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The circadian *Clock* mutant mouse: impaired masking response to light

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Abstract Synchronization of an internal clock (entrainment) and a direct response to light (masking) are complementary ways of restricting activity of an animal to day or night. The protein CLOCK has an important role in the oscillatory mechanism of mammalian pace-makers. Our data show that it is also involved in masking responses. Mice with the *Clock/Clock* mutation reduced their wheel running less than wildtypes when given 1-h light pulses of light (2–1,600 lx) in the night. With dimmer lights (<2 lx), there were no significant differences between mutant and wildtype mice. Impaired masking responses to light in *Clock/Clock* mice were confirmed in tests with ultradian light–dark cycles (3.5:3.5 h and 1:1 h). Tests with pulses of light longer than 1 h revealed that, although the mutants responded more slowly to light, they sustained the suppression of activity over the course of the 3-h tests better than wildtypes.

Keywords Rhythms · Masking · Nonvisual photoreception · *Clock* mutation · Melanopsin

Abbreviations SCN: Suprachiasmatic nucleus · LD: Light–dark · DD: Constant darkness · PBS: Phosphate buffer saline

Introduction

The study of mutant and transgenic mice has been a major force in advancing our understanding of the molecular basis of endogenous circadian rhythms (Dunlap 1999; Panda et al. 2002a, b; Stanewsky 2003). For genes affecting rhythms in mammals, the point mutation in the *Clock* gene was the first to be characterized at both the behavioral and molecular level (Vitaterna et al. 1994; King et al. 1997). *Clock* is an important part of an autoregulatory transcription–translation negative feedback loop, although in certain conditions such as continuous light (LL) it can be compensated for by other mechanisms (Spoelstra et al. 2002).

The possibility has been raised that *Clock* might also be important on the input side of an endogenous oscillator in the suprachiasmatic nuclei (SCN). Shearman and Weaver (1999) found that the induction of *c-fos*, *per1*, and *per2* genes in the SCN by light was diminished in mice homozygous for the *Clock* mutation. They inferred that either altered functioning of the SCN was responsible, or there was a retinal change affecting input to the SCN.

Recent work has shown that, along with the classic rod and cone receptors in the outer retina, melanopsin containing cells in the inner retina are important for entrainment of the circadian oscillator in the SCN (Hattar et al. 2003; Panda et al. 2003). However, melanopsin cells also project to parts of the brain other than the SCN (Hattar et al. 2002), and in addition to being involved in entrainment, melanopsin is also involved in pupillary contraction and the acute suppression of locomotor activity by a light pulse (masking). Both entrainment, that is, synchronization of an internal clock which in turn controls when to be active, and a direct response to light serve to confine animals to nocturnal or diurnal niches. But they depend on different mechanisms in the CNS. Thus, masking responses to light are still present when circadian rhythmicity is lost,

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either following lesions (Redlin et al. 1999) or in arrhythmic mutant mice lacking cryptochromes (Mrosovsky 2001; Selby et al. 2000). However, both entrainment and masking entail detection of the levels of general irradiance. Therefore, if the blunted response of period genes in the SCN of *Clock/Clock* mice results from disruption of this irradiation detection system, deficits in such mice should be evident in their masking response to light. A hint that this is so comes from the finding of Vitaterna et al. (1994) that *Clock/Clock* mice, although predominantly nocturnal, have a significantly greater percent of their wheel running in the light portion of a 12:12 h light–dark cycle (LD) than do wildtypes. In another study, Spoelstra et al. (2002) found that LL leads to less suppression of wheel running in *Clock/Clock* mice than in wildtype controls; this is consistent with reduced masking. The present paper gives results using tests specifically designed to measure masking.

Materials and methods

Animals

Eight heterozygous *Clock* mice (*Clock/+*) (C57BL/6J background) were kindly donated by the laboratory of J. S. Takahashi (Northwestern University, Evanston, IL, USA). These four male and four female mice were mated to provide heterozygous, homozygous, and wildtypes for the *Clock* locus on the same background. Only the male offspring were studied in behavioral experiments.

When the offspring were between 5 weeks and 4 months old, they were transferred to cages (44×23×20 cm) equipped with wheels (diameter = 17.5 cm). Wheel revolutions were monitored with Dataquest III hardware and software (MiniMitter, Bend, OR).

Rhythms of wheel running in constant darkness (DD) were studied for at least 20 and up to 36 days. Homozygous mice (*Clock/Clock*) were identified by a loss of the circadian rhythm of wheel running, often preceded by a number of days with a long free-running period (Vitaterna et al. 1994; Herzog et al. 1998). Animals with a long tau that did not lose their circadian rhythm were not included. Wildtype (+/+) mice were identified by clear circadian rhythms with periods less than 24 h (Vitaterna et al. 1994). Food (#5001 rodent chow) and water were available ad libitum in all experiments. Mice had nesting material in their cages throughout the experiment. Room temperature during the experiments was ca. 20.5°C (range 19–23°C).

