A strong stimulus adjusting the circadian clock to the prevailing light-dark cycle is light. However, the circadian clock is reset by light only at specific times of the day. The mechanisms mediating such gating of light input to the CNS are not well understood. There is evidence that Ca\(^{2+}\) ions play an important role in intracellular signaling mechanisms, including signaling cascades stimulated by light. Therefore, Ca\(^{2+}\) is hypothesized to play a role in the light-mediated resetting of the circadian clock. Calbindin-D28k (CB; gene symbol: \textit{Calb1}) is a Ca\(^{2+}\) binding protein implicated in Ca\(^{2+}\) homeostasis and sensing. The absence of this protein influences Ca\(^{2+}\) buffering capacity of a cell, alters spatio-temporal aspects of intracellular Ca\(^{2+}\) signaling, and hence might alter transmission of light information to the circadian clock in neurons of the suprachiasmatic nuclei (SCN). We tested mice lacking a functional \textit{Calb1} gene (\textit{Calb1}\(^{-/-}\)) and found an increased phase-delay response to light applied at circadian time (CT) 14 in these animals. This is accompanied by elevated induction of \textit{Per2} gene expression in the SCN. Period length and circadian rhythmicity were comparable between \textit{Calb1}\(^{-/-}\) and wild-type animals. Our findings indicate an involvement of CB in the signaling pathway that modulates the behavioral and molecular response to light. (Author correspondence: urs.albrecht@unifr.ch)

**Keywords** Circadian rhythm, Entrainment, Signaling, SCN, CalB, Calb1, Light, Clock genes

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INTRODUCTION

The mammalian circadian clock influences a multitude of physiological processes such as cardiovascular activity, sleep/wakefulness, metabolism, and brain function. An optimal timing of diverse biochemical processes will not only separate incompatible chemical reactions from one other but also allow time-specific activation of enzymatic activities responsible for energy production. Therefore, it is evident that a correctly synchronized circadian clock that is in tune with the environment brings about advantages for an individual to optimally perform in a competitive world (Roenneberg & Merrow, 2003).

Correct synchronization of the circadian clock is mainly mediated by light. Light information is transmitted by the retina via the retinohypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN) harboring the main clock in the brain (Dardente & Cermakian, 2007; Moore, 1996), and thereby the SCN clock is synchronized to environmental time. Light administered in the dark phase is thought to elicit glutamate release in the synapses of the RHT, because glutamate can mimic the effects of light. Glutamate activates ionotropic glutamate receptors in the SCN, which trigger influx of Ca\(^{2+}\) (Ding et al., 1994, 1997; Schurov et al., 1999). This leads to the activation of several signal transduction pathways (reviewed in Hirota & Fukada, 2004) and evokes modification of chromatin (Crosio et al., 2000) and clock proteins (Myers et al., 1996) plus the activation of immediate early genes, such as c-Fos (Kornhauser et al., 1990) and the clock genes Per1 and Per2 (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al. 1997; Yan & Silver, 2002). As a consequence, phase advances or delays in locomotor activity can be observed (Akiyama et al., 1999; Albrecht et al., 2001; Tischkau et al., 2003; Wakamatsu et al., 2001). These input signals to the clock adapt its phase and lead to synchronization of the organism to the environment.

In Syrian hamsters (Mesocricetus auratus), a retinorecipient SCN subregion contains calbindin D-28k (CB)-expressing cells, and the protein appears to influence responses to photic cues as indicated by Calb1 antisense oligonucleotides (Hamada et al., 2003). CB is a high-affinity calcium-binding protein, which is implicated in the regulation of Ca\(^{2+}\) homeostasis by acting as a cytosolic Ca\(^{2+}\) buffer (Schwaller, 2009; Schwaller et al., 2002). Recent studies suggest CB might also act as a Ca\(^{2+}\) sensor (Berggard et al., 2002a; Schmidt et al., 2005). Interestingly, aspects of locomotor activity are influenced by this gene in the mouse (Farre-Castany et al., 2007), and, therefore, we started to investigate the role of CB in circadian behavior using mice lacking a functional Calb1 gene (Airaksinen et al., 1997). We find that Calb1\(^{-/-}\) mice of a C57/B16J background display normal circadian wheel-running activity and period length compared to wild-type littermates. Interestingly, Calb1\(^{-/-}\) mice
show larger phase delays in response to a light pulse applied at circadian time (CT) 14, accompanied by a slight increase in Per2 gene induction. Our studies indicate an important function of Calb1 in the resetting mechanism of the circadian clock.

