Chronobiology International, 22(3): 455–471, (2005)

ISSN 0742-0528 print/1525-6073 online
DOI: 10.1081/CBI-200062353

Taylor & Francis
Taylor & Francis Group

LIGHT PULSE-INDUCED HEME AND IRON-ASSOCIATED TRANSCRIPTS IN MOUSE BRAIN: A MICROARRAY ANALYSIS

Rachel Ben-Shlomo,[1] Ruth A. Akhtar,[2,4] Ben H. Collins,[2,5] David J. Judah,[3] Reginald Davies,[3] and Charalambos P. Kyriacou[2]

[1]*Department of Biology, University of Haifa, Israel*
[2]*Department of Genetics, University of Leicester, Leicester, England*
[3]*MRC Toxicology Unit, University of Leicester, Leicester, England*
[4]*Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, England*
[5]*Department of Biology, New York University, New York, NY, USA*

Synchronization of circadian oscillators with the outside world is achieved by the acute effects of light on the levels of one or more clock components. In mammals the PAS transcription factors Clock, NPAS2, and BMAL1 regulate gene expression as a function of the day-night cycle. Both PAS domains of NPAS2 were found to bind heme as a prosthetic group, form a gas-regulated sensor, and exert heme-status control of DNA binding *in vitro*. In a microarray analysis comparing overall changes in brain transcript levels between mice subjected to light pulses during the dark phase with animals maintained in darkness, we traced consistent changes in more than 200 different transcripts. Of these, 20 are associated with heme and iron biosynthesis and catabolism. A model for the pathway of induction of heme and iron homeostasis-related transcripts resulting from light pulses suggests that light signals (as stressors) induce transcription of *heme oxygenase 2* (*Hmox2*) and *cytochrome P450 oxidoreductase* (*Por*), which may serve as a primary line of cellular defense. HMOX2 degrades heme from proteins such as hemoglobin. This degradation generates CO, a signal molecule, and may also change the redox state of the cell by reducing the NADPH/NADP ratio. This could lead to up-regulation of globin gene transcription, thereby releasing iron that in turn controls production of ferritins, and further up-regulating *aminolevulinate synthase 2* (*Alas2*).

Keywords Heme and Iron Metabolism, Circadian Rhythm, Light Pulses, Molecular Mechanism of Entrainment, Biological Clocks

Received February 2, 2005, Accepted March 3, 2005
Address correspondence to Rachel Ben-Shlomo, Department of Biology, University of Haifa—Oranim, Tivon 36006, Israel. E-mail: ekly@research.haifa.ac.il

INTRODUCTION

Light is an influential regulator of physiology and behavior in mammals. An important aspect of this regulation is the ability of the light environment to provide time-of-day information. This information is used to regulate temporal aspects of physiology and behavior, including entrainment of the circadian clock. Brief light exposure during the early subjective night causes phase delays, while exposure during the late subjective night causes phase advances. Thus, the direction (delay or advance) and magnitude of a phase shift are functions of the time in the daily cycle that the light signal is administered (Pittendrigh, 1981; Rea, 1998).

In mammals, the primary circadian clock is located within the suprachiasmatic nuclei (SCN). Presumably, several additional peripheral oscillators are located within other brain regions, such as the pineal gland, motor cortex, forebrain (Hastings et al., 2003; Mrosovsky et al., 2001; Reick et al., 2001), and retina (Tosini and Menaker, 1996). Clocks in the SCN and peripheral pacemakers do not appear to be directly sensitive to light. Synchronization of circadian oscillators with the outside world is achieved through light's acute effects on the levels of one or more of the clock's components. The consequences are 'ripples' through the interconnected molecular loops leading to a stable phase realignment of the endogenous rhythm generator with the external conditions (Edery, 2000; Reppert and Weaver, 2001).

The molecular mechanisms that enable environmental stimuli to abruptly alter circadian rhythms in mammals remain obscure. Recent findings suggest that modulation of activity of the transcription factors Clock and NPAS2, according to the redox state of the cell, may provide an insight into the molecular mechanism of entrainment (Boehning and Snyder, 2002; Dioum et al., 2002). Both genes contain PAS domains, which are generally correlated with the sensing of a variety of environmental signals such as changes in light, redox potential, oxygen, small ligands, and overall energy level of a cell (Gilles-Gonzalez and Gonzalez, 2004; Taylor and Zhulin, 1999). NPAS2 is a mammalian transcription factor expressed primarily in the forebrain, which binds DNA as an obligate dimeric partner of BMAL1 and is implicated in the regulation of circadian rhythms (Dioum et al., 2002; Dudley et al., 2003; Reick et al., 2001). Like the transcription factor Clock, NPAS2 regulates gene expression as a function of the day-night cycle (Reick et al., 2001). Both PAS domains of NPAS2 were found to bind heme as a prosthetic group, to form a gas-regulated sensor. The heme status controls DNA binding *in vitro*. CO was identified as a reasonable candidate ligand for NPAS2 (Dioum et al., 2002). Dioum and colleagues (2002) suggested a role for the NPAS2-BMAL1 heterodimer in orchestrating circadian oscillations of metabolic pathways,

including heme biosynthesis, which might be reciprocally linked to heme catabolism by the molecular sensor. Recently, Kaasik and Lee (2004) showed that, in mouse liver, heme differentially modulates expression of the mammalian *Period* genes *mPer1* and *mPer2 in vivo* by a mechanism involving NPAS2 and mPER2. Further experiments showed that mPER2 positively stimulates activity of the BMAL1–NPAS2 transcription complex and, in turn, NPAS2 transcriptionally regulates *Alas1*. In a microarray analysis comparing overall changes in brain transcript levels between mice subjected to light pulses during the dark phase with animals maintained in darkness, we traced consistent changes in more than 200 different transcripts. Of these, 20 are associated with heme and iron biosynthesis and catabolism.