Apparatus for tests of masking

A room was equipped with two separate sets of lighting units. Entraining lights (Sylvania Octron 4,100 K/32 W light bulbs, two light bulbs above each set of four cages)

provided approximately 800 lx at cage level. A box with additional light sources (three Sylvania Octron 4,100 K/32 W light bulbs) was suspended ca. 1 m above each set of four cages. This light source could be obscured by the stepwise addition of neutral density filters (Rosco Cinegel) into the light path. Lights in these boxes were controlled independently from the entraining lights by the Dataquest III system from outside the room. Illumination levels were measured at the center of the cage floors for all cage positions, with a Hagner EX2 lux meter (Table 1).

Experiment 1: 1-h light pulses

Male mice (eight *Clock/Clock* and six +/+), ages 99–277 days, were transferred from the breeding room to cages with running wheels in another room. They were kept in LD 16:8 h for 20 days before tests with light pulses started. Illumination of the L portion of the LD cycle was about 800 lx. Homozygous and wildtype mice were placed in alternate positions on tables.

To quantify masking responses, 1-h pulses of light were given starting at zeitgeber time 14 (ZT 14; zeitgeber time 12 is defined as lights off), i.e. the light pulses started 2 h after the entraining lights were switched off. The number of wheel revolutions during the light pulse was compared to the number recorded in the same hour on the previous baseline day when it had been dark at that time; masking results are given in percent of the activity on the baseline day. The level of illumination was varied so that behavior of the two genotypes as a function of changing illumination could be tested. Tests for each illumination level took 3 days: on the first day, filters were installed in the lighting units and general maintenance carried out, on the second day (baseline day), the mice were left undisturbed, and on the third day (masking day), the masking light pulse was given. Sham pulses consisted of a 24-stops filter and an addi-

Table 1 Approximate illumination levels measured with a Hagner EX2 lux meter for entraining lights and light levels of the masking pulses used in the experiments

lux	Light level
800	Entraining light
1,600	Bright
1,000	0 stops
100	3 stops
50	4 stops
12	6 stops
2	9 stops
0.2	12 stops
0.04	15 stops
< 0.01*	18, 21, 24, 27 stops
0	Sham

Readings were made at cage level for all cage positions. Means of these readings were rounded. Readings with a * are for illuminations below the sensitivity of our lux meter; they are given in stop values only

tional opaque cardboard in the light path, bright 1,600-lx light pulses had no filter in the light path (0 stops) and in addition, the entraining lights were switched on during the masking pulse. Pulses were given in the following order: 1,000, 12, 0.2, <0.01 (18 stops), <0.01 (24 stops), sham, 2, 1,600, 0.04, <0.01 (21 stops), <0.01 (27 stops), and 100 lx.

Experiment 2: ultradian 3.5:3.5 h cycle

Single light pulses can only test masking at one specific time (e.g. in experiment 1, ZT 14–15). With ultradian LD cycles, such as an LD 3.5:3.5 h (Fig. 1), to which mice cannot entrain their circadian rhythmicity, in the course of 1 week all circadian phases can be tested for animals with a period close to 24 h (Redlin and Mrosovsky 1999a, b). For such ultradian cycles, a score of 50% of the activity during the L phases indicates no masking, and a score of 0% indicates complete masking. This is different from the scoring procedure in experiment 1, in which masking was measured as the percent of baseline activity, with 100% indicating no masking and 0% complete masking of baseline activity.

Altogether, nine *Clock/Clock* and eight *+/+* mice were studied, including the mice from experiment 1, plus an additional three mice. The former remained in their cages with wheels, the latter were given 18 days of adaptation to such cages before the start of the LD 3.5:3.5 h. At this time, ages of mice were 129–340 days. The LD 3.5:3.5 h provided ca. 50 lx during the L phases and was run for 2 weeks. A chi-square periodogram analysis (Clocklab, Actimetrics) was run (based on 13 d) to characterize rhythmicity. The significance level for the periodogram analysis was set at $P < 0.001$. For scoring masking, seven circadian days were analyzed for each genotype.

Experiment 3: 3-h light pulses

The procedures were the same as for experiment 1, except that the light pulse lasted 3 h, not 1 h, and only

three illumination levels were tested, 1,000, 100, and 12 lx; these were the levels at which light pulses suppressed activity with the 1-h pulses. The nine *Clock/Clock* and eight *+/+* mice from experiment 2 were studied. The mice were kept for 20 days after the end of the LD 3.5:3.5 h (experiment 2) in LD 16:8 h in cages with wheels before the 3-h pulses started. At this time, they were 164–375 days old.

By the time of this experiment, some mice were using their wheels less and inconsistently. It is not possible to calculate meaningful masking scores when baselines are erratic. Therefore, data for any animal making fewer than 100 wheel turns in any 1-h section of the 3-h period on baseline days were excluded completely from the analysis. On the basis of this criterion, three *Clock/Clock* and three *+/+* mice were excluded, leaving six *Clock/Clock* and five *+/+* mice contributing data.