METHODS

Animals

The Calb1−/− mice (systematic name: Calb1tm1Mpin ) were produced from R1 stem cells (Airaksinen et al., 1997) and backcrossed to C57Bl6/J (B6) for eight generations, and are thus considered congenic with B6, B6Calb1−/−, and B6Calb1+/+. Animals used in this study were littermates derived from heterozygous B6Calb1+/− matings. All animals, including wild-type ones, were genotyped by PCR (see genotyping section below) as described previously (Vecellio et al., 2000).

Animals were held under a 12:12 h light-dark cycle (LD), with food and water ad libitum, in transparent plastic cages (267 mm long × 207 mm wide × 140 mm high; Techniplast Makrolon type 2 1264C001) with a stainless steel wire lid (Techniplast 1264C116). Three- to six-month-old males were used for experiments. Animal care and handling were performed according to the Canton of Fribourg's law for animal protection authorized by the Office Veterinaire Cantonal de Fribourg, and all procedures were conducted in accordance with the Declaration of Helsinki and conformed to international ethical standards (Portaluppi et al., 2008).

Genotyping

For the genotyping of all mice, genomic DNA was isolated from small tail biopsies using standard methods. For the PCR, a common forward primer (TCCCTCACCTAGAGATAGAAGCAGCGCAG) was used together with either a reverse primer specific for the wild-type allele (AGACAGCAGAATCGAGGAGTCTGCTGCTC) or a reverse primer annealing to the 5' region of the neo′ cassette, which is present in the mutated allele (GC TAAAGCGCATGCTCCAGACTGCCTTGG). This results in a wild-type amplicon of 254 bp and an amplicon derived from the mutant allele of 192 bp.

Locomotor Activity Monitoring

Analysis of locomotor activity parameters was done by monitoring wheel-running activity, as described in Jud et al. (2005), using the ClockLab software (Actimetrics) for all subsequent calculations. Briefly,
for the analysis of free-running rhythms, animals were entrained to LD 12:12 and subsequently released into constant darkness (DD). Internal period length ($\tau$) was determined from a regression line drawn through the activity onsets of ten days of stable rhythmicity under constant conditions. Total and daytime activity, as well as activity distribution profiles, were calculated using the respective inbuilt functions of the ClockLab software. Activity distribution was also evaluated manually from the actograms of animals kept in LD 12:12. Phase shifts were determined according to the Aschoff Type I protocol (Aschoff, 1965). Animals were kept in DD for at least 21 days to establish a stable free-running rhythm. Then, they were subjected to a light pulse (15 min, 400 lux) at circadian time (CT) 14 and thereafter maintained in DD for 21 days. The procedure was repeated for a light pulse at CT22 and CT10, respectively. For the calculation of phase shifts, regression lines were drawn through ten consecutive onsets before and ten consecutive onsets after light application; the first two days after the light pulse were regarded as transition and not taken into account. Phase shifts were expressed as the differences between the regression lines (and thus the hypothetical onsets of activity) on the first day after a light pulse. Numbers of animals used in the behavioral studies are indicated in the corresponding figure legends.

