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Restoring the molecular clockwork within the suprachiasmatic hypothalamus of an otherwise clockless mouse enables circadian phasing and stabilisation of sleep-wake cycles and reverses memory deficits (25/50).

Abbreviated title: SCN clock can regulate sleep in clockless mice (46/50)

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+ Further information and requests for resources and reagents should be directed to and will be fulfilled by the Submitting Author, Michael Hastings (mha@mrc-lmb.cam.ac.uk).

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Abstract

The timing and quality of sleep-wake cycles are regulated by interacting circadian and homeostatic mechanisms. Although the suprachiasmatic nucleus (SCN) is the principal clock, circadian clocks are active across the brain and the respective sleep-regulatory roles of SCN and local clocks are unclear. To determine the specific contribution(s) of the SCN, we used virally mediated genetic complementation, expressing Cryptochrome1 (Cry1) to establish circadian molecular competence in the suprachiasmatic hypothalamus of globally clockless, arrhythmic male Cry1/Cry2-null mice. Under free-running conditions, the rest/activity behaviour of Cry1/Cry2-null controls expressing EGFP (SCN\textsuperscript{Con}) was arrhythmic, whereas Cry1-complemented mice (SCN\textsuperscript{Cry1}) had coherent circadian behaviour, comparable to that of Cry1,2-competent wild-types. In SCN\textsuperscript{Con} mice, sleep-wakefulness, assessed by electroencephalography/electromyography, lacked circadian organisation. In SCN\textsuperscript{Cry1} mice, however, it matched wild-types, with consolidated vigilance states (wake, REM and NREM sleep) and rhythms in NREMS delta power and expression of REMS within total sleep. Wakefulness in SCN\textsuperscript{Con} mice was more fragmented than in wild-types, with more wake-NREMS-wake transitions. This disruption was reversed in SCN\textsuperscript{Cry1} mice. Following sleep deprivation, all mice showed a homeostatic increase in NREMS delta power, although the SCN\textsuperscript{Con} mice had reduced NREMS during the inactive (light) phase of recovery. In contrast, the dynamics of homeostatic responses in the SCN\textsuperscript{Cry1} mice were comparable to wild-types. Finally, SCN\textsuperscript{Con} mice exhibited poor sleep-dependent memory but this was corrected in SCN\textsuperscript{Cry1} mice. In clockless mice, circadian molecular competence focussed solely on the SCN rescued the architecture and consolidation of sleep-wake and sleep-dependent memory, highlighting its dominant role in timing sleep.
The circadian timing system regulates sleep-wake cycles. The hypothalamic suprachiasmatic nucleus (SCN) is the principal circadian clock, but the presence of multiple local brain and peripheral clocks mean the respective roles of SCN and other clocks in regulating sleep are unclear. We therefore used virally mediated genetic complementation to restore molecular circadian functions in the suprachiasmatic hypothalamus, focusing on the SCN, in otherwise genetically clockless, arrhythmic mice. This initiated circadian activity-rest cycles, and circadian sleep-wake cycles, circadian patterning to the intensity of NREM sleep and circadian control of REM sleep as a proportion of total sleep. Consolidation of sleep-wake established normal dynamics of sleep homeostasis and enhanced sleep-dependent memory. Thus, the suprachiasmatic hypothalamus, alone, can direct circadian regulation of sleep-wake.
Introduction

The timing and quality of sleep are determined by a circadian process that ensures sleep occurs appropriately within the light-dark (LD) cycle and a homeostatic process that tracks sleep need during wakefulness (Borbely and Achermann, 1999; Borbely et al., 2016). Whereas the identity of the homeostat remains unknown, the hypothalamic suprachiasmatic nucleus (SCN), is conventionally thought to mediate circadian control (Saper et al., 2005). At the molecular level, the SCN clock consists of transcriptional/post-translational feedback loops (TTFL) in which Period (Per) and Cryptochrome (Cry) genes are trans-activated by CLOCK and BMAL1 heterodimers (Takahashi, 2017). Following their accumulation over circadian day, the encoded Per and Cry proteins inhibit trans-activation, closing the loop, whilst their subsequent degradation over circadian night allows the cycle to recommence. This TTFL is entrained to solar time by rods, cones and melanopsin-containing retinal ganglion cells innervating the SCN (Berson et al., 2002; Hattar et al., 2002; Hattar et al., 2003). In constant darkness (DD), the TTFL runs to its intrinsic approximately 24h period. Importantly, the TTFL is active in all tissues, including brain regions that regulate the sleep/wake cycle and memory (Hastings et al., 2018). The question arises, therefore, as to whether circadian control of sleep is mediated uniquely by the SCN, or do local brain and peripheral clocks also contribute? Beyond that, the influence of the circadian system (SCN and/or local clocks) on sleep-wake cycles remains to be established: does it only affect timing, or does it modify its temporal architecture and/or homeostatic responses (Gillette, 2004)?

Loss-of-function ablation has demonstrated the necessity of the SCN for circadian timing of sleep under DD, and its redundancy in global sleep homeostasis (Tobler et al., 1983; Mistlberger, 2005), although increased non rapid-eye-movement sleep (NREMS) in SCN-ablated mice suggests a broader role in sleep regulation (Easton et al., 2004). Loss-of-genetic function approaches have also been used to interrogate circadian sleep control. Perl/Per2-null mice have a defective TTFL and arrhythmic sleep-wake patterns under DD, but not LD (Shiromani et al., 2004). Moreover, sleep deprivation induces time of day-dependent expression of Perl and Per2 in the forebrain (Franken et al., 2007; Curie et al., 2013), but Perl/Per2-null mice show normal homeostatic regulation of the daily amounts of waking, NREMS, or REM sleep (REMS) (Shiromani et al., 2004). Conversely, CLOCK mutant mice show altered homeostatic regulation in the amount of sleep, with less NREMS in both LD and DD (Naylor et al., 2000). Finally, Cry1/Cry2-null mice lack TTFL function and so have...
no circadian pattern to sleep-wake in DD conditions, but show higher levels of NREMS and EEG delta power (Wisor et al., 2002). Further, when sleep-deprived under a LD schedule, Cry1/Cry2-null mice show less NREMS recovery than do wild-type (WT) mice, suggesting a requirement for Cry proteins in sleep homeostasis. Altogether, this highlights the disparity in the literature on the precise role(s) of the circadian system in regulating sleep/wake cycles.

Untangling the anatomical (SCN, extra-SCN) and genetic (Per, Cry, Clock) contributions to sleep regulation has therefore been challenging for loss-of-function approaches. Interpretation of results from clock gene mutants is constrained because global mutations compromise both the SCN and local clocks. Furthermore, as transcription factors, their encoded proteins may have non-circadian roles. Equally, SCN ablations disrupt neural circuitry and may compromise non-circadian processes (Mistlberger, 2005). Therefore, we chose a gain-of-function approach. Behavioural arrhythmia in clock mutant mice can be rescued by SCN grafting (Sujino et al., 2003) or by virally mediated genetic complementation targeted towards the SCN (Fuller et al., 2008; Maywood et al., 2018). We therefore tested the hypothesis that a molecularly competent SCN clock would be sufficient to co-ordinate phasing and stabilisation of sleep-wake cycles, leading to improved sleep-dependent memory in an otherwise clockless and arrhythmic mouse. To do this we used adeno-associated viral (AAV) vectors to express control EGFP or a Cry1::EGFP fusion targeted at the SCN of Cry1/Cry2-null mice, leaving local clocks across the brain and periphery dysfunctional.

**Materials and Methods**

**Animals and Housing**

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986, with local ethical approval (MRC LMB, AWERB). We used 4-6 months-old male WT mice and Cry1.Cry2 double knock-out mice (CDKO) (van der Horst et al., 1999), all on a C57/B6J genetic background. There were no significant differences in body weights at the time of surgery (WT =30.5 ±1.6g; CDKO =28.5 ±1.1g, n =5, 18 respectively). Mice were housed individually and their activity patterns were monitored continuously using running-wheels. Food and water were provided ad libitum. Mice were entrained to a 12h:12h light:dim red light cycle (LD) for at least 10 days before transfer to a schedule of continuous dim red light (DD) for 14 days for assessment of (ar)rhythmicity (DD1).
Following surgery (see below), mice were maintained on a 12L:12D photoschedule for recovery before transfer to a second period of DD (DD2). In all of our studies, Zeitgeber time (ZT) 0 denotes the time of lights-on and ZT12 lights-off under LD, whereas circadian time (CT) 0 denotes the start of subjective day and CT12 denotes the start of subjective night in DD, as evidenced by activity onset.

**Stereotaxic injection of AAV vector and implantation of EEG/EMG transmitters**

Mice were anaesthetised using isoflurane (induction 2-4%; maintenance 1%) with body temperature thermostatically controlled using a heating pad. Rimadyl was used for post-operative analgesia. Under aseptic conditions, the animals received bilateral stereotaxic injections (0.3μl/site) into the SCN (±0.25mm medio-lateral to Bregma, 5.5mm deep to dural surface) of a pan-cellular AAV-1 vector encoding pCry1-Cry1::EGFP (3.26x10^{12}cg/ml) for circadian expression of Cry1::EGFP fusion, driven by its minimal promoter (SCN\textsuperscript{Cry1}, n =9), or pCry1-EGFP control (4.8x10^{12}cg/ml; SCN\textsuperscript{Con}, n =9) (Maywood et al., 2013; Edwards et al., 2016). At the same time, a telemetric transmitter (TL11M2-F20-EET, Data Sciences International, St Paul, MN) connected to electrodes for continuous electroencephalography (EEG) and electromyography (EMG) recordings was implanted sub-cutaneously. Two screws were implanted above the dura (+1.5mm anterior to Bregma and +1.7mm lateral to Bregma, the second +1.0mm anterior and +1.7mm lateral to Lambda i.e., over the right hemisphere) around which the electrodes for measuring the EEG were placed and secured using dental cement (RelyX Unicem 2 automix; Henry Schein Animal Health, Dumfries, UK). The two EMG leads were inserted into the trapezius muscle ca. 5mm apart and sutured in place (Hasan et al., 2011; Lang et al., 2011). All mice were allowed 10-14 days of recovery following surgery. To confirm AAV targeting of the SCN, at the conclusion of the study, mice were culled and the brains dissected, fixed in 4% paraformaldehyde in phosphate buffer, cryopreserved overnight in 20% sucrose in PBS and then sectioned (40μm) on a freezing sledge microtome (Bright Instruments, UK). Confocal microscopy (Zeiss 780 inverted confocal system) of the native EGFP signal in control SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} groups was used to identify successful targeting of the SCN. Brain sections were mounted onto slides and coverslipped using Vectashield Hardset mounting medium with DAPI (Vector Labs, RRID:AB_2336788). Cell counts in SCN sections were assessed by the ratio of EGFP positive cells to DAPI positive cells using ImageJ. Due to inefficient targeting, two of the
nine animals were removed from further analysis in the SCN\textsuperscript{Cry1} group and one from the SCN\textsuperscript{Con} group.