MATERIAL AND METHODS

Animals Housing and Experiment Design

Adult C57BL male mice (6 wk old on arrival) were kept in reverse phase 12 : 12 light:dark (LD) cycle for at least 2 weeks of acclimatization. The mice were individually housed in cages supplied with water and food. Feeding and cleaning took place during the light phase, just before light offset. Animal experimentation was licensed by the British Home Office under the Animals (Scientific Procedures) Act, 198b. The research protocol followed the good practice standards for animal studies as recommend by the NIH Guidelines for animal research and by the Journal (Touitou et al., 2004).

A 1 h light pulse was administered at CT14, 18, or 22, while control animals remained in darkness. Whole brain total RNA was collected 15 min, 1, 2, 3, and 4 h after onset of the pulse at CT14 or 18, and 1 h after onset at CT22, giving 11 time points in experimental and time-matched control animals. Two animals were sacrificed at each time point under dark conditions by cervical dislocation. RNA was extracted immediately using RNAZol^{TM} B (Biotecx Laboratories, Texas, USA). RNA was transcribed into cRNA with incorporation of fluorescently labeled dUTP (Cy Dyes) by reverse transcription.

Microarrays Analysis

cDNA microarrays based on the polymerase chain reaction (PCR) products of IMAGE clones and generated by the MRC Toxicology Unit at Leicester University were spotted onto poly-L-lysine-coated glass slides using a Stanford-type arrayer. Labeling, hybridization, and primary analyses were carried out according to the methods of Turton and colleagues (2001) and Akhtar et al., (2002). At each time point, hybridization of

50 μg of brain RNA from experimental mice (labeled with Cy5, red) was compared with 50 μg of RNA from control mice (labeled with Cy3, green). Feature sizes, background, and feature fluorescence were calculated using GenePix 3.0 software (Axon Instruments). Median fluorescence for the pixels within the feature was calculated, and the raw data for each channel were then normalized by reference to the median fluorescence of the total feature set for that channel, and a $\log_2$(Cy5/Cy3) ratio was calculated. About 15% of transcripts appeared on arrays more than once, allowing further testing of the internal consistency of the hybridization. Transcripts were considered as suitable for analysis if they showed an hybridization signal in 10 of 11 slides. Patterns of gene expression were visualized using Cluster and Treeview software (Eisen et al., 1998; http://rana.lbl.gov/EisenSoftware.htm).

Data Analysis

We were interested primarily in any gene that was consistently up- or down-regulated at all time points. The genes reported here all showed consistent positive or negative Cy5/Cy3 $\log_2$ ratios after normalization at the main CT14 and CT18 time points. In addition, using the method of Turton and colleagues (2001), we were able to see within each slide whether this ratio for each probe fell outside appropriate two-tailed confidence intervals. Bearing in mind the variation in biological samples, we considered changes as statistically significant only if a transcript expressed significant change at least twice, either at two different time points (two different arrays) or if a transcript was represented on the same array in duplicate and if the change was significant at the same time point in both. A transcript was also considered as affected by the light pulse, even when the level of change was small, if it showed the same direction of change (either up- or down-regulation) in all but one array (minimum 9 of 10 arrays in cases were the hybridization signals were visualized on 10 arrays, sign test $p = 0.021$, or 10 of 11 arrays in cases were the signals were visualized on 11 arrays). These considerations allowed us to identify transcripts even if the magnitude of change was relatively low. In this way, we gathered from the microarray a list of 20 transcripts that were both associated with heme and iron metabolism and affected by light pulses.

Validation

Changes in level of several transcripts were further validated. In the case of *Hba-a1* (Hemoglobin α), where the total amount of transcripts and the magnitude of change were high, results were validated by Northern blot analysis: 5 μg of total RNA denatured with formaldehyde and formamide for 15 mins at 65°C, resolved by gel electrophoresis in 1% agarose

gel in MOPS/formaldehyde buffer, and transferred onto a nylon membrane (Amersham Hybond-XL, Amersham, Pharmacia Biotech, UK). An insert of ~600b of *Hba-a1* was isolated from a plasmid (PT7T3D-PC) and labeled with ^{32}P-dCTP using random primers pd$(N)_6$ and Klenow Fragment DNA Polymerase (Amersham, Pharmacia Biotech, UK). Hybridization of the labeled probe to the filters was done overnight at 42°C in formamide solution. The filter was then washed in high stringency conditions (65°C; 0.1 SSC; 0.1% SDS),
followed by autoradiography.

Relatively scarce transcripts and lower level of changes were tested by Q-PCR, with *Scamp3* (Secretory carrier membrane protein 3) used as a constitutive control, as it does not express circadian changes in any of the tissues examined by Akhtar and colleagues (2002) or Panda and coworkers (2002). Primers for RT-PCR were designed from the sequence of each gene published in the Gene Bank. cDNA was synthesized from 10 μg of RNA using Reverse Transcriptase (Promega, UK) in a final volume of 20 μL. A set of 1/10 dilutions were made to run the RT-PCR. Standard curves were generated by serial of dilutions of a mix of 2 μL of each cDNA. PCR cycles included 95°C, 10 mins; 95°C, 30 s; 53°C, 30 s; 72°C, 30 s. Test of each transcript was conducted in triplicates. We allowed 30% differences among repetitions within each Q-PCR run; hence, a ratio between control and experiment that is lower than 1.3 can be considered as 'not different'. The validation included one time point for transcripts of *Hmox2* and *Fth* and two time points of *Ldh*.

