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Effects of constant light on circadian rhythmicity in mice lacking functional *cry* genes: dissimilar from *per* mutants

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Abstract Mutations in each of the genes *mPer1*, *mPer2*, *mCry1* and *mCry2* separately cause deviations from the wild type circadian system. Differences between these mutant strains have inspired the hypothesis that the duality of circadian genes (two *mPer* and two *mCry* genes involved) is related to the existence of two components in the circadian oscillator (Daan et al., J Biol Rhythms 16:105–116, 2001). We tested the predictions from this theory that the circadian period (τ) lengthens under constant illumination (LL) in *mCry1* and *mPer1* mutant mice, while it shortens in *mCry2* and *mPer2* mutants. *mCry1*^{−/−} and *mCry2*^{−/−} knockout mice both consistently increased τ with increasing light intensity, as did wild type mice. With increasing illumination, rhythmicity is reduced in *mCry1*, *mCry2* and *mPer1*, but not in *mPer2* deficient mice. Results for *mPer* mutant mice are in agreement with data reported on these strains earlier by Steinlechner et al. (J Biol Rhythms 17:202–209, 2002), and also with the predictions from the model. The increase in cycle length of the circadian system by light in the *mCry2* deficient mice violates the predictions. The model is thereby rejected: the *mCry* genes do not play a differential role, although the opposite responses of *mPer* mutants to light remain consistent with a functional Evening–Morning differentiation.

Keywords Constant light · *Cry1* · *Cry2* · *Per1* · *Per2*

Abbreviations

LL	Constant light
DD	Constant dark
τ	Circadian period
LD	Light-dark cycle
SNR	Signal to noise ratio

Introduction

Continuous illumination (LL) has two classic effects on the expression of circadian rhythms, on the degree of rhythmicity and on the circadian period. High light intensity in LL often causes suppression of rhythmicity (Aschoff 1960; Daan and Pittendrigh 1976). It further tends to lengthen circadian rhythms in mammals. The lengthening of circadian period with increasing levels of constant illumination was originally considered to be specific for night-active animals (Aschoff 1960; 1964). On the basis of accumulating mammalian data Aschoff (1979) later changed this rule into the generalization that all mammals, diurnal as well as nocturnal, lengthen the circadian period (τ) with increasing intensity of illumination. These ubiquitous effects of light have rarely been considered in the context of the molecular biology of circadian rhythms. Yet, the responses may be of considerable interest. So far, three studies have reported exceptional LL phenotypes in animals with mutant circadian genes: recovery from—rather than induction of—arrhythmicity in LL in *mClock* mutant mice (Spoelstra et al. 2002) and *mPer2/mCry1* double mutant mice (Abraham et al 2006), and shortening rather than lengthening of τ in LL in *mPer2* mutant mice (Steinlechner et al. 2002). These results suggest that it may be worthwhile to collect more information on rhythmicity in LL in circadian gene mutants. In particular, a hypothesis on the τ response of

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mPer1, *mPer2*, *mCry1* and *mCry2* to continuous light in subcomponents of the circadian oscillator yields specific predictions for the effect of gene deletions in these responses (Daan et al. 2001).

In the dual oscillator hypothesis of Pittendrigh and Daan (1976) the pacemaker properties are explained by the presence of two functional components: an E (Evening) component with a high velocity in darkness and which can be slowed down by light, and an M (Morning) component that has a low velocity in darkness but which is speeded up by light. In the hypothesis E and M are mutually coupled: the phase relationship is assumed to be restricted to certain limits. The differential properties of E and M explain the responses of the circadian system to different photic conditions. These include the adaptation of the internal circadian program to the seasonal changes in day length, and the suppression of rhythmicity in light conditions where the periods of E and M are too far apart. With this hypothesis, specific predictions can be made for the response to different light conditions of a pacemaker that has a deficit in either E or M. Acceleration by constant light should become visible in an E-deficient system, deceleration by constant light in an M-deficient system. As far as arrhythmicity is attributable to an increased discrepancy between the E and M period lengths in constant light we should expect less rhythm suppression in such deficient.

