Essential elements of radical pair magnetosensitivity in *Drosophila*

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Summary:

Many animals use the Earth's magnetic field (geoMF) for navigation[1]. The favored mechanism for magnetosensitivity involves a blue-light (BL) activated electron transfer reaction between flavin adenine dinucleotide (FAD) and a chain of tryptophan (Trp) residues within the photoreceptor protein, CRYPTOCHROME (CRY). The spin-state of the resultant radical pair (RP) and hence the concentration of CRY in its active state is influenced by the geoMF[2]. The canonical CRY-centric radical pair mechanism (RPM) does not, however, explain many physiological and behavioural observations[2–8]. Here, using electrophysiology and behavioural analyses, we assay magnetic field (MF) responses at single neuron and organismal level. We show that the 52 C-terminal (CT) amino acids of CRY, which are missing the FAD binding domain and the Trp chain, are sufficient to facilitate magnetoreception. We also show that increasing intracellular FAD potentiates both BL-induced and MF-dependent effects on the activity mediated by the CT. Additionally, high levels of FAD alone are sufficient to cause BL neuronal sensitivity and, remarkably, potentiation of this response in the co-presence of a MF. These unexpected results reveal the essential components of a primary magnetoreceptor in flies, providing strong evidence that non-canonical (*i.e.*, non-CRY-dependent) RPs can elicit MF responses in cells.

Introduction

The ability of species to navigate considerable distances has long intrigued the biological community[1]. One of several environmental cues to support these migrations is the geoMF. The identity of the primary magnetoreceptor(s), the mechanism(s) that underlies its reported light dependence and how the magnetic signal is transduced remain unknown[9,10]. A favoured model posits a light-induced electron transfer reaction whereby RPs are formed, the spin-states of which are sensitive to MFs as small as the geoMF (~50 μT)[2]. This so-called RPM canonically requires the flavoprotein CRY, which is best known for its role as a circadian BL-photoreceptor in flies and as a light-insensitive transcriptional regulator in the circadian clock of mammals[2,9].

Absorption of BL by CRY-bound FAD initiates an electron transfer cascade along a conserved chain of Trp residues[2,11–13]. In *Drosophila* this forms a spin-correlated RP comprising the photoreduced FAD (FAD^{o-}) and the terminal oxidised Trp ($TrpH^{o+}$)[14]. The spin-state of the RP is initially polarised as a singlet (S, anti-parallel spins), which then rapidly oscillates between S and the triplet spin states (T, parallel spins). Transiently (*i.e.*, before the system relaxes to equilibrium), this inter-conversion can be sensitive to MF, which in turn can lead to

downstream modifications in the biological activity of CRY, *via* conformational change[2]. In its activated state, the CRY C-terminal 'tail' of ~20 residues (CTT) becomes exposed, allowing interactions with signalling partners including PDZ-domain containing proteins[15–21].

Although there is ample evidence consistent with CRY being both necessary and sufficient for light-dependent magnetosensitivity, there are a number of studies that support exceptions to this mechanism [2–8]. In one of the most striking, Fedele and colleagues used a circadian behavioural assay in *Drosophila* to show that CRY-dependent light and magnetosensitivity could be rescued in CRY-null adult flies *via* expression of the 52 CT residues of CRY fused to GFP (GFP-CT) for stability[3]. Furthermore, CRYΔ, resulting from the deletion of the CTT of CRY, appeared largely insensitive to a MF, although BL sensitivity was maintained[3,4].

The *Drosophila* CRY-CT lacks both a FAD-binding pocket and the Trp residues (W394, W342, W397, W420) necessary for the canonical RPM[2,22–24]. Moreover, mutating these Trp residues, including W420F and W342F, at best attenuates, but does not abolish the magnetic functionality of CRY[3,4,25,26]. These results are inconsistent with current understanding of the RPM and question the identity of the magnetically-sensitive RP in the receptor. Proposed alternatives to a RP between FAD^{o-} and $TrpH^{o+}$ include the formation of a RP between FAD^{o-}/$FADH^{o}$ and O_2^{o-} or another (unknown, Z^{o}) radical. It is a matter of some contention whether these 'unconventional' RPs contribute to magnetoreception or even represent a primary sensor[10,27–29].

Here we report the expression of a new transgene encoding Luc-CT (CRY-CT fused to luciferase), which lacks a FAD-binding pocket and the Trp residues and thus, is unable to support light-induced intramolecular electron transfer. Nevertheless, Luc-CT is sufficient to generate changes in BL and MF-dependent phenotypes in a whole organism circadian behavioural assay and in the electrophysiological activity of a model neuron, the larval aCC motoneuron. We show that the MF-responsiveness of Luc-CT is potentiated by increasing the intracellular concentration of free FAD, to the point where high levels of this flavin alone are capable, in the absence of Luc-CT, to support a MF response. Finally, we confirm by mutational analysis that the integrity of the CTT of CRY correlates with its ability to facilitate sensitivity to a MF. Overall, our results suggest that trafficking of the RP is more important than the specific nature of the RP itself and that the ability to detect MFs by RPs is likely to be a widespread property of cells. It is possible that this logic has been optimised through evolution of molecules such as CRY that function both as a sensor and as a transducer.

