Experimental Eye Research 138 (2015) 42–51

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Distribution of galanin receptors in the human eye

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ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form
15 June 2015
Accepted in revised form 22 June 2015
Available online 26 June 2015

Keywords:
Neuropeptide
Blood flow regulation
Autonomic nervous system
Galanin
G-protein coupled receptor

ABSTRACT

The neuropeptide galanin (GAL) is widely distributed within intrinsic and extrinsic sources supplying the eye. It is involved in regulation of the vascular tone, thus important for ocular homeostasis. Since the presence/distribution of its receptors is unknown, we here screen for the presence of the various GAL receptors in the human eye. Meeting the Helsinki-Declaration, human eyes (n = 6; 45–83 years of age, of both sex, post mortem time 10–19 h) were obtained from the cornea bank and prepared for immunohistochemistry against GAL receptors 1–3 (GALR1–GALR3). Over-expressing cell assays served as positive controls and confocal laser-scanning microscopy was used for documentation. Cell assays reliably detected immunoreactivity for GALR1-3 and cross-reactions between antibodies used were not observed. In the cornea, GALR1-3 were detected in basal layers of the epithelium, stroma, endothelium, as well as in adjacent conjunctiva. In the iris, GALR1-3 were detected in iris sphincter and dilator, while iris vessels displayed immunoreactivity for GALR1 and GALR3. In the ciliary body, GALR1 was exclusively found in the non-pigmented epithelium while GALR3 was detected in the ciliary muscle and vessels. In the retina, GALR1 was present in fibers of the IPL, OPL, NFL, many cells of the INL and few cells of the ONL. GALR2 and GALR3 were present in few neurons of the INL, while GALR2 was also found surrounding retinal vessels. RPE displayed weak immunoreactivity for GALR2 but intense immunoreactivity for GALR3. In the choroid, GALR1-3 were detectable in intrinsic choroidal neurons and nerve fibers of the choroidal stroma, and all three receptors were detected surrounding choroidal blood vessels, while the choriocapillaris was immunoreactive for GALR3 only. This is the first report of the various GALRs in the human eye. While the presence of GALRs in cornea and conjunctiva might be relevant for wound healing or inflammatory processes, the detection in iris vessels (GALR1, 2) and choroidal vessels (GALR1-3) highlights the role of GAL in vessel dynamics. Presence of GALR1 in ciliary body epithelium and GALR3 in ciliary vessels indicates involvement in aqueous humor production, whereas retinal GALR distribution might contribute to signal transduction.

1. Introduction

Conversion of visual input into electrical information and routing this information to the brain is the main function of the eye (Levin et al., 2011). These processes require a microenvironment that offers adequate supply with oxygen and nutrients and also removal of all unwanted and potential toxic metabolites. In order to provide this optimized microenvironment, and in order to provide proper visual function, these homeostatic processes require a stringent control. This control is maintained, besides known local mechanisms, for the most part by the autonomic nervous system (McDougal and Gamlin, 2015; Neuhuber and Schrodl, 2011). While autonomic control utilizes well investigated classical transmitters for signal transduction, such as acetylcholine or norepinephrine, a plethora of neuropeptides is additionally involved in these mechanisms, mainly for signal-modulation (Troger et al., 2007). In many instances, however, the exact mechanisms of signal transduction/-

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http://dx.doi.org/10.1016/j.exer.2015.06.024
0014-4835/

modulation are not understood yet.

Neuropeptides represent a class of small protein-molecules involved in neurosignalling or co-transmission of the classical neurotransmitters (De Wied and De Kloet, 1987). Amongst those, galanin (GAL) has been introduced already some decades ago (Tatemoto et al., 1983), consisting of 29 aminoacids, (30 aminoacids in humans) and found in many species (Lang et al., 2015) in the central as well as peripheral nervous system. Galanin takes part in many aspects of autonomic control (Jobling, 2011; Shanks and Herring, 2013; von Rosenvinge and Raufman, 2010), and it is a key player in developmental processes (Zaben and Gray, 2013). It is also involved in many pathological conditions, including Alzheimer's disease and some forms of epilepsia as well as cerebral ischemia/stroke and several forms of psychiatric disorders (for review see (Lang et al., 2015). GAL is acting via three known receptors GALR1-3 (Webling et al., 2012) which belong to the G-protein coupled receptor superfamily. These receptors show "substantial differences in sites of expression as well as their functional coupling and subsequent signaling activities" (Webling et al., 2012), thus easily contribute to the various physiological and pathological effects observed.