RESULTS

Of 4211 cDNAs on the arrays, 1035 passed the requirement of having an hybridization signal in at least 90% of the arrays (at least 10 tests). Of these transcripts, 30% were consistently up-regulated, and 6 to 7% were down-regulated by light pulses during the dark phase. Among them, 33—representing 20 different genes—are associated with heme and iron biosynthesis and catabolism. Therefore, about 15% of the transcripts that expressed consistent change in their level as a result of light pulses during the dark phase belong to this functional category.

Gene expression profiling of heme and iron-associated transcripts expressed by the normalized Cy5/Cy3 fluorescent ratios are summarized in Table 1. Various patterns of changes in RNA levels resulting from the light pulses are revealed in a hierarchical clustering (Figure 1). The most significant change was a repeated up-regulation found in several hemoglobin transcripts (*Hbb-a1*, *Hbb-b1*, *Hbb-b2*) when the light pulse was administered at CT18. This result was further validated for *Hbb-a1* by Northern blot (Figure 2). Microarray analysis indicated sharp (>2-fold) increases in hemoglobin RNA levels 15 mins after onset of the light pulse, either

TABLE 1 Consistent Changes in Heme and Iron Associated Transcripts with Light Pulses. Ratio of Relative Amount of Transcripts of Light Pulsed Mice/Control (Normalized Cy5/Cy3 Fluorescent Ratios)

Transcript	CT14-0.15	CT14-1	CT14-2	CT14-3	CT14-4	CT18-0.15	CT18-1	CT18-2	CT18-3	CT18-4	CT22-1
Alas2 (x3)	0.47	0.46	0.79	0.82	0.67	0.77	1.26	**5.22**	1.28	0.95	0.49
Aplp2	2.43	2.40	1.93	1.59	1.94	1.73	1.18	1.11	1.77	1.47	3.05
Bmal1	0.38	1.29	0.74	0.46	1.04	0.90	0.90	1.16	0.69	1.06	0.56
Epor (x2)	0	0.65	0.52	0.27	0.50	0	0.35	0.49	**0.21**	0.18	0
Fth (x2)	2.38	1.23	1.22	1.37	1.40	1.69	1.53	1.58	1.71	1.25	3.20
Ftl1	1.51	1.45	1.37	1.74	2.09	1.30	1.47	1.54	1.51	1.00	2.66
Hba-a1 (x3)	3.03	1.73	1.25	2.74	1.80	2.32	**3.07**	**8.06**	**3.68**	1.02	6.41
Hba-x	0	0	0	0	0.71	1.43	**2.66**	**5.66**	1.98	0	1.47
Hbb-b1	2.85	1.25	1.28	3.09	1.79	2.34	**3.13**	**8.48**	**3.68**	0.39	6.30
Hbb-b2 (x2)	2.66	1.97	1.16	3.03	1.70	2.12	**3.13**	**8.08**	3.49	1.15	**7.72**
Hmox1 (x3)	0.46	0	0	0	**0.26**	0.60	0.34	0.65	0.32	0.20	0.36
Hmox2 (x2)	1.71	1.58	1.29	1.73	1.46	1.48	1.41	1.38	1.53	1.42	1.67
Lcp2	2.30	1.47	1.19	1.86	1.63	0	2.11	2.42	2.70	0.63	6.01
Ldh1	2.34	1.52	1.26	1.61	1.71	1.52	1.51	1.45	1.30	1.14	3.27
Ldh2 (x4)	2.40	1.72	1.32	1.72	1.66	1.99	1.59	1.76	1.73	1.47	5.36
Mmp11	2.64	1.62	1.26	2.50	1.77	1.65	1.76	1.64	1.91	1.31	4.47
Mpo	2.32	1.98	1.62	2.04	1.89	1.44	1.54	1.15	2.18	1.54	1.21
Mt1	2.81	1.93	1.43	2.35	1.74	1.85	2.07	1.96	1.62	1.16	5.72
Por (x2)	1.67	1.58	1.19	2.33	1.49	1.26	1.05	0.97	1.48	1.34	0.88
Scd1	1.60	1.22	1.02	1.23	1.34	1.48	1.23	1.49	1.13	1.91	1.56
Stat5a (x2)	**0.15**	**0.23**	0.52	0	0.66	0.37	0.72	0.69	0.56	0.40	0.48

Bold type indicates successfully significant changes (see Materials and Methods) within an array; numbers in parentheses indicate transcripts duplicated within an array.

0 = missing data.

