
RESEARCH ARTICLE

Connexin 36 expression is required for electrical coupling between mouse rods and cones

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Abstract

Rod-cone gap junctions mediate the so-called “secondary rod pathway”, one of three routes that convey rod photoreceptor signals across the retina. Connexin 36 (Cx36) is expressed at these gap junctions, but an unidentified connexin protein also seems to be expressed. Cx36 knockout mice have been used extensively in the quest to dissect the roles in vision of all three pathways, with the assumption, never directly tested, that rod-cone electrical coupling is abolished by deletion of this connexin isoform. We previously showed that when wild type mouse cones couple to rods, their apparent dynamic range is extended toward lower light intensities, with the appearance of large responses to dim flashes (up to several mV) originating in rods. Here we recorded from the cones of Cx36^{del[LacZ]/del[LacZ]} mice and found that dim flashes of the same intensity evoked at most small sub-millivolt responses. Moreover, these residual responses originated in the cones themselves, since: (i) their spectral preference matched that of the recorded cone and not of rods, (ii) their time-to-peak was shorter than in coupled wild type cones, (iii) a pharmacological block of gap junctions did not reduce their amplitude. Taken together, our data show that rod signals are indeed absent in the cones of Cx36 knockout mice. This study is the first direct demonstration that Cx36 is crucial for the assembly of functional rod-cone gap junctional channels, implying that its genetic deletion is a reliable experimental approach to eliminate rod-cone coupling.

Keywords: Retina, Photoreceptors, Electrical coupling, Connexin 36

Introduction

The signals generated by rod photoreceptors take multiple parallel routes as they flow through the retina toward ganglion cells. The “primary pathway” traverses rod bipolar cells, AII amacrine cells, ON- and OFF-cone bipolars. The “secondary pathway” leads from rods to cones and, from these, continues downstream *via* cone bipolars. A growing body of evidence now supports the existence of an additional route, involving direct contact between rod photoreceptors and cone bipolar cells (Soucy et al., 1998; Tsukamoto et al., 2001; Tsukamoto et al., 2007; Cowan et al., 2016). Mammalian connexin isoform 36 (Cx36) is expressed at crucial sites along the primary and secondary rod pathways, namely the gap junctions between AII amacrine cells and both themselves and ON-cone bipolars (Feigenspan et al., 2001; Mills et al., 2001; Dedek et al., 2006), as well as those between rods and cones (Lee et al., 2003; O’Brien et al., 2012; Kantor et al., 2015) (but see Feigenspan et al., 2004). Not surprisingly, Cx36 knockout mice have been used extensively to dissect the relative roles of each rod pathway (Guldenagel et al., 2001; Deans et al., 2002; Robson et al., 2004;

Pang et al., 2007; Abd-El-Barr et al., 2009; Seeliger et al., 2011; further references below). The underlying assumption made in these studies was that genetic deletion of Cx36 abolishes all electrical coupling of the neurone pairs where this connexin is normally expressed. Thus, any rod signals detected in the cone bipolars (Pang et al., 2010; Pang et al., 2012), ganglion cells (Volgyi et al., 2004; Cowan et al., 2016) or higher visual centers (Brown et al., 2011) of these mouse mutants could not have exploited the crucial gap junctional sites mentioned above.

In the case of rod-cone gap junctions qualitative evidence obtained in horizontal cells suggests that electrical coupling is strongly impacted when Cx36 is not expressed (Trumpler et al., 2008). However, direct evidence obtained in cones is still missing. In principle, genetic deletion of Cx36 could lead, during development, to the compensatory expression of other connexins and assembly of functional channels. This possibility is made more concrete by ample evidence suggesting that a still unidentified connexin isoform or splice variant participates in the physiological assembly of rod-cone junctional channels, particularly on the rod side (Lee et al., 2003; Feigenspan et al., 2004; Bolte et al., 2016) (but see Deans et al., 2002; Dang et al., 2004): if this unidentified protein is also expressed by cones, it could substitute Cx36 and rescue rod-cone coupling to some extent. To assess the role of Cx36 at a key site of

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the secondary rod pathway and test the above assumption, we recorded from single cones in a mouse line developed by Klaus Willecke and colleagues, in which exon 2 of Cx36 was replaced with the LacZ reporter gene (Cx36^{del[LacZ]}). Mice homozygous for the mutation lack transcription and expression of Cx36, expressing instead a fusion protein comprised of the N-terminus of Cx36 and of β -galactosidase under the Cx36 promoter. They can thus be regarded as functional knockouts of this connexin isoform (Degen et al., 2004; Feigenspan et al., 2004). We compared Cx36^{del[LacZ]}/del[LacZ] mice to their strain-specific wild type controls, designated Cx36^{+/+}. Moreover, since Cx36^{+/-del[LacZ]} heterozygotes were originally back crossed to the C57BL/6 strain (Degen et al., 2004), we also compared these animals to a larger dataset of wild type C57BL/6J mice previously recorded in our laboratory to investigate rod-cone coupling.

