Light induces chromatin modification in cells of the mammalian circadian clock

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The mammalian circadian clock resides in neurons of the hypothalamic suprachiasmatic nucleus (SCN). Light entrains phase resetting of the clock using the retino-hypothalamic tract, via release of glutamate. Nighttime light exposure causes rapid, transient induction of clock and immediate–early genes implicated in phase-shifting the pacemaker. Here we show that a nighttime light pulse caused phosphorylation of Ser10 in histone H3's tail, in SCN clock cells. The effect of light was specific, and the kinetics of H3 phosphorylation were characteristic of the early response, paralleling c-*fos* and *Per1* induction. Using *fos-lacZ* transgenic mice, we found that H3 phosphorylation and Fos induction occurRed in the same SCN neurons. Systemic treatment with the $GABA_B$ receptor agonist baclofen prevented light-induced c-*fos* and *Per1* expression and H3 phosphorylation, indicating that one signaling pathway governs both events. Our results suggest that dynamic chromatin remodeling in the SCN occurs in response to a physiological stimulus *in vivo*.

The central mammalian circadian clock is located in neurons of the hypothalamic suprachiasmatic nucleus[1–3]. The pacemaker activity of the SCN is autonomous, as it functions in constant darkness and in the absence of external light stimuli[1–3]; however, it can be entrained by environmental light–dark cycles[4]. In rodents, at least three neural pathways link the retina to the SCN: first, the direct, monosynaptic retino-hypothalamic tract (RHT), whose main neurotransmitters seem to be excitatory amino acids such as glutamate[5,6]; second, the indirect multisynaptic projection from the intergeniculate leaflet (IGL), which acts via the release of the protein neurotransmitter neuropeptide Y and the inhibitory neurotransmitter γ-aminobutyric acid (GABA)[7,8]; third, an indirect pathway arising from the dorsal and median raphe nucleus, in which serotonin is the main neurotransmitter[9]. Although the RHT alone seems necessary and sufficient to mediate light entrainment[10], interference with the IGL and/or dorsal raphe nucleus transmission[11] can modify the response of the SCN to light.

In mammals kept in darkness, a light pulse during the subjective night (that is, the time of the day corresponding to the dark period in a normal light–dark cycle) causes phase-shifting of the SCN-controlled rhythms[4]. The phase-shift stimulated by light triggers gene expression within the SCN, including the rapid and transient activation of clock genes, such as *Per1* and *Per2* (refs. 12, 13) and of immediate early genes (IEGs), such as c-*fos*, *fos*-B, *jun*-B, *nur*77 and *zif*268 (refs. 14, 15). These changes in gene expression have been implicated in phase-shifting the pacemaker[16,17]. Although the photic regulation of c-*fos* expression in the SCN has been extensively studied, the pivotal events enabling dynamic regulation at the chromosomal level are not yet known.

Light pulses induce activation of the mitogen activated protein kinase (MAPK)[18], and thus possibly modulate the physiological function of substrates within the SCN. In particular, light pulses induce phosphorylation of transcription factor CREB (cAMP response-element binding protein) on serine 133 (ref. 19), the essential phospho-acceptor site that enables the recruiting of the co-activator CBP, a protein with histone acetyltransferase (HAT) activity[20]. Factors of the CREB family are involved in circadian rhythmicity both in *Drosophila*[21] and mammals[22].

Modifications occurring on histone tails are primary events that contribute to the dynamic process of chromatin remodeling, an essential step in transcriptional regulation[23]. Both chromatin remodeling factors and covalent histone modifications facilitate access of transcription factors to chromatin, and regulate expression of a wide range of genes[24]. A number of post-translational modifications occur on histone tails (for review, see ref. 23). Among these, inducible phosphorylation at serine 10 of the histone H3 N-terminal tail in response to a mitogenic stimulus represents the best characterized link between activation of a signal transduction pathway and chromatin modification. Phosphorylation occurs in a subset of histone H3 molecules in a rapid and transient manner, paralleling the induction profile of early response genes[25,26]. We previously identified the MAPK-activated RSK2 kinase as a candidate for epidermal growth factor (EGF)-induced phosphorylation of H3 (ref. 25). RSK2 is also involved in EGF-induced CREB phosphorylation and c-*fos* expression in fibroblasts[27], establishing a direct link between signaling to chromatin and induction of IEGs. Finally, phosphorylation on Ser10 greatly enhances the subsequent HAT-mediated acetylation on the closely located Lys14 on the H3 tail, underscoring the primary importance of phosphorylation[28]. Our knowledge of histone modifications is mostly based on studies in cell culture systems; histone modification function *in vivo* is basically unexplored.