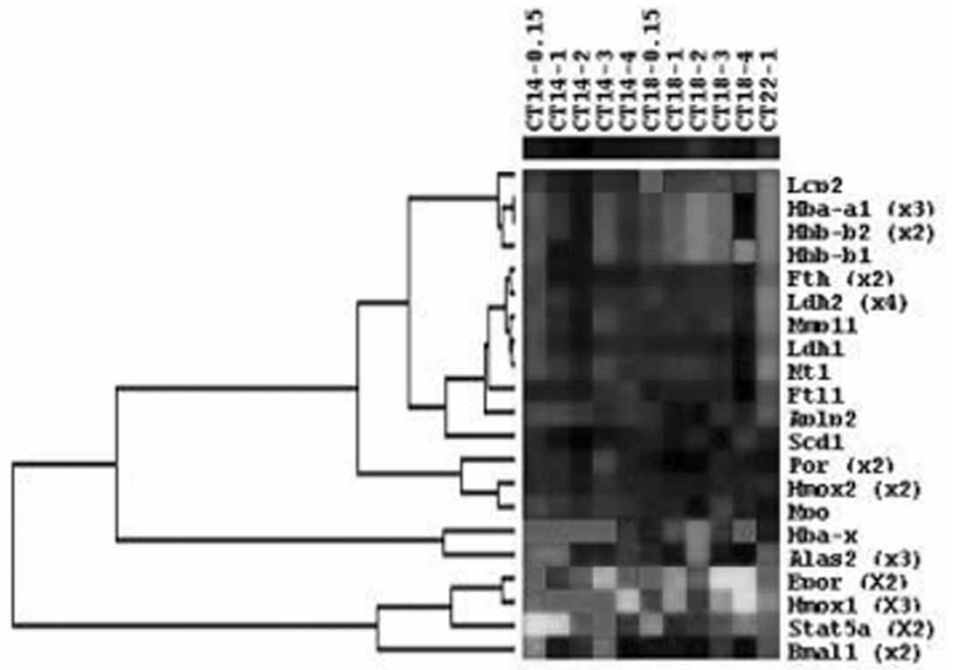

FIGURE 1 Cluster analysis of changes in heme and iron-associated transcripts resulting from light pulse during the dark phase. Each column represent the time of light pulse (CT 14, 18, or 22) and the time of RNA collection after the pulse. Thus CT14−0.15 represents administration of light pulse at CT14, and RNA collection 15 min after the pulse. Red colors represent up-regulation of a transcript, and green down-regulation. Gray color represents missing data.

when administered at CT14 or CT18. Dramatic (>6-fold) increases in hemoglobin transcript level were noted within 1 h after onset of the light pulse at CT22. The hemoglobin mRNA was increase 8-fold 2 h after the CT18 light pulse (see Figure 1 and Table 1).

Changes in the level of *Alas2* RNA differed of through the dark phase. When the light pulse was given at CT18, *Alas2* exhibited a significant 5-fold up-regulation 2 h after the pulse, corresponding to the same time point at which all hemoglobin (*Hbb-a1*, *Hbb-b1*, *Hbb-b2*, and *Hba-x*) transcript levels were maximal. When the light pulse was administered at either CT14 or CT22, *Alas2* levels were slightly down-regulated, while at CT22 the levels of hemoglobin transcripts were considerably up-regulated (Figure 1 and Table 1).

Our microarray analysis showed low, yet consistent, up-regulation of *Hmox2* at all 11 sampled time points (Figure 1 and Table 1). *Hmox2* was present on the array in duplicate spots and expressed a light pulse-induced up-regulation in all 2×11 hybridization signals. *Hmox2* was slightly up-regulated by light as early as 15 min after the pulse. A small ($\approx$>1.5-fold) yet, consistent, increase in the level of *Hmox2* as a

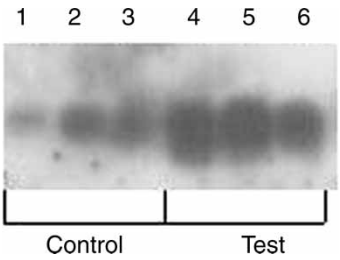

FIGURE 2 Northern blot of *hba-a1* transcript. Samples 1 to 3 are from control mice, and 4 to 6 are from mice subjected to light pulse at CT18. Sample 1 and 4 collected 1 h after the pulse, samples 2 and 5 at 2 h, and samples 3 and 6 collected 4 h after the pulse.

consequence of the light pulse was evident throughout the dark phase (Figure 1 and Table 1). Consistent up-regulation was also noted for *Cytochrome P450 oxidoreductase (Por), matrix metalloproteinase 11 (Mmp11), metallothionein1 (Mt1)*, and the iron-binding *Stearoyl-coenzyme A desaturase 1 (Scd1)* transcripts.

Light pulse during the dark phase resulted in an increase of both ferritin heavy and light chain 1 *(Fth and Ftl1)* transcripts. The increase was noticed through all 11 tested time points, with higher increases when the light pulse was given at CT22. Stronger induction when the pulse was given at CT22 was also found for the two *lactate dehydrogenase* isozymes, *LdhA* and *LdhB*.

While *Hmox2* level increased as a result of the light pulse, *Hmox1* levels decreased dramatically, attaining significant down-regulation within 4 h, when the pulse was given at CT14 or at CT18. The *signal transducer and activator of transcription 5 (Stat5)* was also down-regulated by light, expressing a significant reduction as early as 15 min after pulse onset, when the pulse was administered at CT14. A substantial reduction was noted for erythropoietin receptor (*Epor*) when the pulse was given at CT14 and a further significant reduction was indicated at CT18.

Most of these transcripts were expressed at relatively low abundances in the brain, and their light-induced changes were of low amplitude. The microarray data analysis considered not only significant changes in transcript levels (usually changes >6-fold in amplitude), but also smaller, but consistent, changes in levels throughout all the dark phase. We further successfully validated the results of some of the transcripts by Q-PCR (Table 2). However, *Fth* was not validated.

DISCUSSION

The most significant changes observed were in the up-regulation of several hemoglobin transcripts (*Hbb-a1*, *Hbb-b1*, *Hbb-b2*). Hemoglobin is an extracellular scavenger of both NO and CO, and blocks cholinergic

TABLE 2 Validation of Microarray Results by QPCR

Transcript	Time Point	QPCR-1	QPCR-2	QPCR-3	QPCR-4	Average	Control-*Scamp*	Transcript/ *Scamp* – control	Regulation
Ldh	CT14-0.15	117.40	123.82	123.61	126.65	122.87	121.36	**1.01**	**Up**
	CT14-0.15 Control	52.46	31.19	39.61	53.11	44.09	68.87	**0.64**	
	CT22-1A	250.01	354.73	261.98		288.90	149.31	**1.93**	**Up**
	CT22-1A Control	248.62	353.82	359.25		320.56	233.66	**1.37**	
	CT22-1B	395.01	380.42	328.85		368.09	164.44	**2.24**	**0**
	CT22-1B Control	288.73	231.82	309.96		276.83	145.50	**1.90**	
Hmox-2	CT14-4	630.96	606.91	429.93		555.93	139.09	**4.00**	**Up**
	CT14-4 Control	445.75	422.46	533.45		467.22	158.18	**2.52**	
Fth	CT14-0.15	236.42	323.38	306.21		279.90	121.36	**2.31**	**0**
	CT14-0.15 Control	177.70	216.70	255.88		197.20	68.87	**2.86**	

