Oxytocin Expression and Function in the Posterior Retina: A Novel Signaling Pathway

Patrick Halbach,^{1,2} De-Ann M. Pillers,^{1,3} Nathaniel York,^{1,2} Matti P. Asuma,¹ Michelle A. Chiu,¹ Wenxiang Luo,¹ Sara Tokarz,¹ Ian M. Bird,^{1,2,4} and Bikash R. Pattnaik^{1-3,5}

¹Division of Neonatology, Department of Pediatrics, University of Wisconsin, Madison, Wisconsin, United States

²The Endocrinology-Reproductive Physiology Program, University of Wisconsin, Madison, Wisconsin, United States

³McPherson Eye Research Institute, University of Wisconsin, Madison, Wisconsin, United States

⁴Departments of Obstetrics/Gynecology, University of Wisconsin, Madison, Wisconsin, United States

⁵Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin, United States

Correspondence: Bikash R. Pattnaik, Division of Neonatology, Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Meriter Hospital, 202 South Park Street, Madison, WI, USA; bikashp@pediatrics.wisc.edu.

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PURPOSE. Oxytocin (OXT) is recognized as an ubiquitously acting nonapeptide hormone that is involved in processes ranging from parturition to neural development. Its effects are mediated by cell signaling that occurs as a result of oxytocin receptor (OXTR) activation. We sought to determine whether the OXT-OXTR signaling pathway is also expressed within the retina.

METHODS. Immunohistochemistry using cell-specific markers was used to localize OXT within the rhesus retina. Reverse transcriptase PCR and immunohistochemistry were used to assess the expression of OXTR in both human and rhesus retina. Single-cell RT-PCR and Western blot analyses were used to determine the expression of OXTR in cultured human fetal RPE (hfRPE) cells. Human fetal RPE cells loaded with FURA-2 AM were studied by ratiometric Ca^{2+} imaging to assess transient mobilization of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$).

RESULTS. Oxytocin was expressed in the cone photoreceptor extracellular matrix of the rhesus retina. Oxytocin mRNA and protein were expressed in the human and rhesus RPE. Oxytocin mRNA and protein expression were observed in cultured hfRPE cells, and exposure of these cells to 100 nM OXT induced a transient 79 ± 1.5 nM increase of $[\text{Ca}^{2+}]_i$.

CONCLUSIONS. Oxytocin and OXTR are present in the posterior retina, and OXT induces an increase in hfRPE $[\text{Ca}^{2+}]_i$. These results suggest that the OXT-OXTR signaling pathway is active in the retina. We propose that OXT activation of the OXTR occurs in the posterior retina and that this may serve as a paracrine signaling pathway that contributes to communication between the cone photoreceptor and the RPE.

Keywords: cell-calcium, GPCR, neuropeptide, oxytocin receptor, retinal pigment epithelium, signaling

Oxytocin (OXT) is a nonapeptide hormone synthesized primarily in magnocellular neurons of the paraventricular and supraoptic nuclei in the brain and is released into the systemic circulation via the posterior pituitary.¹ Oxytocin is best known as a maternal birth hormone due to its roles in inducing uterine smooth muscle contraction in parturition and in stimulating lactation.¹ Oxytocin is also implicated in fetal and adult brain, heart, and kidney development and function, suggesting that the role of OXT may be more widespread than originally thought.¹⁻⁵

Oxytocin mediates its effects by binding and activating the rhodopsin-like G protein-coupled oxytocin receptor (OXTR).¹ On activation of the OXTR by OXT, a downstream increase of second messengers occurs, including intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), which facilitates smooth muscle contraction, nitric oxide synthesis, prostaglandin production, activation of the MAP-kinase cascade, and protein synthesis.^{1,6-9} Given the spectrum of events induced by activation of the OXTR, OXT is thought to be involved in a number of physiologic processes.¹

Oxytocin and vasopressin are structurally related cyclic nonapeptide neuropeptides that have been identified by HPLC

in rat, bovine, and human retina.^{10,11} Gauquelin and colleagues^{10,11} suggested that both of these neuropeptides may be important in retinal physiology by virtue of their location. Vasopressin has been well studied in retina, where vasopressin V_{1a} receptors are expressed in the RPE.¹² The RPE is a monolayer of cells interposed between the photoreceptors and the choriocapillaris that maintains the structural and functional integrity of the retina.¹³ Because RPE function is mediated, at least in part, by paracrine signals released from the retina that induce Ca^{2+} mobilization within the RPE, the finding that vasopressin V_{1a} receptor activation is coupled to RPE $[\text{Ca}^{2+}]_i$ mobilization suggests that vasopressin is engaged in retina-RPE signaling.^{12,14,15}

The purpose of this study was to localize OXT and OXTR signaling molecules in the neural retina and the RPE. We used mRNA and protein analyses to evaluate expression of OXT and OXTR in the human and rhesus retina, immunohistochemistry for protein localization, whole-cell patch-clamp electrophysiology for current recording, and live-cell fluorescence imaging to determine whether OXT is capable of inducing $[\text{Ca}^{2+}]_i$ mobilization in cultured RPE cells.