**In situ Hybridization**

Mice were held in DD and exposed to a 15 min light pulse at CT14 and sacrificed at CT15. Specimen preparation and in situ hybridization were carried out as described previously (Oster et al., 2003). Briefly, brains were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin after dehydration. 7 µm paraffin sections were made along the rostrocaudal axis, dewaxed, rehydrated, and fixed in 4% PFA. Sections were subjected to a proteinase K (Roche) treatment and then fixed again and acetylated. After dehydration in graded ethanol series, hybridization was performed overnight at 55°C in a humid chamber. $^{35}$S-rUTP (1250 Ci/mmol, PerkinElmer)-labeled riboprobes were synthesized with the RNAMaxx High Yield transcription kit (Stratagene) according to manufacturer's instructions. Stringency washes were carried out at 63°C and nonspecifically bound RNA was hydrolyzed by ribonuclease A (Sigma) treatment. The in situ hybridization probes ($Per1$, $Per2$, and $c-Fos$) were described previously (Albrecht et al., 1997). Specificity of the riboprobes was verified by hybridizing sense- and antisense-labeled transcripts. Quantification was performed by densitometric analysis (BioRad GS-800) of autoradiographs (Amersham Hyperfilm MP) using the ‘Quantity-One’ Software (BioRad). Optical density values from the SCN were normalized subtracting the optical
density measured in an equal area of the lateral hypothalamus. For each condition, three animals were used, and six sections spanning the central SCN (both nuclei)/animal were analyzed. Relative induction values were calculated by denoting the wild-type dark control value of each experiment as one.

**Immunohistochemistry**

For immunohistochemistry, mice were perfused with 4% paraformaldehyde (PFA) and the brains were removed and immersed overnight in 4% PFA. Free-floating cryo sections (40 μm) were incubated with an antibody against CB (CB38a; 1:10,000, SWant, Bellinzona, Switzerland). After washing, sections were incubated using the Vectastain ABC system (Vector Laboratories, Burlingame, California, USA); for the visualization of the bound antibody complex, sections were incubated with the chromophore 3,3'-diaminobenzidine and hydrogen peroxide.

**Data Analysis**

Statistical analysis of the data of all experiments was performed using Prism4 software (GraphPad Software Inc.). Significant differences between groups were determined using unpaired t-tests. Mean values were considered significantly different with \( p < 0.05 \) (*), \( p < 0.01 \) (**). \( p \) values > 0.05 were considered not statistically significant. Values are presented as mean ± SEM.

**RESULTS**

**Calb1\(^{-/-}\) Mice Lack Expression of Calbindin-D28k in the SCN and Cortex**

To investigate the *Calb1\(^{-/-}\)* mice, we first set out to test the absence of the functional gene in the genome using PCR. We found the wild-type allele was absent in the genome of *Calb1\(^{-/-}\)* mice (see Figure 1A), while a PCR signal for the mutant allele was observed in the samples from these animals. The PCR from genomic DNA of wild-type mice yielded just the opposite, a PCR signal with the wild-type primers and no signal with the primer pair amplifying the mutant allele. We next tested by immunohistochemistry whether the absence of the functional gene affects CB protein expression levels in the brain. We found CB immunoreactivity in the SCN and, as another example, also in the cortex of wild-type mice. In the *Calb1\(^{-/-}\)* mutants, no specific immunoreactivity was detected in both selected areas, indicating that CB is completely absent (see Figures 1B &
FIGURE 1  Genotyping of Calb1−/− and wild-type (WT) mice and expression of calbindin-D28k in mouse SCN and cortex. (A) PCR analysis reveals a 254 nt amplicon of the wild-type Calb1 allele and a 192 nt amplicon of the mutant allele. (B) Protein expression of calbindin-D28k in mouse SCN of wild-type (left) and Calb1−/− (right) mice determined by immunohistochemistry. The inset shows the SCN region of a WT and Calb1−/− mouse at 2.5-times higher magnification. In the WT, distinct neuron somata are labeled (arrowheads), while no specific staining is seen in the section from the Calb1−/− mouse. In comparison to the full picture, the image brightness was increased to the same extent on both images. (C) Expression of calbindin-D28k in mouse cortex of wild-type (left) and Calb1−/− (right) mice. The inset shows a region between cortical layers IV and VI in a WT and Calb1−/− mouse at 2.5-times higher magnification. Distinct neuron somata and neuropil are stained with the anti-calbindin D28k antibody. In the Calb1−/− mouse, the same region shows no specific staining for calbindin. Images were acquired with the same camera settings. Scale bars correspond to 500 μm in the SCN and 1 mm in the cortex.
1C). This is in line with the initial report on the Calb1−/− mice (Airaksinen et al., 1997).

