# Cryptochromes and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation

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**Migratory birds can use a magnetic compass for orientation during their migratory journeys covering thousands of kilometers. But how do they sense the reference direction provided by the Earth's magnetic field? Behavioral evidence and theoretical considerations have suggested that radical-pair processes in differently oriented, light-sensitive molecules of the retina could enable migratory birds to perceive the magnetic field as visual patterns. The cryptochromes (CRYs) have been suggested as the most likely candidate class of molecules, but do CRYs exist in the retina of migratory birds? Here, we show that at least one CRY1 and one CRY2 exist in the retina of migratory garden warblers and that garden-warbler CRY1 (gwCRY1) is cytosolic. We also show that gwCRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, which also showed high levels of neuronal activity at night, when our garden warblers performed magnetic orientation. In addition, there seem to be striking differences in CRY1 expression between migratory and nonmigratory songbirds at night. The difference in CRY1 expression between migrants and nonmigrants is particularly pronounced in the large displaced ganglion cells known to project exclusively to a brain area where magnetically sensitive neurons have been reported. Consequently, cytosolic gwCRY1 is well placed to possibly be the primary magnetic-sensory molecule required for light-mediated magnetoreception.**

Since the description of animal magnetosensory capabilities in the 1960s (1–3), it has been convincingly shown that songbirds can use a magnetic compass for orientation during their migratory journeys (3–6), but the physiological mechanisms enabling migratory birds to sense the reference direction provided by the Earth's magnetic field still remain unknown. Two types of potential magnetoreception mechanisms have been suggested over the past decades: one mechanism that is based on magnetite particles and one mechanism that is based on photoreceptors forming radical-pair intermediates (for summary, see ref. 7). Although no direct physiological or molecular evidence has been reported, numerous orientation cage experiments with captive migratory songbirds have revealed several important characteristics of their magnetic compass.

The magnetic compass of migratory songbirds is an inclination compass; that is, it detects the axis but not the polarity of the magnetic field lines (4–5). Furthermore, magnetic orientation in migratory songbirds depends on the wavelength of the ambient light (8–11). Migratory songbirds are active and orient magnetically under dim blue and green light, whereas they are active but disoriented under dim red light (8–10). These findings strongly suggest that photoreceptor molecules in the eye are involved in magnetoreception and that these photoreceptor molecules should absorb in the blue and green range of the spectrum. The involvement of photoreceptors in the eye is further supported by the finding that birds with their right eye covered seem unable to perform magnetic orientation (12). A recent behavioral experiment (13) testing the magnetic orientation responses of European robins, *Erithacus rubecula* (a night-migrating songbird), exposed to oscillating magnetic fields provided strong indirect evidence that the magnetic-inclination compass of night-migrating songbirds is based on a radical-pair mechanism (7, 13).

Photoreceptor-based radical-pair mechanisms were suggested by Schulten *et al.* (14) and strongly elaborated on by Ritz *et al.* (7). They are based on the fact that radical-pair reactions will be modulated differently depending on the direction of the Earth's magnetic field relative to the orientation of the radical-pair-forming molecule (7, 15, 16). In short, the current hypothesis (7) further suggests that light in the blue-green range will excite photoreceptors forming radical pairs upon photoexcitation in the retina of the migratory bird. Because of the shape of the retina (half ball) and the presumed fixed orientation of the radical-pair-forming photoreceptors inside the cells, the magnetic field would modulate the radical-pair reaction and, thereby, the light sensitivity differently in different parts of the retinas, leading to perception of the magnetic field as visual patterns (7). Radical-pair-mediated magnetoreception would not be able to detect the polarity of the field lines, but only their axis, which is in line with the inclination-based nature of the songbird magnetic compass (4, 10).

Based on these theoretical considerations and behavioral evidence, the primary magnetic-sensory molecule in the retina of migratory songbirds should be a photopigment that is excited by light in the blue-green range and forms radical pairs upon photoexcitation. The cryptochromes (CRYs) (17–22) have been suggested as the most likely candidate class of molecules (7) because they are blue-green photoreceptors in plants (17, 19, 22) and because closely related 6,4-photolyases have been shown to form radical pairs upon photoexcitation (23). Other classes of photoreceptors, such as phototropsins (24) and chlorophylls (25), found in plants can also undergo radical-pair reactions. Rhodopsins should not be able to form radical pairs because photoexcitation leads to cis–trans isomerization of retinal rather than an electron transfer (e.g., ref. 26). Thus, CRYs are the only currently known class of molecules found in the retina of vertebrates that are likely to fulfill the physical and chemical characteristics that are required for function as the primary magnetic sensor (7).