TABLE 1. Antibodies Used in Immunohistochemistry (IHC) and Western Analysis

Antibody		Source
Anti-oxytocin receptor (OXTR)	Primary rabbit polyclonal 1:300 (IHC) 1:1,000 (Western)	Novus Biologicals, Littleton, CO, USA
Anti-oxytocin receptor (OXTR)	Primary mouse monoclonal 1:1,000 (Western)	R&D Systems, Minneapolis, MN, USA
Anti-oxytocin (OXT)	Primary rabbit polyclonal 1:300 (IHC)	Abcam, Cambridge, MA, USA
Anti-RPE65	Primary mouse monoclonal 1:300 (IHC)	Abcam
Anti-cone arrestin 7G6 (7G6)	Primary mouse monoclonal 1:300 (IHC)	Gift from Peter MacLeish, Atlanta, GA, USA
Lectin PNA	Alexa Fluor 594 conjugate	Life Technologies, Grand Island, NY, USA
Anti- β -actin (β -actin)	Primary mouse monoclonal 1:1,000 (Western)	LI-COR Biosciences, Lincoln, NE, USA
Anti-GAPDH 14C10	Primary rabbit monoclonal 1:1,000 (Western)	Cell Signaling, Boston, MA, USA
Anti-mouse Alexa-Fluor 488 (green)	Secondary goat 1:1,000 (IHC)	Invitrogen, Grand Island, NY, USA
Anti-rabbit Alexa-Fluor 594 (red)	Secondary goat 1:1,000 (IHC)	Invitrogen
DAPI nuclear stain (blue)	Secondary 1:1,000 (IHC)	Molecular Probes, Inc., Eugene, OR, USA
Anti-mouse IR dye 680RD	Secondary 1:15,000 (Western)	LI-COR Biosciences
Anti-rabbit IR dye 800CW	Secondary 1:15,000 (Western)	LI-COR Biosciences

MATERIALS AND METHODS

Tissue Collection

Nonhuman primate *Macaca mulatta* (rhesus) eyes were obtained within 30 minutes of euthanasia during the morning hours of 8:30 to 10:30 AM, from the Wisconsin National Primate Research Center (Madison, WI, USA). All studies were in compliance with University of Wisconsin-Madison Animal Care and Use Committee requirements, as well as the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. Human donor eyes were obtained within 12 hours post mortem from the Lions Eye Bank of Wisconsin (Madison, WI, USA).

Reagents

Reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise noted. The HEPES Ringer's (HR) extracellular bath solution contained 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 10 mM glucose, 2.5 mM probenecid (Invitrogen, Grand Island, NY, USA), and adjusted to pH 7.4 with NaOH; ATP 100 μM and OXT 100 nM were prepared in HR solution. The antibodies used in this study are listed in Table 1.

Tissue Preparation for Immunohistochemistry

Rhesus eyes were opened at the pars plana, immersion fixed in 4% paraformaldehyde for 15 minutes, and cryopreserved using a 5%, 10%, and 20% gradient of ice-cold sucrose for 24 hours at each concentration. The eye was hemi-sectioned at the ora serrata, and the vitreous body was removed. The posterior segments were embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA) and cut into 10- μm frozen sections. Human 10- μm retinal sections were bought from the National Disease Research

Interchange (Philadelphia, PA, USA). All sections were stored at -80°C .

Immunohistochemistry

Frozen tissue sections were thawed to 25°C , rehydrated using PBS (Life Technologies, Grand Island, NY, USA), and blocked in PBS containing 10% goat serum, 5% BSA, and 0.3% Triton X-100 for 30 minutes at 25°C . The tissue was incubated with primary antibodies diluted in incubation solution (1:3 blocking solution to PBS) overnight at 4°C in a humidified chamber. The sections were washed three times in PBS and incubated for 1 hour at 25°C with secondary antibodies Alexa-Fluor 488 (1:1000, goat anti-mouse; Invitrogen), Alexa-Fluor 594 (1:100, goat anti-rabbit; Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI) (1:1000; Molecular Probes, Inc., Eugene, OR, USA) diluted in incubation solution. Secondary antibody controls were tested for all experiments. The sections were washed three times in PBS and mounted using Fluoromount (Sigma-Aldrich Corp.). Images were acquired using a Nikon Eclipse Ti-E confocal microscope (Nikon, Melville, NY, USA) equipped with a CoolSnap HQ Photonics camera (Nikon) and the images analyzed with NIS-Elements Advanced Research software (Nikon).

