

LETTERS

Animal cryptochromes mediate magnetoreception by an unconventional photochemical mechanism

Robert J. Gegear¹, Lauren E. Foley¹, Amy Casselman¹ & Steven M. Reppert¹

Understanding the biophysical basis of animal magnetoreception has been one of the greatest challenges in sensory biology. Recently it was discovered that the light-dependent magnetic sense of *Drosophila melanogaster* is mediated by the ultraviolet (UV)-A/blue light photoreceptor cryptochrome (Cry)¹. Here we show, using a transgenic approach, that the photoreceptive, *Drosophila*-like type 1 Cry and the transcriptionally repressive, vertebrate-like type 2 Cry of the monarch butterfly (*Danaus plexippus*) can both function in the magnetoreception system of *Drosophila* and require UV-A/blue light (wavelength below 420 nm) to do so. The lack of magnetic responses for both Cry types at wavelengths above 420 nm does not fit the widely held view that tryptophan triad-generated radical pairs mediate the ability of Cry to sense a magnetic field. We bolster this assessment by using a mutant form of *Drosophila* and monarch type 1 Cry and confirm that the tryptophan triad pathway is not crucial in magnetic transduction. Together, these results suggest that animal Crys mediate light-dependent magnetoreception through an unconventional photochemical mechanism. This work emphasizes the utility of *Drosophila* transgenesis for elucidating the precise mechanisms of Cry-mediated magnetosensitivity in insects and also in vertebrates such as migrating birds.

A wide variety of animals are able to detect the Earth's magnetic field and use it as a source of directional information^{2,3}. In many cases, animal magnetic detection seems to depend on chemical reactions initiated by specific wavelengths of light^{4,5}. The most popular chemical reaction model proposes that light-dependent magnetoreception is mediated by radical-pair reactions that are generated in specialized photoreceptors^{6–8}.

Because of their photoreceptor function and biochemical properties, the DNA photolyase-related Crys have been postulated to be the key photoreceptor molecules that generate magnetically sensitive radical-pair products^{7,9}. However, animal Crys are not all functionally equivalent. In fact, two phylogenetically and functionally distinct groups of animal Crys have been identified and characterized, largely on the basis of their roles in the regulation of circadian clocks^{10,11}. *Drosophila*-like type 1 Crys are sensitive to UV-A/blue wavelengths of light^{12,13} and function mainly as circadian photoreceptors. Vertebrate-like type 2 Crys, in contrast, are thought to function primarily as negative regulators of the clock's transcriptional feedback loop, the quintessential intracellular gear of the molecular clock. Although both types of Cry seem to be widespread in the animal kingdom, there is considerable variation in their distribution among taxa¹¹: insects can have type 1 Cry (the only one found in *Drosophila*), type 2 Cry, or both, whereas vertebrates have only type 2 Crys. Type 2 Cry proteins are thought to mediate light-dependent magnetoreception in many vertebrate groups¹⁴ including migratory birds, but direct evidence for such a role has yet to be established for any animal because of the lack of available tools for genetic manipulation.

Recently we integrated genetic and behavioural approaches to show that light-dependent magnetoreception in *Drosophila* is mediated by its

type 1 Cry (ref. 1). The necessity of *Drosophila* Cry for magnetosensitivity was shown by using two distinct Cry-deficient mutations, which abolished magnetosensitive behavioural responses. This work established *Drosophila* as a viable genetic system with which to delineate the molecular underpinnings of Cry-based magnetoreception in animals.

We began our present investigations by showing that a *Drosophila cry* transgene can rescue magnetosensitivity in Cry-deficient flies. We used the GAL4–UAS system, with *timeless (tim)*–GAL4 as the driver, which drives transgene expression in *tim*-expressing and most *cry*-expressing cells¹⁵. We attempted the rescue of magnetosensitive behavioural responses in the Cry loss-of-function *cry^b* mutant background by expressing a UAS–*Drosophila cry* transgene. Magnetosensitivity was assessed by using our behavioural assay in which flies experience an electric-coil-generated magnetic field and show their behavioural responses in a binary-choice T maze housed in an illuminated black box¹. The two-coil system produces a magnetic field on one side of the T maze but produces no field on the opposite side. Flies were tested for either their response to the magnetic field in the naive state or after a training session in which the field was paired with a sucrose reward. Response to the magnetic field was expressed as a preference index, with negative values representing an avoidance of the field and positive values a preference for the field. As described previously, wild-type Canton-S flies expressing endogenous Cry show differential behavioural responses to the magnet in the T maze: a naive avoidance of the field and a trained preference for the field (white and black bars, respectively, in Fig. 1a, left bar set)¹. This establishes a standard of behavioural responses for the transgenic studies.