EEG/EMG recordings and determination of vigilance states and spectral analysis

Transmitters were activated on the day before data collection and EEG/EMG were recorded continuously from the freely moving animals in both DD (3 days; >21 days post-surgery; Figure 1A) and LD (2 days; >30-37 days post-surgery) using Data Sciences International hardware and Dataquest ART v2.3 Gold software (Data Sciences International, ST Paul, MN). Vigilance states for consecutive 4s epochs were classified by visual inspection according to standard criteria: wakefulness (high and variable EMG signal, low-amplitude EEG signal), NREMS (high EEG amplitude dominated by slow waves, low EMG), and REMS (low EEG amplitude, theta oscillations and muscle atonia). Vigilance states were analysed offline using Neuroscore Software (v.2.1 Data Sciences International) with the EEG and EMG signals modulated with a high-pass (3dB, 0.5Hz) and a low-pass (50Hz) analogue filter and manually assessed. For both LD and DD conditions, continuous recordings were analysed and time spent in each vigilance state was expressed as a percentage of the total recording time over various intervals (1h to 24h). All DD recordings were started after at least 7 days of constant conditions. The mean duration of individual bouts of vigilance states was analysed for the 12h light/subjective day and 12h dark/subjective night periods, and between ZT6-12 on baseline day and following 6h of sleep deprivation (SD). The total amount of NREMS during SD was calculated as well as the latency to the first >25 epochs (100 seconds) of NREMS post 6h of SD. Two mice from the SCN\textsuperscript{Con} group were excluded from the sleep analysis (n=1 LD and DD; and n=1 from DD only) due to the lack of sleep-wake data. Spectral analysis was computed for consecutive 4s epochs by a fast-Fourier transform (frequency range: 0.5 -49.80Hz; resolution 0.24Hz; Hanning window function) on the EEG signal for wakefulness, NREMS and REMS. Genotypic differences were determined in DD over a complete circadian cycle, and expressed as either absolute EEG power or a percentage of total EEG power (i.e., relative EEG power) within all vigilance states for each mouse. Epochs containing EEG artefacts were discarded from the analyses. The time course of EEG delta activity (1-4Hz) during NREMS was also computed in 2h bins during LD and DD and post 6h SD and presented as absolute EEG power and/or relative (i.e., as a percentage of the mean) to 24h baseline for each mouse.

Sleep deprivation and novel object recognition test
Mice were recorded for a 24h baseline day followed by 6h SD and a further 18h recovery sleep. SD (ZT0-6) involved gentle procedures, i.e., introduction of novel objects such as nesting material, “fun tubes” and an initial cage change. Novel object recognition was tested in dim red light (<10lux) between ZT20 and ZT22, in a red Perspex box measuring 50x50x50 cm with an overhead camera (Logitech Carl Zeiss Tessar HD 1080P) placed above the arena. The mice were habituated to the arena without objects for 10 min, followed by an initial familiarisation session 24h later where they were exposed to two identical objects for 10 min (plain or patterned Perspex objects e.g., square, pyramid, oval, egg-cup all of similar sizes). After 24h, the mice were re-tested with one of the objects being replaced by a novel object of similar size. Animals are assumed to have remembered the familiar object if they spend significantly more time investigating the novel object during the test phase. Investigation was considered when the mouse nose-pointed within 1cm of the objects, but was not included if the mouse was climbing on the objects. The discrimination index (DI) was calculated as the difference between the time spent exploring the novel and familiar objects divided by the total time spent exploring the two objects (DI = (TN - TF)/(TN + TF)). The time animals spent exploring each object in both the familiarisation and test sessions were analysed offline from the video recordings, using software designed by the laboratory of Prof W. Wisden, Imperial College, London, UK (Yu et al., 2014) with the experimenter blind to the genotype of the animal.

**Experimental Design and Statistical Analysis**

Analyses were conducted in Prism version 9.1.2 for macOS X (GraphPad software). One or two-way ANOVA, with repeated measures where relevant, and with post-hoc Tukey’s, Dunnett’s or Sidak’s multiple comparisons tests were used to compare changes in sleep/wake parameters across genotypes. Where sphericity of the data was not assumed a Geisser-Greenhouse’s epsilon correction was used (and so fractional degrees of freedom values are used to compute a P value), as recommended in Prism. Data from the running-wheels were analysed using ClockLab (Actimetrics Inc., USA), running within Matlab (Mathworks, USA). Circadian period (chi-squared periodogram analysis; unpaired Student’s t-test between WT and SCN\textsuperscript{Cry1} mice) and mean DD activity profiles were calculated for each animal, where activity was averaged over 8-10 days of activity and organized into 0.1h bins. Comparisons of sleep-wake bouts, duration and frequency were made using ANOVA between genotypes and within genotype. In all cases, the experimental unit was an individual mouse. Male mice were used to avoid the confounding effect of oestrus cycles on circadian
behaviour patterns observed in female mice. Following attrition due to technical difficulties, the three treatment-group sizes were WT n =5; SCN$^{\text{Con}}$ n =7 and SCN$^{\text{Cry1}}$ n =7. Given the variance of our measures, these sample sizes would yield a statistical power of 90-95% (G*Power 3.1, University of Dusseldorf, Germany).

**Results**

*Local expression of Cry1 in the suprachiasmatic hypothalamus initiates circadian wheel-running behaviour in clockless mice*

Local, bilateral AAV-mediated nuclear expression of Cry1::EGFP in the suprachiasmatic hypothalamus of Cry1/Cry2-null mice was evident from the EGFP tag (Figure 1B-D). It was limited to the SCN and the immediately surrounding hypothalamus, in some cases extending into the anterior hypothalamic area and paraventricular nucleus (PVN) and/or posteriorly to the retrochiasmatic area and/or anteriorly to the medial preoptic area (Figure 1E). Cry1 did not extend into the dorsal or lateral hypothalamus, and there was no consistent pattern of extra-SCN expression between animals: the SCN was the only target common to all mice (see overlay in Figure 1E). The total proportion of transfected cells within the paired SCN, together, was 46.5±6.5% (n=7; range 24-74%), as assessed by the ratio of EGFP positive cells to DAPI positive cells using ImageJ, which is in agreement with our previous studies (Maywood et al., 2018; Brancaccio et al., 2019). WT mice exhibited robust circadian cycles of wheel-running behaviour, whereas Cry1,2-null mice targeted with EGFP (SCN$^{\text{Con}}$) were arrhythmic in DD before and after surgery (Figure 2A). In contrast, previously arrhythmic SCN$^{\text{Cry1}}$ mice exhibited robust circadian wheel-running behaviour following expression of Cry1::EGFP, as demonstrated previously (Maywood et al., 2018). The mean post-surgery activity profile between groups shows the significant circadian rhythmicity in both the wild-types and SCN$^{\text{Cry1}}$ mice, whereas activity in SCN$^{\text{Con}}$ mice was distributed evenly across the circadian cycle, phase-referenced to the prior LD cycle (Figure 2B). Furthermore, the period of 24.8 ±0.2h (n =7) (Figure 2C) in SCN$^{\text{Cry1}}$ mice was significantly longer than in WT (n =5; 24.1 ±0.1h) and diagnostic of a Cry1-driven TTFL (van der Horst et al., 1999) (Maywood et al., 2018). Finally, non-parametric analysis of locomotor activity confirmed the excellent circadian organisation of WT mice, and its disorganisation in SCN$^{\text{Con}}$ mice, which had low relative amplitude and stability and high variability (Figure 2D-F). In contrast, previously arrhythmic SCN$^{\text{Cry1}}$ mice showed robust circadian behaviour after surgery, with significantly...
improved organisation comparable to WT mice, and a non-significant trend for lower intra-
daily variability (see Legend Figure 2 for statistical analyses).

Rescue of circadian sleep/wake patterning in SCN\textsuperscript{Cry1} mice

Initiation of circadian control of wheel-running behaviour by AAV-mediated genetic
complementation made it possible to explore the degree of control to sleep mediated by
molecular competence in the suprachiasmatic hypothalamus. EEG spectral analysis showed
that under DD, in the absence of any masking or other effects of light, the different vigilance
states exhibited their characteristic neurophysiological features (Figure 3). Moreover, the
genotype had no significant effect on these parameters, confirming that the absence of Cry
proteins does not affect the core molecular and neural machinery that generates the states of
wakefulness, REMS and NREMS.

The total amount of wake did not vary between groups under LD (24h) or DD (circadian
cycle) (Figure 4A, B) (wake LD: 1xANOVA \(F_{2,16}=1.615, p=0.2298\); wake DD: 1xANOVA
\(F_{2,15}=3.26, p=0.0669\), but compared with WT controls, both SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice
exhibited more NREMS under LD, as reported (Wisor et al., 2002) (NREMS LD: 1xANOVA
\(F_{2,16}=4.7, p=0.024\); Tukey’s multiple comparisons test: WT v SCN\textsuperscript{Con} \(p=0.0347\), WT v
SCN\textsuperscript{Cry1} \(p=0.0397\), SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} \(p=0.9969\); NREMS DD: 1xANOVA \(F_{2,15}=2.2,
p=0.15\)). In DD, but not LD, both SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice showed a small elevation in
REMS (REMS LD: 1xANOVA \(F_{2,16}=2.9, p=0.08\); REMS DD: 1xANOVA \(F_{2,15}=5.7,
p=0.015\); Tukey’s multiple comparisons test: WT v SCN\textsuperscript{Con} \(p=0.0245\), WT v SCN\textsuperscript{Cry1}
\(p=0.0232\), SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} \(p=0.9967\)) (Figure 4C-F). These likely represent Cry-
dependent traits independent of the SCN.

