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Molecular Brain Research 122 (2004) 158–166
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

Further evidence for the role of cryptochromes in retinohypothalamic photoreception/phototransduction

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Accepted 8 December 2003

Abstract

Cryptochrome is a blue-light absorbing photopigment that has been proposed to act as a photoreceptor for a variety of nonvisual light-responsive tasks. While mouse models have suggested an important role for cryptochrome in nonvisual photoreception, there are no biochemical data demonstrating the functional photoreceptive capability of cryptochrome in mice. There are two models that describe the effect of cryptochrome on light responsive events: (1) cryptochrome is a photoreceptor or (2) cryptochrome is required for either normal phototransduction from the retina to the brain or for normal transcriptional regulation in the brain, irrespective of light. To differentiate between these two models, we have examined the integrity of the regulatory mechanism of *c-fos* in cryptochromeless cell lines and in the suprachiasmatic nucleus (SCN) of cryptochromeless mice. Photoinduction of *c-fos* mRNA in the SCN can be used as a marker for circadian photoreception/phototransduction and it is drastically reduced in mice lacking cryptochromes. Our results indicate that light-independent transcription regulatory system of *c-fos* is normal in cryptochromeless mice and that the reduced *c-fos* light responsiveness in the absence of cryptochromes is due to a loss of photoreceptor function.

Theme: Neural basis of behavior
Topic: Biological rhythms and sleep

Keywords: Suprachiasmatic nucleus; Retinal degeneration; *c-fos*; Circadian

1. Introduction

The mammalian retina mediates several nonvisual light-responsive behaviors, including circadian photoreception, pupillary light responsiveness and acute suppression of locomotor activity (masking). Candidate photoreceptors for these behaviors include opsins, such as classical rod and cone opsins as well as the novel photopigment melanopsin [21], and the blue-light photoreceptive pigments, cryptochromes [15]. The *rd* allele in mice results in severe retinal degeneration, with a primary loss of the rod photoreceptors followed by secondary loss of cone photoreceptors [4]. While these *rd*/*rd* mice lack the photopigments required for vision, they retain robust nonvisual responses to light [3,6,22,36], indicating the involvement of other nonclassical photoreceptors.

Analyses of nonclassical photoreceptor mutants have demonstrated that neither melanopsin nor cryptochromes are absolutely required for nonvisual irradiance detection tasks. Melanopsin knockout mice (*Opn4* −/−) exhibited somewhat reduced sensitivity for circadian phase-shifting [19,23] and masking [19], but displayed normal entrainment [19,23], nearly normal pupillary light reflex [14,20] and gene induction by light in the neural center for circadian rhythms, the suprachiasmatic nucleus (SCN) [23]. Mice lacking cryptochromes (*mCry1* −/− *mCry2* −/−) displayed a severe defect in gene induction in the SCN [24,34], but retained normal pupillary light reflex [33] and masking [17]. Because cryptochromes also have a transcriptional regulatory function in the molecular clock mechanism (reviewed in Ref. [28]), cryptochromeless mice could not be assayed for circadian phase-shifting.

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doi:10.1016/j.molbrainres.2003.12.006

These findings suggested that more than one type of photoreceptor was involved in these nonvisual photoreceptive tasks. Indeed, both *rd/rd Opn4 −/−* mice and *rd/rd mCry1 −/− mCry2 −/−* mice displayed severe defects in all photoreceptive tasks tested [9,20,24,33]. While this may be indicative of primary photoreceptive functions for both melanopsin and cryptochromes, there are alternative explanations for the effect of cryptochrome mutation on photoreception. First, cryptochromes may be photoreceptors that require melanopsin as part of their signaling pathway. Second, melanopsin could be the primary photoreceptor and the effect of cryptochrome could be due to its transcriptional regulatory function.

At present, the signaling pathways of melanopsin and cryptochrome in the retina are unknown. The striking reduction in the inducibility of immediate–early genes in the SCN by light in cryptochromeless mice is consistent with either model. Although melanopsin protein expression and retinohypothalamic tract architecture are normal in *rd/rd mCry1 −/− mCry2 −/−* mice [32], it is possible that an effect of cryptochrome mutation on circadian clock function or other signaling pathways may explain the reduced photosensitivity of these mice. To clarify whether the loss of light-responsive gene expression in these mice is merely a result of a defect in the molecular machinery underlying circadian rhythm generation or whether it is due to the loss of a functional photoreceptor, we characterized the mechanistic aspects of light-induced gene expression in cryptochromeless mice.

2. Materials and methods

2.1. Mice

The *mCry1 −/− mCry2 −/−* mice, *rd/rd mCry1 −/− mCry2 −/−* mice, and wild-type mice of similar mixed background were generated as described previously [24,34] and maintained on an LD 12/12-h schedule under ambient room lighting.

2.2. Fibroblast cell lines

Wild-type (B6) and *mCry1 −/− mCry2 −/−* mice were anesthetized with sodium pentobarbitol and a small section of skin was removed for culture. The tissue was trypsinized and plated in DMEM with 10% Colorado calf serum and grown at 37 °C with 5% CO_2. After growth was observed, cells were trypsinized and replated until a viable culture was established.

