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Cryptochrome-based Magnetic Sensing

Hore, Peter
THE UNIVERSITY OF OXFORD
UNIVERSITY OFFICES
OXFORD, ,
GB

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14. ABSTRACT

- For the first time, a cryptochrome (Cry4) from a truly migratory animal (the European robin, Erithacus rubecula, Er) has been expressed in high purity with stoichiometric incorporation of the FAD chromophore. We have also generated associated mutants, eliminating in turn each of the four tryptophans involved in the electron transport chain.
- We have reported the first measurements of magnetic field effects on the photocycle of ErCry4 in both the wild type protein and in Trp to Phemutants.
- Magnetic field effects on ErCry4 are 1-2 orders of magnitude stronger than those on Cry4 from non-migratory chicken and pigeon.
- EPR experiments clearly establish that ErCry4 has a functioning Trptetrad.
- The relative magnetic sensitivities of the 3rd (C) and 4th (D) flavintryptophan radical pairs in ErCry4 suggest that TrpC does most of the magnetic sensing whereas TrpD is mainly responsible for initiating biochemical signalling.
- Molecular dynamics simulations suggest that the 3rd and 4th radical pairs are in rapid dynamic equilibrium.
- We have shown that ErCry4 is specifically localised in the outer segments of double cone and long-wavelength single cone photoreceptor cells in the retinas of several bird species.
- We have suggested why localisation of ErCry4 in the double cones could be advantageous and how the Cry4 molecules could be oriented in the two components of the double cones.
- Quantitative PCR data show that Cry4 is expressed ~2.5 times more in the migratory season in night-migratory robins, whereas there are no seasonal differences in non-migratory chicken.
- The crystal structure of Columba livia (pigeon) Cry4 has been determined. Homology modelling has been used to propose a structure for ErCry4.
- Hydrogen-deuterium exchange mass spectrometry of Drosophila cryptochrome (DmCry) has revealed blue light-dependent conformational changes that could initiate magnetic signal transduction.
- Cry4 has been identified as the most likely of the bird cryptochromes to be responsible for magnetic sensing.
- Broadband radiofrequency electromagnetic fields have a greater disruptive effect on the ability of migratory songbirds to use their magnetic compass than do strong narrow-band fields. These effects are consistent with a spin-coherence time of 2-10 Î¼s.
- Using a new computational approach, we have shown that the current model of radical-pair magnetoreception can qualitatively, but not quantitatively, account for the effects of broadband radiofrequency noise on the orientational abilities of migratory birds.
- We have suggested conditions for behavioural experiments using broadband radiofrequency noise that have the potential to distinguish between the two types of radical pairs currently postulated as magnetic compass sensors.
- Six potential ErCry4 interaction partner proteins have been identified and possible magnetic signaling pathways have been discussed.
- No evidence has been found for an interaction between Cry4 and IscA1 (iron-sulphur cluster assembly 1 protein).
- A unique suite of optical spectroscopic techniques has been developed specifically for the detection of magnetic field effects on cryptochromes and model compounds.
- Studies of DmCry have shed light on the different magnetic sensitivities of radical pairs in proteins that have 3 or 4 tryptophans in the electron transfer chain.
- Four maquettes (non-natural proteins) have been designed, expressed and studied as models of the first of the sequential light-induced electron transfers in cryptochromes. Their magnetic sensitivity shows some surprising similarities to the natural proteins.
- A carotenoid-porphyrin-fullerene model compound has been shown to exhibit pronounced anisotropic magnetic field effects at field strengths relevant to magnetoreception (~50 microtesla).
- No statistically significant magnetic field effects have been found in studies of fruit flies in two behavioural assays. This result casts serious doubt on whether Drosophila can sense magnetic fields at all and thus strongly suggests that night-migratory songbirds remain the organism of choice for elucidating the mechanism of light-dependent magnetoreception.
- Molecular dynamics simulations have been performed to assess the likelihood that the spin-relaxation of flavin-tryptophan radical pairs in cryptochrome is slow enough to allow significant sensitivity to Earthstrength magnetic fields.
- We have shown that modulation of exchange and dipolar interactions could boost the magnetic sensitivity of a cryptochrome-based radical pair magnetoreceptor.
- Spin dynamics calculations on realistic models of cryptochrome-based radical pairs have suggested a new type of magnetic response that would give birds a much more precise compass bearing than hitherto thought possible.
- Chemical mechanisms for the amplifying the effects of magnetic fields on radical pairs in cryptochromes have been studied experimentally and computationally.
- We have proposed a simple solution to the problem of detecting small magnetic field effects in the presence of large variations in the intensity and polarization of the light required to activate the magnetic compass sensor.
- We have proposed an in vivo experiment with the potential to determine whether the involvement of cryptochrome in magnetoreception is as a magnetic sensor or a signal transducer downstream of a different the primary sensor.
- Spin dynamics simulations of superoxide-containing radical pairs argue strongly against the involvement of superoxide radicals in compass magnetoreception.
- In answer the question, How quantum is radical pair magnetoreception? We have shown that accurate simulation of anisotropic magnetic field effects relevant to magnetoreception seems to require full quantum mechanical calculations.
- Results from Information Theory have been used to obtain a lower bound estimate of the precision with which a bird could orient itself using only geomagnetic cues.