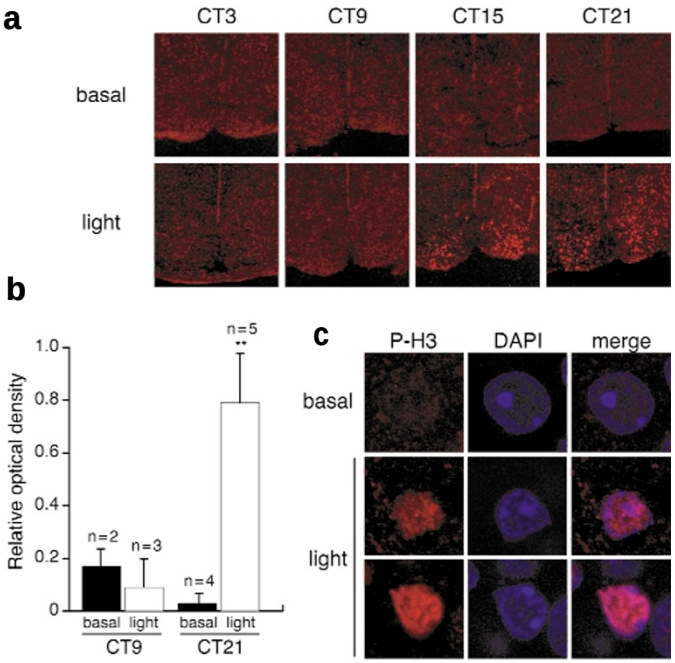


Fig. 1. Time- and light-dependent phosphorylation of histone H3 in the suprachiasmatic nucleus. (**a**) Immunofluorescence on mouse SCN cryosections with the P-H3 specific antibody. Mice were dissected at four different circadian times (CT followed by the number of hours after the beginning of the subjective day), after a 15-min light pulse and 15 min in the dark (light) or at equivalent times but omitting the pulse (basal). (**b**) Quantification of the P-H3 signal in the SCN at CT9 and CT21, after a light pulse (light) or not after a light pulse (basal). The data shown are a compilation of several independent experiments, as indicated. The increase in P-H3 signal upon light stimulation at CT21 is statistically significant (Student's *t*-test, $p < 0.01$). (**c**) Confocal microscopy analysis of independent SCN cells under basal conditions (top) or after a light pulse (middle and bottom). Nuclear signal in light-stimulated mice varies from low staining with punctated pattern (middle) to intense and widespread staining (bottom).

Here we report that the photic stimulus has previously unrecognized profound effects on chromatin remodeling in non-proliferating neurons. A light pulse applied to mice during the subjective night induced the phosphorylation of the histone H3 in the ventrolateral SCN, but not in the pineal gland or retina. This transient modification occurred with early response kinetics, paralleling the induction of the c-*fos* gene. Moreover, H3 phosphorylation and gene induction were blunted by the $GABA_B$ receptor agonist baclofen, which counteracts light effects on the SCN[29,30]. Our results provide *in vivo* evidence of chromatin remodeling in the vertebrate nervous system in response to an external stimulus.

Results

Light-inducible histone H3 phosphorylation in the SCN

Two groups of mice were placed for 2 weeks on a 12–12 hour light–dark (LD) cycle and then transferred to constant darkness (DD) for 4 days. At different circadian times (CT; CT0 corresponds to lights on), one group of mice was directly killed, whereas the second group was exposed to light for 15 minutes and then returned to darkness for 15 additional minutes before dissection. Phosphorylation was determined by immunofluorescence in whole-brain cryosections using a specific antibody directed against a phosphorylated Ser10 H3 peptide (P-H3; ref. 31). There was no spontaneous phosphorylation of H3 in the SCN at any time of the circadian cycle (**Fig. 1a**). A light pulse induces prominent H3 phosphorylation in the SCN, though only during the subjective night (when light causes phase-shift of the clock; for example, a delay at CT15 and an advance at CT21)[4], and densitometric analysis confirmed that the photic stimulation was statistically significant (**Fig. 1b**). No H3 phosphorylation was present in other brain areas. The SCN ventrolateral region, an area that displays maximal induction of c-*fos* in response to light[14], displayed maximal H3 phosphorylation. Therefore, as for immediate early gene expression, H3 phosphorylation was induced by retinal illumination and was gated by the circadian clock.