**Calb1−/− Mice Exhibit Normal Circadian Activity Rhythms**

Under LD conditions, wheel-running activity of Calb1−/− mice (see Figure 2B) was mostly confined to the dark span, as observed in wild-type animals (see Figure 2A). The distribution of activity was similar in both genotypes (see Figure 2E, light period, WT: 1541 ± 303 wheel revolutions/day; Calb1−/−: 1970 ± 366 wheel revolutions/day; dark span,
WT: 19220 ± 1878 wheel revolutions/day; Calb1−/−: 17260 ± 1091 wheel revolutions/day; t-test wt vs. Calb1−/−, \( p > 0.05, n = 12 \), values are represented as mean ± SEM. When placed into DD, wild-type animals free-ran with a period length of 23.65 ± 0.03 h (n = 12) and Calb1−/− mice free-ran with a period length of 23.58 ± 0.09 h (n = 12) (see Figure 2C). Total activity under DD as well as under LD conditions were similar in both genotypes (t-test, \( p > 0.05 \)): wild-type (DD) 21230 ± 1857 wheel revolutions/day, wild-type (LD) 21090 ± 1635 wheel revolutions/day (n = 12) and for Calb1−/− mice (DD), 20340 ± 1406 wheel revolutions/day, Calb1−/− mice (LD) 19370 ± 980 wheel revolutions/day (n = 12) (see Figure 2D).

**Phase Delays Are Elevated in Calb1−/− Mice**

Phase shifting of the circadian locomotor activity rhythm was tested by applying light pulses at three different circadian time points. The light pulse at CT14 evoked a phase delay in wild-type and Calb1−/− mice (see Figure 3A), which was significantly greater in the latter genotype (Figure 3B, WT: −135.1 ± 7.8 min, n = 11; Calb1−/−: −169.4 ± 5.2 min, n = 12; t-test, \( p = 0.0013 \), values are represented as mean ± SEM). The light pulse presented at CT22 induced phase advances of similar magnitude in both genotypes (see Figures 3A and 3B, WT: 40.8 ± 6.4 min, n = 6; Calb1−/−: 48.3 ± 5.7 min, n = 6; t-test, \( p > 0.05 \)). The light pulse administered during the subjective day at CT10 did not evoke a phase shift, neither in wild-type nor in Calb1−/− mice (see Figure 3B, WT: −1.8 ± 4.4 min, n = 4; Calb1−/−: 6.5 ± 3 min, n = 6; t-test, \( p > 0.05 \)). The results indicate that phase delays are increased in Calb1−/− mice at CT14. However, we cannot exclude that this is the result of a shift of the phase-response curve, although the results obtained at CT10 and CT22 would argue against this.

**Lack of CB Affects Light-Induced Per2 Gene Expression**

Because the light pulse applied at CT14 resulted in an increase in the behavioral response of Calb1−/− mice, we investigated its influence on gene expression in the SCN. Per1, Per2, and c-Fos have been previously described as light-inducible genes in the SCN (Albrecht et al., 1997; Rusak et al., 1990; Shearman et al., 1997; Shigeyoshi et al., 1997; Yan & Silver, 2002). The induction of the Per1 and c-Fos genes in response to light at CT14 was comparable between Calb1−/− and wild-type animals (see Figure 4). Interestingly, Per2 gene induction was significantly increased in Calb1−/− compared to WT mice, as revealed by quantification of optical density in the SCN (see Figure 4). After the light pulse at CT14, more silver grains were detected in the SCN of Calb1−/− than in wild-type mice.
This is paralleled by the larger phase delay at CT14 observed in Calb1−/− compared to wild-type animals (see Figure 3). No subregional differences in Per2 mRNA expression between wild-type and Calb1−/− mice were observed in the central part of the SCN.