Therefore, the aims of the present article are to (*i*) test whether CRYs exist in the retina of migratory birds performing a magnetic orientation task at night, (*ii*) elucidate their cellular location within the retina, (*iii*) test whether the CRY-containing cells show neuronal activity when migratory birds perform night-time magnetic orientation behavior, and (*iv*) compare the

Abbreviations: CRY, cryptochrome; gwCRY, garden-warbler CRY; INL, inner-nuclear layer.

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results of the first three aims in migratory songbirds with a nonmigratory songbird.

## Materials and Methods

The migratory behavior of night-migratory songbirds can be studied in caged birds because they are so eager to migrate that they will show migratory-restlessness behavior (wing-flapping and jumping on the perch) that is oriented in the direction in which they want to fly (27–29). In the present study, we tested 30 migratory garden warblers, *Sylvia borin*, and 10 nonmigratory zebra finches, *Taeniopygia guttata*, in orientation cages. The birds were kept indoors in a wooden laboratory under the natural light–dark cycle. At approximately noon, birds that were to be tested were marked with a small stripe of reflective tape on the head and transferred to a circular orientation cage. The timed lights went off at local sunset, reducing the light level in the room to 0.04 lux, originating from four diffused white light bulbs that were not directly visible to the birds. The behavior of the birds inside the orientation cage was monitored by an infrared (840 nm) video camera. The orientation of the birds was tested in the undisturbed geomagnetic field (field strength, 48,300 nT; inclination, 67°) or in an equivalent magnetic field (field strength, 48,300 ± 200 nT; inclination, 67° ± 0.2°) turned 120° ± 0.2° in the horizontal plane by 3D ($2 \times 2 \times 2$ m) Helmholtz coils (28).

During these orientation experiments, we carefully observed the birds on a video screen in real time and collected the retinas of 11 garden warblers immediately after they had shown at least 1 h of consistent migratory-restlessness behavior. All birds spent ≥100 min in the orientation cage after the lights went off before being killed. We also collected retinas from four garden warblers in room-light conditions in which they do not show migratory behavior. As nonmigratory controls, we collected the retinas of 10 zebra finches (five during the day and five at night).

When a given bird had shown ≥1 h of migratory-restlessness behavior (garden warblers at night; garden warblers during the day and all zebra finches just had to stay awake), it was killed according to the German legislation regulating the use of animals in research. Actual collection times varied between 22:23 and 00:40, equivalent to between 1 h and 40 min and 4 h after the lights went off. The day birds were collected between 19:48 and 20:30 having spent the preceding 12–14 h under constant room-light (275 lux) conditions. In all cases, eyecups were prepared within 6–13 min of the death of the bird under dim red light and were fixed in 2% paraformaldehyde, embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and frozen to −80°C.