Human Fetal RPE Cell Cultures

The use of commercial human fetal cell lines was approved by the institutional review board of the University of Wisconsin-Madison. Passage 2 cryopreserved Primary Clonetics Human RPE cells (hFRPE) (LONZA, Walkersville, WA, USA) were cultured in 75- cm^2 flasks in hFRPE culture media (MEM alpha base medium [Gibco, Grand Island, NY, USA]), N1 supplement, glutamine (Gibco), pen-strep (Gibco), MEM nonessential amino acids, taurine, hydrocortisone, and 3,3',5'-triiodothyronine + 10% fetal bovine serum (FBS) (Gibco) for 48 hours. When

TABLE 2. Primers used in PCR

Gene Symbol		Primers	Product Length
Human oxytocin receptor (OXTR)	Forward	5'-ctggagcgcctttcttcttc-3'	256 bp
	Reverse	5'-gacaaaggaggagcaggttg-3'	
Rhesus oxytocin receptor (OXTR)	Forward	5'-gtcaagctcatctccaagg-3'	451 bp
	Reverse	5'-taggggcagaccacttatg-3'	
ATP1A1 (ATPase)	Forward	5'-cttcctcatcggtatcatcg-3'	257 bp
	Reverse	5'-ccactctgattctctgtcg-3'	
Ezrin	Forward	5'-gatttcctacctggctgaag-3'	108 bp
	Reverse	5'-ccacatcttcagggtagaac-3'	
RPE65	Forward	5'-ctgattgtggatctctgctg-3'	379 bp
	Reverse	5'-gcttacagagcctatctgg-3'	
GAPDH	Forward	5'-gacatcaagaaggtagtgaagcag-3'	209 bp
	Reverse	5'-tccacaacctgttgctgta-3'	

at 70% confluence, the cells were exposed to 1X EDTA-trypsin (LONZA) for 4 minutes at 37°C in 5% CO₂. Cells were collected in hRPE culture media + 8% FBS. Cells were seeded at a density of approximately 1×10^4 cells/cm² onto 25-cm² flasks or laminin-coated coverslips (12 mm; Thermo Fischer Scientific, Fitchburg, WI, USA). Cells were cultured in hRPE media + 8% FBS until they attained 95% confluence and were then maintained in hRPE media + 0% FBS with a media change every 2 days. Human fetal RPE cells cultured on coverslips for 4 to 5 weeks were used for single-cell RT-PCR and calcium imaging, whereas cells cultured in 25-cm² flasks were used for Western blot analysis.

RNA Isolation and RT-PCR of Human and Rhesus Ocular Tissue

Rhesus and human eyes were opened at the pars plana and the RPE, retina, and rectus muscle tissues were harvested and placed into RNA stabilizing solution (RNAlater; Ambion, Austin, TX, USA). Total RNA was extracted using the RNeasy Mini kit (cat. 74104; QIAGEN, Valencia, CA, USA). Ten milligrams of tissue was homogenized in 350 μ L RLT lysis buffer (cat. 79216; QIAGEN) supplemented with 20 μ L 2M dithiothreitol (DTT) per milligram. The lysate was centrifuged at 14,400g for 3 minutes. The supernatant was transferred to a tube containing 1 mL 70% ethanol and transferred to an RNeasy spin column (QIAGEN), and centrifuged at 10,000g for 15 seconds. The column was washed with 700 μ L buffer RW1 (cat. 1053394; QIAGEN) and spun at 10,000g for 15 seconds. The column was washed a second time with 500 μ L buffer RPE (cat. 1018013; QIAGEN). The RNA was eluted with 50 μ L RNase-free water (QIAGEN) and stored at -80°C.

Amplification of mRNA was performed by reverse transcription using the SuperScript III First-Strand Synthesis System (Invitrogen) per the manufacturer's protocol. Briefly, 200 ng RNA was incubated for 5 minutes at 65°C in a 10- μ L mixture containing 2.5 μ M random hexamers and 1 mM dNTP. After cooling on ice for 3 minutes, 10 μ L cDNA synthesis mixture containing 2 $\times$ RT buffer, 10 mM MgCl₂, 0.02 M DTT, 40 U RNaseOUT (Invitrogen), and 200 U SuperScript III (Invitrogen) was added to the RNA-primer mixture. Reverse transcription was performed using the GenAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) at 25°C for 10 minutes, 50°C for 50 minutes, 85°C for 5 minutes, and then the reaction was quenched on ice.