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CRYPTOCHROME-BASED MAGNETIC SENSING

FINAL PERFORMANCE REPORT

15 Sep 2014 - 14 Sep 2020

Principal Investigators

P. J. Hore, University of Oxford, UK
C. R. Timmel, University of Oxford, UK
S. R. Mackenzie, University of Oxford, UK
S. Waddell, University of Oxford, UK
H. Mouritsen, University of Oldenburg, Germany
K.-W. Koch, University of Oldenburg, Germany

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SUMMARY

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- The relative magnetic sensitivities of the 3rd (C) and 4th (D) flavin-tryptophan radical pairs in *Er*Cry4 suggest that $\mathrm{Trp_C}$ does most of the magnetic sensing whereas $\mathrm{Trp_D}$ is mainly responsible for initiating biochemical signalling.
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- Spin dynamics simulations of superoxide-containing radical pairs argue strongly against the involvement of $O_2^{\bullet -}$ in compass magnetoreception.
- In answer the question, *How quantum is radical pair magnetoreception?*, we have shown that accurate simulation of anisotropic magnetic field effects relevant to magnetoreception seems to require full quantum mechanical calculations.
- Results from Information Theory have been used to obtain a lower bound estimate of the precision with which a bird could orient itself using only geomagnetic cues.

INTRODUCTION

The sensory mechanisms that allow birds to perceive the direction of the Earth's magnetic field for the purpose of navigation are beginning to be understood. One of the two leading magnetoreception hypotheses is founded on magnetically sensitive photochemical reactions in the retina. It is thought that transient photo-induced radical pairs in cryptochrome, a blue-light photoreceptor protein, act as the primary magnetic sensor. Experimental and theoretical support for this mechanism has been accumulating over the last few years but as described in our recent review articles,[1,2] there are still many open questions.

We are engaged in a diverse set of interlinked experiments and calculations designed to answer the majority of the "ten most important questions in magnetoreception" identified in Ref. 1.

The objectives of the project, as summarized in our research proposal, are:

(1) To determine the detailed physico-chemical mechanism by which cryptochromes respond to the intensity and direction of Earth-strength magnetic fields.
(2) To determine how cryptochromes are immobilized within retinal neurons and to characterize the interactions responsible.
(3) To determine which of the four cryptochromes of migratory birds are involved in magnetoreception.
(4) To identify the cryptochrome binding partners that mediate the first steps in the signal transduction pathway.
(5) To determine whether cryptochrome-based magnetoreception has an absolute requirement for polarized light.
(6) To determine whether cryptochromes are the primary magnetic compass sensors.

Principal responsibility for the six objectives lies as follows:

1 and 6	University of Oxford (Hore, Timmel, Mackenzie, Waddell)
2–5	University of Oldenburg (Mouritsen, Koch).