For the detection of CRY1 transcripts, total RNA was extracted from the retinas by means of a NucleoSpin RNA II Kit according to the manufacturers protocol (Macherey & Nagel), which included treatment with DNase I to exclude contamination with genomic DNA. cDNA synthesis was performed from either 2 μg of RNA with SuperScript II Rnase $H^-$ reverse transcriptase (Invitrogen) or from ≈700 ng of RNA with Revert AID $H^-$ First-Strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocols. First-round hot-start PCRs (total volume, 25 μl) included 2 μl of cDNA/1× reaction buffer (Promega)/1,25 mM $MgCl_2$/0.2 mM each dNTP (Roche Diagnostics, Mannheim, Germany)/0.8 μM each primer (MWG Biotech, Ebersberg, Germany)/1 unit of *Taq* polymerase (Promega). cDNA synthesis and contamination with genomic DNA was controlled by using intron-spanning β-actin-specific primers [USP, 5′-GGCATGTGCAAGGCCGGCTTC-3′ (exon 2); and DSP, 5′-GGATGGCATGAGGGAGCGCGT-3′ (exon 4)] with the following conditions: 2 min at 95°C; 35 cycles of 30 s at 95°C, 1 min at 64°C, and 1.5 min at 72°C; and 15 min at 72°C). For the detection of the 642-bp CRY1 transcripts in the left and right retina, degenerate primers [USP, 5′-TTGTCGACAATGCTGGAAG(CT)TGGA-3′; and DSP, 5′-TTGAATTCTTCTTC(CT)(GT)GA(CT)T(AT)GG(AG)CG-3′] were used with the following conditions: 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C; and 2 min at 72°C). The entire coding region of garden-warbler CRY1 (gwCRY1) cDNA was obtained by means of long PCR (3 min at 94°C; and 40 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C) with 2 units of Taq plus 0.04 plaque-forming units of polymerase (Stratagene) and the two following additional sets of specific primers: $USP^N$, 5′-AGCAAGGTCTCCTTTCATCCTCTCAATATTCAGA-3′; $DSP^N$, 5′-AGCACGGTCTCCCATGGGGGTGAACGCCGTGCACTGGTT-3′ (amplification of the 5′ end); $USP^C$, 5′-AGCAAGCGGCCGCTTATCAATTTGTGCTCTGCCGCTGGACTTT-3′; and $DSP^C$, 5′-AGCACGGTCTCCGAAACAGATCTACCAGCAGCTT-3′ (amplification of the 3′ end). The latter primers were designed to get overlapping fragments encoding the N- and C-terminal regions of CRY1, which were then used to reconstruct the full-length gwCRY1 coding sequence. The same procedures were applied to detect a 237-bp fragment of CRY2 by using the following primer pair: USP, 5′-ACGAAGAATTCGACGAAAGCCACATCCAG-3′; and DSP, 5′-AGCACGTCGACGAACCCCATCTGCATCCA-3′. cDNA encoding the C-terminal part of CRY2 (777 bp) was obtained by means of nested PCR using the following primer pairs: USP, 5′-TTATGAGGCCCCCGATTTTCTGTTG-3′; DSP, 5′-AATCCCCTGAGACAGGAACCCTGAAGCC-3′; $USP^{nest}$, 5′-GTTCCTGCTCCTCTGGTCACCTCTT-3′; and $DSP^{nest}$, 5′-AGGAACCCTGAAGCCTTGGCAAAG-3′. All cloned fragments were subjected to nucleotide sequencing, and DNA similarities and identity scores were analyzed by FASTA and WU-BLAST2 algorithms.

Frozen cryostat sections (12–15 μm) of the retinas, which were mounted on gelatin-coated slides, were used for immunohistochemical labeling with CRY1 antibodies directed against the near-C-terminal part of the CRY1-protein by following standard protocols (30). Primary-antibody incubation was carried out overnight at 4°C for CRY1/sc-5953 (diluted 1:100), Egr-1/sc-189 (diluted 1:500), and c-Fos/sc-253 (diluted 1:200/1:500). All antibodies were purchased from Santa Cruz Biotechnology and diluted in PBS/0.3% TX-100. Immunoreactivity was visualized by indirect immunofluorescence using FITC-conjugated donkey anti-goat IgG for CRY1 detection and Cy3-conjugated donkey anti-rabbit IgG for Egr-1 and c-Fos detection. In control experiments, primary antibodies were omitted or preabsorbed with the appropriate inhibitory peptide (sc-5953 P, sc-189 P, or sc-253 P), which was diluted 1:50 in the incubation buffer. In double-labeling experiments, incubations with either both primary or both secondary antibodies were carried out at the same time. Labeled sections were analyzed by using a TSC confocal microscope (Leica, Nussloch, Germany), and brightness and contrast of the images were adjusted to the same background level by using PHOTOSHOP software (Adobe Systems, San Jose, CA). The immunosignal intensity was quantified relative to background intensity at the confocal microscope by encircling relevant areas on retina slices from different experimental groups incubated on the same slide, such that the intensity index = {[2× immunostaining intensity (ISI) in ganglion cells/(ISI in inner plexiform + ISI in GCaxon layer)] −1}. Specificity of the antibodies was tested by means of immunoblotting using standard protocols (30). Immunoreactive proteins were detected by using the chemiluminescence method (ECL-system; Amersham Pharmacia Buchler, Braunschweig, Germany). The Western blot analyses were replicated four times with different birds and positive rat-brain controls. The strengths of the bands were in line with the immunosignal quantifications. In control experiments, the primary antibody (diluted 1:500 in PBS) was preadsorbed by its inhibitory peptide (diluted 1:50).

