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Activation of rhodopsin gene transcription in cultured retinal precursors of chicken embryo: role of Ca²⁺ signalling and hyperpolarization-activated cation channels

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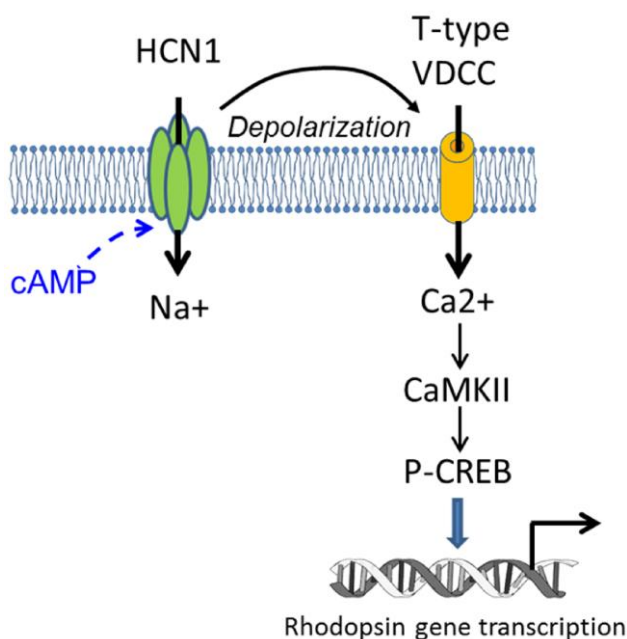
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ABSTRACT

The present study reports that the spontaneous 50-fold activation of rhodopsin gene transcription, observed in cultured retinal precursors from 13-day chicken embryo, relies on a Ca^{2+} -dependent mechanism. Activation of a transiently transfected rhodopsin promoter (luciferase reporter) in these cells was inhibited (60%) by cotransfection of a dominant-negative form of the cAMP-responsive element binding protein (CREB). Both rhodopsin promoter activity and rhodopsin mRNA accumulation were blocked by Ca^{2+} /calmodulin-dependent kinase II (CaMKII) inhibitors, but not by protein kinase A inhibitors, suggesting a role of Ca^{2+} rather than cAMP. This was confirmed by the inhibitory effect of general and T-type selective Ca^{2+} channel blockers. Oscillations in Ca^{2+} fluorescence (Fluo8) could be observed in 1/10 cells that activated the rhodopsin promoter (DsRed reporter). A robust and reversible inhibition of rhodopsin gene transcription by ZD7288 indicated a role of hyperpolarization-activated channels (HCN). Cellular localization and developmental expression of HCN1 were compatible with a role in the onset of rhodopsin gene transcription. Together, the data suggest that the spontaneous activation of rhodopsin gene transcription in cultured retinal precursors results from a signalling cascade that involves the pacemaker activity of HCN channels, the opening of voltage gated Ca^{2+} -channels, activation of CaMKII and phosphorylation of CREB.



INTRODUCTION

The mature retina displays a laminar organization consisting of three cellular layers : outer nuclear layer (composed of rod and cone photoreceptors), inner nuclear layer (composed of horizontal cells, bipolar cells, amacrine cells and Müller glial cells) and ganglion cell layer (composed of the output neurons whose axons give rise to the optic nerve). All these cell types originate from a common pool of embryonic progenitors and differentiate in a tiling sequence, conserved among vertebrate species: ganglion cells first, followed by horizontal cells, photoreceptors, amacrine cells, bipolar cells and finally Müller glial cells (Belecky-Adams *et al.*, 1996; Cepko *et al.*, 1996). As evidenced by lineage-tracing studies, two daughter cells derived from the same progenitor often differentiate into distinct cell types, a result indicating that these cells retain a certain degree of plasticity until their last mitosis (Belecky-Adams *et al.*, 1996; Cepko *et al.*, 1996). Nevertheless, as far as photoreceptors are concerned, cell-fate commitment appears to take place soon after proliferation has ended, because some photoreceptor-specific markers, such as the transcription factor “neural retina leucine zipper” (Nrl) in mouse rods, or the calcium-binding protein visinin in chicken cones, are expressed within one day of exiting mitosis (Bruhn and Cepko, 1996; Akimoto *et al.*, 2006). One aspect of photoreceptor differentiation that is conserved in mammals and birds is the existence of a long time lag between commitment, revealed by Nrl or visinin expression, and overt differentiation, reflected by opsin expression. For example in mouse and chicken, significant rhodopsin expression is detected about 8 days after photoreceptor commitment (Treisman *et al.*, 1988; Belecky-Adams *et al.*, 1996; Bruhn and Cepko, 1996; Cailleau *et al.*, 2005; Akimoto *et al.*, 2006; Voisin *et al.*, 2012). The nature of this long time lag between commitment to a photoreceptor fate and opsin gene expression has not been elucidated. It is not known whether it reflects the time required for the implementation of a differentiation program acquired postmitotically, or if it reflects a standby state of committed precursors, waiting for a signal to activate opsin gene expression. Hypothetically, this signal might correspond to spontaneous oscillations in Ca²⁺ and cAMP levels that have been described in developing retinas (Catsicas *et al.*, 1998; Wong *et al.*, 1998; Firth *et al.*, 2005; Dunn *et al.*, 2006). In chicken retina, Ca²⁺ oscillations are maximal when opsin gene expression begins (E14-E18, Wong *et al.*, 1998) and they have been shown to reach the outer nuclear layer (Catsicas *et al.*, 1998). In keeping with this hypothesis, we previously reported that chicken retinal precursors isolated in culture at E8 are not capable to spontaneously activate rhodopsin gene transcription, but can be induced to

do so in response to cAMP or membrane depolarization (Voisin and Bernard 2009). More recently, we observed that chicken retinal precursors isolated in culture after E10 spontaneously activate rhodopsin gene transcription (Voisin *et al.*, 2012). Therefore, the present study sought to examine the possible role of cAMP- and Ca²⁺-dependent signalling pathways in the spontaneous activation of rhodopsin gene transcription in chicken retinal precursors cultured after E10.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs were purchased from Anjou-Accouvage (Le-Louroux-Béconnais, France) and incubated at 37°C, 70% humidity. Embryos were sacrificed by decapitation between embryonic day 8 (E8) and E13. Retinas were immediately dissected in culture medium, and processed for cell culture. Chickens (postnatal day 1 (P1) to P4) were sacrificed by CO₂ and decapitation. Animal care followed the European Community and French Ministry guidelines (decree 87848, license E 86-040). The experiments complied with the ARRIVE guidelines.

Embryonic retinal cell cultures

Primary cultures of embryonic chicken retinal cells were prepared as previously described (Adler, 1990), with slight modifications (Voisin *et al.*, 2012). Neural retinas from E13 embryos, free of pigment epithelium, were dissociated by trypsin-DNase digestion, seeded at high density ($1.25 \times 10^6/\text{cm}^2$) in plastic plates pre-coated with polyornithine ($20 \mu\text{g}/\text{cm}^2$) and cultured 1 to 5 days (see figure legends) at 37°C, 5% CO₂, constant darkness, in medium 199 supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L glutamine and with either 0.5% bovine serum albumin (BSA) (all from Sigma-Aldrich, Saint-Quentin-Fallavier, France) or 10% newborn calf serum (NCS; Life technologies, Saint-Aubin, France).

Pharmacological treatments

For real time RT-PCR analysis, E13 retinal cells were cultured for 4 days and were treated with drugs for the last 2 days, before RNA extraction. For rhodopsin promoter analysis, E13 retinal cells were treated with pharmacological compounds immediately after seeding, transfected with promoter-reporter plasmids 4 h later, and assayed for luciferase activities 24 h later. The following compounds were dissolved in double-distilled water: Rp-8Br-cAMPS (BioLog, Bremen, Germany), H89, CdCl₂, NiCl₂, tubocurarine (Sigma-Aldrich), ω -conotoxin GVIA, SNX 482, kynurenic acid (R&D Systems, Lille, France). The following compounds were dissolved in dimethyl sulfoxide (DMSO): ML218 (Sigma-Aldrich), KN-62, KN-93, bepridil, cilnidipine, ω -agatoxin IVA, mibefradil, ryanodine, 2-APB, ZD7288, CNQX, MK801 (R&D Systems), KN-92 (Calbiochem). Controls received an equal volume of solvent (water or DMSO).

