Cryptochrome 2 mediates directional magnetoreception in cockroaches

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The ability to perceive geomagnetic fields (GMFs) represents a fascinating biological phenomenon. Studies on transgenic flies have provided evidence that photosensitive Cryptochromes (Cry) are involved in the response to magnetic fields (MFs). However, none of the studies tackled the problem of whether the Cry-dependent magnetosensitivity is coupled to the sole MF presence or to the direction of MF vector. In this study, we used gene silencing and a directional MF to show that mammalian-like Cry2 is necessary for a genuine directional response to periodic rotations of the GMF vector in two insect species. Longer wavelengths of light required higher photon fluxes for a detectable behavioral response, and a sharp detection border was present in the cyan/green spectral region. Both observations are consistent with involvement of the FADox, $FAD^{\bullet-}$ and $FADH^{-}$ redox forms of flavin. The response was lost upon covering the eyes, demonstrating that the signal is perceived in the eye region. Immunohistochemical staining detected Cry2 in the hemispherical layer of laminal glia cells underneath the retina. Together, these findings identified the eye-localized Cry2 as an indispensable component and a likely photoreceptor of the directional GMF response. Our study is thus a clear step forward in deciphering the in vivo effects of GMF and supports the interaction of underlying mechanism with the visual system.

magnetoreception | cryptochrome | light spectrum | locomotor activity | circadian genes

Behavioral evidence for sensitivity to geomagnetic fields (GMFs) has been found in numerous vertebrate and invertebrate taxa (1); however, the underlying mechanisms remain a biological and biophysical enigma. In the late 1970s, the effect of light on the orientation of birds inspired Schulten and colleagues (2) to suggest that reactions of radical pairs (RPs) formed by photosensitive biological processes may be susceptive to external magnetic fields (MFs), and thus provide the basis for in vivo chemical magnetoreception. Since then, ample studies have supported this hypothesis (reviewed, e.g., in refs. 3 and 4).

In the past decade, proteins from the Cryptochrome/Photolyase family (CPF) have been widely discussed as being relevant to the light-dependent biological compass relying on the RP mechanism (5–7). Plant Crys mediate sensitivity to blue/UVA light (8), and this sensitivity was reported to be influenced by a MF (9), although later verification failed (10). Crys are essential for circadian clock function in mammals, but are likely not directly involved in light reception (11). In the fruit fly, *Drosophila melanogaster*, Cry mediates the light entrainment of the circadian clock (12). Both fly circadian rhythmicity and geotaxis turned out to be Cry-dependent and were also affected by a MF (13–15). Curiously, some insect species have only a *Drosophila*-type of Cry (Cry1 or animal type I Cry), whereas others have a mammalian-type of Cry (Cry2 or animal type II Cry) or both (16).

The validity of the RP mechanism was proven in the carotenoid–porphyrin–fullerene triad (17). In CPF proteins, the change in redox state of their flavin adenine dinucleotide (FAD) cofactor can result in magnetosensitive RPs (18). Although the RPs studied in two CPF proteins were magnetosensitive (19, 20), RP-based GMF effects and anisotropic MF effects have not been shown in CPF proteins. In contrast, ultrafast GMF effects on transient FAD fluorescence in an apparently purified Cry from birds was reported in a recent study (21), suggesting the existence of a GMF-sensitive reaction that differs from spin-selective RP recombination.

The biological output of the RP–GMF interaction might hypothetically be generated when a particular redox status of a FAD cofactor is reached, changing the configuration of the Cry protein (22) or its C terminus, which switches the Cry to a signaling state (23). Concerning possible downstream effects, Cry activation was shown to control permeability of potassium channels in *Drosophila* (24).

In terms of Cry-mediated in vivo chemical magnetoreception in general, an organism's sensitivity to the presence of GMF should be considered separate from its sensitivity to the GMF's orientation (25). Although the sole detection of the presence or intensity of a GMF can be accomplished in vitro via a disordered RP system (17), a number of additional critical requirements should be met to function as a sensor of magnetic direction, from the anisotropy of electron–nucleus interactions to the anatomy of a sensory organ (see *Discussion*).

Significance

The photosensitive protein Cryptochrome (Cry) is involved in the detection of magnetic fields (MFs) in *Drosophila*. However, Cry-dependent responses to natural MF intensities and to the direction of the MF vector have not been demonstrated previously in any insect. Birds, monarch butterflies, and many other species perceive the direction of geomagnetic field (GMF) lines, but the involvement of Cry has not been rigorously proven using genetic tools. In this study, by combining behavioral and genetic approaches, we provide the first unambiguous evidence to our knowledge of a Cry-dependent sensitivity to the direction of GMF in two cockroach species. Furthermore, by eye-covering experiments and by immunolocalization of a crucial mammalian-type Cry2 under the retina, we clearly show that the eye is an indispensable organ for the directional GMF response.

