The Circadian Clock regulates RPE-mediated Lactate Transport via *SLC16A1* (MCT1)

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Highlights

The *SLC2A1* and *SLC16A1* transporter genes are highly expressed in ARPE-19 cells.

RPE monolayer organization is necessary for rhythmic transporter expression.

POS-incubation affects transporter expression in a time-dependent manner.

RPE-mediated transport is rhythmic for lactate, but not for glucose.

Abstract

Multiple retinal cells were shown to harbor a circadian oscillator, including retinal pigment epithelial cells (RPE). However, little is known about the functions that are regulated by the RPE clock. The aim of this study was to investigate whether the circadian clock in the RPE regulates the transport of glucose and its glycolytic metabolic by-product - lactate. To that end, we first characterized the mRNA expression profile of glucose and monocarboxylate transporters in ARPE-19 cells. We found that *SLC2A1* and *SLC16A1* were, respectively, the most abundantly expressed glucose and lactate (monocarboxylate) transporters. We further observed that the protein products of *SLC2A1* (encoding GLUT1) and *SLC16A1* (encoding MCT1) localize on the apical membrane of ARPE-19 monolayers. In a subsequent time-course experiment, we found that *SLC2A1* and *SLC16A1* mRNA oscillated in ARPE-19

monolayers, but not in dispersed cells, suggesting that monolayer cellular organization is necessary for rhythmic regulation of these transporters. In these monolayers, we found that MCT1 proteins varied over time in contrast to GLUT1 proteins which did not vary over time. Spectrophotometric measurements of supernatants sampled from ARPE-19 monolayer cultures revealed that glucose concentrations did not significantly differ between apical (Api) supernatants and basolateral (BL) ones. In addition, we did not find rhythms in Api or BL glucose concentrations. Conversely, we found higher lactate concentrations in Api supernatants than BL ones. Further, we found that Api lactate concentrations were rhythmic. Pearson's *r* revealed that the concentration gradients (Api - BL) of glucose and lactate correlated with the gene expression of respective *SLC2A1* and *SLC16A1* transporters. Incubation with photoreceptor outer segments (POS) affected the mRNA expression of *SLC16A1* and *SLC2A1* in ARPE-19 monolayers in a time-dependent manner, thus suggesting that the retina might modulate the RPE clock-controlled expression of transporters via interactions with POS. In conclusion, this work provides evidence that the transport of lactate is regulated by the circadian clock in the RPE.

Keywords: Retinal Pigment Epithelium, Glucose, Lactate, Epithelial Transport, Circadian Clock, Retina

1. Introduction

The mammalian retina contains a complete circadian clock system which regulates numerous physiological, biochemical and cellular processes within the eye (McMahon et al., 2014). These include melatonin synthesis, dopamine release, photoreceptor disc shedding and regulation of extracellular pH (McMahon et al., 2014). Virtually all retinal cells display cyclic functions, including photoreceptors (Tosini et al., 2007) as well as the adjacent monolayer of retinal pigment epithelial (RPE) cells (Baba et al., 2010). The existence of a self-sustained RPE clock is a relatively recent discovery (Baba et al., 2010). Earlier work demonstrated that cultured human RPE cells are capable of generating rhythms of clock gene transcription and adenylyl-cyclase activity (Pavan et al., 2006; Yoshikawa et al., 2008). While recent studies investigated various mechanisms of regulation of the RPE clock (Baba et al., 2017; Ikarashi et al., 2017; Morioka et al., 2018; Milićević et al., 2019), little is known about processes and functions it regulates.

The retina is among the most energy demanding tissues in the human body (Ames et al., 1992; Anderson and Saltzman, 1964; Yu and Cringle, 2001) with the photoreceptors being the most metabolically active cells in the retina (Narayan et al., 2017). Photoreceptors metabolize glucose via

glycolysis and oxidative phosphorylation (Ames et al., 1992; Narayan et al., 2017). Glycolysis is considered as an energy-inefficient metabolic pathway as it yields 2 ATP/glucose and generates lactate as a metabolic byproduct. In contrast, oxidative phosphorylation produces 34 ATP/glucose (Ng et al., 2015). In photoreceptors, glucose metabolism exhibits day/night variations, in which glycolysis occurs primarily during light exposure and oxidative phosphorylation in darkness (Lin et al., 2015). This shift creates considerable disparities in daily photoreceptor glucose demand and production of subretinal lactate as a metabolic byproduct of glycolysis.

To meet the metabolic needs of the retina, the RPE supplies glucose and removes subretinal lactate via transporters expressed on the RPE (Adijanto and Philp, 2012). On the basolateral side of the RPE, the GLUT1 transporters move glucose from the blood in choriocapillaris through the RPE cytoplasm. Glucose is then transferred via apically expressed GLUT1 to the subretinal space. Lactate is removed from the subretinal space via apical monocarboxylate transporter (MCT) 1, partially metabolized within the RPE and the remaining lactate is secreted to the choriocapillaris via basolaterally expressed MCT3 (Adijanto and Philp, 2012). Considering that the RPE harbors a circadian oscillator (Baba et al., 2017; Baba et al., 2010; Baba and Tosini, 2018), we hypothesize that the RPE circadian clock meets the daily metabolic needs of photoreceptors via rhythmic expression of glucose and monocarboxylate transporters.