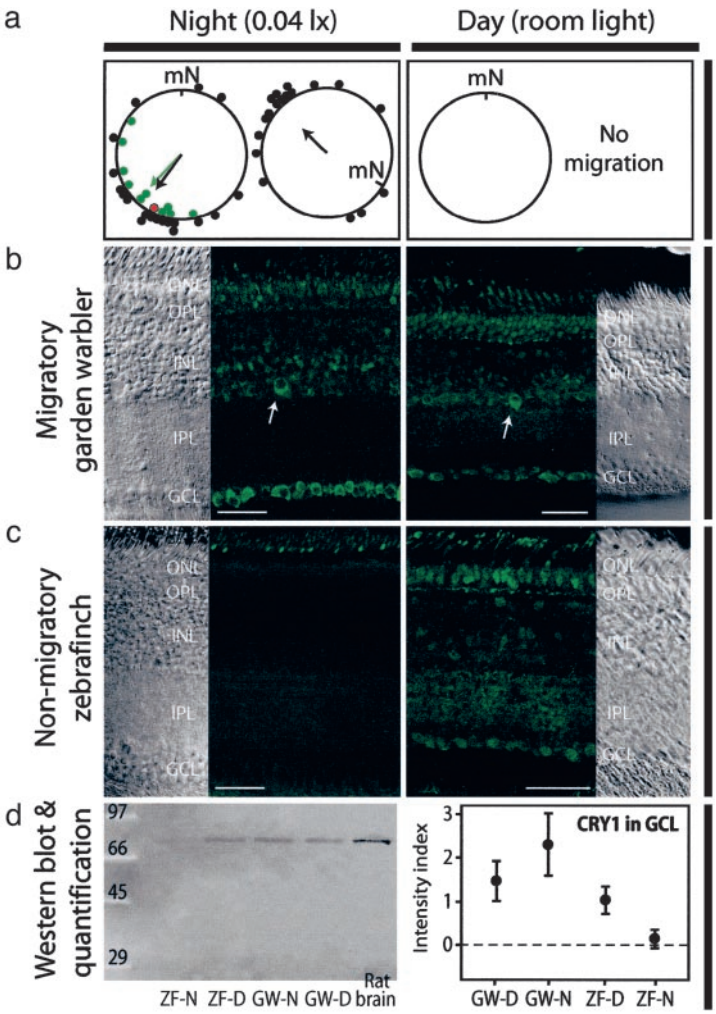


**Fig. 1.** CRY protein expression in the retina of migratory garden warblers and nonmigratory zebra finches. (*a*) Magnetic orientation of garden warblers. Each data point indicates the mean orientation of one individual. The arrows indicate group mean vector lengths (*r*). The orientation of the night-birds (Night) from which retinas were collected (green symbols) were highly oriented in the normal migratory direction ($n = 11$, mean = 223°, $r = 0.77$, and $P < 0.001$). The red dot represents the night bird whose retina is shown in *b*. During the day, the birds do not show orientation behavior (Day). mN, Magnetic North. (*b*) Immunohistochemical staining of CRY1 protein during nocturnal magnetic orientation (*Left*) and during the day (*Right*) in migratory garden warblers (images from the same slide taken with identical settings). Large displaced ganglion cells marked with arrow. (*c*) Immunohistochemical staining of CRY1 protein during the night (*Left*) and day (*Right*) in nonmigratory zebra finches (images from same slide taken with identical settings). Labeling of photoreceptor outer segments and Müller cell end-feet in *b* and *c* is unspecific. (*d*) Example of Western blot analysis confirming specificity of the antibody and quantification of CRY1 expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. (Scale bar, 40 $\mu$m.)

## Results

The magnetic-orientation responses of 30 migratory garden warblers were tested at night in circular orientation cages inside a wooden laboratory. The birds showed clear magnetic orientation (Fig. 1*a*) in their species-specific migratory direction (normal field: mean = 215°, $n = 30$, $r = 0.62$, and $P < 0.001$), and they significantly (Mardia–Watson–Wheeler test; $n = 48$, $\chi^2 = 19.6$, and $P < 0.001$) changed their orientation as predicted (mean = 316°, $n = 18$, $r = 0.51$, and $P < 0.01$) in a turned magnetic field (magnetic North = 120°).