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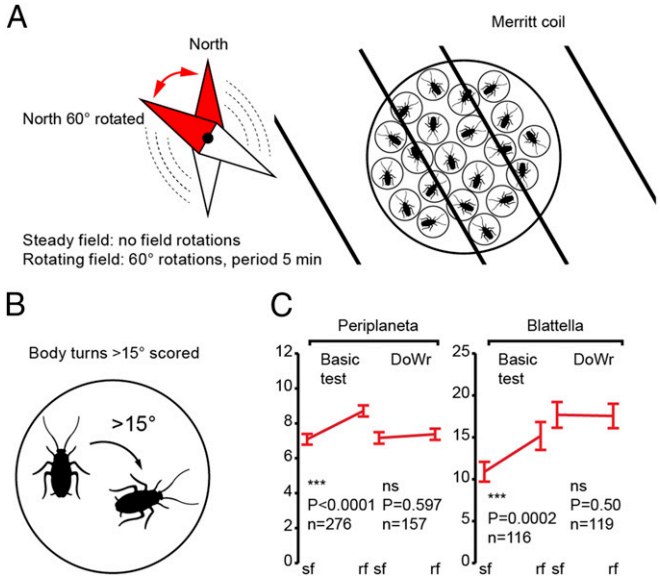

Fig. 1. MIR. (*A*) Schematic illustration of the magnetoreception assay: When the geomagnetic horizontal vector is rotated back and forth 60° periodically, the cockroaches change their resting positions more frequently compared with during steady MF periods. Magnetoreception assay setup: Cockroaches were placed individually into Petri dishes with opaque walls (small circles) accommodated in arena (large circle). On the next day, MF was changing its direction every 5 min during 135-min intervals. The four black lines depict the position of the Merritt coil frames. (*B*) Body turn was scored if body rotation exceeded 15°. (*C*) MIR essay is selective to magnetic direction: The activity was scored as a number of body turns per 135-min interval during control (steady field, sf) and treatment (rotating field, rf) periods. Red line depicts average paired levels of all individual activities (±SEM) between control and treatment periods. In the Basic test setup, significant elevation of activity (Wilcoxon Match Pair Test) was found. Nonspecific effects of electric feeding of coils were eliminated using a double-wrapped design (DoWr) that allowed us to feed the coils without producing any external MF. The red lines indicate the mean values of all animals between control and treatment; intra- and intersample variations are irrespective of pair test significance. n, number of animals.

The most convincing evidence of Cry-dependent magnetosensitivity was provided on *Drosophila* (26, 27). The ability to recognize the presence of a MF relies on functional Cry1, and this magnetoreception in Cry-deficient fruit fly mutants could be rescued using mammalian-like Cry2 (28). The flies were trained to recognize the local magnetic anomaly up to 10 times stronger than the natural GMF in T-shape maze experiments. Although the choice of one of two arms involved orientation, the actual physiological effect was consistent with nondirectional magnetic sensitivity (29), as was discussed for plants (9, 10), fruit fly geotaxis (14), and the fruit fly circadian clock (13, 15). Therefore, rather than demonstrating a genuine directional sensor serving as a GMF compass, these studies proved that Cry mediated detection of a rather intense, artificial magnetic anomaly.

Here, we have taken advantage of an assay enabling us to test directional magnetic sensitivity in insects at naturally occurring GMF intensities (30) and functionally confirmed that mammalian-like Cry2 is necessary for sensing the directional component of MFs of natural intensities by two different species of cockroaches.

Results

Previously we developed an assay enabling us to test directional magnetic sensitivity in insects at natural GMF intensity (30) (Fig. 1 *A* and *B*) in a nonconditioned, spontaneous behavioral reaction to slow shifts of the magnetic North position. We first used the American cockroach, *Periplaneta americana*, but soon realized that this species most likely contains only Cry2. Thus, we added another cockroach species, *Blattella germanica*, which has both Cry types (see *SI Appendix*, Fig. S1 for phylogenetic analysis and *SI Appendix*, Fig. S2 for details on Cry1 search in *P. americana*).