Results and Discussion

CRY-CT is sufficient to support magnetosensitivity consistent with a RPM.

To validate our electrophysiological assay we expressed full-length *Drosophila* CRY (DmCry) in the aCC motoneuron; this supported a BL-induced increase in action potential (AP) firing by 1.7-fold and by 2.4-fold, in the co-presence of a MF (BL+MF,100 mT, Fig.1Aii,iii-B, p=0.005, see also Extended Data Fig.1)[4]. Expression of CRY-CT (fused to GFP) in a *cry-null* background supports a MF-induced shortening of circadian period[3]. To eliminate the possibility that GFP might, like CRY, support intramolecular light-induced electron-transfer, we fused CRY-CT to Luciferase (Luc-CT) and maintained the flies in the absence of luciferin substrate. BL lengthened the free-running period of *tim-GAL4 > UAS-Luc-CT; cry^{02}/cry^{02}* flies compared to those in constant darkness (DD) (23.75 v 24.50 h respectively, p=0.019) revealing the BL light-sensitivity of CRY-CT. As with the GFP-CT construct[17], Luc-CT exposure to a MF (300 µT, 3 Hz) was sufficient to shorten the free-running circadian period in the MF exposed but not the sham group (Fig.1Ci, pre-exposure/post-exposure x sham/MF interaction $F_{1,377}$=7.6, p=0.006, Extended Data Fig.2A-D). Remarkably, expression of Luc-CT in aCC supported a BL-induced increase in AP firing (1.4-fold), which was increased further in the co-presence of a MF (Fig.1Cii, 2-fold, p=0.002).

CRY CT is not a RP partner

Although CRY-bound FAD may be dispensable, it is possible that free FAD in proximity could interact by forming a RP with the sole Trp in the CRY CT. This alternative mechanism may explain why mutations of single Trp residues that constitute the Trp-tetrad are not significantly detrimental to CRY dependent magnetoreception[3,4,25,30]. The Trp residue in CRY-CT has not been implicated in the canonical RPM. However, theoretically it is capable of generating a RP with free FAD reminiscent of the interaction between Flavin mononucleotide and the surface Trp of lysozyme[31]. Thus, we substituted this residue (W536) for a less redox-active phenylalanine.

Expression of Luc-CT$_{W(536)F}$ was sufficient to lengthen circadian period in BL *vs.* DD (24.47 v 23.95 h, respectively, p=0.0017) indicating it supports circadian light-responsiveness. A 2-way ANOVA revealed a significant interaction between the pre/ exposure and MF/sham treatment ($F_{1,198}$=5.1 p=0.025, Fig.1Di). Expression of this variant also shortened the free-running circadian period when exposed to a MF (300 µT, 3 Hz) compared to their pre-exposure and to the sham exposed flies (p=0.023, p=0.015, respectively, Fisher LSD test, whilst the stringent Newman-Keuls test narrowly missed significance for both comparisons (p=0.063, p=0.074, Extended Data Fig.2E-F).