Each PCR reaction used 1 $\times$ ImmoMix PCR mix (Bioline USA, Inc., Taunton, MA, USA), 150 nM forward primers, 150 nM reverse primer, and 1 μ L reverse transcription product brought to a total volume of 10 μ L with RNase-free water. Denaturing, annealing, and extension began with one cycle at

95°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, a ramp from 57°C to 72°C for 60 seconds, and 72°C for 10 minutes, respectively. The PCR products were resolved in a 2% agarose gel (BIO-41026; Bioline, Taunton, MA, USA), and documented using the FOTO Dual Trans-illuminator (FOTODYNE, Hartland, WI, USA). All PCR primers are shown in Table 2.

Sequencing of RT-PCR Products

Reverse transcription-PCR products were sequenced by Sanger DNA sequencing at the University of Wisconsin Biotechnology Center (Madison, WI, USA) and compared with the published human OXTR sequence to confirm that it represented OXTR (NCBI BLAST#P30559).

Human Fetal RPE Single-Cell RNA Isolation and Amplification

Single hRPE cells were collected with a micropipette under microscope visual control and expelled into a PCR tube containing a multiplex PCR reaction mixture (50 nM each nested PCR external primers, 1 $\times$ ImmoMix, and reverse transcription product). The thermal cycle conditions are as stated above for OXTR amplification. We used only 25 cycles in the first round of amplification. In the second round of PCR amplification, expression of each target gene was individually detected. The reaction contained 2 μ L the first-round amplification PCR product, 500 nM each internal primer, and 1 $\times$ ImmoMix PCR mix. The second round of thermal cycling conditions were 95°C for 10 minutes, followed by 30 to 40 cycles at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and 72°C for 10 minutes. Products of PCR were resolved in 2% agarose gel (Bioline) and visualized using 0.5- μ g/mL ethidium bromide staining. The Na-K-ATPase and Ezrin mRNA internal markers confirmed that the culture hRPE cells were genetically representative of RPE cells.¹³ In an attempt to avoid amplification of genomic DNA, all primers were designed to span introns (Table 2).

Western Blot Analysis

Human fetal RPE cells were lysed in radio immunoprecipitation assay buffer containing a protease inhibitor cocktail (P2714; Sigma-Aldrich Corp.) and centrifuged for 2 minutes at 10,000g. Denaturing of the protein lysate was done by adding 1X Laemmli buffer containing 5% β -Mercaptoethanol and incubating at 99°C for 10 minutes. Protein samples were separated in a 4% to 20% Tris-Cl gradient gel (Bio-Rad, Hercules, CA, USA), electroblotted onto a PVDF LI-COR membrane (EMD Millipore,

FIGURE 1. Oxytocin localized to the cone photoreceptors in rhesus retinal tissue. Confocal images of (A) OXT (red), (B) 7G6 (green), and (C) colocalization with DAPI (blue) signal in the rhesus retina. Layers of retina are labeled in (C). (A) Scale bar: 50 μm (A–C). Confocal images of rhesus outer retina layer for (D) OXT (green), (E) PNA (red), and (F) colocalization with DAPI. Scale bar: 60 μm. (G) Confocal image of processed tissue without any primary antibody. (H) Confocal image of rhesus tissue after preabsorbing OXT antibody with OXT peptide. (I) Plot of pixel distribution for PNA (red) and OXT (green) in (F) to determine colocalization of OXT in the cone photoreceptors. The measurements were carried out for the white line in (F). INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PR, photoreceptors. Representative of four eyes.

BillERICA, MA), and stained with Ponceau S to confirm the success of the transfer. After Ponceau S was rinsed out from the membrane with water, the membranes were incubated in blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour. The membranes were incubated with the primary antibodies, anti-β-actin (1:1000) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000) plus anti-OXTR (1:1000; see Table 1), in blocking buffer containing 0.1% Tween at 4°C overnight. The membranes were washed five times in PBS containing 0.2% Tween for 5 minutes, and incubated with the secondary antibodies IR Dye 680RD anti-mouse (1:15,000) and IRDye 800CW anti-rabbit (1:15,000) in blocking buffer containing 0.1% TWEEN and 0.02% SDS for 1 hour in the dark (Table 1). The membranes were then washed five times in PBS containing 0.2% Tween and once in PBS for 5

minutes. Protein bands were visualized using the Odyssey Infrared Imager (LI-COR Biosciences).