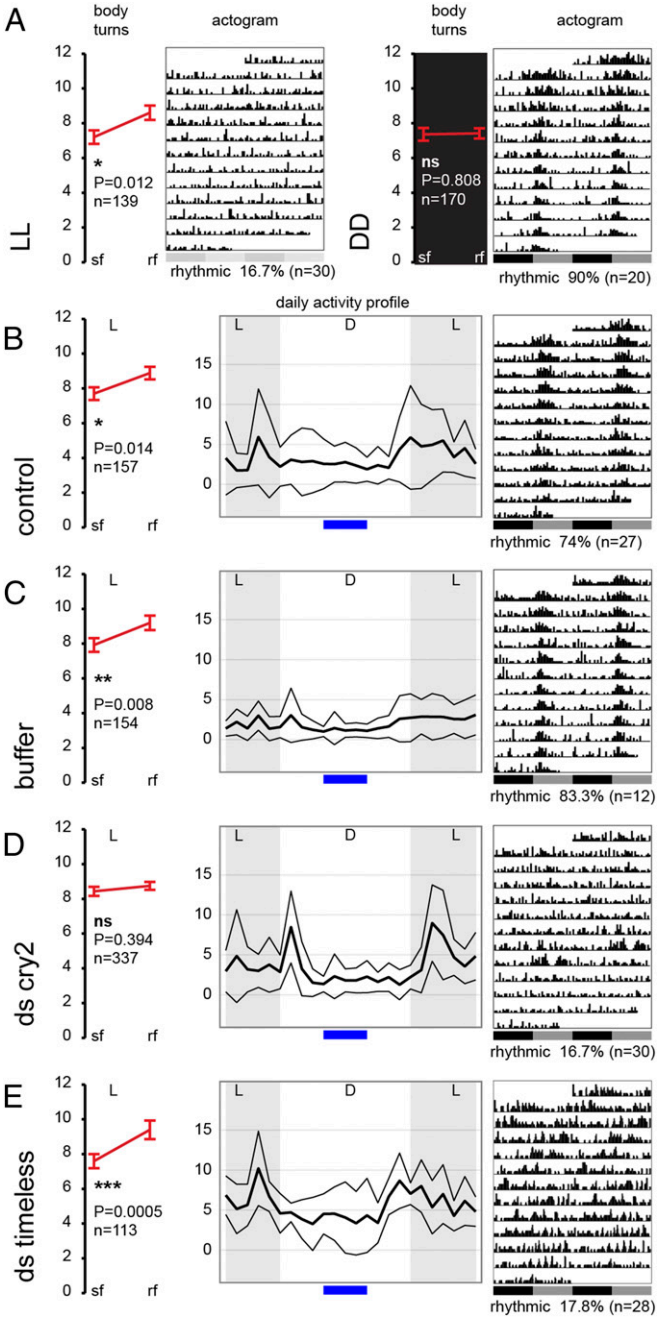

Fig. 2. Phenotypes of *P. americana*. Magnetoreception scored as body turns under sf and rf (*A–E*, *Left*); red line depicts the average change of body turns (±SEM). Daily activity profiles at a 12 h light:12 h dark cycle are shown as a black line (±SD, thinner lines); horizontal blue bar under daily activity profiles indicates the time during which magnetoreception was assayed (*B–E*, *Middle*). Circadian activity in constant conditions is shown as double-plotted actograms (*A–E*, *Right*). (*A*) Constant light (LL) abolished circadian rhythmicity, leaving magnetic sensitivity intact, whereas DD abolished magnetic sensitivity with unaffected circadian locomotor activity. (*B*) The control RNAi and (*C*) buffer injected animals displayed normal magnetoreception, as well as circadian rhythmicity. (*D*) *cry2* RNAi-treated animals lost both magnetoreception and circadian rhythmicity. (*E*) *timeless* RNAi cockroaches showed unaffected magnetoreception, but their circadian behavior was disrupted.

During their resting time around noon, cockroaches display minimal locomotor activity (Fig. 2*B*). However, when the direction of the horizontal magnetic vector of the natural GMF is rotated periodically (Fig. 1 *A* and *B*), the animals change their resting positions more frequently. We term this phenomenon magnetically induced restlessness (MIR) (Fig. 1*C*). As a control, a double-wrapped coil that does not generate any MF had no effect on MIR in any of these two species (Fig. 1*C*).

Cry2 Is Necessary for Directional Sensitivity. The fact that no MIR was observed in complete darkness (DD; Fig. 2*A*, *Right*) supports the involvement of photosensitive processes in *P. americana* magnetoreception. To test the causal involvement of Cry2 in cockroach directional magnetoreception, we used our behavioral assay under 365 nm UV light in combination with a reverse genetic approach. Injections of double-stranded (ds) cry2 RNA (RNAi) significantly reduced the cry2 mRNA and protein levels (*SI Appendix*, Figs. S5 and S8) and completely abolished MIR behavior (Fig. 2*D*). Control injections of the nonspecific dsRNA or buffer alone (Fig. 2 *B* and *C*) had no effect on the response to changes of the MF vector. Importantly, the assays were conducted during the middle of the photophase, when a drop of activity was observed in all treatment groups. This indicated that the MIR was not an artifact caused by endogenous activity patterns of the cockroaches (Fig. 2 *B–E*, *Middle*). The silencing of Cry2 was also accompanied by severe disruption of circadian rhythmicity in constant dark conditions (Fig. 2*D*, *Right* and *SI Appendix*, Table S1) compared with control animals (Fig. 2 *B* and *C*). The overlap of the circadian clock mechanism and magnetic sensing was further tested under constant light conditions known to interfere with proper clock function. As expected, the constant light regime resulted in arrhythmic circadian behavior, but the sensitivity of the animals to the changes in the MF direction was unaffected (Fig. 2*A*, *Left*). In addition, injection of dsRNA targeting the clock gene *timeless* had no effect on MIR, but did abolish cockroach circadian rhythmicity (Fig. 2*E* and *SI Appendix*, Table S1). These results clearly support the separation of GMF sensing from the circadian clock.

