Many animals are able to sense the Earth’s geomagnetic field to enable behaviors such as migration. It is proposed that the magnitude and direction of the geomagnetic field modulates the activity of cryptochrome (CRY) by influencing photochemical radical pair intermediates within the protein. However, this proposal will remain theoretical until a CRY-dependent effect on a receptor neuron is shown to be modified by an external magnetic field (MF). It is established that blue-light (BL) photoactivation of CRY is sufficient to depolarize and activate Drosophila neurons. Here, we show that this CRY-dependent effect is significantly potentiated in the presence of an applied MF (100 mT). We use electrophysiological recordings from larval identified motoneurons, in which CRY is ectopically expressed, to show that BL-dependent depolarization of membrane potential and increased input resistance are markedly potentiated by an MF. Analysis of membrane excitability shows that these effects of MF exposure evoke increased action potential firing. Almost nothing is known about the mechanism by which a magnetically induced change in CRY activity might produce a behavioral response. We further report that specific structural changes to the protein alter the impact of the MF in ways that are strikingly similar to those from recent behavioral studies into the magnetic sense of Drosophila. These observations provide the first direct experimental evidence to support the hypothesis that MF modulation of CRY activity is capable of influencing neuron activity to allow animal magnetoreception.

Key words: action potential; cryptochrome; depolarization; Drosophila; magnetic field; radical pair

Significance Statement

The biophysical mechanism of animal magnetoreception is still unclear. The photoreceptor protein cryptochrome has risen to prominence as a candidate magnetoreceptor molecule based on multiple reports derived from behavioral studies. However, the role of cryptochrome as a magnetoreceptor remains controversial primarily because of a lack of direct experimental evidence linking magnetic field (MF) exposure to a change in neuronal activity. Here, we show that exposure to an MF (100 mT) is sufficient to potentiate the ability of light-activated cryptochrome to increase neuronal action potential firing. Our results provide critical missing evidence to show that the activity of cryptochrome is sensitive to an external MF that is capable of modifying animal behavior.
exposed and on the presence of a circadian photoreceptor protein, cryptochrome (CRY) (Gegeear et al., 2008). CRYs are widespread throughout nature and, in animals (including migratory birds), have well described circadian roles as either photoreceptors or light-independent transcriptional regulators (Ceriani et al., 1999; Griffin et al., 1999). CRYs with known photoreceptor function contain a noncovalently bound, blue-light (BL) chromophore called flavin adenine dinucleotide (FAD) (Zolowski et al., 2011; Levy et al., 2013) and are putative magnetoreceptors (Dodson et al., 2013). CRY-dependent magnetoreception is currently proposed to be a result of light-initiated electron transfer chemistry in the protein, which might be magnetically sensitive by virtue of the radical pair mechanism (RPM) (Rodgers and Hore, 2009; Dodson et al., 2013; Hore and Mouritsen, 2016; Jones, 2016). Indeed, magnetically sensitive radical pair reaction dynamics have been observed in the photoresponse of isolated CRYs from Arabidopsis (Maeda et al., 2012; Kattig et al., 2016) and extensive theoretical work indicates that CRY photochemistry has a number of properties ideal for a biological magnetic sensor (Dodson et al., 2013). However, there is as yet no direct in vivo evidence that CRY is a magnetoreceptor and the possible identity of the magnetically sensitive radical pair in CRY is currently a matter of considerable debate (Hogben et al., 2009; Solov’yov and Schulten, 2009; Nießner et al., 2014; Wiltschko et al., 2016).