2.3. c-fos mRNA induction in fibroblast cell lines

Fibroblasts were grown to confluence on 6-cm^2 dishes in DMEM with 10% fetal bovine serum (FBS) and were then maintained in serum-free medium for 24 h prior to induction. The serum-free medium was either replaced with fresh DMEM supplemented with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) or 20% FBS or 10 μM forskolin to induce *c-fos* expression. Samples were harvested at the indicated time points directly in TRIzol reagent (Gibco/BRL) and the *c-fos* and *gapdh* mRNA levels were quantified from 6 μg total RNA using an RNase protection assay kit according to the manufacturer's recommendations (Roche). The ^{32}P-labeled riboprobes were directed against *c-fos* nucleotides 919–1151 (pBluescript) and *gapdh* nucleotides 345–660 (pTRI). Quantification of the protected fragments was done using a phosphorimager (Molecular Dynamics) with ImageQuant5.0 software (Molecular Dynamics) and statistical analysis of significance was done by Student's *t*-test. All *c-fos* mRNA levels are shown as fold induction relative to the untreated control, normalized to the *gapdh* present in each sample.

2.4. Light induction of c-fos in the SCN

For the age correlation experiment, mice were exposed to a broad-spectrum fluorescent light at a rate of 43.9 $\mu mol \cdot m^{-2} \cdot s^{-2}$ for 30 min (total irradiance 7.9×10^4 $\mu mol \cdot m^{-2}$) at ZT18 and were sacrificed at 30–45 min after the start of irradiation. For the kinetics of induction experiment, mice were exposed to the same light intensity, but were sacrificed at 0, 5, 15, 30, 45, 60 and 120 min after the start of irradiation. For circadian gating of *c-fos* induction in wild-type and *mCry1 −/− mCry2 −/−* mice after 32 and 44 h in constant darkness, mice were exposed to 43.9 $\mu mol \cdot m^{-2} \cdot s^{-2}$ for 30 min (total irradiance 7.9×10^4 $\mu mol \cdot m^{-2}$). For the induction of *c-fos* over time in darkness to examine circadian gating, after an initial 12-h light/12-h dark regimen, mice entered constant darkness at ZT12. At every 4 h for 44 h, mice were induced with 5 min of light (14 $\mu mol \cdot m^{-2} \cdot s^{-2}$) and sacrificed 25 min later. Brains were dissected under yellow light.

2.5. In situ hybridization

Coronal sections of frozen brain were made (18 μm thick) and sections were fixed in formalin and hybridized with ^{35}S-labelled *c-fos* and *mPer3* riboprobes antisense to nucleotides 855–1627 and 480–824, respectively. The ^{35}S-labeled probe was prepared by in vitro transcription of linearized pBluescript SK+ plasmids containing these gene fragments with T7 or T3 RNA Polymerase (Promega). Fixation and hybridization of the slides and autoradiography were performed as described [15]. A Leica M420 macroscope was density-calibrated with a Kodak Control Scale T-14 and was used to capture SCN images with an Optronics DE 1750 camera. Quantitation was performed with Scion Image 3.0a (Scion version of NIH Image). The nonspecific background signal in brain was subtracted from the signal for each SCN section to normalize for differences in hybridization quality in individual slides. In addition, mice used as dark, unin-