Predictably, Cry-deficient *cry^b* lines expressing either the *tim*–GAL4 driver alone or the *Drosophila cry* transgene alone did not respond to the magnetic field (Fig. 1a, middle two sets of bars). However, *cry^b* flies expressing the *Drosophila cry* transgene under the control of the *tim*–GAL4 driver now showed significant naive and trained responses to the field (*tim*–GAL4/UAS–*cry*; Fig. 1a, right bar set), similar in both direction and magnitude to those of wild-type flies (Fig. 1a, left bar set). Thus, the *tim*–GAL4 driver expresses Cry appropriately so that it can rescue the magnetosensitive behavioural responses in the *cry^b* background, making possible a study of the biochemical mechanism through which animal Crys mediate magnetosensitivity.

We next used the *tim*–GAL4 driver in *cry^b* flies for evaluating the functional roles of monarch butterfly Crys in magnetosensitivity. Monarchs have both a type 1 Cry (designated Cry1) and a type 2 Cry (designated Cry2). Studies *in vitro* and *in vivo* have shown that they are functionally distinct¹⁶; Cry1 functions as a circadian photoreceptor, whereas Cry2 functions as a core clock component. Similarly, monarch Cry2 does not show sensitivity to light, at least in the assay systems studied so far^{10,11}. Monarch butterfly Crys thus provide a unique opportunity to compare the functional equivalence for magnetosensitivity of type 1 and type 2 Crys directly from the same species *in vivo*, using transgenesis in *Drosophila*.

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A monarch *cry1* transgene driven by *tim-GAL4* successfully restored both naive and trained responses to the magnetic field in the *cry^b* background (*tim-GAL4/UAS-dpcry1*; Fig. 1b). Furthermore, we showed that monarch Cry1 rescues magnetosensitivity in a wavelength-dependent manner (Fig. 1c, d). Similarly to *Drosophila* Cry (ref. 1), monarch Cry1-rescued flies showed significant naive and trained responses to a magnetic field under full-spectrum light (about 300–700 nm) and under UV-A/blue light (less than 420 nm), but the behavioural responses were abolished when only long-wavelength light (more than 420 nm) was available. Thus, at the intensities used, light in the UV-A/blue range is both necessary and sufficient for monarch Cry1 to function in the