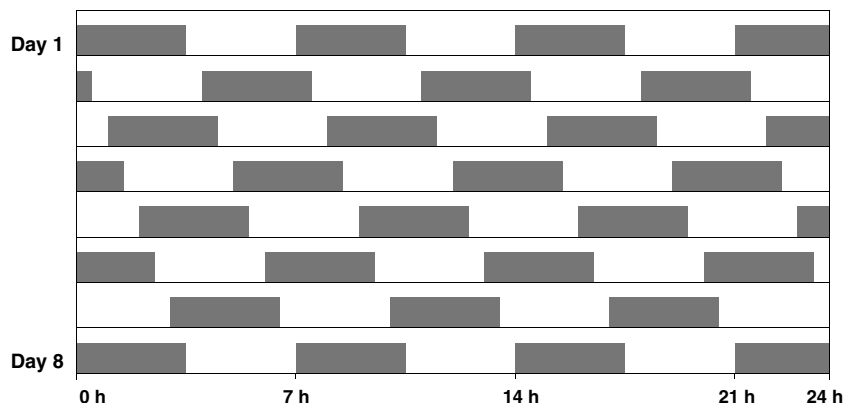
Experiment 4: ultradian 1:1 h cycle

The same mice from experiment 3 (nine *Clock/Clock* and eight *+/+* mice), age now 178–389 days, were kept 8 days in LD 16:8 h. They were then given an LD 1:1 h for 12 days to test whether or not the differences between the genotypes found with the 1-h pulse tests can be replicated under a cycle with repeated 1-h pulses. Again, as with the LD 3.5:3.5 h, illumination during the L phases was ca. 50 lx. The last 1 week of data were used to analyze masking, and all animals contributed data. As in experiment 2, the distribution of activity between the light and dark phases of the ultradian 1:1 h cycle was used as a measure for masking by light.

Melanopsin immunocytochemistry

Four *Clock/Clock* and four *+/+* mice, age 171–352 days, were used to compare the density of melanopsin-immunopositive ganglion cells in the retina. In short, animals were sacrificed and eyes removed and lifted with a suction pipette filled with phosphate buffer saline (PBS, pH 7.4). Eyes were fixed in 4% parafor-

Fig. 1 Diagram of an LD 3.5:3.5 h cycle. Gray bars indicate dark phases. Note that after 1 week initial phase relationships are regained



maldehyde at 4°C. The cornea was slit open to allow better penetration of the fixative. Fixed specimens were then shipped from Toronto to Baltimore for all further steps. After fixation, retinas were removed and flattened by four radial cuts. Retinas were rinsed thoroughly in PBS with 0.1% Triton X-100 followed by incubation in the blocking solution containing 5% inactivated serum/PBS with 0.1% Triton X-100 at room temperature for 1 h. Peptide-affinity-column purified rabbit anti mouse melanopsin antibody was then incubated with the free floating whole retinas at 1:100 dilution in the blocking solution with gentle agitation at 4°C overnight. After washing, the retinas were incubated with Alexa Fluor 488 conjugated antirabbit secondary antibody (Molecular Probes, OR, USA) 1:1,000 dilution in PBS with 0.1% Triton X-100 at 4°C overnight. For counting of immunopositive cells, only one eye from each animal was used. Areas of continuous retina undamaged by the radial cuts were studied, and the number of melanopsin immunopositive cells was counted by an observer blind to the genotype.

Statistics

Data are given as means \pm standard error (SE). The effect of genotype was assessed by two-tailed *t*-tests or repeated measures two-way ANOVA, if appropriate. The level of significance was set at $P < 0.05$.

Results

Experiment 1: 1-h light pulses

In wildtype mice, bright light pulses (1,600, 1,000 and 100 lx) led to a complete suppression of wheel running compared to the baseline scores (Fig. 2). In contrast, *Clock/Clock* mice reduced their activity levels, but did not completely suspend wheel running at these brightness levels. With dim light pulses, activity during the masking pulses actually increased over the baseline level (this phenomenon is referred to as “positive masking”, Mrosovsky 1999), both in wildtype *+/+* and *Clock/Clock* mice (Fig. 2). It is apparent that the response of the mice changed direction, from negative to positive masking, as the lights during pulses were made progressively dimmer. Therefore, the results were analyzed separately for the ranges of illumination resulting in negative masking (2–1,600 lx) and in positive masking (<0.01–0.2 lx). Negative masking responses to bright light pulses were significantly different between genotypes ($P < 0.0001$, repeated measures two-way ANOVA). The two-way repeated measures ANOVA detected no difference between the genotypes with dim light pulses (<0.01–0.2 lx; $P > 0.89$). This response of the *Clock/Clock* mice is consistent with a lowered sensitivity to bright light. Responses to dim light pulses were apparently not affected.