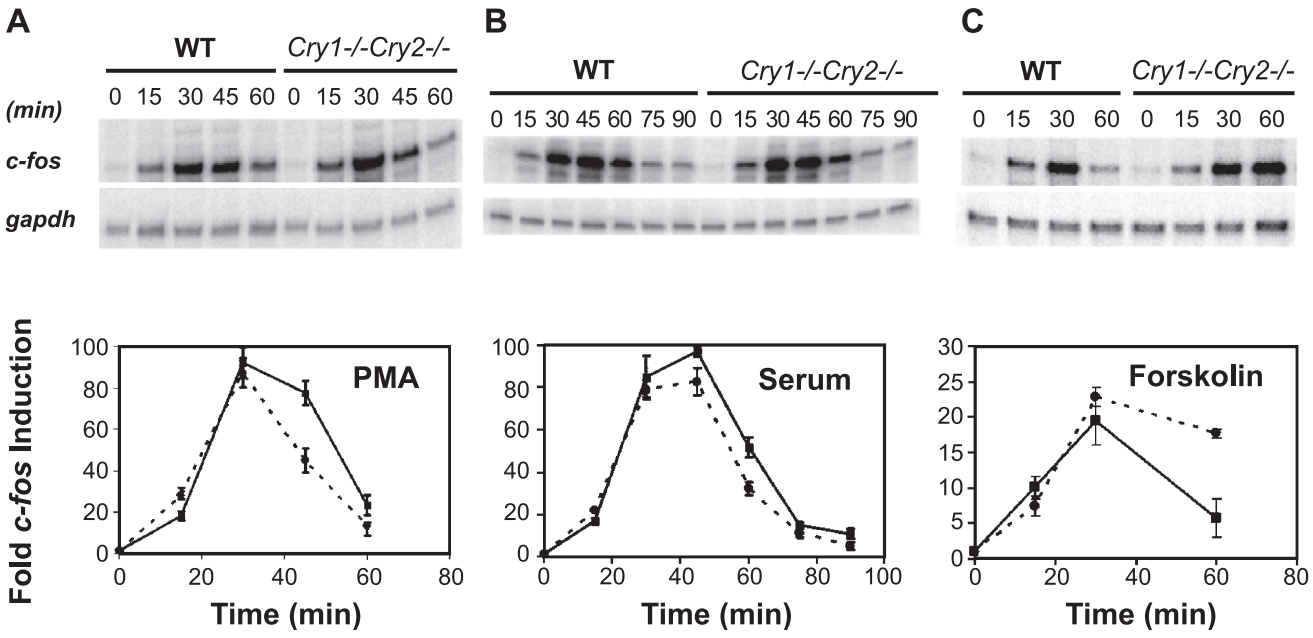

Fig. 1. Induction of *c-fos* by chemical agents in primary fibroblasts. Wild-type (solid lines) and *mCry1 −/− mCry2 −/−* (dashed lines) fibroblasts derived from mouse skin were induced with 100 ng/ml PMA (A), 20% fetal bovine serum (B) or 10 μM forskolin (C). *c-fos* mRNA was analyzed at indicated times by RNase protection. Data were normalized to levels of *gapdh* mRNA. Points indicate means of three (A, C) or four (B) experiments and error bars represent S.E.M. No significant difference was found between wild-type and knockout cell lines at any time point or condition as determined by paired, two-tailed Student's *t*-test.

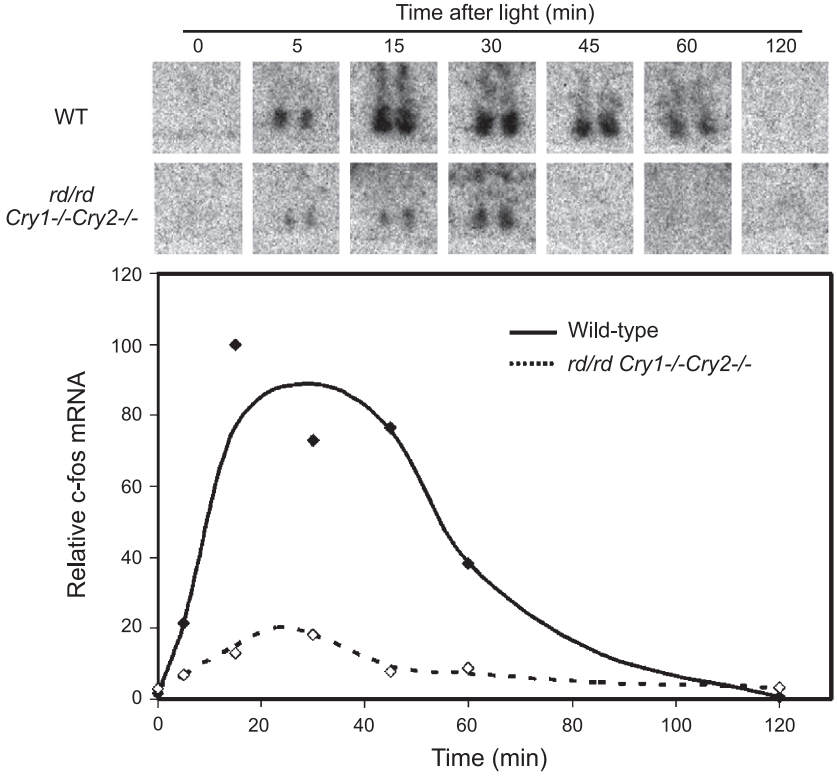

Fig. 2. Kinetics of *c-fos* induction by light in mouse SCN. At ZT18, mice were exposed to 43.9 μmol·m^{-2}·s^{-2} photons of light and were sacrificed at 0, 5, 15, 30, 45, 60 and 120 min after the beginning of light, and *c-fos* mRNA was examined by in situ hybridization. Representative photomicrographs of the region of peak *c-fos* induction in the SCN for each time point are shown (top) as well as a graphical representation (bottom). Induction levels were normalized to peak induction in wild-type (100%). Each point represents a single mouse. WT, wild-type; Rd12, *rd/rd mCry1 −/− mCry2 −/−*.

duced controls were quantitated for basal levels of *c-fos* and averaged for each genotype. Values were normalized to wild-type gene induction (100%). Statistical analyses included the use of the Student's *t*-test, two-tailed, both paired and unpaired. For investigation of circadian gating of *c-fos* induction in wild-type and *rd/rd Cry1 −/− Cry2 −/−*, both single-factor and two-factor ANOVA were performed.