The following experiments confirmed that the locomotor activity in photophase is lower after any treatment in the second cockroach species, *B. germanica* (Fig. 3 *A–E*, *Middle*). Although control dsRNA or buffer injection had no effect on MIR and circadian phenotypes in DD (Fig. 3 *A* and *B*), cry2 knockdown abolished MIR and reduced circadian rhythmicity (Fig. 3*C*). Remarkably, cry1 reduction did not affect MIR, whereas circadian rhythmicity was reduced even more than in cry2 RNAi animals, indicating that cry1 RNAi was efficient (Fig. 3*D* and *SI Appendix*, Fig. S5). Notably, cry1 knockdown resulted in significant up-regulation of cry2 levels (*SI Appendix*, Fig. S5); nevertheless, the detailed mechanism, how cry1 depletion affects behavioral rhythmicity, is beyond the scope of this study. Consistently, cry1+cry2 double RNAi abolished MIR and reduced circadian rhythmicity (Fig. 3*E*). Cry2 therefore represents a prerequisite for magnetic susceptibility in both cockroach species.

Magnetoreception Is Dependent on Light from UV to Cyan/Green 505 nm. To further characterize magnetoreception, we tested seven wavelengths of light under different intensities (*SI Appendix*, Table S7). The minimal light intensity needed for MIR was at UVA 365 nm. The sensitivity of the MIR gradually dropped to 4×10^{16} photons $m^{-2} \cdot s^{-1}$ under two blue light wavelengths, followed by a local boost of sensitivity at 505 nm. Under 505 nm, the MIR was still significant under dim light (7×10^{15} photons $mm^{-2} \cdot s^{-1}$), but the MIR response dropped precipitously at higher wavelengths; no MIR was observed at 528 nm, even with 1,000× stronger light (6×10^{18} photons $m^{-2} \cdot s^{-1}$; Fig. 4*A*). Enlarged samples were tested to exclude possible errors (*SI Appendix*, Table S7), but the steep threshold of MIR sensitivity was upheld. There was also a negative result at 407 nm light and its maximal intensity