Whole-Cell Electrophysiology and $[Ca^{2+}]$ Imaging

Human fetal RPE cells grown on coverslips to confluency (5–10 weeks) were enzymatically dissociated and whole-cell electrophysiology performed as previously described.^{16,17} For Ca^{2+} imaging, coverslips were washed once with ice-cold PBS and incubated in 5 μM FURA-2 penta-acetoxymethylester (AM; Molecular Probes) in hRPE culture media + 0% FBS for 45 minutes at 37°C. Following incubation with FURA-2 AM, coverslips were rinsed four times in HR solution (see reagents) and transferred to the recording chamber. Human fetal RPE cells were maintained in HR solution; ATP and OXT solutions were exchanged using a gravity-feed eight-valve solution

FIGURE 2. Expression of OXTR mRNA in the eye tissues of humans and rhesus monkeys. **(A)** Oxytocin receptor mRNA in RPE cells, neural retina, and rectus muscles of human eyes. **(B)** Oxytocin receptor mRNA in rhesus RPE, retina, and rectus muscles. Also shown are expression of RPE65, Ezrin, and GAPDH mRNA in rhesus tissue. **(C)** DNA sequencing showed that the PCR product from human RPE shares 100% homology with the published human OXTR (NCBI BLAST #P30559). Samples were verified in three separate experiments.

exchange system with a ValveLink Pinch Valve controlled through ValveLink8.2 (Automate Scientific, Berkeley, CA, USA). Images were acquired at 10-second intervals using a $\times 10$ objective and Lambda LS lamp (Sutter Instruments, Novato, CA, USA). The 340- and 380-nm excitation wavelengths, as well as the 100-ms shutter speed, were controlled by a Lambda 10-2 controller (Sutter Instruments). Emission was set to 518 nm. Image frames from the CoolSnap HQ Photonics camera (Nikon) were digitized and stored for off-line analysis. Background and calibration images were similarly acquired and used to obtain the absolute changes in fluorescence values. The amplitudes of the responses induced by 100 μM ATP and 100 nM OXT were compared and $[\text{Ca}^{2+}]_i$ was calculated using FURA-2AM imaging standards (Invitrogen). All experiments were conducted in triplicate.

Statistical Methods

Data were analyzed using the Student's *t*-test in Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and expressed as mean $\pm$ SEM. The $[\text{Ca}^{2+}]_i$ was calculated according to the FURA-2AM calibration standards (Invitrogen). Significance was defined as $P < 0.05$.

RESULTS

Oxytocin Expression in the Rhesus Cone Photoreceptors

The identification of OXT in HPLC fractions of rat, bovine, and human retina without defined tissue localization by Gauquelin and colleagues¹⁰ prompted us to delineate its location. When examined by confocal microscopy, anti-OXT signal was visible in the posterior rhesus retina (red staining, Fig. 1A) and exhibited a robust transverse staining pattern between the outer nuclear layer (ONL) and RPE within the photoreceptor layer. To determine whether the anti-OXT staining pattern was in the photoreceptor layer, the sections were stained with a monoclonal anti-cone arrestin antibody (7G6) that showed staining of inner and outer segments of cone photoreceptors; 7G6 staining was also visible for the cell body and cone synaptic terminal (green staining, Fig. 1B).¹⁸ Colocalization of OXT and 7G6 revealed that OXT staining was membrane extensions of 7G6-positive cells without any overlap, indicating OXT is not cytoplasmic in the cone photoreceptor outer segment (Fig. 1C). Specific localization of OXT to the cone photoreceptors was further confirmed by co-staining with OXT (Fig. 1D, green), and the cone sheath marker peanut agglutinin (PNA; Fig. 1E, red). The PNA specifically labels the extracellular sheath of inner and outer segments of cone photoreceptors and hence is used as a marker of the cone photoreceptor extracellular matrix.^{19–21} Superimposition of these images (Fig. 1F with DAPI) revealed that both PNA and OXT relatively colocalized (Fig. 1D). We did not visualize any OXT labeling when either primary antibody was omitted from the immunostaining procedure (Fig. 1G) or when OXT antibody was preincubated with an OXT polypeptide before tissue incubation (Fig. 1H). Our results confirm the findings of Gauquelin and colleagues¹⁰ that OXT is present in the posterior eye. Furthermore, we also illustrate the presence of OXT in the proximity of the cone photoreceptor extracellular domain.