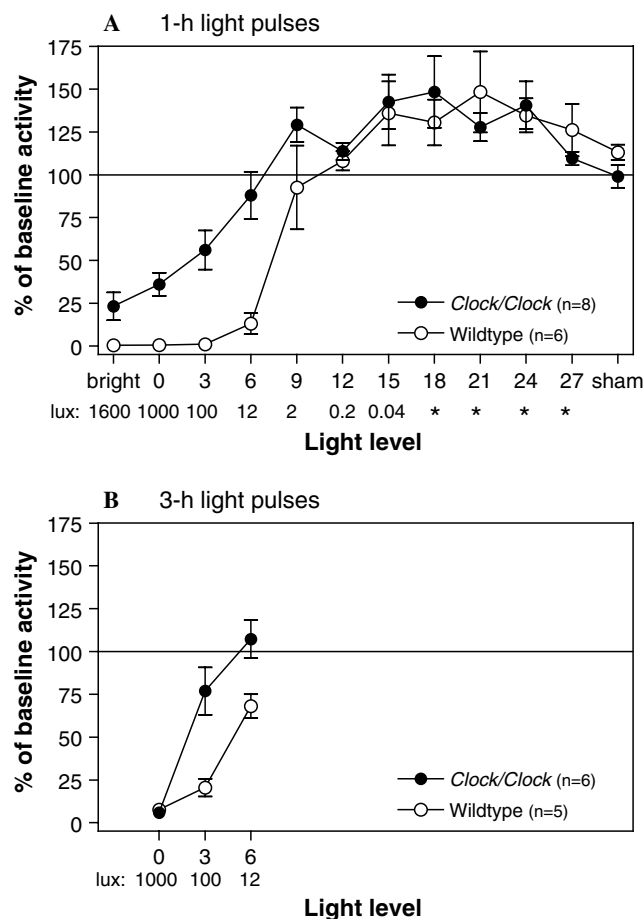


Fig. 2 Masking of *Clock/Clock* (filled circles) and wildtype mice (open circles), given as percentage of baseline activity (means \pm SE), as a function of illumination levels. **a** Experiment 1, 1-h light pulses given from ZT 14 to ZT 15; **b** experiment 3, 3-h light pulses, given from ZT 14 to ZT 17. Lux values are given for pulses within the range of our lux meter (Hagner EX2); * beyond measuring range (<0.01 lx), light level is indicated by the number of stops of neutral density filters in the light path. *Bright 0* stops plus additional entraining lights; *sham* with cardboard blocking light path

Experiment 2: ultradian 3.5:3.5 h cycle

Periodogram analysis showed significant peaks in the 7-h range, reflecting the external LD 3.5:3.5 h. In addition, a peak in the circadian range (mean 24.42 ± 0.09 h) was found in the wildtype mice. Eight and nine *Clock/Clock* mice had an additional peak with an average period of 31.46 ± 0.14 . Figure 3 gives representative actograms and periodograms.

To ensure that masking scores can be compared for animals with sizable differences in period, two analysis intervals were chosen and used for both genotypes. The analysis of the 49 3.5-h bins (=171.5 h/24.5 h=7 days) of the LD 3.5:3.5 h ensures that masking for the wildtype mice was scored for 7 circadian days, and the analysis of the 63 3.5-h bins (=217 h/31 h=7 days) covers the same number of circadian days for the mice with periods of ca. 31 h. In both cases, analysis was

