Title

- Full title: **Clock gene-independent daily regulation of haemoglobin oxidation in red blood cells**
- Short title: RBC haemoglobin oxidation rhythms

Authors

Andrew D. Beale[1], Priya Crosby[1,8], Utham K. Valekunja[2,3], Rachel S. Edgar[4], Johanna E. Chesham[1], Elizabeth S. Maywood[1], Fatima H. Labeed[5], Akhilesh B. Reddy[2,3], Kenneth P. Wright Jr.[6], Kathryn S. Lilley[7], Michael H. Hastings*[1], John S. O'Neill*[1]

Affiliations

[1] MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, UK

[2] Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Pennsylvania, PA 19104, USA.

[3] Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[4] Department of Infectious Diseases, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, UK.

[5] Faculty of Engineering and Physical Sciences, University of Surrey, Stag Hill, Guildford, GU2 7XH, UK

[6] Department of Integrative Physiology, Sleep and Chronobiology Laboratory, University of Colorado Boulder, Boulder, CO 80309-0354, USA

[7] Cambridge Centre for Proteomics, Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK

[8] Present address: Department of Chemistry and Biochemistry, University of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA.

* Correspondence: oneillj@mrc-lmb.cam.ac.uk; mha@mrc-lmb.cam.ac.uk

Abstract

Cellular circadian rhythms confer daily temporal organisation upon behaviour and physiology that is fundamental to human health and disease. Rhythms are present in red blood cells (RBCs), the most abundant cell type in the body. Being naturally anucleate, RBC circadian rhythms share key elements of post-translational, but not transcriptional, regulation with other cell types. The physiological function and developmental regulation of RBC circadian rhythms is poorly understood, however, partly due to the small number of appropriate techniques available. Here, we extend the RBC circadian toolkit with a novel biochemical assay for haemoglobin oxidation status, termed "Bloody Blotting". Our approach relies on a redox-sensitive covalent haem-haemoglobin linkage that forms during cell lysis. Formation of this linkage exhibits daily rhythms *in vitro*, which are unaffected by mutations that affect the timing of circadian rhythms in nucleated cells. *In vivo*, haemoglobin oxidation rhythms demonstrate daily variation in the oxygen-carrying and nitrite reductase capacity of the blood, and are seen in human subjects under controlled laboratory conditions as well as in freely-behaving humans. These results extend our molecular understanding of RBC circadian rhythms and suggest they serve an important physiological role in gas transport.

Introduction

Daily rhythms in behaviour and physiology are observed in all kingdoms of life and are of fundamental importance for understanding human health and disease (Patke et al., 2020). Daily organisation of cellular homeostasis results in oscillations of protein abundance, firing rate, and motility. Physiology is affected by the mutation of a number of proteins including kinases (e.g. CK1, CK2 and GSK3), transcription factors (e.g. CLOCK and BMAL1), and repressors such as PER and CRY (Ko and Takahashi, 2006). In most cell types, whilst the identity of 'clock-controlled genes' varies with tissue context, the rhythmic regulation of clock-controlled gene expression is proposed to be the central mechanism by which cellular biology and function manifests as a daily rhythm (Ruben et al., 2018; Zhang et al., 2014).

However, oscillations in cellular biology exist in the naturally anucleate red blood cell (RBC), which cannot be attributable to rhythms in gene expression. Circadian regulation of a number of cellular processes, such as metabolism, redox balance, proteasomal degradation, and membrane electrophysiology all exist within RBCs (Ch et al., 2021; Cho et al., 2014; Henslee et al., 2017; Homma et al., 2015; O'Neill and Reddy, 2011). The period of these circadian rhythms is sensitive to inhibition of proteasomal degradation and the activity of casein kinase 1 (Beale et al., 2019; Cho et al., 2014). In the absence of transcription or translation, RBC circadian rhythms are hypothesised to be representative of a post-translational oscillator (PTO) conserved from the last eukaryotic common ancestor that involves CK1, a ubiquitous component of circadian rhythms across the eukaryotic lineage (Causton et al., 2015; O'Neill et al., 2020). RBCs can therefore serve as a tractable model for delineation of the PTO mechanism and the post-translational rhythmic regulation of cellular processes (Wong and O'Neill, 2018).

Observations of circadian rhythms in RBCs raise two important questions. First, since anucleate RBCs derive from nucleated precursors during erythropoiesis, do TTFL-regulated rhythms in erythroid progenitors influence time-keeping in isolated RBCs once they are terminally differentiated and anucleate? Second, in what way might circadian rhythms in RBCs impact upon critical physiological functions, such as gas transportation? Here, we address these questions in three ways: (1) characterising a novel rhythmic process in RBCs to aid investigation of RBC circadian mechanisms; (2) using this tool to describe oscillations in RBCs derived from mice harbouring well-characterised circadian mutations; and (3) investigating this functionally relevant process in free-living human subjects. Taken together, we explore this process as a novel biomarker for circadian phase *in vivo* that may be relevant for understanding daily variation in oxygen delivery, peripheral blood flow, and body temperature.

Results

An additional circadian marker in RBCs

Circadian rhythms of NAD(P)H concentration, PRX-$SO_{2/3}$ abundance and membrane physiology are observed in isolated mammalian red blood cells (Cho et al., 2014; Henslee et al., 2017; O'Neill and Reddy, 2011). While investigating PRX-$SO_{2/3}$ rhythms in isolated human red blood cells, we noticed faint chemiluminescent bands at ~16 kDa and ~32 kDa which exhibited more robust circadian rhythmicity than PRX-$SO_{2/3}$ (O'Neill and Reddy, 2011). This uncharacterised, rhythmic chemiluminescence was readily observed upon incubating membranes with enhanced chemiluminescence (ECL) reagent immediately after transfer from polyacrylamide gel, without any antibody incubations or exogenous source of peroxidase activity (Figure 1A, Supplementary Figure 1A). Recognising the utility of an additional marker for RBC circadian rhythms, we sought to characterise the source of this chemiluminescence.

The presence of chemiluminescence in the absence of antibody suggested that some RBC protein species possess an intrinsic peroxidase activity that is tolerant to the denaturing buffer (containing 1% dodecyl sulphate pH 8.5) used for cell lysis. The ECL peroxidase reaction employed in modern immunoblotting is usually catalysed by horseradish peroxidase, but can in fact be catalysed by any haem group (Das and Hecht, 2007; Keilin and Hartree, 1950). At high concentrations, sodium azide (NaN_3) inactivates peroxidase activity of haem groups through azidyl radical addition to the prosthetic group, and is a common means of inactivating peroxidase activity in immunoblotting (Montellano et al., 1988). To confirm that this SDS-resistant species indeed catalyses the ECL reaction through an intrinsic peroxidase activity, we incubated freshly transferred membranes with 30 mM (0.2%) NaN_3 for 30 minutes prior to assaying peroxidase activity *via* ECL (Figure 1B).