CRY expression at the mRNA level was first analyzed by RT-PCR. First, a gwCRY1 fragment of ≈642 bp (Fig. 2), including the region encoding the FAD-binding region thought to be involved in the radical-pair reaction (18, 23), was amplified with degenerated CRY1 primers. Subsequently, the complete coding sequence was reconstructed (GenBank accession no. AJ632120), and FASTA and WU-Blast2 analysis of the gwCRY1 nucleotide sequence revealed the highest homology (93%) with chicken CRY1 (GenBank accession no. AY034432). We also amplified a 777-bp fragment encoding the C terminus of gwCRY2 (GenBank accession no. AY739908), which showed highest homology (89%) with chicken CRY2 (GenBank accession no. AY034433). Both gwCRYs belong to the animal CRY family (31). Subsequent immunohistochemistry revealed that gwCRY1 was located in the cytosol, whereas gwCRY2 was located in the nucleus. Because a CRY working as the primary magnetoreceptor must be oriented in the cells and, therefore, is most likely linked to cytosolic skeleton proteins, a nucleic location is very unlikely. For these reasons, we focused subsequent experiments on gwCRY1 only.

The specificity of the CRY1 antibodies was confirmed by four independent sets of immunoblots [all showing only one immunoreactive protein at the appropriate molecular mass of ≈70 kDa (Fig. 1*d*)], which disappeared when the antibody was preabsorbed with the corresponding peptide. In migratory garden warblers, immunolabeling for CRY1 was found in specific cell populations across the retina. Invariably, 95–100% of garden-warbler ganglion cells showed high levels of cytosolic CRY1 with a tendency toward higher expression in night birds than in day birds (*t* test, df = 8, $t = 2.06$, $P = 0.07$; Fig. 1 *b* and *d*). All large, displaced ganglion cells were also strongly labeled for CRY1 in garden warblers in both day and night. The photoreceptor layer of garden warblers showed high expression of CRY1 particularly in day birds. In the inner-nuclear layer (INL) of garden warblers, 10–15% of the cells were CRY1-positive, and night garden warblers tended to show higher levels of CRY1 in a cell population with elongated somata. In nonmigratory zebra finches, the expression in day birds closely resembles that found in garden warblers during the day, except that the large displaced ganglion cells were always CRY1-negative in zebra finches (Fig. 1*c*). However, during the night, CRY1 expression in nonmigratory zebra finches dropped dramatically to nonspecific background level (night mean of intensity index [(signal/background) − 1] = 0.14 ± 0.21 (SD); day mean of intensity index = 1.04 ± 0.31;

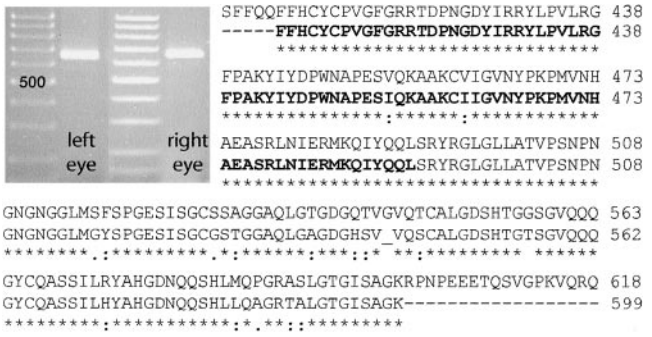


**Fig. 2.** Detection of a CRY1 transcript with the expected size of ≈642 bp in the left and right retinas of garden warbler (lower sequence). The deduced amino acid sequence for this fragment reveals 91% identity with the specific C-terminal region of chicken CRY1 (upper sequence). The FAD-binding domain thought to be involved in radical-pair reactions is shown in bold. Upper left, 5′ end; lower right, 3′ end.

*t* test, df = 8, $t = 5{,}35$, and $P < 0.001$; Fig. 1 *c* and *d*). This decrease in CRY1 expression is in line with the normal circadian oscillation found previously in CRY1 mRNA in nonmigratory domestic chicken and quail (20, 21) but strikingly different from the unusually high expression levels [mean intensity index = 2.31 ± 0.72 (SD)] found in migratory garden warblers at night (Fig. 1*d*). There were no obvious differences in CRY1 patterns between the right and left eye (12).