3. Results

3.1. Normal c-fos inducibility in cryptochromeless fibroblasts

In *mCry1 −/− mCry2 −/−* and in *rd/rd mCry1 −/− mCry2 −/−* mice, light induction of *c-fos* in the SCN is drastically reduced [24]. The light-inducible genes *mPer1*, *mPer2* and *c-fos* are presumably activated through a synergistic action of the MAP kinase cascade and cAMP pathways by light in the SCN [5,16,30]. It is possible that in cryptochromeless mice activation of one or both of these pathways and consequent *c-fos* induction by any stimulus may be impaired, resulting in reduction of light-responsiveness in these mice. Therefore, we first examined whether these different signaling pathways for *c-fos* induction were operational in the absence of cryptochromes.

The *c-fos* promoter contains at least four response elements, including the serum response element (SRE) and the cAMP response element (CRE). A single stimulus can activate multiple signaling pathways, either PKC-dependent or -independent, which converge upon the *c-fos* promoter and result in induction of *c-fos* through multiple promoter elements (reviewed in Ref. [10]). In the SCN, the CRE is believed to be the prime candidate for light regulation of *c-fos* transcription [26] although the SRE has been implicated as well [2]. We generated primary fibroblast cell lines from wild-type and *mCry1 −/− mCry2 −/−* mice to use as a model system for the integrity of transcriptional regulation of *c-fos* in cryptochromeless mice. These signaling pathways are conserved between SCN neurons and

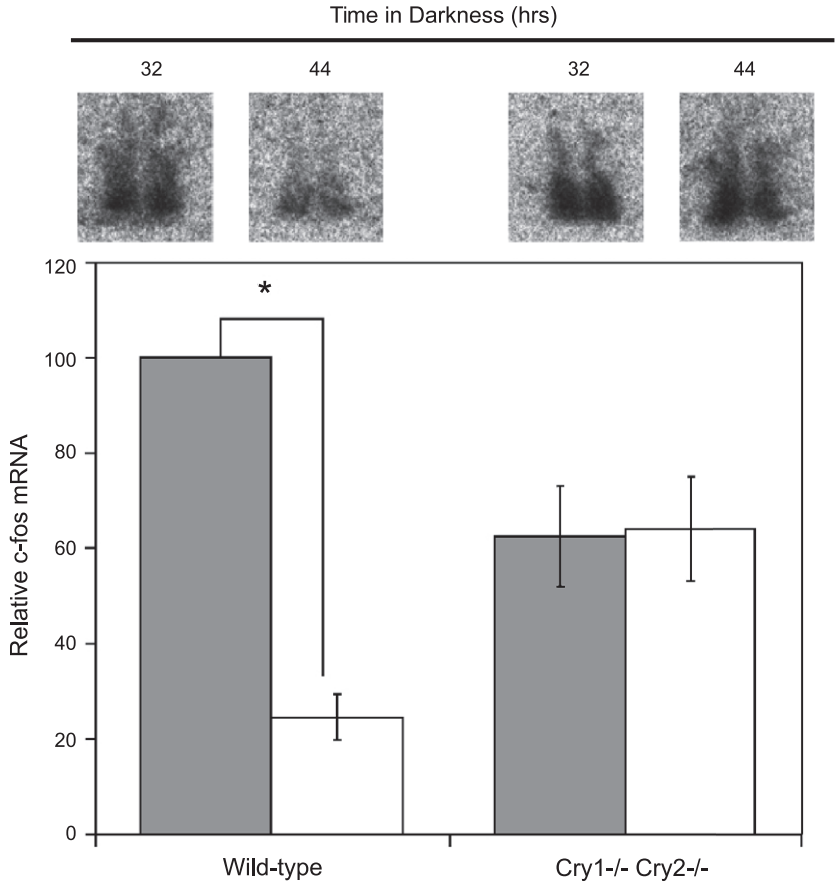

Fig. 3. Lack of circadian gating of *c-fos* induction by light in *mCry1 −/− mCry2 −/−* mice. Wild-type and *mCry1 −/− mCry2 −/−* mice entered constant darkness at ZT12. Mice were induced with light (43.9 μmol·m^{-2}·s^{-2} photons) at 32 or 44 h after entry into darkness and sacrificed at 30 min following the start of irradiation. *c-fos* mRNA in the SCN was measured by in situ hybridization and levels of induction were normalized to wild-type induction at 32 h. Representative photomicrographs of peak *c-fos* signal in the SCN (top) and graphical representation (bottom) are shown. Gray bars represent subjective night (32 h) and white bars represent subjective day (44 h). Data represent means from three experiments ($n=3$) and error bars represent S.E.M. Asterisk denotes significance, $p<0.01$, Student's paired *t*-test.

fibroblast cells [35], and hence analysis of transcriptional regulation in fibroblasts should be applicable to SCN neurons.