Pre-treatment with azide elicited a marked reduction in chemiluminescence, indicating that a haem-mediated (non-HRP) peroxidase activity was indeed adsorbed to the nitrocellulose membrane following transfer after SDS-PAGE. Haemoglobin (Hb, MW 16KDa) is the most common haem protein in RBCs, comprising 97% of all RBC protein (Pesciotta et al., 2015; Roux-Dalvai et al., 2008) with a small minority of Hb monomers being observed to run as a cross-linked dimer (~32 kDa) by SDS-PAGE (Fuhrmann et al., 1988). We therefore considered it plausible that the rhythmic non-specific peroxidase activity might be attributable to a covalent haem-Hb moiety: a linkage that would be intrinsically resistant to denaturation *via* SDS and subsequent polyacrylamide gel electrophoresis. In

RBCs under physiological conditions, however, Fe-haem is well characterised as being bound to Hb protein through non-covalent co-ordination by proximal and distal histidine residues (Perutz et al., 1960). Therefore, it should not be resistant to denaturation by SDS. Importantly, however, covalent linkage of haem to haem-binding proteins, including globins, has been reported to occur under several non-physiological conditions (Catalano et al., 1989; Deterding et al., 2004; Enggist et al., 2003; Reeder, 2010; Reeder et al., 2008).

To assess whether some fraction of Fe-haem is covalently bound to Hb protein we employed Ni-NTA affinity chromatography under native and denaturing conditions. Under native conditions, haem proteins are readily bound by Ni-NTA (Pesciotta et al., 2015). Under denaturing conditions, we reasoned that only proteins that are covalently linked to haem should show high affinity for the Ni-NTA matrix. Commensurately, we found that the non-specific peroxidase bound to, and eluted from, Ni-NTA under both native and denaturing conditions (Figure 1C). Furthermore, mass spectrometry of the eluted 16kDa protein revealed the major species to be haemoglobin beta and alpha ($\geq$ 95% coverage) (Supplemental Figure 2A). Moreover, mass spectrometry of full length HbA, purified under denaturing conditions, revealed additional peaks at +614 Da compared with the HbA polypeptide, which corresponds to the mass of deprotonated haem b (Supplemental Figure 2B).

We noted that, as expected, the total amount of Hb detected by Coomassie was invariant throughout the RBC circadian cycle (Figure 1A, Supplementary Figure 1,3); whereas the peroxidase activity associated with Hb (Hb*) showed a clear circadian rhythm (Figure 1A, Supplementary Figure 1, 3). Moreover, the proportion of Hb protein that was covalently linked to haem-Hb was very low compared with the total amount of RBC Hb (Figure 1C) and was completely SDS-resistant (Figure 1D). Finally, we considered that the unsaturated bonds in the haem porphyrin ring should be an attractive target for Click chemistry through base-catalysed Michael addition from a cysteinyl thiol under the denaturing and basic RBC lysis conditions we employed (Nair et al., 2014). This reaction would be expected to produce a thioester-linked adduct of Fe-haem to Hb protein, though likely with a poor yield since this reaction would be competing with mixed disulphide bond formation under these lysis conditions.

To test this, we included high concentrations of strong reductants (DTT and NH_3OH) in the lysis buffer, which reduce thioester (but not thioether) bonds under aqueous conditions (Pedone et al., 2009). We found that the residual peroxidase activity of Hb on nitrocellulose membranes was completely abolished under these conditions (Figure 1E, F), supporting a thioester linkage. To validate this, we pre-incubated RBCs with N-ethylmaleimide (NEM) to alkylate Hb cysteine residues prior to

lysis - effectively blocking *de novo* thioester bond formation without affecting any thioester bonds that might exist prior to lysis. NEM pre-treatment abolished subsequent peroxidase activity (Figure 1G), indicating that thioester bond formation is facilitated by protein denaturation during cell lysis.

Overall, our observations accord with a model whereby the rhythmic peroxidase activity we detect arises from a small proportion of Hb becoming thioester-linked to haem during cell lysis, but does not explain why the formation of this bond might exhibit a circadian rhythm. Previous work has indicated that RBCs exhibit a circadian rhythm in the oxidation and over-oxidation of peroxiredoxin proteins, the latter being degraded by the proteasome (Cho et al., 2014), as well as rhythms in Hb-quaternary structure and the cellular reducing equivalents (NAD(P)H), that are normally used by methaemoglobin (metHb) reductase to reduce metHb back to the ground state (O'Neill and Reddy, 2011). These are suggestive of a circadian rhythm in the rate of oxidation of oxyhaemoglobin to form ferric (inactive, deoxy, Fe(III)) metHb, and subsequent rate of H_2O_2 formation, possibly as a consequence of daily variation in Hb dimer-tetramer equilibrium (O'Neill and Reddy, 2011).

In light of these observations, and the strong evidence for dynamic regulation of Hb redox state in RBCs under physiological conditions (Umbreit, 2007), we asked whether the redox state of haem-Hb at the point of cell lysis might affect the level of thioester bond formation. Deoxygenated ferrous (Fe(II)) Hb is readily susceptible to oxidation to ferric (Fe(III)) metHb by nitrite at low millimolar concentrations that will not oxidise cysteine (Cortese-Krott et al., 2015). Conversely, millimolar ascorbate reduces metHb back to the ferrous state (Eder et al., 1949; Gibson, 1943), whereas similar concentrations of azide, a less favourable electron donor or acceptor, should have less effect on Hb redox state under these conditions. Acute treatments of intact RBCs prior to lysis with sodium nitrite and sodium ascorbate dose-dependently decreased and increased, respectively, the peroxidase activity detected at Hb molecular weight on nitrocellulose membranes (Figure 1H), compared with a much more modest effect of sodium azide. To validate this finding, instead of transfer to nitrocellulose, we also performed an in-gel colourimetric haem stain on these samples which yielded similar results to the ECL assay for peroxidase activity (Figure 1H).

By mechanisms that remain to be firmly established, in isolated RBCs there is a rhythm in the rate of Hb auto-oxidation and metHb formation (O'Neill and Reddy, 2011). Our observations support a model whereby a small proportion of ferrous haem spontaneously crosslinks with Hb via a thioester bond upon cell lysis. This proportion is influenced by the redox state of haem-Hb in the RBCs prior to lysis and is stable during subsequent SDS-PAGE and transfer to nitrocellulose, allowing detection by ECL

reagent. Since Hb redox state is circadian regulated, the Hb-based marker demonstrated here represents a novel report for RBC circadian rhythms, a second facet of the redox rhythm in RBCs. We coin this technique "Bloody Blotting", distinguishable from immunoblotting by the lack of any antibodies, blocking step or exogenous source of peroxidase activity, and only requires RBC lysis.