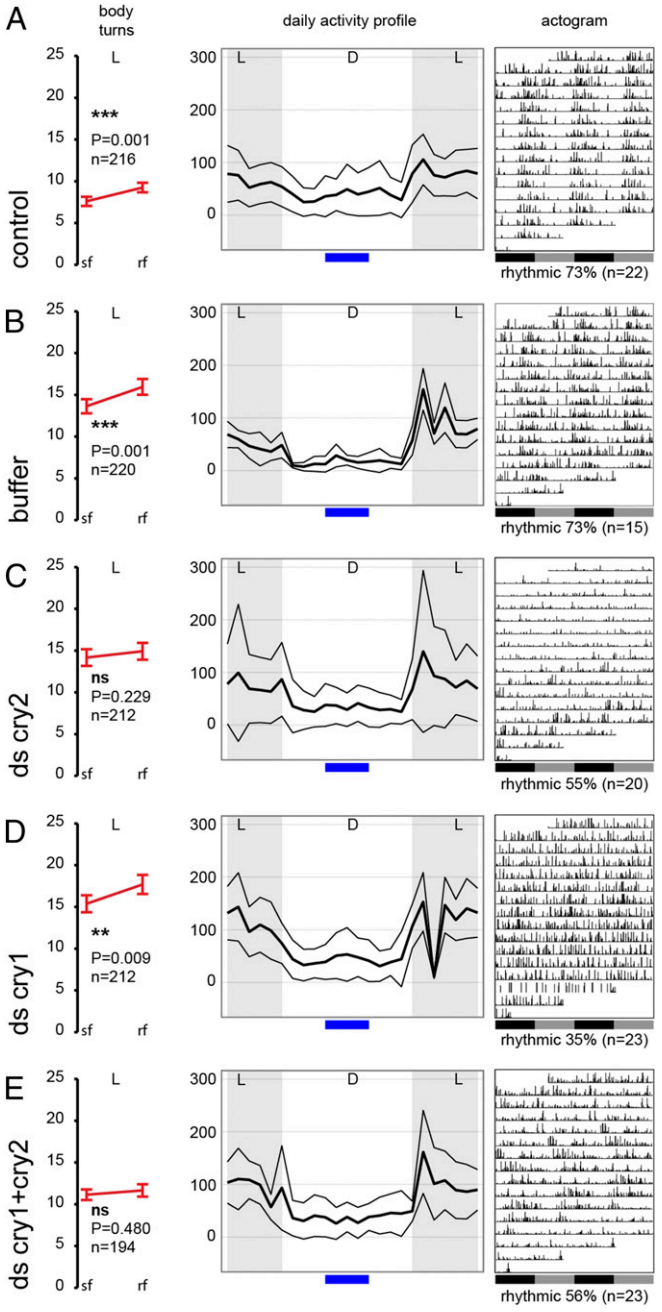

Fig. 3. Phenotypes of *B. germanica*. Magnetoreception scored as body turns under sf and rf; red line depicts the average change of body turns (±SEM). Daily activity profiles (*Middle*) at LD 12:12 are shown as a black line (±SD, thinner lines); horizontal blue bar indicates the time during which magnetoreception was assayed. Circadian activity (*Right*) in DD is shown as double-plotted actograms. (*A*) The control RNAi- and (*B*) buffer-injected animals displayed normal magnetoreception as well as circadian rhythmicity. (*C*) *cry2* RNAi-treated animals lost magnetoreception and have reduced rhythmicity in DD. (*D*) *cry1* RNAi cockroaches showed unaffected magnetoreception, but their circadian behavior was disrupted. (*E*) *cry1, cry2* double RNAi-treated animals lost magnetoreception and had reduced rhythmicity in DD.

2×10^{17} photons $m^{-2} \cdot s^{-1}$, likely showing the upper border of a functional window shown previously [e.g., from experiments on birds (31)].

Magnetoreceptor Is Most Likely Located in the Compound Eyes. Because light is necessary for magnetoreception (Fig. 2*A*), we attempted to localize the magnetoreceptive organ anatomically by shielding/painting the compound eyes. The animals with compound

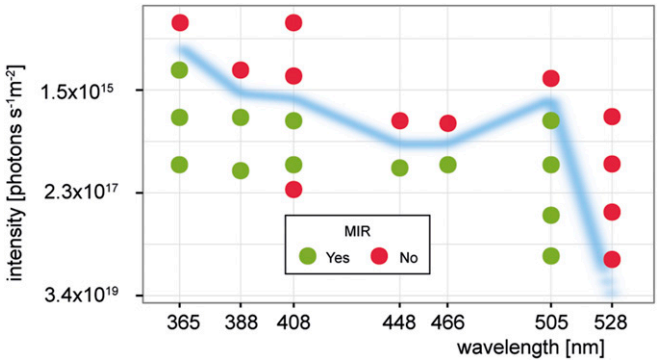

Fig. 4. Light sensitivity of *Blattella* magnetoreception is wavelength-dependent and restricted to the region from UV to cyan/green light. Green dots indicate functional MIR; red dots no MIR reaction. Blue line approximates low threshold of illumination necessary for MIR. *y* axis, light intensity; *x* axis, wavelength of light used in experiment. For details, see *Discussion, Spectral Effects and Magnetic Signaling*.

eyes covered by transparent enamel retained MIR, whereas black enamel prevented any magnetoreceptive response, suggesting eyes as the necessary organ for magnetoreception (Fig. 5*A*).

Laser-scanning confocal microscopy localized Cry2 to a hemispherical multicellular structure immediately beneath the cockroach retina (Fig. 5 *B* and *C*). Colocalization of Cry2 with antisera raised against the alpha subunit of Na/K-ATPase (32) (*SI Appendix*, Fig. S10 *A–C*) resulted in a pattern similar in structure to *Drosophila* epithelial glial cells (33, 34), suggesting these cells are glia localized underneath the retina between the two basement membranes (35). Higher magnification revealed an orderly columnar alignment of the Cry2-positive cells (Fig. 5 and *SI Appendix*, Fig. S9), with the strongest signal localized in the close vicinity of the plasma membrane (*SI Appendix*, Fig. S10).

Discussion

Cry Phylogeny in Blattodea. In this study, we explored the role of Crys in magnetoreception, using two cockroach species. Cry1 was not found in *P. americana* by examination of its assembled transcriptome, or in raw RNA reads even when Cry2 coding sequences or Cry unrelated genes were identified (*SI Appendix*, Fig. S2). Because the Cry protein structure is remarkably well conserved, we suggest that Cry1 is absent in *P. americana*. This hypothesis is further supported by phylogenetic analysis of insect Crys, where Cry1 was not identified either in cockroach *Cryptocercus* or in two termite species, suggesting the loss of Cry1 in multiple species of the Blattodea lineage. Therefore, the most plausible explanation is the existence of different *Cry* genes in *B. germanica* and *P. americana.*

Detection of Directional Changes of MF in Cockroaches. Even though the MIR assay does not monitor directional locomotion from point A to point B, which might be expected if compass abilities are investigated (e.g., in migratory birds), it gives unequivocal evidence of sensitivity to the direction of the magnetic vector. The simplicity of the behavioral output is a particular strength of this assay compared with orientation tests made on birds, where unimodal versus axial orientation under different light conditions are results that should be considered separately (36). Another merit of our unconditioned assay is its relative insensitivity to changes in motivation under different light regimens, which may alter more complex behavioral programs such as migration behavior.