Members of the University of Oxford involved in the project:

Prof. Peter Hore, PI (Chemistry)
Prof. Christiane Timmel, PI (Chemistry)
Prof. Stuart Mackenzie, PI (Chemistry)
Prof. Scott Waddell, PI (Physiology, Anatomy and Genetics)
Dr Christian Kerpal, postdoctoral researcher financed from other sources until 31 October 2015 and then by AFOSR (now Adjunct Professor, Furman University, USA)
Marcin Konowalczyk, DPhil student, financed by AFOSR (started 1 October 2015, degree awarded 2020)
Marco Bassetto, DPhil student, financed by AFOSR (started 1 October 2015, degree awarded 2020)
Dr Kevin Henbest*, postdoctoral researcher
Dr Daniel Kattnig*, postdoctoral researcher (now at the Living Systems Institute and Department of Physics, University of Exeter)
Dr Lauren Jarocha*, postdoctoral researcher (now Assistant Professor, Furman University, USA)
Dr Jonathan Storey*, postdoctoral researcher
Dr Charlotte Dodson*, postdoctoral researcher
Emrys Evans*, DPhil student (degree awarded 2016)

Dean Sheppard*, DPhil student (degree awarded 2017)
Cat Gunn*, DPhil student (degree awarded 2016)
Susannah Worster*, DPhil student (degree awarded 2018)
Hamish Hiscock*, DPhil student (degree awarded 2018)
Tilo Zollitsch*, DPhil student (degree awarded 2018)
Katie Buckton*, DPhil student (degree awarded 2021)
Victoire Déjean*, DPhil student
Shane Chandler*, DPhil student (degree awarded 2019)
Matthew Golesworthy*, DPhil student
Thomas Player*, DPhil student
Jiate Luo*, DPhil student
Hang Zhou*, DPhil student
Angela Gehrkens*, DPhil student
Jamie Gravell*, DPhil student
Patrick Murton*, DPhil student

*Financed from other sources

Members of the University of Oldenburg involved in the project:

Prof. Dr Henrik Mouritsen, PI (Neurosensory Sciences)
Prof. Dr Karl-Wilhelm Koch, PI (Biochemistry)
Dr Haijia Wu, postdoctoral researcher, (financed by AFOSR, started 15 May 2015)
Dr Arndt Meyer, postdoctoral researcher (financed by AFOSR, started 1 January 2015, succumbed to cancer)
Dr Alexander Scholten, permanent position paid by University of Oldenburg
Dr Karin Dedek, permanent position paid by University of Oldenburg
Rabea Bartölke*, postdoctoral researcher
Anja Günther, PhD student, (financed by AFOSR, started 15 November 2014, degree awarded 2020)
Angelika Einwich, PhD student (financed by AFOSR from 1 January 2019 to 14 September 2020, pregnancy leave from 17 November 2019 onwards, before that she was funded from other sources)
Dmitry Kobylkov*, PhD student (degree awarded 2020)
Jingjing Xu*, PhD student
Pranav Seth, PhD student (financed by AFOSR, February-September 2020, before that he was funded from other sources)
Stefan Tetenborg (financed by AFOSR for 2 months in 2020, before that he was funded from other sources, degree awarded 2020)
Katharina Goertemaker*, PhD student
Chad Yee*, PhD student

*Financed from other sources

Collaborators involved in the project:

Prof. David Manolopoulos (University of Oxford)
Prof. Justin Benesch (University of Oxford)
Prof. Ilia Solov'yov (University of Southern Denmark, now University of Oldenburg)
Prof. Dr Stefan Weber (University of Freiburg)
Dr Erik Schleicher (University of Freiburg)

Prof. P. Leslie Dutton (University of Pennsylvania)
Dr Christopher Moser (University of Pennsylvania)
Prof. Can Xie (Peking University)
Dr Daniel Kattnig (University of Exeter)
Prof. Joseph S. Takahashi (University of Texas Southwestern)
Dr Yoga Chelliah (University of Texas Southwestern)
Prof. Dr Sascha Laubinger (University of Oldenburg)
Prof Dr Franz Bairlein (Institute of Bird Research, Wilhelmshaven)