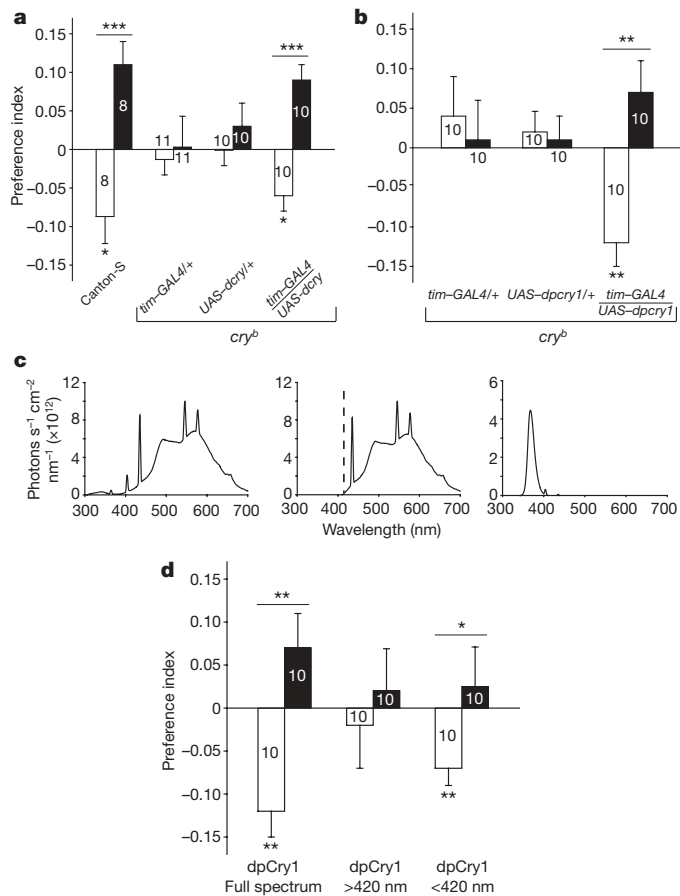


Figure 1 | Type 1 Cry rescues light-dependent magnetoreception in Cry-deficient flies. **a**, A *tim-GAL4*-driven *Drosophila* transgene (*tim-GAL4/UAS-dcry*) rescues magnetic responses in *cry^b* flies, in a similar manner to the responses of wild-type Canton-S flies, whereas *tim-GAL4/+* or *UAS-dcry/+* alone does not. Bars show preference index values for naive (white) and trained (black) groups. Numbers represent the sizes of groups tested. Asterisk, $P < 0.05$; three asterisks, $P < 0.001$. Genotypes in parentheses: *tim-GAL4/+* ($y w; tim-GAL4/+; cry^b$), *UAS-dcry/+* ($y w; UAS-mycdcry/+; cry^b$) and *tim-GAL4/UAS-dcry* ($y w; tim-GAL4/UAS-mycdcry; cry^b$). **b**, A *tim-GAL4*-driven monarch (*dp*)*cry1* transgene (*tim-GAL4/UAS-dpcry1*) rescues magnetic responses in *cry^b* flies, whereas *tim-GAL4/+* or *UAS-dpcry1/+* alone does not. Bars show preference index values for naive (white) and trained (black) groups. Two asterisks, $P < 0.01$. Genotypes in parentheses: *tim-GAL4/+* ($y w; tim-GAL4/+; cry^b$), *UAS-dpcry1/+* ($y w; UAS-mycdpCry1\#15b/+; cry^b$) and *tim-GAL4/UAS-dpcry1* ($y w; tim-GAL4/UAS-mycdpCry1\#15b; cry^b$). **c**, Irradiance curves for different light conditions: left, full spectrum; middle, above 420 nm; right, below 420 nm. Light measurements were taken from inside the training tube and test tube. The full-spectrum and above-420-nm irradiance curves were reported previously¹. **d**, Wavelength dependence of magnetic responses is rescued by monarch (*dp*)*Cry1* ($y w; tim-GAL4/UAS-mycdpCry1\#15b; cry^b$). The full-spectrum data are the same as in **b**. Bars show preference index values for naive (white) and trained (black) groups. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$. Values in **a**, **b** and **d** are means \pm s.e.m.

Drosophila magnetoreception system. Taken together, these results indicate that type 1 Cry has a key function in the magnetic transduction pathway of *Drosophila* and provide evidence for the functional equivalence of type 1 Cry in magnetoreception across insect species.

A monarch *cry2* transgene driven by *tim-GAL4* also successfully restored both naive and trained responses to the magnet in the *cry^b* background (*tim-GAL4/UAS-dpcry2*; Fig. 2a) and did so in a wavelength-dependent manner similar to that of the monarch *cry1* transgene (Fig. 2b). The reliance of monarch Cry2-induced magnetosensitivity on UV-A/blue light is consistent with the predicted spectral sensitivities of chicken type 2 Cry, as measured from the embryonic iris *ex vivo*¹⁷. These genetic data show that a type 2 Cry can function in a light-dependent behavioural response; another transgenic study has suggested a light-induced proteolytic response for a type 2 protein¹⁸. The light dependence of magnetosensitivity in flies expressing monarch *cry1* or *cry2* transgenes suggests that both proteins undergo the photochemical reactions necessary for magnetic sensing.

The widely held radical-pair model for Cry-mediated magnetoreception is derived from the biochemical reactions proposed for the photocycle of plant Crys (ref. 6). In this model, magnetically sensitive radical-pair products are generated when the fully oxidized FAD chromophore is photoreduced through a sequence of intraprotein electron transfers along a chain of three tryptophan residues; these three residues are conserved in photolyases and Cry (the so-called 'Trp triad'; Supplementary Fig. 1)⁶. It is well established that blue light in the 450-nm range is sufficient for the photoreduction of oxidized flavin and the generation of Trp-triad-mediated radicals^{6,12,18–20}. The lack of a magnetic response for both type 1 and type 2 Cry under light at wavelengths above 420 nm suggests that neither type signals through the photoreduction of oxidized flavin. The results further imply that the photoactivation of either Cry type for magnetosensing does not depend on the presence of Trp-triad-mediated radical pairs.