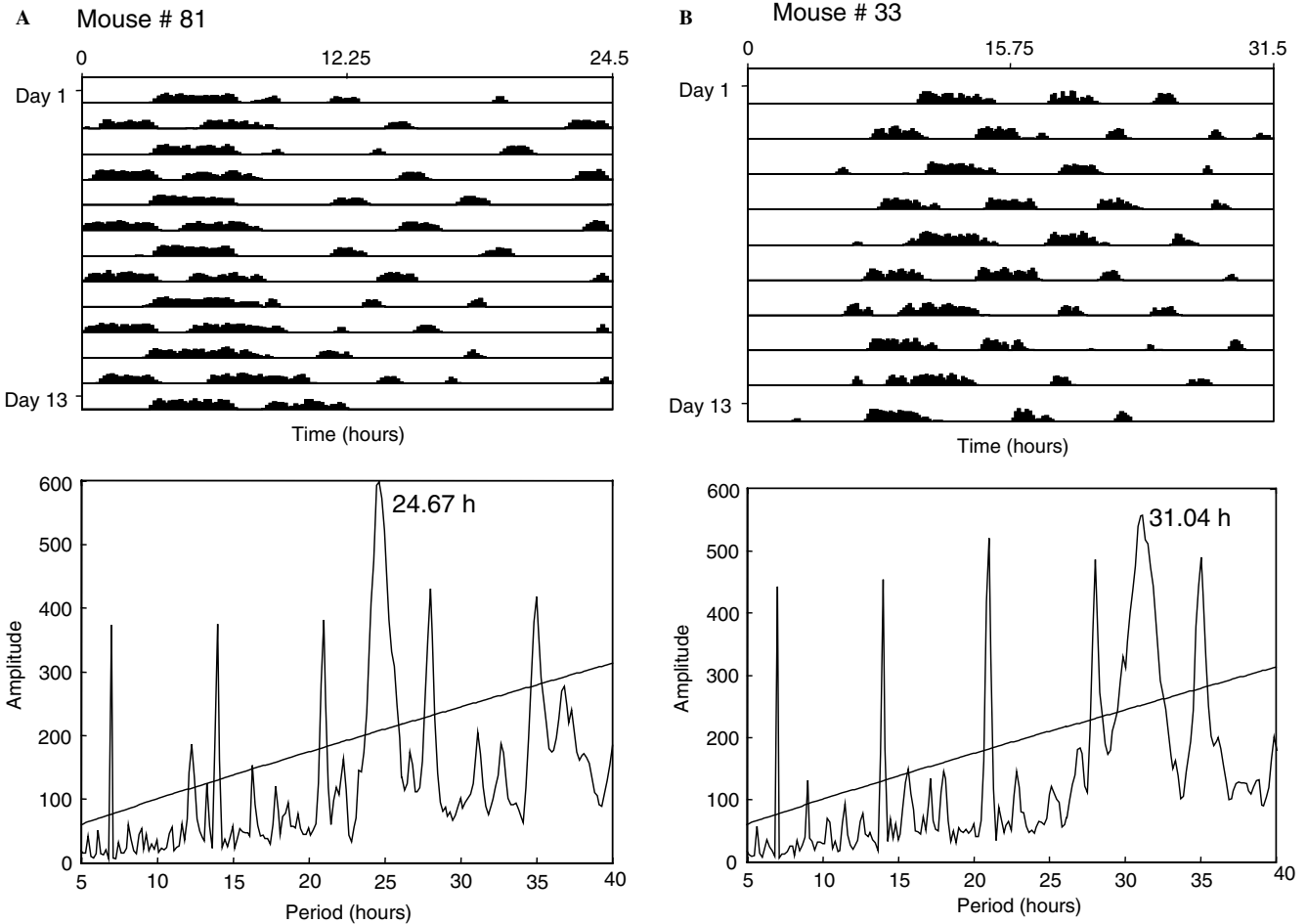


Fig. 3 Representative actograms and chi-square periodograms (Clocklab, Actimetrics) for one wildtype mouse (a) and one *Clock/Clock* mouse (b) during 13 days of the LD 3.5:3.5 h. Actograms use different time scales (24.5 h in a, 31.5 h in b) to adjust for the notable differences in period. Periodograms show peaks resulting from the LD cycle (multiples of 7 h) and an additional peak corresponding to the endogenous period (value given in the graph). Actograms give wheel revolutions for 10-min bins with the first quartile including 0–80 revolutions, etc

based on the last time bins available to meet these requirements within the 2 weeks in LD 3.5:3.5 h.

Wildtype mice undertook $16.9 \pm 2.4\%$ (time corresponding to 7 circadian days for wildtype) and $16.8\% \pm 2.6\%$ (time corresponding to 7 circadian days for *Clock/Clock*) of their wheel running during the L phases of the LD 3.5:3.5 h; this was not different from the scores of $15.5 \pm 3.5\%$ (time corresponding to 7 circadian days for wildtype) or $15.4 \pm 3.4\%$ (time corresponding to 7 circadian days for *Clock/Clock*) for the *Clock/Clock* mice (two-tailed *t*-tests, $P > 0.7$). Thus, both genotypes showed similar substantial masking to the repeated 50-lx light pulses and most wheel running took place during the dark phases. This result was unexpected, given the results of experiment 1 with sizable differences between the genotypes for light pulses in the range between 12 and 100 lx.

To further analyze the response of the mice during the LD 3.5:3.5 h, a time course of the masking response during the repeated 3.5 h of light was constructed. Wheel turns during 30-min bins (0–30, 30–60 min, etc.) of every 3.5-h light pulse during the 1-week test interval were added, and activity distribution between these 30-min bins and the respective 30-min bins during the 3.5-h dark phases were calculated (Fig. 4). For 30-min bins,

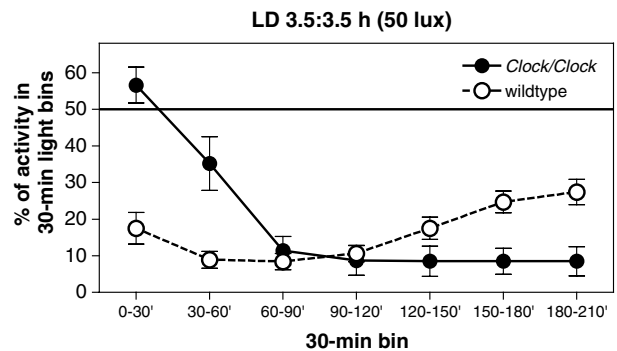


Fig. 4 Masking of *Clock/Clock* (filled circles) and wildtype mice (open circles) in experiment 3 during specific 30-min light bins of the LD 3.5:3.5 h as a percentage of the total activity during those specific 30-min bins of the light and dark portions combined, i.e. the score reflects the distribution of activity between e.g. the first 30 min of all light phases and the first 30 min of all dark phases

the score indicating no masking was again 50%. Even though the masking scores were remarkably similar between genotypes when data for all 30-min bins of the 3.5-h light phase are considered (repeated measures two-way ANOVA, $P > 0.43$), the time course (Fig. 4) reveals that the genotypes differed in their response pattern. This was reflected in the interaction of the repeated measures ANOVA between genotype and 30-min bins, which was highly significant ($P < 0.0001$). A separate analysis for the different parts of the curve gave significant differences between the genotypes for the first 90 min ($P < 0.001$) and also for the last 120 min ($P < 0.025$; repeated measures two-way ANOVA for both analyses). Therefore, *Clock/Clock* mice responded slower to the light pulses with a reduction of their wheel running, but the reduction was sustained longer than by the wildtype mice.

Experiment 3: 3-h light pulses

As with 1-h light pulses, *Clock/Clock* mice suppressed their wheel running significantly less than the wildtype mice in response to the 3-h pulses (Fig. 2b; repeated measures two-way ANOVA, $P < 0.01$). However, differences to the results with 1-h pulses were obvious. In particular, the 1,000-lx pulse resulted in stronger masking (lower masking percentage scores) in the *Clock/Clock* mice when pulses were 3 h long than when they were 1 h long (two-tailed t -test, $P < 0.01$).