Materials and methods

Dissection, recordings and light stimulation

All procedures involving the handling of experimental animals were approved by the ethical committee of the University of Pisa (prot. n. 2891/12) and were conducted in accordance with Italian (D.lgs.vo 116/92) and EU regulations (Council Directive 86/609/EEC). Dissection and recordings of Cx36^{del[LacZ]}/del[LacZ] and Cx36^{+/+} mice were performed as previously described by the authors for C57BL/6J (Cangiano et al., 2012; Asteriti et al., 2014). In brief, adult animals were dark-adapted for at least 3 h and anesthetized by i.p. injection of urethane 20% w/v in 0.9% saline. Under dim near infrared illumination (≈ 720 nm) each retina was extracted through a corneal incision into ice-cold bicarbonate-buffered Ames' medium (A1420; Sigma-Aldrich, St. Louis, MO), flattened on filter paper through gentle transparietal suction and sectioned at 250 μ m intervals on a manual tissue chopper (Leica Biosystems, Wetzlar, Germany). Slices were transferred to the recording chamber and thereafter superfused with Ames' at around 24°C. "Blind approach" recordings were obtained from rods (with perforated patch clamp or a loose seal technique) or from cones (perforated patch clamp). Cone sampling was random along the dorso-ventral axis of the retina and thus across the well known gradient of S/M opsin expression (Applebury et al., 2000). The intracellular solution contained: (in mM) 90 Kaspertate, 20 K₂SO₄, 15 KCl, 10 NaCl, 5 K₂Pipes and was corrected to a pH of 7.20 with KOH/HCl. The pipette backfilling solution also contained 0.4 mg/ml amphotericin-B pre-dissolved in DMSO at 60 mg/ml. As previously discussed by the authors in these recording conditions the liquid junction and Donnan potentials can be expected to partly cancel each other (Cangiano et al., 2012), leading us to report uncorrected values of membrane potential. Where stated gap junctions were blocked with meclofenamic acid (M4531; Sigma-Aldrich, St Louis, MO). Recordings were made with an Axopatch 1D amplifier having its low pass filter set at 500 Hz and acquired at 5 kHz by a Digidata 1320A using pClamp 9 software (Axon Instruments, Foster City, CA). Full field flashes of 1–10 ms were delivered with a green LED (peak emission 520 nm) or an ultraviolet LED (peak emission 365 nm) driven by computer-controlled current sources. The photon flux densities reaching the photoreceptors for different LED drive levels were separately estimated using a calibrated low power detector positioned at the recording chamber (1815-C/818-UV; Newport, Irvine, CA).

Animal models

Heterozygous Cx36^{+/-del[LacZ]} mice (Degen et al., 2004; Feigenspan et al., 2004) were obtained from Dr. Karin Dedek and mated. From their progeny two separate colonies were established, one consisted of functional connexin 36 knockout mice (Cx36^{del[LacZ]}/del[LacZ]), while the other represented their wild type controls (Cx36^{+/+}). Genotyping was done by PCR on 0.5 μ g of DNA extracted from neonatal tail samples using a RedTaq PCR mix (Sigma-Aldrich, St. Louis, MO) and the primer sets: (Cx36 intron lacZ) 5'-TGC ATT TGC CAG AGT AAA GGT GCG (Cx36 branch lacZ) and 5'-TTC TGT TTC AGC GCT TAC CAG TCC. PCR products were visualized by gel electrophoresis with Safeview Classic Nucleic acid stain (ABM, Richmond, BC, Canada). The Cx36^{del[LacZ]} "knockout" amplicon had the expected size of 220 bp and the Cx36⁺ "wild type" amplicon one of 330 bp. The C57BL/6J cones included in this study were recorded previously by our laboratory under the same experimental conditions and flash strengths.

Analysis and statistics

The recordings were analyzed with AxographX software (Axograph Scientific, Sydney, Australia). Statistical significance was assessed with the Mann–Whitney–Wilcoxon test (MWW) (either paired or unpaired, as specified in the text) or the Kruskal–Wallis test (KW) when comparing more than two groups of data. Data groups were considered significantly different when $P < 0.05$.