RNA extraction and real time RT-PCR

Cells cultured for 4 days in serum-free medium were sonicated in LiCl 3M/urea 6M and RNA was extracted as previously described (Cailleau *et al.*, 2005). Random hexamer-primed reverse transcription and real time PCR on LightCycler® (Roche Applied Science, Meylan, France) were as previously described (Cailleau *et al.*, 2005). The primer pairs for chicken rhodopsin (RH1) and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as previously described (Voisin *et al.*, 2012). Sequences of the primers for chicken HCN1 were: sense = 5'-TTACGCTTATTACGCCTTTCAAG-3'; antisense = 5'-GGTGGGAAATCCTGGAGTAAC-3' (GenBank XM_429145). Primer pairs were validated as follows: single PCR product (melting curve, gel electrophoresis, DNA sequencing), linear distribution of the PCR sigmoids ($r \geq 0.99$) with an efficiency of 1.85 to 1.98. We verified that 2- to 10-fold dilutions of the RNA introduced into an RT-PCR reaction caused the expected critical cycle (Ct) increments. Reverse transcripts copy numbers were calculated from standard curves established with known quantities of each template.

Transfections with luciferase reporter plasmids

Cells cultured in NCS-supplemented medium were transfected by the CaPO₄ method, as previously described (Voisin and Bernard, 2009). Cells (2.5×10^6 in 600 μ L medium) were transfected with 0.6 μ g of plasmid containing 1.6 kb of the chicken rhodopsin promoter, upstream of the firefly luciferase reporter (pRho1.6kb, previously described in Voisin and Bernard, 2009) or with 0.6 μ g of promoterless pGL3-Basic plasmid (Promega, Charbonnières, France) as negative control. Variations in transfection efficiency were normalized by cotransfection with 0.2 μ g of pRL-TK plasmid (Promega), containing HSV thymidine kinase promoter upstream of *Renilla* luciferase. In cells treated with H89 (Fig.2B), normalization was performed with 0.15 μ g of pRL-SV40 plasmid (Promega), containing the enhancer and early promoter of SV40 upstream of the *Renilla* luciferase, because we observed that co-transfection with pRL-TK introduced a correction bias. Expression of a dominant-negative form of the transcription factor CREB (cAMP-response element binding protein), with a mutation in the phosphorylation site, was performed by co-transfection with 0.6 μ g of pCMV-CREB133 (Clontech, Saint-Quentin-en-Yvelines, France). When different pharmacological agents were tested in the same experiment, the different experimental groups were always transfected with the same CaPO₄/DNA precipitate, to minimize variations due to qualities of precipitates.

Firefly and *Renilla* luciferase activities were assayed with the Dual-Glo™ luciferase assay system (Promega) on a GloMax™20/20 luminometer (Promega).

Transfection with a DsRed reporter plasmid and co-labelling of lipid droplets

The 1.6 kb chicken rhodopsin promoter was subcloned into the promoterless vector pDsRed2-1 (Clontech), to generate the "pDsRed-pRhodo1.6kb" plasmid. Sequence and orientation were verified by sequencing (BigDye and ABI 3130, Applied Biosystems, Courtaboeuf, France). E13 retinal cells, seeded in chamber slides (Lab-Tek Permanox®, NUNC) pre-coated with polyornithine, were transfected with 2 µg of "pDsRed-pRhodo1.6kb". Two days post-transfection, cells were fixed (4% paraformaldehyde, 20 min, 4°C), washed twice with phosphate-buffered saline (PBS), and oil droplets were labelled with Bodipy® 493/503 (Molecular probes, Montluçon, France) 0.5 µg/mL in PBS 1% DMSO (20 min, 20°C). After a final wash in PBS, slides were mounted in Mowiol® 4-88 (Sigma-Aldrich) and examined on a fluorescence microscope (Leica DMI6000) equipped with a digital camera (Leica DFC350FX with LAS AF software).

Viability assays

Cell viability in the presence of the different pharmacological agents was evaluated by measuring the yield of RNA extraction per 10⁶ cells, and with a classical cytotoxicity assay (Fig. S1). To determine more specifically whether the pharmacological treatments may have selectively affected the viability of the rod precursors, we verified that the effects of the drugs on rhodopsin promoter activity were reversible upon rinsing. This test could not be applied to RNA analysis, because 4 days of culture were required for accumulating enough rhodopsin mRNA, and cell survival spontaneously declined beyond 4 days of culture. The reversibility protocol was as follows: E13 cells received pharmacological treatments immediately after seeding and were transfected 4 h later. Luciferase assay was performed 24 h post-transfection/treatment on a first experimental group, while a second group was transferred to control culture medium and assayed for luciferase 3 days later.

Immunodetection of HCN1 on western blots

Embryonic chicken retinas (40 mg) were sonicated in 700 µL of TPE Buffer (50 mM Tris pH 7.4 ; 0.5 M NaCl ; 1 mM phenylmethanesulfonylfluoride ; 1 mM EGTA) cleared from cell debris at 1000 x g (20 min, 20°C) and membranes were recovered by centrifugation at 100 000 x g (1 h, 4°C). Pellets were washed with 1 mL of TE buffer (10 mM Tris pH 7.5 ; 1 mM EDTA),

resuspended in Laemmli buffer (Laemmli 1970) and heated at 37°C for 20 min. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred on nitrocellulose membrane. Non-specific sites were blocked in PBS containing 0.05% Tween-20 (PBS-Tween) and 5% BSA. The membranes were incubated (overnight, 4°C) with mouse monoclonal anti-HCN1 primary antibody (clone S70-28, Acris Antibodies, Herford, Germany) diluted 1:1000 in PBS-Tween, 1% BSA. The secondary antibody (CyTM3-conjugated goat-anti-mouse IgG antibody, Jackson, Newmarket, UK), diluted 1:400 in PBS-Tween, 1% BSA, was incubated 1h at 20°C. As negative control, a membrane was incubated with the secondary antibody alone. Fluorescence was detected on a laser scanner (Typhoon Trio, GE Healthcare) coupled to the ImageQuant software (GE Healthcare).

Detection of HCN1 by immunohistochemistry

Retinas fixed in Bouin's solution were dehydrated in ethanol, then butanol, and embedded in paraffin. Sections (6 µm) were blocked in PBS-Tween, 5% BSA and incubated (overnight, 4°C) with the anti-HCN1 antibody described above (1:1000 in PBS-Tween, 1% BSA). Immunocomplexes were detected with biotinylated horse anti-mouse IgG (Vector laboratories, Paris, France) diluted 1:500 in PBS-Tween and with avidin-biotin-peroxidase complexes (Vectastain Kit; Vector Laboratories). Peroxidase activity was revealed with 0.05% diaminobenzidine, 38 mmol/L ammonium nickel sulfate and 0.003% H₂O₂ in 0.15 mol/L sodium acetate buffer. Sections were observed on a light microscope (Zeiss Axioplan) equipped with a digital camera (Olympus LC20 with LCmicro software).

Calcium imaging

Embryonic chicken retinal cells (E13) were cultured on glass-slides (3 cm diameter) precoated with polyornithine. Cells were washed with medium 199 without phenol red (Sigma-Aldrich) and then loaded with 1 mM Fluo-8 (Interchim, Montluçon France) at 37°C for 30 min. Images were obtained with a spinning disk confocal system from Andor technology (Belfast, Ireland) equipped with Andor Ixon+897 back illuminated EMCCD camera (16 µm² pixel size), Olympus inverted IX81S1F-ZDC microscope (Olympus, France) and an incubation chamber (37°C and CO₂ enrichment, Solent Scientific, UK). For calcium imaging, cells were illuminated at 488 nm and emitted fluorescence was collected through a passband filter at 525 nm (30 nm width). IQ2 acquisition software (Andor technology) was used to acquire images (1 image every 2 sec during 10 to 20 min) of 512x512 pixels (0.33 µm per pixel with x40 objective lens).

Fluorescence was recorded in cells from 11 individual microscope fields (approximately 150 cells per field), in 3 independent experiments.

Statistical analyses

For real-time RT-PCR analyses, statistics were performed on the normalized Ct values (for each individual sample, the Ct value for rhodopsin was normalized to the Ct value for GAPDH). For luciferase assays, statistics were performed on the normalized luciferase activities (*firefly*/Renilla ratios measured in each individual culture well). Statistical analyses were performed with the two-tailed Student's *t*-test. Data are presented as mean \pm SEM. Statistical significance is indicated in each figure legend. For all experiments, differences were considered significant at $p < 0.05$.