We examined induction of *c-fos* transcript by three different stimuli in wild-type and *mCry1 −/− mCry2 −/−* fibroblast cell lines. Serum can induce *c-fos* through the SRE promoter element through both PKC-dependent and -independent pathways, but does not activate PKA pathways, and PMA can induce *c-fos* expression through both the SRE and CRE elements through both PKC-dependent and −independent pathways [10]. When either wild-type or *mCry1 −/− mCry2 −/−* cells were exposed to 20% serum, induction of *c-fos* mRNA was robust and peaked at 30–45 min with similar induction kinetics, as shown in Fig. 1 by RNase protection assay. Similarly, PMA induced *c-fos* in both cell lines with a peak at 30 min. Forskolin, an adenylate cyclase activator, activates the CRE element in the *c-fos* promoter through the cAMP pathway, which is most representative of the events occurring within the SCN to initiate immediate–early gene induction. Treatment with forskolin induced *c-fos* with normal induction kinetics and amplitude in cryptochromeless fibroblasts (Fig. 1C). Thus we concluded that the signaling pathways upstream of *c-fos* and the basal *c-fos* transcription machinery are intact in the absence of cryptochromes.

3.2. Lack of effect of cryptochrome defect on kinetics and gating of c-fos induction in the SCN

We previously reported that *c-fos* induction in the SCN was severely reduced in *rd/rd mCry1 −/− mCry2 −/−* mice [24]. However, those data were obtained at a single time point after the pulse. It was conceivable that in these animals the induction kinetics were altered. To address this issue, we examined the kinetics of *c-fos* induction by light in the mouse SCN. We found that although induction of *c-fos* was greatly reduced in the triple mutant mouse, the kinetics of induction in wild-type and *rd/rd mCry1 −/− mCry2 −/−* mice were similar, with peak signal at 15–30 min after initiation of the light exposure (Fig. 2), indicating that the loss of sensitivity to photoinduction is real and not an artifact of different induction kinetics in the mutant.

Next we examined whether there might be an effect of circadian clock disruption on the timing of *c-fos* induction. The light sensitivity of gene induction in the SCN is gated by circadian time, with maximal induction occurring during subjective night (and early subjective morning) and very little induction occurring during subjective day [8]. We first examined circadian gating in wild-type and in *mCry1 −/− mCry2 −/−* mice by comparison of *c-fos* induction achieved by a light pulse after either 32 h in darkness (subjective night) or 44 h in darkness (subjective day). We found that although wild-type mice showed clear gating of *c-fos* inducibility ($p < 0.005$; Student's *t*-test), the *mCry1 −/− mCry2 −/−* mice had equivalent levels of inducibility at either time of day (Fig. 3). Thus, even though gating is lost in the absence of cryptochromes, the *mCry1 −/− mCry2 −/−* mice still exhibited significant and robust induction at high irradiance (60% of wild-type), indicating that the gate is not "shut" in the absence of cryptochromes. We previously reported that lack of cryptochromes reduces photosensitivity of *c-fos* induction 10–20-fold in animals with rods and cones and more than 3000-fold in rodless coneless (*rd*) mice, with respect to the light dose required to reach half-