Results

We first performed a survey of Cx36^{del[LacZ]}/del[LacZ] rods to ensure that their light sensitivity was not different from that in wild type rods. We recorded rod photovoltages generated in response to sequences of dim green/uv flashes (g/uv; 16.6 photons/ μ m²) and bright green rod-saturating flashes (G; 3140 photons/ μ m²). These two flash strengths were chosen, respectively, to stimulate rods with minimal cone activation (dim), and to saturate rods while moderately stimulating cones (bright flashes) (Asteriti et al., 2014). Cx36^{del[LacZ]}/del[LacZ] rods expressed qualitatively similar light responses to wild type rods (Fig. 1A). We quantified their light sensitivity as the % ratio of their dim flash response *peak amplitudes* over those to bright flashes ($g_{\text{peak}}/G_{\text{peak}}$). This parameter was a reasonable proxy for rod light sensitivity, since its typical values of around 40% (Fig. 1B) implied that g flashes activated rods in a steep section of their dose-response function. $g_{\text{peak}}/G_{\text{peak}}$ was not significantly different ($P = 0.70$; KW test) between the rods of Cx36^{+/+} ($n = 10$), Cx36^{del[LacZ]}/del[LacZ] ($n = 9$) and C57BL/6J ($n = 45$) mice.

Having established that Cx36^{del[LacZ]}/del[LacZ] rods have a normal light sensitivity, we went on to assess whether Cx36 is a necessary component of rod-cone gap junctions by looking for rod signals, generated in the scotopic regime, in the cones of Cx36^{del[LacZ]}/del[LacZ] mutants. Sequences of dim and bright green/uv flashes, of the same photon densities used in rods, were delivered immediately after seal formation and throughout the recordings. These sequences were originally employed in Asteriti et al. (2014) to rapidly assess both the presence of rod signals (dim g and uv flashes) and each cone's intrinsic spectral preference (bright G and UV flashes after a bright G rod-saturating preflash). Qualitatively we found that Cx36^{+/+} cones frequently expressed a time-dependent

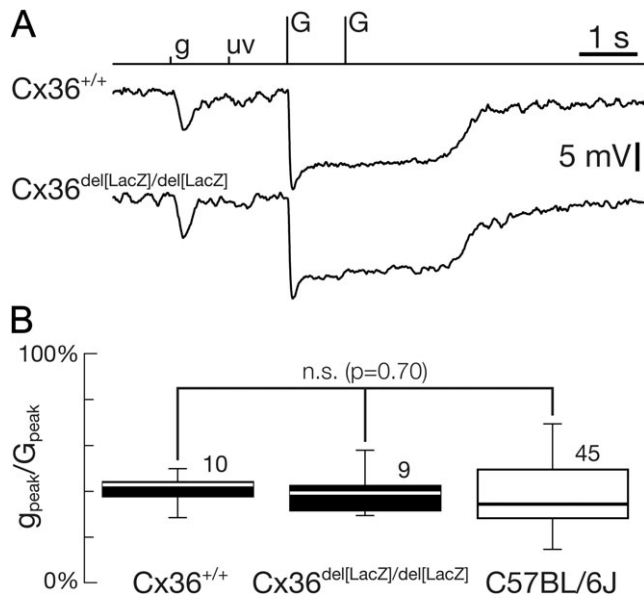


Fig. 1. Functional knockout of Cx36 does not affect rod light sensitivity. (A) Current clamp responses in two rods from $Cx36^{+/+}$ and $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ mice to a sequence of dim flashes (g, green; ultraviolet, uv: 16.6 photons/ μm^2) and bright rod-saturating flashes (green, G: 3140 photons/ μm^2). Records are not averages. Dark membrane potentials (V_{dark}) were -40.4 and -34.9 mV, respectively. (B) Plot of the % ratio of dim over bright green flash response peak amplitudes ($g_{\text{peak}}/G_{\text{peak}}$) of rods from $Cx36^{+/+}$, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ and C57BL/6J mice. Data are shown as median, interquartile range, minimum and maximum values.

increase in rod-cone coupling (Fig. 2A), similarly to what previously reported in C57BL/6J cones (Asteriti et al., 2014). In contrast, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ cones did not display this phenomenon and maintained very small responses to dim g flashes throughout the recordings, which required the averaging of multiple sweeps to clearly emerge above baseline noise (Fig. 2B).