RESULTS

***In vitro* activation of the rhodopsin gene promoter in developing rods.**

Retinal precursor cells were isolated from chicken embryos aged E8 to E13 and transiently transfected *in vitro* with a luciferase reporter plasmid harbouring a 1.6 kb rhodopsin promoter. As illustrated in figure 1A, rhodopsin promoter activity in cultured cells markedly increased with the age of donor embryos. Cells obtained from E8 embryos did not activate the rhodopsin promoter, a small (6-fold) increase was observed in cells from E10 embryos and a robust (30- to 100-fold) activation of the promoter was observed in cells obtained at E12-E13 (Fig. 1A). Rhodopsin promoter activity faithfully reflected the accumulation of rhodopsin mRNA measured in parallel cultures (Fig. 1B), thus confirming our previous report on the spontaneous activation of rhodopsin gene transcription *in vitro* (Voisin *et al.*, 2012). In addition, rhodopsin promoter activation appeared to take place in rod precursors, because transfection with a plasmid containing the same promoter driving a Dsred fluorescent reporter, allowed us to observe labelled cells with the characteristic shape of photoreceptors (Fig. 1C-E) and lacking the cone-specific lipid droplet (Fig. 1F-H). The labelled cell frequency was about 1/1000, which is coherent with the following proportions: 30% photoreceptors in the culture (Xie and Adler, 2000), 15% of the photoreceptors being rods in chicken retina (Voisin *et al.*, 2012), 1-2% transfection efficiency (our observations with constitutive promoters). Assuming the differentiation of cultured precursors replicates to some extent the *in vivo* situation, we sought to characterize the regulation pathway involved in the spontaneous activation of rhodopsin gene transcription.

Rhodopsin promoter activity is inhibited by a dominant-negative form of CREB, but not by PKA inhibitors.

Based on our previous work indicating that exogenous cAMP stimulates rhodopsin gene transcription in retinal cells isolated from E8 embryos (Voisin and Bernard, 2009), we examined whether an endogenous activation of the cAMP-dependent pathway could be responsible for the spontaneous activation of the rhodopsin gene, observed in cells isolated at E13. A non-phosphorylatable form of CREB lacking the critical serine 133 (CREB133), was co-transfected with the promoter-reporter plasmid. This resulted in a 60% inhibition of

rhodopsin promoter activity (Fig 2A), thus indicating that the phosphorylated form of CREB is required for rhodopsin gene activation. Due to the low transfection efficiency (around 1%), the effect of CREB133 could not be detected on rhodopsin mRNA levels (data not shown).

The PKA inhibitors Rp-8Br-cAMPS and H89 did not prevent rhodopsin promoter activation in the cultured cells (Fig 2B). Indeed, Rp-8Br-cAMPS could even cause a slight stimulation of the rhodopsin gene promoter (Fig 2B). A partial degradation of Rp-8Br-cAMPS may have generated trace amounts of 8Br-cAMP (as mentioned by the manufacturer), which is an activator of rhodopsin gene transcription (Voisin and Bernard, 2009). Rp-8Br-cAMPS and H89 also failed to interfere with rhodopsin mRNA levels (data not shown). Together, the data would suggest that CREB, but not its phosphorylation by PKA, is required for rhodopsin promoter activation.

***In vitro* activation of rhodopsin gene transcription is dependent on CaMKII.**

A possible role of Ca²⁺/calmodulin-dependent kinase II (CaMKII) was investigated, because this enzyme is known to phosphorylate and activate CREB in different tissues (Dash *et al.*, 1991; Sheng *et al.*, 1991). The CaMKII inhibitors KN-62 and KN-93 prevented the spontaneous rise in rhodopsin mRNA levels (Fig. 3A). The selectivity of KN-62 for CaMKII has been established more thoroughly than that of KN-93, but the latter was reported to be more potent (Tokumitsu *et al.*, 1990; Sumi *et al.*, 1991; Davies *et al.*, 2000) and this was reflected on the inhibition of rhodopsin mRNA accumulation (Fig. 3A). KN-92, a KN-93 analogue that lacks activity on CaMKII (Tombes *et al.*, 1995) did not significantly inhibit rhodopsin mRNA expression (Fig. 3A), indicating that the effect of KN-93 was mostly due to CaMKII inhibition. Coomassie blue G at 2 μ M had no effect (data not shown), thus ruling out a possible action of KN-62 through the P2X₇ purinergic receptor (Gargett and Wiley, 1997). The effects of KN-62 and KN-93 and the lack of effect of KN-92 could be confirmed at rhodopsin promoter level (Fig. 3B). The faster response of the promoter-reporter system over endogenous mRNA accumulation, allowed us to observe that the effect of KN-62 was reversible upon rinsing (Fig. 3C). In addition, general cell viability was not affected by the presence of KN compounds, thus confirming that the observed effects were not due to toxicity (Fig. S1). We also verified that KN62 inhibited CREB phosphorylation in cultures of chicken retinal precursors (Fig. S2).

***In vitro* activation of rhodopsin gene transcription depends on voltage-gated Ca²⁺ channels.**

Based on our previous work indicating that membrane depolarization stimulates rhodopsin gene transcription in retinal cells isolated at E8 (Voisin and Bernard, 2009), we examined a possible contribution of voltage-gated calcium channels to the spontaneous activation of the rhodopsin gene in E13 cells. General inhibitors of voltage-activated Ca²⁺ channels (Cd²⁺, bepridil) prevented the rise in rhodopsin mRNA levels (Fig. 4A). Inhibition was also observed with Ni²⁺ and mibefradil that preferentially target T-type and R-type Ca²⁺ channels (Mehrke *et al.*, 1994; Randall and Tsien, 1997; Lee *et al.*, 1999; Catterall *et al.*, 2005) and with ML218 that selectively targets T-type Ca²⁺ channels (Xiang *et al.*, 2011). In contrast, the L-type Ca²⁺ channel blocker nifedipine (Catterall *et al.*, 2005) had no significant effect (Fig. 4A). The prominent role of T-type Ca²⁺ channels was confirmed by the observation that rhodopsin promoter activity was not affected by different blockers with selectivity for L-type, N-type, R-type, P/Q-type channels (Catterall *et al.*, 2005), whereas it was dose-dependently inhibited by bepridil, mibefradil and ML218 (Fig. 4 B, C). As illustrated for mibefradil in figure 4D, the effects of these three compounds were reversible upon rinsing. In addition, we verified that bepridil, mibefradil and ML218 had only minor effects on general cell viability (Fig. S1).

Ca²⁺ oscillations in cultured retinal precursors

Because Ca²⁺ waves can be observed in the developing chicken retina (Catsicas *et al.*, 1998; Wong *et al.*, 1998), we sought to observe Ca²⁺ oscillations in cultured chicken retinal precursors loaded with Fluo-8. Strictly dependent on the presence of 5% CO₂ in the incubation chamber, 6% of the cells displayed spontaneous oscillations of Ca²⁺ fluorescence, with amplitudes ranging from 150% to 650% of baseline intensity (average 210% ± 10, n=84 cells) and a duration of 0,5 to 2 min (Fig. 5). These oscillations (Fig. 5A-C) were very similar to the Ca²⁺ transients described in cultured rat retinal cells (Firth and Feller, 2006), and resembled the “spikes” and “waves” previously described in cultured embryonic spinal cord neurons (Gu *et al.*, 1994). Ca²⁺ oscillations in individual cells were not evenly distributed throughout the 20 min recording period and there was no evidence of synchronization between cells within the same field. To determine whether Ca²⁺ oscillations could occur in rod precursors, we

monitored Fluo-8 fluorescence in cells expressing the DsRed reporter protein under control of the rhodopsin promoter. As illustrated in figure 5D-F, one out of 10 DsRed-positive cells exhibited spike-like oscillations. Our attempts to pharmacologically interfere with Ca²⁺ oscillations were inconclusive, due to disturbance of the CO₂ flow.

***In vitro* activation of rhodopsin gene transcription depends on HCN channels.**

We investigated whether the Ca²⁺ influx required for rhodopsin gene activation was due to depolarizing neurotransmitters or to hyperpolarization-activated cation channels (HCN). Based on previous studies indicating that waves of depolarization in the developing retina are generated by acetylcholine nicotinic and glutamate ionotropic neurotransmissions (Catsicas *et al.*, 1998; Wong *et al.*, 1998; Firth *et al.*, 2005), we tested the corresponding antagonists: tubocurarine 1 μM (nicotinic antagonist), kynurenic acid 1 mM (excitatory amino-acid antagonist), CNQX 10 μM (AMPA/kainate antagonist) and MK801 10 μM (NMDA antagonist). None of them caused any reduction in rhodopsin promoter activity (data not shown). In contrast, ZD7288, a potent inhibitor of hyperpolarization-activated cation channels (Harris and Constanti, 1995) inhibited rhodopsin mRNA accumulation in a dose-dependent manner (Fig. 6A) and also dose-dependently and reversibly blocked rhodopsin promoter activation (Fig. 6B, C). We also verified that the highest dose of ZD7288 did not significantly affect cell viability (Fig. S1).