Figure 1

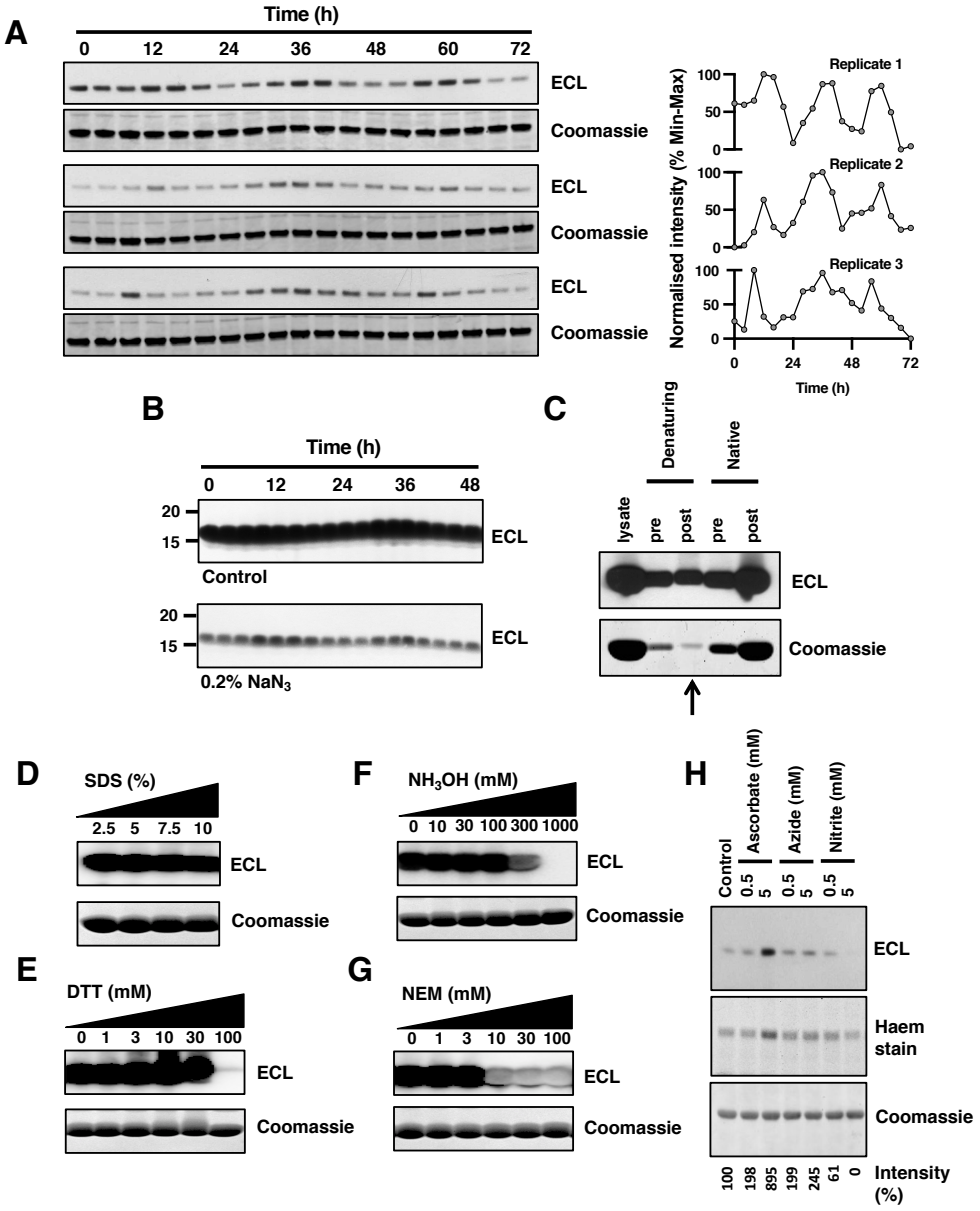

Figure 1. Biochemical characterization of a protein-bound peroxidase activity in isolated RBCs following detergent lysis and SDS-PAGE.

(A) Circadian rhythm of peroxidase activity in 3 independently isolated sets of human red blood cells sampled every 4 hours under constant conditions, quantifications shown on right hand side. After lysis, SDS-PAGE and transfer to nitrocellulose, membranes were immediately incubated with ECL reagent to reveal peroxidase activity bound to the membrane. (B) Representative blots performed in parallel following SDS-PAGE of human erythrocyte time course samples and transfer to nitrocellulose membranes. Before incubation with ECL reagent,

membranes were incubated for 30 minutes in PBS ± 0.2% sodium azide. (C) Ni-NTA affinity purification of human RBC lysates under denaturing and native conditions with samples show pre- and post-elution. The indicated band (arrow) was excised for mass spectrometry. (D-F) RBC samples were lysed with a buffer containing increasing concentrations of (D) SDS; (E) DTT; or (F) hydroxylamine (NH_3OH, pH 7) and incubated for 30 minutes at room temperature. Samples were incubated with ECL reagent following SDS-PAGE and transfer to nitrocellulose. (G) Intact RBCs were incubated with increasing concentrations of N-ethylmaleimide (NEM) for 30 minutes at room temperature before lysis. Samples were incubated with ECL reagent following SDS-PAGE and transfer to nitrocellulose. (H) RBCs were pre-incubated for 30 minutes in Krebs buffer at room temperature containing sodium ascorbate, sodium azide, sodium nitrite or sodium chloride (5 mM) as a control and subject to SDS-PAGE and nitrocellulose transfer. Representative ECL-only blot and in-gel haem staining is shown. ECL staining intensity relative to control is shown. Coomassie stains of the protein remaining in gels subsequent to transfer onto nitrocellulose membranes are shown as loading controls in all panels.

Clock mutations do not affect RBC circadian rhythms

Oscillations in cellular processes in RBCs cannot be attributable to transcriptional repression by PER and CRY proteins since these proteins are not present in RBCs, nor do RBCs possess the capacity for transcriptional feedback (Bryk and Wiśniewski, 2017; O'Neill and Reddy, 2011). Consistent with this, circadian rhythms in mature isolated RBCs are also insensitive to inhibition of nascent transcription and translation. However, the RBC develops from nucleated precursors, the normoblasts, during erythropoiesis and we therefore considered whether the developmental expression of circadian cycles in erythroid precursors might affect circadian phenotype of mature RBCs, once isolated from the body. To answer this question, we isolated RBCs from mice harbouring well-characterised circadian mutations of the post-translational regulators of PER and CRY, CK1$\varepsilon^{Tau/Tau}$ (Lowrey et al., 2000; Meng et al., 2008) and FBXL3$^{Afterhours/Afterhours}$ (Godinho et al., 2007) respectively, and examined circadian rhythms under constant conditions using PRX-$SO_{2/3}$ abundance and Bloody Blotting as rhythmic reporters.

Mice homozygous for these mutations exhibit behavioural circadian periods that are shorter (*tau*) and longer (*afterhours*, *afh*) than wildtype (Godinho et al., 2007; Lowrey et al., 2000; Meng et al., 2008). Since circadian timekeeping occurs cell-autonomously, the short and long period phenotypes observed at the whole organism level are readily recapitulated in fibroblasts isolated from homozygous mutant PER2::LUC mice (Figure 2A). Fibroblasts harbouring *tau* or *afh* mutations exhibit circadian periods 2.4 h shorter and 6.7 h longer, respectively, than wildtype (Figure 2A). In RBCs isolated from the same mice and cultured *ex vivo*, high amplitude oscillations were observed in PRX-$SO_{2/3}$ abundance (Figure 2B, C) and Hb* (Figure 2D, E, Supplementary Figure 3A, B, C). However, unlike

fibroblasts, no significant difference in circadian period was seen between the three mouse genotypes (Figure 2F). Therefore, the altered timing observed in mutants of nucleated cells is not inherited by RBCs and the timekeeping role of CK1 in RBCs (Beale et al., 2019) is within a PTO that does not involve PER, CRY, CK1ε or FBXL3 (Bryk and Wiśniewski, 2017; O'Neill and Reddy, 2011). Interestingly, compared with human RBC time courses (Henslee et al., 2017; O'Neill and Reddy, 2011), we observed that murine PRX-$SO_{2/3}$ immunoreactivity was extremely high during the first 24 hours of each 72-hour time course (Figure 2B). We attribute this to the different conditions under which blood was collected: blood was collected from mice during their habitual rest phase by cardiac puncture and exposed immediately to atmospheric oxygen levels, whereas human blood was collected from subjects during their habitual active phase through venous collection into a vacuum-sealed collection vial. Furthermore, the same circadian period between these reporters and genotypes is suggestive that they are two outputs of the same underlying oscillation, but with a higher relative amplitude and an earlier phase in the novel bands.

