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Circadian Clock Control of Connexin36 Phosphorylation in Retinal Photoreceptors of the CBA/CaJ Mouse Strain

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Abstract

The gap-junction-forming protein connexin36 (Cx36) represents the anatomical substrate of photoreceptor electrical coupling in mammals. The strength of coupling is directly correlated to the phosphorylation of Cx36 at 2 regulatory sites: Ser110 and Ser293. Our previous work demonstrated that the extent of biotinylated tracer coupling between photoreceptor cells, which provides an index of the extent of electrical coupling, depends on the mouse strain. In the C57Bl/6J strain, light or dopamine reduces tracer coupling and Cx36 phosphorylation in photoreceptors. Conversely, darkness or a dopaminergic antagonist increases tracer coupling and Cx36 phosphorylation, regardless of the time of day. In the CBA/CaJ strain, photoreceptor tracer coupling is regulated by light and dopamine, but also by a circadian clock, a type of oscillator with a period close to 24 h and intrinsic to the retina, so that under prolonged dark-adapted conditions tracer coupling is broader at night compared to daytime. In the current study, we examined whether the modulation of photoreceptor coupling by a circadian clock in CBA/CaJ mouse photoreceptors reflected a change in Cx36 protein expression and/or phosphorylation. We found no significant change in Cx36 expression or in the number of Cx36 gap junction among the conditions examined. However, we found that Cx36 phosphorylation is higher under dark-adapted conditions at night than in the daytime, and is the lowest under prolonged illumination at any time of the day/night cycle. Our observations are consistent with the view that the circadian clock regulation of photoreceptor electrical coupling is mouse strain-dependent and highlight the critical position of Cx36 phosphorylation in the control of photoreceptor coupling.

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Keywords

retina; gap junction; electrical coupling; photoreceptors; circadian clock

Introduction

Anatomical and functional evidence indicates that vertebrate photoreceptors are electrically coupled to each other through gap junctions (Bloomfield et al., 2009; Li and O'Brien, 2012 for reviews). Connexin36 (Cx36) or Gjd2, or its non-mammalian ortholog Cx35, is a major component of gap junctions in photoreceptors (Li and O'Brien, 2012). The phosphorylation state of Cx35/36 at 2 primary regulatory sites, Ser110 and Ser276 (or Ser293 in mammals), is directly correlated to the conductance of the Cx35/36-forming junctions so that electrical coupling is strong when the connexin is phosphorylated and weak when it is unphosphorylated (Kothmann et al., 2007, 2009; Li et al., 2013). The use of phospho-specific antibodies against S110 or S276 (or S293) has proven a useful tool to advance our understanding of the regulation of photoreceptor coupling and thereby of the plasticity of this unique neuronal network. For instance, this immunohistochemical approach helped to demonstrate that photoreceptor coupling is modulated by light, dopamine, and adenosine in zebrafish retina (Li et al., 2009; Li et al., 2014).

In mammals, details on the dynamic regulation of the network of electrically-coupled photoreceptors have started to emerge as well. Using a technique of cut loading, we recently reported that biotinylated tracer coupling between mouse photoreceptors is sensitive to light/dark-adaptation and the light effector dopamine (Ribelayga et al., 2008; Li et al., 2013). In addition, this approach revealed differences between mouse strains. A circadian regulation of photoreceptor tracer coupling with the largest extent of coupling at nighttime was observed in the CBA/CaJ mouse (Ribelayga et al., 2008) whereas it was not observed in the C57Bl/6J strain, in which tracer coupling did not significantly change with time of day (Ribelayga et al., 2008; Li et al., 2013). Consistent with the latter findings, our previous work also showed that the phosphorylation state of Cx36 in C57Bl/6J photoreceptors is controlled by light and dopamine but provided little support for a control by a circadian clock (Li et al., 2013). Although our work indicated that the number of Cx36 gap junctions in C57Bl/6J photoreceptors does not fluctuate with lighting conditions or time of day (Li et al., 2013), a recent study suggested that Cx36 transcript and protein may fluctuate on a daily or circadian time scale depending on the mouse strain (Katti et al., 2013). Here we sought to determine which of Cx36 expression and phosphorylation is the major event controlling photoreceptor coupling on a circadian manner in CBA/CaJ mice. We report that Cx36 is highly phosphorylated during the night or subjective night compared to the day under dark-adapted conditions or the subjective day or under light-adapted conditions. The data further demonstrate that neither Cx36 protein expression nor the number of Cx36-containing gap junctions in photoreceptor cells change according to time of day or lighting conditions. Thus, our work strongly suggests that the modulation of Cx36 phosphorylation is an important process in the circadian clock pathway that controls photoreceptor coupling in CBA/CaJ mouse and that the circadian control of photoreceptor coupling is mouse strain-dependent.