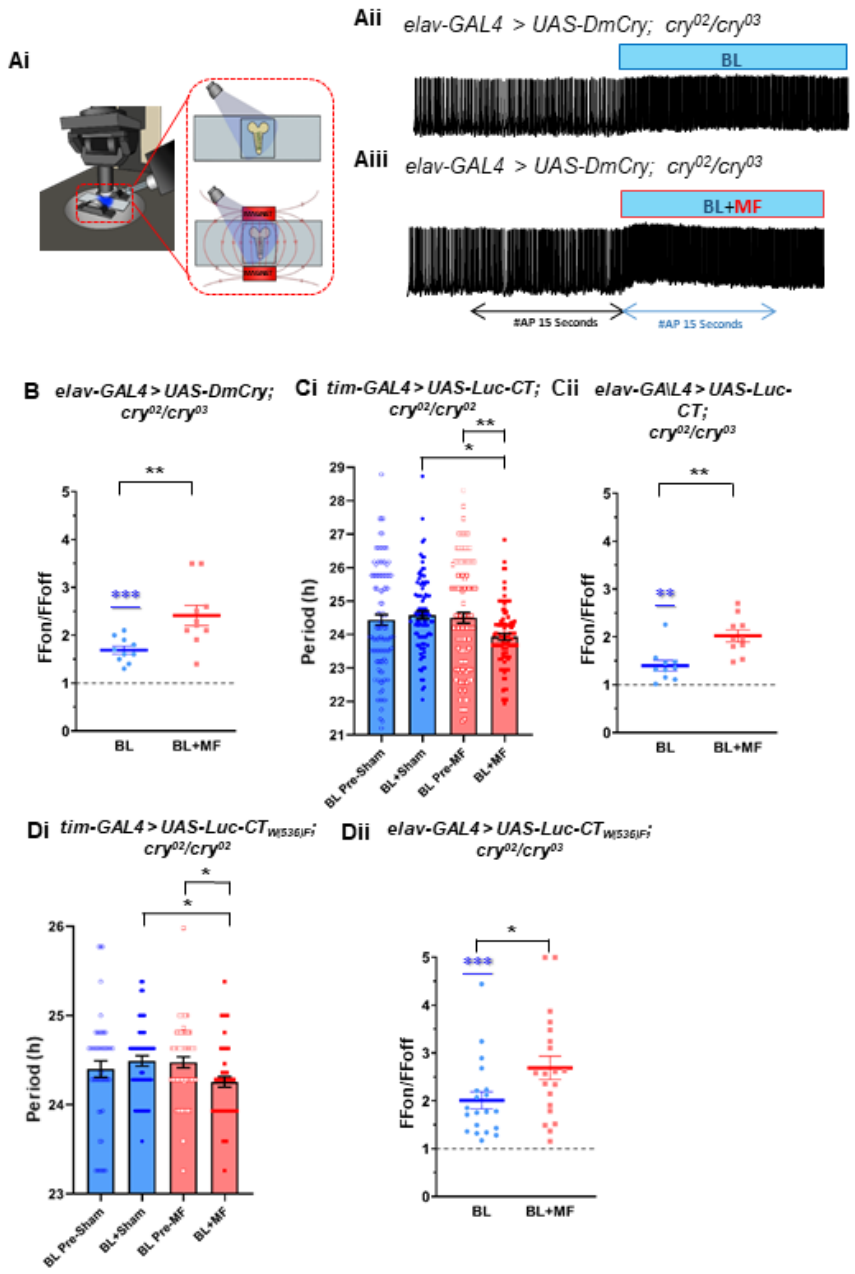

Figure 1. Luc-CT is sufficient to support magnetosensitivity.
(Ai). Electrophysiological setup in presence/absence of 100 mT MF (Aii) BL-exposure of aCC neurons expressing DmCry increase firing. (Aiii) Co-presence of MF (100 mT) potentiates BL effect. Traces represent aCC from different preparations. (B). Relative firing frequency of aCC expressing DmCry. BL increases firing 1.69-fold ($t_{(9)}$=7.72, p=<0.0001, n=10, Firing-Frequency$_{on}$/Firing-Frequency$_{off}$). External MF (BL+MF, 100 mT) potentiates effect to 2.41-fold (BL *vs* BL+MF, $t_{(18)}$=3.2, p=0.005, n=10, Extended Data Fig.1A). (Ci). *tim-GAL4>UAS-Luc-CT; cry^{02}/cry^{02}* show period shortening under MF (3-way ANOVA gives significant Sham/MF x pre/post-exposure interaction ($F_{1,377}$=7.6, p=0.006) with MF-exposed flies showing significantly shorter period than sham exposed. Each of 4 replicates (Replicates, third factor in ANOVA) showed the same period shortening under MF (Extended data Fig.2A-C). (Cii). Luc-CT supports increased aCC firing in BL (1.4-fold, $t_{(9)}$=4.01, p=0.003 n=10, Extended Data Fig.1B) and 2-fold in BL+MF (BL *vs* BL+MF, p=0.002, $t_{(df,18)}$ =3.71, n=10). (Di). Luc-CT$_{W(536)F}$ revealed significant period

shortening under exposure to MF (2-way ANOVA revealed significant pre/post-exposure x MF/sham interaction $F_{1,198}$=5.1 p=0.025, *post-hoc* tests in Extended Data Fig.2E-F). (Dii). aCC expressing Luc-$CT_{W(536)F}$ exhibit 2-fold change in response to BL ($t_{(19)}$=6.06, p=<0.0001, n=20). Response to BL+MF is more variable but significantly greater than BL alone (2.69-fold, 2-way ANOVA, Replicates as factor, p=0.03, $F_{(1,16)}$=5.09, Extended Data Fig.1C). Raw data used to derive electrophysiological fold-change/BL responses are shown in Extended Data Fig.1. Controls are reported in Extended Data Fig.3. For FFon/FFoff data, blue asterisks represent significance for aCC comparing before *vs*. after BL exposure (same cells, paired t-test); black asterisks represent comparisons of BL *vs.* BL+MF (different cells, unpaired t-test). * p=≤0.05, ** p=≤0.01, *** p=≤0.001.

Expression of Luc-$CT_{W(536)F}$ also supported a strong (2-fold) BL response on AP firing in aCC and, again a significant, albeit more variable potentiation in BL+MF (Fig.1Dii, 2.69-fold, p=0.03, 2-way ANOVA replicates as a factor, Extended Data Fig.1C). We conclude that Luc-$CT_{W(536)F}$ reproduces the activity of Luc-CT at the level of both single neuron and whole organism even though the effects appear less robust.

