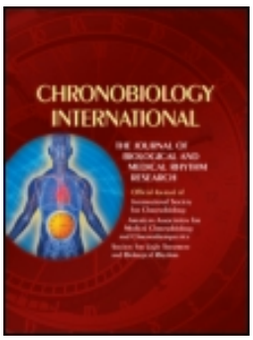

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Impaired Masking Responses to Light in Melanopsin-Knockout Mice

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ABSTRACT

There are two ways in which an animal can confine its behavior to a nocturnal or diurnal niche. One is to synchronize an endogenous clock that in turn controls the sleep–wake cycle. The other is to respond directly to illumination with changes in activity. In mice, high illumination levels suppress locomotion (negative masking) and low illumination levels enhance locomotion (positive masking). To investigate the role of the newly discovered opsin-like protein melanopsin in masking, we used 1h and 3h pulses of light given in the night, and also a 3.5 : 3.5h light–dark (LD) cycle. Mice lacking the melanopsin gene had normal enhancement of locomotion in the presence of dim lights but an impaired suppression of locomotion in the presence of bright light. This impairment was evident only with lights in the order of 10 lux or brighter. This suggests that melanopsin in retinal ganglion cells is involved in masking, as it is in pupil contraction and phase shifts. Melanopsin is especially important in maintaining masking responses over long periods.

Key Words: Melanopsin; Irradiance detection; Masking; Rhythms; Retina; Diurnality.

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INTRODUCTION

Mice with outer retina damage are capable of entraining their circadian rhythms to light–dark (LD) cycles and of phase shifting in response to pulses of light (Ebihara and Tsuji, 1980; Foster et al., 1991). This has led to a search for non-rod non-cone receptors mediating responses of the circadian system to photic input. However, it has been realized that a number of other responses to light are also spared following retinal degeneration. These include pupillary contraction (Keeler, 1927), direct suppression of locomotion by light, i.e., masking (Aschoff, 1960; Mrosovsky, 1994), preferences for dark areas (Mrosovsky and Hampton, 1997), probably anterior chamber associated immune deviations (review in Van Gelder, 2001), and even arbitrary avoidance responses using light as a cue (Mrosovsky and Salmon, 2002). Therefore, it is likely that non-rod non-cone receptors are not dedicated only to the synchronization of rhythms but have a more general function in irradiance detection.

Recent work has implicated melanopsin retinal ganglion cells in the inner retina as strong candidates for irradiance detectors (Provencio et al., 1998, 2002; Berson et al., 2002; Hattar et al., 2002). Melanopsin cells project to the suprachiasmatic nucleus, intergeniculate leaflet, pretectum, and various other brain areas (Hattar et al., 2002), making melanopsin a good candidate for detection of general irradiance. Melanopsin-knockout mice have diminished phase shifting, diminished period lengthening in constant light (Panda et al., 2002; Ruby et al., 2002) and diminished pupillary constriction in response to light (Lucas et al., 2003). The phase shifting and period lengthening responses are presumably dependent on projections to the suprachiasmatic nucleus, and the pupillary response on projections to the pretectum. The central areas involved in masking of locomotion light are unknown, but if melanopsin cells provide the input, then masking should also be impaired in melanopsin-knockout mice. The present paper tests this proposition.

MATERIALS AND METHODS

Animals and Housing Conditions

Melanopsin-knockout mice (*mop* $-/-$) were produced as described in Hattar et al. (2002). Genotype was validated by Southern blotting and PCR (Hattar et al., 2002). In addition, the intrinsic photosensitivity of melanopsin-positive cells was lost in melanopsin-knockout mice (Lucas et al., 2003). Twelve melanopsin knockouts and 10 wildtype controls (*mop* $+/+$) of the same C57BL6/129 strain were studied. These mice, all males, were bred at the Johns Hopkins University School of Medicine (Baltimore, MD) and taken to the University of Toronto at the age of 124–179 days (21.5 ± 4 weeks). Some of these mice had previously been tested for pupillary responses to dim lights (Lucas et al., 2003). Food (Purina Mills #5001 chow) and water were provided ad libitum; nesting material (nestlets) was also given.