Materials and Methods

The care and use of the mice are in accordance with federal and institutional guidelines. The experiments have been proposed, reviewed and approved by the local IACUC (protocol # HSC-AWC-12-066). Adult male or female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME), 2–6 month-old, were housed in a 12 hours (h) light/12 h dark cycle (with lights-on at 7.00 a.m.) for at least 2 weeks prior to an experiment. Circadian conditions were created by keeping the mice in the dark for up to 30 h, with dark adaptation starting at the end of the light phase (7.00 p.m.). Mice were sacrificed either in the middle of the subjective day, that is after 18 hrs of dark-adaptation, or in the middle of the subjective night after 30 hrs of dark-adaptation. Manipulation of the animals and retinal tissue during the night or under circadian conditions were conducted under infrared light with the help of infrared goggles (D-321G-A; Night Optics USA, Huntington Beach, CA). In some experiments, the animals were exposed to room lights (300–500 lux) for 1–3 hrs prior to sacrifice. Light-exposed animals and dark-adapted controls were sacrificed at the same time in the circadian cycle.

Mice were anesthetized with a mixture of ketamine and xylazine (100/10 mg/kg, I.P.), decapitated, and one eye was rapidly enucleated and placed in 2% carbodiimide (Sigma-Aldrich, St-Louis, MO) in 10 mM phosphate buffer saline (PBS) for 30 min. Eyeballs were rinsed in PBS and placed in 30% sucrose overnight to develop cryo-protection and sectioned (12 μ m-thick sections) with a cryostat. Retinal sections were reacted to a solution containing primary antibodies against pan-Cx36 (1/800, clone 8F6.2, Millipore) and a phospho-antibody that specifically recognizes Cx36 phosphorylated at S293 (1/400, PS293Cx36) (Kothmann et al., 2007). Because S110 and S293 phosphorylation vary in parallel in the vertebrate retina (Li et al., 2012), we restricted our analysis to S293 as in our previous work in rabbit (Kothmann et al., 2009, 2012) and mouse (Li et al., 2013) retinas. The specificity of the pan-Cx36 and PS293Cx36 antibodies in mouse has been described previously (Li et al., 2013). Imaging and quantification of the phosphorylation state of Cx36 was done as described previously (Kothmann et al., 2007, 2009, 2012; Li et al., 2009, 2013, 2014). Briefly, immunostaining of Cx36 and P293Cx36 was imaged on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) using 12 bit data acquisition. For each experiment, once settings were optimized, they remained unchanged for all conditions and treatments to avoid imaging biases. Five confocal slices at 0.3 μ m intervals were taken at randomly selected locations in the outer plexiform layer (OPL) in the region from 50 to 80% of the distance from the optic nerve head to the periphery. The five slices were stacked to generate a $35 \times 16 \times 0.9 \mu\text{m}$ ($504 \mu\text{m}^3$) volume, which was exported into one 12-bit tiff image. Five images were collected from three sections of one eyecup to represent one animal. The confocal images were analyzed with Simple PCI software (Hamamatsu Corporation, Middlesex, NJ). In each image, a measure of the mean pixel intensity in the red channel was obtained from the entire sampling volume. The average value from 5 images was used to represent the level of Cx36 expression in the OPL of one animal. To estimate Cx36 phosphorylation level, regions of interest (ROI) were defined as contiguous pixels whose Cx36 intensity was >20% of the total intensity range and whose size was no smaller than $0.1 \mu\text{m}^2$. The phosphorylation level was estimated by taking a ratio

of the intensity of phospho-S293 to that of Cx36 antibody within each ROI. Since the population of phosphorylation state ratios is not normally distributed, the median intensity ratio was calculated from all Cx36 plaques in 5 images to represent the phosphorylation level of one animal. Averages of at least 5 animals from each experimental condition were compared between treatments and conditions using two-way ANOVA followed by Tukey's Test. The number of Cx36 plaques was estimated by counting the total number of ROI in each image. The average ROI count over 5 images was used to represent one animal. Experiments were duplicated. Representative data are shown in figures 1 and 2.