That Luc-$CT_{W(536)F}$ does not obliterate a MF response argues against a significant role for a hypothetical RP between W536 and FAD. Indeed, the weaker MF-response may be structural in origin[32]. Evidence suggests that an arginine (R532) in close proximity could form a cation-π interaction with W536 to stabilise an alpha helical conformation[33] that would be affected by the W536F substitution. Thus, the structure of the CTT seems important to ensure a robust MF response even when it is likely that the RP is not (even in part) carried by the same molecule. Control genotypes for all experiments described above showed no response to either BL or BL+MF (Extended Data Fig.3).

Free FAD supports magnetoreception.

The fact that Luc-$CT_{W(536)F}$ is sufficient to support magnetosensitivity implies that a different, non-CRY, RP is involved. In this regard, it is notable that free FAD is capable of generating a magnetically sensitive RP *via* intramolecular electron transfer[7,34]. To explore this, we supplemented additional FAD to aCC *via* the internal patch saline. Increasing the concentration of FAD (10 to 50 µM, in the patch pipette) potentiates the efficacy of Luc-CT to mediate BL-dependent increases in AP firing (Fig.2A, R^2=0.71, p=0.034), an effect that is enhanced in the presence of BL+MF (100 mT, p=0.015). Significantly, MF potentiation is by a fixed proportion relative to BL at each FAD concentration tested (evidenced by equal gradients of lines of best fit). This is a prediction of the RPM, reflected by these data, in that providing biological saturation is not limiting, the magnetically-induced change should remain constant[35]. In the absence of the Luc-CT construct, FAD (up to 50 µM) induced a weak, but significant, BL response (p=0.03, Fig.2B, Extended Data Fig.4A); however, no potentiating effect was observed in BL+MF.

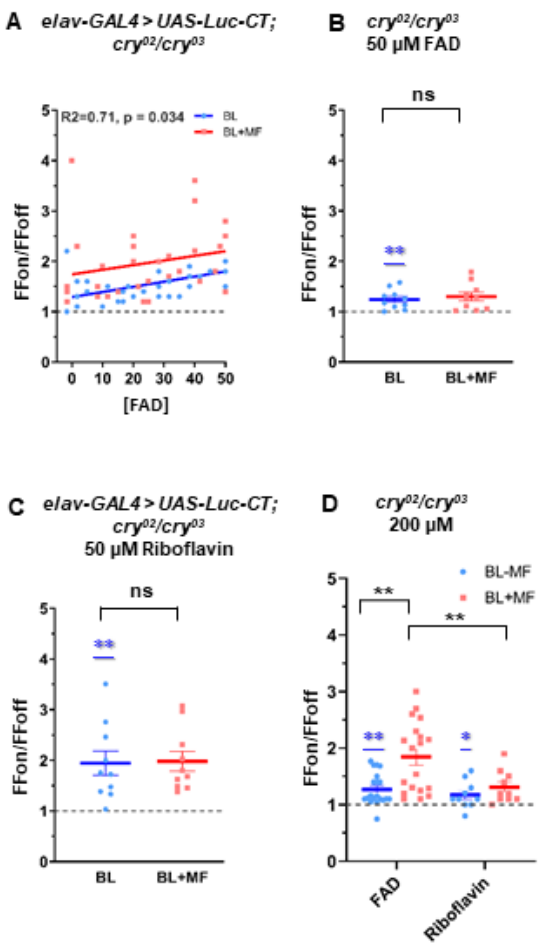

Figure 2. Free FAD potentiates the effect of Luc-CT and at high concentration supports magnetosensitivity alone.
(A). Increasing FAD in aCC (*via* the recording pipette) expressing Luc-CT increases the response to BL, demonstrated by linear regression analysis (R^2=0.71, $F_{(1,4)}$=10.1, p=0.03). The co-presence of a MF (100 mT) further increases the response ($F_{(1,9)}$=9.06, p=0.015) by a consistent amount across concentrations tested. n=5 for all points except BL 30 µM and 50 µM [FAD] for which n=6. (B). Addition of FAD (50 µM) supports BL sensitivity (see also Extended Data Fig.4A), but not magnetosensitivity in the absence of Luc-CT and in a CRY null (p=0.609, $t_{(18)}$=0.521, n=10). (C). Addition of riboflavin (50 µM), supports the response to BL in aCC expressing Luc-CT, but not MF potentiation ($t_{(18)}$=0.12, p=0.91, n=10). (D). Increased FAD (200 µM), in *cry*^{0}, supports BL-induced change in firing (1.27-fold, $t_{(19)}$=4.29, p=0.0004, n=20, Extended Data Fig.4C). In the co-presence of a MF (n=19), the FAD effect on BL is significantly potentiated (1.84-fold, 2-way ANOVA ($F_{(1,55)}$=3.51, p=0.066), Newman-Keuls *post-hoc* p=0.003). Riboflavin (200 µM) shows a similar BL effect (1.17-fold, $t_{(9)}$=2.33, p=0.045, Extended Data Fig.4C) but no MF potentiation (1.31-fold, Newman-Keuls *post-hoc* p=0.67). Raw data can be found in Extended Data Fig.4. Blue asterisks represent significance values for aCC before *vs*. after BL exposure (paired t-test, same cells); black asterisks (or ns = not significant) represent comparisons of BL *vs* BL+MF (unpaired t-test, different cells). ns p=>0.05, * p=≤0.05, ** p=≤0.01, *** p=≤0.001.