On the basis of known properties of the circadian system of *mPer* and *mCry* mutant mice, a molecular specification of this hypothesis has been formulated by Daan et al. (2001). This includes non-redundant roles for the *mPer1* and *mCry1* genes in the function of the M component, and for the *mPer2* and the *mCry2* genes in the E component. Both *mPer* and *mCry* genes belong to a set of known genes that form the molecular autoregulatory transcription–translation feedback loops underlying circadian rhythms generation. Transcription of both sets of genes is activated by the CLOCK/BMAL1 protein dimer, and in turn negatively feeds back on the CLOCK/BMAL1 mediated transcription (e.g., King and Takahashi 2000; Shearman et al. 2000; Kume et al. 2004). According to the hypothesis by Daan et al. (2001) the properties of the circadian system of mice with a deficit in one of these genes should correspond with the properties of a circadian system with a deficit in the E or M component. The results obtained by Steinlechner et al. (2002) have confirmed these predictions for *mPer1^{Brdm1}* and *mPer2^{Brdm1}* in constant light; the *mCry1* and *mCry2* mutant circadian properties in LL have not yet been studied. Here we test the predictions for both *mCry1* and *mCry2* knockout mice: *mCry1^{-/-}* mice were predicted to lengthen τ with increasing light intensity as found in *mPer1^{Brdm1}*, while *mCry2^{-/-}* mice were predicted to shorten τ as observed in *mPer2^{Brdm1}*. We also repeat the study by Stein-

lechner et al. (2002) to be able to compare the *Cry* and *Per* effects quantitatively in the same study design, with both increasing and decreasing LL intensity.

Methods

The experiment included eight *mPer1^{Brdm1}*, eight *mPer2^{Brdm1}*, eight wild type mice; and six *mCry1^{-/-}*, 8 *mCry2^{-/-}* and eight wild type mice. The *mPer* mice originated from a C57BL/6 $\times$ 129SvEvBrd genetic background, and were backcrossed once to C57BL/6 and were on average 115 days old (SD = 22 days). The *mCry* mice originated from a C57BL/6 $\times$ 129ola genetic background, and were backcrossed four times to C57BL/6 and were on average 76-days-old (SD = 14 days); all mice in the experiment were males. For both *mCry* and *mPer* mutant strains, wild type control mice were from the same background. The generation of the mutants has been described by van der Horst et al. (1999) for the *mCry* knockout strains and by Zheng et al. (1999) for the *mPer* mutants. Both *mPer1^{Brdm1}* and *mPer2^{Brdm1}* mutations are considered null mutations (Zheng et al. 2001).

Animals were housed individually in 25 $\times$ 25 $\times$ 40 cm cages, with food and water ad libitum. Spontaneous locomotor activity was recorded with running wheels (14 cm diam) connected to an Event Recording System (ERS) storing wheel revolutions in 2 min intervals. Temperature was maintained at 23 $\pm$ 1°C throughout the entire experiment. All cages were placed in a custom designed experimental setup with 24 compartments (75 $\times$ 50 $\times$ 70 cm). All compartments are illuminated with two fluorescent tubes (Philips fluotone TLD85 W/83°). Directly above this partition a horizontal shutter closes off the light source by a computer-controlled electric motor, to ensure continuous control of light intensity without spectral change.

All mice were entrained to LD 12:12 (L 1000 lx) for 14 days, and then exposed successively for 15 days to DD, 10 days to LL 1 lx, 17 days to LL 200 lx, 14 days to LL 1,000 lx, and 14 days in LL 10 lx. All mice were then re-entrained to LD 12:12 for 42 days and then exposed to constant illumination in 14 day sections with consecutive light intensities of 1,000, 100, 10, and 1 lx (2,586, 514, 254, 27 and 3 m W m⁻², respectively).

To assess period length, individual activity data from each section of the record were subjected to χ^2 periodogram analysis (Sokolove and Bushell 1978). We further evaluated the effects of different light intensities on the level of activity (average number of wheel revolutions per hour) and on the degree of rhythmicity in the circadian activity pattern recorded. For this last purpose, we determined the Signal to Noise Ratio (SNR) for the most prominent rhythm in the activity pattern. The SNR has been used to quantify

the strength of a circadian rhythm previously (White et al. 1992; Ruf 1999) and is calculated by dividing the variance of the signal by the variance of the noise. The signal is composed of the mean activity counts in each 2-min bin over the circadian period, itself established by periodogram analysis. The noise is the difference between the original raw data and the signal values. The SNR was always based on all days in each light condition, except for the first 3 days to avoid inclusion of remnant rhythmicity or arrhythmicity of the previous light condition.