We directly tested whether the Trp triad pathway is necessary for Cry-mediated magnetoreception by mutating the terminal tryptophan to phenylalanine in *Drosophila* Cry (W342F; two independent *UAS* lines) and also in monarch Cry1 (W328F; three independent lines) and monarch Cry2 (W345F; two independent lines). Magnetosensitivity was then assessed in *cry^b* flies expressing each of the Trp-mutated Crys as *UAS* transgenes under control of the *tim-GAL4* driver. These mutations were chosen because previous biochemical studies had

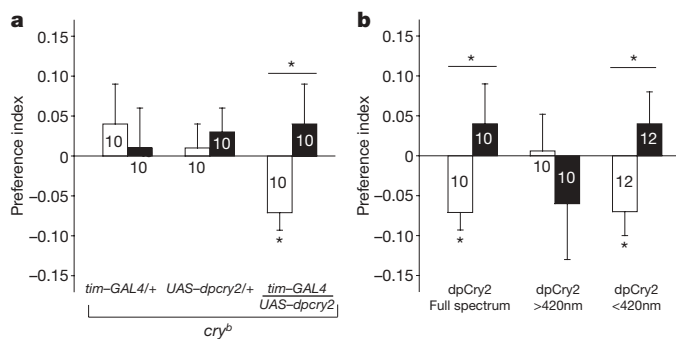


Figure 2 | Monarch type 2 Cry rescues light-dependent magnetosensitivity in Cry-deficient flies. **a**, A *tim-GAL4*-driven monarch (*dp*)*cry2* transgene (*tim-GAL4/UAS-dpcry2*) rescues magnetic responses in *cry^b* flies, whereas *UAS-dpcry2/+* alone does not. The *tim-GAL4/+* data are replotted from Fig. 1b. Bars show preference index values for naive (white) and trained (black) groups. Numbers represent the sizes of groups tested. Asterisk, $P < 0.05$. Genotypes in parentheses: *tim-GAL4/+* ($y w; tim-GAL4/+; cry^b$), *UAS-dpcry2/+* ($y w; UAS-mycdpCry2\#125a/+; cry^b$) and *tim-GAL4/UAS-dpcry2* ($y w; tim-GAL4/UAS-mycdpCry2\#125a; cry^b$). **b**, Wavelength dependence of magnetic responses is rescued by monarch (*dp*)*Cry2* ($y w; tim-GAL4/UAS-mycdpCry2\#125a; cry^b$). The full-spectrum data are the same as in **a**. Bars show preference index values for naive (white) and trained (black) groups. Asterisk, $P < 0.05$. Values in both panels are means \pm s.e.m.

shown that mutating this tryptophan residue identically in *Drosophila* Cry and in monarch Cry1 blocks the photoreduction of oxidized FAD *in vitro*^{12,20}, thus preventing the generation of Trp-triad-mediated radical-pair products. No such biochemical data exist for any type 2 Cry.

Mutating the terminal tryptophan in *Drosophila* Cry and monarch Cry1 did not affect the ability of transgenic flies to respond to the magnetic field, because lines of both *tim-GAL4/UAS-dcryW342F* and *tim-GAL4/UAS-dpcry1W328F* flies showed appropriate, significant naive and trained responses in the *cry^b* background (Fig. 3a, b), as expected based on the lack of magnetic sensing under light above 420 nm. The Trp triad is important but not essential for *Drosophila* Cry to function in the circadian system, because the Trp mutant transgenes modestly rescued circadian photoreceptive responses in *cry^b* flies (Supplementary Fig. 2). Although other ways of generating radical pairs in Cry have been proposed, for example through superoxides, all described radical-pair mechanisms depend on a functional Trp triad^{6,21,22}. Radical pairs generated through those mechanisms are therefore not necessary for magnetoreception through type 1 Crys.