To produce a magnetically induced behavioral response, it is expected that CRY activity should be altered by the presence of a magnetic field (MF) that, in turn, will be transduced to a change in neuronal activity (magnetic field (MF) that, in turn, will be transduced to a change in neuronal activity) (Kattnig et al., 2016; Fedele et al., 2014b). Magnetic field exposure on the circadian period (see Materials and Methods) is sufficient to ensure adequate Drosophila CRY photostimulation (Fogle et al., 2011). Each cell was recorded both in darkness and under light exposure. These experiments were repeated in the presence of an MF from a pair of NeFeB permanent magnets placed at a distance of 1.5 cm from the sample, where the MF was measured to be 100 ± 6 mT. For electrophysiological investigations, a larval CNS was positioned at the center between the magnets with the same anterior–posterior orientation, which was aligned parallel to the magnet separation axis. Membrane potential changes in response to CRY photoactivation were calculated as ∆Vm = (Vm + BL) − (Vm − BL). Resting membrane potential (RMP) was assessed for each cell in zero current-clamp mode before any stimulation. Input resistance was measured from the steady-state responses to a series of hyperpolarizing and subthreshold depolarizing current steps (4 pA/1 s long) as the slope of a linear fit to the resulting voltage–current relationship (from −12 pA to +8 pA). The same stimulation protocol was repeated twice, before and after constant BL exposure. To confirm the involvement of potassium currents in this process (Fogle et al., 2011), a set of recordings was repeated in the presence of potassium channel inhibitors (50 mM tetrodotoxin, 10 mM 4-aminoypyridine, and 5 mM cesium chloride).

Membrane excitability was determined as the number of action potentials fired by a series of rectangular depolarizing current pulses (4 pA steps, from 0 to 60 pA/s long). The mean number of evoked action potentials elicited by incremental current injections was counted for each event. Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism version 6. Sample size (n) is reported in each bar. Statistical significance was determined between pairwise comparisons only (e.g., ≥2 MF or ±BL) using Student’s paired or unpaired t tests, as appropriate. Results from different genotypes are shown together in figures to conserve space. To assess membrane excitability, the input–output relationship was evaluated by linear fitting using GraphPad Prism version 6 and analyzed for significance using an ANCOVA model. Significance levels were set at p < 0.05.

Results

To determine whether exposure to an MF can influence firing activity of Drosophila neurons, we mounted a pair of NeFeB permanent magnets around the electrophysiological preparation (see Materials and Methods). We used the same MF intensity, 100 mT, as used previously for our experiments performed on embryonic and neonatal culture (Marley et al., 2014). An MF of this magnitude will result in a magnetic flux density (MF) of approximately 100 mT, which is on the order of typical Earth’s magnetic field. The MF is oriented parallel to the stack center, which is expected to ensure adequate Drosophila CRY photostimulation (Fogle et al., 2011). Each cell was recorded both in darkness and under light exposure. These experiments were repeated in the presence of an MF from a pair of NeFeB permanent magnets placed at a distance of 1.5 cm from the sample, where the MF was measured to be 100 ± 6 mT. For electrophysiological investigations, a larval CNS was positioned at the center between the magnets with the same anterior–posterior orientation, which was aligned parallel to the magnet separation axis. Membrane potential changes in response to CRY photoactivation were calculated as ∆Vm = (Vm + BL) − (Vm − BL). Resting membrane potential (RMP) was assessed for each cell in zero current-clamp mode before any stimulation. Input resistance was measured from the steady-state responses to a series of hyperpolarizing and subthreshold depolarizing current steps (4 pA/1 s long) as the slope of a linear fit to the resulting voltage–current relationship (from −12 pA to +8 pA). The same stimulation protocol was repeated twice, before and after constant BL exposure. To confirm the involvement of potassium currents in this process (Fogle et al., 2011), a set of recordings was repeated in the presence of potassium channel inhibitors (50 mM tetrodotoxin, 10 mM 4-aminoypyridine, and 5 mM cesium chloride).

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Magnetic Field Exposure Affects Neuronal Firing

Figure 1. MF exposure potentiates the BL-evoked depolarization of the membrane potential. A, Representative electrophysiological recordings from L3 aCC/RP2 ectopically expressing wild-type cry. Reversible changes in \( V_m \) were observed after BL exposure in presence or absence of a 100 mT MF. No detectable change in \( V_m \) was exhibited by the control group (Gal4 ElaVC155). B, Quantitative analysis of MF effect on the BL-induced \( V_m \) changes to control cells and cells expressing CRY. C, No difference in RMP, which was measured before light stimulation, was observed in any experimental group. All values shown are means ± SEM and n is shown in each bar. *\( p < 0.01 \).