Spectral Effects and Magnetic Signaling. The dependence of magnetoreception on specific wavelengths and intensities of light has been documented extensively in birds (summarized in refs. 31, 36, 37). The FAD cofactor of CPF proteins is capable of both ground-state (dark) and light-driven redox reactions. One-electron reduction of neutral fully oxidized state (FAD_{ox}) and one-electron oxidation of anionic fully reduced state ($FADH^-$) produce anionic semiquinone ($FAD^{\bullet -}$) and neutral semiquinone ($FADH^{\bullet}$) radicals, respectively, that are capable of magnetosensitive spin-correlated RPs (18) (*SI Appendix*, Fig. S11). The most frequently discussed model supposes spin-correlated RPs consisting of $FAD^{\bullet -}$ and a cationic radical of Trp or Tyr initiated by the UVA/blue light excitation of FAD_{ox} (*SI Appendix*, Fig. S11, Scheme 1). MF modulates interconversion of the singlet and triplet states of RP, hence changing the proportions of two competing pathways yielding different reaction products (5).

FAD changes its spectral absorption properties as it goes through several redox states, during its redox cycle (38) (*SI Appendix*, Fig. S12). Examination of spectral limits during in vivo magnetoreception may help clarify which redox forms of FAD and which radical partners participate in the signaling conformation of Cry proteins (39).

Our MIR spectrum for *Blattella* (Fig. 4) shows a steep decline of sensitivity in the cyan region 505–528 nm, remarkably matching the decline of light absorption of three redox forms of flavin: FAD_{OX}, $FADH^-$ and $FAD^{\bullet -}$ (40) (*SI Appendix*, Fig. S11). Such a coincidence points to possible involvement of flavin in cockroach magnetoreception. The role of the $FADH^{\bullet}$ redox form, which absorbs in wide spectrum including wavelengths above cyan (*SI Appendix*, Fig. S10), is not clear. The MIR spectrum (Fig. 4), however, does not fully conform to a direct light absorption by FAD_{ox} (*SI Appendix*, Fig. S11). Although two peaks of magnetosensitivity of *Blattella* at UV 365 and green/cyan 505 nm are apparent, the absorbance of FAD_{ox} peaks at about 450 nm. As a possible explanation, an indirect FAD_{ox} excitation via energy transfer from a UVA-absorbing antenna cofactor may occur. Such a cofactor (e.g., methenyltetrahydrofolate) is well-known for many CPF proteins, and was discussed also for *Drosophila* Cry (41). Because both wavelength peaks of MIR response exactly match the peaks of visual sensitivity of *Periplaneta* (42) and *Blattella* (43), they might reflect a generally close association between magnetoreception and vision.

The drop in MIR sensitivity is surprisingly sharp in the wavelength range between 505 and 528 nm, where magnetoreception is lost even under exposure to three orders magnitude of light intensity (Fig. 4 and *SI Appendix*, Table S7). Such a sharp cutoff cannot easily be attributed to the spectral sensitivity limit of a single crucial photopigment. As Wiltschko et al. (44) state from a comparable phenomenon in birds: "It rather seems to reflect some

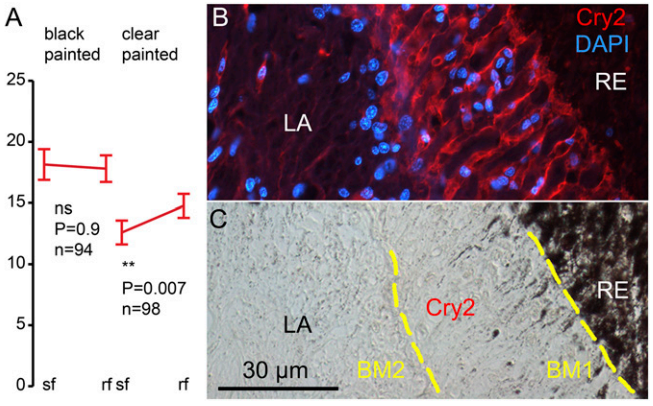

Fig. 5. *P. americana* eye participates in magnetoreception and expresses Cry2. (*A*) Cockroaches with clear-painted eyes responded to MF rotation, whereas individuals with black-painted eyes did not. (*B*) Immunofluorescence and (*C*) Nomarski contrast of *P. americana* eye. Cry2 immunoreactivity (in red) is localized underneath the retina (RE) between the two basement membranes (BM1, BM2), according to Ribi (35). BM1, first basement membrane; BM2, second basement membrane; LA, lamina; RE, retina; blue, cell nuclei (DAPI stained).