Numbers represent the estimated amount of specific transcript RNA in ng. The final column gives the relative amount normalized in comparison to the constitutive *Scamp* control (see Materials and Methods).

stimulation of cGMP synthesis (Artinian et al., 2001). Heme controls translation of the globin mRNAs and formation of heme commences with the rate-limiting enzyme ALAS (Dzikaite et al., 2000). ALAS2, an erythroid specific ALAS, is believed to be the key control point for regulating heme synthesis in reticulocytes in response to heme, iron, or other physiological stimuli. Heme also exerts a negative feedback effect on its own synthesis; increasing heme levels appear to block import of the ALAS2 protein into mitochondria by binding to a specific cysteine-proline–rich sequence in the signal peptide (Dzikaite et al., 2000, and reference therein). The microarray results may suggest that the signal for globin transcription is more intimately regulated by light and may be a part of the entrainment input pathway. Both *globin* and *Alas* transcripts exhibit additional post-transcriptional regulation, where *globin* translation is controlled by heme availability, and *Alas* translation is controlled by iron availability (Eisenstein, 2000). Heme synthetic capacity declines more rapidly than globin synthetic capacity during reticulocyte maturation *in vitro* (Ponka, 1997 and references there in). This may explain the differences in duration of increase of *Alas2* and hemoglobin transcripts level following the light pulse at CT18.

A possible means of reciprocally connecting CO production to the regulatory system controlling circadian rhythms has been suggested by Zheng and colleagues (2001), who found that the genes encoding *Alas* are under circadian control in the mouse liver. Circadian expression of *mAlas1* and *mAlas2* was completely disrupted in mutant mice that were deficient in both *mPer1* and *mPer2*, indicating that mPER1 and mPER2 regulate the availability of heme. NPAS2 knockout mice, on the other hand, fail to exhibit rhythmic *mPer2* gene expression in regions of the forebrain known to express NPAS2 (Reick et al., 2001). These same regions of the forebrain exhibit enhanced expression of the gene for heme oxygenase 2 (*Hmox2*), an enzyme that generates CO using heme as a substrate (Vincent et al., 1994). Photic regulation of *Hmox2* enzymatic activity was also found in hamster retina, where under LD conditions activity was significantly higher at mid-day than at midnight (Sacca et al., 2003). In DD, however, these differences disappeared. In hamster SCN, HMOX enzymatic activity was significantly higher during the night, suggesting an endogenous circadian control, although light stimuli did not reveal significant changes (Rubio et al., 2003).

The heme oxygenase system consists of two major forms: the oxidative stress-inducible protein Hmox1 (HSP32) and the constitutive isozyme Hmox2 (Maines, 1997). In adult animals, Hmox predominantly functions in the generation of CO, producing bile pigment, and releasing heme iron for gene regulation. Hmox2/CO activity was found to be both necessary and sufficient to induce clock resetting (Artinian et al., 2001). In mammalian cells, the only source of CO is heme following its cleavage by Hmox

(Ponka, 1997). If CO serves as a signal molecule for generating cGMP, hemoglobin can be one of the sources for heme substrate (Maines, 1997). A 3-fold decrease in cGMP level was found upon application of hemoglobin to SCN slices (Artinian et al., 2001).

If oscillatory expression of *mAlas* were to direct a corresponding oscillation in heme biosynthesis, then heme catabolism by *Hmox2* to generate CO might also vary as a function of the day-night cycle (Dioum et al., 2002). If so, the role of NPAS2-BMAL1 heterodimer in orchestrating circadian oscillation of metabolic pathway, including heme biosynthesis, might be reciprocally linked to heme catabolism by the molecular sensor. Microarray analysis indicated that light pulses generate two different patterns of change for *Hmox* and *Alas*.

Hmox1 and *Hmox2* encode different gene products. HMOX1, also known as HSP32, is exquisitely sensitive to all kinds of stimuli and agents (Maines, 1997). The consensus sequences necessary for binding several regulatory factors are present in the promoter of *Hmox1*, whereas only a single glucocorticoid response element, which is not a strong transcriptional enhancer, is present and functional within the *Hmox2* promoter region (Maines, 1997). In neonatal rat hippocampus high levels of *Hmox1* are expressed in neuronal cell populations, but when treated with glucocorticoid, the population of *Hmox1* expressing neurons decreases (Maines, 1997). The different regulation of *Hmox2* and *Hmox1*, as evident by the microarray results, may also reflect changes in the level of glucocorticoids in the mouse brain resulting from light pulses during the dark phase.

Fluctuations in cellular redox have been proposed to act directly to entrain the molecular clock (Boehning and Snyder, 2002; Dioum et al., 2002; Rutter et al., 2001; Schibler and Sassone-Corsi, 2002). The reduced cofactors NADH and NADPH (nicotinamide adenine dinucleotide and its phosphate) greatly enhance binding of Clock-BMAL1 and NPAS2-BMAL1 heterodimers to DNA; whereas, the oxidized forms of the same molecules, NAD and NADP, inhibit the DNA binding of these dimers (Boehning and Snyder, 2002; Rutter et al., 2001). If active NPAS2, in the presence of BMAL1 and pure NADPH, is exposed to CO, the NPAS2-BMAL1 heterodimer do not form. This inactivation by CO requires the presence of heme in the protein and is entirely reversible on removal of CO (Dioum et al., 2002; Gilles-Gonzalez and Gonzalez, 2004). Although the physiological effects of heme ligands on NPAS2 are not yet known, it is already clear that heme status couples directly to DNA binding in this transcription factor (Gilles-Gonzalez and Gonzalez, 2004). The HMOX system requires the concerted activity of NADPH-cytochrome P450 reductase, which transfers an electron from NADPH to heme and utilizes molecular oxygen for cleavage of heme (Maines, 1997). Interestingly, *Por* and *Hmox2* show a very similar pattern of

expression on the microarray (see cluster analysis in Figure 1). Thus, the induction of heme degradation by *Hmox2* (and *Por*) may inhibit NPAS2 (or Clock) and BMAL1 heterodimer formation and/or binding to DNA, and consequently it may block the positive regulation of *Per* transcription. The microarray analysis conducted on whole brain did not show any significant nor consistent change in *Bmal1* level as a result of light pulses.