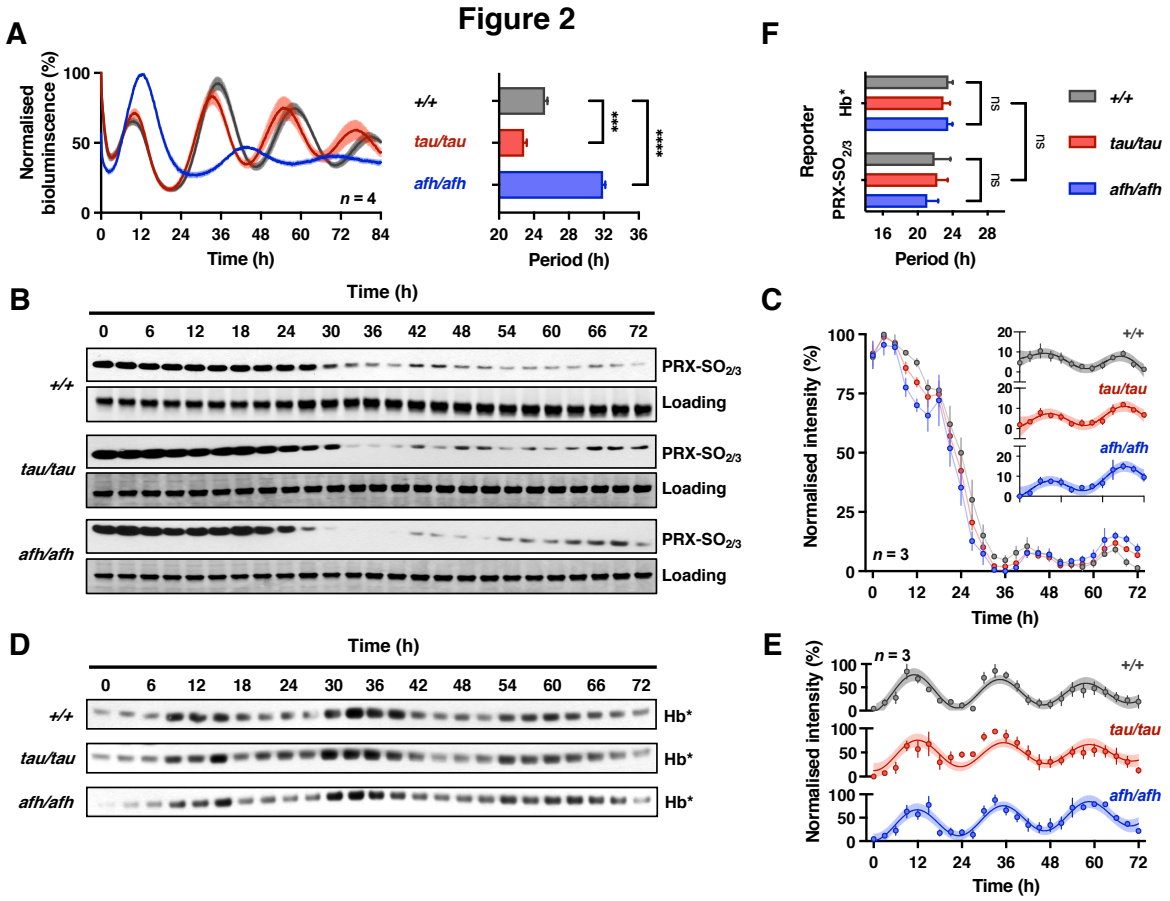

Figure 2. The Clock in Mouse Erythrocytes is Unaffected by Mutations that Affect Circadian Period in Nucleated Cells

(A) Primary fibroblasts isolated from CK1ε$^{Tau/Tau}$ or Fbxl3a$^{Afh/Afh}$ mutant mice, also transgenic for the *Cry1:luc* reporter, exhibit bioluminescence rhythms that are shorter and longer than wild type control, respectively. Normalised mean±SEM are shown as line and shading respectively (*n*=4). Grouped quantification of circadian period from A. Mean±SEM, p<0.0001 by 1-way ANOVA; Sidak's multiple comparisons test displayed on graph: ***p<0.001, ****p<0.0001. (B) RBCs from CK1ε$^{Tau/Tau}$, Fbxl3a$^{Afh/Afh}$ and wild type mice were incubated at constant 37°C from the point of isolation. Single aliquots were lysed every three hours. Representative immunoblot showing PRX monomer over-oxidation over 72 hours under constant conditions. Coomassie-stained gels indicate equal loading for each immunoblot. (C) Grouped quantification from B (mean±SEM, n=3 per genotype). 2-way ANOVA, p<0.0001 for time effect, but not significant for genotype or time/genotype interaction (p>0.05); inset shows the final 36 hours with expanded y-axis. Damped cosine wave using least squares non-linear regression fitted to final 36 hours shown as line, with SEM error shown as shading. (D) Representative blots showing peroxidase activity detected without antibody following transfer to nitrocellulose membrane, at the same molecular weight as hemoglobin dimer (~32 kDa), indicated as Hb*. (E) Grouped quantification from D (mean±SEM, n=3 per genotype). 2-way ANOVA, p<0.0001 for time effect, but not significant for genotype or time/genotype interaction (p>0.05). (F) Circadian periods of PRX overoxidation and peroxidase activity, Hb*, derived from damped cosine fits from C and E respectively. Mean±SEM, no significant difference by 2-way ANOVA.

Daily variation in redox status in humans *in vivo*

In vivo, haem-Hb occurs in human RBCs in two oxidation states: as ferrous Hb(II) and ferric metHb(III). The transport of oxygen requires oxygen reversibly bound to ferrous Hb(II). Oxygenated Hb(Fe(II))O_2 is a very stable molecule but does slowly auto-oxidize at a rate of about 3-4 %/day (Eder et al., 1949; Johnson et al., 2005) a rate that is accelerated at lower partial pressures of O_2 (pO_2) when the haemoglobin is partially oxygenated. In a healthy individual, metHb reductase ensures that < 1 % of RBC Hb exists in the Fe(III) state, although disease, dietary nitrites and inherited conditions such as methemoglobinemia (Cawein et al., 1964) can cause this level to be higher. Given the daily variation of Hb redox status suggested by this and previous studies in mouse and human RBCs *in vitro* (Cho et al., 2014; O'Neill and Reddy, 2011), we used "Bloody Blotting" to determine whether daily variation in Hb(II):metHb(III) ratio occurs *in vivo*. We therefore collected and flash froze blood samples every 2 hours from 4 healthy volunteers, beginning at habitual wake time, over a complete circadian cycle under controlled laboratory conditions (Figure 3A).