To back this clear qualitative impression with quantitative evidence, we compared the amplitudes of cone responses to dim green flashes: (i) across $Cx36^{+/+}$, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ and C57BL/6J mice, and (ii) within each group at increasing times from seal formation. Our recordings included, within each group of mice, both green-dominant ($G/UV \geq 1$; $Cx36^{+/+}$ $n = 4$, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ $n = 14$) and uv-dominant cones ($G/UV < 1$; $Cx36^{+/+}$ $n = 4$, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ $n = 4$) (see also Asteriti et al., 2014). Suspected pure S-cones, which represent a small minority of cones (Haverkamp et al., 2005), were identified as those having a G/UV ratio < 0.05 and were one for each group (indicated in Fig. 3B with triangles). Since the trial-to-trial dim flash responses in uncoupled wild type cones and the majority of mutant cones were too small to be unambiguously identified above baseline noise, for all three mouse groups we measured the average amplitude between 150 and 210 ms after the flash ($g_{150-210}$): this range straddled the peak of rod-derived dim flash signals in wild type cones (see Discussion in Asteriti et al., 2014). As previously reported by the authors, patching on wild type cones evoked a progressive increase in their electrical coupling to rods with a time course of minutes to tens of minutes (Asteriti et al., 2014). We captured this process by averaging the dim flash response amplitudes within each of four time ranges from seal formation: 1–5 min, 6–15 min, 16–35 min and 36–60 min. Since not all recordings lasted 1 h or more, a decreasing number of cones contributed data to each subsequent time range.

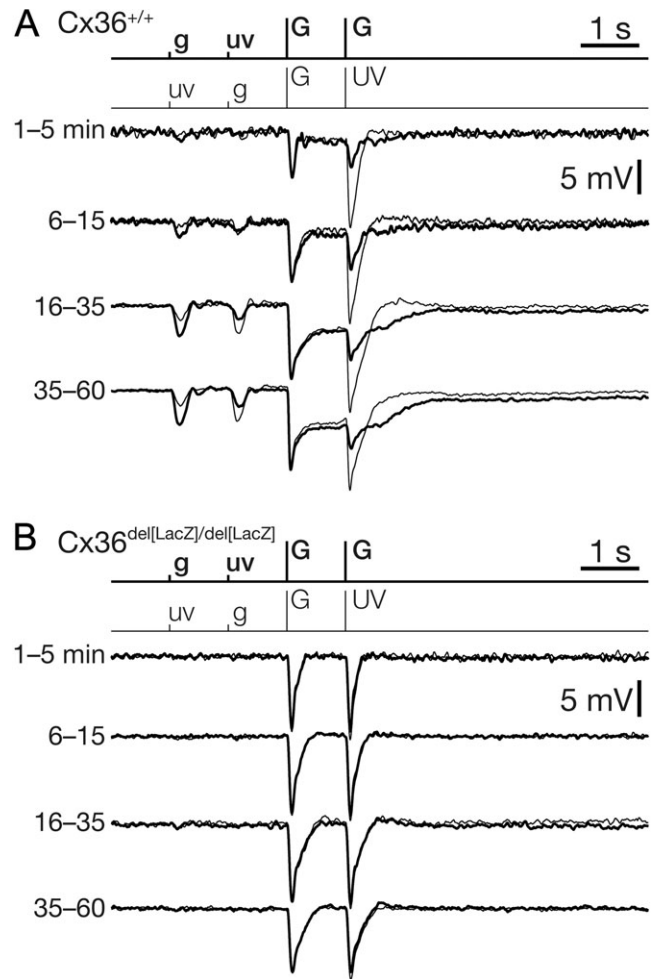


Fig. 2. Functional knockout of Cx36 abolishes the spontaneous increase in rod-mediated signals observed in wild type cones (sample records). (A) Current clamp response of a cone from a $Cx36^{+/+}$ mouse to a sequence of dim flashes (g, green; ultraviolet, uv: 16.6 photons/ μm^2) and bright rod-saturating flashes (green, G: 3140 photons/ μm^2). Records are averages of multiple responses obtained in the specified time ranges after seal formation. V_{dark} values were -40.6 , -43.4 , -45.1 and -43.3 mV, respectively. (B) Responses to the same stimulation protocol recorded in a cone from a $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ mouse. V_{dark} values were -38.2 , -37.3 , -41.7 and -48.4 mV, respectively.