On arrival in Toronto, the mice were kept in a 16 : 8h LD cycle, with ca 750 lux in the L phase as measured with a Hagner E2X luxmeter. After 3 days they were separated into single cages; 8 days after arrival they were placed in cages ($44 \times 23 \times 20 \times$ cm) equipped with running wheels (17.5 cm diameter). Revolutions were monitored with Dataquest III

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hardware and software. Room temperature was $22 \pm 3^{\circ}C$. The mice were kept in accordance with the guidelines of the Canadian Council on Animal Care. All data from one *mop* $-/-$ mouse were excluded because its activity at night was low and erratic.

Starting after 27 days of adaptation to the wheels, masking was assessed in three different ways. The first experiment (1h pulses) was designed to discover at what range of illuminations interesting effects occurred. The other two experiments investigated the generality of these observations using different test methods.

1h Light Pulses

To examine the masking effects of light during the night, sets of three fluorescent tubes (Sylvania Octron 32 watt 4100 K) were positioned above the cages; these lights were controlled separately from the entraining lights, and were programmed to come on for 1h, starting at Zeitgeber time (ZT) 14, i.e., 2h after dark onset (ZT 12 = dark onset). The illumination level during the pulses was altered by adding neutral density filters (Rosco Cinegel) below the light tubes. These filters were designated in photographic stops: the greater the number of stops, the dimmer the light (for further details and discussion of different ways of measuring light in this apparatus, see Mrosovsky et al., 1999).

The illumination during the light pulse was varied from 1650 lux to complete darkness. The former was achieved by additional lights (extra lights test), the latter by a screen of opaque cardboard (sham pulse test). The three dimmest light levels were beyond the sensitivity of our meter, and are specified only in stop units. It is emphasized that the units of measurement are not critical to a comparison between genotypes. The order of tests was: 0, 6, 12, 18, 24 stops, sham pulse (no light), 3, 9, 15 stops, extra light, 21, 27 stops.

The response was scored as the number of wheel revolutions during the light pulse expressed as a percentage of the number of revolutions made by the same animal during the same hour on the previous night when there was no light pulse. Each test took 3 days: baseline day, pulse day, and maintenance day on which filters were changed in preparation for the next pulse. The tests took place over 43 days, which included a few repeat tests to make up for cases of data lost on an account of malfunction of individual microswitches.

3h Light Pulses

Starting 15 days after the last 1h pulse, tests with 3h pulses were given over a period of 8 days. The 3h pulses started at ZT 14. Other than pulse duration, procedures were the same as for the 1h pulses. The order of the tests was 0, 3 stops, extra light.

3.5 : 3.5h LD Cycle

This ultradian cycle is useful for assessing masking because it is difficult to entrain circadian rhythms to days of 7h or multiples of 7h. As a result, the dark and light portions of the cycle move across the circadian cycle, coming to their initial positions after 1 week (Redlin and Mrosovsky, 1999). Masking assessed with this cycle does not depend on one being confident that a light pulse is falling at the same circadian time each time it is given.

On the contrary, maintaining this schedule for a week ensures that all phases of a circadian cycle are tested.

Starting 5 days after the end of the 3h pulse tests, the LD cycle was altered to 3.5 : 3.5h with 800 lux in the L portion. The mice were kept in this cycle for 14 days. Only the last week was used for analysis. The percentage of total activity in that week that occurred in the dark was calculated for each animal.

Release to DD

To check for similarity of entrainment between the genotypes, after the last 1h pulse the mice were placed in continuous darkness (DD) for 2 days. The difference in onset of wheel running between the last day in LD and the first in DD was calculated. Onset was defined as the time when wheel revolutions in a 10 min bin exceeded 55 and was followed by at least this amount within the next 40 min (Janik and Mrosovsky, 1993); occasional short bouts of running separated from the main running bout by >1h were not counted as onsets.