Because the LD 3.5:3.5 h cycle results had revealed different time courses of the masking response, the 3-h pulses were also analyzed with 30-min bin resolution (Fig. 5). Masking scores reported here are the percentage of wheel running during the respective 30-min bin of the light pulse compared to the same 30-min bin on the baseline day, with a 0% score indicating complete suppression of wheel running and 100% indicating no change compared to baseline. At the 1,000-lx level, a similar effect as with the LD 3.5:3.5 h cycle was apparent (inset in upper panel of Fig. 5): a different response pattern was seen between wildtype and *Clock/Clock* mice. Overall, there was no difference between the genotypes (repeated measures two-way ANOVA, $P > 0.47$, interaction between genotype and light level: $P < 0.01$). However, when the first and second halves of this 3-h pulse were analyzed separately, genotypes could be distinguished statistically (repeated measures two-way ANOVA results for bins 0–30, 30–60 and 60–90 min: $P < 0.05$; for bins 90–120, 120–150 and 150–180 min: $P < 0.02$). Thus, *Clock/Clock* mice were slow in suppressing their wheel running to the light pulse but sustained the suppression until the end of the pulse. The wildtype mice, in contrast, showed a rapid and complete suppression of wheel running within the first 30 min of the 1,000-lx pulse but increased activity again towards the end of the pulse. Repeated measures two-way ANOVA on the 30-min bin results for the 100-lx pulse ($P < 0.05$) and for the 12-lx pulse ($P < 0.05$) confirmed a

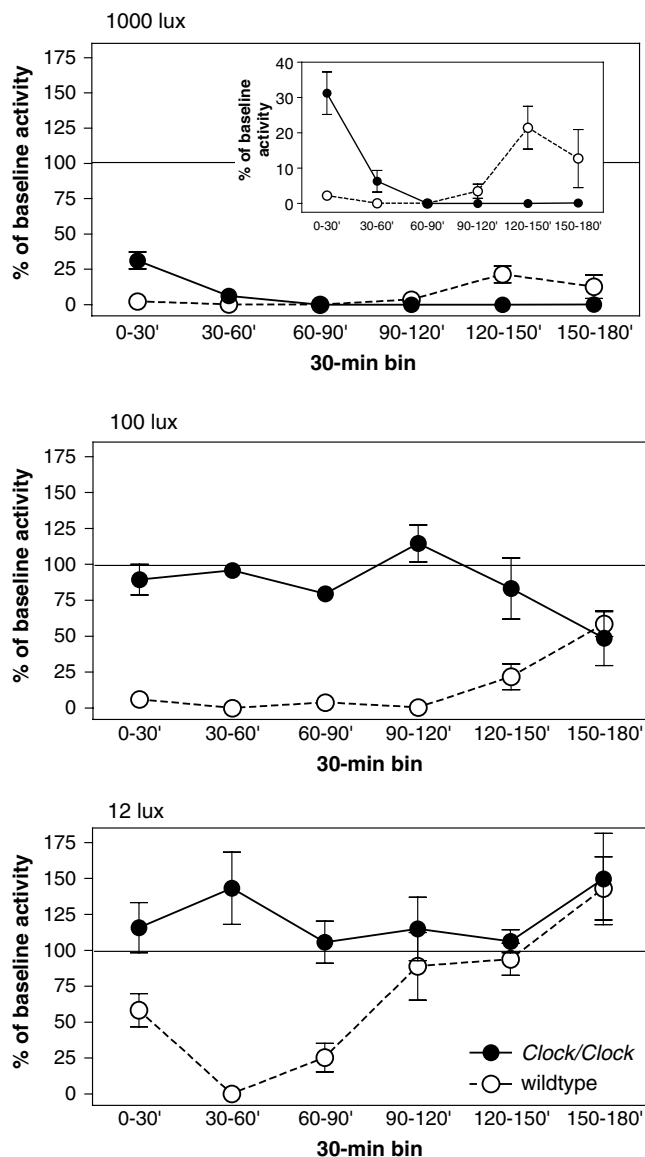


Fig. 5 Masking of *Clock/Clock* (filled circles) and wildtype mice (open circles) in experiment 3, given as percentage of baseline activity (means \pm SE) as a function of 30-min bins of the 3-h pulse for 1,000 lx (top), 100 lx (middle), and 12 lx (bottom). Inset in upper figure duplicates the 1,000-lx masking results with finer y-axis resolution

masking impairment in the *Clock/Clock* mice which showed little or no suppression of wheel running with 100- or 12-lx 3-h pulses. This is in contrast to the wildtype mice, which suppressed wheel running early on during the pulses but started to run again later in the 12- and 100-lx 3-h pulses (Fig. 5).