Few reports of GAL in the uvea of humans (May et al., 2004; Selbach et al., 2000) and non-human primates (Firth et al., 2002) exist, and these have been linked to a more sensory function and in line with these observations is the presence of GAL in the human trigeminal ganglion (Del Fiacco and Quartu, 1994). However, in various different mammals GAL is also widely distributed within other parts of the cranial autonomic nervous system (Troger et al., 2007), and known species differences in neuropeptide distribution easily contribute to these contrary observations. Nevertheless, GAL has been also detected in human aqueous humor (Ortego and Coca-Prados, 1998), and further in human ciliary epithelium in-vitro (Ortego and Coca-Prados, 1998) indicating that other ocular sources of GAL exist besides the autonomic nervous system. While these GAL origin(s) especially in the human eye have been reported, and since further also systemic active serum levels of GAL are known (Legakis et al., 2007; Slopien et al., 2004), we here focus on the different targets of GAL-action and therefore describe the various GAL-receptors in the human eye with morphological methods. Part of the manuscript has been published in abstract form (Schroedl et al., 2015).

2. Methods

2.1. Tissue preparation

The study on human tissues was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee being no clinical drug trial or epidemiological investigation. Furthermore, the study did not extend to examination of individual case records. The anonymity of the patients has been ensured. Human tissue samples ($n = 6$; 45–83 years of age, of both sex, post mortem time 10–19 h) were obtained from the cornea bank of the University Eye Clinic, Salzburg, Austria, and were prepared for cryosections and subsequent immunohistochemistry.

Eyes were dissected free, cut into anterior and posterior half by circumferentially following the ora serrata and fixed by immersion in phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA; 2 h at room temperature, RT). They were rinsed in PBS (24–48 h) and transferred into PBS containing 15% sucrose (24 h at 4 °C). Tissues were embedded in tissue embedding medium (NEG50, Fisher Scientific, Vienna, Austria) and frozen at −80 °C by using liquid nitrogen-cooled methylbutane and stored at −20 °C for further processing.

2.2. Immunohistochemistry

Tissues were mounted in a cryostat (HM 550, Microm, Walldorf, Germany) and serial sections of 16–20 μm were collected on adhesion slides (Superfrost Plus; Thermo Scientific, Wien Austria) and air-dried for 1 h at RT. After a 5 min rinse in tris-buffered saline (TBS; Roth, Karlsruhe, Germany) slides were incubated for 1 h at RT in TBS containing 5% donkey serum (Sigma–Aldrich, Wien, Austria), 1% bovine serum albumin (BSA; Sigma–Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5 min rinse, slides were incubated with rabbit antisera selected from preliminary experiments and generated against human GALR1 (1:400; Genetex, Irvine, USA), GALR2 (1:500; LifeSpan Biosciences, Seattle, USA) and GALR3 (1:500; Genetex), all diluted in TBS, containing 1% BSA and 0.5% Triton X-100, 12 h at RT. After a rinse in TBS (three times 5 min) binding sites of primary antibodies were visualized by Alexa555-tagged antisera (1:1000; Invitrogen, Karlsruhe, Germany) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 h at RT) followed by another rinse in TBS (three times 5 min). Whenever necessary, slides received an additional nuclear staining using 4′,6-Diamidino-2 phenylindol dihydroclorid (DAPI). For that, slides were incubated 10 min (1:4000, stock 1 mg/ml, VWR, Vienna, Austria) followed by a rinse in PBS (three times 5 min) and slides were embedded in TBS-glycerol (1:1 at pH 8.6). Negative controls in tissues were performed by omission of the primary antibodies during incubation and resulted in no staining.

2.3. Documentation

In order to document single and double label immunohistochemistry, a confocal laserscanning unit (Axio ObserverZ1 attached to LSM710, Zeiss, Göttingen, Germany; × 20 dry or ×40 and ×60 oil immersion objektive lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss) was used. Sections were imaged using the appropriate filter settings for Alexa555 (555 nm excitation, coded red) and DAPI (345 nm excitation, coded blue), and additionally background illumination was used whenever appropriate (488 nm excitation, coded green). All images presented here represent confocal images in single optical section mode. A semiquantitative evaluation of the detected immunoreactivity has been performed and graded into strong immunoreactivity (+++), medium immunoreactivity (++), weak immunoreactivity (+), and absent immunoreactivity (o) by two independent observers.

2.4. Control of galanin-receptor antibodies on GALR expressing cell lines

For testing the respective antibodies specificity, inducible cell lines expressing the GAL receptors were used. The neuroblastoma cell lines SH-SY5Y hGalR1/hGalR2 (SY5Y-R1/R2) and the Flp-in T-REx 293hGalR3 (HEK-R3) were cultured as described (Berger et al., 2004; Runesson et al., 2009). For immunofluorescence staining, the cells were grown in chamber slides (Corning Life Sciences, Amsterdam, The Netherlands) and receptor expression was induced by addition of 1 μg/ml doxycycline for 24 h. After a brief rinse in PBS, cells were fixed with 4% PFA in PBS for 10 min, followed by three washes with PBS (all steps at RT). Unspecific binding sites were blocked by incubation with TBS containing 5% donkey serum (Sigma–Aldrich), 1% bovine serum albumin (BSA; Sigma–Aldrich), and 0.5% Triton X-100 (Merck). Subsequent immunohistochemistry used the identical protocol as described here in Section 2.2.

To detect possible cross-reactions of the antibodies, all receptor