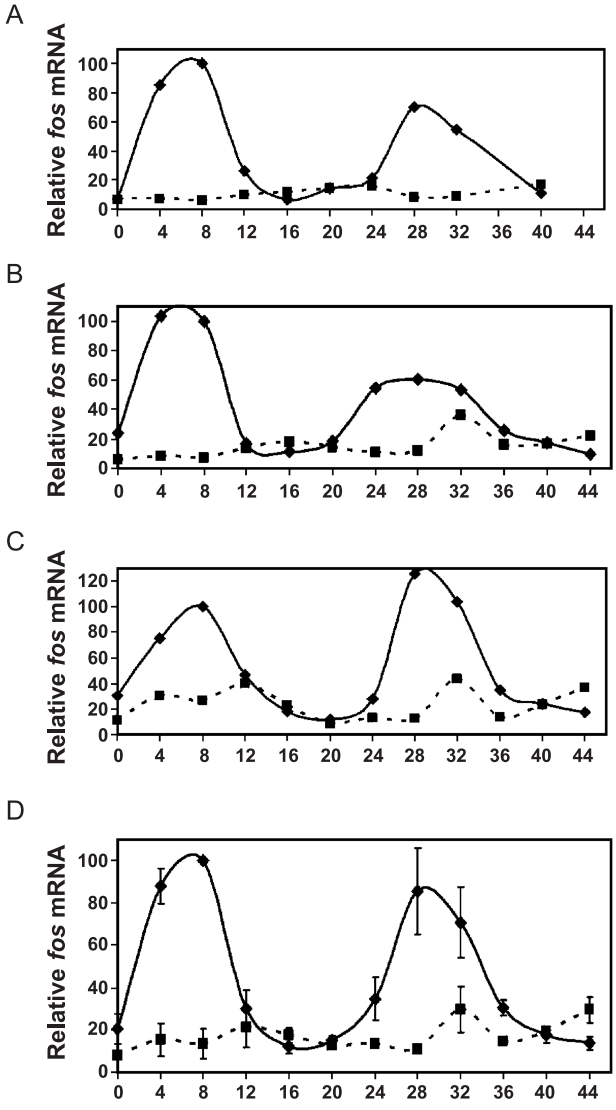

Fig. 4. Circadian gating of *c-fos* induction by light in mouse SCN. Wild-type (solid lines) and *rd/rd mCry1 −/− mCry2 −/−* (dashed lines) mice were placed into constant darkness at ZT12, exposed to 4200 μmol/m^2 light and sacrificed every 4 h. Each of three individual experiments is shown (A–C) where each point represents a single mouse, and the average of all experiments is shown as mean ± S.E.M. ($n = 3$) (D). Values for *c-fos* mRNA were normalized within each experiment to the value for *c-fos* induction at 8 h in darkness. Although there was an apparent peak for induction at 32 h in two out of three trials, this peak was not statistically different from other time points when all three trials were averaged (Student's paired *t*-test, $p > 0.15$) and did not appear to be circadian in nature.

maximal response [24]. Therefore, we performed an in-depth study of circadian gating in these mice to determine if there is any circadian time at which *c-fos* inducibility is enhanced. Wild-type and *rd/rd mCry1 −/− mCry2 −/−* mice were maintained on a 12-h light/12-h dark cycle under ambient room lighting and then were released into constant darkness for 44 h. At 4-h intervals, mice were exposed to 30 min of broad-spectrum cool white fluorescent light, which achieves maximal *c-fos* induction in *rd* mice and sacrificed at the end of the light exposure. Induction of *c-fos* mRNA was examined in the SCN by in situ hybridization.

In three separate trials, wild-type mice exhibited clear gating of *c-fos* inducibility, with maximal induction at 4, 8, 28 and 32 h in constant darkness corresponding to subjective night, which was significantly different from trough levels of induction (Student's paired *t*-test, $p < 0.05$; Fig. 4). In *rd/rd mCry1 −/− mCry2 −/−* mice, there was no consistent peak for induction, which remained extremely low. Wild-type and *rd/rd mCry1 −/− mCry2 −/−* mice exhibited significantly different responses overall (two-factor ANOVA without replication, $p < 0.02$). Single factor ANOVA indicated that wild-type mice had significant changes in response to light over different circadian times ($p < 0.001$) and that *rd/rd mCry1 −/− mCry2 −/−* mice did not ($p = 0.30$). Therefore, while we cannot rule out the possibility that some slight regulation of *c-fos* inducibility may remain in *rd/rd mCry1 −/− mCry2 −/−* mice, any such moderation of gating in this genotype does not substantially alter *c-fos* photoinducibility.

3.3. Lack of residual clock function in cryptochromeless mice

Although we could not detect gating under constant darkness in either *mCry1 −/− mCry2 −/−* or *rd/rd mCry1 −/− mCry2 −/−* mice, we sought to determine whether partial circadian clock function might exist in the absence of cryptochromes under a light/dark regimen. While mice lacking cryptochromes are arrhythmic when maintained in constant darkness, they exhibit apparently normal rhythmicity of locomotor activity, as well as oscillating *Per2* gene expression, under light/dark conditions [31,34]. This phenotype suggests that cryptochromes are absolutely required for genuine circadian rhythmicity, i.e., maintenance of rhythm in the absence of environmental cues, and the rhythmic behavior of these mice under light/dark conditions appears to be merely "masking" or an acute suppression of activity in response to light. However, an alternate explanation exists: the loss of gating would allow acute induction of *c-fos* and m*Per2* (which remains inducible in *mCry1 −/− mCry2 −/−* and *rd/rd mCry1 −/− mCry2 −/−* mice [29,34]) throughout the light period, and therefore it is conceivable that acute induction of these genes under a light/dark regimen may generate a molecular oscillation sufficient to "drive" the circadian clock machinery for the