We observed a significant variation in Hb-linked peroxidase activity that peaked shortly after waking and reached its nadir 12 hours later (Figure 3B and C, Supplementary Figure 1B). This, together with our biochemical and *in vitro* data, suggests that the Hb(II):metHb(III) ratio varies with time of day, with the proportion of metHb in the blood highest (peroxidase activity lowest) towards the end of the habitual waking period. To test whether daily variation in metHb levels occurs in a real-world setting, we assessed metHb levels and other blood parameters in 4 free-living healthy human subjects non-invasively, using pulse co-oximetry. As expected, pulse rate (PR) exhibited a clear daily variation in these subjects (Figure 3D, left), with significant autocorrelation in the circadian range (Figure 3D, right). Perfusion index (PI, a measure of peripheral blood flow) (Goldman et al., 2000) also exhibited a clear daily variation, peaking around midnight, approximately antiphasic to the daily variation in pulse rate. Remarkably, in contrast to total Hb (SpHb) that displayed no significant 24h variation, the proportion of metHb (SpMet) in the blood exhibited a striking daily variation that peaked during the night in broad agreement with the increased haem-Hb activity rhythm observed in acute samples (Figure 3B). The oxygen saturation of the blood (SpO_2) varied in antiphase with the metHb rhythm, peaking during the active phase (Figure 3D), consistent with the previously established relationship between Hb oxygenation and metHb formation (Umbreit, 2007).

Taken together, our results establish a novel reporter for circadian rhythms in RBCs *in vitro* and provide further insight into the determinants and physiological outputs of the circadian clock in the

most abundant cell of the human body (Alberts et al., 2017; Sender et al., 2016). This novel reporter is dependent on the redox status of Hb, which alters the proportion of haem that is covalently linked to Hb on cell lysis, and therefore free to catalyse an ECL reaction without antibodies. The redox status of haemoglobin is under circadian control *in vitro* and *in vivo* and is independent of the TTFL-based timing mechanism of RBC precursors, but is clearly synchronised by systemic cues *in vivo*.

Figure 3

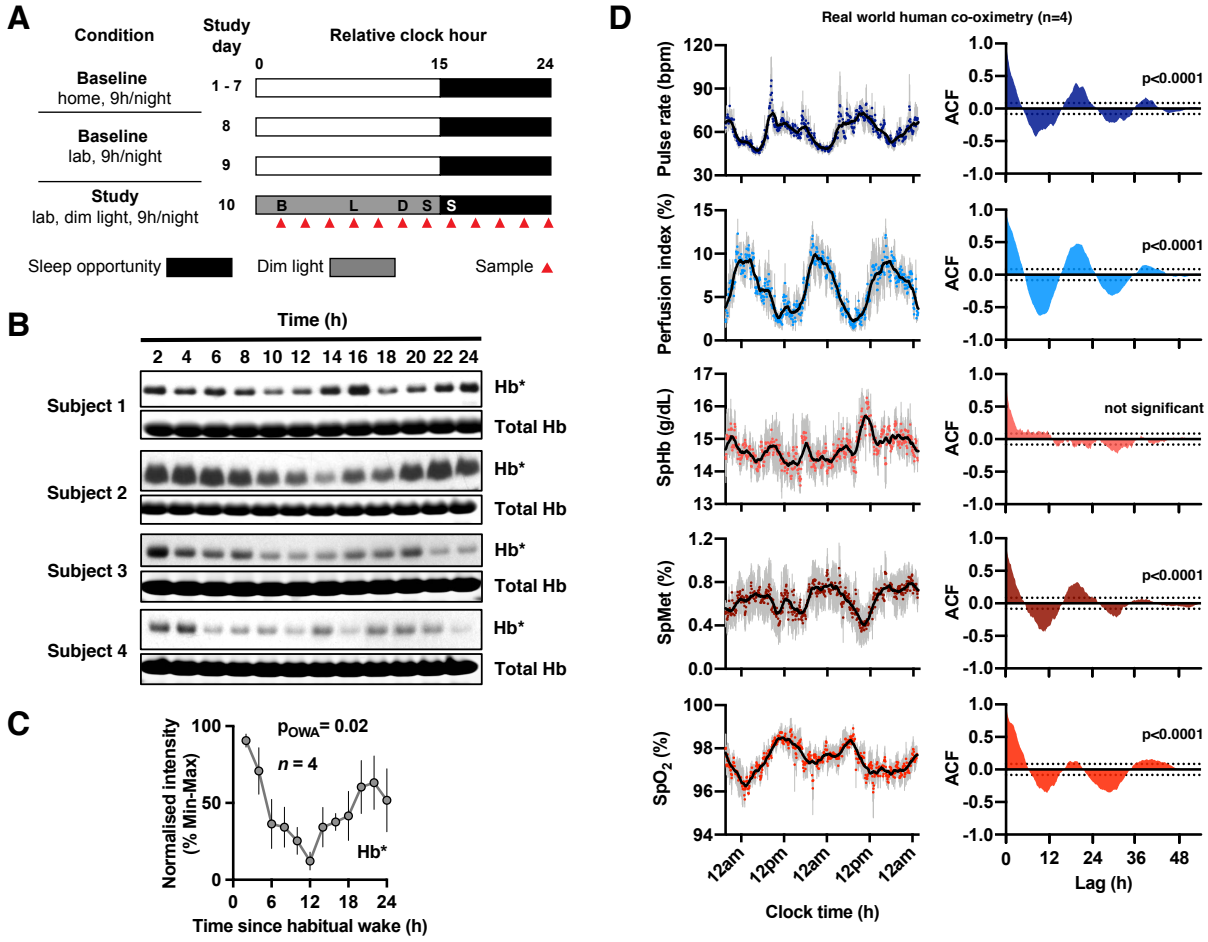

Figure 3. Daily variation in redox status in humans *in vivo*

(A) Experimental protocol for four healthy participants, relative to waketime at 0. Participants maintained consistent 15h:9h days consisting of wake (white) and sleep opportunity (black) at home for at least one-week prior to entering the laboratory. Following two baseline laboratory days, participants were awakened at habitual waketime and studied under dim light conditions (< 10 lux in the angle of gaze during wakefulness and 0 lux during scheduled sleep), given an energy balanced diet (standardised breakfast, B, lunch, L, and dinner, D, meals given at 2h, 8h and 12h respectively, and two after dinner snacks, S_1 and S_2, at 14h and 16h, water available *ad libitum*). Blood samples were collected every two hours (red triangles). Daytime and light exposure during scheduled wakefulness is depicted as white; night-time and sleep opportunity is depicted as black; dim light during wakefulness is depicted as dark grey. (B) Whole blood samples from 4 volunteers were subject to SDS-PAGE and immunoblot. Representative Hb* peroxidase activity (upper). Total Hb from Coomassie staining serves as a loading control (lower). (C) Quantification of 4 subjects. One way ANOVA, p<0.05. (D) Pulse co-oximetry

data from four healthy male volunteers under normal daily life. (Left) Pulse rate, perfusion index, total haemoglobin concentration SpHb, methaemoglobin proportion SpMet, and oxygen saturation SpO_2 are plotted against local time (coloured dots = grouped means; grey lines = error bars; solid black line = 1-hour moving average). (Right) Autocorrelation function (ACF) against time lag (h) of the grouped means. 95% confidence bands of the ACF are plotted as dotted lines on y-axis, p value represents extra sum-of-squares F test comparing a straight line fit (H_0) with a damped cosine fit.