Results

Figures 1 and 2 show actogram examples representative for the six genotypes analyzed. The *mCry1*^{-/-} mice shortened circadian period length in DD, and lengthened it with increasing light intensity. Circadian period of *mCry2*^{-/-} mice in DD was longer than 24 h, and lengthened even more in constant light. In all three *mCry* strains rhythmicity was reduced but preserved in both DD and LL. Circadian

rhythms in *mPer1*^{Brdm1} mice had a normal period in DD and lengthened in LL. Circadian rhythmicity gradually disappeared in *mPer1*^{Brdm1} mice when exposed to bright light, and was restored with decreasing light intensity. Opposite trends in circadian period length and rhythmicity are observed in *mPer2*^{Brdm1} mice. These mice lost rhythmicity in low light intensity and regained their circadian rhythm with shortened period length in LL.

Average τ values are presented in Fig. 3. These are based on the average τ per individual for equal light intensity conditions. Individuals were only included if a significant rhythm between 20 and 30 h was detected by periodogram analysis in both conditions. The values obtained in 100 and 200 lx were pooled. The response to light intensity in τ , SNR and activity level was tested in a multiple regression model with log light intensity and genotype as independent variables (Table 1). Wild type mice increased their period length from 24.1 h in DD to 25.9 h in 1,000 lx. For all light intensities, *mCry1*^{-/-} mice had a significantly shorter, and *mCry2*^{-/-} mice had a significantly longer period than wild type control mice, corresponding to

Fig. 1 Actogram examples of *mCry* strains in light with increasing and decreasing intensity. Horizontal lines delimit LL light intensities (lx) denoted on the left. The actograms in the upper and lower panel are from the same animal

Fig. 2 Actogram examples of *mPer* strains in light with increasing and decreasing intensity. Horizontal lines delimit LL light intensities (lx) denoted on the left. The actograms in the upper and lower panel are from the same animal

the difference in DD. SNR values for all *mCry* strains were on average highly similar across all LL intensities, and gradually decreased with increasing light intensity. Average activity levels were equal in LD, DD and in all LL light intensities in all *mCry* strains. Increasing light intensity increasingly suppressed activity levels.

A significant and differential effect of light intensity on circadian period was present in the *mPer* mice (see Table 1). *mPer1^{Brdm1}* mice lengthened their circadian period more strongly from 24.2 h in DD to 27.6 h in 1,000 lx. When exposed to constant light, none of the *mPer2^{Brdm1}* mice lengthened circadian period, instead the *mPer2^{Brdm1}* fluctuate around 23 h, with the shortest period in 1 and 10 lx. *mPer1^{Brdm1}* mice show low SNR values in all LL light intensities, however in *mPer2^{Brdm1}* mice a significant effect by light intensity is present on the level of SNR. These mutants show a restoration of SNR in low LL light intensities compared to DD (Table 1); the SNR of the most prominent rhythm detected between 20 and 30 h in DD was close to zero. Wild type mice were on average more rhythmic than *mPer* mutant mice, with SNR values gradually decreasing with increasing light intensity. The average activity level (wheel revolutions $\times$ h⁻¹) in entrainment was reduced in *mPer2^{Brdm1}* mice and even more reduced in

mPer1^{Brdm1} mice compared to wild type mice (Fig. 3). This difference was retained in DD and in LL at all light intensities. All three *mPer* strains showed an overall decrease in hourly activity in LL. The sharpest decrease was observed in *mPer2^{Brdm1}* mice.

Discussion

The two *mCry* mutants express their differences in period length equally under different intensities of constant illumination. The increasing τ values both in *mCry1^{-/-}* and *mCry2^{-/-}* with increasing LL light intensity refute the prediction concerning the *mCry* genes derived from the molecular two-component theory proposed by Daan et al. (2001). Apparently neither *mCry* gene separately has to be functional for the lengthening of τ in response to continuous light. The propensity of *mCry2^{-/-}* mutant mice to show attenuated delays, as observed in phase responses elicited by brief light pulses (Spoelstra et al. 2004) does not prevent strong τ lengthening in constant illumination.