Fig. 3A shows the main statistics of dim green flash responses ($g_{150-210}$) of $Cx36^{+/+}$, $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ and C57BL/6J cones in each of the four recording time ranges. $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ cones had much smaller dim flash responses than both $Cx36^{+/+}$ and C57BL/6J. With $Cx36^{+/+}$ cones this became significant starting from the 16–35 min range, while with the larger sample of C57BL/6J cones it was significant from the 1–5 min range (significance levels and sample numbers are reported in Fig. 3A; unpaired MWW test). As expected no significant difference was detected between $Cx36^{+/+}$ and C57BL/6J cones in any time range. These results show that when Cx36 is absent, rod-cone coupling is impaired. However, despite being dim green flash responses in $Cx36^{\text{del[LacZ]}/\text{del[LacZ]}}$ cones greatly reduced in amplitude, they were still significantly greater than zero in each of the first three time ranges ($P < 0.001$, $P < 0.01$, $P < 0.05$ respectively; single group MWW test). To find whether this residual signal originated in rods and was fed to cones *via* gap junctions formed by other connexin

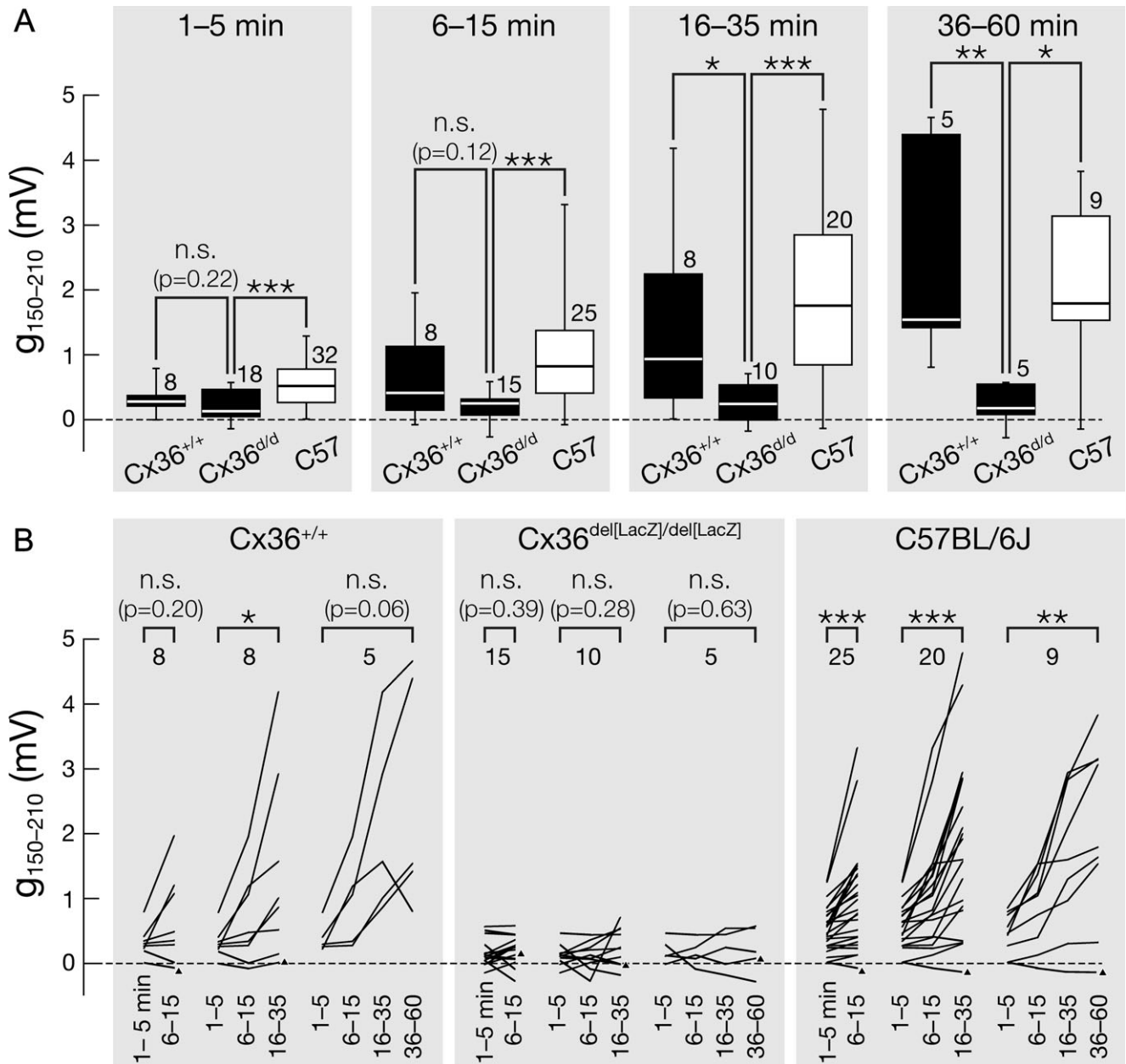


Fig. 3. Functional knockout of Cx36 abolishes the spontaneous increase in rod-mediated signals observed in wild type cones (quantification and statistics). **(A)** Dim green flash response amplitudes, measured as the average value in the range 150–210 ms after the flash ($g_{150-210}$, see Results for an explanation of this choice), are compared in the same time ranges after seal formation, between different groups of cones in Cx36^{+/+}, Cx36^{del[LacZ]/del[LacZ]} and C57BL/6J mice. Data are shown as median (thick lines), interquartile range (boxes) and min/max values (error bars). The number of cones contributing to the data are reported above the interquartile range. **(B)** Dim green flash response amplitudes ($g_{150-210}$) are compared in the same groups of cones, be they from Cx36^{+/+} or Cx36^{del[LacZ]/del[LacZ]} or C57BL/6J mice, between the first minutes after seal formation and later time ranges. Each line shows the trend in a single cone and the total number of cones is reported above. Strongly uv-dominant cones ($G/UUV < 0.05$) are indicated with a triangle. In both panels statistical significance is reported as follows: * is $P < 0.05$, ** is $P < 0.01$, *** is $P < 0.001$.