To be potentially relevant for magnetoperception, the CRY-containing retinal cells of garden warblers must be active at night when the birds perform magnetic orientation. We tested the neuronal-activity pattern of retinal cells by using the markers c-Fos and ZENK (*z*if268, *E*gr-1, *N*GF-1A, *K*rox-24). The transcription factors (immediate early genes) c-Fos and ZENK are known to show vision-dependent expression in the retina of many animals (32–35), including chicken (32), and their expression requires neuronal activity (36–37). The c-Fos and ZENK proteins were detectable in a cell 15–20 min after neuronal activity occurred, and peak protein expression was detected after 45–60 min (38). Thus, c-Fos and/or ZENK protein detected immunohistochemically in a cell was produced by neuronal activity 15–90 min before the tissue was fixed, which matches the time frame in which our birds performed magnetic orientation. Consequently, colocalization of c-Fos and ZENK protein in garden-warbler retinal cells showing high CRY levels would show that these cells were sending neuronal information to the brain while our birds oriented to the geomagnetic field at low night light levels (0.04 lux).

As expected, immunostaining and Western blot analysis revealed higher c-Fos expression in both zebra finches (*t* test, df = 8, $t = 7.18$, and $P < 0.001$) and garden warblers (*t* test, df = 8, $t = 3.25$, and $P < 0.02$) during the day, when vision is more active, than during the night (Fig. 3*a*). But whereas c-Fos expression during the day was similar in garden warblers and zebra finches, at night it was significantly higher (*t* test, df = 8, $t = 4.474$, and $P < 0.01$) in the migratory garden warblers. In these night garden warblers, c-Fos is strongly expressed in ganglion cells and in ≈5% of the cells within the INL (Fig. 3*b*). ZENK was strongly expressed in the nuclei of garden-warbler ganglion cells (Fig. 3*c*). The c-Fos and ZENK signals colocalized with CRY1 in all garden-warbler ganglion cells and large displaced ganglion cells but only sporadically in other cell types at night during magnetic orientation (Fig. 3 *b* and *c*).

In their theoretical article, Ritz *et al.* (7) specifically suggested that the large, morphologically distinct (39, 40), and displaced ganglion cells are likely to be a location for magnetoreception because they project exclusively to the nucleus of the basal optic root (39, 40), where magnetically sensitive neurons have been reported (41) and visual flow-fields arising from self-motion are processed (42). The scarce, large CRY1-positive cells found in the INL of the garden warbler (Figs. 3 and 4) share the features reported for displaced ganglion cells in birds (39, 40) (in particular, their large size, disk-shaped nuclei, and large cytosolic space) and, thus, are easy to locate by Nomarski optics. Thy1, a general marker for ganglion cells, also in birds (43), further confirmed their identification as displaced ganglion cells. We analyzed >100 of these large cells in the garden warbler and >30 in the zebra finch, and we found striking differences in CRY1 and c-Fos expression. In the migratory garden warbler (day and night), the large displaced ganglion cells always contained strong CRY1 immunolabeling and a strong label for c-Fos (Fig. 4). In contrast, the large displaced ganglion cells of nonmigratory zebra finches (day and night) were always CRY1-negative (Fig. 4). Furthermore, all of the >20 large displaced ganglion cells that were analyzed in night zebra finches were c-Fos-negative. In zebra finches during the day, as expected because of normal visual processes, both c-Fos-positive (active) and c-Fos-negative (inactive) large displaced ganglion cells were found, but even when active, they never expressed CRY1.