FIGURE 3 Calbindin-D28k (Calb1)−/− mice show larger phase delays compared to wild-type (WT) littermates. (A) Examples of activity records in constant darkness or DD (black bar above actograms). Black arrows point to the day when the light pulse was applied at circadian time (CT) 14 or CT22, respectively. (B) Quantification of resetting behavior in wild-type (grey) and Calb1−/− (white) mice at CT10 (n = 4 and 6, respectively), CT14 (n = 11 and 12, respectively; unpaired t-test, **p = 0.0013), and CT22 (n = 6). Negative values indicate phase delays, positive values phase advances.
FIGURE 4  Per1, Per2, and c-Fos gene induction after light pulse at CT14. (A) Quantification of gene expression in the SCN of wild-type (grey bars, n = 3) and Calb1−/− mice (white bars, n = 5). Comparable levels of the three genes are observed when no light pulse is applied (control). The light pulse at CT14 leads to induction of the three genes. The level of induction is comparable in the two genotypes, except for Per2, which is more induced in Calb1−/− SCN (unpaired t-test, *p = 0.043). (B) Representative sections illustrating Per2 mRNA levels with or without light pulse applied at CT14. Scale bars: 500 μm.
DISCUSSION

The circadian clock in the SCN encompasses a dynamic system of regulatory mechanisms that respond differentially to light. Light-induced phase shifts of behavior occur only during the night or subjective night. At these times (between CT12 and CT24), light also induces Per genes (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Yan & Silver, 2002). At the behavioral level, alterations in Per1 or Per2 gene expression correlate with alterations in phase advances or delays, respectively (Albrecht et al., 2001; Gau et al., 2002; Oster et al., 2003; Yan et al., 2006).

Because Ca^{2+} impinges on many signaling pathways, including those mentioned above, we investigated mice deficient in the gene coding for calbindin D-28k (Calb1^{-/-}). CB is a Ca^{2+} binding protein involved in the regulation of Ca^{2+} homeostasis by acting as a cytosolic Ca^{2+} buffer, but likely also as a Ca^{2+} sensor (Schmidt et al., 2005; Schwaller et al., 2002, 2009). We found that Calb1^{-/-} mice display normal circadian rhythmicity, with period length, total activity, and activity distribution comparable to wild-type animals (see Figure 2). These findings differ from a previous report in which 40% of Calb1^{-/-} mice were described as arrhythmic and the other 60% showing low-amplitude circadian rhythmicity and marked activity during the subjective day (Kriegsfeld et al., 2008). Furthermore, in the latter group with low rhythmicity, no significant differences in phase shifting were observed between wild-type and Calb1^{-/-} mice at all the time points investigated (CT4, CT16, CT22). The authors do not discuss the point that within their Calb1^{-/-} mouse population, there exist two subpopulations with two discrete, discernable circadian phenotypes, albeit a common genotype. The original mutation (Airaksinen et al., 1997) was made in embryonic stem cells of the 129 strain. Kriegsfeld et al. (2008) consider it rather unlikely that in their back-crossed strain (for seven generations to B6) that the region flanking the targeted Calb1 gene, which most likely maintains some of the Sv129 alleles, is responsible for the observed effects. Animals used in our study are derived from the initial Calb1^{-/-} mice generated by Airaksinen et al. (1997) with a mixed R1 × B6 genetic background. They were then back-crossed for eight generations to B6 mice in Fribourg, Switzerland, and Calb1^{+/-} siblings were used to generate wild-type and Calb1^{-/-} littermates. Thus, the most likely explanation for the discrepancy between the results of the study by Kriegsfeld et al. (2008) and ours lies in subtle genetic differences between the two theoretically “identical” Calb1^{-/-} strains.