Another possibility is that there are light-sensitive biochemical reactions for magnetosensing that involve type 1 Crys but do not

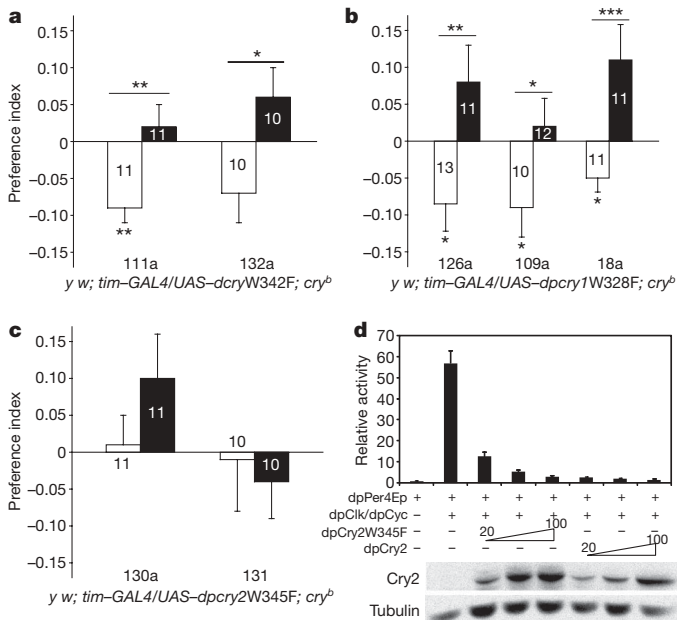


Figure 3 | Effects of terminal tryptophan mutations on type 1 and type 2 Cry-mediated magnetosensitivity. **a**, The *Drosophila* CryW342F mutation rescues magnetosensitive responses. Two mutant lines (111a and 132a) of *y w; tim-GAL4/UAS-dcryW342F; cry^b* were tested. Bars show preference index values for naive (white) and trained (black) groups. Numbers represent the sizes of groups tested. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$. **b**, The monarch Cry1W328F mutation rescues magnetosensitive responses. Three mutant lines (126a, 109a and 18a) of *y w; tim-GAL4/UAS-dpcry1W328F; cry^b* were tested. Bars show preference index values for naive (white) and trained (black) groups. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. **c**, The monarch Cry2W345F mutation does not rescue magnetosensitive responses. Two mutant lines (130a and 131) of *y w; tim-GAL4/UAS-dpcry2W345F; cry^b* were tested. Bars show preference index of the naive (white) and trained (black) groups. For **a–c**, none of the *UAS-trp mutant/+* lines (without driver) restored magnetosensitive behavioural responses (data not shown). Values in **a–c** are means \pm s.e.m. All Cry mutations were sequenced and confirmed from all of the different transgenic lines used. **d**, Cry2W345F still inhibits Clock:Cycle-mediated transcription in Schneider 2 cells. Top: the dpPer4Ep reporter (20 ng) was tested in the presence (+) or absence (-) of dpClk/dpCyc expression plasmids (10 ng each); wild-type dpCry2 (20, 50 and 100 ng) or dpCry2W345F (20, 50 and 100 ng) was used^{10,11}. Luciferase activity was computed relative to β -galactosidase activity. Values are means \pm s.e.m. for three independent experiments. Bottom: representative western blot of Cry2 (dpCry2 or dpCry2W345F) and tubulin expression.

require a functional Trp triad, such as those that may occur in a photolyase-like photocycle^{12,20}. In this photocycle, UV-A/blue light (below 420 nm) induces reduced flavin to initiate electron transfer to an unknown substrate that generates a radical and initiates the signal; back transfer turns off the signal²³. Whether this photocycle could generate radical pairs appropriate for magnetoreception merits consideration.