The direction of change in circadian period length for *mPer1^{Brdm1}* mice in increasing light intensities in our study was similar to those reported for increasing light intensities

Fig. 3 Average τ values (upper graph); level of rhythmicity expressed by the Signal to Noise Ratio (SNR, middle graph; a stable rhythm is reflected by a high signal to noise ratio); average activity level in wheel revolutions/min (lower graph). Error bars always indicate 1 SEM. Average values and SEM plotted are based on averages per animal per light intensity. The data points for different genotypes are laterally shifted for clarity

by Steinlechner et al. (2002). Since we observed the light dependency in both directions this cannot be attributed to a sequence effect. Our results confirm their finding that period length shortens in *mPer2^{Brdm1}* in constant light relative to the period length of wild type mice. While general in (diurnal) birds, this shortening of τ in response to constant light is unique among mammals, where so far all species measured exhibit an increase in circadian period in LL (Aschoff 1979). The shortened τ in *mPer2^{Brdm1}* mice in LL in two studies is a remarkable confirmation of the deviant phenotype predicted by the molecular two-component model (Daan et al. 2001). In our study there appeared to be no further shortening of the circadian cycle with increasing intensity beyond 1 lx in the *mPer2^{Brdm1}* strain as was observed by Steinlechner et al. (2002). An even shorter circadian period of circa 20 h in LL of circa 400 lx has been observed in *mPer2^{Brdm1}mCry1^{-/-}* mice (Abraham et al. 2006).

It is of interest that the shortening of τ in *mPer2^{Brdm1}* in LL is accompanied by a decrease in activity level, where all

other strains shorten τ from LL to DD in combination with an increase in activity level. There is a rather general negative association between the amount of locomotor activity and circadian period (Aschoff 1960; Aschoff et al. 1973; Turek 1989). This may be caused by additional variables, such as testosterone titers (Daan et al. 1975), acting on both activity and the circadian system. It may also be due to a feedback effect from activity on the pacemaker. The present results demonstrate that the period shortening in LL cannot be attributable to such feedback, since activity levels dropped systematically with increasing light levels in all strains, including *mPer2^{Brdm1}*. The extra lengthening in circadian period in *mPer1^{Brdm1}* mice and the shortening in circadian period in *mPer2^{Brdm1}* mice suggests distinct roles for the *mPer1* and *mPer2* gene in accelerating and decelerating the circadian system, respectively.

The activity level in all strains tested decreased with increasing light intensity. Activity levels in *mCry1^{-/-}* and *mCry2^{-/-}* mice were quite similar to those of wild type control mice in all photic conditions including LD and DD.

Table 1 Circadian period (τ), Signal to Noise Ratio (SNR) and activity explained by multiple regression analysis of log light intensity (Log I), genotype and their interaction