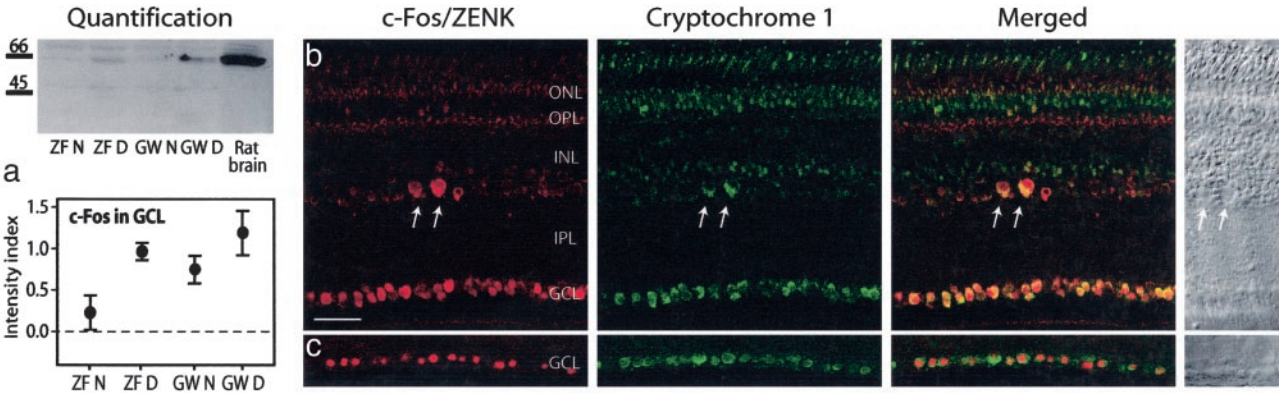


**Fig. 3.** Colocalization of CRY1 and neuronal-activity markers in the same 300-nm-thick retina slice from a magnetically orienting garden warbler. (*a*) Example of Western blot analysis confirming specificity of the antibody and quantification of c-Fos expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. (*b* and *c*) Immunohistochemical double labeling of CRY1 (cytosolic) with the neuronal-activity markers c-Fos (*b*; cytosolic and nucleic) and ZENK (*c*; exclusively nucleic; only the ganglion cell layer is shown) reveal that CRYs are found in night-active ganglion cells and in large displaced ganglion cells of the INL (arrows). (Scale bar, 40 μm.) GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