Confocal analysis of SCN sections from mice light-stimulated at CT21 showed a nuclear staining pattern specific for the euchromatin (**Fig. 1c**). The staining, as previously observed in EGF-induced fibroblasts[25], was not uniform over the nucleus, although it appeared more diffused as compared to the nuclear speckles observed in fibroblasts. This may be ascribed to the distinct experimental settings, as well as to the diverse signaling pathways involved; in our previous study, growth factors led to cell proliferation[25], whereas here light elicited a dynamic response in non-proliferating neurons.

No H3 phosphorylation in retina and pineal gland

In mammals, the SCN receives photic information from the retina, which also contains a circadian pacemaker[3]. Circadian timing information is then relayed from the SCN to the pineal gland, thereby controlling the rhythmic release of the hormone melatonin[1,3]. We thus studied the consequence of photic stimulation on H3 phosphorylation in these areas. Western blot analyses were

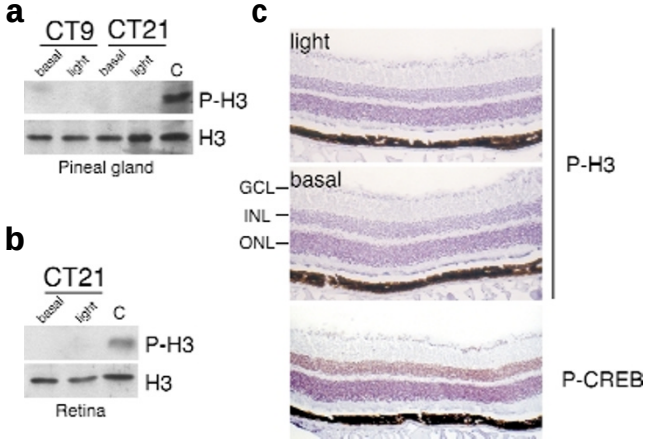


Fig. 2. Photic stimulation did not induce H3 phosphorylation in the pineal gland and in the retina. Western blot analyses were performed on pineal gland (**a**) isolated before (basal) and after a 15-min light pulse followed by 15-min in the dark (light) at CT9 and CT21, and on retina (**b**) isolated before and after a light pulse at CT21. Control of H3 phosphorylation in EGF-stimulated mouse fibrolablasts, third lane (C). (**c**) Immunohistochemistry with P-H3 antibodies on retina sections. Mice were dissected at CT21 before or after a light pulse. No induction of histone H3 phosphorylation is detected. Use of P-CREB antibodies as control for presence of phosphoproteins shows a signal in the inner nuclear layer (INL). GCL, ganglion cell layer; ONL, outer nuclear layer.