Based on the notion that amphibian and mammalian photoreceptors preferentially express channels of the HCN1 subtype (Demontis *et al.*, 2002; Knop *et al.*, 2008; Barrow and Wu, 2009), we investigated whether the development and localization of HCN1 in the chicken retina would be compatible with a role in rhodopsin gene expression. As illustrated in figure 7A, development of HCN1 mRNA levels was biphasic, with a first plateau between E11 and E17 and a second one after E19. HCN1 protein became faintly detectable on western blot at E14 and its concentration steadily increased to give a strong monospecific signal at one day post-hatch (P1) (Fig. 7B). Immunohistochemistry of HCN1 channels at P1 gave a strong signal in the upper half of the outer plexiform layer (Fig. 7C), where the synaptic spherules of rods and the synaptic pedicles of double cones are normally found (Morris and Shorey, 1967). A fainter labelling was also observed in the inner plexiform layer (Fig. 7C). At higher magnification, HCN1 immunolabelling in photoreceptors appeared concentrated in a round-shaped area, directly under the nucleus, that closely matched the description of rod synaptic spherules

(Morris and Shorey, 1967). The frequency of labelled synaptic terminals was around 25 per 100 μm (Fig. 7C).

DISCUSSION

The present study provides evidence that spontaneous activation of rhodopsin gene transcription in cultured precursor cells of the chicken retina is driven by a Ca²⁺-dependent mechanism that involves CREB, CaMKII, voltage-gated Ca²⁺ channels and HCN channels. Ca²⁺ spikes and waves could be observed in a small proportion of cultured precursors, although a causal link between these Ca²⁺ transients and rhodopsin gene activation could not be established. This Ca²⁺-dependent component in rhodopsin gene activation had not been detected before and its discovery raises questions as to the exact mechanism involved and its physiological relevance to the *in vivo* situation.

Many studies aimed at understanding the molecular basis of photoreceptor differentiation have led to the identification of transcription factors and diffusible signals that govern different steps of this developmental process, including precursor proliferation, commitment to the photoreceptor cell fate and expression of the opsins (Levine *et al.*, 2000; Swaroop *et al.*, 2010). All these informations are valuable, because they open new possibilities of retinal therapy (Forrest and Swaroop, 2012; Montana *et al.*, 2013). By focusing on the spontaneous expression of the rhodopsin gene in retinal precursors isolated from E13 chicken embryos, we have identified a role for Ca²⁺ signalling at a late stage of rod differentiation. In a previous study we had observed that retinal precursors isolated at E8 did not spontaneously activate rhodopsin gene transcription in culture, but could be instructed to do so by treatments that caused membrane depolarization and/or elevation of cAMP levels (Voisin and Bernard, 2009). We therefore hypothesized that this mechanism might be involved in the spontaneous activation of rhodopsin gene transcription observed in retinal precursors isolated at E13. This hypothesis was also based on the notion that terminal differentiation of ganglion cells in the retina is regulated by waves of depolarization that induce a Ca²⁺/Calmodulin-dependent activation of adenylate cyclase (Torborg and Feller, 2005; Dunn *et al.*, 2006; Nicol *et al.*, 2006; Nicol *et al.*, 2007). Our results show that rhodopsin promoter activation requires CREB phosphorylation, because it is strongly inhibited by dominant-negative CREB133. However, rhodopsin gene activation appears to rely on Ca²⁺ but not on cAMP, because it is inhibited by CaMKII inhibitors, but not by PKA inhibitors. The signalling pathway involves an influx of extracellular Ca²⁺, because rhodopsin gene expression is strongly inhibited by general blockers of voltage-gated Ca²⁺ channels (cadmium, bepridil). A contribution of Ca²⁺-induced Ca²⁺ release could be ruled out, because ryanodine (50 μ M) clearly had no effect on rhodopsin promoter activation (data not shown). As to a possible contribution of inositol 1,4,5-

trisphosphate (IP₃)-induced Ca²⁺ release, the results of 6 independent experiments mostly argue against it, because the inhibitor 2-APB (40-100 μM, Maruyama *et al.*, 1997) caused a 50% decrease in one experiment, a 100% increase in another experiment, and had no significant effect in the other 4 experiments (data not shown). However this may be for want of a reliable inhibitor, because 2-APB was reported to be an “inconsistent inhibitor of IP₃-induced Ca²⁺ release” and is also known to have other effects (Bootman *et al.*, 2002; Hu *et al.*, 2004). The finding that CaMKII participates to the transcriptional activation of the rhodopsin gene in cultured embryonic retinal cells, raises the question of the molecular mechanisms that are activated downstream of this kinase. Our results indicated that phospho-CREB is involved in the activation of the rhodopsin gene promoter, and they also showed that CaMKII phosphorylates this transcription factor in cultured retinal cells. However, since the rhodopsin gene is activated selectively in rods, its transcription cannot rely only on CREB, which is ubiquitously expressed. CREB could possibly act by binding to one of three “CRE-like” sequences detected in the rhodopsin gene promoter (Voisin and Bernard, 2009) and thereby facilitate the binding of rod-specific transcription factors to nearby *cis*-regulatory elements. CREB might also act more indirectly, by increasing the expression and/or transcriptional activity of rod-specific transcription factors. A detailed analysis of the rhodopsin gene promoter will be required to address these different questions.

Our results showing the dependence of rhodopsin gene expression on voltage-gated Ca²⁺ channels prompted us to identify more precisely the channel subtype that might be involved. Because dihydropyridine-sensitive L-type channels have been described in cultured embryonic chicken photoreceptors (Gleason *et al.*, 1992; Uchida and Iuvone, 1999; Ko *et al.*, 2007), we repeatedly tested the effects of nifedipine and cilnidipine on rhodopsin gene transcription and consistently obtained negative results. In contrast, rhodopsin gene transcription was efficiently inhibited by T-type channel blockers. The reason for this apparent discrepancy may be that previous studies describing L-type channels had been performed on cones, identified by the presence of a lipid droplet (Gleason *et al.*, 1992; Uchida and Iuvone, 1999; Ko *et al.*, 2007). Because cones differentiate earlier than rods in this culture system (Voisin *et al.*, 2012) and because L-type Ca²⁺ channels have also been reported to mediate neurotransmission in mature rods (Akopian and Witkovsky, 2002), we suggest that the predominant contribution of T-type channels in rhodopsin gene activation may reflect the immature state of rod precursors in our cultures. This would be in keeping with previous observations in different tissues and in some retinal neurons, indicating that T-type channels

are frequently expressed before L-type during development (Yaari *et al.*, 1987; Schmid and Guenther, 1999; Bringmann *et al.*, 2000; Chemin *et al.*, 2002; Levic *et al.*, 2007) and are subsequently down-regulated in adult cells (Gonoi and Hasegawa, 1988; McCobb *et al.*, 1989; Gu and Spitzer, 1993). Therefore, even though T-type channels have not been clearly described in mature photoreceptors, further studies would be required to determine whether they are transiently expressed in rod precursors. In agreement with this notion, T-type channels have been described in the human Y-79 retinoblastoma cell line, which bears characteristics of an immature retinal precursor (Hirooka *et al.*, 2002). Interestingly, T-type channels were expressed only when Y-79 cells were cultured in suspension, a condition that favours the expression of photoreceptor markers in these cells (Albini *et al.*, 1992; Bernard *et al.*, 1995 ; Bernard *et al.*, 1996). Although the pharmacological profile of the Ca²⁺ channels involved in rhodopsin gene activation corresponds to T-type, a contribution of R-type channels cannot be completely ruled out. Indeed, the T-type blocker mibefradil is also a potent inhibitor of R-type channels (Randall and Tsien, 1997) and to our knowledge, a possible effect of ML218 on R-type voltage-gated Ca²⁺ channels has not been evaluated (Xiang *et al.*, 2011). In addition, although the selective R-type blocker, SNX482, did not inhibit rhodopsin promoter activity at 300 nM, it has also been reported to be relatively inactive on R-type currents in some rat central neurons (Newcomb *et al.*, 1998).