In a screen for genes activated by induction of NPAS2:BMAL1, the *lactate dehydrogenase A* (*LdhA*) gene was identified as a direct and *bona fide* transcriptional target. LDH reversibly catalyzes the enzymatic conversion of pyruvate to lactate. In the forward direction the reaction consumes reduced NADH and evolves balanced levels of the oxidized form of the cofactor NAD (Rutter et al., 2001). Two *Ldh* isozymes—*LdhA* and *LdhB*—were presented on the arrays, and both were consistently up-regulated by the light pulses.

HMOX activity gives rise to CO and iron. Under stress conditions, enhanced production of CO could be considered to be a component of the cellular defense mechanism (Maines, 1997). Iron can be toxic to the cell because of its ability to promote oxidation of lipids, proteins, and other cellular components (Eisenstein, 2000). The released iron would induce the synthesis of ferritin, the iron storage protein. Light pulse during the dark phase resulted in an increase of both ferritin heavy and light chain 1 (*Fth* and *Ftl1*) transcripts.

Erythropoietin (EPO) and its receptor (EPOR) are required for the production of mature red blood cells. *Epor* expression was induced by anemic stress in human-*Epor* transgenic mice (Chin et al., 2000). The microarray results indicate down-regulation in *Epor* transcripts that may be associated with increase of hemoglobin transcripts in the brain. Furthermore, *signal transducer and activator of transcription 5 (Stat5)*, which was found to be important in EPO-induced hemoglobin synthesis in erythroid cells (Wakao et al., 1997), was also down-regulated by light.

Nitric oxide (NO) is also an established regulator of clock resetting by a nocturnal light/glutamatergic signal (Ding et al., 1994). Myeloperoxidase (MPO) can serve as a catalyst for generating NO-derived oxidant. The enzyme contains heme prosthetic groups that are ligated through a histidine nitrogen and use H_2O_2 as the electron acceptor in the catalysis of oxidative reactions (Abu-Soud et al., 2001). Heme reduction causes a dramatic change in the heme pocket electronic environment that alters the affinity and/or accessibility of heme iron toward NO. A slight induction of the *Mpo* transcript by light pulses was evident on the array throughout the dark phase. Additional transcripts that may be associated with heme or iron biosynthesis and catabolism were present on the array. Up-regulation was noted both in the transcripts for amyloid beta precursor-like protein 2 (*Aplp2*), which binds to HMOX and may inhibit its activity (Takahashi et al., 2000), and for Lymphocyte cytosolic protein 2 (*Lcp2* also known as

SLP-76), which is needed for the hematopoietic signaling pathway (Abtahian et al., 2003).

The evolution of complex cellular and developmental processes depends on the maintenance and regulation of orchestrated responses of large amount of genetic information. Most genes are regulated by mixing and matching different types of activators and repressors in coordinated fashion (Tacchini et al., 2002). Neurons containing the molecular clock might be entrained by neuronal activity through fluctuations in the ratios of reduced-to-oxidized NAD cofactors (Rutter et al., 2001). Sensing of redox potential may provide a versatile measure of cellular energy. Recent studies suggest that heme-containing PAS-domain proteins may sense oxygen, light, redox potential, or proton motive force as a way of monitoring energy changes in living cells (Gilles-Gonzalez and Gonzalez, 2004; Taylor and Zhulin, 1999).

In case of abnormal conditions, which are usually accompanied by changes in oxidation/reduction status of the cell and an induction of *Hmox* activity, HMOX becomes a prominent component of the heme degradation process. Hemoglobin is the most abundant hemoprotein, containing as much as 70% of the total iron content of a normal adult. Heme substrate becomes available from additional sources that can affect the rate of hemoglobin denaturation and that may make available extracellular heme for degradation (Maines, 1997).

The human ß-globin gene is abundantly expressed specifically in adult erythroid cells, and daily variation in blood and serum concentrations of hemoglobin have been reported (Wisser and Breuer, 1981). The ß-globin promoter contains a TATA-like motif, an initiator and a downstream E-box element that are all required for high level transcription *in vitro*. Helix–loop–helix (HLH) proteins contribute to the formation of transcription complexes on the adult human ß-globin gene, and the differential association of HLH proteins with the basal promoter contributes to the stage-specific expression of the gene (Leach et al., 2003). Interestingly, the transcription factors Clock (NPAS2) and BMAL1 that are positively regulated clock genes belong to the bHLH-PAS transcription factors that bind to E-box enhancers. Moreover, *globin* RNAs robustly cycle in mouse SCN (Panda et al., 2002; http://expression.gnf.org/circadian), with a similar phase to *Bmal1*.