FAD autoreduction occurs following electron transfer from the adenine side chain to the photoexcited isoalloxazine, generating an intramolecular RP[7,36]. Although riboflavin contains the same isoalloxazine chromophore and can populate photoexcited triplet states[37], it lacks

an adenine diphosphate side chain (Extended Data Fig.5) and is thus unable to generate the same intramolecular RP[38]. Riboflavin (50 µM), in the presence of Luc-CT, supported a BL effect (~1.94-fold, Fig.2C, Extended Data Fig 4B), but there was no additional increase under BL+MF (100 mT, Fig.2C, p=0.9).

Our results are consistent with an interaction between FAD and Luc-CT, possibly in complex with other, unknown, molecules, which together may facilitate transduction of a magnetic signal. Furthermore, our data suggest that molecules other than CRY are able to generate magnetically-sensitive RPs and produce a biological effect under appropriate conditions. *In vitro* spectroscopy has shown that BL photoexcited FAD generates RPs that are responsive to MFs,[39] and it appears likely that FAD is responsible for MF effects recently observed on cellular autofluorescence[34]. Thus, FAD (but not riboflavin) at higher concentrations may act as a magnetoreceptor. To test this, we recorded from aCC in a *cry* null background, which shows no overall BL or MF response (Extended Data Fig.3). We observed that high levels of FAD in the internal patch saline (200 µM) was sufficient to support a BL-dependent increase in AP firing without need for Luc-CT (Fig.2D, 1.27-fold, Extended Data Fig.4C). Remarkably, this effect was potentiated, in the presence of a MF (100 mT, Fig.2D, 1.84-fold, p=0.003). Cells supplemented with riboflavin (200 µM) showed an increase in APs in response to BL (Fig.2D, Extended Data Fig.4C) but did not show potentiation of the response in a MF (100 mT, p=0.67).

The integrity of the CTT is crucial for magnetic sensitivity.

Activity of Luc-CT$_{W(536)F}$ (Fig.1D) highlights that the integrity of the CTT appears to be important to its role in facilitating magnetosensitivity. The CTT of CRY also contains several linear motifs including putative PDZ-binding sequences[40] (*e.g.*, EEEV 528-531, Fig.3A). PDZ proteins function as modular scaffolds that direct the cellular localisation of signalling molecules such as ion channels (for instance, Shaker K^+) [41,42] and assembly of signalling partners (including CRY) into a 'signalplex' of the photo-transduction cascade in the *Drosophila* eye[20,21]. To explore the importance of CTT structure and, specifically, to determine whether the putative PDZ-binding motif at residues 528-531 regulates magnetosensitivity, we mutated valine to lysine in position 531 (V531K)[40] in full-length DmCry. Pan-circadian expression (*i.e.*, using the *tim-GAL4* driver) of DmCry$_{V(531)K}$ in a *cry^{02}* background retained a circadian light sensitivity with a slight period shortening (Fig.3B, DD=24.51 *vs* BL=24.20 h, p=0.005, Grubbs outlier test excluded a single very weakly rhythmic short period (20.3 h) fly in DD, see Methods), but failed to support a whole-organism behavioural response to MF (Fig.3B, $F_{1,55}$=0.932, p=0.76, Extended Data Fig.6A). Expression of DmCry$_{V(531)K}$ in aCC showed the expected effect of BL

on AP firing (Fig.3C, 1.76-fold, Extended Data Fig.6B). However, as in the circadian assay, this variant was unable to support magnetosensitivity (100 mT, Fig.3C, p=0.77). The loss of a MF effect, but retention of a BL response for $DmCry_{V(531)K}$ is reminiscent of the CRYΔ mutant[3,4], which lacks the CTT entirely. These results confirm the CTT as a probable mediator of the MF response, where it likely serves to facilitate formation of protein complexes that transduce a magnetic signal.