isoforms or, instead, if it originated in the cones themselves, we performed some further analyses.

We looked at whether dim green flash responses increased in amplitude during the recordings by comparing the second, third and fourth time ranges to the first (Fig. 3B). In Cx36^{del[LacZ]/del[LacZ]} cones no significant differences were detected ($P = 0.39$, $P = 0.28$, $P = 0.63$ respectively; paired MWW test), while in both Cx36^{+/+} and C57BL/6J cones at least one comparison was significantly different

(significance levels and sample numbers are reported in Fig. 3B). Therefore, within the statistical power reached with our sample size, in Cx36^{del[LacZ]/del[LacZ]} mutant cones we found no evidence of the time-dependent increase in coupling observed in wild type cones, and between other cells expressing Cx36 (Zoidl et al., 2002; Hornstein et al., 2005; Veruki et al., 2008; Del Corso et al., 2012).

Cx36^{del[LacZ]/del[LacZ]} mutant rods and cones could, however, be residually coupled by connexin isoforms that do not express

this phenomenon. To exclude this possibility, first we examined more closely three Cx36^{del[LacZ]/del[LacZ]} cones that showed an intrinsic preference for ultraviolet over green light (i.e. G/UV bright flash amplitude <1 when delivered after a G rod-saturating pre-flash) and in which sufficiently large average dim flash responses were observed. We computed their ratios of *g/uv* dim flash peak amplitudes and found them also to point toward a uv-preference: *g/uv* = 0.05 and G/UV = 0.04, *g/uv* = 0.45 and G/UV = 0.92, *g/uv* = 0.37 and G/UV = 0.56 (Fig. 4A), respectively. This was clearly consistent with their dim flash responses being generated in their own outer segments and not in hypothetically coupled rods, which have *g/uv* values of around 2.7 (Asteriti et al., 2014). In contrast, in two intrinsically uv-preferring Cx36^{+/+} cones coupled to rods, dim flashes showed non-concordant spectral preferences: *g/uv* = 2.65 and G/UV = 0.29 (Fig. 2A), *g/uv* = 1.82 and G/UV = 0.55, respectively. This was entirely consistent with a rod origin of their dim flash responses, as previously shown in a larger sample of C57BL/6J