MFs potentiate the CRY-induced increase in membrane potential

CRY mediates a light-evoked increase in membrane potential in large lateral ventral neurons (ILNv’s) in the adult Drosophila brain and also when ectopically expressed in nonclock neurons (Fogle et al., 2011). Similarly, we found that ectopic expression of CRY in larval aCC/RP2 motoneurons is similarly sufficient to produce an appreciable and reversible depolarization in membrane potential on exposure to BL (470 ± 40 nm; \( \Delta V_m = 0.193 ± 0.028 \) mV, \( n = 13 \); Fig. 1A, B). The effect of BL is CRY specific and no effect was observed in wild-type animals (parental line ElaVC155), which instead showed a slight, but steady, hyperpolarization of the membrane potential (\( \Delta V_m = -0.72 ± 0.31 \) mV, \( n = 5 \); Fig. 1A, B), which occurs independently from light stimulation (we frequently observed a slow hyperpolarization in wild-type recordings). Our recording saline contained the cholinergic blocker mecamylamine, which removes all excitatory synaptic drive to motoneurons (Baines et al., 1999). Therefore, we discount the possibility that CRY-dependent effects observed in aCC/RP2 are due to change in activity of presynaptic neurons.

Significantly, repeating the BL illumination in the presence of a static MF potentiated the effect of CRY to produce almost double the level of depolarization (\( \Delta V_m = 0.47 ± 0.03 \) mV, \( n = 11 \); Fig. 1A, B). An unpaired \( t \) test revealed a significant difference between the −MF and +MF groups (\( p = 0.0056 \); Fig. 1B). No MF-related effect was observed in the control group not expressing CRY (ElaVC155; −MF = −0.72 ± 0.31 mV, \( n = 5 \), vs +MF = −0.45 ± 0.24 mV, \( n = 6 \)). The mean cell capacitance of motoneurons (a measure of cell size) in −MF and +MF was not statistically different (12.73 ± 0.78 vs 12.90 ± 0.80 pF, −MF vs +MF, respectively).

Oxidized CRY, which action spectra suggest is the resting state in vivo (Ahmad et al., 2002), does not significantly absorb wavelengths of light longer than ~530 nm (VanVickle-Chavez and Van Gelder, 2007). Therefore, as a further control, we tested the effect of MF exposure on membrane potential after exposure to OL (590 ± 18 nm). We observed no detectable light or light+MF-induced changes (+OL, −MF = −0.35 ± 0.15 mV, \( n = 10 \), vs +OL+MF = −0.55 ± 0.33 mV, \( n = 10 \), \( p = 0.96 \)), obtaining values similar to those measured in the control group (ElaVC155). We showed previously that exposure to OL does not
MF exposure potentiates the BL-evoked increase in input resistance. A, Series of current steps (4 pA/1 s) ranging from -12 to +8 pA applied before (gray traces) and during (black traces) BL exposure in the absence (-MF) and presence (+MF) of a 100 mT MF. B, C, Effect of BL exposure on input resistance in the absence or presence of the MF.

MF exposure potentiates CRY-induced increase in input resistance

The input resistance of a neuron reflects the extent to which membrane channels are open. Therefore, we investigated how this intrinsic membrane property is influenced by both CRY photoactivation and the co-presence of an MF. CRY-expressing aCC/RP2 motoneurons were tested before and during BL exposure by applying a series of hyperpolarizing and subthreshold depolarizing current steps (4 pA/1 s long; Fig. 2A). In the absence of an MF, CRY photoactivation induces a significant increase in input resistance from 1.10 ± 0.18 to 1.20 ± 0.20 GΩ (p = 0.03, paired t test, n = 12; Fig. 2B). Similarly, an increase from 1.08 ± 0.18 to 1.52 ± 0.35 GΩ (p = 0.04, paired t test, n = 10; Fig. 2C) was measured in the presence of an MF. Comparing the two datasets, we found that the BL-induced change in input resistance is significantly potentiated by the co-presence of an MF (ΔRes_input = 0.09 ± 0.59 vs 0.59 ± 0.18 GΩ, -MF vs +MF, p = 0.0097, unpaired t test).