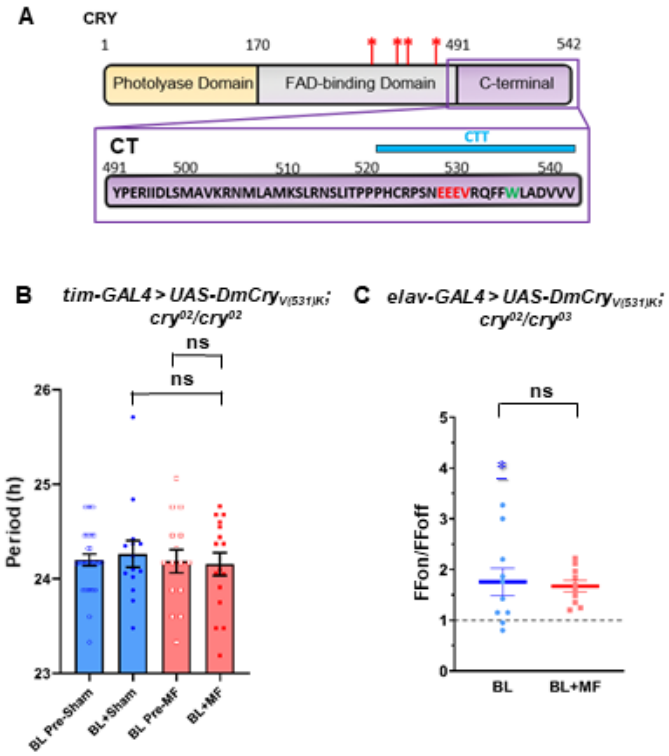

Figure 3. Integrity of the CTT is required for magnetosensitivity.
(A). Cartoon of the domain structure of CRY including the CT (aa 491-542) and CTT (aa 521-542). The four Trp residues, essential for the canonical RPM, are indicated by red asterisks. A putative PDZ binding site (EEEV 528-531) was mutated (V531) $CRY_{V(531K)}$. The terminal Trp (W536) mutated in Luc-$CT_{(W536F)}$ is shown in green. (B). $CRY_{V(531)K}$ expressed in clock neurons (*tim-Gal4*) does not support magnetosensitivity in the circadian assay. 2-way ANOVA revealed no significant main, or interaction effects (interaction, $F_{1,52}$=0.09, p=0.77, Extended Data Fig6.A). (C). Expression of $CRY_{V(531)K}$ in aCC neurons is sufficient to support a response to BL (p=0.017, $t_{(9)}$=2.934, n=10) but not a potentiation in BL+MF (100 mT, p=0.768, $t_{(18)}$=0.299, n=10). Raw data are shown in Extended Data Fig.6. Blue asterisks represent significance values for aCC before *vs*. after BL exposure (paired t-test, same cells); ns (not significant) represent comparisons of BL *vs* BL+MF (unpaired t-test, different cells). ns p=>0.05, * p=≤0.05, ** p=≤0.01, *** p=≤0.001.

Conclusions.

We have observed that contrary to several reports[2,13], but not others[3], full length CRY may be sufficient, but is not strictly necessary, to mediate *Drosophila* magnetosensitivity. The

expression of the C-terminal 52 residues of CRY is sufficient to support magnetosensitivity in both single neuron and whole animal assays. Despite being different measures, it is notable that a 300 µT MF results in a *decrease* in circadian period whereas a 100 mT MF results in an *increase* in AP firing. The RPM mechanism predicts biphasic responses to µT MF and mT MF that are opposite in this way[35]. Thus, whilst our data challenge the canonical CRY-dependent RPM model of animal magnetoreception, they are nonetheless consistent with a RPM. Although animals are not likely to sense a 100 mT MF in nature, in the context of MF effects on RP reactions, mechanistic insight such as this is only possible when working in both µT MF and mT MF regimes.

Luc-CT neither binds the FAD cofactor directly nor does it contain the chain of Trp residues considered essential for the canonical CRY-based RPM. However, the Luc-CT response is potentiated by increasing the cytosolic availability of FAD, a common biological redox cofactor, confirming that redox reactions are at the core of magnetosensitivity[43]. The finding that alternative RPs can transduce physiological MF effects also suggests that other, non-photochemical RPs, may contribute to magnetoreception, which is consistent with a growing list of examples reporting RP mediated magnetoreception in darkness[29,44–46]. The synergistic interaction between Luc-CT and free FAD suggests the former facilitates formation of a complex that enables the transduction of a magnetic signal by the latter. However, given that free FAD itself can mediate a magnetic response *in vivo,* we suggest that CRY has evolved to bring the RP and effectors in close proximity in order to potentiate the comparatively weak activity of the geoMF. In this regard CRY may not be the primary magnetoreceptor. This view is in contrast to a recent *in vitro* spectroscopic study that suggested that full length CRY itself is the magnetoreceptor[47]. By contrast, our observations suggest an ancient and ubiquitous effect of MFs on biological RPs that evolution has optimised, through CRY and partners, to function as a magnetoreceptor.

This paper is supported by an extended data file

Materials and Methods:

Fly stocks

For larval recordings, embryos were raised at 25°C in a 12:12 light / dark cycle until 3rd instar wall climbing larvae (L3) emerged, these were then kept in darkness through the day of recording to minimise light dependent CRY degradation. Recordings were conducted between circadian time hours: 2-10. Flies were maintained on standard corn meal medium at 25°C. The driver line *elav*^{C155}*-GAL4*; ; *cry*^{03} was obtained from Bloomington Stock centre (#BL458) and crossed into a *cry*^{0} background as described[48]. *cry*^{0} flies were obtained from Bloomington Stock centre (#BL86267). *UAS-cry; cry*^{02} is already described[3,49].