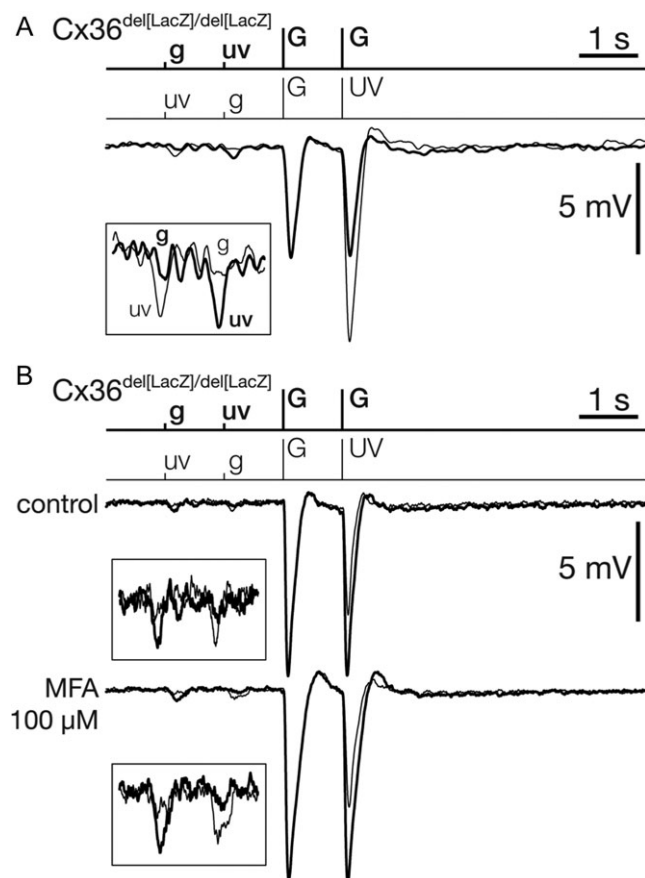


Fig. 4. The dim flash spectral preference of uv-dominant cones and the lack of an effect of the gap junction blocker MFA confirm that deletion of Cx36 abolishes rod-cone coupling. (A) Current clamp response to dim and bright flashes of a uv-dominant cone from a Cx36^{del[LacZ]/del[LacZ]} mouse (same protocol as in Fig. 2). The small dim flash responses show a similar uv-dominance to that expressed by the cone with bright flashes (delivered after a rod-saturating preflash). This does not occur in wild type cones coupled to rods (Fig. 2A). Records are averages. V_{dark} was -41.8 mV. (B) Current clamp responses to dim and bright flashes of a green-dominant cone from a Cx36^{del[LacZ]/del[LacZ]} mouse in control and >20 min after superfusion of the gap junction blocker meclofenamic acid (MFA; $100 \mu\text{M}$). The persistence of dim flash responses shows that they are not fed into the cone by gap junctions. Records are averages. V_{dark} values were -47.4 and -42.4 mV, respectively.

cones that had *g/uv* > 1.8 when electrically coupled to rods (Asteriti et al., 2014). Second, we examined the time-to-peak (TTP) of dim flash responses in Cx36^{del[LacZ]/del[LacZ]} cones and found them to be on average 137 ms (SEM 8 ms; $n = 14$), significantly shorter ($P < 0.01$; unpaired MWW test) than the 181 ms (SEM 6; $n = 5$) of Cx36^{+/+} coupled cones and also shorter than the 197 ms in C57BL/6J cones (Asteriti et al., 2014). Again, the faster kinetics of the very small dim flash responses displayed by Cx36^{del[LacZ]/del[LacZ]} cones are consistent with those signals originating in the same photoreceptors. Third, in three Cx36^{del[LacZ]/del[LacZ]} cones we succeeded to test the effect of the gap junction blocker meclofenamic acid ($100 \mu\text{M}$), which is effective on rod-cone coupling in the mouse (Asteriti et al., 2014). In neither of them did we observe a significant change in dim green flash response amplitudes between control conditions and after 20 min from the start of blocker superfusion. Average values in each cone were, respectively, 0.55 mV (SEM 0.05, control) and 0.56 mV (SEM 0.08, MFA; $P = 0.84$, unpaired MWW test), 0.39 mV (SEM 0.04, control) and 0.47 (SEM 0.03, MFA; $P = 0.21$) (Fig. 4B), 0.44 mV (SEM 0.11, control) and 0.35 mV (SEM 0.09, MFA; $P = 0.49$). These data are supported by previous evidence that C57BL/6J cones in MFA maintain a residual dim G flash response (fig. suppl. 2 in Asteriti et al., 2014). Taken together, these three independent tests argue against the presence of residual electrical coupling in Cx36^{del[LacZ]/del[LacZ]} cones.

Discussion

Rod-cone gap junctions represent the key element of what is commonly referred to as the “secondary pathway” of rod signal flow toward the inner retina. The actual impact of this route in vision remains a subject of debate, although the most accredited theory is that it mediates the transfer of rod signals at light levels above the single photon regime (Smith et al., 1986; Sharpe & Stockman, 1999 and references in the Introduction). In addition, more recent evidences suggest that it is under circadian control (Ribelayga et al., 2008) and is able to undergo rapid and strong upregulation (Asteriti et al., 2014). Interestingly, telodendrial processes and electrical coupling between rod-like and cone-like photoreceptors were recently shown by our group to be present also in lampreys (Asteriti et al., 2015), our phylogenetically most distant vertebrate relatives. This finding strongly suggests that rod-cone coupling was emplaced soon after the high sensitivity rod photoreceptor had evolved from a cone progenitor in the early Cambrian period. Its persistence throughout more than 500 million years of vertebrate diversification and across classes, implies that it must confer significant advantages. Essential tools in the quest to identify these advantages are animal models in which rod-cone coupling is genetically altered. Here we verified, for the first time, the assumption made in many studies, either explicitly or implicitly (see the Introduction), that rods and cones are completely uncoupled in Cx36 knockout mice. We used the Cx36^{del[LacZ]} model of functional knockout: within the sample size and sensitivity of our recordings we did not find any evidence of rod signals in the cones of animals homozygous for the mutant allele. In contrast, in cones from wild type controls of the same strain we frequently observed the same rod signals expressed in C57BL/6J cones and previously shown by the authors to be mediated by gap junctions (Cangiano et al., 2012; Asteriti et al., 2014).