Magnetosensitivity was not restored in either of the two transgenic lines expressing the monarch Cry2 Trp mutation (*tim-GAL4/UAS-dpcry2W345F*) in the *cry^b* background (Fig. 3c), even though protein expression was stable (Supplemental Fig. 3). The Trp-mutated Cry2 did alter the ability of the protein to function as a transcriptional repressor, although the mutant protein could still repress Clock:Cycle-mediated transcription in a dose-dependent manner in cell culture (Fig. 3d). Because monarch Cry2 cannot rescue magnetic sensing under light above 420 nm (Fig. 2b), the lack of a magnetic response in Cry2 Trp mutant flies under full-spectrum light is unlikely to be caused by the absence of radical pairs generated through the Trp-triad pathway. Rather, the Cry2 Trp mutation may have caused a non-specific effect on magnetosensing function as a result of protein misfolding. Thus, Cry2 may use a photochemical mechanism common to both type 1 and type 2 Cry, such as the photolyase-like photocycle mentioned above. Alternatively, Cry2 could use a photochemical mechanism that is dependent on the terminal Trp but is independent of Trp-triad-generated radical pairs.

A recent report described a Cry-mediated effect of magnetic fields on circadian period in *Drosophila* studied under constant light²⁴. In the circadian system of *Drosophila*, Cry interacts with the key clock protein Timeless (Tim) in a light-dependent manner, leading to its degradation and subsequent effects on circadian period²⁵. Thus, this reported light-induced magnetosensitive effect of Cry on circadian period is dependent on its interaction with Tim. The behavioural responses to a magnetic field in the present study do not depend on Tim; Tim-deficient *tim⁰* mutant flies showed robust naive and trained responses to the magnetic field in the T maze (Fig. 4).

Our *tim⁰* results also show that light-dependent magnetoreception through type 1 Crys is not mediated through photochemical interactions between Cry and Tim. In addition, other core clock proteins are not likely to be involved in magnetoreception. Indeed, flies deficient in the essential clockwork transcription factor Cycle (homozygous *cyc⁰* mutant flies²⁶) also showed a strong response to the magnetic field (Fig. 4), and we have shown previously that disrupting the molecular clock under constant light conditions does not affect magnetosensitivity in wild-type Canton-S flies¹.

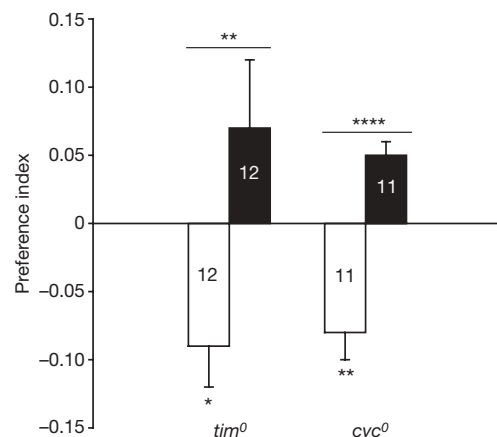


Figure 4 | The clock proteins Tim and Cyc are not required for *Drosophila* magnetoreception. Naive and trained responses to a magnetic field are not impaired in either Tim-deficient *tim⁰* mutant flies or Cyc-deficient *cyc⁰* flies. Bars show preference index values for naive (white) and trained (black) groups. Numbers in bars represent the sizes of groups tested. Values are means \pm s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; four asterisks, $P < 0.0001$.

Our results advance the field of magnetoreception in animals by indicating that both type 1 and type 2 Crys can function in magnetosensitive responses without generating radical pairs through the Trp triad, which is widely thought to be the critical element of a Cry-based magnetoreception mechanism^{6,7,9}. The future challenge will be to understand more precisely how magnetosensitive chemical reactions are actually generated, and to determine how, once generated, they are transduced into neural signals that lead to behavioural responses. A recent study has suggested that light-activated *Drosophila* Cry can directly modulate ion channel activity in fly brain, independently of its light effects on the molecular clock mechanism²⁷. Although more work is needed to address the mechanistic details of this possibility, it is one way in which magnetosensitive Cry of either type could transduce directional information to the brain for behavioural responses.

Our research into animal magnetoreception was initiated on the idea that monarch butterflies, like other long-distance migrants, use several navigational strategies, including magnetoreception²⁸. Indeed, our transgenic results now show that both types of monarch Cry protein have the molecular capacity to transduce magnetic information, and the search is now on for a light-sensitive behavioural correlate of magnetosensitivity in migrating monarchs. This study highlights how the unique biology of monarch butterflies continues to advance our understanding of general biological principles.