We then examined the temporal distribution of sleep/wake. Under LD, WT mice showed
appropriate nocturnal wakefulness and more NREMS and REMS in daytime (Figure 4A, C,
E). Equally, both Cry\textsuperscript{I}, Cry\textsuperscript{2}-null groups, SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1}, had clearly defined
light/dark differences in wake and NREMS (Figure 4A, C), whereas SCN\textsuperscript{Con} mice did not
show significant light/dark differences in REMS in LD (Figure 4E). Despite the light/dark
organisation of the sleep/wake patterns, both the SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice spent
significantly less time in wake in the dark phase compared to WT (Figure 4A,C,E; as
assessed by post-hoc Sidak’s and Tukey’s multiple comparisons tests within genotype and
between genotypes, respectively, where there was a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 4 for details).

Under DD, WT mice again showed clear differences between subjective day (rest phase; higher levels of NREMS and REMS) and subjective night (active phase; more wake) (Figure 4B, D, F). In contrast, under DD, the SCN\textsuperscript{Con} mice showed no significant circadian patterning to vigilance states (Figure 4B, D, F; no significant rest/active differences within genotype assessed using 2xRMANOVA with post-hoc Sidak’s multiple comparisons tests- see Legend Figure 4 for statistical analyses). Unlike in LD, where the light imposed a level of organisation on the sleep/wake profiles, under DD the SCN\textsuperscript{Con} mice spent significantly more time in wake (Figure 4B) and less time in NREMS in the circadian day (Figure 4D), and significantly less time in wake and more NREMS and REMS in circadian night, compared to WT (Figure 4B, D, F; as assessed by post-hoc Tukey’s multiple comparisons between groups where there was a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 4 for details). In contrast, SCN\textsuperscript{Cry1} mice showed robust circadian organisation to the sleep-wake cycle, with clear subjective day and night differences comparable to those of WT controls across all vigilance states. Nevertheless, these mice did spend significantly less time in wake and more time in NREMS and REMS during the circadian night compared to WT (Figure 4B, D, F; as assessed by post-hoc Tukey’s multiple comparisons between genotypes where there was a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 4 for details). The loss of circadian organisation to sleep in SCN\textsuperscript{Con} mice was therefore reversed in SCN\textsuperscript{Cry1} mice, enabling the de novo establishment of a more WT-like organisation to the diurnal/circadian patterning to sleep/wake.

A finer-grained, 2h resolution, analysis of the 24h distribution of sleep/wake emphasised further the effects on sleep-wake patterning of global Cry1,2 deficiency and local restoration of clock function in the SCN region (Figure 5; as assessed by post-hoc Tukey’s multiple comparisons tests between genotypes where there was either a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 5 for details). Under LD, all groups exhibited a daily pattern of vigilance states, but with a reduced amplitude in the SCN\textsuperscript{Con} mice, likely reflecting their poor behavioural entrainment to, and/or masking by, the photoschedule (Figure 5A-D). Furthermore, in the second half of the light phase (ZT6-12), SCN\textsuperscript{Con} mice had significantly more wakefulness (+50min) and less NREMS (-40min) compared with WT.
mice. Expression of Cry1 in the SCN region reversed these deficits (1xANOVA ZT6-12: Wake amount: F_{2,16} =8.3, p<0.005; post-hoc Tukey’s multiple comparison test: WT v SCN^{Con} p=0.003; WT v SCN^{Cry1} p =0.36; SCN^{Cry1} v SCN^{Con} p=0.035; NREMS amount: F_{2,16} =7.2, p =0.0059; post-hoc Tukey’s multiple comparison test: WT v SCN^{Con} p=0.007; WT v SCN^{Cry1} p =0.55; SCN^{Cry1} v SCN^{Con} p=0.036). Conversely, at the end of the dark phase, WT mice showed more wake and less NREMS and REMS than both CDKO groups (Figure 5A-C). Finally, the amount of REM as a proportion of total sleep (TS= NREMS + REMS) was high in day and low at night in WT mice and this pattern was replicated by SCN^{Con} and SCN^{Cry1} (Figure 5D). Under LD, therefore, loss of Cry proteins altered the temporal distribution of vigilance states across the 24h, and this was partially restored by Cry1 expression focussed on the SCN.

The group differences between WT and SCN^{Con} mice were amplified in DD (Figure 5E-H, see Legend Figure 5 for 2xRMANOVA analyses between genotypes, although not possible to do statistics including the group SCN^{Cry1} as these mice have a different endogenous free-running period and so a different timescale). Whereas WT exhibited robust circadian patterning, SCN^{Con} mice failed to show any significant organisation of wake, NREMS or REMS across the circadian cycle (Figure 5E-G). Similarly, in WT mice the amount of REM sleep as a proportion of total sleep, which under DD is a measure of circadian control independent of changes in the absolute amount of wakefulness, was highly circadian, whereas it was not in SCN^{Con} mice (REM/TS: WT:1xANOVA F_{11,48} =5.8, p<0.0001; SCN^{Con}: F_{11,60} =0.9) (Figure 5H). Expression of Cry1 had a marked restorative effect on sleep/wake patterns in DD. The SCN^{Cry1} mice showed a more WT-like organisation of the sleep/wake cycle across circadian day and night (Figure 5E-G), although this rescue was not complete as they did have slightly less wakefulness (ca. 12%) and more NREMS (ca. 10%) and REMS (ca. 2%) in the circadian night (CT12-24) compared with WT. Levels of REMS/TS were higher overall in SCN^{Cry1} mice, but they nevertheless showed a significant circadian rhythm in the distribution of REMS/TS over the circadian cycle (REMS/TS: SCN^{Cry1}: 1xANOVA F_{12,78} =7.72, p<0.0001) (Figure 5H) and, as with the WT mice but not the SCN^{Con} mice, had significantly lower overall levels of REMS/TS in circadian night (total CT12-24) than in circadian day (total CT0-CT12) (Total REMS/TS in circadian day/circadian night: 2xRMANOVA: Interaction F_{2,15} =21.12, p<0.0001, Genotype F_{2,15} =5.18, p=0.0195, Time F_{1,15} =31.94, p<0.0001; post-hoc Sidak’s multiple comparison test: total in circadian day v total in circadian night: WT p=0.0056; SCN^{Con} p=0.38; SCN^{Cry1} p<0.0001). Together, the
LD and DD analyses confirm the interaction between light and the circadian system in
organising sleep/wake cycles (Tsai et al., 2009), and suggest that the suprachiasmatic
clockwork has a sleep-promoting/wake-suppressing effect in the second half of the light/rest
phase of the LD and DD cycles (ZT/CT 6-12). Nevertheless, in the absence of a lighting
cycle, a molecularly competent SCN is able to impose a circadian distribution of sleep-wake
patterns in an otherwise clockless mouse.

Consolidation of disrupted sleep/wake architecture in SCN^{Cry1} mice
Loss of circadian patterning to sleep-wake in SCN^{Con} mice and its restoration to WT-like
organisation in SCN^{Cry1} mice were indicators of the autonomous power of the
suprachiasmatic clock. We then examined its effect on sleep/wake architecture, as there was
no *a priori* reason to expect that a functional SCN could reinstate a WT-like structure. When
entrained to an LD cycle, WT mice showed a longer duration of wake in the dark (active)
phase, and correspondingly fewer episodes of NREMs and REMS at night and more in the
light (rest) phase (with no systematic changes in their duration) (Figure 6A, B; as assessed by
post-hoc Sidak’s and Tukey’s multiple comparisons tests within genotype and between
genotypes, respectively, where there was a significant Interaction and/or Genotype effect in a
2xRMANOVA; see Legend Figure 6 for details). In comparison, SCN^{Con} mice showed
weaker consolidation. They did not exhibit longer wake bouts during the dark (active) phase
than during the light (rest) phase in LD, with nocturnal wake bouts being shorter than in WT
mice, and NREMS bouts longer in both the light and dark phases, as reported previously for
Cry1, Cry2-null mice (Wisor et al., 2002). Nevertheless, in SCN^{Con} mice, even though bouts
of NREMS and REMS were more frequent in the light compared with the dark phase, they
had significantly fewer bouts during the daytime (rest phase) than WT mice (Figure 6B; as
assessed by post-hoc Tukey’s multiple comparisons between genotypes where there was a
significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 6 for
details). Local expression of Cry1 in the SCN region reversed the deficits of SCN^{Con} mice in
LD: the duration of wake bouts was significantly longer at night compared with SCN^{Con}
mice, and no different from WT measures, and the duration and number of NREMS and
REMS bouts was comparable to WT mice (Figure 6A, B; as assessed by post-hoc Tukey’s
multiple comparisons between genotypes where there was a significant Interaction and/or
Genotype effect in a 2xRMANOVA; see Legend Figure 6 for details).
The differences between groups in sleep consolidation were even more stark under circadian free-running conditions. WT mice retained their longer duration of nocturnal wake bouts (active phase) and more bouts of NREMS and REMS in circadian daytime (rest phase) (Figure 6 C, D; 2xRMANOVA with post-hoc Sidak’s multiple comparisons tests within genotype- see Legend Figure 6 for statistical analyses). SCN\textsuperscript{Con} mice, however, exhibited no significant rest/active differences in the duration or number for any vigilance state. The durations of wake bouts in circadian night were significantly shorter than in WT mice, and the number of nocturnal bouts of all three states were more numerous than in WT mice, reflecting the loss of consolidated wake in circadian night. Equally, SCN\textsuperscript{Con} exhibited significantly more episodes of wake in circadian daytime than did WT mice (as assessed by post-hoc Sidak’s and Tukey’s multiple comparisons tests within genotype and between genotypes, respectively, where there was a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 6 for details). Importantly, all of these deficiencies in SCN\textsuperscript{Con} mice were reversed by expression of Cry1 in the SCN and adjacent tissue. In SCN\textsuperscript{Cry1} mice, nocturnal wake bout duration was significantly longer than in subjective day, albeit not as long as in WT mice, and bouts of NREMS and REMS were significantly more numerous in circadian day than in circadian night (as assessed by post-hoc Sidak’s and Tukey’s multiple comparisons tests within genotype and between genotypes, respectively, where there was a significant Interaction and/or Genotype effect in a 2xRMANOVA; see Legend Figure 6 for details). Thus, global loss of Cry proteins destabilises sleep-wake structure on LD and even more so under DD, while restoring local expression of Cry1 in the SCN region, resulting in a molecularly competent SCN, reverses the loss of sleep-wake architecture and consolidates vigilance states in SCN\textsuperscript{Cry1} mice.