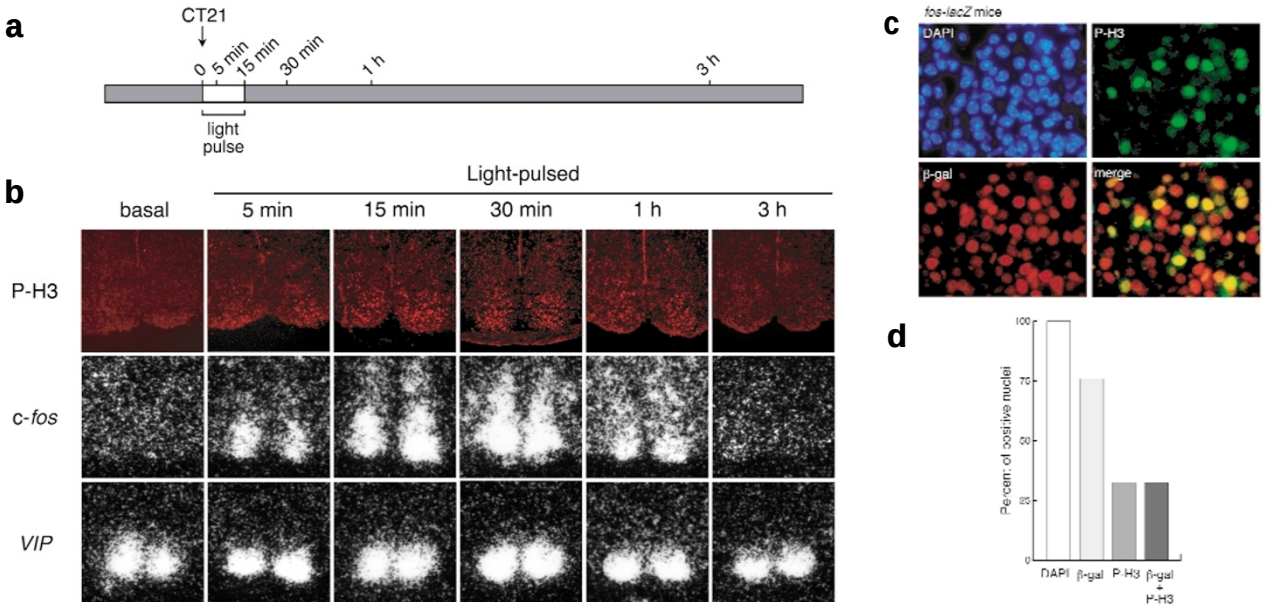


Fig. 3. Timing correlation of histone H3 phosphorylation and c-*fos* induction in the SCN. (**a**) Time course experiment presented in (**b**). Mice, entrained on a light–dark cycle and then placed in constant darkness for five days, were given a light-pulse at CT21. Mice were dissected after 5 or 15 min of lighting, or at different times after a 15-min light pulse. Brain cryosections were processed for either immunohistochemistry with P-H3 specific antibody or *in situ* hybridization with c-*fos* or *VIP* probes. (**c**) Immunofluorescence on SCN cryosections (enlarged) from *fos-lacZ* transgenic mice. Double labeling with P-H3 and β-galactosidase antibodies of a single cryosection is shown, along with a combination of the P-H3 and β-gal images (merge). Mice were given a light-pulse at CT21 as described in (**a**) and dissected after two hours. (**d**) Quantification of the data in (**c**). Neurons with the different stainings were counted and plotted as a percentage of the total number of neurons in the area (as assessed with DAPI). The procedure was repeated for several independent experiments, with analogous results.

done on pineal gland extracts during a normal light–dark cycle (data not shown), and after a light pulse at CT9 and CT21 (**Fig. 2a**). In both cases, no H3 phosphorylation was detectable. In eye samples collected at CT21, following or not following a light pulse, no H3 phosphorylation was observed (**Fig. 2b**). Also in retinal sections from mice stimulated or not stimulated with light at CT21 (**Fig. 2c**), CT3, CT9 and CT15 (data not shown), no signal was present, although control CREB phosphorylation was detected in the inner nuclear and ganglion cell layers (**Fig. 2c**). Thus, light-induced H3 phosphorylation seemed to be restricted to the cells of the central clock. These results stressed the specificity of SCN cells as receptors of the light signal, and of their capability to transduce the signal into a selected phosphorylation of one histone tail.

Coupling H3 phosphorylation with c-*fos* expression

To study the time course of H3 phosphorylation after photic induction, we stimulated animals with light for 15 minutes and returned them to the dark for various lengths of time before dissection. Other mice were exposed to light for five minutes and then killed immediately (**Fig. 3a**). We used serial sections from individual mice to probe c-*fos* gene expression and H3 phosphorylation in parallel. Expression of *VIP* was used as a control[32]. As for c-*fos* inducibility[14], short light pulses were sufficient to induce H3 phosphorylation. Histone H3 phosphorylation peaks 30 minutes after the onset of light, whereas three hours later it is no longer detectable. Thus, the timing of H3 phosphorylation closely coincides with the early gene response (**Fig. 3b**).

To verify that induction of H3 phosphorylation and early gene expression occur concomitantly in the same SCN neurons in response to the light stimulus, we used transgenic *fos-lacZ* mice[33]. In these mice, β-galactosidase (β-gal) expression driven by the c-*fos* promoter was inducible by a light-pulse at CT21, during the subjective night[33]. Nuclei positive for H3 phosphorylation, but not for β-gal, were detected one hour after the CT21 light pulse (data not shown). Two hours after the light pulse, β-gal-positive and double-labeled nuclei were visible (**Fig. 3c**). Almost half of β-gal-positive nuclei (Cy3 labeled) also exhibited P-H3 staining (FITC labeled), whereas all P-H3 positive nuclei were also stained with the anti-β-gal antibody (**Fig. 3d**). These data provide strong evidence that the two events occur concurrently in a large subset of SCN neurons.