A few points regarding our data and their broader implications must be discussed. First, when the original Cx36^{+/del[LacZ]} heterozygotes were developed they were back crossed to the C57BL/6

strain (Degen et al., 2004). The absence of a difference in behavior between the two groups of wild type cones (those from Cx36^{+/+} and those from C57BL/6J mice) was thus in line with our expectations. Second, our approach to detecting rod-cone coupling was mainly based on the large difference in intrinsic light sensitivity between the two photoreceptors. Therefore, a pre-requisite for the present study and, in fact, for all studies investigating the three rod pathways, was an assessment of the light sensitivity of Cx36 mutant rods. Reassuringly, we found it to be indistinguishable from wild type rods, which supports past evidence based on electroretinogram recordings of the scotopic a-waves and b-waves in Cx36 knockouts (Guldenagel et al., 2001; Robson et al., 2004; Pang et al., 2007). Third, three different Cx36 knockout mouse lines have been developed and used to investigate retinal processing (Deans et al., 2001; Guldenagel et al., 2001; Dang et al., 2004) (incidentally, two of these contain reporter genes driven by the Cx36 promoter). While our present data was obtained in the Cx36^{del[LacZ]} model, we are not aware of any circumstance that would make our conclusions inapplicable to the other two lines. Fourth, in the macaque it appears that blue cones (i.e. pure S-cones) form fewer junctional contacts with rods compared to other cones (O'Brien et al., 2012). If this phenomenon occurred also in mouse it would imply that the present work should be conducted preferentially in cones having some degree of M-opsin expression. This was precisely the case, as reported in the Results (see spectral preference distribution). Fifth, it is worth assessing the potential impact of circadian rhythmicity on our lack of detection of residual coupling in mutant cones. As mentioned above, the Cx36^{del[LacZ]} mouse line was originally obtained after back crossing to the C57BL/6 strain. Based on tracer diffusion and dopamine release measurements Li et al. (2013) concluded that in C57BL/6 mice, which are melatonin-deficient, dark adaptation during daytime “results in a state that is qualitatively similar to the nighttime state in that photoreceptor coupling is increased”. Our recordings were made after at least 3 h of dark adaptation of the animals, so based on their findings we would be led to *exclude the possibility* that our mutant cones didn't display coupling to rods because any hypothetical residual junctional channels were kept closed by the retinal neuromodulatory systems. It must be stressed that our own data (Asteriti et al., 2014) (Fig. 3 in the present study) clearly show that under the same daytime/dark adapted conditions C57BL/6J and Cx36^{+/+} cones keep a large proportion of their rod-cone coupling potential unexpressed, its underlying extent being revealed by a phenomenon of spontaneous coupling increase triggered by the recording pipette. Overall, the possibility that Cx36^{del[LacZ]/del[LacZ]} cones maintain some latent junctional channels to rods that are neither gated by prolonged dark adaptation nor by the perturbation of the local milieu caused by the recording electrode, seems highly improbable.

There is significant evidence indicating that rod-cone gap junctions are heterotypic, in that Cx36 is not the only connexin taking part in their assembly, an unidentified isoform or splice variant being likely expressed on the rod side (Lee et al., 2003; Feigenspan et al., 2004; Bolte et al., 2016). Since rods evolved from a cone ancestor (Lamb, 2013), it cannot be excluded that also cones express this unknown connexin protein to some degree (in addition to Cx36). Nonetheless, our findings indicate that, in the absence of Cx36, functional channels cannot be assembled and inserted between rods and cones. Moreover, they provide the necessary support for many studies that have used Cx36 knockout mice to examine the relative roles in vision of the three rod pathways (references in the Introduction) or assessed a possible involvement of rod-cone coupling in degenerative retinal diseases (Striedinger et al., 2005; Kranz et al., 2013).

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