These differences in sleep-wake consolidation between groups were emphasised further by the number of transitions between wake-NREMS and NREMS-wake (there were no significant differences in transitions between NREMS-REMS, REMS-NREMS or REMS-Wake following a 1xANOVA in either LD or DD). In both LD and DD, SCN\textsuperscript{Con} mice showed more transitions than did WT mice, and this was reversed in SCN\textsuperscript{Cry1} mice in both LD (Figure 6E) and DD (Figure 6F) compared with WT and SCN\textsuperscript{Cry1} mice (as assessed by post-hoc Tukey’s multiple comparisons tests within genotype following a 1xANOVA; see Legend Figure 6 for details). This further confirms that rhythmic expression of Cry1 targeted to the SCN of Cry1/Cry2-null mice stabilises the sleep-wake over both the 24h LD cycle and across circadian time, consistent with the view that the suprachiasmatic pacemaker drives the
maintenance of wakefulness and the consolidation of sleep, as appropriate, across the LD cycle and across subjective day and night.

**Temporal control of sleep homeostasis in SCN<sup>Cry1</sup> mice**

To what extent can SCN-mediated consolidation of sleep timing and patterning affect homeostasis? EEG delta power (1-4Hz) during NREMS is a commonly used index of sleep homeostasis, with higher levels indicating increased sleep need. Under LD, absolute and relative EEG delta power in NREMS (normalised to total power to correct for electrode placement) in WT mice declined spontaneously across the inactive light phase and increased during the dark phase (Figure 7A, B), coincident with increased wake. This pattern was clearly under circadian regulation in DD (Figure 7C, D). In contrast, SCN<sup>Con</sup> mice revealed only a low amplitude pattern on LD, and no significant circadian pattern in DD (Figure 7C, D). As reported previously (Wisor et al., 2002), however, Cry1/Cry2-null mice exhibited higher levels of absolute NREMS EEG delta power, in particular during the light/inactive phase when compared with WT mice (Figure 7A, C). SCN<sup>Cry1</sup> mice had a significant rhythm in relative delta power not only in LD but also in DD (Figure 7B, D; as assessed by post-hoc Dunnett’s multiple comparisons tests against the time point before transition to lights off (LD)/activity onset (DD) following a 1xANOVA; see Legend Figure 7 for details). In LD, the peak amplitude of EEG delta power of SCN<sup>Cry1</sup> mice was the same as in WT, and although in DD the peak amplitude was reduced compared to WT, it was nevertheless phased appropriately (Figure 7D). This lower peak may reflect the slightly higher levels of NREMS in the early circadian active phase in DD compared to WT, a difference which was not evident in LD (Figure 5B, F). Finally, all groups showed a similar latency to first NREMS episode >100s on the dark-to-light transition (Latency to first NREMS episode >100s: WT =18.9 ±8.9min, n =5; SCN<sup>Con</sup> =18.0 ±6.8min, n =7; SCN<sup>Cry1</sup> =17.5 ±5.2min, n =7; 1xANOVA: F<sub>2,16</sub> =0.01, p=0.99). Together these data demonstrate that rescuing Cry1 in the SCN region restores rhythmic expression of NREMS EEG delta power in both LD and DD, confirming the appropriate phasing and organisation of sleep-wake across the 24h/circadian cycle. SCN-mediated circadian organisation and consolidation were therefore accompanied by appropriate dynamic signalling of sleep need.

Having shown that the suprachiasmatic clock can direct the circadian patterning and stabilisation of sleep-wake cycles, we next tested whether restoring a molecularly competent clock in the SCN region has an effect on the homeostatic regulation of sleep by measuring
sleep and the EEG responses following 6h sleep deprivation (SD) that started from lights onset (ZT0-6). SD was equally effective across the three groups, with no significant differences in the small amount of NREMS during SD (WT 9.8 ±3.5min, SCN\textsuperscript{Con} 13.6 ±2.7min, SCN\textsuperscript{Cry1} 7.4 ±2.5min, n =5, 7, 7 respectively; 1xANOVA: F\textsubscript{2,16} =1.61, p=0.2311) or the latency to sleep post-SD (time to first NREMS bout >100s duration, WT 12.9 ±6.8min; SCN\textsuperscript{Con} 10.4 ±3.4min; SCN\textsuperscript{Cry1} 10.3 ±3.7min; 1xANOVA: F\textsubscript{2,16} =0.18, p=0.8354). In the 2 hours immediately after SD, all groups showed a significant increase in delta power when NREMS occurred in that interval reflecting greater homeostatic sleep pressure (Figure 8A; no significant Interaction or Genotype effect following 2xRMANOVA showing that all genotypes responded to the 6h SD in the same way; see Legend Figure 8 for statistical analyses). The circadian timing system regulates sleep-wake cycles. The hypothalamic suprachiasmatic nucleus (SCN) is the principal circadian clock, but the presence of multiple local brain and peripheral clocks mean the respective roles of SCN and other clocks in regulating sleep are unclear. We therefore used virally mediated genetic complementation to restore molecular circadian functions in the suprachiasmatic hypothalamus, focussing on the SCN, in otherwise genetically clockless, arrhythmic mice. This initiated circadian activity-rest cycles, and circadian sleep-wake cycles, circadian patterning to the intensity of NREM sleep and circadian control of REM sleep as a proportion of total sleep. Consolidation of sleep-wake established normal dynamics of sleep homeostasis and enhanced sleep-dependent memory. Thus, the suprachiasmatic hypothalamus, alone, can direct circadian regulation of sleep-wake. Genotype did not, therefore, affect the neurophysiological capacity to sense and respond to sleep deprivation during subsequent NREMS when it did occur (Wisor et al., 2002). Furthermore, recovery from SD, compared to baseline, was not different between genotypes, insofar as accumulated sleep loss increased during SD, but then decreased at the same rate in all three groups over the subsequent 18h (Figure 8B). In addition, over the 6 hours immediately following SD (ZT6-12), the genotype of the mice did not significantly affect the change in NREMS bout duration compared to baseline, although the individual WT and SCN\textsuperscript{Cry1} mice all showed an increase following 6h SD, the response was more variable in the SCN\textsuperscript{Con} animals (Figure 8C; no significant Interaction or Genotype effect following 2xRMANOVA; see Legend Figure 8 for statistical analyses) (Figure 8C). By whatever mechanism, all three groups recovered lost sleep i.e., exhibited effective homeostasis. Notwithstanding overall comparability between genotypes, there were also informative differences. The time-course for the decline in EEG delta power in NREMS after SD was
It declined progressively across the light phase in WT mice, but more slowly in SCN<sup>Con</sup> mice and, unlike in WT mice, did not reach baseline levels during the light phase (Figure 8D, E, F). Expression of Cry1 in the SCN region corrected these deficits (Figure 8C, D, G). Thus, although there were no significant differences in the overall recovery of sleep loss after SD (Figure 8B), there were significant differences in its time-course during the light phase (ZT6-12) (Figure 8H-J). Whereas WT and SCN<sup>Cry1</sup> mice showed a sustained absence of wake (Figure 8H) and elevation of both NREMS and REMS in the light phase (Figure 8I, J), the SCN<sup>Con</sup> mice exhibited significantly less (ca. 70min) NREMS and more (ca. 60min) wakefulness between ZT6-12 following 6h SD (1xANOVA: wake: F<sub>2,16</sub>=6.72, p=0.0076, Tukey’s post-hoc multiple comparisons tests: WT v SCN<sup>Con</sup> p=0.017, WT v SCN<sup>Cry1</sup> p=0.9527, SCN<sup>Con</sup> v SCN<sup>Cry1</sup> p=0.017; NREMS: F<sub>2,16</sub>=5.76, p=0.013, Tukey’s post-hoc multiple comparisons tests: WT v SCN<sup>Con</sup> p=0.034, WT v SCN<sup>Cry1</sup> p=0.9995, SCN<sup>Con</sup> v SCN<sup>Cry1</sup> p=0.022). This suggests that SCN<sup>Con</sup> mice had a decreased sleep pressure and/or an inability to maintain consolidated NREMS at this phase of the LD cycle. Given that analysis of NREMS EEG delta power indicated that decreased sleep pressure was not the case in SCN<sup>Con</sup> mice (Figure 8A, D, F), consolidation of NREMS bout duration may have been limiting, as all WT and SCN<sup>Cry1</sup> mice increased the NREMS bout duration following 6h SD (Figure 8C), overall the SCN<sup>Con</sup> mice did not show a significant difference in this parameter ±6h SD (two-tailed paired t-test t<sub>6</sub>=1.15, p=0.2925) (Figure 8C). It may be that poor consolidation and/or less time in NREMS at this phase (ZT6-12) altered the time course to recovering sleep loss, and may reflect an interaction between the circadian and homeostatic processes regulating sleep-wake at the end of the light phase (Figure 8D, F, I). Loss of Cry proteins did not, therefore, globally affect neurophysiological mechanisms of homeostatic sleep recovery, but in SCN<sup>Con</sup> mice with an ineffective SCN clock, the dynamics of recovery were altered and the expression of Cry1 in the SCN region corrected this (Figure 8 C-J). Cry proteins and a competent SCN clock are not, therefore, necessary components of the fundamental sleep homeostatic mechanism, but they do regulate its time-course.

Rescue of sleep-dependent memory in the novel object test in SCN<sup>Cry1</sup> mice

To determine whether SCN-mediated circadian control over phasing and consolidation of sleep/wake cycles has consequences for brain function, we assessed cognitive performance in the Novel Object Recognition (NOR) task, a recognised sleep-dependent behaviour (Palchykova et al., 2006). There were no statistically significant differences between groups in time spent exploring the objects during training (Figure 9A, B) (2xRMANOVA:
WT mice demonstrated robust memory for the familiar object by spending significantly more time investigating the novel object when tested (positive Discrimination Index). In contrast, SCN\textsuperscript{Con} mice failed to discriminate between the novel and familiar objects, with an overall null preference between the two objects (Figure 9C). Thus, the global absence of Cry proteins compromised performance in a test of memory known to be sleep-dependent. The initiation of circadian competence in SCN\textsuperscript{Cry1} mice, and thereby organisation of sleep/wake cycles, resulted in all 7 SCN\textsuperscript{Cry1} mice showing a preference for the novel object (1xANOVA $F_{2,16} = 11.58$, $p=0.0008$; post-hoc Tukey’s multiple comparison test: WT v SCN\textsuperscript{Con} $p=0.0015$, WT v SCN\textsuperscript{Cry1} $p=0.7493$, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} $p=0.0035$). These results demonstrate that global loss of Cry1 proteins compromises NOR performance, and that a molecularly competent suprachiasmatic pacemaker can not only establish the necessary organisation and consolidation of sleep/wake, but also sustain sleep-dependent memory.