In conclusion, our low-resolution microarray analysis on murine brains has nevertheless revealed a number of heme- and iron-associated genes whose expression is modulated by light pulses in a global manner. We present a rational, and we believe a reasonably compelling, explanation of how these genes may be functionally inter-related within this metabolic pathway. A model for the pathway of induction of heme and iron homeostasis-related transcripts resulting from light pulses is summarized in Figure 3. At CT14, the light pulse up-regulated *globin* transcripts

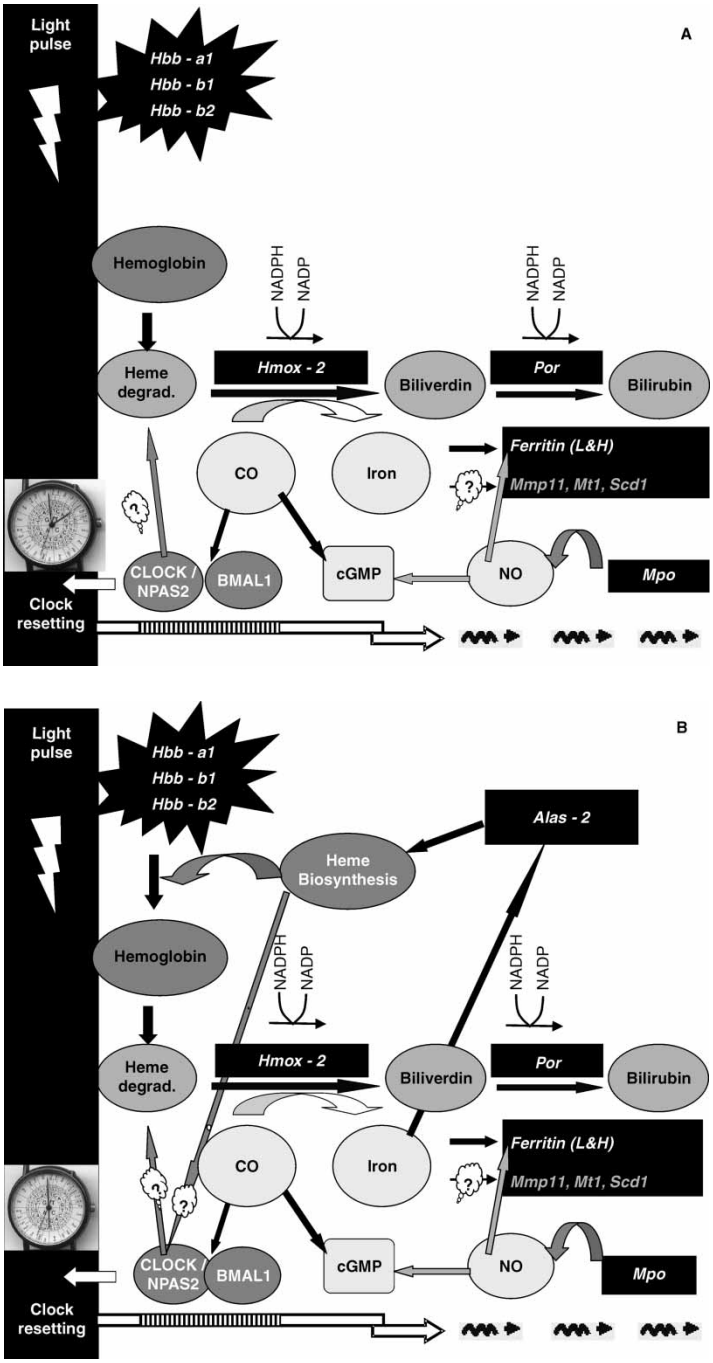

FIGURE 3 A model of light entrainment pathways of molecular changes in brain cells. In black frame transcripts that were up-regulated by light pulse [A.] Light pulse is administered at CT14: *Hmox2 Por* and *Globin* transcripts are up-regulated but not *Alas2*. Heme is degraded, CO and iron are produced and the cellular level of heme may decline. [B.] Light administered at CT18: *Alas2* is also up-regulated which result in heme biosynthesis and synthesis of hemoglobin.

but not *Alas2* (Figure 3a). Thus the total level of heme may decline. At CT18 *Alas2* was also up-regulated, which results in heme biosynthesis and synthesis of hemoglobins (Figure 3b). The light signal (as a stressor) induces transcription of *Hmox2* and *Por*, which may serve as a primary line of cellular defense, whereby the prooxidant toxic heme molecule is degraded. HMOX2 protein degrades heme from proteins such as hemoglobin and perhaps NPAS2 that have heme as a prosthetic group. This degradation generates CO—a signal molecule, and may also:

a. up-regulate transcription of globin genes;
b. releases iron that controls production of ferritins and at CT18 also up-regulates *Alas2*;
c. change the redox state of the cell by reducing the ratio NADPH/NADP.

Finally, the CO gaseous neurotransmitter may regulate transcription factors NPAS2-BMAL1 (and probably also Clock-BMAL1) via redox changes, thereby inhibiting formation of the heterodimers and thus altering their affinity to DNA (Boehning and Snyder, 2002).

ACKNOWLEDGMENTS

We are pleased to acknowledge a BBSRC grant and a Royal Society Wolfson Research Merit Award to CPK. We thank the MRC for a studentship to BHC.

REFERENCES

Abtahian, F., Guerriero, A., Sebzda, E., Lu, M.M., Zhou, R., Mocsai, A., Myers, E.E., Huang, B., Jackson, D.G., Ferrari, V.A., Tybulewicz, V., Lowell, C.A., Lepore, J.J., Koretzky, G.A., Kahn, M.L. (2003). Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science* 299:247–251.

Abu-Soud, H.M., Hazen, S.L. (2001). Interrogation of heme pocket environment of mammalian peroxidases with diatomic ligands. *Biochemistry* 40:10747–10755.

Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H., Kyriacou, C.P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr. Biol.* 12:540–550.

Artinian, L.R., Ding, J.M., Gillette, M.U. (2001). Carbon monoxide and nitric oxide: interacting messengers in muscarinic signaling to the brain's circadian clock. *Exp Neurol.* 171:293–300.