Evidence for a role of voltage-gated Ca²⁺ channels in rhodopsin gene activation prompted us to identify signals that could cause membrane depolarization in rod precursors. While acetylcholinergic and glutamatergic transmissions have been shown to play a role in retinal waves of depolarization (Firth *et al.*, 2005; Catsicas *et al.*, 1998; Wong *et al.*, 1998), their contribution to rhodopsin gene activation could be ruled out by the absence of effect of selective antagonists of nicotinic receptors and glutamate ionotropic receptors. Naturally, a possible role of other neurotransmitters in rhodopsin gene activation would deserve further analysis, especially with the notion that some receptors that have hyperpolarizing effects in the adult may be depolarizing in the embryo, due to an inverted gradient of chloride ions in immature neurons (Blankenship and Feller, 2010; Cherubini *et al.*, 2011). Nevertheless, our data positively identified HCN channels as a likely cause of membrane depolarization leading to rhodopsin gene activation, because both rhodopsin promoter activity and rhodopsin mRNA levels were strongly and reversibly inhibited by ZD7288. HCN channels are members of the six-transmembrane-domain superfamily and are activated upon hyperpolarization (Biel *et al.*, 2009). The depolarizing current they carry destabilizes the resting membrane potential and is

responsible for the repetitive activity of cardiac pacemaker cells (DiFrancesco, 2010). HCN channels play an essential role in the functional differentiation of neonatal cardiomyocytes, (Er *et al.*, 2003). They also play an important role in the functional maturation of neurons and in the establishment of neuronal networks (Bender and Baram, 2008). HCN channels have been detected in the retina, particularly in photoreceptors of salamander, mouse, rat and rabbit (Moosmang *et al.*, 2001; Demontis *et al.*, 2002; Knop *et al.*, 2008; Barrow and Wu, 2009; Della Santina *et al.*, 2010). In these cells, the predominant HCN isoform is HCN1 (Demontis *et al.*, 2002 ; Knop *et al.*, 2008 ; Barrow and Wu, 2009). In mature photoreceptors, HCN channels help shorten the response to light (Knop *et al.*, 2008; Barrow and Wu, 2009). Our study provides the first description of HCN1 in the chicken retina. The data presented here suggest that HCN channels may play a role in rod photoreceptor differentiation, long before the onset of photosensitivity, by initiating a signalling pathway that activates rhodopsin gene transcription. HCN1 expression during development shows only traces of HCN1 mRNA in the retina at E8, when retinal precursors are not capable of spontaneously activating rhodopsin gene transcription in culture (Voisin and Bernard, 2009; Voisin *et al.*, 2012). Then, HCN1 mRNA levels reach a half-maximal plateau at E11, when retinal cells acquire the ability to spontaneously express the rhodopsin gene in culture (Voisin *et al.*, 2012). At this stage however, the HCN1 protein must be very low because it is hardly detectable on western blots and not at all by immunohistochemistry. Finally, a second rise in HCN1 mRNA levels, accompanied by a rise in HCN1 protein levels on western blots, can be observed after E17, when the burst of rhodopsin mRNA expression occurs *in vivo* (Bruhn and Cepko, 1996 ; Voisin *et al.*, 2012). At this stage, the HCN1 protein becomes detectable by immunohistochemistry and appears mainly concentrated in the upper half of the outer plexiform layer, where synaptic terminals of rods and double-cones are found (Morris and Shorey, 1967). Localization and shape of HCN1 immunolabelling strongly argues for its presence in synaptic spherules of rods, as they have been described by electron microscopy (Morris and Shorey, 1967). However, the frequency of HCN1-labelled synaptic terminals (about 25 per 100 μm) is somewhat higher than the rod frequency we observed in a previous study (about 14 per 100 μm : Voisin *et al.*, 2012), suggesting some of the labelled cells may be double cones. Evidence that HCN1 channels are expressed in rods raises the possibility that E13 retinal cells isolated in culture might activate the rhodopsin gene in a cell-autonomous manner, even if a possible involvement of cell-contacts cannot be ruled out, due to the relatively high-density of our cell cultures. Nevertheless, our results suggest that the long time-lag between commitment of retinal precursors to a photoreceptor fate (around E6) and onset of rhodopsin gene expression (around E15-E16) may

be due to the late expression of HCN channels. Indeed, the limiting factor is unlikely to be the expression of Ca²⁺ channels, because E8 retinal cells are able to activate rhodopsin gene transcription in response to depolarizing treatments (Voisin and Bernard, 2009). If the expression of HCN channels is the limiting factor for rhodopsin gene activation, then forced expression of cloned HCN1 in E8 embryonic retinal cells should be sufficient to trigger rhodopsin transcription. Conversely, knocking down HCN1 channels should block the spontaneous activation of rhodopsin transcription in cultured E13 retinal cells. This should be tested in future studies. It is worth noting that the involvement of HCN channels in rhodopsin gene activation offers another possible explanation to the previously reported cAMP-dependent stimulation of rhodopsin gene transcription (Voisin and Bernard, 2009). Indeed, cAMP can directly bind to HCN channels and facilitate their opening (Biel et al., 2009), a mechanism by which it might trigger the Ca²⁺-dependent activation of the rhodopsin gene described herein. This mechanism might also contribute to the paradoxical stimulation of rhodopsin gene transcription by the PKA inhibitor Rp-8Br-cAMPS, because this compound also acts as a direct activator of HCN channels (Bois et al., 1997).

Evidence that both HCN and Ca²⁺ channels play a role in the transcriptional activation of the rhodopsin gene raised the possibility that these channels might generate Ca²⁺ transients in rod precursors. This hypothesis appeared plausible, because in chicken retina, Ca²⁺ waves have been observed not only in amacrine and ganglion cells, but also in the photoreceptor layer (Catsicas *et al.*, 1998). In addition, spontaneous Ca²⁺ oscillations have been reported in cultures of dissociated chick pineal photoreceptors (D'Souza and Dryer, 1994). Since Ca²⁺ transients have previously been shown to play a crucial role in the early differentiation of *Xenopus* spinal cord neurons (Rosenberg and Spitzer, 2011) and in retinal ganglion cell maturation (Firth *et al.*, 2005), they might be involved in rhodopsin gene activation. In good agreement with a previous study showing that spontaneous Ca²⁺ transients could still be observed in cultures of dissociated rat retinal cells (Firth and Feller, 2006), we show here that ~ 6% of the embryonic (E13) chicken retinal cells isolated in culture display Ca²⁺ oscillations. The patterns and kinetics of these oscillations are very similar to those described in rat retinal cells. These spontaneous oscillations had not been mentioned in a previous study of Ca²⁺ fluorescence in cultures of embryonic chicken retinal cells (Uchida and Iuvone, 1999). However, this may be due to a number of technical differences, including: 1) the age of embryonic cells, 2) cell-density in the cultures and, above all, 3) absence or presence of CO₂ in the incubation chamber (which we found was essential for oscillations to occur). Our results indicate that Ca²⁺ spikes

can actually occur in rod precursors expressing the DsRed fluorescent reporter, under control of the rhodopsin promoter. The percentage of DsRed cells exhibiting Ca²⁺ oscillations (10%) is close to that observed for the whole population of cultured cells (6%). However, this also means that 90% of the DsRed-positive cells did not display Ca²⁺ oscillations, although they activated the rhodopsin promoter. This result might be interpreted in different ways. Firstly, it may indicate that Ca²⁺ oscillations are simply not required for rhodopsin promoter activation. Secondly, it may be that the amplitude and/or the duration of the Ca²⁺ oscillations produced by 90% of the precursors are too small to be detected with our experimental setup. This may be the case if the rod precursors present in our cultures are still “immature” and express only T-type Ca²⁺ channels. Indeed, studies on spinal neurons of *Xenopus* embryo have shown that T-type Ca²⁺ channels must be relayed by high-voltage-activated Ca²⁺ channels, for oscillations in Ca²⁺ fluorescence to be detectable (Holliday and Spitzer, 1990; Gu and Spitzer, 1993). Thirdly, it may be that Ca²⁺ oscillations are only transiently required for rhodopsin gene activation in the course of rod differentiation and that 90% of the DsRed-positive cells in our cultures were already past this stage. Further studies dedicated to the pharmacological characterization of Ca²⁺ oscillations in identified rod precursors will be required to evaluate these different possibilities. This should also help determine whether these Ca²⁺ oscillations may rely on an interplay between HCN channels and T-type Ca²⁺ channels, similar to the mechanism described in cells of the sinoatrial node (Robinson and Siegelbaum, 2003).