In this study, we characterized the mRNA expression profile of glucose and monocarboxylate transporters in an ARPE-19 cell culture model and the protein localization of the most abundantly expressed transporters GLUT1 and MCT1. We found rhythmicity of mRNA expression of *SLC2A1* and *SLC16A1* transporters as well as MCT1 proteins in monolayer but not in dispersed cultures. We probed whether the clock mediated regulation of transporter mRNA affects the glucose and lactate translocation by measuring glucose and lactate concentrations from sampled supernatants from our ARPE-19 monolayers. Finally, by incubating ARPE-19 monolayers with POS, we show that photoreceptors might affect clock-controlled mRNA expression of transporters via POS shedding.

2. Materials and methods

2.1. Cell culture

Culturing of ARPE-19 cells was performed essentially as described in (Milićević et al., 2019). In brief, ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium:

Nutrient Mixture F-12 with L-glutamine and Hepes (DMEM/F12, Gibco life technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (PS) (Gibco BRL, Grand Island, NY, USA) at 37°C and 5% CO₂. After 5 passages, the cells were either grown as monolayers or as dispersed cells. Dispersed cells were plated on 12-well culture plates (#3513 Costar, Kennebunk, ME, USA) at a density of 1.6×10^5 cells/cm² in a volume of 1.5 ml and cultured for 4 days.

Monolayers were grown by plating on Matrigel-coated (160 µl of 1:40 diluted GFR Matrigel, Corning, NY, USA) transwell filters (#3460 Costar, Kennebunk, ME, USA, 12-well plate, 12 mm diameter, 0.4 µm pore size). The seeding density was 1.6×10^5 cells/cm² in a total volume of 0.5 ml/filter. Monolayers were cultured using DMEM/F12 medium supplemented with 1% FCS and PS for 28 days. Growth and morphology was monitored using microscopy. Before applying lysis buffer (PCR and WESTTM experiments), matrigel was first removed from the cells by incubating in Cell Recovery solution (Corning, Bedford, MA, USA) for 30 min at 4°C.

Synchronization of cells was performed by applying 50% FCS in DMEM/F12 between time-points 0-2h ("serum-shock"), followed by a medium change using serum-free DMEM/F12 as described previously (Balsalobre et al., 1998).

We measured transepithelial electrical resistance (TEER) of monolayer samples prior to experiments using an ohmmeter (Millicell ERS-2, Billerica, MA, USA). We performed a background correction of TEER measurements by subtracting the value of a Matrigel coated filter without cells from TEER measurements with cells. The resulting values were multiplied by transwell filter area. We chose TEER of 20 Ωcm² as a threshold value for intact monolayers.

2.2. RNA isolation, cDNA synthesis and mRNA quantification

We extracted total RNA using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. We used 50 ng of total RNA to generate complementary DNA with oligo(d.T) primed reactions using Superscript III reverse transcriptase (Life technologies, Waltham, MA, USA). The synthesized cDNA was amplified with transcript-specific, intron-spanning primers. We used PCR programs optimized for transporter mRNA expression profiling (**Fig. 1**) and quantification (**Fig. 2, Table 2 and 5**). Table 1 shows the sequences of used primers. After electrophoresis on 2% agarose gels, PCR products were imaged using the FujiFilm LAS300 (Tokyo, Japan) and quantified using Aida image

analyzer v4.26. Normalization was performed by division with the signal obtained from the PCR product of the reference gene *EF1 α* and by using average expression values within an experiment as a calibrator between experiments.

Gene	Forward Primer	Reverse Primer
Glucose transporter genes		
<i>SLC2A1</i>	CACTGTCGTGTCGCTGTTTG	TCCGGCCTTTAGTCTCAGGA
<i>SLC2A2</i>	CAGTCAAGGACCACGTCCTG	CAGTCTCTGTAGCTCCTAGG
<i>SLC2A3</i>	ACTTACTCCCGGAACGCTGG	TGGCTGGTAGAGCCCACTTC
<i>SLC2A4</i>	CATGGGTTTCCAGTATGTTGC	GGTGAAGGCAGCTGAGATC
<i>SLC2A5</i>	CCACGTCATAGGACATGCC	TGGTGAGGAGGCAGATCACG
Monocarboxylate transporter genes		
<i>SLC16A1</i>	TGTTGACATGGTAGCCCGAC	TGAGCCGACCTAAAAGTGGT
<i>SLC16A7</i>	AACTCTCATGGACCTCGTGG	GCCAATGAGCAGCCACACGC
<i>SLC16A8</i>	GCCTCTCCTACGGCATGGTG	GGCGCTGAGCACCTCCAGG
<i>SLC16A4</i>	TGCCTGTACTGGTTGATCTGT	TGCGATGTGTTTCTTTCAAGC
<i>SLC16A5</i>	TCTTCCTCATCTCAGCTGCC	CACTCATGGCTGCTCCTGGA
Clock genes		
<i>REV-ERBα</i>	TAAGCCGCACCACCTACAGC	GGCCAGAGGCTCATCTTGGA
<i>PER2</i>	GCGTCATGATGACGTACCAG	TCGCTGAGTCCCAGAGAAGG
Reference gene		
<i>EF1α</i>	AAATAAGCGCCGGCTATGCC	CAAAGCGACCCAAAGGTGGAT