Boehning, D., Snyder, S.H. (2002). Circadian rhythms. Carbon monoxide and clocks. *Science* 298: 2339–2340.

Chin, K., Yu, X., Beleslin-Cokic, B., Liu, C., Shen, K., Mohrenweiser, H.W., Noguchi, C.T. (2000). Production and processing of erythropoietin receptor transcripts in brain. *Brain Res. Mol. Brain Res.* 81:29–42.

Ding, J.M., Chen, D., Weber, E.T., Faiman, L.E., Rea, M.A., Gillette, M.U. (1994). Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science* 266: 1713–1717.

Dioum, E.M., Rutter, J., Tuckerman, J.R., Gonzalez, G., Gilles-Gonzalez, M.A., McKnight, S.L. (2002). NPAS2: a gas-responsive transcription factor. *Science* 298:2385–2387.

Dudley, C.A., Erbel-Sieler, C., Estill, S.J., Reick, M., Franken, P., Pitts, S., McKnight, S.L. (2003). Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. *Science* 301: 379–383.

Dzikaite, V., Kanopka, A., Brock, J.H., Kazlauskas, A., Melefors, O. (2000). A novel endoproteolytic processing activity in mitochondria of erythroid cells and the role in heme synthesis. *Blood* 96: 740–746.

Edery, I. (2000). Circadian rhythms in a nutshell. *Physiol. Genomics* 3:59–74.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 95:14863–14868.

Eisenstein, R.S. (2000). Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu. Rev. Nutr.* 20:627–662.

Gilles-Gonzalez, M.A., Gonzalez, G. (2004). Signal transduction by heme-containing PAS-domain proteins. *J. Appl. Physiol.* 96:774–783.

Kaasik, K., Lee, C.C. (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature* 430:467–471.

Hastings, M.H., Reddy, A.B., Maywood, E.S. (2003). A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat. Rev. Neurosci.* 4:649–661.

Leach, K.M., Vieira, K.F., Kang, S.H., Aslanian, A., Teichmann, M., Roeder, R.G., Bungert, J. (2003). Characterization of the human beta-globin downstream promoter region. *Nucleic Acids Res.* 31: 1292–1301.

Maines, M.D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* 37:517–554.

Mrosovsky, N., Edelstein, K., Hastings, M.H., Maywood, E.S. (2001). Cycle of *period* gene expression in a diurnal mammals (*Spermophilus tridecemlineatus*): implications for nonphotic phase shifting. *J. Biol. Rhythms* 16:471–478.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109:307–320.

Pittendrigh, C.S. (1981). Circadian systems: Entrainment. In: Ashoff, J., ed. *Handbook of Behavioral Neurobiology*. New York: Plenum, pp. 95–124.

Ponka, P. (1997). Specific regulation of iron metabolism and heme synthesis: distinct tissue-control mechanisms in erythroid cells. *Blood* 89:1–25.

Rea, M.A. (1998). Photic entrainment of circadian rhythms in rodents. *Chronobiol. Int.* 15:395–423.

Reick, M., Garcia, J.A., Dudley, C., McKnight, S.L. (2001). NPAS2: an analog of clock operative in the mammalian forebrain. *Science* 293:506–509.

Reppert, S.M., Weaver, D.R. (2001). Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.* 63:647–676.

Rubio, M.F., Agostino, P.V., Ferreyra, G.A., Golombek, D.A. (2003). Circadian heme oxygenase actitivy in the hamster suprachiasmatic nuclei. *Neurosci. Lett.* 353:9–12.

Rutter, J., Reick, M., Wu, L.C., McKnight, S.L. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* 293:510–514.

Sacca, G.B., Saenz, D.A., Jaliffa, C.O., Minces, L., Keller Sarmiento, M.I., Rosenstein, R.E. (2003). Photic regulation of heme oxygenase activity in the golden hamster retina: involvement of dopamine. *J. Neurochem.* 85:534–542.

Schibler, U., Sassone-Corsi, P. (2002). A web of circadian pacemakers. *Cell* 111:919–922.

Tacchini, L., Fusar-Poli, D., Bernelli-Zazzera, A. (2002). Activation of transcription factors by drugs inducing oxidative stress in rat liver. *Biochem. Pharmacol.* 3:139–148.

Takahashi, M., Dore, S., Ferris, C.D., Tomita, T., Sawa, A., Wolosker, H., Borchelt, D.R., Iwatsubo, T., Kim, S.H., Thinakaran, G., Sisodia, S.S., Snyder, S.H. (2000). Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron.* 28:461–473.

Taylor, B.L., Zhulin, I.B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* 63:479–506.

Tosini, G., Menaker, M. (1996). Circadian rhythms in cultured mammalian retina. *Science* 272: 419–421.

Toutiou, Y., Portaluppi, F., Smolensky, M.H., Rensing, L. (2004). Ethical principles and standards for the conduct of human and animal biological rhythm research. *Chronobiol. Int.* 21:161–170.

Turton, N.J., Judah, D.J., Riley, J., Davies, R., Lipson, D., Styles, J.A., Smith, A.G., Gant, T.W. (2001). Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance. *Oncogene* 20:1300–1306.

Vincent, S.R., Das, S., Maines, M.D. (1994). Brain heme oxygenase isoenzymes and nitric oxide synthase are co-localized in select neurons. *Neurosci.* 63:223–231.

Wakao, H., Chida, D., Damen, J.E., Krystal, G., Miyajima, A. (1997). A possible involvement of Stat5 in erythropoietin-induced hemoglobin synthesis. *Biochem. Biophys. Res. Commun.* 234:198–205.

Wisser, H., Breuer, H. (1981). Circadian changes of clinical chemical and endocrinological parameters. *J. Clin. Chem. Clin. Biochem.* 19:323–337.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, C.C., Lee, A. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 105:683–694.