Experiment 4: ultradian 1:1 h cycle

Wildtype mice undertook $16.0 \pm 3.2\%$ of their wheel revolutions during the 1-h light phases of the LD 1:1 h, which was significantly less than the $28.7 \pm 2.3\%$ of the *Clock/Clock* mice (two-tailed t -test, $P < 0.01$). Masking

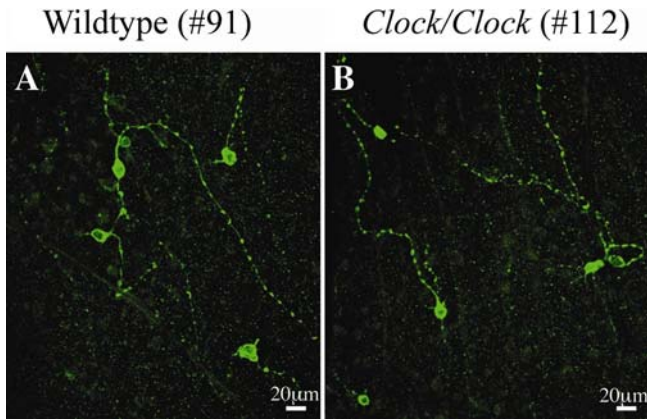


Fig. 6 Melanopsin-immunopositive cells in the retina of a wildtype (a) and a *Clock/Clock* (b) mouse

scores of the wildtype mice were similar under LD 1:1 h and under LD 3.5:3.5 h (paired two-tailed *t*-test, $P=0.58$). In contrast, masking of the *Clock/Clock* mice was poorer under the LD 1:1 h schedule ($28.7 \pm 2.3\%$ score) compared to the LD 3.5:3.5 h cycle ($16.0 \pm 3.6\%$ score; paired two-tailed *t*-test, $P < 0.005$). This result corroborated the impaired masking of the *Clock/Clock* mutant during the single 1-h pulses and during the early phases of the longer pulses. Furthermore, the similar masking scores for *Clock/Clock* and wildtype mice under LD 3.5:3.5 h may therefore be attributed to pulse duration, and it seems unlikely that the repetitive pattern of light stimulation contributed to the good overall masking of the *Clock/Clock* mice under the LD 3.5:3.5 h.

Melanopsin immunocytochemistry

Analysis of immunopositive ganglion cells in the retinas did not show any differences between the genotypes (Fig. 6). *Clock/Clock* mice had a melanopsin-immunopositive cell density of 12.90 ± 1.30 cells/mm²; this was similar to the density of 13.25 ± 0.88 cells/mm² for the wildtype mice ($P > 0.83$, two-tailed *t*-test).

Discussion

Sensitivity change probable

The impaired negative masking in *Clock/Clock* mice is consistent with there being a reduction in sensitivity to light. The modest suppression of wheel running in the mutant mice could be made greater by making the illumination during the 1-h light pulse stronger. Reduced sensitivity echoes the view of Shearman and Weaver (1999) that “it seems most likely that the *Clock* mutation may reduce sensitivity of the circadian system to light”. However, an increased phase-shifting response has been

reported for a single light level in *Clock* mutants (Spoelstra et al. 2002).

Decreased sensitivity to light is unlikely to be the result of gross losses of melanopsin-containing cells. The immunocytochemistry showed that melanopsin cells in the retina of *Clock/Clock* mice are comparable in number to those of wildtypes. Moreover, the shape of the function relating masking responses to illumination levels in the pulse is different in the *Clock* mice studied here from that found in melanopsin knockout mice. In *Clock* mutants, the curve appears to be shifted toward higher light levels compared with the wildtypes. In melanopsin knockout mice, the impaired masking responses appear to remain at a fairly constant level, despite increases in the light levels (Mrosovsky and Hattar 2003).

Time course of suppression of locomotion

The data from the 3-h pulses and for the 3.5:3.5 h LD cycle add information on the time course of the masking in the two genotypes. When the full 3-h or the full 3.5-h periods of light are analyzed, it becomes evident that the cessation of running after lights come on is more abrupt in the wildtypes; however, toward the end of these longer pulses, running in the wildtypes tends to pick up again. In the mutants, in contrast, the decrease in wheel running when the lights come on is slower, but once suppressed, locomotion tends to remain more or less suppressed until the end of the longer pulses. Thus, the time course of masking is altered in the *Clock* mutants. This means that when light pulses are used to test masking, differences between genotypes in their percentage masking scores, relative to comparable baseline periods without light, will depend on how long the pulse is, or what periods within a longer pulse are analyzed.

Apart from the methodological implications, in particular, a recommendation to include tests with longer pulses when studying masking, it is possible that the time-course data would be instructive about mechanisms. For example, it is interesting to compare the time course of masking in the *Clock* mutants to that found in the melanopsin knockout mice. In the latter, suppression of activity when lights come on appears to be as rapid as in wildtypes, but in the knockout mice, the activity gradually comes back over a course of about 2 h (Mrosovsky and Hattar 2003). Thus, the deficit in melanopsin knockout mice is one in the ability to maintain masking. It is in the initiation of masking that the *Clock* mutants are deficient. In contrast, maintenance of masking is as strong or stronger in the *Clock* mice than in wildtypes. This is a further reason, in addition to their normal complement of melanopsin cells, for thinking that masking deficits in *Clock* mutant mice are not the result of dysfunctional melanopsin.