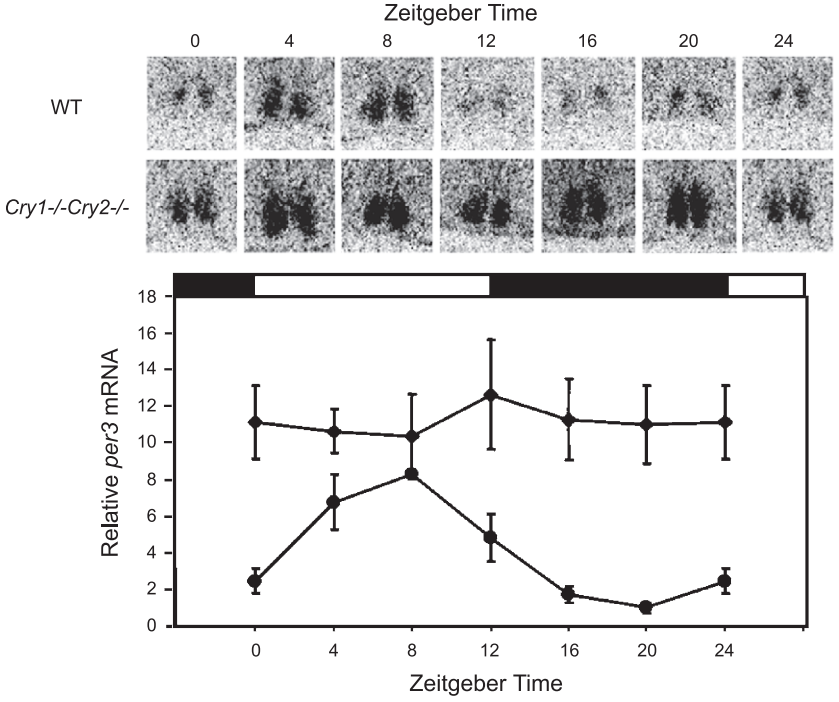

Fig. 5. *mPer3* gene oscillation in response to light/dark cycles. Representative SCN images of *mPer3* expression are shown (top) every 4 h from ZT0 to ZT20. Quantitation is shown (bottom) as mean ± S.E.M. ($n = 3$). Open markers, *mCry1 −/− mCry2 −/−*; solid markers, wild-type. The horizontal bar indicates lighting: white, light; black, dark.

span of 1 day and generate a behavioral rhythm, despite the lack of maintainable rhythm in darkness. Therefore, we examined whether a clock-controlled gene, *mPer3*, which oscillates with circadian rhythmicity and is not light-inducible in wild-type mice [27], was expressed in a circadian manner under a light/dark photoperiod in cryptochromeless mice.

Wild-type mice exhibited robust *mPer3* oscillation under the light/dark cycle, with peak and trough expression at ZT8 and ZT20, respectively (Fig. 5). However, *mCry1 −/− mCry2 −/−* mice lacked any detectable variation in *mPer3* levels over time, and *mPer3* levels were greatly elevated in the mutant mice, consistent with the negative transcriptional activity of cryptochromes on *Period* genes [12,34]. These data indicated that modest *c-fos* and/or *mPer2* induction is not sufficient to "drive" the molecular machinery and affect expression of other clock-controlled genes. Indeed, in *mCry1 −/− mCry2 −/−* mice *Per2* protein levels are reduced despite the elevated level of *Per2* transcription [13].

3.4. Photoreceptor for residual photoresponses in rd/rd mCry1−/− mCry2−/− mice

Lastly, we sought to determine which photoreceptor was responsible for the residual *c-fos* induction at high doses in *rd/rd mCry1 −/− mCry2 −/−* mice. In the absence of rods, cones and cryptochromes, the only known remaining candidate photoreceptive pigment for this response is melanopsin. However, with the *rd* phenotype, there is also the possibility that residual cones remain. In the *rd* animal, rods are destroyed by 3 weeks of age but some cone nuclei are present in young mice and continue to degenerate throughout the lifetime of the animal [4]. It is important to note that despite the presence of cone nuclei in *rd* mice, no outer segments are detectable and there is no evidence that these nuclei exhibit any photoreceptor function in *rd/rd* mice [4,7]. We measured *c-fos* induction by light in *rd/rd mCry1 −/− mCry2 −/−* mice of varying ages and found that *c-fos* induction could not be correlated to the age of the animal nor to the previously reported degeneration rate of cone nuclei as determined by light microscopic analysis (Fig. 6) [4] suggesting that melanopsin, and not residual cones, is likely to be responsible for the residual gene photoinduction observed in these mice. In comparison, the *rd/rd* mice used to breed the triple mutants exhibit induction at levels close to wild-type even at 6–8 months [24], an age by which some loss of circadian photosensitivity has been observed [3,36], although the decline in sensitivity appears to be dependent upon genetic background [37].

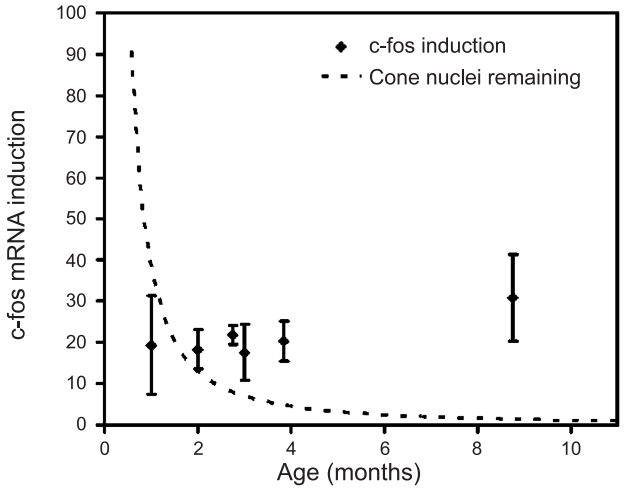

Fig. 6. Lack of effect of age on *c-fos* induction in SCN of *rd/rd mCry1 −/− mCry2 −/−* mice. Data points represent mean *c-fos* induction by light ($n=2–3$). Induction was normalized to *c-fos* induction in wild-type mice (100%). The dotted line represents remaining cone nuclei detectable at different ages in the *rd/rd* mouse as described in Ref. [4], and they are plotted as a percentage of cones detectable in wild-type. Vertical bars represent S.E.M. of *c-fos* induction. Points shown are averages of animals at indicated ages except the oldest point, which represents average induction in animals between 6.75 and 10.75 months of age.