The observations made in the present study raise new questions as to the physiological role of HCN channels and voltage-gated Ca²⁺ channels in retinal development. To date, photoreceptor differentiation has not been analyzed in detail in mice bearing targeted mutations in HCN or T-type Ca²⁺ channel genes, but the structure and functionality of their mature retinas were close enough to normality to suggest that rod differentiation was not deeply affected (Knop *et al.*, 2008; Alnawaiseh *et al.*, 2011; Della Santina *et al.*, 2012), and the same observation was made in R-type Ca²⁺ channel-deficient mice (Alnawaiseh *et al.*, 2011). However, as in many other instances, the impact of a single gene inactivation on photoreceptor development may be compensated by the upregulation of other genes with related functions (Weiergräber *et al.*, 2005). Therefore, our observations call for further experiments to determine: 1) whether the role of HCN channels and T-type Ca²⁺ channels in rhodopsin gene expression can be extended to the mouse retina, 2) whether retinas of mice bearing mutations in HCN channels, T-type or R-type Ca²⁺ channels show a delay in the timing of photoreceptor differentiation, 3) whether selective blockers of HCN channels and T-type or R-type Ca²⁺

channels can impair photoreceptor development when injected in the embryonic eye. By disclosing the role of HCN channels, voltage-gated Ca²⁺ channels, CaMKII and phospho-CREB in rhodopsin gene activation, the present study may contribute to a better understanding of rod photoreceptor differentiation and help unify this process with similar mechanisms of neuronal maturation in other regions of the central nervous system (Rosenberg and Spitzer, 2011). If the regulation pathway described herein can be extended to the human retina, it may help improve therapies against retinal degenerations, for example by providing new ways of promoting photoreceptor differentiation from stem cells, in a context of cell-replacement strategies.

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FIGURE LEGENDS

Figure 1. Spontaneous activation of rhodopsin promoter and mRNA expression in cultured embryonic chicken retinal cells. **A.** Retinal precursors (from E8 to E13) were cultured for 24 h in control conditions before transfection with either pRho1.6kb or pGL3-Basic plasmids, and with the pRL-TK normalizing-plasmid. Luciferase activities were measured 48 h post-transfection and expressed as firefly/*Renilla* ratios (mean \pm SEM, n=3). Similar results were obtained in 2 to 5 independent experiments. ** p<0.02 ; *** p<0.01. **B.** Retinal precursors were cultured for 4 days in control conditions. Rhodopsin mRNA levels were measured by real-time RT-PCR and normalized to GAPDH (mean \pm SEM, n=4). **C-H.** E13 retinal precursors were transfected with pDsRed-pRhodo1.6kb and were observed by fluorescence microscopy, 2 days post-transfection. **F-H.** Transfected cells incubated with Bodipy to label oil droplets. None of the 100 DsRed-fluorescent cells observed contained a lipid droplet. F, DsRed2-1 (red); G, Bodipy (green); H, merged images of F and G. Magnification bars = 10 μ m

Figure 2. Rhodopsin promoter activity is inhibited by a dominant-negative form of CREB, but not by PKA inhibitors. **A.** Retinal cells from E13 embryos were cotransfected with either pRho1.6kb or pGL3-Basic, and with a plasmid expressing CREB133. Normalization was performed with pRL-TK. Results are firefly/*Renilla* luciferase ratios measured 72 h post-transfection (mean \pm SEM, n=6). *** p<0.001. Similar results were obtained in 4 independent experiments. **B.** Cells cultured for 24 h with the indicated compounds were transfected with pRho1.6kb and with either pRL-TK (Rp-8Br-cAMPS) or pRL-SV40 (H89). Results are firefly/*Renilla* luciferase ratios measured 24 h post-transfection and expressed as percent of control group (mean \pm SEM, n=3). **p<0.02; NS, non-significant.

Figure 3. Rhodopsin mRNA levels and promoter activity are inhibited by CaMKII inhibitors. **A.** E13 retinal cells were cultured for 4 days and treated with the indicated compounds for the last 2 days. “E13 t0” corresponds to cells before culture. Rhodopsin mRNA levels (real-time RT-PCR) are normalized to GAPDH and expressed as percent of control (mean \pm SEM, n=4). ***p<0.00005; NS, non-significant. **B.** Cultured cells were treated with the indicated compounds and transfected with either pRho1.6kb or pGL3-Basic, and with pRL-TK (for

normalization). Results are firefly/*Renilla* luciferase ratios measured 24 h post-transfection/treatment and expressed as percent of control (mean \pm SEM, n=3). ***p<0.005; NS, non-significant. **C.** Transfected cells treated with KN-62 (or DMSO) were assayed for luciferase activities at 24 h post-transfection/treatment or transferred to fresh control medium and assayed 3 days later (“wash”: note the different scale). Results are mean \pm SEM (n=3). ***p<0.05. Similar results were obtained with KN-93.

Figure 4. Rhodopsin mRNA levels and promoter activity are inhibited by blockers of voltage-gated Ca²⁺ channels. **A.** E13 cells were cultured for 4 days and treated for the last 2 days with: 5 μ M bepridil (Bepr.), 200 μ M CdCl₂, 200 μ M NiCl₂, 10 μ M mibefradil (Mibef.), 15 μ M ML218 or 20 μ M nifedipine (Nifed.), water or DMSO. “E13 t0” corresponds to cells before culture. Rhodopsin mRNA levels (real-time RT-PCR) were normalized to GAPDH and expressed as percent of control (mean \pm SEM, n=4). **p<0.002; ***p<0.0001; NS, non-significant. **B, C.** Cells were treated with either 20 μ M bepridil (Bepr), 20 μ M nifedipine (Nifed), 5 μ M cilnidipine (Cilnid), 1 μ M ω -conotoxin GVIA (ω -Cono), 300 nM SNX482 (SNX) or 30 nM ω -agatoxin IVA (ω -Aga) (panel B) or with increasing concentrations of bepridil, ML218 or mibefradil (panel C) and transfected with pRho1.6kb and with pRL-TK (for normalization). Results are firefly/*Renilla* luciferase ratios measured 24 h post-transfection and expressed as percent of control (mean \pm SEM, n=3). ***p<0.00005; NS, non-significant. In **A, B,** and **C,** drug selectivities for Ca²⁺ channel are in parentheses. **D.** Transfected cells treated with mibefradil (or DMSO) were assayed for luciferase activities at 24 h post-transfection/treatment or transferred to fresh control medium and assayed 3 days later (“wash”: note the different scale). Results are mean \pm SEM (n=3). ***p<0.00005. Similar results were obtained with bepridil and ML218.

Figure 5. Calcium oscillations in E13 retinal precursors cultured for 2 days in control conditions and loaded with Fluo-8. Fluorescence was monitored every 2 s, during 20 min (A-C) or 10 min (D-F). Panel **A** shows an image of the fluorescent cells. Panels **B** and **C** show examples of spike-like (B) and wave-like (C) fluorescence oscillations in isolated cells. Panels **D-F** show the recording of an E13 retinal precursor transfected with pDsRed-pRhodo1.6kb. **D,**

DsRed fluorescence (red); **E.** Fluo-8 fluorescence (green), the DsRed-expressing cell is shown by an arrow; **F.** Spike-like Ca²⁺ oscillations in the DsRed cell. Magnification bars 20 μ m.

Figure 6. Rhodopsin promoter activity and mRNA levels are inhibited by the HCN channel blocker ZD7288. **A.** E13 cells were cultured for 4 days and treated with ZD7288 (or DMSO) for the last 2 days. Rhodopsin mRNA levels (real-time RT-PCR) were normalized to GAPDH and expressed as percent of control (mean \pm SEM, n=4). **B.** E13 cells were treated with ZD7288 (or DMSO) and transfected with pRho1.6kb and with pRL-TK (for normalization). Results are firefly/*Renilla* luciferase ratios measured 24 h post-transfection and expressed as percent of control (mean \pm SEM, n=3). **C.** Transfected cells treated with ZD7288 (or DMSO) were assayed for luciferase activities at 24 h post-transfection/treatment or transferred to fresh control medium and assayed 3 days later (“wash”: note the different scale). Results are mean \pm SEM (n=3). ***p<0.0001.

Figure 7. Developmental expression of HCN1 mRNA and protein in the chicken retina. **A.** HCN1 mRNA levels in chicken retina at indicated developmental stages (real time RT-PCR, mean \pm SEM, n=3). **B.** HCN1 immunodetection on western blots of membrane proteins from chicken retina at indicated developmental stages (Cy3-coupled secondary antibody). Specific HCN1 signal indicated by arrow. Non-specific bands were also detected with the secondary antibody alone (not illustrated). **C, D.** HCN1 immunodetection on sections of P1 chick retina (biotinylated secondary antibody, vectastain kit). **C,** low magnification image of the whole retina. **D,** higher magnification of photoreceptors with labelling in rod spherules. OLM, outer limiting membrane; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; N, nuclei; IS, inner segments. Magnification bars: 50 μ m in C; 10 μ m in D.