Previous work shows that light-evoked and CRY-mediated membrane depolarization in ILNv’s is dependent on inactivation (i.e., closure) of Hyperkinetic (HK), a β-subunit of voltage-gated potassium channels (Shaker and Ether-a-go-go) that contains a conserved redox sensor. The presence of potassium channel inhibitors (tetraethylammonium, 4-aminoypyridine, and cesium chloride) is sufficient to block this effect of CRY in ILNv’s (Fogle et al., 2015). To determine whether CRY acts via a similar mechanism in aCC/RP2, we repeated our experiments in the presence of these blockers. BL activation of CRY produced no change to membrane potential in the presence of blockers (Δt = 0.11 ± 0.37 mV, n = 6), supporting the hypothesis that CRY-mediated potassium channel inactivation underlies increased membrane depolarization in all neuron types in which it is expressed.

MFs potentiate the CRY-induced increase in membrane excitability

CRY increases the spontaneous action potential firing rate in response to BL in Drosophila circadian neurons (Sheeba et al., 2008; Fogle et al., 2011). An increased hyperactivity of neuronal circuitry is also consistent with an increased seizure phenotype in larvae derived from MF coupled to BL exposure (Marley et al., 2014; Giachello and Baines, 2015). Excitatory synaptic inputs to Drosophila motoneurons show considerable variability, making determination of effect to spontaneous firing more challenging. To overcome this, we injected above-threshold depolarizing current pulses (4 pA steps, 0–60 pA/1 s long; Fig. 3) in aCC/RP2 and counted the number of action potentials fired (i.e., a standard input–output determination). This was determined in the presence of mecamylamine to block the endogenous synaptic excitation of these neurons. An ANCOVA revealed significant differences in the regression coefficients (slopes) of the input–output curves measured during BL exposure in the absence and presence of an MF (+BL, -MF vs +BL, +MF; F[1,274] = 26.51, p < 0.0001; Fig. 3A). Conversely, no detectable effect of MF was observed comparing the -MF and +MF groups in darkness (-BL, -MF vs -BL, +MF; p > 0.99, data not shown). These results suggest that exposure to a moderate MF is sufficient to potentiate the effect of CRY photoactivation in increasing neuron action potential firing rate.

Trp triad is not required for CRY-dependent sensitivity to an MF

The precise mechanism of magnetoreception by CRY remains unknown. A prevailing view implicates a triad of tryptophan residues to facilitate a RPM that is sensitive to external MFs. To determine whether a Trp triad is required for the potentiation of CRY activity in motoneurons, we expressed the CRY_W342F variant, which replaces a conserved tryptophan (W342) with a redox inactive phenylalanine, thus disrupting the Trp triad responsible for donating electrons to the FAD cascade during light activation (Kao et al., 2008; Immeln et al., 2015). To determine whether CRY acts via a similar mechanism in aCC/RP2, we repeated our experiments in the presence of these blockers. BL activation of CRY produced no change to membrane potential in the presence of blockers (Δt = 0.11 ± 0.37 mV, n = 6), supporting the hypothesis that CRY-mediated potassium channel inactivation underlies increased membrane depolarization in all neuron types in which it is expressed.
al., 2012). The W342 radical cation is proposed to be a member of the magnetically sensitive radical pair in CRY (Maeda et al., 2012). Expression of CRY<sup>W342F</sup> in aCC/RP2 failed to produce a detectable change in membrane potential when exposed to BL. However, in the presence of an MF, BL was able to evoke a significant depolarization (+BL<sub>- MF</sub> = Δ<sub>-0.01 ± 0.29 mV</sub>, <i>n</i> = 10, vs +BL<sub>+ MF</sub> = Δ<sub>1.45 ± 0.37 mV</sub>, <i>p</i> = 0.006, <i>n</i> = 10; Fig. 4A, B), suggesting that a single mutation to the Trp triad is sufficient to alter the response of CRY to BL, but does not prevent activation in the co-presence of an MF.

Previous studies of circadian behavior have similarly shown a marked reduction in the response of cry-null adult flies expressing CRY<sup>W342F</sup> to BL, whereas, paradoxically, sensitivity to MF exposure is maintained (Gegear et al., 2010; Fedele et al., 2014b). No changes in RMP were observed between the −MF and +MF conditions in the absence of light exposure (Fig. 4C).