Oxytocin Receptor mRNA Expression in the Human and Rhesus Retina

Oxytocin typically acts via binding to OXTR. To address the role of cone photoreceptor OXT, we sought to determine whether there was expression of OXTR in the retina. Human and rhesus RPE, retina, and rectus muscle were collected and assessed for OXTR mRNA using RT-PCR. Visualization of the RT-PCR products from human RPE revealed a 256-bp consistent with OXTR (Fig. 2A) and a 451-bp band in rhesus RPE cells (Fig. 2B). In the rhesus tissue, no OXTR mRNA was detected in the neural retina. Levels of cross-contamination between the RPE and retina were verified by the detection of Ezrin and RPE65 transcripts. Sequencing the OXTR mRNA PCR fragment product obtained from the human RPE showed 100% homology with the published sequence (NCBI BLAST #P30559; Fig. 2C). Our results demonstrate the novel finding that the OXTR mRNA is expressed in the human and rhesus retina.

Oxytocin Receptor Localization in the Human and Rhesus RPE

To localize OXTR protein expression within the retina, we performed immunohistochemistry with anti-OXTR using frozen rhesus and human retinal sections. Oxytocin receptor immunoreactivity was observed in a thin, horizontal band posterior to the retinal photoreceptors in both the human and rhesus sections (red staining, Figs. 3A, 3B, left). Anti-RPE65 exhibited a staining pattern specific to the RPE (green staining,

FIGURE 3. Oxytocin receptor localized to the RPE in human and rhesus retinal tissue. (A) Oxytocin receptor (red, left), RPE65 (green, middle), and DAPI (blue) signal in the rhesus retina. (B) Oxytocin receptor (red), RPE65 (green), and DAPI (blue) signal in the human retina. Oxytocin receptor and RPE65 colocalize to the RPE in both monkeys and humans ([A, B], right). Scale bar: 50 μ m. (C) Western blot from RPE, retina, and placental extracts showing OXTR (700 nm) and GAPDH (800 nm). (D) Fold expression of OXTR protein in RPE versus retina extract represented as a ratio (mean $\pm$ SEM). $P < 0.05$ paired t -test. Representative of four replicates.

Figs. 3A, 3B, middle). Co-staining with anti-OXTR and anti-RPE65 revealed colocalization of OXTR and RPE65 to the RPE (Figs. 3A, 3B, right). The RPE-specific staining for OXTR in rhesus was also demonstrated by nonfluorescent hematoxylin and eosin and AEC (3-amino-9-ethylcarbazole) staining methods (data not shown). In addition to the localization of OXTR in the RPE, a few punctate OXTR-positive signals were visible within the retinal tissue. Western blot analysis of extracts from rhesus RPE and human placenta (control) showed an approximately 62-kDa band corresponding to OXTR protein expression in these tissues (Figs. 3C, 3D). A very faint band, several folds less intense than the band seen for the RPE, was present in the retinal extract, and was attributed to RPE contamination (Figs. 3C [lane 2], 3D).

Cultured hRPE Cells Express OXTR mRNA and Protein

Given the proximal localization of OXT to the cone photoreceptor sheath, we sought to determine whether RPE OXTR could be activated by OXT. First, we wanted to show that cultured hRPE cells were a useful model for our findings in human and rhesus RPE tissue, so OXTR mRNA expression was assessed using single-cell RT-PCR. Detection of RPE-specific Kir7.1 current was performed by single-cell electrophysiology (Fig. 4). As shown in Figure 4A, confluent monolayers of pigmented hexagonal RPE cells were routinely obtained. On dissociation with papain, individual cells appeared figure-8 shaped (Fig. 4B), suggesting that we were successful in obtaining cells with an intact apical and basolateral structure.²² These cells were labeled with Wheat Germ Agglutinin Alexa-

Fluor 350 to mark the intact membrane; Rb⁺ increased Kir7.1 current more than 4-fold in all 15 cells recorded, whereas high K⁺ had no significant increase in current (Figs. 4C–E). These results suggest that these commercially available cells are an appropriate source of hRPE for use in research.

Consistent with our findings in isolated retinal tissue (see above), single hRPE cell RT-PCR products contained the expected 256-bp OXTR band (data from one cell shown in Fig. 5Aia). The expression of OXTR mRNA in hRPE cells, in conjunction with the expression of Na-K-ATPase (Fig. 5Aii) and Ezrin (Fig. 5Aiii), which are known markers for RPE cells, indicated that cultured hRPE exhibited normal RPE cell gene expression. Expression of OXTR protein in the hRPE was demonstrated by the presence of an approximately 62-kDa band that corresponded to a similar band in the control human placental tissue (Fig. 5B) and OXTR localized to pan-cytokeratin-positive hRPE cell membrane (Fig. 5C).¹