Results

We first compared the phosphorylation state of Cx36 in the outer plexiform layer (OPL) of mouse retinas that were harvested in the middle of the day following 1 hr of dark-adaptation (Zeitgeber Time 06 [ZT06]) or in the middle of the night (ZT18) or in the middle of the subjective day (CT06) or the middle of the subjective night (CT18). Figure 1A shows that the total amount of Cx36 (red, pan-Cx36) in the OPL was similar at all time points tested. The vast majority of Cx36-immunoreactive puncta in the OPL are associated with photoreceptors (Li et al., 2013). Averaged values of the mean pixel intensity in the red channel, which reflects the level of expression of Cx36 in the OPL, are illustrated in Figure 1B. A two-way ANOVA of the data was performed with time in the cycle as the first factor (i.e., daytime, nighttime) and the nature of the cycle as the second factor (i.e., light/dark, circadian). ANOVA of the data presented in figure 1B revealed no significant time in the cycle [$F_{(1,21)} = 1.19$, $P = 0.289$], nature of the cycle [$F_{(1,21)} = 0.193$, $P = 0.666$], or time in the cycle x nature of the cycle [$F_{(1,21)} = 1.86$, $P = 0.190$] effects. The average number of gap junction plaques in each condition is illustrated in Figure 1C. ANOVA of the data presented in figure 1C revealed no significant time in the cycle [$F_{(1,21)} = 0.160$, $P = 0.694$], nature of the cycle [$F_{(1,21)} = 0.005$, $P = 0.944$], or time in the cycle x nature of the cycle [$F_{(1,21)} = 0.007$, $P = 0.935$] effects. The phosphorylation state of Cx36 in the OPL was quantified by the ratio of PS293Cx36/pan-Cx36 (see Kothmann et al., 2009 or Li et al., 2013 for details). We found changes in the phosphorylation state of Cx36 according to the time of day (Fig. 1A, 1D). ANOVA of the data presented in figure 1D revealed significant time in the cycle [$F_{(1,21)} = 22.5$, $P < 0.0001$] effect, and no significant nature of the cycle [$F_{(1,21)} = 0.045$, $P = 0.0834$] or time in the cycle x nature of the cycle [$F_{(1,21)} = 0.327$, $P = 0.575$] effects. Thus the data demonstrate that a circadian clock in CBA/CaJ mouse controls the phosphorylation state of Cx36 so that the connexin is highly phosphorylated during the night or subjective night and poorly phosphorylated during the day in the dark or subjective day. In addition, our data indicate that neither Cx36 expression nor the number of Cx36 gap junctions in the OPL change with time of day.

Next, we tested the effects of light adaptation on the phosphorylation state of Cx36 in the middle of subjective day and subjective night. Animals were exposed to room lights (300–500 lux) 1 h or 3 h before sacrifice. Examples of immuno-staining of Cx36 and PS293Cx36 under dark-adapted or light-adapted conditions during subjective day or night are shown in figure 2A. Averaged values of the mean pixel intensity in the red channel under the various experimental conditions are illustrated in Figure 2B. A two-way ANOVA of the data was performed with time in the cycle as the first factor (i.e., daytime, nighttime) and the lighting

conditions as the second factor (i.e., dark-adapted, light-adapted for 1 h, light-adapted for 3 h). ANOVA of the data presented in figure 2B revealed no significant time in the cycle [$F_{(1,33)} = 0.00264$, $P = 0.959$], lighting conditions [$F_{(1,33)} = 1.99$, $P = 0.155$], or time in the cycle x lighting conditions [$F_{(1,33)} = 0.250$, $P = 0.781$] effects. Averaged values of the total number of Cx36 plaques and of the phosphorylation states of OPL Cx36 under the various experimental conditions are shown in figures 2C and 2D, respectively. ANOVA of the data presented in figure 2C revealed no significant time in the cycle [$F_{(1,33)} = 0.033$, $P = 0.857$], lighting conditions [$F_{(1,33)} = 0.582$, $P = 0.565$], or time in the cycle x lighting conditions [$F_{(1,33)} = 0.444$, $P = 0.646$] effects. Thus, Cx36 expression and the number of Cx36 plaques in the OPL were not altered by the lighting conditions. ANOVA of the data presented in figure 2D revealed significant time in the cycle [$F_{(1,27)} = 8.88$, $P < 0.01$] and lighting conditions [$F_{(1,27)} = 39.48$, $P < 0.0001$] effects, but no time in the cycle x lighting conditions [$F_{(1,27)} = 1.40$, $P = 0.268$] effects. These data demonstrate that photopic light decreases Cx36 phosphorylation in CBA/CaJ photoreceptors on a time scale of hours, a result in line with our previous observations in the C57Bl/6J (Li et al., 2013).