Baclofen blocks light-induced H3 phosphorylation

The administration of the $GABA_B$ receptor agonist baclofen is known to inhibit phase-shifts induced by light during the subjective night, as well as c-*fos* gene expression[29,30]. We thus considered whether a pharmacological treatment modulating light-dependent effects on the clock would also interfere with the signaling routes required for H3 phosphorylation. Mice, entrained on a L12–D12 cycle for two weeks and housed in DD for four days, were injected intraperitoneally with baclofen or a saline solution at CT3, CT9, CT15 or CT21. After 10 minutes, animals were exposed to a 15-minute light pulse, and then placed in the dark for 15 minutes before dissection. We then analyzed gene expression and histone H3 phosphorylation in the SCN of these mice (**Fig. 4**).

Baclofen alone had no effect on IEGs or clock gene expression in the SCN at any time. On the other hand, baclofen strongly reduced c-*fos* induction in response to light at CT15 and CT21. A similar effect was observed on the light-induced expression of clock gene *Per1*. Transcript levels of *Per1* oscillate during the day,

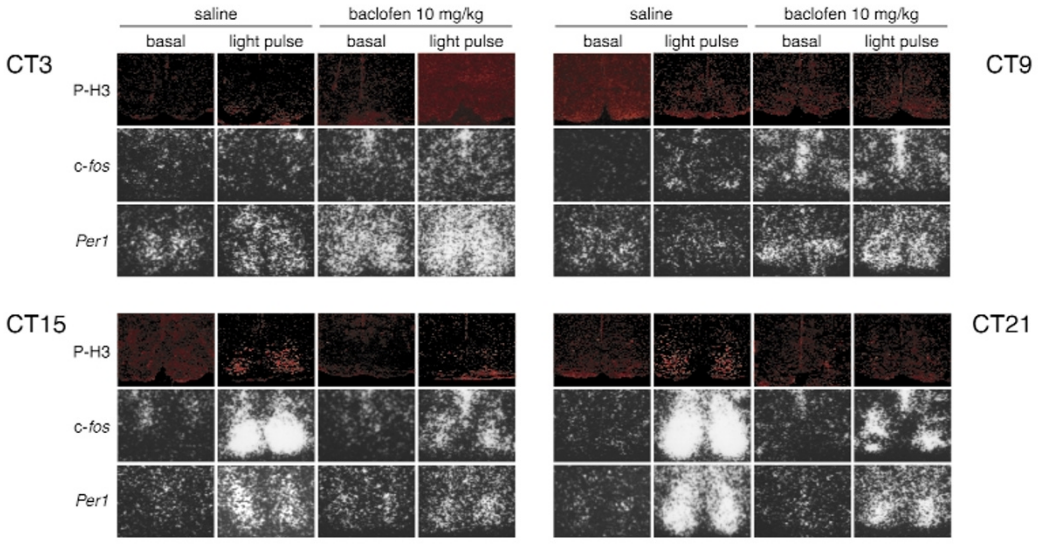


Fig. 4. Baclofen inhibits light-induced phosphorylation of histone H3 and induction of c-*fos* and *Per*1 gene expression in the SCN. Mice entrained as described in Fig. 1 were injected with saline solution or baclofen (10 mg/kg) 10 min before a light pulse at CT3, CT9, CT15 and CT21. Brain cryosections were processed for either immunohistochemistry with P-H3 specific antibody or *in situ* hybridization with c-*fos* or *Per1* probes.