Discussion

RBCs have been an interesting model for circadian rhythms in the absence of transcription for a number of years. Here we have extended understanding of RBC circadian physiology and function, and uncovered a novel RBC clock marker based upon a redox-sensitive covalent haem-Hb linkage that forms during cell lysis/protein denaturation. We show that RBC rhythms are independent of their developmental pathway and have potential functional significance in their O_2-carrying and NO-generating capacity.

Many biomarkers for assessing circadian phase exist, but are often slow, cumbersome or expensive to process, or have limited resolution, even though some progress is being made (Wu et al., 2020). “Bloody Blotting” is fast and inexpensive; however, its utility is likely limited to research contexts. In contrast, we demonstrate here that pulse co-oximetry, a wearable technology, accurately reports diurnal variation in several physiologically relevant, haemodynamic parameters simultaneously over many days.

Physiological Relevance

Circadian rhythms have previously been described in isolated human and mouse RBCs (Beale et al., 2019; Ch et al., 2021; Cho et al., 2014; Henslee et al., 2017; O’Neill and Reddy, 2011) under comparable free run conditions to those employed here. Those experiments employed *ex vivo* entrainment by applied 12h:12h temperature cycles to mimic body temperature rhythms. They revealed that PRX-$SO_{2/3}$ immunoreactivity in isolated RBCs from both species peaked approximately 10-14h after the subjective transition to warm temperature, equivalent to the end of the active phase *in vivo*. In our mouse RBC time course, cells were collected shortly after lights on (subjective dawn, when mouse body temperature cools), thus the observed PRX-$SO_{2/3}$ rhythms are broadly consistent with previous reports. In contrast, metHb levels were elevated (Hb* activity lowest) during the subjective rest phase in mouse RBCs *ex vivo* and humans *in vivo*. PRX-$SO_{2/3}$ and Hb redox state oscillated with the same circadian period, as has been shown for rhythms in PRX-$SO_{2/3}$ abundance membrane physiology and central carbon metabolites (Ch et al., 2021; Henslee et al., 2017). This suggests that there is a single

underlying molecular oscillator in RBCs, whose activity is dependent on ion transport, the 20S proteasome and CK1 (Beale et al., 2019; Cho et al., 2014; Henslee et al., 2017) and whose rhythmic outputs include Hb redox state, PRX-$SO_{2/3}$ degradation, metabolic flux and K^+ transport. As with other cell types, RBC rhythms *in vivo* are presumably synchronised by systemic cues (Dibner et al., 2010) which continue under constant routine conditions with respect to posture, food intake and sleep (Skene et al., 2018). Whilst we cannot rule out therefore that systemic cues, or indeed body temperature or sleep-wake rhythms, are driving our observed metHb rhythms in humans *in vivo*, our observation, taken together with *ex vivo* data, are consistent with the interpretation that RBC rhythms are under cell-autonomous circadian control and synchronised by systemic timing cues.

Daily regulation of Hb redox status has functional significance in at least two ways. Since metHb cannot bind O_2, metHb rhythms may affect the oxygen-carrying capacity of the blood. We found a daily rhythm in SpO_2 in antiphase with metHb (Figure 3D). Though this is correlational, the phase relationship between the rhythm in metHb and SpO_2 is consistent with the influence of metHb on Hb's allosteric Hill co-efficient. MetHb stablises the R state of Hb (Gladwin and Kim-Shapiro, 2008) meaning that when metHb is higher, transition to the Hb T-state will occur at relatively lower pO_2 and thereby facilitate increased oxygen supply to peripheral blood vessels/tissues during the rest phase. However, the circadian variation in proportion of metHb in the blood reported here is relatively small, and so the physiological significance on oxygen capacity in a healthy individual is likely limited.

A more important potential consequence of daily cycles of Hb redox status is the regulation of vascular tone and peripheral blood flow through nitric oxide (NO) signalling (DeMartino et al., 2019; Grubina et al., 2007; Huang et al., 2005; Umbreit, 2007). Indeed, a secondary function of RBCs is to store, carry and release NO equivalents (nitrite, nitrosyl adducts, and N_2O_3 respectively). This complements local NO synthesis by tissue-resident nitric oxide synthases through RBC-dependent NO generation when pO_2 falls, which in turn stimulates peripheral vasodilation (Basu et al., 2007; Cosby et al., 2003; Grubina et al., 2007). MetHb reacts with nitrite to yield an intermediate that rapidly reacts with NO to yield N_2O_3, which is proposed as the essential vector for release of NO from RBCs that stimulates hypoxic vasodilation in all peripheral blood vessels (Basu et al., 2007); with the level of NO signalling/vasodilation being dependent on metHb levels. At high pO_2, oxygenated Hb(II) inactivates local NO, resulting in vasoconstriction (Doyle and Hoekstra, 1981; Umbreit, 2007). Thus, based on current understanding of human physiology, increased metHb during the rest phase (at night) should contribute to increased peripheral blood flow. This can be clearly discerned from the high amplitude diurnal variation in perfusion index we observed (Figure 3D).

The fundamental mechanism underlying daily rhythms in body temperature is not currently understood. It is known not to be solely attributable to activity or feeding-dependent thermogenesis (Krauchi and Wirz-Justice, 1994), though another possibility is a rhythm in brown adipose tissue (BAT) thermogenesis controlled by the ventromedial hypothalamus (VMH) (Orozco-Solis et al., 2016). We note that increased peripheral blood flow at night is the primary mechanism allowing the heat release that, in humans, is essential for lowering core body temperature at night and habitually accompanies the transition from wake to sleep. Our observations lead directly to the hypothesis that RBC metHb rhythms function to maximise the oxygen-carrying capacity of blood during the active phase (daytime in humans), whilst facilitating increased peripheral blood flow and vasodilation at night. The latter allows core body and brain temperature to cool and thereby facilitate the transition into sleep (Rzechorzek et al., 2021). A direct prediction from this hypothesis is that perturbation of daily metHb rhythms will have commensurate effects on daily rhythms in core body temperature and the sleep-wake cycle (Figure 4). This will be investigated in our future work.