Discussion

To address the specific role of the SCN in sleep regulation we used genetically clockless Cry1/Cry2-null mice that in the absence of LD have no circadian patterning to sleep-wake cycles, poorly consolidated sleep and wake, compromised dynamics of homeostatic recovery sleep, and impaired sleep-dependent memory (van der Horst et al., 1999; Wisor et al., 2002; Wisor et al., 2008; Maywood et al., 2018). We initiated \textit{de novo} circadian rhythmicity locally to the SCN and closely adjacent hypothalamus by virally mediated expression of Cry1 (Edwards et al., 2016; Maywood et al., 2018). Importantly, the SCN was the only tissue successfully targeted in all mice, but for caution we refer to the SCN region. The rest of the brain, and the periphery remained circadian-incompetent. In agreement with previous studies we established circadian locomotor activity rhythms in SCN\textsuperscript{Cry1} mice (Maywood et al., 2018) comparable to the effect of WT SCN grafts in Cry1/Cry2-null mice (Sujino et al., 2003). This allowed us to test the contribution of the suprachiasmatic molecular clockwork to the temporal regulation of sleep. Genetic rescue established sleep-wake cycles that were appropriately phased and consolidated, and accompanied by improved performance in a test of sleep-dependent memory. On some measures SCN\textsuperscript{Cry1} mice did not reach those of wild-types, which may reflect the incomplete targeting of the paired SCN. Nevertheless, the data suggest that the circadian system promotes wake in subjective night and facilitates sleep, by
promoting its intensity and consolidation, in subjective daytime. Although the neural circuits
and neurophysiological processes underlying sleep homeostasis are not compromised by
global Cry1,2-deficiency, our data do illustrate an interaction between the homeostatic and
circadian mechanisms during the recovery from SD. We conclude that the SCN has
continuous influence on sleep-wake organisation across the circadian cycle and, directly or
indirectly, modulates sleep consolidation and homeostatic regulation. Further dissection of
the relevant neural pathways would require Cre-dependent targeting of specific sub-
populations, such as VIP and VIP- or dopamine-receptor-expressing cells ((Patton et al.,
2020; Hamnett et al., 2021; Maywood et al., 2021) within the SCN and/ or its targets.
Moreover, analysis of female mice, in which the SCN direct oestrous variability in arousal
state, may provide additional insight.

Overall sleep-wake distributions across the entrained and free-running cycles showed modest
differences between groups, with Cry1, Cry2-null mice (SCN\textsubscript{Con} and SCN\textsubscript{Cry1}) showing an
overall increase in the amount of NREMS in LD (Wisor et al., 2002). Studies using other
global circadian mutations/deletions or ablation of the SCN in otherwise intact animals
(Naylor et al., 2000; Easton et al., 2004; Laposky et al., 2005; Mistlberger, 2005) have
demonstrated either no differences or an increase/decrease in NREMS, making it difficult to
interpret whether any phenotypes are due to an extra-SCN circadian effect, or a more global
effect on the dynamics of the complex neural circuitry underlining the control of sleep-wake
states (Saper et al., 2010). Nevertheless, the SCN\textsubscript{Con}, but not the SCN\textsubscript{Cry1} mice, did show a
significant decrease in the amount of NREMS in the second half of the light/rest phase,
suggesting that restoring rhythmicity to the SCN enables promotion of sleep/inhibition of
wake at a time when the homeostatic pressure to sleep has declined. Furthermore, whereas
the rescued animals showed a WT-like consolidation of wake episodes in the dark/active
phase in both LD and DD, the SCN\textsubscript{Con} mice did not, and this was reflected in their reduced
amplitude or absence of rhythmic time-course for NREMS delta power in LD and DD,
respectively. Together these results show the mouse SCN clock has opposing influences on
sleep-wake organisation across the cycle: promoting wakefulness in the dark/subjective day
and sleep in light/subjective night.

The dynamics of sleep homeostasis were examined following SD, to assess whether there is
an interaction between circadian and homeostatic regulatory processes. Evidence suggests
influences of sleep homeostasis on the functioning of the circadian clock (Deboer et al.,
2003; Deboer et al., 2007; Schmidt et al., 2009), and that these processes can act independently (Tobler et al., 1983; Shiromani et al., 2004). Conversely, global circadian clock mutants can also show altered changes in NREMS EEG delta power in response to SD, consistent with a role for clock genes in sleep homeostasis (Naylor et al., 2000; Wisor et al., 2002; Laposky et al., 2005; Dijk and Archer, 2010; Curie et al., 2013). As discussed above, however, this does not necessarily indicate a role for the SCN in regulating homeostasis. Indeed, Cry1, Cry2-null mice, and mice lacking the Cry2 gene (SCN-Cry1) have an intact sleep homeostatic response (Wisor et al., 2002; Wisor et al., 2008), suggesting that the SCN is not necessary for the expression of the initial neurophysiological response to SD. Nevertheless, SCN-Con mice had an altered time-course in their recovery from SD, most notably between ZT6-12 when the SCN is exerting a sleep-promoting influence. This suggests a role, direct or indirect, for the circadian timing system in modulating the recovery from SD, and implies an interaction between the homeostatic and circadian processes, to ensure prolonged and consolidated sleep at a phase when sleep pressure is low. Studies in humans have similarly postulated a role for the circadian system in influencing sleep homeostasis (Lazar et al., 2015), although it remains to be established whether the central circadian clock in the SCN, and/or clocks in other brain areas underlie these mechanisms.

How might the SCN exert its effects? The sleep-wake regulatory circuit has been described as a “flip-flop” switch, whereby sleep-wake transitions are regulated by a reciprocal inhibition between sleep-promoting and wake-promoting nodes of the hypothalamus and brainstem (Saper et al., 2010). Multiple direct and indirect pathways from the SCN to both sleep- or wake-promoting nodes could therefore influence state-switching over the circadian cycle, which would be expected if the circadian clock has an ongoing active role in regulating sleep-wake (Deurveilher et al., 2002; Schwartz et al., 2011). Recent work has further confirmed that the circadian regulation of wakefulness is modulated by the SCN-paraventricular nucleus (PVN)-lateral hypothalamic (LH) pathway (Ono et al., 2020). Activation of LH-GABA neurons can exert direct synaptic control over the sleep-promoting-galaninergic neurons in the ventrolateral preoptic nucleus (VLPO) promoting arousal during NREMS in the light phase (Venner et al., 2019). In addition, a LH-thalamic reticular nucleus (TRN)-GABAergic-thalamocortical inhibitory circuit may be involved in the rapid arousal during NREMS-wake transitions (Herrera et al., 2016). Similarly, the loss of the widely projecting orexin/hypocretin neurons results in more frequent transitions into sleep and so would prevent prolongation of wake episodes (Hara et al., 2001). It may be that changes in
GABAergic/glutamatergic drive impose the rapid changes in state, whereas neuropeptides such as galanin/orexin act as neuromodulators influencing the stabilisation of sleep-wake states, and low (no?) amplitude and/or phasing of output from these cells underlie the sleep phenotypes in Cry1,Cry2-null, which are ameliorated following rescue of rhythmicity in the SCN^{Cry1} mice (Willie et al., 2003; Herrera et al., 2016; Venner et al., 2019). In addition, the SCN may also act indirectly; initiation of behavioural rhythmicity in SCN^{Cry1} mice will in turn regulate their metabolic demands, providing feedback from the periphery and/or brain regions. These could in turn influence, indirectly, the timing and homeostatic regulation of sleep (Ehlen et al., 2017; Northeast et al., 2020). For example, overexpression of BMAL1 in skeletal muscle (but not the brain) is reported to influence the daily amount of NREMS, but not the 24h pattern to sleep/wake, nor the homeostatic responses to SD (Ehlen et al., 2017).

Sleep-dependent memory was severely compromised in SCN^{Con} mice, consistent with other reports of cognitive impairment in Cry1/Cry2-nulls (Van der Zee et al., 2008; De Bundel et al., 2013). This could be a result of arrhythmia (in the SCN and/or hippocampal formation), or a non-circadian, molecular consequence of local Cry deficiency. Restoration in SCN^{Cry1} mice refuted the latter, emphasising the central importance of circadian organisation to cognitive function, be it in the SCN and/or locally in the hippocampal formation, and driven by the SCN. The role of the SCN may, however, be bivalent. In hamsters made arrhythmic using a light pulse paradigm, memory was impaired (Ruby et al., 2008), but the effect was reversed by SCN ablation (Fernandez et al., 2014), suggesting that a dysfunctional SCN signal is more cognitively debilitating than no signal at all. Similarly, in a mouse model of down syndrome, impaired object recognition is restored by SCN ablation (Chuluun et al., 2020). These observations raise the possibility that cognitive deficits might be mitigated by improving circadian amplitude when it is disrupted as, for example, in patients with Alzheimer’s disease (Hatfield et al., 2004) (Leng et al., 2019).

In conclusion, by adopting a gain-of-function approach, we have shown that the suprachiasmatic clockwork can impose temporal organisation on the sleep-wake cycle, facilitating circadian initiation and maintenance of wake, promoting sleep consolidation, the dynamics of homeostatic recovery, and sleep-dependent memory. Thus, expression of Cry proteins outside the SCN region is not necessary to sustain these processes. Our results therefore add to understanding of the relative contributions of the SCN, extra-SCN clocks and circadian clock genes in the temporal organisation of sleep and wake.
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Figure 1: Histological confirmation of the expression of pCry1-Cry1-EGFP in the suprachiasmatic hypothalamic SCN of Cry1,2-null mice used in the sleep studies

(A) Timeline showing the order of experimental procedures and interventions. The number of days between each stage is listed

(B) Fluorescence confocal images from the brains of 2 mice injected with an AAV (pCry1-CRY1::EGFP) to restore CRY1 into the SCN (SCN^{Cry1}), x20, scale bar=150um.

(C) Tiled (4x4) fluorescence confocal image from the same animal as in the upper panel of (A), x20, scale bar=150um.

(D) High power fluorescence confocal image from the same animal as in the upper panel of (A), showing the nuclear localisation of the GFP signal; x63, scale bar= 10um.