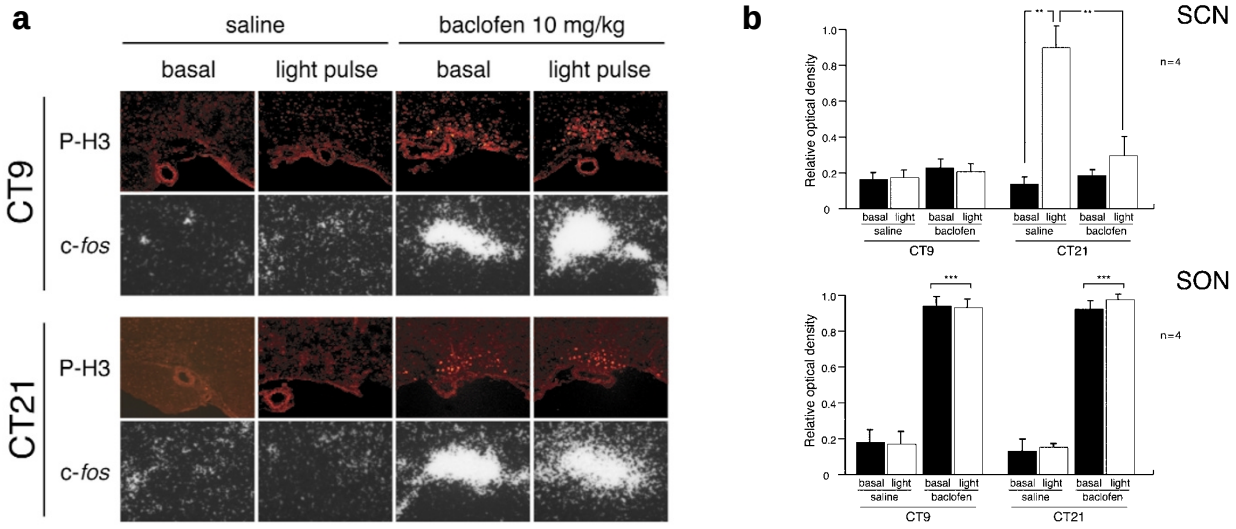
peaking during the light period in the SCN[12,13]. As for c-*fos, Per 1* expression is induced by light during the subjective night[12,13]. Daytime high levels of *Per1* were not blunted by baclofen (**Fig. 4**; CT3 and CT9), demonstrating that the effect of the drug is specific for the light-induced expression.

H3 phosphorylation closely followed c-*fos* and *Per1* expression, as baclofen powerfully reduced the light-induced effect (**Figs. 4** and **5b**). Some P-H3 positive nuclei remained in the mediolateral part of the SCN, which is consistent with what we and others[29] have observed for c-*fos* induced expression. These results strongly suggest that the same signaling routes within the SCN are involved in this chromatin modification as in IEG and clock gene expression.

H3 phosphorylation in the supraoptic nucleus

GABA mediates neurotransmission from the SCN to magnocellular neurons in other hypothalamic nuclei, such as the paraventricular nucleus and the supraoptic nucleus (SON)[34,35].

Fig. 5. Baclofen induces histone H3 phosphorylation and c-*fos* expression in the supraoptic nuclei (SON) in a light-independent manner. (**a**) Brain cryosections from mice treated as described in Fig. 4, at CT9 or CT21, were processed for either immunohistochemistry with P-H3 specific antibody or *in situ* hybridization with the c-*fos* probe. Representative images of the SON regions are shown. Similar results were obtained at CT3 and CT15 (data not shown). (**b**) Quantification of the P-H3 signal in the SCN at CT9 and CT21, after saline or baclofen injection, followed by a light pulse (light) or not (basal). The data shown are a compilation of several independent experiments, as indicated. In the SCN, the increase in P-H3 signal upon light stimulation at CT21, as well as the inhibition of this response by baclofen, are statistically significant (Student's *t*-test, $p < 0.01$). In the SON, the increase in P-H3 signal upon treatment with baclofen is statistically significant (Student's *t*-test, $p < 0.001$).



Moreover, various treatments induce Fos protein expression in the SON[36,37]. We asked whether induction of H3 phosphorylation accompanies c-*fos* gene activation in other neuronal settings. Thus, we explored the effect of baclofen administration and found that it triggered c-*fos* gene inducibility in cells of the SON (**Fig. 5a**). This induction was concomitant with a robust induction of H3 phosphorylation. Importantly, the baclofen-dependent induction of both c-*fos* gene expression and H3 phosphorylation was not clock regulated; it was observed at any time of the day. Moreover, the two regulatory events in the SON were independent of the light effect. Thus, although regulation was very different in the SON compared to the SCN (**Fig. 5b**), c-*fos* expression and H3 phosphorylation were still tightly coupled. These observations provide another independent and strong physiological link between these molecular events.