Dependent Variable	Parameter	Δ Residual SS	Δ df	P	Coefficient	Dependent Variable	Parameter	Δ Residual SS	Δ df	P	coefficient
T	Null model	308.67	114			T	Null model	264.05	105		
	Final model	45.01	109				Final model	62.35	100		
	Constant		+1		24.27		Constant		+1		24.66
	Log I	+16.87	+1	<0.00001	0.46		Log I	+23.79	+1	<0.0001	0.51
	<i>mCry1</i> ^{-/-}	+40.24	+1	<0.00001	-1.88		<i>mPer1</i> ^{Brdm1}	+4.13	+1	<0.05	0.59
	<i>mCry2</i> ^{-/-}	+16.71	+1	<0.00001	1.12		<i>mPer2</i> ^{Brdm1}	+18.35	+1	<0.0001	-1.68
	<i>mCry1</i> ^{-/-} * Log I	+0.30	+1	n.s.	0.09		<i>mPer1</i> ^{Brdm1} * Log I	+5.29	+1	<0.005	0.41
	<i>mCry2</i> ^{-/-} * Log I	+2.30	+1	<0.05	0.24		<i>mPer2</i> ^{Brdm1} * Log I	+5.46	+1	<0.005	-0.46
SNR	Null model	17.26	131			SNR	Null model	28.20	143		
	Final model	11.57	126				Final model	9.36	138		
	Constant		+1		0.62		Constant		+1		0.86
	Log I	+1.52	+1	0.0001	-0.13		Log I	+11.68	+1	<0.001	-0.13
	<i>mCry1</i> ^{-/-}	+0.09	+1	n.s.	0.09		<i>mPer1</i> ^{Brdm1}	+14.25	+1	<0.00001	-0.54
	<i>mCry2</i> ^{-/-}	+0.02	+1	n.s.	-0.03		<i>mPer2</i> ^{Brdm1}	+12.44	+1	0.0001	-0.40
	<i>mCry1</i> ^{-/-} * Log I	+0.04	+1	n.s.	-0.03		<i>mPer1</i> ^{Brdm1} * Log I	+10.32	+1	n.s.	0.04
	<i>mCry2</i> ^{-/-} * Log I	+0.02	+1	n.s.	-0.02		<i>mPer2</i> ^{Brdm1} * Log I	+11.96	+1	<0.001	0.19
Activity	Null model	6072.53	131			Activity	Null model	7049.40	143		
	Final model	4586.43	126				Final model	3415.48	138		
	Constant		+1		14.37		Constant		+1		16.25
	Log I	+481.84	+1	<0.001	-2.30		Log I	+111.69	+1	<0.05	-1.11
	<i>mCry1</i> ^{-/-}	+4.87	+1	n.s.	-0.65		<i>mPer1</i> ^{Brdm1}	+1380.31	+1	<0.00001	-10.11
	<i>mCry2</i> ^{-/-}	+82.77	+1	n.s.	-2.48		<i>mPer2</i> ^{Brdm1}	+342.98	+1	<0.0005	-5.04
	<i>mCry1</i> ^{-/-} * Log I	+15.01	+1	n.s.	-0.62		<i>mPer1</i> ^{Brdm1} * Log I	+10.48	+1	n.s.	-0.48
	<i>mCry2</i> ^{-/-} * Log I	+7.82	+1	n.s.	0.41		<i>mPer2</i> ^{Brdm1} * Log I	+69.74	+1	n.s.	-1.24

τ final model: $r^2 = 0.85$ (*mCry*); $r^2 = 0.31$ (*mPer*). SNR final model: $r^2 = 0.33$ (*mCry*); $r^2 = 0.31$ (*mPer*). Activity final model: $r^2 = 0.24$ (*mCry*); $r^2 = 0.52$ (*mPer*). Changes in residual sum of squares (Δ Residual SS) were computed by excluding parameter values from the final model

In agreement with the masking responses observed in *mCry1*^{-/-}*mCry2*^{-/-} double mutant mice during light pulses of different intensity (Mrosovsky 2001), activity levels in all three genotypes were increasingly and evenly reduced. Apparently, none of the *mPer* or *mCry* genes is necessary for the suppressing effect of light on general activity.

The study shows that after loss of rhythmicity in DD renewed entrainment to LD 12:12 is not required for *mPer2*^{Brdm1} mice to become rhythmic again. Constant light either initiates the circadian oscillation, or the pacemaker regains control over its behavioral output.

Low levels of rhythmicity are generally associated with low activity (Aschoff 1960; Turek 1989). This holds also for all genotypes tested here, except for *mPer2*^{Brdm1} mice exposed to dim constant light. Although *mPer2*^{Brdm1} mice are arrhythmic in DD, their activity level in DD is similar to that in LD. Arrhythmic *mPer2*^{Brdm1} mice in DD are also much more active than rhythmic *mPer1*^{Brdm1} mice. Only when exposed to high LL light intensities, the SNR in *mPer2* mutants may be reduced by severely suppressed

activity levels. Their loss of rhythmicity in DD was not predicted by the model.

mPer1^{Brdm1} mice were least rhythmic in LL and showed least locomotor activity of all genotypes. In contrast to *mPer2*^{Brdm1} mice, rhythmicity in *mPer1*^{Brdm1} mice is almost entirely lost in high intensity LL but recurs with decreasing light intensity. The low SNR in *mPer1*^{Brdm1} mice under exposure of bright light does not support the interpretation by Steinlechner et al. (2002), that rhythmicity is sustained in *mPer1*^{Brdm1} in these conditions. In our study, wild type mice were most rhythmic in all conditions, but with a clear suppression in level of rhythmicity by high light intensities. Abraham et al. (2006) show disruption of rhythmicity in both *Per1* and *Bmal1* expression in the SCN in DD, and restoration in LL in *mPer2*^{Brdm1}*mCry1*^{-/-} mice. Like the *mPer2*^{Brdm1} mice, this double mutant is arrhythmic in DD, but it shows a 20 h circadian period in locomotor behaviour in constant light of ~300 lx. Whether also *mPer2*^{Brdm1} mice have restoration of both *Per1* and *Bmal1* expression in LL is not known.