antagonistic interactions with receptor activated by longer wavelength light." As short and long wavelengths may have opposite effects on the relative concentration of FAD_{OX} versus semiquinone $FADH^{\bullet}$ redox states, changes in illumination color can also result in antagonistic changes of cryptochrome activation (36). If wavelengths from UV to blue stimulate MIR while green light 528 nm suppresses it, then the semiquinone $FADH^{\bullet}$ (or other still-unknown pigment) could be considered an antagonistic candidate in our MIR assay.

Reports of light-dependent magnetoreception beyond 528 nm are not unique within behavioral reports on birds (green/yellow) (45). However, the finding that preexposure of European robins to white light not earlier than 30 min before testing is necessary for proper orientation suggests a dependence on the short-wavelength part of spectra as well (39), meaning that orientation under green/yellow light is only a transient phenomenon (45).

A contrasting situation was reported in the fruit fly (*D. melanogaster*) and monarch butterfly (*Dannaus plexippus*), which lost magnetic compass orientation if the illumination wavelength was already above 420 nm, which is still well within the absorption range of FAD_{OX} (26, 27, 46). Because the studies did not seek thresholds for separate colors, the question remains whether the diurnal species such as *Dannaus* or *Drosophila* had already reached the absolute threshold of their sensitivity to light-driven magnetoreception. *Blattella* lost its magnetoreception under only 10-fold dimmer light than *Dannaus* or *Drosophila.* Provided they have denser cuticle or eye pigment shielding than night-active *Blattella*, brighter illumination might induce magnetoreception even for green and longer wavelengths. The inconsistencies among *Blattella*, *Drosophila*, *Dannaus*, and birds show that taxa-specific Crys may use different redox cycles and that the analysis of the long-wavelength tail of the light spectrum may be of particular interest to identify other yet-uncharacterized signaling partners. In summary, our behavioral data on the spectral dependence of Cry2-dependent insect magnetoreception are consistent with the involvement of electron transfer reactions of FADox, $FADH^{-}$, and $FAD^{\bullet -}$.

Directional Sensitivity. Local magnetic conditions that possibly control RP reactivity are a result of interplay between external MFs, nuclear spins of RP partners (47, 48), and electron spins of nearby radicals (49). To function as a directional sensor that changes the product yield solely after a shift in the magnetic vector, the RPs must respond anisotropically (2, 50). So far, there has been no demonstration that any Cry radical reaction responds to an Earth-strength MF, or that this response is anisotropic, as would be required for a magnetic compass (18).

Another important condition is that sensory molecules should optimally be aligned in the same direction within the receptor cells, in order that the stochastic effects of freely rotating molecules would be eliminated (51). Such a condition could be met by cytoskeletal anchoring to the membrane, or by at least partial immobilization within the cells (52).

Moreover, for sufficiently fast compass orientation, the magnetic direction-sensitive structure should ideally consist of spatially organized receptor cells, so that the signal from differentially activated cells can be compared, and the GMF vector can be directly identified. As suggested (48), the retina forms an ideal hemispherical organ for photoreception, with a potential for directional magnetoreception. If the Cry reaction products are involved in the visual signaling pathway, they could modulate the rate of light transduction to a neural signal (5).

Our behavioral–genetic evidence provides a link between truly directional magnetic sensitivity and mammalian Cry protein, supporting the so-far-hypothetical predictions of directional magnetic sensitivity discussed earlier.

Anatomical Localization of the Magnetoreceptor. The experiment with painted eyes suggested that the magnetoreceptive tissue is localized either within the compound eye or underneath it (Fig. 5*A*). Cry2-immunoreactive cells were localized immediately beneath the pigmented layer of the cockroach retina in a hemispherical multicellular structure (Fig. 5*B*). The cells were spread between the two basement membranes (Fig. 5*C*) separating the retina from the fenestrated layer of lamina neuropil (35). High-magnification analysis identified Cry staining strongest in the close vicinity of the plasma membrane (*SI Appendix*, Fig. S10). Indeed, association of Cry with the scaffolding protein close to the membrane was reported recently for *Drosophila* (53). Such immobilization would further support Cry as a directional magnetoreceptor, as discussed earlier. Furthermore, microinjection of a fluorescent neuronal tracer into the cockroach ocular photoreceptor cells revealed an intimate contact between the neuronal output from the retina and the Cry2-positive cells (*SI Appendix*, Fig. S9), suggesting their possible interplay.

