. At the transcriptional level, while the expression of ORβ was relatively constant, much more variability between different specimens was observed for ORα. This suggests the possibility of variation in the ratio of ORα *v* ORβ mRNA expression.

The precise effects of oestrogens on the retina are largely unexplored and a detailed discussion about their potential action in retinal pathology is obviously speculative even

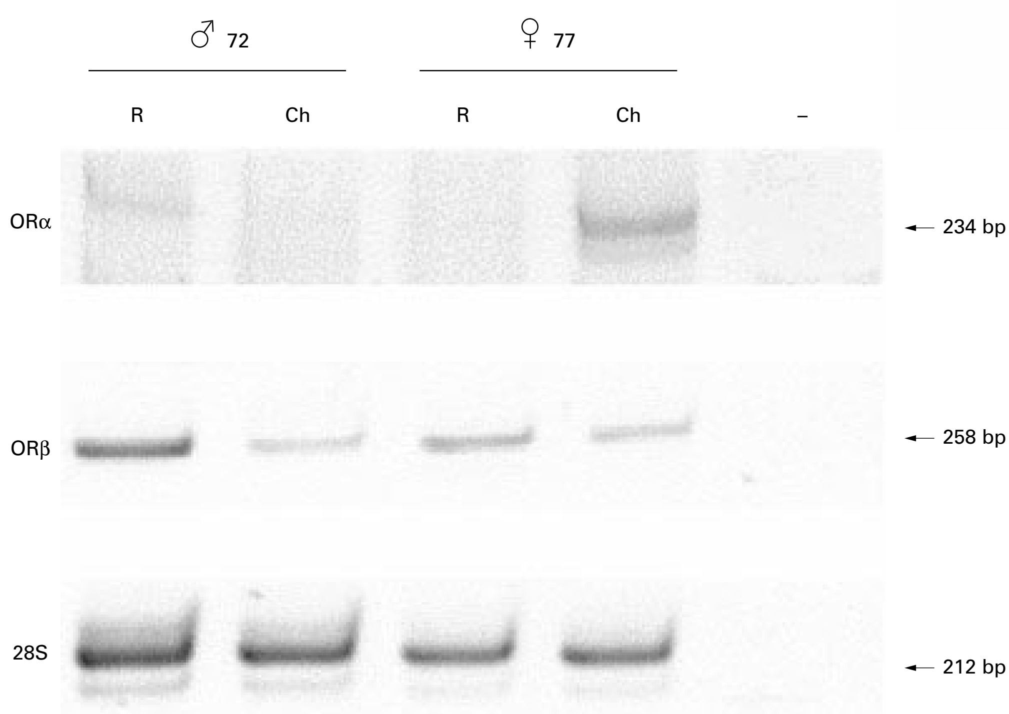

Figure 4 ORα and ORβ mRNA expression in human retinas (R) and choroidal (Ch) regions of a representative couple of male and female donors. RT-PCR was performed as described in Materials and methods. The 28S rRNA is used to assess the total amount of RNA loaded.

if some epidemiological data suggest a protective effect.

Nevertheless, our results contribute to explain, at least partly, the beneficial effects observed in retinal pathology with genistein treatment. Genistein is a naturally occurring phyto-oestrogen with a 20-fold affinity difference for ORβ *v* ORα.[15] Recent studies have focused on the role of phyto-oestrogens on angiogenesis and tumour development.[16] Genistein in vitro inhibits the proliferation of brain derived endothelial cells.[17] Oestradiol 17-β is able to completely inhibit microvessels growth from explants of rat aorta embedded in collagen gel.[18] Genistein effects described on retinal degeneration after ischaemia reperfusion in rat[19] or on experimental choroidal regeneration[20] were attributed to its inhibitory action on tyrosine kinases. Tyrosine kinase inhibition requires rather high local concentration of genistein (>10 μM) whereas a 0.1 μM concentration is sufficient to exert an OR mediated effects.[21] In female rats receiving dietary genistein in high amounts (daily dose of 20 mg/kg of body weight), the concentration in serum was only 2.2 μM.[22] In a rat model of vascular endothelial injury associated with smooth muscle cell proliferation, a vasculoprotective effect of genistein without effect on the reproductive system was demonstrated by preferential targeting to ORβ. This observation was explained by the predominance of the ORβ subtype in the vascular wall, with a >40-fold upregulation of ORβ expression after injury, while ORα expression remained unchanged.[23]

Increasing experimental evidence demonstrate that oestrogens are neuroprotective[24 25] and that oestrogen replacement therapy may contribute to the prevention of, or even delay the onset of, Alzheimer's disease.[26] In the eye, macular degeneration has been associated with early menopause.[27] Precise biological mechanisms of oestrogen action on the retina still remain to be elucidated, but it is tempting to assume that one of the ways by which oestrogen could influence retinal biology is through RPE function. Relatively few genes are proved to be under the control of ORs. Among these is cathepsin D, an aspartic protease highly expressed in human retinal pigment epithelial lysosomes with transcription regulated by oestrogen.[28] Experimental impairment of cathepsin D results in accumulation of rod outer segment debris in the RPE.[29] Another possibility would be the systemic or local regulation of apolipoprotein E (Apo E) metabolism. Apo E and its alleles (apoE2, apoE3, apoE4) are believed to play a part in cardiovascular disease,[30] Alzheimer's disease,[31] and in AMD.[32] Mice on hypercholesterolic diet[33] or deficient for apoE[34] develop ultrastructural changes in Bruch's membrane similar to human basal linear deposits. Drusen, the hallmark of AMD, share some major components with atherosclerotic plaques, including among others apoE.[35] After brain injury, apoE is increased in areas of synaptic remodelling, and oestrogen increase apoE mRNA in astrocytes and microglia.[36] Importantly, it has been shown that oestrogen prevent atherosclerosis in apoE deficient male mice.[37]

Finally, our observations suggest that under certain circumstances, the ORβ/ORα expression ratio could vary in the eye. This provides a mechanism by which oestrogen could exert different effects on the same cell type. Indeed, it has recently been demonstrated in an experimental model using transient transfection that ORα and ORβ, when complexed with oestrogen, were signalling in opposite ways (turning on/off gene transcription) from an AP1 transcription site.[38]

Additional experimental and epidemiological studies about the roles of oestrogens in normal and diseased retina are obviously mandatory. The demonstration of two different receptor subtypes raises a perspective for preferential targeting.

Proprietary interest category: Nil.

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The first two authors contributed equally to this paper.

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