Table 1. List of primers used

2.3. Western Blotting

ARPE-19 monolayers were “serum-shocked” (t=0-2h), given serum-free medium (t=2h) and harvested at t=12h and t=24h. Matrigel was removed, and cells were lysed with RIPA buffer (10 mM Tris/HCl pH 8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% Triton X-100) supplemented with phosphatase (PhosSTOP, Roche, Mannheim, Germany) and protease inhibitors (cOmplete, Roche). We used a modified Lowry assay (DC protein assay, Bio-Rad laboratories, USA) to determine total protein concentrations. Concentrations varied between 0.55 and 0.76 mg/ml. Protein detection and quantification was performed using the capillary-based automated WESTM Simple Western (ProteinSimple, San Jose, USA). All procedures were performed according to the manufacturer’s instructions (Harris, 2015) with the following modifications: For GLUT1 detection and quantification, we prepared samples using 3.5 μ l of protein lysates, 0.5 μ l of 0.1X sample buffer and 1 μ l of fluorescent marker. Samples were heated at 55°C for 15 min and loaded into each WESTM plate

well. For MCT1 detection and quantification, we used 3 μ l of protein lysates, 1 μ l of 1X sample buffer supplemented with 2.5% β -mercaptoethanol and 1 μ l of fluorescent marker. Samples were heated at 37°C for 30 min and loaded into each WES™ plate well. We used the following primary antibodies: GLUT1 (R&D Systems, 40 μ g/ml, MAB14181) and MCT1 (Abcam, 1:15, AB90582). After WES™ quantification, we normalized the protein expression profiles of GLUT1 and MCT1 with total protein concentrations of the respective sample lysate, as previously described (Milićević et al., 2019)

2.4. Photoreceptor Outer Segment isolation

Bovine eyes were obtained from the slaughterhouse. After cutting the eyes open, we scrapped the retinas from posterior eye cups. Retinas were homogenized in 42% sucrose, 1 M NaCl, 0.1 M MgCl₂ and 1 M Tris-acetate. The supernatant was centrifuged, filtered through a sterile gauze and diluted in 2 volumes of 0.01 M Tris-acetate. POS were separated in a sucrose gradient by ultracentrifugation (Optima ultracentrifuge, Beckman Coulter, Inc., Brea, CA) at 25,000 RPM for 40 min at 4°C. POS were recovered in 0.01 M Tris-acetate, ultracentrifuged at 20,000 RPM for 30 min at 4°C, and dissolved in DMEM:F12 medium. The number of POS particles / ml was determined by manual counting using a haemocytometer.

2.5. Photoreceptor Outer Segment incubation assay

After synchronization of ARPE-19 monolayers (t = 2h), serum-containing medium was removed and 0.4 ml and 1 ml of serum-free medium was administered to apical and basolateral compartments, respectively. At the intervention time-point (either t = 9h, 15h, 21h, 27h or 33h) 100 μ l of medium or POS was administered. Cells were incubated with 1.2×10^7 POS particles for 3h (or 6h, see Supplementary file) at 37°C in 5% CO₂. After a 3h incubation, the cells were washed, harvested, cleaned from Matrigel and lysed.

2.6. Immunocytochemistry and imaging

Cells were fixed with 4% paraformaldehyde for 10 min at RT. Transwell filters with cells were cut and mounted on poly-L-lysine coated glass coverslips. Blocking was performed using 1% BSA, 10% normal donkey serum, 0.2% Triton X-100 in PBS. Incubation with primary antibodies (MCT1, 1:250, Abcam, AB90582; GLUT1, 1:250, ThermoFisher, MA5-11315) was performed for 16h at 4°C. Incubation with

Cy3 conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch, 715-165-150, USA) was performed for 60 min at RT. Samples were mounted with Vectashield antifade medium with DAPI (Vector laboratories, Burlingane, CA, USA). Confocal stacks were acquired with a Leica TSC SP-8 mounted on a Leica DMI6000 inverted microscope using a HC Plan Apochromat x63 objective.