(E) Colour-coded depiction of the area of AAV-pCry1-CRY1::EGFP expression within the SCN and surrounding hypothalamus represented on coronal schematics modified from a mouse brain atlas (Paxinos and Franklin, 2001). Each colour represents a single mouse used in the sleep studies. The grey shaded area represents the SCN. The yellow shaded area in the overlay plot shows the targeted area common to all mice. (AC=anterior commissure; AH=anterior hypothalamus; MPOA=medial preoptic area; OC=optic chiasm; OT=optic tract; PVN=paraventricular nucleus; VLPO=ventrolateral preoptic nucleus).

Figure 2: Local expression of Cry1 in the suprachiasmatic hypothalamus initiates circadian wheel-running behaviour in clockless mice

(A) Double-plotted wheel-running traces from a wild-type (top left), two SCN^{Con} (middle and top right) and three representative traces from SCN^{Cry1} mice (bottom panel). Grey shaded areas represent darkness (LD; DD) before (DD1) and after (DD2) surgery (denoted by the red Asterix). The CDKO mice were arrhythmic pre-surgery, and the SCN^{Con} mice remained
arrhythmic post-surgery, whereas SCN\textsuperscript{Con} mice showed significant circadian rhythmicity post-surgery. The yellow bar highlights the 6h sleep deprivation and the green box the timing of the novel object recognition (NOR) testing.

(B) Mean activity (±SEM) counts over a circadian cycle in wild-type (n=5; blue), SCN\textsuperscript{Con} (n=7; green) and SCN\textsuperscript{Cry1} (n=7; magenta). (2xRMANOVA: Interaction F\textsubscript{90,630} =4.0, p<0.0001; Genotype F\textsubscript{2,14} =0.09, p=0.9138, Time F\textsubscript{45,630} =10.77, p<0.0001).

(C-F) Mean (±SEM) and individual values of period (C), relative amplitude (D), interdaily stability (E) and intradaily variability (F) in wild-type (n=5; blue), SCN\textsuperscript{Con} (n=6; green) and SCN\textsuperscript{Cry1} (n=7; magenta). Open bars are pre-surgery, shaded bars post-surgery. (Period: 2-tailed unpaired Student’s t-test: t\textsubscript{10} =4.03; p<0.005; 2xRMANOVA comparison of pre- and post-surgery measures between SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} genotypes: Relative amplitude: Interaction F\textsubscript{1,12} =29.6 p=0.0002, Genotype F\textsubscript{1,12} =70.35 p<0.0001; Surgery F\textsubscript{1,12} =56.17 p<0.0001; Interdaily stability: Interaction F\textsubscript{1,12} =7.0 p=0.021, Genotype F\textsubscript{1,12} =6.4 p=0.0265; Surgery F\textsubscript{1,12} =8.19 p=0.0143; Intradaily variability: Interaction F\textsubscript{1,12} =2.15 p=0.17, Genotype F\textsubscript{1,12} =0.036 p=0.852 Surgery F\textsubscript{1,12} =0.36 p=0.5586; 1xANOVA comparing WT vs. post-surgery of SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} genotype: Relative amplitude: F\textsubscript{2,16} =59.9 p<0.0001, post-hoc Tukey’s multiple comparisons tests WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.8419, SCN\textsuperscript{Cry1} v SCN\textsuperscript{Con} p<0.0001; Interdaily stability: F\textsubscript{2,16} =23.15 p<0.0001, post-hoc Tukey’s multiple comparisons tests WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.0006, SCN\textsuperscript{Cry1} v SCN\textsuperscript{Con} p=0.11; Intradaily variability: F\textsubscript{2,16} =6.3 p=0.0098, post-hoc Tukey’s multiple comparisons tests WT v SCN\textsuperscript{Con} p=0.0072, WT v SCN\textsuperscript{Cry1} p=0.1247, SCN\textsuperscript{Cry1} v SCN\textsuperscript{Con} p=0.2765).

***, **** p<0.01, p<0.0001; ++++, ++++, ++ p<0.0001, p<0.001, p<0.01 v WT; xxxx p<0.0001 v SCN\textsuperscript{Cry1}).

Figure 3: Local expression of Cry1 in the suprachiasmatic hypothalamus organises circadian sleep/wake patterning

(A-C) Mean baseline raw EEG spectral power in NREMS (A), REMS (B) and wake (C) over a circadian cycle (DD) (2xRMANOVA: NREMS: Interaction: F\textsubscript{120,900} =1.0, p=0.43, Genotype F\textsubscript{2,15} =1.12, p=0.3555, Frequency F\textsubscript{1,6,23.4} =143.2, p<0.0001; REMS: Interaction:
F_{120,900} = 1.02, Genotype F_{2,15} = 1.25, p = 0.3151, Frequency F_{1,9.25.9} = 98.69, p < 0.0001, p = 0.44; Wake: Interaction: F_{120,900} = 0.68, p > 0.99, Genotype F_{2,15} = 1.52, p = 0.2506, Frequency F_{1,6.23.4} = 55.74, p < 0.0001.

(Wake: Interaction: F_{120,900} = 0.68, p > 0.99, Genotype F_{2,15} = 1.52, p = 0.2506, Frequency F_{1,6.23.4} = 55.74, p < 0.0001).

(D-F) Mean baseline relative EEG spectral power (relative to total power) in NREMS (D), REMS (E) and wake (F) over a circadian cycle (DD). (2xRMANOVA: NREMS: Interaction: F_{120,900} = 0.52, p > 0.99, Genotype F_{2,15} = 1.28, p = 0.3064, Frequency F_{1.6.23.4} = 98.69, p < 0.0001; REMS: Interaction: F_{120,900} = 0.91, p = 0.7926, Genotype F_{2,15} = 0.4, p = 0.6768, Frequency F_{2.3,31.9} = 214.0, p < 0.0001; Wake: Interaction: F_{120,900} = 0.32, p > 0.99, Genotype F_{2,15} = 0.60, p = 0.5615, Frequency F_{1.6.23.4} = 160.5, p < 0.0001). Wild-type (n=5); blue; SCN^Con (n=6); green: SCN^{Cry1} (n=7); magenta.

Figure 4: Local expression of Cry1 in the suprachiasmatic hypothalamus organises circadian sleep/wake patterning.
post-hoc Tukey’s multiple comparisons test, day: WT v SCN\textsuperscript{Con} p=0.0174, WT v SCN\textsuperscript{Cry1} p=0.3224, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.2648, night: WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.0085, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.1521)

(B, D, F) Time spent in wakefulness (B), NREMS (D) and REMS (E) in DD (% time; mean ±SEM and individual points). Dark shaded bars on LHS show time over a circadian cycle (1xANOVA: REMS DD, post-hoc Tukey’s multiple comparisons test +p<0.05 v WT), with the circadian day/rest phase (clear) and circadian night/active phase (lightly shaded) bars on the RHS. There is clear circadian day (rest)/night (active) organisation in all vigilance states in both WT and SCN\textsuperscript{Cry1} mice but not only in the SCN\textsuperscript{Con} mice. (2xRMANOVA: Wake: Interaction: F\textsubscript{2,15} =35.74, p<0.0001, Genotype: F\textsubscript{2,15} =3.106, p=0.0744, Time: F\textsubscript{1,15} =117.2, p<0.0001, post-hoc Sidak’s multiple comparisons test day-night: WT and SCN\textsuperscript{Cry1} p<0.0001, SCN\textsuperscript{Con} p=0.9957, post-hoc Tukey’s multiple comparisons test, day: WT v SCN\textsuperscript{Con} p=0.0013, WT v SCN\textsuperscript{Cry1} p=0.4087, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0175, night: WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.0023, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0005; NREMS: Interaction: F\textsubscript{2,15} =25.3, p<0.0001, Genotype: F\textsubscript{2,15} =2.196, p=0.1457, Time: F\textsubscript{1,15} =80.98, p<0.0001, post-hoc Sidak’s multiple comparisons test day-night: WT p<0.0001, SCN\textsuperscript{Cry1} p= 0.0002, SCN\textsuperscript{Con} p=0.9996, post-hoc Tukey’s multiple comparisons test, day: WT v SCN\textsuperscript{Con} p=0.0005, WT v SCN\textsuperscript{Cry1} p=0.0522, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.1075, night: WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.0021, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0036; REMS: Interaction: F\textsubscript{2,15} =19.67, p<0.0001, Genotype: F\textsubscript{2,15} =4.735, p=0.0255, Time: F\textsubscript{1,15} =78.41, p<0.0001, post-hoc Sidak’s multiple comparisons test day-night: WT and SCN\textsuperscript{Cry1} p<0.0001, SCN\textsuperscript{Con} p=0.9999, post-hoc Tukey’s multiple comparisons test, day: WT v SCN\textsuperscript{Con} p=0.9361, WT v SCN\textsuperscript{Cry1} p=0.1428 SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0542, night: WT v SCN\textsuperscript{Con} p<0.0001, WT v SCN\textsuperscript{Cry1} p=0.0432, SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0144. *p<0.05, ***p<0.001, ****p<0.0001 within genotype; +, ++, ++++, +++++ p<0.05, <0.01, <0.001, p<0.0001 vs WT; x, xx, xxx p<0.05, <0.01, <0.001 vs SCN\textsuperscript{Cry1}. Wild-type (n=5); blue; SCN\textsuperscript{Con} (n=6); green: SCN\textsuperscript{Cry1} (n=7); magenta.