Discussion

Gene regulation requires condensation and decondensation of chromatin[23,24]. One key step in this process is regulation of histone modifications, of which acetylation and phosphorylation have been the most studied cases[23]. Clock function relies greatly on transcriptional regulation. The generation of physiological circadian rhythms involves transcriptional regulatory feedback loops[2,3], and one initial step in the integration of environmental stimuli is the rapid induction of a panel of genes[12–15]. Here, phase-shift-inducing light stimulation profoundly affected SCN cell physiology by triggering a major histone modification, possibly a primary event of the transcriptional cascade leading to regulated clock function.

Light treatment of mice during the subjective night elicits powerful induction of immediate-early genes, an event that seems to be important for clock phase-shifting[16,17,38], although a more complex scenario is possible[39,40]. The data presented here provide evidence for a tight link between this transcriptional event and H3 phosphorylation in the SCN. First, the accumulation of c-*fos* mRNA and of P-H3 present strikingly overlapping kinetics. Second, both events occur in the same region of the SCN, mainly in the ventrolateral region. Third, a high proportion of SCN neurons show colocalization of β-galactosidase and P-H3 signal in light-treated *fos-lacZ* transgenic mice. Fourth, induction of c-*fos* (and *Per1*) and H3 phosphorylation in reponse to light are severely blunted upon treatment with baclofen. These notions suggest that H3 phosphorylation is involved in the transcriptional response to light in the SCN, and therefore significantly participates in the phase-shift events induced by this stimulus. However, additional experiments are needed to causally link H3 phosphorylation to these processes. Previous studies in EGF-stimulated fibroblasts indicated that the same signal transduction pathway leads to a concerted phosphorylation of CREB and the H3 tail[25,27,41]. A parallel with the SCN is interesting, as CREB phosphorylation is also induced in response to light[19]. The panel of genes activated by light in the SCN is likely to be different because P-H3 labeling covers large patches throughout the nucleus, which contrast with the speckles observed in fibroblasts upon EGF stimulation[25](**Fig. 1c**).

Using c-*fos-lacZ* transgenic mice, we showed that H3 phosphorylation and c-*fos* promoter induction occurred in the same SCN neurons (**Fig. 3c** and **d**). However, some uncoupling was present. The most likely explanation was that scoring by immunoreaction only partially reflected the overlapping of the two events. Indeed, whereas the P-H3 antibody revealed an early response event, the anti-β-gal signal was the result of accumulated β-gal protein generated with slower kinetics. Thus, many β-gal-positive nuclei were not P-H3 positive, because the phosphorylation signal was long gone while the highly stable β-gal protein was still immunoreactive.

Induced H3 phosphorylation in the SCN and SON constituted a demonstration of non-mitotic H3 phosphorylation *in vivo*. The same modification was elicited, in each case, by very different stimuli. In the SCN, the light signal is transmitted through the RHT via the neurotransmitter glutamate and the ionotropic NMDA receptor[6]. In the SON, metabotropic GABA receptors are likely involved, because the response is stimulated by the $GABA_B$ receptor agonist baclofen. Given the distinct effectors used by these two types of receptors[42,43], the intracellular pathways leading to H3 phosphorylation *in vivo* are likely to differ profoundly. Consistent with this notion is the observation that the mode of regulation in the two hypothalamic nuclei is distinct: in the SCN, H3 phosphorylation was light dependent, gated by the clock, and blocked by baclofen; in the SON, the response was equivalent at any time of the day, was independent of lighting conditions, and was induced by baclofen. The SON has an important involvement in osmoregulation, through osmosensitivity of its magnocellular neurosecretory cells and neurohypophysial hormone release[44]. H3 phosphorylation, and in general chromatin remodeling, may be involved in hormonal response governed by this physiological pathway.

In addition to counteracting the effect of light on IEG gene expression, baclofen blunts both the *Per1* gene response and H3 phosphorylation. Several studies have addressed the question of the physiological role of GABA in the SCN[45,46]. GABA may be a coupling agent between SCN cells[45], as it is able to acutely inhibit SCN neuron firing rate (through $GABA_B$ receptors) and shift the phase of these neurons (through $GABA_A$ receptors). In addition, it synchronizes SCN cells in culture when administered at regular times[45]. Finally, GABA seems to modulate gap junction communication between SCN neurons[46]. The involvement of additional chromatin modifications in a number of neuronal settings, and in response to a number of stimuli, is still to be explored.