**C-terminal is essential for CRY-dependent sensitivity to an MF**

Because disruption to the Trp triad did not affect CRY sensitivity to an MF, we examined additional regions of the protein. Dele-
tion of residues 521–540 from the C-terminal (CRY\,514\,6) confers a constitutively active status to CRY (Dissel et al., 2004), which can be further activated by BL (Dissel et al., 2004; Fedele et al., 2014b). The effect of MF observed on the circadian period of wild-type flies, however, is not evident in those expressing CRY\,514\,6, whereas an effect of MF is retained on locomotor hyperactivity (Fedele et al., 2014b). This suggests that the C terminus is required for some aspects of CRY signaling, but not others. Expression of CRY\,514\,6 in aCC/RP2 neurons showed a marked depolarization of membrane potential consequent to BL exposure, which is slightly increased compared with expression of wild-type CRY (+ BL, + MF = Δ2.63 ± 0.35 mV, n = 10). Strikingly, the same exposure to BL in the presence of an MF produced no potentiation of the effect on membrane potential (+ BL, + MF = Δ2.29 ± 0.35 mV, n = 10, p = 0.50; Fig. 4 A, B).

Expression of CRY\,514\,6 results in a modest, but not statistically significant, depolarization of basal membrane potential compared with controls (Ela\,V\,C155) in the absence of BL exposure (Fig. 4C), which is consistent with a constitutively active state. Therefore, residues within the C-terminal of CRY are required for neuronal sensitivity to an MF, which mirrors the requirement of this region for magnetic sensitivity, negative geotaxis (Fedele et al., 2014a), and circadian period (Fedele et al., 2014b).

Discussion

The precise biophysical origin of animal magnetosensitivity remains unclear. Among the proposed primary magnetoreceptors is the flavoprotein CRY, which is thought to provide geomagnetic information via a quantum effect in a light-initiated radical pair reaction. However, the involvement of this mechanism remains hypothetical until the physical process in a magnetoreceptor is shown to result directly in a physiological response, like an electrical response in a receptor cell (Lohmann, 2010). We showed previously that the Drosophila CNS, when exposed to BL during embryogenesis, develops heightened seizure susceptibility at postembryonic stages. MF exposure coupled with BL illumination significantly potentiates this effect. Experiments performed on cry-null mutants, or when stimulating at wavelengths not overlapping with the absorption spectrum of oxidized CRY (e.g., 590 nm), showed no impact of an MF on seizure duration. We postulated that MF exposure resulted in circuit instability due to potentiation of a CRY-mediated disturbance of neuronal activity during the embryonic stage, when the CNS is particularly sensitive, which is slightly increased compared with expression of wild-type CRY (+ BL, + MF = Δ2.63 ± 0.35 mV, n = 10). Strikingly, the same exposure to BL in the presence of an MF produced no potentiation of the effect on membrane potential (+ BL, + MF = Δ2.29 ± 0.35 mV, n = 10, p = 0.50; Fig. 4 A, B).

Expression of CRY\,514\,6 results in a modest, but not statistically significant, depolarization of basal membrane potential compared with controls (Ela\,V\,C155) in the absence of BL exposure (Fig. 4C), which is consistent with a constitutively active state. Therefore, residues within the C-terminal of CRY are required for neuronal sensitivity to an MF, which mirrors the requirement of this region for magnetic sensitivity, negative geotaxis (Fedele et al., 2014a), and circadian period (Fedele et al., 2014b).

Interestingly, however, opposing effects of MF have also been observed—for example, on the circadian period of Drosophila (Yoshii et al., 2009; Fedele et al., 2014b)—that appear to result from differing wavelengths of light rather than different magnitudes of MF. Whereas a 300 μT MF produced a period shortening under light of 430–470 nm (Fedele et al., 2014b), under light of 445–495 nm (Yoshii et al., 2009) or 500 ± 20 nm (Fedele et al., 2014b), a period shortening was observed. A similar influence of wavelength could be at play here: the antagonist effect of μT MF observed on the negative geotaxis of adult flies was conducted under 450 ± 20 nm and our agonist effects reported here and on the seizure response (Marley et al., 2014) was <470 ± 20 nm. These and other (Phillips and Sayeed, 1993) effects of wavelength have been discussed in the context of the photochemical interconversion of different oxidation states of the FAD bound to CRY and are also considered to be consistent with an underlying RPM (Phillips et al., 2010).