RESULTS

1h Light Pulses

The wildtypes behaved as found with other strains of mice (Mrosovsky et al., 1999). During pulses of higher illumination they decreased their activity (negative masking), but with dimmer lights their wheel running increased (positive masking; Fig. 1). The knockout mice behaved in similar ways with the dim stimuli, also becoming more active. With the brighter stimuli (10 lux or more), however, they differed from wildtypes, in that they showed an impaired masking response to light. The effects of genotype, light level, and their interaction were all highly significant ($p < 0.0001$, 2-way ANOVA). However masking responses to light were still present in *mop* $-/-$ mice, although diminished.

3h Light Pulses

For the 3h pulses, tests were made only with light levels that had been found to reveal impairments in the knockout mice when given 1h pulses. With these relatively bright lights the melanopsin-knockout mice had reduced masking responses (Fig. 2; effect of genotype $p < 0.0001$, 2-way ANOVA). This confirms the results with the 1h pulses of similar brightness. An additional finding with the 3h pulses was that wildtype mice with their melanopsin system intact refrained from running much for most of the 3h, especially in illuminations of 820 lux or greater (panels on right, in Fig. 3). In contrast, the melanopsin knockouts, despite the initial inhibition, gradually resumed running even though the light remained on. By about 100 min into the 3h light pulses, the mice were as active as they had been in the dark on the previous night (i.e., baseline) at that time (panels on left in Fig. 3). Thus, melanopsin was required for maintaining the inhibitory effects of light on locomotion rather than for mediating the initial response, which remained strong in *mop* $-/-$ mice (see first 10 min bins in left panels in Fig. 3).

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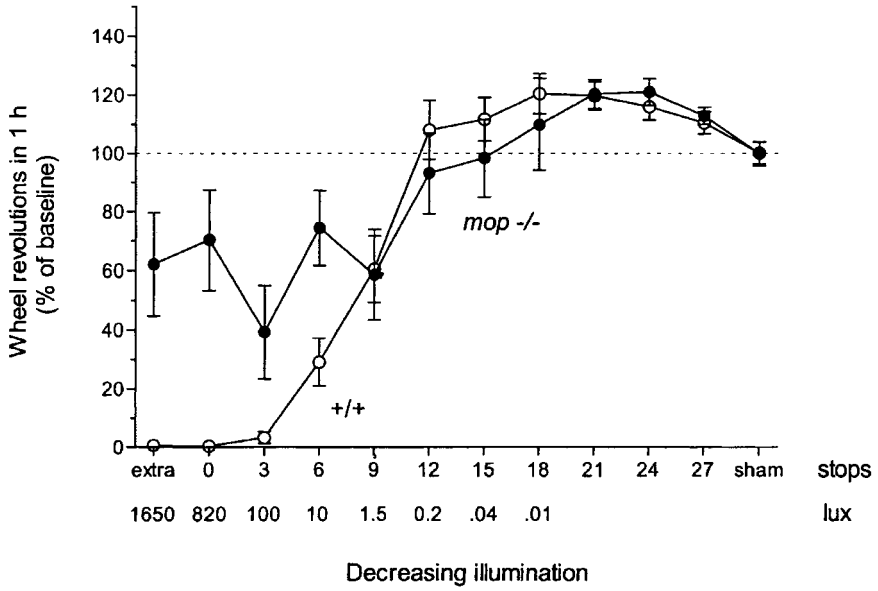

Figure 1. Mean ± SEM of suppression of wheel running during 1h pulses of light, expressed as a percentage change from baseline running. Different light levels during tests with pulses on different days are given in lux and photographic stops (see Materials and Methods).