Oxytocin Induces a Transient Rise of hRPE [Ca²⁺]_i

Activation of OXTR induces a transient increase of [Ca²⁺]_i due to Ca²⁺ release from the endoplasmic reticulum.¹ Therefore, if RPE OXTR is functional, application of OXT should trigger an increase in [Ca²⁺]_i through the activation of OXTR receptors. We used hRPE cells cultured for 4 weeks at 100% confluence and used FURA-2AM ratiometric fluorescence imaging to assess hRPE [Ca²⁺]_i. In Figure 6Ai, the basal [Ca²⁺]_i level (represented by dark blue) was determined in HR extracellular solution. When 100 nM OXT was perfused through HR, a blue to light-green color change was observed (Fig. 6Aii), which was completely reversed on OXT removal (Fig. 6Aiii). The ATP was

FIGURE 4. Characterization of hRPE cells. (A) Monolayer of pigmented hexagonal RPE cells grown on glass coverslips. (B) Papain digested individual figure-8-shaped cells seen with patch pipette on the *left* and membrane labeling on the *right*. (C) Representative current-voltage curve displaying whole-cell current measured in presence of 5 mM K⁺ (black trace) and 135 mM Rb⁺ (red trace). (D) Average increase in inward current in presence of external 135 mM Rb⁺ and 135 mM K⁺. (E) Current responses to step voltage pulses (+60, -60, and -100 mV) in presence of 5 mM K⁺, 135 mM Rb⁺, and 135 mM K⁺ in a representative cell.

FIGURE 5. Oxytocin receptor mRNA and protein expressed in cultured hRPE cells. (A) Single hRPE cells were evaluated for OXTR (lane *i*), ATPase (lane *ii*), and Ezrin (lane *iii*) mRNA using single-cell RT-PCR. Lane *a* is the sample with superscript III, lane *b* is the sample without superscript III, and lane *c* is water with superscript III. Human fetal RPE cells expressed the expected 256-bp OXTR band. (B) Western blot analysis revealed the expression of an approximately 62 kDa OXTR protein in two separate hRPE cultures (hRPE1 and hRPE2) grown at 100% confluence for approximately 4 weeks. A similar approximately 62 kDa band was also observed in human placental tissue (control); β-actin was used as a control and was observed as an approximately 42 kDa band in all samples. Representative of three replicates. (C) Human fetal RPE cells in the microscope field showing OXTR (green) antibody labeling of cell membrane; DAPI (blue) was used to label the cell nucleus of pan cytokeratin (red)-positive cells. Scale bar: 5 μm.

FIGURE 6. Transient rise of hRPE $[Ca^{2+}]_i$ in response to OXT treatment. Human fetal RPE cells were cultured at 100% confluence and treated with FURA-2 AM to assess flux in $[Ca^{2+}]_i$, represented by a change in R (340/380) on stimulation with 100 μ M ATP and 100 nM OXT. (A) A qualitative colorimetric representation of the increase in 340/380 ratio for hRPE cells in HR (blue, left, i), treated with 100 nM OXT (green, middle, ii), and then returned to HR wash (blue, right, iii). (B) A representative trace from a randomly selected single hRPE cell treated with 100 μ M ATP after 4 minutes and 100 nM OXT after 10 minutes of the experiment. The increase in R (340/380) ratio represents an increase in $[Ca^{2+}]_i$. (C) The mean R (340/380) $\pm$ SEM change in response to ATP treatment and OXT treatment ($n = 282$). (D) A representative trace of OXT-induced Ca^{2+} response R (340/380) versus time before, during, and after treatment of cells with OXTR antagonist L-371,257 (gray bar). (E) Average increase in $[Ca^{2+}]_i$ inhibited by L-371,257 in 69 cells from three separate experiments. Horizontal bars in (B) and (D) indicate the duration of application.

used as a positive control because it is known to induce a transient rise in hRPE $[Ca^{2+}]_i$.²³ Figure 6B shows the time course of a representative 340/380-ratio output from an individual cell. When 100 μ M ATP was added to the HR extracellular bath ($t = 2.66$ minutes), the 340/380 ratio increased 0.09 units (87 nM Ca^{2+}), before returning to baseline (152 nM Ca^{2+}) on ATP wash ($t = 4$ minutes). After a 5.5-minute wash period in HR bath solution, 100 nM OXT was added to the solution bath ($t = 9.5$ minutes), which induced a reversible increase in the 340/380-ratio output of 0.083 units (79 nM Ca^{2+}) that returned to baseline (148 nM Ca^{2+}). The mean increase in the 340/380 ratio due to ATP and OXT treatment was plotted for 282 cells randomly selected from three separate experiments (Fig. 6D). The average 340/380-ratio output for ATP and OXT treatment was similar: 0.092 ± 0.003 (87 ± 2 nM Ca^{2+}) and 0.83 ± 0.0024 (79 ± 1.5 nM Ca^{2+}), respectively. To confirm that OXT activates OXTR, we used a specific OXT-antagonist, L-371, 257 (500 nM), which reversibly inhibited the OXT-induced Ca^{2+} rise during OXT (6 μ M) treatment (Fig. 6D) by more than 80% of the OXT response (Fig. 6D). Therefore, OXT specifically activates OXTR and effectively induces a transient rise in hRPE $[Ca^{2+}]_i$.