2.7. Biochemical analysis

After performing the “serum-shock” synchronization on ARPE-19 monolayers (Balsalobre et al., 1998), we applied exactly 0.5 ml of serum-free medium apically and 1ml basolaterally. Apical and basolateral supernatants were sampled immediately before cell harvesting (for PCRs). An aliquot of 100 µl was submitted to biochemical analysis (Cobas 8000 ISE900 Chemistry Analyzer, Roche Diagnostics, Rotkreuz, Switzerland) at the Department of Clinical Chemistry, Amsterdam UMC. Glucose (kit: GLUC3 glucose HK gen.3, Roche 05168791190) and lactate concentrations (kit: LACT2 Lactate Gen.2, Roche 05171881190) were measured according to manufacturer’s instructions (Roche Diagnostics, Rotkreuz, Switzerland).

2.8. Data analysis

2.8.1 Analysis of glucose and lactate concentrations.

Data analysis was performed on raw and detrended glucose and lactate concentrations. To remove the trend (continuous decrease/increase in measurements) of glucose and lactate concentrations, we first performed linear regression on the raw glucose ($p < 0.0001$, $r^2 = 0.84$, $y = -0.1196 \cdot t + 17.4$) and lactate data ($p < 0.0001$, $r^2 = 0.84$, $y = 0.2263 \cdot t + 0.4362$). To detrend the data, we subtracted the respective calculated y values from each glucose and lactate measurement. To remove negative y values after detrending, the minimum y value was set to zero. In that regard, we added, respectively, 0.7296 and 1.5674 to each detrended glucose and lactate measurement. Further analysis was performed on detrended data. Concentration gradients were calculated by subtracting basolateral from apical concentrations.

2.8.2 Statistics

Data were obtained from at least 3 biological replicates and expressed as mean±SEM. Plots were generated using GraphPad Prism software (La Jolla, CA, USA) or SigmaPlot (Systat Software, San Jose,

CA, USA). Normality of distribution was confirmed using the Kolmogorov-Smirnov test. Circadian gene expression profiles were determined by nonlinear least squares fitting to a sine wave function using SigmaPlot: $y = y_0 + c \cdot \cos [2\pi (t-\phi)/\tau]$, where τ represents period, ϕ acrophase and c amplitude. The function featured the following constraints: $20h < \tau < 28h$; $\phi < \tau$ and $c > 0$. Where indicated analysis was performed with a 2-way ANOVA followed by Holm-Sidak's post-hoc tests or 1-way ANOVA. Correlations between gene expression and solute concentration gradients were performed using Pearson's r .

3. Results

3.1. Glucose and lactate transporter expression profile in ARPE-19 cell cultures

We first characterized the mRNA expression profile of glucose transporters in ARPE-19 cell cultures. RT-PCR analysis revealed that in both ARPE-19 dispersed cells and monolayers, the expression of *SLC2A1* mRNA was considerably high, while expression of other glucose transporters was remarkably low (*SLC2A5*) or below detection (*SLC2A2-4*) (Fig. 1a).

Next, we examined the monocarboxylate transporter mRNA expression profile of ARPE-19 cells (Fig. 1b). RT-PCR analysis revealed detectable expression of *SLC16A5* and *SLC16A7*, but not of *SLC16A4* and *SLC16A8*, in both ARPE-19 cultures. Of all monocarboxylate transporter genes, the mRNA expression of *SLC16A1* was the highest in our ARPE-19 cultures.

Finally, we aimed to find the localization of proteins encoded by the most abundant glucose (*SLC2A1*) and monocarboxylate (*SLC16A1*) transporters in ARPE-19 monolayers, and compare these with the *in vivo* RPE situation. Confocal microscopy revealed that GLUT1 (Fig. 1c) and MCT1 (Fig. 1d) localized on the apical membrane of ARPE-19 monolayers. Therefore, our ARPE-19 monolayers displayed MCT1 localization similar to *in vivo* RPE (Philp et al., 1998). However, they lacked basolateral expression of GLUT1 (Sugasawa et al., 1994).

Figure 1. Expression profile of glucose and monocarboxylate transporters in ARPE-19 cell cultures. (a) The positive controls used in RT-PCR analysis were: cortex (C), retina (R), placenta (P) and liver (L) cDNA. W – water. The ARPE-19 dispersed cells (Disp. c.) and monolayer (Monolay.) samples were obtained from 4 independent cultures. These cells expressed the *SLC2A1* glucose transporter. Gene expression of *SLC2A5* was very low and *SLC2A2-4* mRNA was not detectable in ARPE-19 cells. (b) *SLC16A1-8* transporter expression profile in ARPE-19 cells. Immunohistochemical localization of (c) GLUT1 and (d) MCT1 in ARPE-19 monolayers. Nuclei were labeled with DAPI. Confocal microscopy revealed that GLUT1 and MCT1 localized on the apical membrane of ARPE-19 monolayers (white arrows).