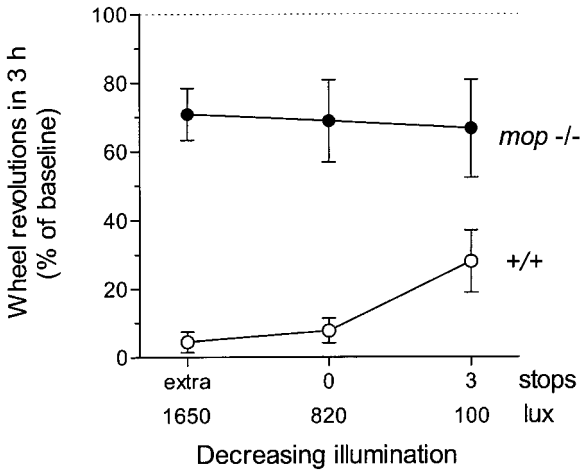

Figure 2. Mean ± SEM of suppression of wheel running during 3h pulses of light. Other details the same as for Fig. 1.

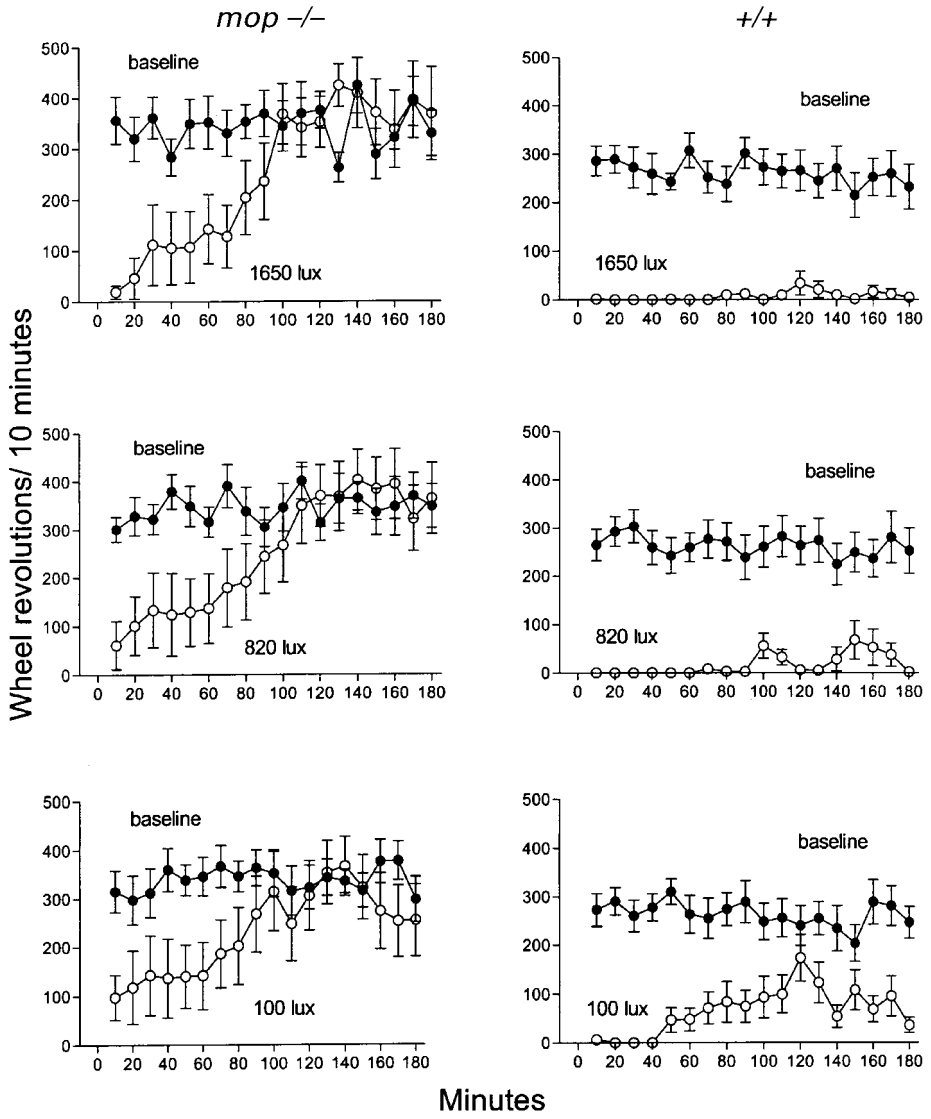

Figure 3. Data for 3h pulses plotted in 10 min time bins. Solid symbols are wheel revolutions (means ± SEMs) over the 3h period in the dark on the baseline day when it remained dark. Open symbols are for revolutions during the light pulse.