Behavioral studies with migratory birds have also revealed that monochromatic light intensity can have a significant impact on magnetoreception (Wiltschko et al., 2007). Among other wavelengths, increasing the intensity of BL ultimately disrupted orientation along the migratory direction and the effect speculated by the investigators might be due to photoactivation of the birds’ color cones above a certain threshold. If this were indeed the reason, it would not be a consideration in the experiments described here, where our measurements are on single motoneurons.

The fact that expression of CRY\,W342F removes the effect of BL but not of MF is intriguing. It is possible that, although the MF effects appear to require light, photoactivation and the influence of MF are in some way separated? It has been proposed that the magnetically sensitive radical pair is generated in the dark during reoxidation of the flavin bound to CRY after photoreduction (Wiltschko et al., 2016). However, such an explanation would still require photoreduction and alternative photo-induced electron transfer pathways have been identified in Trp triad variants in vitro (Biskup et al., 2013) and FAD photoreduction observed in
similar variants in vivo (Engelhard et al., 2014). Interestingly, these alternative pathways appear to be less efficient than the Trp triad and produce lower concentrations of radicals compared with wild-type CRY. Because the flavin radical is proposed to correspond to the signaling state in Drosophila CRY (Vaidya et al., 2013), this lower radical concentration could in itself explain our observations. It is possible that CRY W342F is still photostable, but to a level that induces a change in membrane potential below our detection limit, resulting in no apparent effect of BL. When this photoactivation is potentiated by MF, however, it might increase the concentration of activated CRY W342F above a threshold to a point where there is a measurable effect on membrane potential.

In contrast, CRY A414S removes the potentiation by MF of the increase in membrane potential by BL. The deletion of residues 521–540, which confer a constitutively active status to CRY (Dissel et al., 2004), may disrupt a possible interaction with another unknown effector(s) or prevent conformational changes within the protein that are essential for the transduction of signal. The signaling interactions involving CRY here might therefore bear a closer resemblance to those involved in the control of circadian period and negative geotaxis—both of which require an intact C terminus to exhibit magnetosensitivity (Fedele et al., 2014a; Fedele et al., 2014b)—than the interactions that result in locomotor hyperactivity, for which the N-terminal FAD-binding region appears to be sufficient (Fedele et al., 2014b). Alternatively, magnetoreception by CRY may require cyclic changes between an active and inactive state, which this deletion might preclude. This seems less likely in light of the effect of MF on locomotor hyperactivity (Fedele et al., 2014b). Indeed, it is possible that CRY might only be necessary for signaling in animal magnetoreception rather than acting as a magnetoreceptor. We feel that this is unlikely because, if true, it would suggest that Drosophila motoneurons are inherently magnetosensitive, but lack CRY to enable this sensory modality. The precise role of CRY is still uncertain, as demonstrated by the striking observation that the isolated C-terminal region, which lacks the FAD chromophore thought necessary for radical pair photochemistry, is sufficient to facilitate a change in period by an applied MF (Fedele et al., 2014b).

Our results represent an important step in elucidating the link between magnetoreception and neuronal activity to facilitate a better understanding of the mechanisms used by organisms to sense the geomagnetic field. We show for the first time that an MF can affect neuronal activity directly through potentiation of phototransduced CRY. Our data obtained using CRY W342F support the existing hypothesis that disruption of the Trp residue at position 342 is not crucial for flies to sense an MF (Gegear et al., 2010; Fedele et al., 2014b). Our data are also consistent with exposure to an MF increasing the sensitivity of CRY W342F to BL, possibly by increasing its stability (Fedele et al., 2014b) or by increasing the concentration of flavin radicals. In contrast, the C-terminal would seem to have a prominent role in enabling CRY to facilitate modulation of neuronal activity by MFs. Exploitation of Drosophila genetics offers the exciting prospect to establish whether CRY is indeed a primary magnetoreceptor and to advance our understanding of the molecular basis of that activity and the mechanism of signal transduction.

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