Discussion

Sensory organs are able to encode amounts of information far greater than their output capacity through the process of adaptation or gain control. The complexity of adaptation is exemplified in the retina, which faces the challenge to operate and transmit information over 10 log units of light intensity during the course of the day/night cycle with a given ganglion cell output capacity of approximately 2 log units (Dowling, 2012). This amazing capability depends on a specific functional architecture, including 2 types of photoreceptors, rods and cones, and a wide variety of neuronal mechanisms encompassing both adaptive mechanisms driven by ambient light intensity and endogenous mechanisms, such as circadian clocks (Mangel and Ribelayga, 2010; Dowling, 2012; McMahon et al., 2014). The regulation of photoreceptor electrical coupling is an important adaptive mechanism in the vertebrate retina because it affects many of the properties of the photoreceptor light response, including response threshold and kinetics, spectral sensitivity and the degree of rod and cone signal mixing (Yang and Wu, 1989; Krizaj et al., 1998; Ribelayga et al., 2008; Heikkinen et al., 2011; Jin et al., 2015). Evidence indicates that the regulation of photoreceptor electrical coupling relies on Cx35/36 and on the modulation of its phosphorylation state (Li and O'Brien, 2012).

In the present study, we show that both light/dark adaptation and a circadian clock control the phosphorylation state of Cx36 in photoreceptors in the CBA/CaJ mouse retina, so that Cx36 phosphorylation is minimal under daytime illumination and maximal at night in the dark. We also show that neither light/dark adaptation nor the circadian clock significantly alters the expression level or the number of Cx36 gap junctions in the OPL. These results are consistent with the dopamine and circadian modulation of the rod input to goldfish cones (Ribelayga et al., 2008) and, by way of electrically coupled cones, to horizontal cells (Wang and Mangel, 1996; Ribelayga et al., 2002) and to rabbit horizontal cells (Ribelayga and Mangel, 2010). Our results are also consistent with the circadian variation in tracer coupling observed in CBA/CaJ photoreceptors (Ribelayga et al., 2008), although it remains unclear to what extent relative tracer permeabilities mirror relative degrees of Cx36 phosphorylation.

In addition to the gap junction conductance, which is closely associated to Cx36 phosphorylation, the extent of tracer coupling depends on many factors, among them the size, lipophilicity and electrical charge of the tracer molecule, the resistance of the plasma membrane, and the time between loading/diffusion and fixation. Therefore, although we can safely conclude that the nocturnal increase in Cx36 phosphorylation we report here is consistent with the nocturnal increase in tracer coupling we previously reported (Ribelayga et al., 2008), we should restrain from interpreting the data quantitatively.

Rhythmic processes in the retina emanate from clock neurons located in the retina and that express clock genes and proteins (Liu et al., 2012; McMahon et al., 2014). The identity of the clock cell types and of the clock pathway involved in the circadian control of photoreceptor coupling remains incomplete but the neurohormone melatonin appears to play a key role in this pathway (Ribelayga et al., 2004; McMahon et al., 2014). Melatonin is a nocturnal effector of a retinal clock, with low levels during the day and high levels at night (Iuvone et al., 2005; McMahon et al., 2014). Melatonin exerts pleiotropic effects on retinal cells, directly via activation of its membrane receptors (Huang et al., 2013) and indirectly via the suppression of dopamine release (McMahon et al., 2014). The rhythmic production of melatonin is absent or of very low amplitude in most mouse strains, including the common C57Bl/6J strain, because of null mutations in the genes coding the key enzymes of the melatonin synthetic pathway (Iuvone et al., 2005; Kasahara et al., 2010). Few strains, such as C3H and the CBA strain used here, show normal high-amplitude rhythms of melatonin (Iuvone et al., 2005; Kasahara et al., 2010). Under constant darkness conditions, retinal dopamine release is circadian in melatonin-proficient mouse strains (Iuvone et al., 2005; Z.Z. and C.P.R. unpublished observations), while it is constitutively low in melatonin-deficient strains (Iuvone et al., 2005; Li et al., 2013; Z.Z. and C.P.R. unpublished observations). The clock-controlled increase in dopamine release during the subjective day or the day under dark-adaptive conditions in CBA/CaJ mice is sufficient to activate D₄ dopamine receptors on the photoreceptors and reduce coupling, a state that can be reversed by a dopaminergic antagonist (Ribelayga et al., 2008; Jin et al., 2015). The presence of rhythms of photoreceptor tracer coupling and Cx36 phosphorylation in CBA/CaJ photoreceptors and their absence in C57/Bl6J photoreceptors are consistent with the presence of a rhythm of dopamine release in CBA/CaJ retinas and its absence in C57/Bl6J retinas. The mechanisms controlling Cx36 phosphorylation and dephosphorylation may have different kinetics. Indeed, although 1 hr of dark adaptation was sufficient to increase Cx36 phosphorylation (Fig. 1D), phosphorylation barely dropped after 1 h of illumination (Fig. 2D). In Li et al., 2013, we reported that in C57Bl/6J mice the phosphorylation of Cx36 in photoreceptors can recover by 50% its nighttime level after 2 hrs of dark adaptation during the day. Similarly, dopamine release can recover to its dark-adapted (low) values within 2 hrs of dark adaptation (Li et al., 2013). Although we did not test shorter times in the dark, these data are consistent with our observations in CBA/CaJ mice that 1 h of darkness is sufficient to increase Cx36 to 50% of its nighttime value (Fig. 1D). The decrease in phosphorylation triggered by light has obviously slower kinetics (Fig. 2D). Although the mechanisms regulating the decrease in phosphorylation remain to be studied in details, they probably involve dopamine release triggered by light. In our experiments, we used constant room light and constant light is known to be poorly efficient at stimulating dopamine