3.5 : 3.5h LD Cycle

Because mice cannot readily entrain their circadian rhythms to a cycle of this periodicity, differences between the amount of activity in the dark and in the light are likely to represent masking effects rather than instructions from an endogenous oscillator. Actograms plotted on a 7h *X* axis provide an easy way of visualizing the amount of

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masking (Fig. 4). Masking was weaker in the melanopsin knockouts: $75.3 \pm 5.2\%$ SEM ($n = 9$) of their wheel running occurred in the dark, compared to $97.8 \pm 0.7\%$ SEM ($n = 12$) for the controls (no masking gives a 50% score on this measure). The difference between the genotypes was highly significant ($p < 0.0001$ two-tailed t-test).

Release to DD

The onset of activity on the first day of DD was 1.5 ± 0.43h SEM earlier than the previous onset for the *mop* $-/-$ mice, and 1.6 ± 0.34h SEM earlier for the wildtypes. The lack of significant difference ($p = 0.86$ two-tailed t-test) provides further assurance that the two groups were entrained in a similar way, and that therefore the light pulses occurred at the same phase of their circadian cycles.

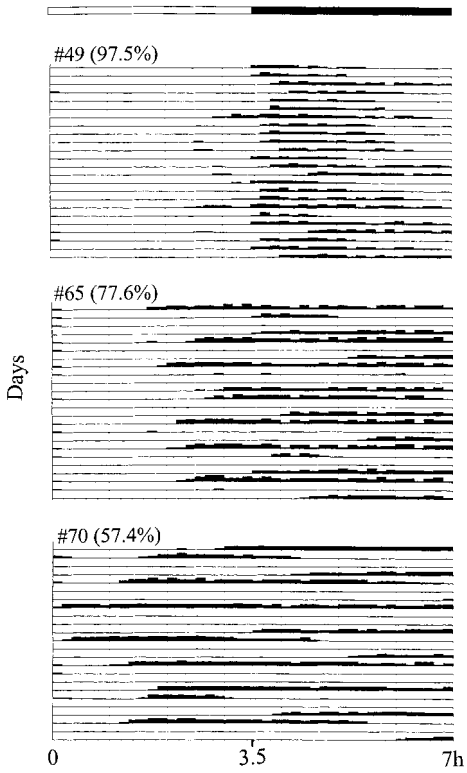

Figure 4. Actograms of wheel running for mice on a 3.5 : 3.5h LD schedule, plotted on a 7h time scale (X axis). Y axis shows successive 7h spans for the last week on this LD cycle. Top, wildtype; middle and bottom *mop* $-/-$. Numbers in parentheses are masking scores (percentage of total revolutions that were made in the dark; 100% = total masking, 50% = no masking). Activity levels are given in five quantiles, with the first showing 1–155 revolutions, the second 156–310, etc. The gaps in activity in some of the dark periods represent times when the circadian cycle is specifying rest, even though it is dark.

DISCUSSION

The results of the masking tests with both 1 and 3h single pulses in the night were consistent with those from the 3.5 : 3.5h cycle. Taken together they establish that masking is definitely not normal in melanopsin-knockout mice. This qualifies the statement of Panda et al. (2002) that "masking is preserved" in melanopsin-knockout mice, although subsequent opinions (Panda et al., 2003) appear to differ on this point. Panda et al. (2002) used a 300 lux white light for their test. In our tests at this illumination there is some impairment of masking. Although different light meters and the exact way of measuring the light within a cage preclude close comparisons, our results conclusively demonstrate that melanopsin is important for masking in mice.