3.2. Expression profile of glucose and monocarboxylate transporter genes is rhythmic in monolayer, but not in dispersed ARPE-19 cell cultures

Our previous work indicated that cellular organization of the RPE affects circadian parameters of clock and clock-controlled gene expression in ARPE-19 cultures (Milićević et al., 2019). To investigate

whether the ARPE-19 monolayers have enhanced rhythmicity of transporter gene expression with respect to dispersed ARPE-19 cells, we performed a series of RT-PCR analyses on mRNA from both cultures. We synchronized ARPE-19 cells using the serum-shock procedure by giving them 50% serum (t = 0 - 2h), then serum-free medium (t = 2h). We harvested cells at 3h intervals for 54h and we characterized the circadian expression profile of *SLC2A1* and *SLC16A1* by RT-PCR.

The clock genes *REV-ERB α* and *PER2* were shown to be among the most rhythmically expressed in ARPE-19 dispersed and monolayer cultures (Milićević et al., 2019). Thus, in this study, we performed a RT-PCR analysis on these genes to confirm the rhythmicity of our ARPE-19 cultures. As expected, RT-PCR analysis showed that *REV-ERB α* and *PER2* were *indeed* rhythmic in both dispersed and monolayer cultures (Fig. 2; Table 2). Dispersed ARPE-19 cell cultures did not show rhythmic mRNA expression of *SLC2A1* (Fig. 2; Table 2) and *SLC16A1* (Fig. 2; Table 2). In contrast, ARPE-19 monolayers did show rhythmic mRNA expression of *SLC2A1* (Fig. 2; Table 2) and *SLC16A1* (Fig. 2; Table 2). These results suggest that the RPE monolayer cellular organization is necessary for rhythmic mRNA expression of *SLC2A1* and *SLC16A1* transporters.

Figure 2. The monolayer cellular organization of the ARPE-19 is necessary for rhythmic mRNA expression of the *SLC2A1* glucose and *SLC16A1* monocarboxylate transporters. Time is represented on the x-axis, relative mRNA expression is on the y-axis. Dispersed (Top) and monolayer (Below) ARPE-19

cells were cultured. Cells were synchronized using 50% serum (t = 0-2h), then the medium was changed to serum-free medium (t = 2h), followed by cell harvest at 3h intervals and RT-PCR analysis. Traces represent the best fitted sinusoidal regressions supporting rhythmic mRNA expression (dashed lines when non-rhythmic expression).

Table 2. Statistical analysis of transporter and clock gene mRNA rhythmicity

Non-linear regression analysis					1-way ANOVA	
Gene	P value	Period (h)	Acrophase (h)	Amplitude	P value	F value
Dispersed ARPE-19 cells						
Transporter genes						
<i>SLC2A1</i>	0.3818	21.00±2.38	18.51±2.10	0.52±0.29	0.0033	2,93
<i>SLC16A1</i>	0.2827	20.00±1.85	9.10±2.33	0.12±0.06	0.2076	1.37
Clock genes						
<i>REV-ERBα</i>	<0.0001	21.64±0.81	18.18±0.69	0.58±0.10	<0.0001	5.14
<i>PER2</i>	0.0010	20.95±1.12	2.05±1.61	0.66±0.15	0.0170	2.31
ARPE-19 monolayers						
Transporter genes						
<i>SLC2A1</i>	0.0218	22.05±1.54	15.94±1.46	0.31±0.10	<0.0001	4.49
<i>SLC16A1</i>	0.0198	28.00±2.60	18.59±1.78	0.19±0.06	<0.0001	6.18
Clock genes						
<i>REV-ERBα</i>	<0.0001	23.37±1.20	15.44±1.02	0.40±0.08	0.0024	2.97
<i>PER2</i>	0.0475	23.48±1.63	20.80±1.25	0.33±0.11	0.1117	1.59

Table 2. Statistical analysis of transporter and clock gene rhythmicity in ARPE-19 dispersed and monolayer cultures. Data were fitted to the equation $y = y_0 + c \cdot \cos [2\pi (t-\phi)/\tau]$ by non-linear regression. Bold values represent p<0.05

3.3. Do transporter proteins show temporal variations in ARPE-19 monolayers?

Considering that the monolayer cellular organization is necessary for rhythmic expression of transporter mRNA, we hypothesized that in monolayers, transporter proteins also show temporal variations. To test this hypothesis, we synchronized ARPE-19 monolayers (t = 0-2h) and harvested these cells at 2 time-points (t = 12h and t = 24h). Using the WESTTM capillary based protein quantification technique, we found that GLUT1 protein levels did not show statistically significant differences

between $t = 12\text{h}$ and $t = 24\text{h}$ (Student's t -test, $p = 0.94$). Conversely, differences in MCT1 protein levels between these 2 time-points were significantly different (Student's t -test, $p = 0.01$). These results suggest that in ARPE-19 monolayers, *SLC16A1* rhythmicity is translated to the protein level, in contrast to *SLC2A1* which shows rhythms only on the mRNA level.