Taken together, the results of our study establish the role of a mammalian-type Cry in light-dependent magnetoreception in two insect species phylogenetically distant to *Drosophila.* More important, our study delivers original evidence that the Cry2 protein is involved in the detection of GMF vector direction at natural intensities, which is a crucial feature of a biological receptor providing genuine compass bearings; by means of immunolocalization, we identified the eye and subretinal region as a likely site of magnetoreception in insects; and we show that spectral dependency of mammalian Cry-linked magnetoreception is in line with involvement of flavins. Altogether, the work provides original genetic proof of the previously hypothesized chemical reactions that may underlie a functional compass in animals.

Materials and Methods

Behavioral Tests: MIR. Cold-immobilized cockroaches, regardless of sex, were transferred individually into glass Petri dishes with white opaque walls and placed into a white arena with a translucent lid. On the next day, a camera–PC system underneath a glass pane holding the dishes sampled the silhouettes of the animals illuminated from above every 1 min. Frames taken between 10:00 and 14:30 were downloaded and divided into six 45-min intervals: the first two (1, 2; 10:00–11:30) before magnetic North rotation, the middle three intervals (3–5; 11:30–13:45) when the field was periodically rotated, and the last interval (6; 13:45–14:30) after this magnetic treatment. The temperature varied between 21 °C and 24 °C in the testing room.

Photic Conditions. A set of three UV LEDs 365 nm (Nichia NCSU033A) illuminated the arena through a translucent lid that diffused light so that its intensity was 4.04×10^{16} quanta $m^{-2} \cdot s^{-1}$ in the center of the arena and 3.12×10^{16} quanta $m^{-2} \cdot s^{-1}$ along the wall line (radiometer International Light IL700, SHD 033 probe). Types and spectral characteristics of all LEDs used are given in *SI Appendix*, Fig. S6 and Table S7.

Magnetic Conditions. The natural geomagnetic background within the testing space was as follows: horizontal component 18 μT, total vector 45 μT, and inclination 66°. The spatial variation in the arena region was <2% (measured by HMR 2300 magnetometer; Honeywell). During periods of magnetic North rotations (periods 3–5), only the horizontal component of local GMF was rotated by 60° by means of a horizontal four-element double-wrapped Merritt coil, making an angle with the *N*–S axis of 120° (*SI Appendix*, Fig. S3).

Evaluation and Statistics. For both MIR and circadian activity, the number of body axis changes >15° was determined visually using Screen Protractor software (Iconico.com Software) for *Periplaneta*. In the case of *Blattella*, MIR recordings were done automatically with image analysis software RoachLab. In both cases, the personnel scoring the activity and doing the statistical analysis were not aware of which set of images they were evaluating. For MIR analysis, the activity of every animal was given as a pair of numbers: activity in steady field – sf (control periods 1 + 2 + 6) versus activity in rotated field – rf (treated periods 3 + 4 + 5). The experiment was principally designed as "paired," where individual animals represented objects producing mutually consecutive outcomes from measurement periods. In such a design, the outcomes must be compared on the basis of paired statistical tests, which can then adjust the internal correlation (individuality) of primary data. Because of the pair design of the test, we did not compare statistically groups among each other.

Antibodies and Immunodetection. The synthetic peptide CHSPSYRENIKSGIHFHR corresponding to the C-terminal region of the *Periplaneta* Cry2 was used to generate a custom-made specific antibody (Moravian Biotech). Cry2 primary antibody was used at a dilution of 1:1,000 or 1:1,500, with similar results. Immunofluorescent detection and microinjection of Alexa Fluor-conjugated dextran neuronal tracer were carried out as described earlier (54). The alpha5 mouse monoclonal antibody (DSHB Hybridoma Product a5; *SI Appendix*, Fig. S10) was used at a dilution of 1:50, as described in refs. 32 and 34.

Verification of RNAi Efficiency. *cry2*, *cry1*, *timeless*, or *lacZ* dsRNA was injected, and the cockroaches were euthanized 14 d later. Their brains were dissected and used for RNA isolation and subsequent quantitative RT-PCR analysis (*SI Appendix*, Fig. S5). Alternatively, dissected brains were surgically divided into two hemispheres, including the optic lobes and eyes. One hemisphere was used for RNA isolation and subsequent qRT-PCR analysis (*SI Appendix*, Fig. S8*B*). The second hemisphere was fixed and used for immunocytochemistry (*SI Appendix*, Fig. S8 *A* and *C–F*). In this case, both control (*lacZ* RNAi) and *cry2* RNAi brain sections were processed on the same microscope slide. The analysis was performed double blind, and the reduction of protein was evaluated by Image J analysis software (*SI Appendix*, Fig. S1*E*).

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