Molecular Cloning of Luc-CT

Luciferase CDS was cloned from the *UAS-Luc-CRY* fly line[17] and subsequently amplified with the following primers to include overhangs compatible for the NEB Gibson Assembly assay
F: *TATCCTTTACTTCAGGCGGCCGCATGGAAGACGCCAAAAACATAAAGAAAGG;*
R: *TCCGGATACTCGAGCACGGCGATCTTTCCGCCC.*
The CT portion of *cry* was produced by gene synthesis (GeneArt, ThermoScientific) based on the original *GFP-CT* construct[3]. *CT* was designed to include 5' and 3' overhangs compatible with subsequent NEB Gibson Assembly assay (Seq:*AAAGATCGCCGTGCTCGAGTATCCGGAGCGGATCATTGATTTGTCCATGGCCGTGAAGCGCAACATGCTGGCCATGAAGTCCCTGCGCAACAGCCTGATCACCCCCCCACCACATTGCCGCCCCAGCAATGAGGAGGAAGTGCGCCAGTTCTTCTGGCTGGCCGATGTGGTGGTGTAATCTAGAGGATCTTTGTGAAGGA*).
pJFRC2-10XUAS-IVS-mCD8::GFP (Addgene #26214) plasmid was digested with *NotI* and *XbaI.* A Gibson Assembly assay was performed to ligate pJFRC, *Luc* and *CT* in a single step reaction. A Myc tag was produced by annealing single Oligos designed using The Oligator software (11avidson.edu) and ligated 5' of Luc-CT. Briefly, 5 µM of Oligos
<u>47-mer Top1</u>
5'-GATCTCACAATGGAACAGAAGCTGATCTCCGAGGAGGACCTGGGCGC
<u>47-mer Bottom1</u>
5' GGCCGCGCCCAGGTCCTCCTCGGAGATCAGCTTCTGTTCCATTGTGA
Resulting in 5' *BglII* and 3' *NotI* over-hangs once annealed, were diluted in 1X annealing buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 7.4), boiled in 500 µl of H_2O for 10 minutes and left overnight to cool down to room temperature to hybridise. The pJFRC2-10XUAS-Luc-CT plasmid was then digested with *BglII* and *NotI.* The fragment encoding the Myc tag was then ligated using

standard methods. After sequence validation, the plasmid was injected into the *y w, M(eGFP, vas-int, dmRFP)ZH-2A; P{CaryP}attP40* (Stock 13-40, Cambridge University Fly facility) using the Phi31 integrase system for insertion. The resulting transformants were subsequently backcrossed into the w^{1118} background for 7 generations.

The Luc-$CT_{W(536)F}$ transgene was generated by gene synthesis (Eurofins, Ebersberg, Germany) and sub-cloned into pJFRC-MUH via 5' *NotI* and 3' *XbaI* restriction sites. Transgenic injections for Luc-$CT_{W(536)F}$ were carried out by Manchester University Fly Facility using the same *y w M(eGFP, vas-int, dmRFP)ZH-2A; P{CaryP}attP40* line (Stock 13-40, University of Cambridge Fly Facility).

HA-cry$_{(V531K)}$ (containing a HA tag at the N-terminal of the encoded protein) was already available as a clone into the yeast plasmid pEG202[40]. It was released by *EcoRI-XhoI* digestion and subcloned into pUAST[50]. Transgenics were produced by P-element transformation by the University of Cambridge Fly Facility using the line S(6)1 inserted on chromosome 2.

Electrophysiology

The experimenter was blinded to genotype during both recordings and subsequent data analysis. L3 larvae were dissected under extracellular saline as described[51] with the only modification being a red filter applied to both the dissecting light and compound microscope to minimise CRY degradation prior to, and during, recordings. Thick-walled borosilicate glass electrodes (GC100F-10; Harvard Apparatus, UK) were fire polished to resistances of 10 -15 MΩ. Recordings were made using a Multiclamp 700B amplifier controlled by pCLAMP (version 10.4) *via* a Digidata 1440A analog-to-digital converter (Molecular Devices, CA, USA) and only cells with an input resistance of ≥500 MΩ were used. Traces were filtered at 10 kHz and sampled at 20 kHz. The extracellular saline solution contained the following (in mM): 135 NaCl, 5 KCl, 4 $MgCl_2.6H_2O$, 2 $CaCl_2.2H_2O$, 5 TES, and 36 sucrose, pH 7.15. The intracellular patch solution contained the following (in mM): 140 K-D-gluconate, 2 $MgCl_2.6H_2O$, 2 EGTA, 5 KCl, and 20 HEPES, pH 7.4. KCl and $CaCl_2$ were from Fisher Scientific (UK); sucrose was from BDH (UK); all remaining chemicals were from Sigma-Aldrich (UK). Mecamylamine (1 mM) was applied to all preparations to isolate the aCC motoneurons from excitatory cholinergic synaptic input. For recordings supplemented with additional FAD or riboflavin (Sigma-Aldrich, UK) dilutions were made up in intracellular saline and kept in the dark.