Figure 5: Local expression of Cry1 in the suprachiasmatic hypothalamus organises circadian sleep/wake patterning

(A-D) show the 2h profiles (mean ±SEM) of wakefulness (A), NREMS (B), REMS (C) and REMS/Total sleep (TS; D) in wild-type (n=5; blue), SCN\textsuperscript{Con} (n=7; green) and SCN\textsuperscript{Cry1} (n=7;
magenta) under a 12h light:12h dark photoschedule (2xRMANOVA wake: Interaction: F22,176 =2.23, p=0.0022, Genotype F2,16 =0.9038, p=0.4247, Time F11,176 =30.82, p<0.0001; NREMS: Interaction: F22,176 =2.0, p=0.0072, Genotype F2,16 =1.99, p=0.1673, Time F11,176 =31.17, p<0.0001; REMS: Interaction: F22,176 =2.85, p<0.0001, Genotype F2,16 =0.17, p=0.8497, Time F11,176 =26.4, p<0.0001; REM/TS: Interaction: F22,176 =0.93, p=0.55; Genotype: F2,16 =0.65, p=0.5377, Time F5,176 =12.51, p<0.0001; post-hoc Tukey’s multiple comparison tests *, **, *** P<0.05, 0.01, 0.001 WT vs SCN; x, xx P<0.05, <0.01 WT vs SCN

(E-H) show the 2 circadian hour profiles (mean ±SEM) of wakefulness (E), NREMS (F), REMS (G) and REMS/TS (H) in wild-type (n=5; blue), SCNCon mice (n=6; green) and SCN Cry1 (n=7; magenta) under free-running constant conditions (N.B. Not possible to do statistics including SCN Cry1 as these mice have a different endogenous free-running period and so a different timescale). (2xRMANOVA wake: Interaction: F11,99 =7.6, p<0.0001, Genotype F1,9 =5.073, p=0.0508, Time F11,99 =9.75, p<0.0001; NREMS: Interaction: F11,99 =7.5, p<0.0001, Genotype F1,9 =2.5, p=0.1481, Time F11,99 =9.3, p<0.0001; REMS: Interaction: F11,99 =4.6, p<0.0001, Genotype F1,9 =5.26, p=0.0474, Time F11,99 =7.5, p<0.0001; REM/TS: Interaction: F11,99 =2.8, p=0.0033, Genotype F1,9 =5.84, p=0.0388, Time F11,99 =6.68, p<0.0001; post-hoc Tukey’s multiple comparison test *, **, *** P<0.05, 0.01, 0.001 WT vs SCNCon). Grey shaded area represents darkness.

Figure 6: Local expression of Cry1 in the suprachiasmatic hypothalamus consolidates sleep/wake architecture

(A) Mean duration (s, mean ±SEM) of bouts in wakefulness, NREM and REM in 12h light (open circles) and 12h dark (closed circles) in wild-type (n=5, blue), SCNCon (n=7, green) and SCN Cry1 (n=7, magenta) mice in entrained (LD) conditions. Whereas the WT and SCN Cry1 mice have a significant increase in wake duration during the dark/active phase compared with the light/inactive phase, the SCNCon animals have significantly fewer nocturnal bouts and so do not have a significant LD organisation. (Episode duration: 2xRMANOVA Wake: Interaction: F2,16 =8.5, p=0.003; Genotype: F2,16 =1.97, p=0.1715, Time: F1,16 =61.38, p<0.0001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p<0.0001; SCNCon p=0.4397; SCN Cry1 p<0.0001; and Tukey’s (Genotype): Light: WT v SCNCon p=0.8393; WT
v SCN\textsuperscript{Cry1} p=0.8368; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p>0.9999; Dark: WT v SCN\textsuperscript{Con} p=0.0044; WT v SCN\textsuperscript{Cry1} p=0.7266; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0161; NREMS: Interaction: F\textsubscript{2,16}=0.0665, p=0.9359; Genotype: F\textsubscript{2,16}=4.393, p=0.0302; Time: F\textsubscript{1,16}=0.4075, p=0.5323; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p=0.9798; SCN\textsuperscript{Con} p=0.9991; SCN\textsuperscript{Cry1} p=0.8986; and Tukey’s (Genotype): Light: WT v SCN\textsuperscript{Con} p=0.026; WT v SCN\textsuperscript{Cry1} p=0.4817; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.2106; Dark: WT v SCN\textsuperscript{Con} p=0.037; WT v SCN\textsuperscript{Cry1} p=0.415; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.3408; REMS: Interaction: F\textsubscript{2,16}=3.185, p=0.0685; Genotype: F\textsubscript{2,16}=3.041, p=0.076; Time: F\textsubscript{1,16}=0.4075, p=0.5323.

(B) Mean number of bouts (mean ±SEM) of wakefulness, NREMS and REMS in 12h light (open circles) and 12h dark (closed circles) in wild-type (n=5, blue), SCN\textsuperscript{Con} (n=7, green) and SCN\textsuperscript{Cry1} (n=7, magenta) mice in LD. All three genotypes have a significant LD organisation of NREMS and REMS episodes, although the SCN\textsuperscript{Con} mice have fewer bouts of NREMS and REMS in the light period i.e., the rest period. (Episode number: 2xRMANOVA Wake: Interaction: F\textsubscript{2,16}=0.635, p=0.5427; Genotype: F\textsubscript{2,16}=1.17, p=0.335, Time: F\textsubscript{1,16}=0.93, p=0.341; NREMS: Interaction: F\textsubscript{2,16}=3.688, p=0.048; Genotype: F\textsubscript{2,16}=1.779, p=0.2006, Time: F\textsubscript{1,16}=99.36, p<0.0001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p<0.0001; SCN\textsuperscript{Con} p=0.0019; SCN\textsuperscript{Cry1} p<0.0001; and Tukey’s (Genotype): Light: WT v SCN\textsuperscript{Con} p=0.0091; WT v SCN\textsuperscript{Cry1} p=0.1234; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.4282; Dark: WT v SCN\textsuperscript{Con} p=0.9661; WT v SCN\textsuperscript{Cry1} p=0.9778; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.9985; REMS: Interaction: F\textsubscript{2,16}=5.573, p=0.0146; Genotype: F\textsubscript{2,16}=2.259, p=0.1367, Time: F\textsubscript{1,16}=115.3, p=0.001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p<0.0001; SCN\textsuperscript{Con} p=0.0019; SCN\textsuperscript{Cry1} p<0.0001; and Tukey’s (Genotype): Light: WT v SCN\textsuperscript{Con} p=0.0023; WT v SCN\textsuperscript{Cry1} p=0.0543; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.3507; Dark: WT v SCN\textsuperscript{Con} p=0.9347; WT v SCN\textsuperscript{Cry1} p=0.9904; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.969).

(C) Mean duration (s, mean ±SEM) of bouts in wakefulness, NREM and REM in subjective day (open circles) and subjective night (closed circles) in wild-type (n=5, blue), SCN\textsuperscript{Con} (n=6, green) and SCN\textsuperscript{Cry1} (n=7, magenta) mice in free-running conditions (DD). Both the WT and SCN\textsuperscript{Cry1} mice have a significant circadian organisation to wake duration but the SCN\textsuperscript{Con} animals do not, due to significantly fewer wake episodes during the circadian night/active phase when compared with the WT and SCN\textsuperscript{Cry1} mice. (Episode duration: 2xRMANOVA Wake: Interaction: F\textsubscript{2,16}=26.33, p<0.0001; Genotype: F\textsubscript{2,16}=7.14, p=0.0066, Time: F\textsubscript{1,16}=104.4, p<0.0001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p<0.0001;
SCN<sub>Con</sub> p=0.3901; SCN<sub>Cry1</sub> p=0.0008; and Tukey’s (Genotype): Light: WT v SCN<sub>Con</sub> p=0.9564; WT v SCN<sub>Cry1</sub> p=0.9421; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.7902; Dark: WT v SCN<sub>Con</sub> p<0.0001; WT v SCN<sub>Cry1</sub> p=0.0004; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.0472; NREMS: Interaction: F<sub>2,16</sub> =0.072, p=0.9306; Genotype: F<sub>2,16</sub> =2.17, p=0.1485. Time: F<sub>1,16</sub> =11.19, p=0.044; REMS: Interaction: F<sub>2,16</sub> =1.543, p=0.2458; Genotype: F<sub>2,16</sub> =0.5545, p=0.5857. Time: F<sub>1,16</sub> =0.089, p=0.792).

(D) Mean number of bouts (mean ±SEM) of wakefulness, NREMS and REMS in subjective day (open circles) and subjective night (closed circles) in wild-type (n=5, blue), SCN<sub>Con</sub> (n=6, green) and SCN<sub>Cry1</sub> (n=7, magenta) mice in DD. In the absence of light, the SCN<sub>Con</sub> mice no longer show a circadian organisation in the number of NREMS and REMS episodes, with significantly more episodes in the dark/active period compared with the WT mice. (Episode number: 2xRMANOVA Wake: Interaction: F<sub>2,16</sub> =0.8968, p=0.4266; Genotype: F<sub>2,16</sub> =4.52, p=0.0291, Time: F<sub>1,16</sub> =4.714, p=0.0464; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p=0.29569; SCN<sub>Con</sub> p=0.2809; SCN<sub>Cry1</sub> p=0.09948; and Tukey’s (Genotype): Light: WT v SCN<sub>Con</sub> p=0.0272; WT v SCN<sub>Cry1</sub> p=0.95879; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.1467; Dark: WT v SCN<sub>Con</sub> p=0.0232; WT v SCN<sub>Cry1</sub> p=0.1612; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.54; NREMS: Interaction: F<sub>2,16</sub> =19.42, p<0.0001; Genotype: F<sub>2,16</sub> =1.756, p=0.2065, Time: F<sub>1,16</sub> =80.45, p<0.0001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p<0.0001; SCN<sub>Con</sub> p=0.9967; SCN<sub>Cry1</sub> p<0.0001; Tukey’s (Genotype): Light: WT v SCN<sub>Con</sub> p=0.294; WT v SCN<sub>Cry1</sub> p=0.5757; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.0279; Dark: WT v SCN<sub>Con</sub> p=0.0056; WT v SCN<sub>Cry1</sub> p=0.058; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.4883; REMS: Interaction: F<sub>2,16</sub> =4.184, p=0.036; Genotype: F<sub>2,16</sub> =2.032, p=0.1656, Time: F<sub>1,16</sub> =42.26, p<0.0001; post-hoc multiple comparisons tests: Sidak’s (light-dark): WT p=0.0006; SCN<sub>Con</sub> p=0.4367; SCN<sub>Cry1</sub> p=0.0005; Tukey’s (Genotype): Light: WT v SCN<sub>Con</sub> p=0.9696; WT v SCN<sub>Cry1</sub> p=0.7682; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.8841; Dark: WT v SCN<sub>Con</sub> p=0.0103; WT v SCN<sub>Cry1</sub> p=0.4043; SCN<sub>Con</sub> v SCN<sub>Cry1</sub> p=0.119). (2xRMANOVA post-hoc Sidak’s multiple comparisons test (Light-dark difference within genotype) ****p<0.0001, ***p<0.001, **p<0.01; post-hoc Tukey’s multiple comparisons test (Genotype difference) ****p<0.0001 ***p<0.001, **p<0.01, *p<0.05).