Figure 3. Time affects the protein expression of MCT1 but not GLUT1 in ARPE-19 monolayers. ARPE-19 monolayers were synchronized by “serum-shock” ($t = 0 - 2\text{h}$), given serum-free medium ($t = 2\text{h}$) and harvested at $t = 12\text{h}$ and $t = 24\text{h}$. (Top) Quantification of proteins using WESTM revealed no statistically significant differences in GLUT1 proteins between $t = 12\text{h}$ and $t = 24\text{h}$ (Student's t -test, $p = 0.94$). In contrast, MCT1 protein levels differed between $t = 12\text{h}$ and $t = 24\text{h}$ (Student's t -test, $p = 0.01$). Individual values, means and SEM are plotted. (Bottom) Cropped digitally generated WESTM images are shown (uncropped are provided in Figs S2-S3). GLUT1 was detected at 61 kDa and MCT at 47-48 kDa.

3.4. The circadian regulation of glucose and lactate transport.

Since we observed rhythmic mRNA expression of the main retinal glucose transporter *SLC2A1* and lactate transporter *SLC16A1*, we wanted to determine whether this rhythm also has functional consequences. We opted for an indirect approach by measuring remaining glucose and accumulating lactate in ARPE-19 monolayer supernatants sampled from apical (Api) and basolateral (BL) compartments.

ARPE-19 monolayers were synchronized using the serum-shock procedure as previously described. We sampled Api and BL supernatants at 6h intervals for 30h. As expected, a two-way ANOVA analysis revealed that glucose concentrations continuously decreased over sampling time (Fig. 4a, $F(4, 20) = 29.54$, $p < 0.0001$), but were not affected by compartment ($F(1, 20) = 1.54$, $p = 0.23$) or time x compartment ($F(4, 20) = 0.98$, $p = 0.44$). To determine whether the concentration of glucose oscillated in supernatants, we performed non-linear regression on detrended data (Fig. 4c). We did not find rhythms in Api ($p = 0.11$) or BL ($p = 0.52$) glucose concentrations. We then calculated glucose gradients for each time-point by subtracting Api – BL glucose concentrations. We found that Pearson's linear regression revealed a significant negative relationship between the relative mRNA expression of *SLC2A1* and calculated glucose gradients ($r = -0.60$, $p < 0.05$). Taken together, these results suggest that RPE-mediated glucose transport is not rhythmic.

We then measured lactate concentrations from Api and BL supernatants from our time-course experiment. Two-way ANOVA testing showed that lactate concentrations were significantly increased over sampling time (Fig. 4b, $F(4, 20) = 317$, $p < 0.0001$) and were affected by sampling compartment ($F(1, 20) = 315.5$, $p < 0.0001$) and had a tendency for time x compartment ($F(4,20) = 2.50$, $p = 0.08$). These results suggest that lactate was either preferentially secreted in, or translocated to the Api side of ARPE-19 monolayers. Non-linear regression analysis on detrended data showed that Api lactate was rhythmic ($c = 0.78 \pm 0.15$; $\phi = 12.24 \pm 0.80h$; $\tau = 26.54 \pm 1.99h$ and $p < 0.01$) and BL showed a tendency ($p = 0.06$). To confirm if the lactate transfer rate is correlated with the mRNA of the *SLC16A1* transporter we performed Pearson's r analysis. Pearson's linear regression showed a significant relationship between the relative gene expression of *SLC16A1* and lactate concentration gradients ($r = 0.68$, $p < 0.05$). In summary, these results suggest that the circadian clock regulates the translocation of lactate.

Figure 4. Temporal analysis of supernatant glucose and lactate concentrations of ARPE-19 monolayers. Apical (Api) and basolateral (BL) supernatants were sampled from ARPE-19 monolayers at 6h intervals. Cells were synchronized using medium supplemented with 50% fetal calf-serum at t=0-2h followed by a medium change at t=2h. (a) Glucose and (b) lactate concentrations were measured from Api and BL supernatants. Non-linear regression was performed on detrended (c) glucose and (d) lactate concentrations. Glucose and lactate concentration gradients were calculated by subtracting BL concentrations of respective solutes from Api ones. Results of Pearson's r correlation revealed a

relationship between (e) glucose and (f) lactate transporter mRNA and concentration gradients of respective solutes. Values are plotted on graphs or shown as means $\pm$ SEM, n = 3 / time-point / treatment. **** p<0.0001

3.5. Incubation with POS regulates the mRNA expression of the *SLC2A1* and *SLC16A1* transporters.

Our previous experiments showed that *SLC2A1* and *SLC16A1* exhibit rhythmic mRNA expression in the RPE (Fig. 2). Considering that the RPE and photoreceptors lie in close contact and are metabolically coupled (Kanow et al., 2017); (Adijanto and Philp, 2012), we speculate that the interactions with photoreceptors may regulate the mRNA expression of glucose and monocarboxylate transporters in the RPE.

To test this hypothesis, we first synchronized ARPE-19 monolayers by serum-shock treatment (t = 0 – 2h) and gave them serum free medium (t = 2h). We then incubated cells with POS or medium at time points t = 9 - 12h; t = 15 - 18h; t = 21 - 24h; t = 27 - 30h; t = 33 - 36h, harvested the cells and performed RT-PCR analysis.