4. Discussion

We have investigated the effect of the loss of cryptochromes on the mechanistic integrity of light-mediated *c-fos* induction, which is used as a marker for phototransduction to the SCN. We found that the various intracellular mechanisms mediating *c-fos* induction are preserved in cryptochromeless mice. We also determined that kinetics of *c-fos* induction is normal in these animals, although the induction level is severely compromised. Therefore, we conclude that the impaired photoresponse in these mice is not due to defects in intracellular signaling or in transcription regulation.

We also present evidence that reduced circadian photosensitivity of cryptochromeless mice is not due to an elevated gate of inducibility caused by the absence of cryptochrome. We show that in *rd/rd mCry1 −/− mCry2 −/−* mice "gating" is lost and *c-fos* induction is severely impaired at all circadian times. The mechanism of gating of *c-fos* induction is not known, and therefore, loss of gating can have two different interpretations regarding the light responsiveness of these mice. First, it may imply the "gate" has been compromised in arrhythmic animals in such a way that it is always "closed", and thus the reduction in gene responsiveness in the absence of cryptochromes would be at the level of transcriptional machinery and not necessarily due to a loss of a photoreceptive pigment. However, we consider this model unlikely because in *Clock/Clock* mutant mice, which lose rhythmicity in constant darkness, *c-fos* inducibility by light is essentially normal [25]. In contrast, the photosensitivity for *c-fos* induction in *mCry1 −/− mCry2 −/−* mice is reduced by 10–20-fold compared to wild-type [24], and this reduction becomes more pronounced in the *rd/rd mCry1 −/− mCry2 −/−* background, resulting in more than 3000-fold reduction in sensitivity compared to *rd/rd* alone [24], suggesting that loss of rhythmicity and gating cannot explain the extent of impairment of *c-fos* induction in

the absence of cryptochromes. The second and more likely interpretation is that loss of gating reflects removal of a transcriptional inhibitory mechanism by the core molecular components, and gene photoinducibility is at the maximal level possible at all circadian timepoints in mice lacking cryptochromes. Therefore, the drastic reduction in *c-fos* induction in the SCN by light in these animals is most likely due to reduced photoreception caused by the ablation of the cryptochrome photopigment.

Although gating and circadian clock function were clearly lost in constant darkness in cryptochromeless mice, it remained possible that some partial circadian clock function might be driven by light/dark cycles, resulting in gating under light/dark cycles. Although we could not test directly for gating under these conditions, we sought to determine whether cryptochrome-deficient mice, which maintain rhythmic locomotor activity under light/dark cycles, may undergo daily re-entrainment of the molecular clock mechanism by light, driving the clock through the duration of one circadian cycle [1,34]. Our data demonstrate that rhythmic expression of *mPer2* in these mice under LD [34] does not translate into rhythmic oscillation of another clock-controlled gene, *mPer3*, consistent with a previous report that *mPer3* oscillation is lost in another arrhythmic mouse model, *Clock/Clock* mice [11]. However, anticipatory wheel-running activity, where mice exhibit increased locomotor activity in the hours just prior to lights off, has been reported in cryptochrome-deficient mice [17,34], and therefore the existence of a partially functioning clock, though unlikely, cannot be completely ruled out. Indeed, recently some evidence for partial clock function in the absence of cryptochromes was reported [18]. The issue of whether a rudimentary circadian clock remains in the absence of cryptochromes has yet to be resolved.

Mouse knockout studies have provided intriguing data regarding nonvisual photoreception. *Rd* mice that lack functional rods and cones exhibit increased sensitivity to photoinduction of *c-fos* in the SCN. The elimination of cryptochromes in *rd/rd* mice results in more than 3000-fold decrease in the sensitivity of *c-fos* induction in the SCN [24] and 20-fold reduction in pupillary response, compared to *rd/rd* alone [33]. Finally, ablation of melanopsin, which only moderately affects non-visual photoresponses, when combined with the *rd* mutation virtually abolishes all visual and non-visual photoresponses [9,20]. It is premature to fit all these data into a coherent mechanistic model at present, but one must conclude that none of the photopigments studied in mammals thus far, including rods, cones, melanopsin and cryptochrome, is in and of itself essential for circadian photoreception, and that the circadian photoreceptive system appears to be composed of multiple, redundant, signaling pathways. However, as more biochemical data become available, the interplay between the three types of photoreceptors, classical opsins, cryptochromes and melanopsin, may be more amenable to analysis at the molecular level.

Acknowledgements

We would like to thank Cheng Chi Lee for the plasmid containing the *mPer3* sequence used for in situ hybridization. CPS was responsible for providing the mice used in this work.

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