In the specification of the molecular two-oscillator model as proposed by Daan et al. (2001) a distinct role for *mPer1* and *mCry1* in the M- and for *mPer2* and *mCry2* in the E-component of the circadian oscillator was suggested. These two components were predicted to respond oppositely in velocity and hence phase when the circadian system is exposed to light. These opposing influences could also possibly account for arrhythmicity in wild type mice in LL (Daan et al. 2001). Mice, single mutant for *mPer1*, *mPer2*, *mCry1* or *mCry2*, or mice double mutant for *mPer1mCry1* or *mPer2mCry2* would then be exempted from these opposite forces in LL conditions and might be expected to more readily preserve rhythmicity. This is not the case. Compared to *mPer1^{Brdm1}* and *mPer2^{Brdm1}* mutants, wild type mice have a more stable rhythm that is less disturbed by increasing light intensity than *mPer1^{Brdm1}* mice. Wild type control mice are equally rhythmic as *mCry1^{-/-}* or *mCry2^{-/-}* mice in any condition. The finding by Abraham et al (2006) that knocking out the *Cry1* gene in the *mPer2^{Brdm1}* mouse has virtually no effect on the LL phenotype is fully consistent with this result.

The possibility exists that the circadian phenotype of the mutants used in this study results from changes in light input to the SCN. The cryptochromes are not strictly required for inner retinal phototransduction as pupil responses in mice lacking both cryptochromes are only attenuated in retinal degenerate mice (*rd/rd*) (Van Gelder et al. 2003; Van Gelder 2005). Effects of non-functional *Per* genes on retinal gating of light input to the SCN have not been reported. Another issue is the role of genetic background of the mice in responses to light. Zheng et al. (1999) and Bae and Weaver (2001) have demonstrated similar effects of the *mPer2* mutation on rhythmicity in two different background strains, but an *mPer2* knockout in a C57BL/6J background shows normal circadian rhythmicity in DD with normal τ (Xu et al. 2007). Whether background also affects the light responses remains to be tested.

In summary, mutations of *mPer1* and *mPer2* have opposite effects on the influence of constant light on the circadian system, while the deletions of *mCry1* and *mCry2* cause opposite effects on circadian period independent of the light intensity. The lengthening of τ by light in both *mCry1* and *mCry2* knockouts is not in agreement with the predictions from the model, which therefore has to be rejected: there is no evidence for functional differentiation of the *mCry* genes as postulated, in spite of the different periods in DD and the differences in phase responses in the *mCry1^{-/-}* and *mCry2^{-/-}* mice (Spoelstra et al 2004). The hypothesis is also refuted for the *Cry* genes by the persistence of rhythmicity in DD in juvenile *mPer1^{Brdm1}mCry2^{-/-}* in DD (Oster et al 2003) and in adult *mPer2^{Brdm1}mCry1^{-/-}* in LL (Abraham et al 2006). In contrast, the short circadian period in constant light, the elevated PRC for light pulses in

mPer2 mutant mice and the strong lengthening of τ in *mPer1^{Brdm1}* relative to wild type in LL have confirmed predictions from the model. Nonetheless, the potential involvement of *mPer1* in an M-component and of *mPer2* in an E-component of the system must root in a different molecular mechanism than postulated by Daan et al (2001). The analysis of rhythmicity by SNR suggests that these actions are not exerted through masking, i.e. via behavioural responses, but directly by light on the pacemaker. On a more general note, the observation that mouse strains become arrhythmic in constant darkness but rhythmic in constant light (*mPer2^{Brdm1}*; Steinlechner et al 2002; this study; *Clock*: Spoelstra et al 2002) questions the validity of constant darkness as the golden standard for circadian system assessment.

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