(E) Mean number of transitions between sleep-wake states in LD reveals an increase in the numbers of transitions between wake-NREMS-wake in the SCN<sub>Con</sub> mice suggesting a lack of consolidated sleep/wake. (1xANOVA: LD: Wake-NREMS: F<sub>2,16</sub> =5.8, p=0.012; Tukey’s
multiple comparisons test WT v SCN\textsuperscript{Con} p=0.033; WT v SCN\textsuperscript{Cry1} p=0.9996; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.021; NREMS-wake: F\textsubscript{2,16} =5.5, p =0.0148; Tukey’s multiple comparisons test WT v SCN\textsuperscript{Con} p=0.0203; WT v SCN\textsuperscript{Cry1} p=0.788; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0481).

(F) Mean number of transitions between sleep-wake states in DD shows a significant increase in NREMS-wake transitions in the SCN\textsuperscript{Con} mice compared with both the WT and SCN\textsuperscript{Cry1} mice. Therefore, restoration of rhythmicity to the region of the SCN in SCN\textsuperscript{Cry1} mice restores sleep/wake consolidation. (1xANOVA: DD: Wake-NREMS: F\textsubscript{2,15} =1.1, p =0.3; NREMS-wake: F\textsubscript{2,15} =10.8 p=0.0012; Tukey’s multiple comparisons test WT v SCN\textsuperscript{Con} p=0.0044; WT v SCN\textsuperscript{Cry1} p=0.9995; SCN\textsuperscript{Con} v SCN\textsuperscript{Cry1} p=0.0021) *p<0.05, ** p< 0.01.

Figure 7: Characterisation of the effect of local Cry1 expression in the suprachiasmatic hypothalamus on delta power (1-4Hz) in NREMS in entrained and free-running conditions

(A) 2h profiles (mean ±SEM) of NREMS EEG raw delta power in WT, SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice in entrained (LD) conditions.

(B) 2h profiles (mean ±SEM) of NREMS EEG relative delta power (relative to total power) in WT, SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice in entrained (LD) conditions reveals both the WT and SCN\textsuperscript{Cry1} mice have significant, appropriately phased rhythms in NREMS EEG relative power, whereas the SCN\textsuperscript{Con} mice have a low amplitude rhythm. (WT:1xANOVA: F\textsubscript{11,47}=15.8, p<0.0001; post hoc Dunnett’s multiple comparisons test vs ZT10: p<0.0001 ZT2, p=0.1405 ZT4, p=0.7954 ZT6, p=0.9825 ZT8, p=0.9995 ZT12, p=0.9995 ZT14, p<0.0001 ZT16, p<0.0001 ZT18, p<0.0001 ZT20, p=0.0024 ZT22, p=0.0136 ZT24; SCN\textsuperscript{Con}: F\textsubscript{11,68} =3.9, p=0.0165; post hoc Dunnett’s multiple comparisons test vs ZT10: all not significant; SCN\textsuperscript{Cry1}: F\textsubscript{11,70} =14.5, p<0.0001, post hoc Dunnett’s multiple comparisons test vs ZT10: p=0.1387 ZT2, p=0.9811 ZT4, p=0.9993 ZT6, p>0.9999 ZT8, p>0.9999 ZT12, p=0.9815 ZT14, p<0.0001 ZT16, p<0.0001 ZT18, p<0.0001 ZT20, p=0.3489 ZT22, p=0.1845 ZT24.

(C) 2h profiles (mean ±SEM) of NREMS EEG raw delta power in WT, SCN\textsuperscript{Con} and SCN\textsuperscript{Cry1} mice in constant (DD) conditions.
Figure 8: Effect of rescue of rhythmic Cry1 expression in the suprachiasmatic hypothalamus on the homeostatic response to 6h sleep deprivation

(A) Individual changes in NREMS delta power (1-4Hz) in the first 2h of recovery sleep (ZT6-8; open circles) compared with baseline sleep (ZT6-8; closed circles) in WT, SCNCon and SCN^Cry1 mice shows a significant effect of the treatment (6hSD) with all 3 genotypes showing the SD was effective in initiating a significant homeostatic response, but no genotype or genotype x treatment effects (2xRMANOVA: Interaction: F_{2,16}=0.19, p=0.83; Genotype: F_{2,16}=1.19, p=0.33; Treatment (±6h SD): F_{1,16}=83.91, p<0.0001).

(B) No significant differences between genotypes in the cumulative decrease in total sleep time (NREMS + REMS) over the 6h of SD with subsequent partial recovery of sleep during the 18h post-SD in wild-type (n=5, blue), SCNCon (n=7, green) and SCN^Cry1 (n=7, magenta) mice. (2xRMANOVA: Interaction F_{22,176} =0.39, p=0.99; Genotype: F_{2,16} =0.43, p=0.65; Time: F_{2,24}=58.5, p<0.0001).

(C) Individual changes in NREMS bout duration (minutes) between ZT6-12 on baseline day (closed circles) and after 6h SD (open circles); although there was a significant effect of the treatment (6hSD) in increasing the duration of the NREMS bout duration there was no
genotype or genotype x treatment effect (2xRMANOVA: Interaction F_{2,16}=1.3, p=0.29; Genotype: F_{2,16}=2.25, p=0.14; Treatment (±6h SD): F_{1,16}=20.15, p=0.0004).

(D) Comparison of the time course of EEG delta power in NREMS following 6h of SD between WT (blue), SCN^{Con} (green) and SCN^{Cry1} (magenta) mice. The decline in EEG delta power in NREMS was significantly slower in SCN^{Con} mice when compared with WT and SCN^{Cry1} animals (2xRMANOVA: Interaction: F_{16,123}=2.33, p=0.0049, Genotype F_{2,16}=0.91, p=0.4235, Time F_{4,55}=27.22, p<0.0001; post-hoc Tukey’s multiple comparison test *, ** p<0.05, 0.01 WT vs SCN^{Con}; ++ p<0.01 SCN^{Con} vs SCN^{Cry1}).

(E-G) Time course (mean±SEM) of EEG delta power relative to baseline during NREMS on baseline day (closed circles) and following 6h SD (open circles; data replotted from 8D) in WT (E; n=5, blue), SCN^{Con} (F; n=7, green) and SCN^{Cry1} (G; n=7, magenta) mice (2xRM ANOVA: WT: Interaction F_{8,27}=14.63, p<0.0001, Time F_{8,32}=6.43, p<0.0001, Treatment (+6hSD) F_{4,4}=6.34, p<0.0001; SCN^{Con}: Interaction F_{8,4}=13.54, p<0.0001, Time F_{8,4}=7.54, p<0.0001, Treatment (+6hSD) F_{4,4}=6.0, p=0.0498; SCN^{Cry1}: Interaction F_{8,4}=17.84, p<0.0001, Time F_{8,4}=12.14, p<0.0001, Treatment (+6hSD) F_{1,6}=60.28, p=0.613; post-hoc Sidak’s multiple comparison test baseline v +6h SD *, **, ***, **** p<0.05, 0.01, 0.001, 0.0001).

(F-H) Percentage time (mean±SEM) spent in wakefulness, NREMS and REMS during 6h of sleep deprivation (SD) and 18h of recovery in WT (n=5, blue), SCN^{Con} (n=7, green) and SCN^{Cry1} (n=7, magenta) mice. The SCN^{Con} mice show significant increases in wake with concomitant decreases in NREMS and REMS during the recovery from 6h SD, in particular during the light phase (ZT6-12), and at the end of the dark phase (2xRMANOVA: Wake: Interaction F_{22,176}=3.05, p<0.0001, Genotype F_{2,16}=0.76, p=0.49, Time F_{6,89}=40.96, p<0.0001; NREMS: Interaction F_{22,176}=3.43, p<0.0001, Genotype F_{2,16}=1.54, p=0.2436, Time F_{6,87}=46.45, p<0.0001; REMS: Interaction F_{22,176}=2.97, p<0.0001, Genotype F_{2,16}=0.11, p=0.8982, Time F_{6,93}=24.01, p<0.0001; post-hoc Tukey’s multiple comparison test *, **, *** p<0.05, 0.01, 0.001 WT vs SCN^{Con}, x p<0.05 WT vs SCN^{Cry1}, + p<0.05 SCN^{Con} vs SCN^{Cry1}).

Figure 9: Cry1 expression in the suprachiasmatic hypothalamus rescues performance in the novel object test
(A) Protocol for the sleep-dependent memory test where mice are habituated to the test arena, 24h later mice investigate 2 identical objects then 24h later one object is replaced with a novel object. Training and testing were done during the dark/active phase between Zeitgeber time 20-22h.

(B) The amount of time (mean ±SEM) the mice spent on the objects during the training phase was not significantly different between groups (see Results).

(C) The Discrimination Index (mean ±SEM and individual points) revealed the SCN$^{\text{Con}}$ mice had an overall null preference for the novel object, whereas both the wild-type and SCN$^{\text{Cry1}}$ groups of mice all showed a significant preference for the novel object (see Results; 1xANOVA with post-hoc Tukey’s multiple comparison test: WT v SCN$^{\text{Con}}$ ** $p<0.01$, WT v SCN$^{\text{Cry1}}$ n.s., SCN$^{\text{Con}}$ v SCN$^{\text{Cry1}}$ ** $p<0.01$) (Wild-type (n=5) blue: SCN$^{\text{Con}}$ (n=7) green: SCN$^{\text{Cry1}}$ (n=7) magenta.)
### Experimental Protocols

| Protocol                  | Timing       |
|---------------------------|--------------|
| I2L:12D and DD pre-surgery assessment | 35-45 days   |
| I2L:12D surgery (transmitter implantation and AAV injection) | 21-28 days   |
| post-operative recovery (>14d) | 9 days       |
| DD EEG/EMG recording (after >7d of DD) | 3-6 days     |
| I2L:12D EEG/EMG recording LD-SD (after >9d) |            |
| I2L:12D Novel object recognition test |            |

### Imaging Data

#### (B) DAPI, GFP, Merge

#### (C) PVN, 3V, SON, OC

#### (D) Merge, GFP

#### (E) Bregma -0.1, -0.46, -0.94 Overlay

AC, MPOA, VLPO, SCN, LH, AH, OC
Spectral power ($\mu V^2/0.5Hz*10^4$)

- A) NREMS
- B) REMS
- C) Wake

- D)
- E)
- F)
A) Habituation Training ZT20-22 Test ZT20-22

10 minutes 10 minutes 10 minutes

24h 24h

Left Right

0

5

10 

15 

20

Time spent on object, s

A)

C)

B)

20

15

10

5

0

Time spent on object, s

Left

Right

C)

ns

Discrimination Index

WT SCNCon SCN^Cry1

-0.5

0.0 

0.5

0.5

0.0

-0.5