CB expressing neurons in the SCN of hamsters receive direct input from the retina via the retinohypothalamic tract (Bryant et al., 2000). Experiments using Calb1-antisense oligodeoxynucleotides suggest that these neurons gate photic entrainment of cellular circadian oscillators.
and thereby influence the timing of locomotor activity (Hamada et al., 2003). Similarly, we find that Calb1−/− mice respond with a larger behavioral phase-delay to a light pulse at CT14 than do wild-type animals (see Figure 3). However, at CT10 and CT22, which correspond to the subjective day and phase-advance portion of the phase-response curve, respectively, no difference between Calb1−/− and wild-type mice is observed (see Figure 3), suggesting the phase-response curve is unlikely to be shifted. These findings reinforce previous observations that indicated a tendency for slightly larger phase delays in Calb1−/− mice (Kriegsfeld et al., 2008), although in that study, CT16 was investigated for phase delays in contrast to CT14 in our study. Comparable to the results in mice, a role for CB in phase shifting was observed in hamsters (LeSauter et al., 1999). This correlation between our observation in mice and the one in hamsters would suggest similar functions of CB in both species. However, differences in the neuroanatomical organization of the SCN in these species have been noted. Therefore, CB might act differently in hamsters and mice, despite the apparent correlation observed here.

To determine whether Per gene expression parallels the behavioral observations, we tested gene induction in the SCN of wild-type and Calb1−/− mice. We found Per2, but not Per1 and c-Fos, gene expression to be significantly more induced by the light pulse applied at CT14 in Calb1−/− mice (see Figure 4). This is in agreement with findings that indicate a correlation between the magnitude of phase delays and the amount of Per2 gene induction (Albrecht et al., 2001; Oster et al., 2003; Yan et al., 2006). Consistent with our findings, the study by Kriegsfeld et al. (2008) reports no difference between wild-type and Calb1−/− mice in light-dependent Per1 gene induction. However, they observe a reduction in c-Fos inducibility in Calb1−/− mice. Unfortunately, light-dependent Per2 gene induction was not investigated in that study.

In the circadian system, light-induced phase-shifts are dependent on extracellular Ca2+ influx and intracellular Ca2+ levels regulated by intracellular Ca2+ channels, involving inositol trisphosphate (Hamada et al., 1999) and ryanodine receptors (Ding et al., 1998). Interestingly, neuronal ryanodine receptors mediate light-induced phase-delays of the circadian clock (Ding et al., 1998). These receptors regulate the release of Ca2+ from the endoplasmic reticulum, resulting in a spatiotemporally restricted rise in cytosolic Ca2+. This might lead to the activation of Ca2+-dependent kinases that could activate a signaling cascade leading to the induction of target genes, such as Per2. As UV flash photolysis experiments revealed that CB acts in the first 100 ms as a Ca2+ buffer, reducing the initial Ca2+ amplitude by approximately 50%, both in vitro (Nagerl et al., 2000) and in soma (Airaksinen et al., 1997) and dendrites (Schmidt et al., 2003) of Purkinje cells, we hypothesize that CB might act as a short-term sink for Ca2+ released by ryanodine or possibly also inositol trisphosphate.
receptors. The latter hypothesis is based on CB’s ability to interact and to activate myo-inositol monophosphatase, a key enzyme of the inositol-1,4,5-trisphosphate signaling cascade (Berggard et al., 2002b; Schmidt et al., 2005). The absence of CB might lead to an increase in Ca$^{2+}$ amplitude after a light pulse at CT14 because buffering of Ca$^{2+}$ released by ryanodine receptors is reduced. The increase in Ca$^{2+}$ amplitude would activate signaling pathways more efficiently. This would lead to stronger transcriptional initiation of target genes and, as a consequence, altered behavioral response. Although our findings support this model, future experiments will reveal whether this hypothesis is valid.

Taken together, we show that Calb1$^{-/-}$ mice lacking CB display larger light-induced phase-delays of the circadian rhythm. This is accompanied by a small increase in Per2 gene induction in the SCN. Our results indicate an important function of CB in the resetting mechanism of the circadian clock in response to light seen during the subjective night.

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