The impairment of masking seen in the present experiments was more prominent in two circumstances. First, it became more evident relative to controls as the duration of the light pulses increased (Fig. 3). After 100 min at light levels exceeding 800 lux, masking was virtually absent in the *mop* −/− mice but still strong in the wildtypes. This large difference provides compelling evidence for a role for melanopsin in masking. Initially the presence of light depresses locomotion in mice lacking melanopsin through some mechanism that partially compensates for the lack of melanopsin, but mice lacking melanopsin cannot sustain the initial response. This indicates that the compensating systems adapt to light after the initial response. But this adaptation is not rapid; it continues over a span of about 100 min (Fig. 3).

The relaxation of masking in the *mop* −/− mice suggests that one function of melanopsin is to maintain a response to illumination over long periods. A similar conclusion can be drawn from studies of melatonin suppression in people. When light of a bluish hue near the peak of the melanopsin spectral response curve is used, melatonin suppression can be maintained for more than 6h. When longer wavelength light is used, melatonin suppression does not persist as long (Lockley et al., 2003). For studies of tonic responses to irradiance levels, masking of locomotion may have some advantages. It provides a more or less continuous index of the state of the animal without the need for interventions such as taking blood samples or restraining an animal to record pupil size.

The second circumstance in which the impairment of masking was more prominent was when higher light levels were used in the 1h tests. This is similar to what Lucas et al. (2003) found for pupillary responses to light with the same strain of melanopsin knockouts; reduced pupil contraction was evident only at their higher light levels. Such similarities are consistent with the view that melanopsin-based irradiance detectors in the inner retina provide input to a variety of non-image-forming response systems.

In contrast to the impairment in *mop* −/− mice of masking responses to light at the higher illumination levels in our experiments, the responses at low illumination levels were indistinguishable from those of the wildtypes. It should be noted, however, that at high illuminations locomotion was decreased, but at low illuminations locomotion was increased, as illustrated in Fig. 1. This positive masking (see Mrosovsky, 1999, for terminology) at low illuminations is dependent on the rod/cone system; in mice with degeneration of the outer retina, positive masking is eliminated or greatly attenuated. This has been found with *rd*/*rd* mice of three strains (Mrosovsky et al., 1999) and with *dta* mice (Mrosovsky et al., 2000). The preservation of masking responses in *mop* −/− mice to dim lights may be attributed to preservation of rods and cones, just as the preservation of the

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pupillary response to dim light in these animals has been attributed to the functioning of the rod/cone system (Lucas et al., 2003).

If the rod/cone system is intact in *mop* −/− mice, then it becomes a potential candidate for mediating their residual masking response to lights of higher illuminations. Conceivably there may be yet other photopigments involved, perhaps cryptochrome (Miyamoto and Sancar, 1998; Van Gelder et al., 2003), but rods or cones are more probable as triple knockout mice, lacking functional rods, cones, and melanopsin appear to lack any negative masking responses (Hattar et al., 2003; Panda et al., 2003).

There is a potential problem to invoking the rod/cone system to account for residual masking responses in melanopsin-knockout mice. At low illuminations, rod/cone activity promotes locomotion (positive masking). Therefore, invoking rod/cone activity to account for residual suppression of locomotion (negative masking) in *mop* −/− mice at higher illuminations would seem paradoxical. However, this may be resolved; it may indeed be that light falling on rods and cones can have both inhibitory and stimulatory effects on locomotion but that the effects are of a completely different nature. Motivationally inhibitory effects might provide some partial functional compensation, allowing *mop* −/− mice to show some residual suppression of locomotion at higher irradiances, as in the present experiment. The stimulatory effects might depend not on irradiance detection but on form vision. Being able to discern outlines and shapes, even if only dimly, may assist the animal in moving around faster and more easily at night (Mrosovsky et al., 2000). Thus, rods and cones might have two mechanistically and functionally different effects on locomotion. The first could be an inhibitory effect on the motivation to move around and would provide partial compensation for melanopsin-dependent irradiance detection at high illuminations. The second, operating at low illuminations, could be a consequence of having enough light for some form vision, and would enable an animal—one already motivated to move at night—to do so more vigorously than possible in complete darkness. Whatever the explanation may be for the residual masking in *mop* −/− mice, our results establish that melanopsin plays a major role in negative masking, especially in the maintenance of suppressed locomotion in light.

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