Photoactivation and Magnetic Field application

Light stimulation was supplied by a blue LED (470 nm, Cairn Research, UK) at a power of ~2.2 mW/cm^2, a value used previously to stimulate CRY[52]. Each cell was injected with a variable amount of constant current until threshold potential was reached and the neuron was allowed to settle until AP firing was ~5-7 Hz. Once a stable firing rate was achieved, each neuron was recorded for at least 20 s before exposure to BL illumination for 30 s. Magnetic exposure was provided by two NeFeB static magnets mounted around the preparation at a distance that provided a MF of 100 (± 5) mT. Field strength was measured using a 5180 Gauss/Tesla Meter (F.W. Bell. USA). This method is essentially identical to that used previously[4].

Statistical analysis of electrophysiological recordings

A D'Agostino & Pearson analysis showed our data to be normally distributed, thus parametric tests were applied in all cases. Data are shown as mean ± SEM. To determine BL sensitivity a paired t-test (two-tailed) was used to compare the number of APs a neuron fired in the 15 s following light stimulation to the number of APs in the preceding 15 s before light exposure. For comparison between BL and BL+MF the number of APs in the 15 s preceding and proceeding BL or BL+MF exposure was used to determine the firing-fold change (FF_{on}/FF_{off}) for each cell. Statistical significance for MF potentiation against BL effect alone was determined using an unpaired t-test (two-tailed) to compare the firing-fold change for the BL population to the BL+MF population. Where multiple genotypes/conditions were tested simultaneously, a 2-way ANOVA with Newman-Keuls *post-hoc* testing was used. For the FAD dose response curve, a BL effect of [FAD] was determined by linear regression fitting and significance determined using an ANCOVA model. Average MF potentiation of the Luc-CT FAD dose response (Fig.2A) was determined based on the intercept of the Y axis. Unpaired t-tests (two-tailed) were also applied in the Extended Data figures to compare the number of APs in the 'before' BL±MF conditions, as well as to BL and BL+MF exposures. Control lines were also compared to their respective experimental genotype by both 1-way ANOVA (with BL and BL+MF recordings separated) and by 2-way ANOVA. Raw data are reported in the Extended Data.

Behavioural analyses and statistics

Circadian locomotor activity was recorded using a *Drosophila* Trikinetics Monitor 2 (Waltham, MA, USA)[3]. To test the effects of MF on the free-running circadian period of locomotor activity, we used a modified version of the Schuderer apparatus, which consists of two independent

double wrapped coils placed inside two μ–metal boxes within a commercial incubator. The shielded, four quadratic Helmholtz coil systems produce a homogenous, linearly polarized *B* field with perpendicular orientation to the horizontal plane of the Trikinetics monitors. Each coil is formed with a pair of wires, with the current passing in the same direction through both wires for MF exposure but in opposite directions to provide a sham exposure condition. A computer randomly assigns the MF and Sham exposed chambers and the experiment is performed blind from the experimenter[17].

One to three day old flies were first entrained at 20 ^{o}C in the apparatus under a dim BL: darkness 12 h cycle (BL: DD = 12:12) for three full days, before being pre-exposed to continuous BL for 7 days, followed by exposure to BL+MF or BL+Sham for a further 7 days[3]. Thus, there were 4 measurements, the pre-exposure (BL) period of flies that were subjected to a MF or sham, plus the exposure period for both (BL+MF and BL+Sham). A fifth control condition examined the period of Luc-CT*; cry^{02}* in DD without exposure. All experiments were performed using a low frequency 3 Hz field at 300 μT and dim BL at 0.15 - 0.25 μW/cm^2, wavelength 450 nm, 40 nm broad range (RS Components, UK).

Rhythmicity and period were determined using spectral analysis employing a MatLab-based version of the BeFly program[53]. Statistical analysis of period was performed using ANOVA with either Statistica (Statsoft, CA, USA) for factorial analyses or Prism (Graphpad) for 1-way ANOVA. Although there was a clear prediction that Luc-CT flies would have a shorter period under a MF[3] we nevertheless used the stringent Newman-Keuls *post-hoc* test to compare groups after factorial ANOVA buttressed by the more liberal Fisher LSD test for the circadian Luc-CT$_{W(536)F}$ results. To compare the DD periods with those from the BL pre-exposure conditions we used an unpaired t-test. Circadian data were first tested using a Grubbs outlier test (GraphPad Prism, alpha =0.01 two-sided, Z=5.3). One datum from the DD data of CRY$_{V(531)K}$ which represented the least robust single period in the dataset with an anomalous period of 20.3 h (8 sd away from the mean) was identified and removed.

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