Which H3 kinase is acting in response to light in the SCN? RSK2 is the H3 kinase in EGF-stimulated fibroblasts[25]. Other kinases, including MSK1 and PKA, have the capacity to phosphorylate H3 *in vitro* and are therefore among possible SCN candidates[26,47]. All the above-mentioned kinases belong to the AGC group of protein kinases[23]. The search for the light-induced H3 kinase may reveal essential elements of the light transducing pathway and their links to central clock elements.

The molecular pathways by which light influences the circadian cycle are not fully recognized, although advances in various organisms implicate light-transducing molecules in clock function[48–50]. Here we demonstrate that light acts directly on nuclear function by inducing a distinct histone modification, thus influencing the state of higher chromatin organization. This light-induced chromatin modification is gated by the clock. Our results also demonstrate that multiple signaling pathways may converge to specific chromatin sites to elicit the same histone modification.

Methods

Animals and tissue preparation. We used wild-type 129/Sv mice, although similar results were obtained using other mouse strains. Trangenic mice (*fos-lacZ*) were obtained from T. Curran[33]. All animals were 7–12 week-old males. Mice were entrained on a L12–D12 (12 h light–12 h dark) for two weeks, and then placed in constant darkness (DD) for four days. They were then exposed to a 15-min light pulse and placed back in the dark for 15 min (unless otherwise mentioned). Baclofen (10 mg/kg), dissolved

in 0.9% NaCl, or saline solution alone, was administered intraperitoneally 10 min before the light pulse. Mice were decapitated and dissected under dim red light conditions. Tissues to be assayed by western blot analysis were homogenized in boiling Laemmli buffer. For immunohistochemistry and *in situ* hybridization, tissues were placed in OCT and frozen on dry ice and 10-µm thick coronal cryosections were prepared.

***In situ* hybridization.** *VIP* (cloned by PCR from genomic mouse DNA, nucleotides 3095–3262 of the Genbank sequence X74297), c-*fos* and *mPer1* (nucleotides 1–336 of ORF, Genbank sequence AF022992) [^{35}S]-labeled antisense riboprobes were prepared by *in vitro* transcription (Promega, Madison, Wisconsin). As a control for nonspecific signal, consecutive sections were hybridized with c-*fos*, *VIP* and *mPer1* sense probes.

Immunohistochemistry. Brain sections were fixed in 1× PBS solution (pH 7.5) containing 4% paraformaldehyde, and then blocked for 1 h in 5% normal goat serum in 1× PBS/0.05% Tween-20. For Ser-10-phosphorylated H3 (P-H3) immunolabeling, sections were incubated overnight at 4°C with a rabbit polyclonal antibody (1:1000 final dilution, Upstate Biotechnology, Lake Placid, New York). Slides were washed and incubated with a secondary antibody (CY^{TM}3 conjugated anti-rabbit serum, 1:1000; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Immunostained sections were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Roche, Mannheim, Germany) before microscopy.

For double-labeling experiments, sections were incubated with a mouse monoclonal β-galactosidase-specific antibody (1:500, Sigma-Aldrich, St. Quentin Fallavier, France) and the P-H3 rabbit polyclonal antibody, and then incubated with a CY^{TM}3 conjugated anti-mouse serum and a fluorescein (FITC) conjugated anti-rabbit serum (1:100 and 1:200 respectively, Jackson ImmunoResearch Laboratories).

We acquired images with DMLB Leica microscope with a HBO100W lamp. Confocal images were acquired on a Leica DMRE microscope. We analyzed retinal sections by the peroxidase-labeled detection method, and we introduced an additional step to block endogenous peroxidase activity.

Western blot analysis. Protein extracts were resolved by standard SDS-PAGE. Samples were electroblotted onto Protan nitrocellulose (Schleicher & Schuell, Dassel, Germany). Membranes were incubated in PBS/5% low-fat milk and specific antibody for 12 h at 4°C. Donkey, anti-rabbit HRP antibodies were used to reveal immunocomplexes by enhanced chemiluminescence (Pierce, Rockford, Illinois).

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