Figure 1

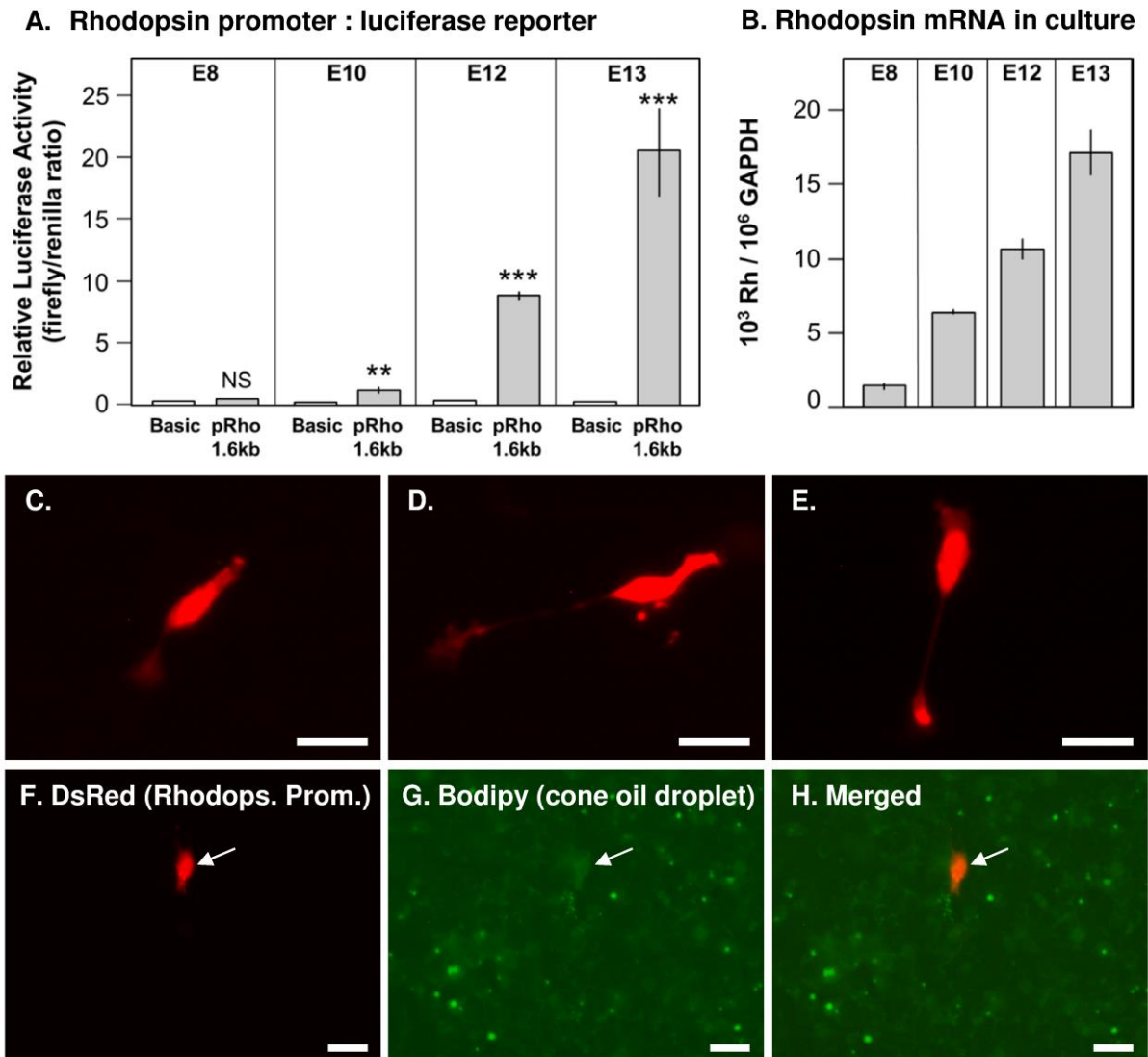
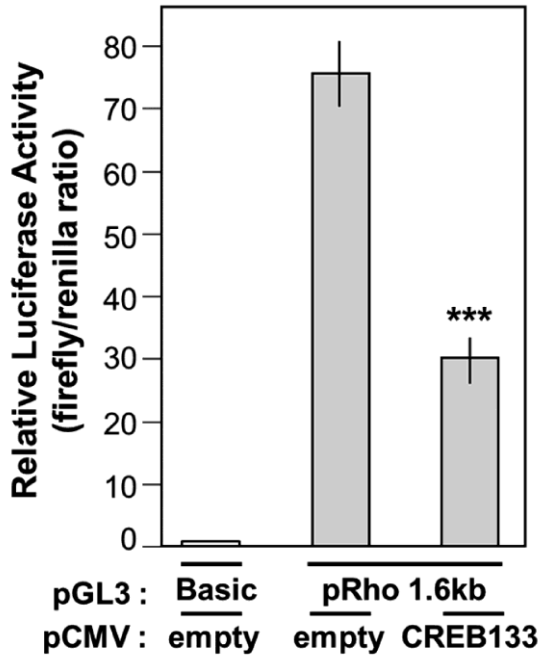