DISCUSSION

Paracrine signaling often involves the binding of a ligand to its appropriate cell surface G-protein-coupled receptor, resulting

in the downstream activation of signaling molecules that mediate a broad spectrum of processes within the cell. We describe the findings that OXT is present in the cone, but not the rod, photoreceptor extracellular matrix, evidenced by its colocalization with the cone-specific marker, PNA.^{19–21} OXTR is present in the adjacent RPE cell, and extracellular OXT induces an increase in hRPE $[Ca^{2+}]_i$ via activation of OXTR.⁹ Taken together, our results suggest that OXT is a paracrine signaling molecule of the RPE.

Oxytocin as a Ligand at the Cone Photoreceptors

Our specific colocalization of OXT with PNA to the cone photoreceptor extracellular matrix not only supports, but extends, the findings by Gauquelin and colleagues.^{10,11} Although this colocalized staining pattern also may implicate the presence of OXT in the RPE apical processes, the important finding here is that OXT is exclusively associated with cone photoreceptors, but not rod photoreceptors. The blood-retina barrier, much like the blood-brain barrier, separates the systemic and retinal domains, and restricts the diffusion of molecules to those smaller than 500 Da.^{13,24,25} Because OXT has a molecular weight of 1007 Da, it is too large to freely diffuse across the RPE.²⁶ Further studies are needed to determine how OXT arrives in the cone photoreceptor environment, and whether it is ferried to the retina via the systemic circulation, or is synthesized in the photoreceptor.^{10,11} Using arginine-vasopressin transgenic rats, Moritoh and

colleagues²⁷ demonstrated the presence of cells in the ganglion and inner nuclear layers that endogenously produce vasopressin. Given that OXT and vasopressin expression are closely related, we speculate that OXT is also endogenously produced within the retina.

Oxytocin Receptor is Present in the RPE

Expression of the OXTR in the human and rhesus retina adds it to the collection of peptide-hormone receptors that have been identified in the RPE, including the vasopressin V_{1a} receptor.^{12,15,28–30} We have shown that the OXTR is localized within the RPE, which is adjacent to the OXT-labeled cone photoreceptors. Given the proximity of OXT and OXTR, we propose that OXT is a candidate for retinal signaling between the cone photoreceptor and the RPE.

Oxytocin Receptor in RPE Triggers Cellular Signaling

Exogenous vasopressin, ATP, acetylcholine, neuropeptide Y, dopamine, serotonin, and epinephrine have been implicated in regulating RPE function due to their ability to induce an increase in RPE [Ca²⁺]_i via activation of their respective receptors.^{12,15,28,30,31} The ATP is thought to be a particularly important mediator of RPE function because the ATP-induced increase in RPE [Ca²⁺]_i facilitates regulated fluid flux across the RPE. This signaling pathway controls the hydration and chemical composition of the subretinal space, which is important for maintaining the RPE-retina interface.^{32,33} Therefore, ATP was used as an internal control for our study.

In previous reports, 100 μM ATP has been shown to induce increases in [Ca²⁺]_i in the range of 60 to 500 nM, depending on the imaging methods, culture conditions, and the RPE cell type used.^{32–34} In our hRPE cells, exogenous ATP increased hRPE [Ca²⁺]_i, which confirms that the population of hRPE cells used are characteristic of RPE cells. Although OXT did not induce an increase in hRPE [Ca²⁺]_i that was comparable in magnitude to that of ATP, the increase was within the range of previously reported increases in RPE [Ca²⁺]_i.^{32–34} Based on OXT specificity for OXTR at physiologic concentrations, as well as the finding that an OXTR antagonist significantly diminished the OXT-induced increase in hRPE [Ca²⁺]_i, the increase in hRPE [Ca²⁺]_i is likely via hRPE OXTR activation.

In summary, we have shown that OXT is exclusively associated with cone photoreceptors and OXTR is present in adjacent RPE cells. Because OXT also induces a physiologic response in cultured hRPE cells, as measured by [Ca²⁺]_i, OXT is a logical RPE paracrine signaling molecule. Given the plethora of RPE cell functions mediated by paracrine signaling molecules, our findings suggest that an OXT signaling pathway may play a critical role in the establishment of the posterior retina cell-signaling component critical for vision.^{1–5}

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