A 2-way ANOVA analysis showed a treatment effect on *SLC2A1* (Fig. 5a, F(1, 20) = 5.74, p < 0.01), but not on *SLC16A1* (Fig. 5b, F(1, 20) = 0.49, p = 0.49) mRNA expression levels. Time was a significant factor for *SLC2A1* (Fig. 5a, F(4, 20) = 5.74, p < 0.01), but not for *SLC16A1* (Fig. 5b, F(4, 20) = 0.86, p = 0.51) mRNA expression levels. Time x treatment was significant for *SLC2A1* (Fig. 5a, F(4, 20) = 4.33, p < 0.05) and had a tendency for *SLC16A1* (Fig. 5b, F(4, 20) = 2.56, p = 0.07) mRNA expression levels. Further, Holm-Sidak's post-hoc test revealed that POS incubation performed at t = 27 - 30h, significantly decreased *SLC2A1* (p < 0.001) and *SLC16A1* (p < 0.05) mRNA expression. In contrast, we did not observe any effects of a 3h or 6h POS incubation on transporter gene expression in non-synchronized ARPE-19 monolayers (Fig. S1). These results suggest that POS can exert a phase-dependent effect on gene expression of glucose and monocarboxylate transporters in the RPE.

Figure 5. POS incubation has a phase dependent effect on transporter gene expression in ARPE-19 monolayers. (a, b) Cells were synchronized using medium containing 50% serum for 2h ($t = 0 - 2h$) and received serum-free medium at $t = 2h$. Cells were incubated with POS (red lines) either between $t = 9-12h$, $t = 15-18h$, $t = 21-24h$, $t = 27-30h$ or $t = 33-36h$. Incubation with medium (black lines) at the same time-points was used as a control. At $t = 27-30h$ POS significantly down-regulated the mRNA expression of *SLC2A1* (a, Holm-Sidak's post-hoc test, $p < 0.001$) and *SLC16A1* (b, Holm-Sidak's post-hoc test, $p < 0.05$). Values are plotted as means $\pm$ SEM, $n=3$ / time-point / treatment. * $p < 0.05$, *** $p < 0.001$

4. Discussion

In this study, we tested the hypothesis that the RPE clock regulates RPE-mediated glucose and lactate transport. We report rhythmic mRNA expression of the most abundant glucose (*SLC2A1*) and monocarboxylate (*SLC16A1*) transporters in ARPE-19 monolayers. In these cells, time did not affect GLUT1 protein levels, in contrast to MCT1 proteins which *did* vary over time. Our results suggest that rhythmic expression of these transporters is influenced by RPE-intercellular coupling and interactions with photoreceptors via POS. Finally, our results suggest that RPE-mediated lactate transport is rhythmic, but we found no evidence of rhythmicity of RPE-mediated glucose transport.

We first characterized the glucose (*SLC2A*) and monocarboxylate (*SLC16A*) transporter gene expression profile in a human RPE cell line (ARPE-19). We found that *SLC2A1* was the most abundant glucose transporter in ARPE-19. This finding is in agreement with previously published work using fresh RPE cultures (Senanayake et al., 2006), laser capture microdissected RPE from human donor eyes (van

Soest et al., 2007) and relevant data from mouse RPE (analysis of the GEO data set GSE10246 (Lattin et al., 2008) performed by Swarup et al., 2019). We report apical localization of the protein product of *SLC2A1*, GLUT1 in our ARPE-19 monolayers corroborating previous results (Senanayake et al., 2006), but lacking basolateral expression which is an *in vivo* characteristic (Philp et al., 2003a; Philp et al., 2003b; Sugawara et al., 1994; Swarup et al., 2019). The uptake of glucose by monolayer cultures from apical and basolateral supernatants was similar. Thus, it is possible that basolateral glucose might be ingested by other transporters which were not studied in this paper.

The monocarboxylate transporter *SLC16A1* was the highest expressed lactate transporter in our ARPE-19 cultures. We localized the corresponding protein, MCT1, to the apical RPE membrane of monolayers. These findings are also in agreement with the literature (Philp et al., 2003a b). However, in contrast to the findings of Philp and coworkers, we were not able to show *SLC16A8* (encoding MCT3) or *SLC16A4* (encoding MCT4) mRNA expression in our cultures (Philp et al., 2003a). This inconsistency may be due to differences in culturing conditions as Philp and coworkers cultured ARPE-19 for an extended period (up to 270 days) without using Matrigel coated transwell inserts (Philp et al., 2003b). Our data suggests that cultures secreted lactate in the apical supernatants, suggesting that the MCT1 transporter plays a prominent role in lactate trafficking in our cultures. The gradual increase in basolateral lactate concentrations might be mediated via other MCT transporters expressed by our cultures.