release. In contrast, flickering light is much more efficient (see Kolbinger and Weiler, 1993, for instance). Thus, it is possible that the slow kinetics of dephosphorylation reflects the poor efficiency of steady background illumination to trigger dopamine release. In agreement with this, Cx36 phosphorylation is decreased by ~ 75% in the C57Bl/6J OPL following 10 min of flashing (3 Hz) bright light stimulation (H.L. and J.O.B., unpublished data). In addition, Cx36 phosphorylation is regulated by adenosine (Li et al., 2013), whose levels are under the control of light and a circadian clock (Ribelayga and Mangel, 2005). The precise mechanisms behind the slow kinetics of Cx36 dephosphorylation remain to be determined in future studies.

A recent publication reported that Cx36 transcript and protein expression are also regulated by light/dark-adaptation or a circadian clock in mouse photoreceptors, although with some interesting differences depending on the mouse strain (Katti et al., 2013). Cx36 transcript expression was found to be rhythmic with high nocturnal values under daily or circadian conditions in the outer nuclear layer (ONL) of both a melatonin-proficient (C3H^{+/+}) and a melatonin-deficient (C57Bl6/FVB) strain. In addition, a daily rhythm of Cx36 protein expression in the OPL with high values during subjective night was observed in the OPL of both strains but the rhythm persisted under circadian conditions only in the C3H^{+/+} strain. The reason why Katti et al. were able to detect a difference in the amount of Cx36 protein in the OPL while we have been consistently unable remains unknown. However, the nocturnal accumulation of Cx36 protein in the OPL they observed is consistent with the view that photoreceptor electrical coupling is maximal at nighttime and is controlled by a circadian clock in melatonin-proficient mouse strains and not in melatonin-deficient strains.

In conclusion, our study helps place the phosphorylation state of Cx36 as a key biochemical step at the crossroad of light-dark and circadian regulatory pathways that control photoreceptor coupling. Our results also support the requirement of melatonin and dopamine rhythms in the circadian control of photoreceptor coupling.

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Figure 1.

Daily and circadian regulation of Cx36 phosphorylation in the OPL of the CBA/CaJ mouse.

A) Examples of immuno-staining of Cx36 and PS293Cx36 in the OPL obtained under dark-adapted conditions in daytime (ZT06) and nighttime (ZT18) and in subjective daytime (CT06) and nighttime (CT18). Bar is 2 μm . B) Mean pixel intensity in the red channel per 504 μm^3 sampling volume under the conditions described in (A). C, D) Averaged values of the total number of Cx36 plaques per 504 μm^3 sampling volume (C) and of the phosphorylation state of OPL Cx36 (D) under the conditions described in (A). D: day dark-adapted; SD: subjective day; N: night; SN: subjective night. $n = 5-6$ animals, 3 sections/animal, 5 measurements/section. ***: $P < 0.001$.

Figure 2.

Effect of acute light exposure on Cx36 phosphorylation in the OPL of the CBA/CaJ mouse during subjective day and subjective night. A) Examples of immuno-staining of Cx36 and PS293Cx36 in the OPL obtained in subjective daytime (CT06) and nighttime (CT18) under dark-adapted conditions or after 1 h of light adaptation. Bar is 2 μm . B) Mean pixel intensity in the red channel per 504 μm^3 sampling volume under the conditions described in (A). C, D) Averaged values of the total number of Cx36 plaques per 504 μm^3 sampling volume (C) and of the phosphorylation states of OPL Cx36 (D) under the conditions described in (A). CDA: control dark-adapted; SD: subjective day; SN: subjective night; +1hL: light-adapted for 1 h; +3hL: light-adapted for 3 h. $n = 5-6$ animals, 3 sections/animal, 5 measurements/section. ***: $P < 0.001$.