What else then in *Clock* mutant mice might be dysfunctional? There appear to be two main players involved in masking: the classic rod/cone photorecep-

tors in the outer retina, and melanopsin-containing cells in the inner retina. If both of these are impaired, as in melanopsin knockout mice also lacking rod and cone transduction, then masking is absent (Panda et al. 2003; Hattar et al. 2003). But if either melanopsin is still present (as in *rd/rd* mice with degeneration of the outer retina), or rods and cones are still present (as in melanopsin knockout mice), then some masking responses to light remain (Mrosovsky et al. 1999; Mrosovsky and Hattar 2003). This supports the idea of redundancy in photoreceptors (Selby et al. 2000; Van Gelder 2003), but with emphasis on rods and/or cones and melanopsin, rather than on cryptochrome.

A further point, inferred from the rapid initial masking of melanopsin knockout mice but their inability to maintain the response, is that the classic photoreceptors are involved in the initial response, and melanopsin in its maintenance. With that in mind, it is tempting to attribute the opposite deficits seen in *Clock* mice (i.e. slow initial response, but strong maintenance) to deficits in the classical rod/cone system.

Rod/cone function apparently normal

Attractive as such an explanation may seem, there are at least two potential objections to attributing impairments in *Clock* mice to rod/cone dysfunction. The first is that deficits in masking are not seen in *rd/rd* mice with degeneration of the classic photoreceptors in the outer retina. Indeed, if anything, suppression of activity by light in such animals is enhanced (Mrosovsky et al. 1999, 2000). We have gone back to the data for these two papers and reanalyzed them in 10-min time bins. There is no indication that the masking response is slow in these animals.

A second reason for not invoking a deficit in rod function to account for slow masking in the *Clock* mutant mice comes from consideration of the positive masking responses to dim lights. As is evident in Fig. 2, when pulses of dim light are given, locomotor activity increases rather than decreases, i.e. there is positive masking (see Mrosovsky 1999 for terminology). This positive masking is dependent on the integrity of the outer retina, as it is absent or greatly attenuated in retinally degenerate mice (Mrosovsky et al. 1999, 2000). Because positive masking—unlike negative masking—is also absent in animals with lesions at the level of the geniculate (Redlin et al. 1999; Edelman and Mrosovsky 2001), it seems likely that it depends on form vision and thus is a different phenomenon from negative masking. Being able to perceive the outlines of forms, even if faintly, may permit the mouse to move around more readily than in complete darkness, thus resulting in increased activity in dim lights. Whether this speculation is correct or not, there is a lack of positive masking in retinally degenerate mice. Therefore, the fact that positive masking is normal in *Clock* mutant mice may be taken as an indication that functioning of

the outer retina is normal or at least not greatly impaired.

Perhaps there are yet other photopigments involved. The loss of masking in mice lacking melanopsin and rod and cone transduction shows only that functioning of these systems is necessary for masking. It does not preclude a role for other pigments in an intact animal. It may be that the *Clock* mutation affects such a role.

Interaction between retinal systems

Alternatively, it may be that deficits in masking in *Clock* mice are not explicable in terms of a dysfunction of an entire class of photoreceptive cells. They may depend instead on changes in the interactions between different retinal systems. Rods and cones play a fundamental role in vision, but they also serve as photoreceptors involved for irradiance detectors, as shown by studies in which entrainment, masking responses, and pupil contraction persist, even if in impaired form, in melanopsin knockout mice (Panda et al. 2002a, b; Lucas et al. 2003; Mrosovsky and Hattar 2003). A double role for classic photoreceptors is consistent with anatomical data demonstrating that some rods project to melanopsin positive ganglion cells, and others to melanopsin negative cells (Belenky et al. 2003). If the *Clock* mutation exclusively affected the connections of rods that project to melanopsin positive cells, altering sensitivity or kinetics, or affected the processing of such rod-originating irradiance signals, then a situation could arise in which an irradiance detection role of rods is impaired, leaving uncompromised input to the classical visual system from other rods. This could leave positive masking intact in the presence of impaired negative masking. The impairment in irradiance system might be one in the shaping and modification of irradiance detection signals at a stage past the photoreceptors. Such a role in modifying the primary sensory signal has been proposed for cryptochromes (Van Gelder 2003). It should be kept in mind that *Clock* is widely expressed in the CNS (strongest expression in hypothalamus and eye) and other tissues (heart, lung, liver, intestine; King et al. 1997). Although the deficit seen here appears to be primarily one of reduced sensitivity, it is possible that there are some effects on responsiveness. Unfortunately, retino-reipient areas of the brain that are necessary for masking of locomotion have not yet been identified, although it has been shown that a number of areas are not essential, namely the SCN, the intergeniculate leaflet, dorsal lateral geniculate, and visual cortex (Redlin and Mrosovsky 1999a, b; Redlin et al. 1999, 2003; Edelman and Mrosovsky 2001).

Conclusion and context

Although much remains to be clarified, the present data establish that a mutation, with a central role in the

molecular oscillatory mechanism (Gekakis et al. 1998) and which was originally found by screening for rhythm anomalies, also has an influence on the direct masking response to light. A dual role for *Clock* as a component of the molecular oscillator and in influencing direct effects of light on locomotor activity has also been reported in *Drosophila* (Kim et al. 2002). Masking responses, like phase-shifting responses, require detection only of overall irradiance. Not only is there functional overlap between these responses, in that both result in confining behavior to a particular portion of the LD cycle—darkness in the case of mice—but also there may be physiological and molecular commonalities.

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