Figure 2

A. Dominant-negative CREB



B. PK_A inhibitors

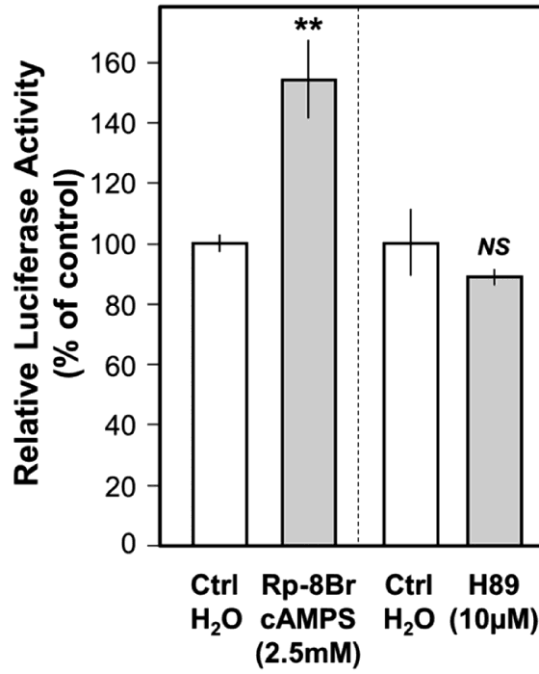


Figure 3

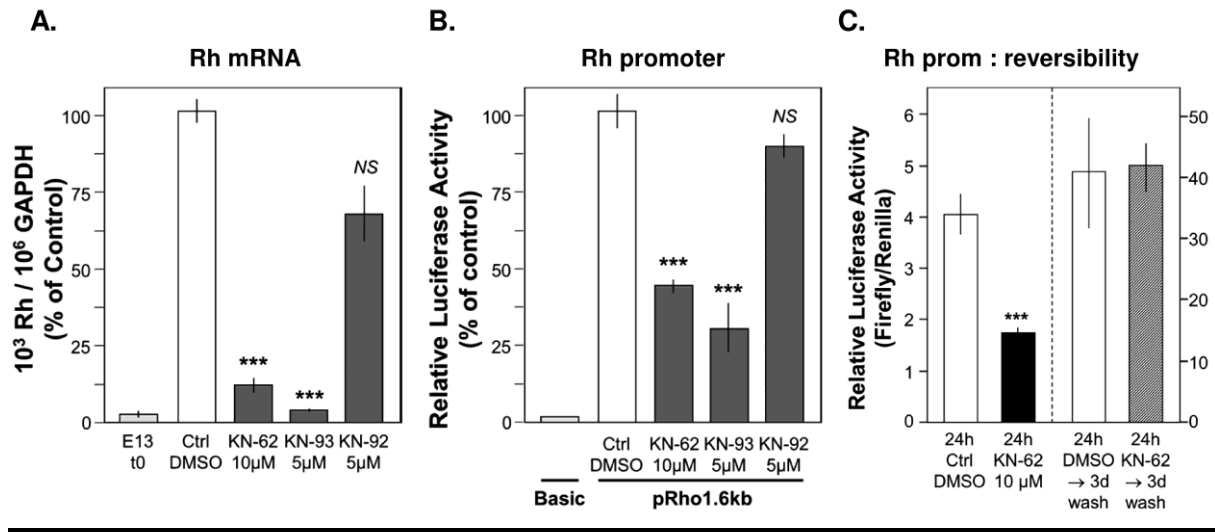


Figure 4

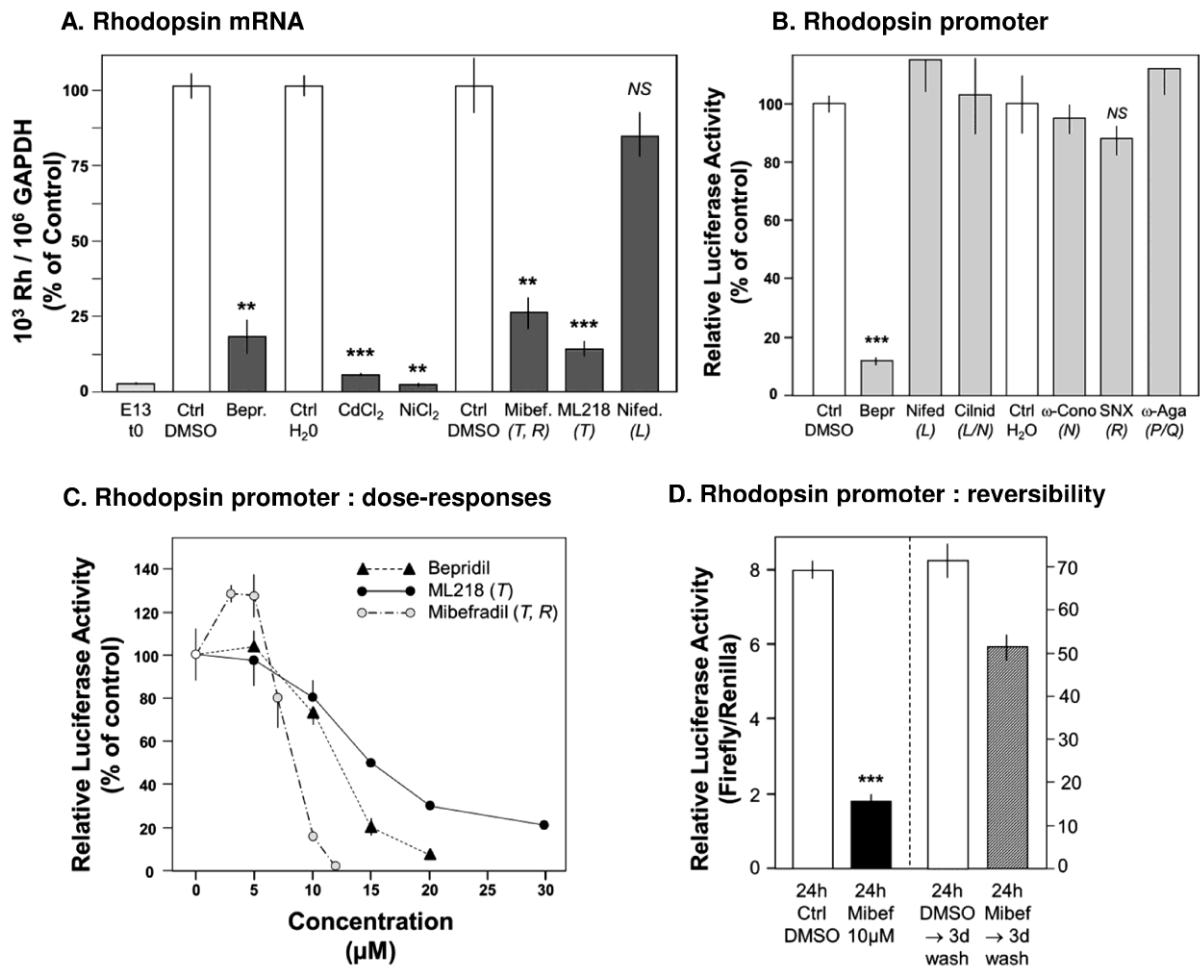
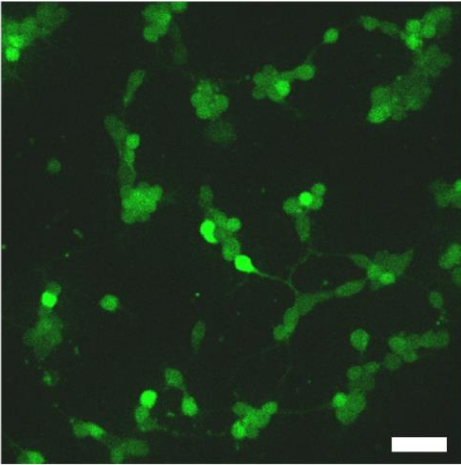
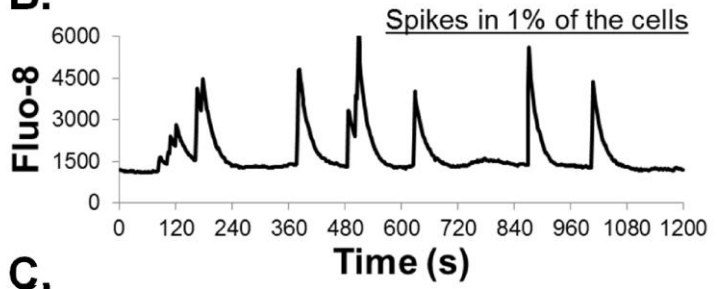


Figure 5

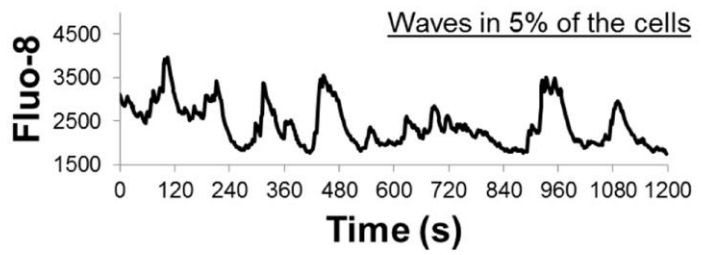
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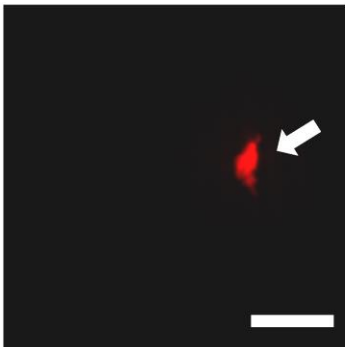
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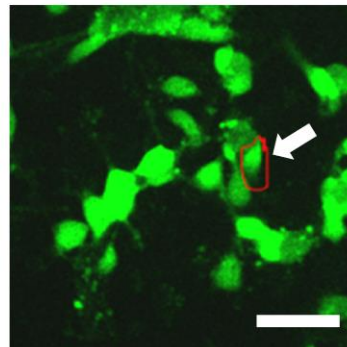
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D.



E.



F.

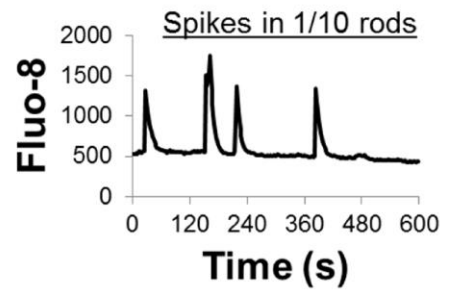


Figure 6

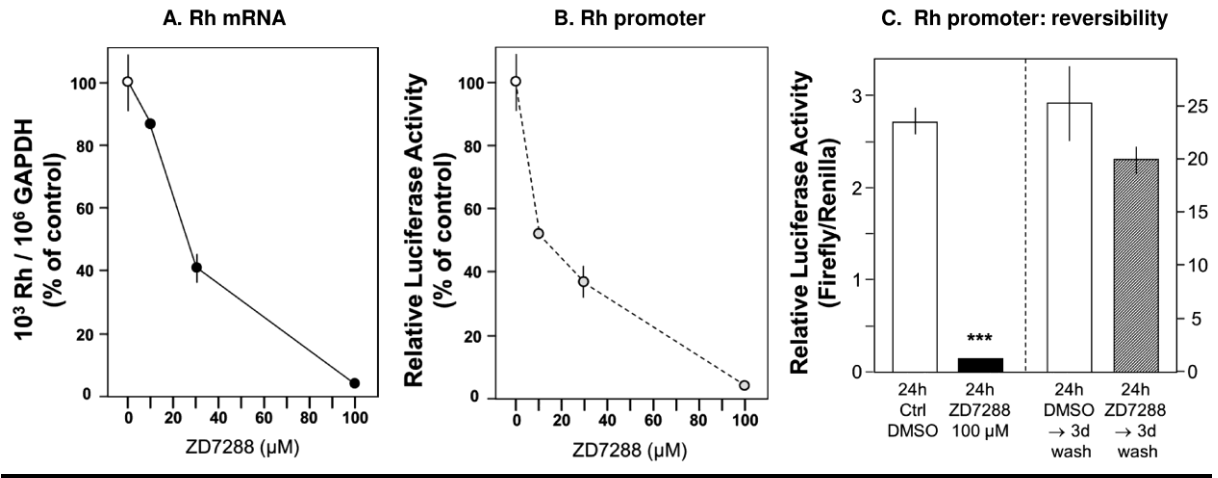


Figure 7