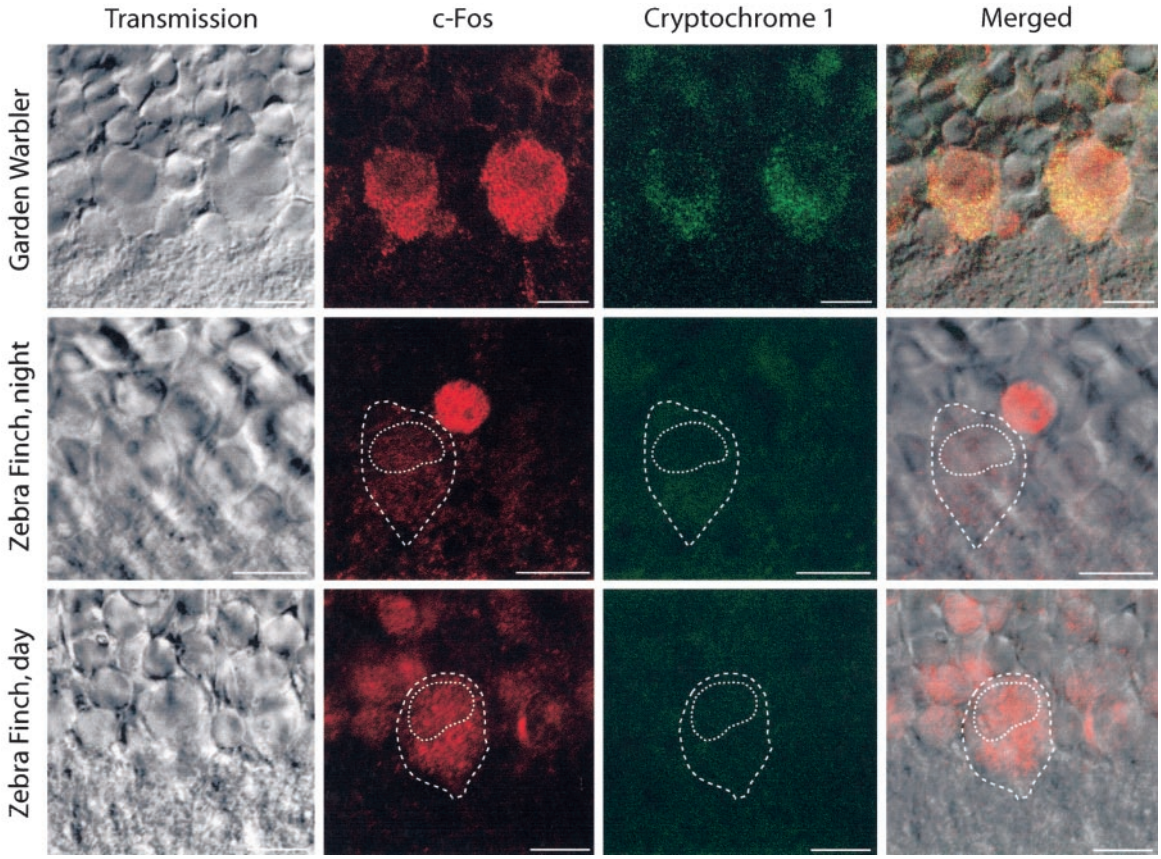


**Fig. 4.** High magnification confocal images confirm the colocalization of CRY1 with the neuronal-activity marker c-Fos in large displaced ganglion cells in the garden warbler [during both night (shown) and day (data not shown)]. In contrast, the large displaced ganglion cells never express CRY1 in zebra finches, neither during the night nor during the day, when both active (c-Fos-positive, as shown) and inactive (c-Fos-negative) large displaced ganglion cells occur in zebra finches.

## Discussion

Although no direct link between CRY-containing cells and magnetoreception is currently known, we present here a number of strong correlations supporting such a link. We have shown that at least one CRY1 exists in the retina of migratory birds and that this gwCRY1 is apparently predominantly or even exclusively located in the cytosol. We have shown that CRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, and we have shown that these cells also show high levels of neuronal activity at night during magnetic orientation in migratory garden warblers. That is, the CRY-containing cells in the eye send information to the brain at night when garden warblers orient to the geomagnetic field. Consequently, cytosolic CRY1 is well placed to possibly take over the role of the required substrate for magnetoreception according to the Ritz *et al.* model (7). We also show that there seem to be striking differences in CRY expression between migratory and nonmigratory birds at night. As in domestic chicken, there seems to be virtually no CRY1 in the retina of nonmigratory zebra finches at night, whereas it is strongly expressed in migratory garden warblers at night. The differences in CRY1 expression between migrants and nonmigrants are particularly pronounced in the large displaced ganglion cells that are known to project exclusively to the nucleus of the basal optic root, where magnetically sensitive neurons have been reported (41). Furthermore, the neuronal activity of the ganglion cells is significantly lower at night in nonmigratory zebra finches than in migratory garden warblers, suggesting that the ganglion cells send more information to the brain at night in migratory birds. Because CRYs are found in the retina of both night-migratory and nonmigratory birds during the day, our data do not exclude that a CRY-mediated magnetic compass could be used also during the day, when wavelength-dependent magnetic-compass orientation has also been observed (10).

CRYs in other vertebrates have been shown to be involved in the inhibitory branch of the autoregulatory transcriptional loop controlling the circadian clock (19), whereas the presumed photoreceptor function of vertebrate CRYs is still debated (17, 19). Our data do not exclude a clock function of the gwCRYs, and the high CRY1-expression in night-migratory garden warblers could be a result of their round-the-clock activity during the migratory season. However, garden warblers taken on nights when they do not show migratory restlessness also show high levels of CRY1-expression (H.M., U.J.-B., M.L., G.F., and R.W., unpublished data), and the cytosolic localization of CRY1 that we found in the garden warbler does not seem to favor a role in the clock transcriptional loop because these processes occur in the nucleus (17).

In conclusion, our results support the hypothesis that CRYs, in addition to their circadian functions (17–22), could indeed play a role in magnetic field reception. Having made this statement, we stress that there are still many questions that must be answered before we can state conclusively that the CRYs are the primary magnetic sensor in migratory birds. Although it has recently been shown that the radical pair of 6,4-photolyase, which is highly homologous to the CRYs, live long enough (>5 $\mu$s) for geomagnetic field effects to take place (44), it, for instance, still needs to be shown how the orientation of the CRYs is fixed in cells so that light-induced signals will be modulated

differently by the magnetic field depending on location in the retina. However, the location of gwCRY1 in the cytosol means that it could be fixed to the cytoskeleton. In any case, having identified the CRY-containing cells in the retina opens a number of experimental possibilities, which we believe will ultimately lead to an understanding of the molecular and physiological mechanisms underlying magnetic-compass orientation in migratory birds.

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