METHODS SUMMARY

Fly stocks were raised on standard cornmeal/agar medium at 25 °C and 60% relative humidity under a 12 h light:12 h dark cycle. The *cry^b*, *UAS-dcry* (*UAS-mycdcry*), *tim⁰* and *cyc⁰* lines were a gift from P. Emery. The generation of *UAS-dpcry1* (*UAS-mycdpCry1#15b*) and *UAS-dpcry2* (*UAS-mycdpCry2#125a*) lines were described previously¹⁶. The *tim-GAL4* (*tim-GAL4/CyO⁺*) driver line was used for all *Drosophila* and monarch transgene expression in *cry^b* flies. The apparatus and behavioural assay used to test for magnetosensitivity in flies has been described¹. The apparatus consisted of a choice chamber and an illuminated black box containing the two-coil system connected to an adjustable direct-current power supply. The two-coil system permits the production of a magnetic field on one side while producing no field on the opposite side, and alternation of the field between sides. Coils were positioned at 45° to the horizontal. We adjusted the power supply so that the magnetic field intensity ranged from 0.1 G (10^{-5} T) at the tube entrance to 5 G (5×10^{-4} T) at the end of the tube. For each population of flies tested (100–150 individuals) we calculated a preference index value from the following equation: $(P_m - 0.5) / [(P_m + 0.5) - (P_m)]$, where P_m is the proportion of flies on the magnetic field side of the T maze. To test whether flies responded to the experimental magnetic field, we used either a Student's *t*-test to compare preference index values between trained and naive groups or a one-sample *t*-test to compare preference index values with zero (that is, preference index value expected with no response to the magnetic field).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions All authors contributed to experimental design, execution, data analysis and writing the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.M.R. (steven.reppert@umassmed.edu) or R.J.G. (robert.gegear@umassmed.edu).

METHODS

Fly lines. For generating *UAS-dcryW342F* transgenic lines, the *dcry* open reading frame containing a W342F mutation was amplified by PCR from pAC5.1V5/His-*dcryW342F* and subcloned into the *XhoI* and *XbaI* sites of the pUAST vector. To generate the *UAS-dpcry1W328F* and *UAS-dpcry2W345F* transgenic lines, the tryptophan-to-phenylalanine mutations were introduced into the pUAST-*dpcry1* and pUAST-*dpcry2* constructs, respectively, with the Stratagene QuikChange II XL site-directed mutagenesis kit. The mutated open reading frames were amplified by PCR and subcloned into the *KpnI* and *XbaI* sites of new pUAST vectors to ensure that there were no vector mutations. All constructs contained a full Kozak sequence. All constructs were sequenced before injection into *w¹¹¹⁸* embryos by Genetic Services. During balancing, the *w¹¹¹⁸* X chromosome was replaced with the *y w*-containing chromosome.

Behavioural apparatus. The choice chamber was composed of a one-tube training section, a two-tube choice section (T maze), and an elevator section to transfer flies between the training and T-maze sections. The housing box was constructed such that the choice chamber could be placed between the two coils in either an upright position (used during training) or a horizontal position (used during testing). The box containing the coil system was open on the top so that the chamber, regardless of position, could be illuminated by one ZooMed

Reptisun 10.0 UV-B fluorescent tube (F20T12) and one Agrobrite full-spectrum fluorescent grow tube. The wavelength dependence of magnetosensitivity was examined by either covering the top of the box with a long-wavelength filter that transmitted wavelengths of light above 420 nm (E420; Gentex) or using a General Electric Black Light 20 to transmit wavelengths below 420 nm.

Behavioural procedure. Flies were starved for 18 h before their magnetic response was tested. All experiments were performed between 8:00 and 12:00 Eastern Standard Time. Flies in the trained group were moved into the training section facing one of two adjustable coils with no field for 2 min and were then reloaded into the training tube containing sucrose reinforcement and a magnetic field for an additional 2 min. After being held for 1 min in the elevator section, flies were tested for their magnetic preference by transferring them to the T-maze section and allowing them to choose for 2 min between the sides with and without a magnetic field. Flies in the naive group were loaded into the elevator section and immediately transferred to the T maze and allowed to choose for 2 min between the sides with and without a magnetic field. Trained and naive groups were tested consecutively and with the magnetic field on the same side. As an additional control for side preferences independent of magnetic stimuli, we alternated the side of the T maze containing the field after each consecutive set of trained and naive flies.