RBCs and the TTFL-less clock mechanism

A number of recent observations have challenged the traditional transcriptional feedback circuit model of circadian rhythm generation and focused instead on the role of post-translational regulation in signalling biological timing information (Crosby and Partch, 2020; Wong and O'Neill, 2018) The PTO model is compatible with nucleate and anucleate cell types, where phosphorylation and dephosphorylation, via CK1 (Etchegaray et al., 2009; Hirota et al., 2010; Lee et al., 2009; Meng et al., 2008), CK2 (Maier et al., 2009; Tsuchiya et al., 2009), GSK3a/b (Hirota et al., 2008; Iitaka et al., 2005) and PP1 (Lee et al., 2011; Virshup et al., 2007), as well as proteasomal degradation (Busino et al., 2007; Eide et al., 2005; Reischl et al., 2007; Siepka et al., 2007) are key components. Recently, a significant regulatory role for phosphorylation on circadian proteins has been demonstrated (Robles et al., 2017; Wong et al., 2020), with oscillations in phosphorylation status highly suggestive that this post-translational modification may regulate a clock without the need for abundance oscillations. Accordingly, roles for CK1, CK2 and proteasomal degradation have been demonstrated in RBCs (Beale et al., 2019; Cho et al., 2014), suggesting that their role within an oscillator is not restricted to the regulation of "clock proteins". The two mutations used here are commonly linked to the regulation of their clock protein target: *Tau*, a mis-sense mutation (R178C) in CK1ε (Lowrey et al., 2000), results in a short period effect that has been tied to the increased degradation of its PER2 target by favouring a rare CK1 conformation (Philpott et al., 2020); *Afh*, a substitution (C358S) in the gene encoding the E3 ubiquitin ligase adaptor *fbxl3*, results in a long period effect that has been tied to delayed degradation

of CRY proteins (Godinho et al., 2007). By choosing mutations in proteins that are not expressed in RBCs from humans (Beale et al., 2019), we delineated the PTO of the RBC from the TTFL/PTO of the nucleated precursor, and demonstrate that the roles of CK1, CK2 and the proteasome shown previously are specific to the RBC and independent of the erythropoietic lineage. Our novel reporter of RBC circadian rhythms, which relies solely on cell lysis and gel electrophoresis without antibodies, will greatly aid the further teasing apart of the PTO of RBCs.

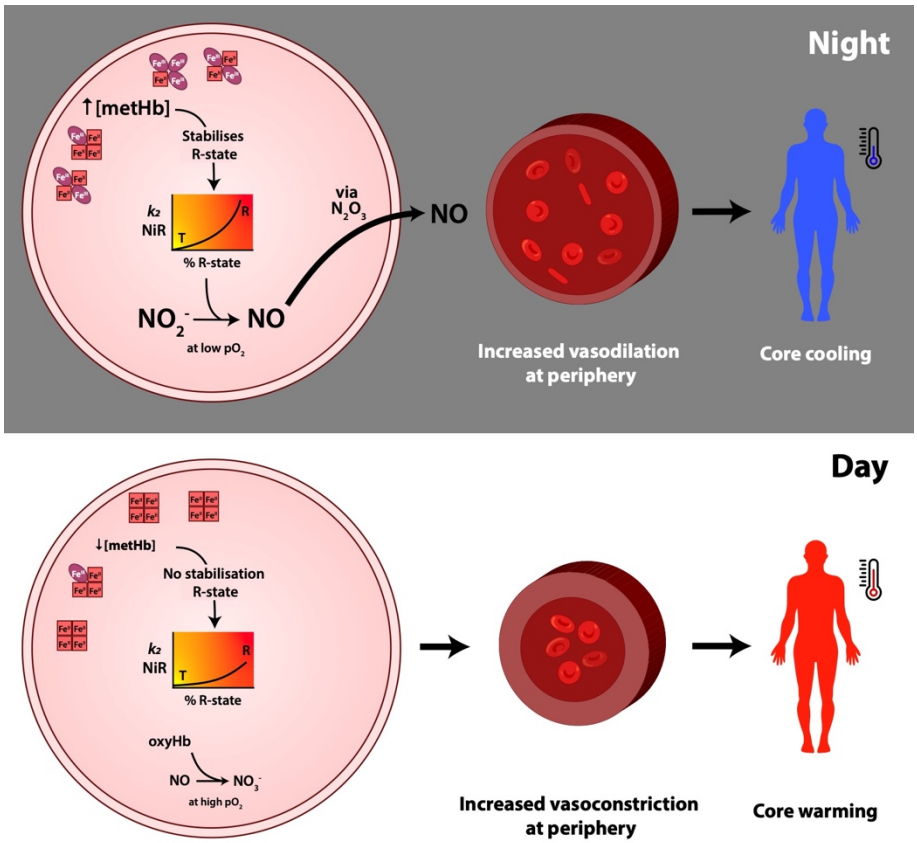

Figure 4. Proposing a model for the contribution of daily metHb rhythms to daily core body temperature rhythms in humans. At night, stabilisation of the R state of haemoglobin as levels of metHb rise (Bunn and Forget, 1986) leads to an increase in affinity for O_2 (Monod et al., 1965; Perutz, 1970) and an increased nitrite reductase activity of deoxyHb at low pO_2 (Grubina et al., 2007; Huang et al., 2005). The subsequent export of increased concentrations of NO, via an N_2O_3 intermediate (Basu et al., 2007), leads to increased vasodilation (Cosby et al., 2003; Crawford et al., 2006) and thus increased heat loss. In the day, the concentration of metHb is lower, reducing deoxyHb nitrite reductase activity and thus RBC-produced NO. When fully oxygenated, oxyHb inactivates NO, or NO_2^-, to nitrate, NO_3^- (Herold and Shivashankar, 2003), reducing vasodilation. Taken together, the daily variation in metHb and its effect on the production and export of NO, and subsequent vasodilation, is predicted to lead to a daily variation in body temperature due to daily variation in vasodilation-mediated cooling (Krauchi and Wirz-Justice, 1994).

Experimental Procedures

Mouse RBC collection for *in vitro* experiments

All animal work was licensed under the UK Animals (Scientific Procedures) Act of 1986 with local ethical approval. Mice were entrained under 12:12 h light:dark cycles from birth prior to being killed at Zeitgeber time 4 (ZT4) by a schedule one method and exsanguinated by cardiac puncture. Blood was immediately transferred to a collection tube (Sarstedt, Nürnbrecht, Germany) containing sodium citrate anti-coagulant, with approximately 8 ml total blood being collected and pooled from 10 isogenic animals (C57BL/6 background, aged 3-4 months) with equal numbers of males and females for each genotype. RBCs were isolated from anticoagulant-treated whole blood by density gradient centrifugation and washed twice in PBS before resuspension in modified Krebs buffer (KHB, pH 7.4, 310 mOsm to match mouse plasma) as described previously (O'Neill and Reddy, 2011). RBC suspensions were aliquoted into single 0.2 mL PCR tube per time point per mouse and incubated at constant 37°C. At each time point the red cell pellet was resuspended by trituration and 50 µl was removed and added to 450 µL 2x LDS sample buffer (Life Technologies, Carlsbad, CA) supplemented with 5 mM DTPA as described previously (Milev et al., 2015).

Human RBC collection for *in vitro* experiments

Studies were conducted in accordance with the principles of the Declaration of Helsinki, with ethical approval from the Local Research Ethics Committee (Cambridge, UK). Participants in the study were screened for health (by history, physical examination, and standard biochemistry and haematology), and did not suffer from sleep disorders or excessive daytime sleepiness. All participants provided written, informed consent after having received a detailed explanation of the study procedures. ~10 ml of blood was collected from each subject in the morning (8-10AM) using tubes containing sodium citrate anticoagulant (Sarstedt). Red cell pellets were obtained using the same method described above, except that Krebs buffer osmolarity was adjusted to 280 mOsm/L to match conditions normally found in human plasma. 100 µl of re-suspended RBCs were dispensed into 0.2-ml PCR tubes (Thermo), and then placed in a thermal cycler (Bio-Rad Tetrad) at constant 37°C for time course sampling, or else maintained at room temperature for immediate experimentation, as described in the text. At each time point the red cell pellet was resuspended by trituration and 50 µl was removed and added to 450 µL 2x LDS sample buffer (Life Technologies, Carlsbad, CA) supplemented with 5 mM DTPA as described previously (Milev et al., 2015).