Our study is the first to show rhythmic gene expression of *SLC2A1* and *SLC16A1* in the RPE. This result is expected since the rhythmic expression of these genes was demonstrated in various tissues in previous studies. For instance, studies have shown rhythmic mRNA expression of *Slc2a1* in rat cerebellum (Soltesova et al., 2013) and in numerous baboon organs and tissues including: the brain, bladder, thyroid, duodenum, kidney medulla and omental fat (Mure et al., 2018). In addition, a time-dependent regulation of glucose uptake was suggested in ARPE-19 cells (Kim et al., 2007). Likewise, the *Slc16a1* mRNA is rhythmically expressed in rat jejunal mucosa (Stearns et al., 2008) and baboon bladder, preoptic area, stomach fundus, omental, retroperitoneal and perirenal fat (Mure et al., 2018). However, the necessity of a monolayer RPE cellular organization for promoting rhythmic gene expression of *SLC2A1* and *SLC16A1* is not expected. Our recent work showed that the monolayer cellular organization of the RPE enhances the amplitudes and shortens the periods of clock gene oscillations, but is otherwise dispensable for the rhythmicity of these cells (Milićević et al., 2019). It is known that in RPE monolayers cells communicate via gap junctions (Ning et al., 2013; Pearson et al., 2004; Vainio et al., 2015) through which secondary messengers such as Ca^{2+} and cAMP can freely permeate (Geletu et al., 2012). It may be possible that the rhythmicity of *SLC2A1* and *SLC16A1*

transporters is driven by a secondary messenger-triggered molecular cascade. However, this rhythmicity is translated only for MCT1 protein and not GLUT1 protein. Inconsistencies between mRNA and protein oscillations were reported in literature, which may be explained by differences in degradation kinetics of studied proteins (Jang et al., 2015; Mauvoisin et al., 2014). Further studies are necessary to elucidate these unexpected effects of culturing conditions on transporter mRNA and protein expression.

We report discrepancies in RT-PCR results between our ARPE-19 monolayer samples obtained at 3h intervals for 54h (Fig. 2) and the control group in our phase-dependent intervention experiments (Fig. 5). In our previous work we also observed similar inconsistencies (Milićević et al., 2019). It is well-known that a medium change affects the results of bioluminescence recordings of explant cultures obtained from *Per2^{LUC}* transgenic mice (Baba et al., 2015; Guenthner et al., 2014). Thus, the inconsistencies observed between Fig. 2 and 5 in this study are likely due to a phase-dependent effect of medium administration.

We hypothesized that rhythmic regulation of these transporters in the RPE is necessary to meet the time-dependent metabolic needs of the retina. In agreement with this view, we observed in our cultures, that the acrophases of mRNA expression of *SLC16A1* (18.59 ± 1.78 h) and *SLC2A1* (15.94 ± 1.46 h) were similar. The expression of these transporters was affected by POS at the same time-point after synchronization ($t = 27 - 30$ h), suggesting that interactions with photoreceptors regulate RPE-mediated metabolite transport. Further, lactate concentrations oscillated in apical supernatants. In addition, the concentration gradients of glucose and lactate correlated with the mRNA expression of respective transporters. However, we did not observe significant differences between apical and basolateral glucose concentrations, and no rhythm of glucose concentrations in supernatants. Thus, results from our *in vitro* assay suggest that the rhythmic regulation of *SLC16A1* is reflected on a functional level, whereas we find no evidence for rhythmic glucose transport.

Previous research reported that inhibition of monocarboxylate transport disrupts retinal function, in particular b-wave and oscillatory potential amplitudes in rats (Bui et al., 2004). Considering that we have shown rhythmic regulation of the *SLC16A1* gene (encoding MCT1) in the RPE, we speculate that the rhythmicity of this gene plays a role in maintaining retinal health. For instance, during aging nearly all aspects of clock-regulated physiological processes deteriorate. Dysregulation of circadian clocks is considered as a potential mechanism that underlies age-associated changes in physiology and behavior (Hood and Amir, 2017). Accordingly, retinal and RPE clock alterations were reported in aged mice (Baba

and Tosini, 2018). In addition, clock disruption was shown to exacerbate age-related retinal cell death in mice (Baba et al., 2018). It is therefore, reasonable to speculate that aging disrupts clock regulated metabolite transport, thus accelerating retinal cell death.

5. Conclusion

In conclusion, our work provides evidence that RPE-mediated transport of lactate is regulated by the circadian clock. This finding broadens the scope of physiological functions regulated by the circadian clock in the RPE-photoreceptor functional unit and its potential role in maintaining retinal health.

Acknowledgements

We thank Hella Aberson and Angelica Duursma for technical assistance. This project has been funded with support from the NeuroTime Erasmus+ of the European Commission, RSB Rotterdam, Nelly Reef foundation, Foundation Ooglijders and Het Lot SVLB.

Author Contributions

NM designed, performed experiments, analyzed data, wrote the manuscript; J.t.B and A.t.A. provided technical assistance; A.B. and M.P.F.S. designed, analyzed and wrote the manuscript.

Competing interests statement

The authors declare no potential competing interests with respect to the research, authorship, and/or publication of this article.

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