Gel electrophoresis and immunoblotting

Western blotting and SDS-PAGE was performed, under reducing conditions, as described previously (Milev et al., 2015). For bloody blotting, membranes were quickly washed with deionised water and then immediately incubated with ECL reagent (Millipore). Coomassie stained gels were imaged using an Odyssey scanner (Licor). Chemiluminescence was detected by x-ray film, except for the data in Figure 2D-G which were collected on a ChemiDoc XRS+ (Bio-rad).

Protein digestion and mass spectrometric analysis

Peptides were prepared from excised bands from Coomassie stained gel as follows. Gels slices were first destained in 50% acetonitrile/50% 100mM NH_4HCO_3. After washing in 2 times 100µl dH2O, gel slices were dehydrated by the additional of 100µl of acetonitrile, and the solvent completely evaporated by lyophilising. Proteins within the bands were first reduced by the addition of 50µl 10mM dithiothreitol in 100mM NH_4HCO_3 and incubated at 37°C for 1 hour. The supernatant was then removed and replaced by 50µl of 55mM iodoacetamide in 100mM NH_4HCO_3 and incubated at room temperature in the dark for 45 minutes. After removal of the supernatant the bands were washed in 100mM NH_4HCO_3 followed by 100% acetonitrile prior to dehydration as described above.

Trypsionlysis was carried out by first rehydrating the bands in 300ng trypsin (Sequencing Grade Modified Trypsin (Promega Product no. V5111) in 60µl 100mM NH_4HCO_3 and incubated overnight at 37°C. The supernatant was then acidified to 0.1% formic acid. Peptide analysis was performed by matrix assisted laser desorption ionisation (MALDI) mass spectrometry (Waters Micromass MaldiMX Micro) using a-cyano-4-hydroxycinnamic acid matrix (10 mg ml−1 in 50% aqueous acetonitrile/0.1% trifluroacetic acid).

Human whole blood collection in controlled laboratory conditions

Studies were conducted with approval of the local research ethics committee. Participants (N=4 [2m, 2f], aged 27.3 ± 5.4 years [mean ± SD]) were healthy based on medical and sleep history, physical exam, normal BMI, 12-lead electrocardiogram, blood chemistries, clinical psychiatric interview, polysomnographic sleep disorders screen, and provided written, informed consent. Participants were excluded from study for current or chronic medical or psychiatric conditions, pregnancy, shift work, or dwelling below Denver altitude (1600 m) the year prior to study. Travel across more than one time zone in the three weeks prior to the laboratory study was proscribed. Participants were medication and drug free based on self-report and by urine toxicology and breath alcohol testing upon admission to the laboratory. Participants maintained consistent 9h sleep schedules for at least one-week prior to the laboratory protocol verified by wrist actigraphy and call-ins to a time stamped recorder. Following two baseline laboratory days, participants were awakened at habitual waketime and studied

under dim light conditions (< 10 lux in the angle of gaze during wakefulness and 0 lux during scheduled sleep), given an energy balanced diet (standardised breakfast, lunch and dinner meals given at 0800, 1400 and 1800 respectively, and two after dinner snacks at 2000 and 2200h, water available *ad libitum* for someone with a 0600h waketime). Blood samples (10 ml) were collected every two hours from each of the four participants into lithium heparin Vacutainers (BD Biosciences, Franklin Lakes, NJ) and separated by centrifugation. Cell fractions were flash frozen in liquid N_2 and stored a -80°C until lysis with 2x LDS sample buffer.

Hb biochemistry

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Direct staining of sodium dodecyl sulfate (SDS)-containing polyacrylamide gels with o-dianisidine, to detect protein-bound to haem was performed as described previously (Maitra et al., 2011). All RBC incubations in Figure 2 were performed in Krebs buffer. All stock solutions were prepared at 2 M in deionised water except N-ethylmaleimide, which was prepared in ethanol. Native purifications were performed using Ni-NTA agarose according to (Ringrose et al., 2008), denaturing purifications in 8M urea were performed according to manufacturer's instructions.

Bioluminescence recording

Primary fibroblasts carrying a *Cry1:luciferase* reporter (Maywood et al., 2013) were isolated from the lung tissue of adult males and cultured as described previously (O'Neill and Hastings, 2008). Cells were synchronized by temperature cycles of 12 h 32°C followed by 12 h 37°C for 5 days, then changed to ''Air Medium'' (Bicarbonate-free DMEM (D5030, Sigma) dissolved in dH_2O, modified to a final concentration of 5mg/mL glucose, 0.35 mg/mL sodium bicarbonate, 20 mM MOPS, 2 mg/mL pen/strep, 2% B27, 1% Glutamax, 1mM luciferin, and adjusted to pH 7.6 and 350 mOsm; adapted from (O'Neill and Hastings, 2008)), and dishes sealed. Bioluminescence recordings were performed using a Lumicycle (Actimetrics, Wilmette, IL, USA) under constant conditions as described previously (Causton et al., 2015). Lumicycle data were detrended to remove baseline changes and then fit with a damped sine wave to determine circadian period as in (Causton et al., 2015).

Pulse co-oximetry

Pulse co-oximetry was performed using a Masimo Radical 7 (Masimo, Irvine, CA, USA) according to manufacturer's instructions using a finger probe to measure blood parameters in the periphery. Co-oximetry records pulse rate (PR), perfusion index (PI, the ratio of the pulsatile blood flow to the nonpulsatile static blood flow in a peripheral tissue), and the proportion of total Hb (SpHb),

Oxygenated Hb (SpO2) and MetHb (SpMet) in the blood. Four freely-behaving, healthy, age-matched male volunteers were monitored over a 3-day period during a normal working week. Data was collected in Cambridge, UK between September and November, 2012. Data collection was conducted in accordance with the principles of the Declaration of Helsinki, with approval/favourable opinion from the Local Research Ethics Committee (University of Cambridge, UK). Participants in the study were screened for relevant self-reported health issues, including sleep disorders or excessive daytime sleepiness.

Statistical Analysis

All graphs and analyses were performed in Prism 8 (Graphpad Software Inc, La Jolla, CA) and R (version 3.6.3) in R Studio (version 1.2.5033, RStudio Inc). Least squares non-linear regression curve fitting in GraphPad Prism (v7.03, GraphPad; La Jolla, CA) was used to determine whether circadian rhythms were present in quantified data by comparing straight line fit and damped cosine fit with the extra sum-of-squares F test as described previously (Beale et al., 2019). Pulse co-oximetry data was analysed for significant rhythmicity by autocorrelation in R and subsequent damped cosine curve fitting as above. Mean ± SEM is reported throughout. Analyses are reported in figure legends.

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Author contributions

JON and MHH designed experiments; JEC performed all animal work and collected tissues; PC performed fibroblast assays, KAF, RSE and JON performed RBC time courses and biochemistry; ABR performed phlebotomy, KSL performed mass spectrometry; UV, GR, JON and ABR collected and analysed co-oximetry data; ADB performed data analysis; FHL and ABR contributed resources and

discussion; KPW collected human blood samples in controlled laboratory conditions; MHH and JON supervised the work; ADB & JON wrote the paper.

Competing interests

No competing interests exist.

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