METHODS, ASSUMPTIONS and PROCEDURES

We have adopted a multidisciplinary, collaborative approach that combines many different techniques and approaches, including: novel cavity-enhanced spectroscopic methods (absorption and fluorescence), pulsed electron spin resonance, H/D exchange mass spectrometry, protein expression in *E. coli* and Sf9 insect cells, quantitative Western blotting, surface plasmon resonance, affinity chromatography, polyacrylamide gel electrophoresis, RNAi knock-down techniques, genetically-based screening using a yeast-two-hybrid system, electrophysiological recordings using a 96-electrode array, behavioural assays, quantum simulations of radical pair spin dynamics, molecular dynamics simulations of cryptochromes, and a *Drosophila* geotaxis assay.

Further information on specific techniques, including outlines of methods developed specifically to address the objectives of the project, can be found in the following pages and in more detail in the published papers resulting from this funding.

RESULTS and DISCUSSION

REVIEW ARTICLES

Hore and Mouritsen have written a tutorial-type review article on radical pair magnetoreception (*Annual Review of Biophysics*, 2016).[2] Our primary aims were to explain the chemical and physical aspects of the radical-pair mechanism to biologists and the biological and chemical aspects to physicists and, in the process, to review the current state of knowledge on magnetoreception mechanisms. As we said in the Abstract, “We dare to hope that this tutorial will stimulate new interdisciplinary experimental and theoretical work that will shed much-needed additional light on this fascinating problem in sensory biology.” At the time of writing, the article has garnered 187 citations and, according to *Web of Science*, is a “Highly Cited Paper”.

Mouritsen has written a more general review of long-distance navigation and magnetoreception in migratory animals (*Nature*, 2018).[3] With 79 citations to date, it too is a “Highly Cited Paper”.

Mouritsen has written a detailed review on the neural basis of long-distance navigation in birds (*Annual Review of Physiology*, 2016),[4] which summarised everything we know about the neural pathways in the brains of birds which are involved in processing navigation-relevant information. Furthermore, it suggested where to look for the needed multisensory integration areas that bring the navigational information together from all the senses and decide in which direction a bird will fly. 51 citations to date.

EXPERIMENTAL METHODS

Throughout the period of the grant, we have developed a unique suite of optical spectroscopic techniques specifically for the detection of magnetic field effects on cryptochromes and model compounds. They include (1) cavity ring-down spectroscopy,[5] CRDS, which affords high temporal resolution in single-wavelength pump-probe type experiments, (2) broadband cavity-enhanced absorbance spectroscopy,[6] BBCEAS, a continuous-wave technique offering sensitive detection across the visible spectrum, (3) transient absorption (TA) methods to monitor radical pair reactions on a picosecond timescale, (4) a variety of highly sensitive fluorescence detection techniques, and (5) evanescent wave CEAS for studying anisotropic magnetic field effects on aligned molecules. Some of the developments in fluorescence detection are summarised in the following paragraphs. Applications of the other techniques are described in later sections.

Fluorescence detection of magnetic field effects

We have shown that the effects of applied magnetic fields on radical pair reactions can be sensitively measured from sample volumes as low as ~100 femtolitres using total internal reflection fluorescence microscopy.[7] Development of such fluorescence-based microscope methods is likely to be a key step in further miniaturisation that will ultimately allow detection of magnetic field effects on single molecules.

Separately, a novel detection scheme for investigating magnetic field effects on flavin-based radical pair reactions has been developed.[8] Its exceptional sensitivity enables the study of samples containing as little as 1 nM flavin. The technique involves monitoring magnetic field-dependent changes in the prompt fluorescence of a continuously photo-excited system. The high detection sensitivity has been used to characterize the influence of external factors on the spin dynamics and magnetic field effects in intermolecular reactions of flavins. We have demonstrated that the technique also works well applied to the intramolecular radical pair reactions in cryptochrome.[8]

Finally, confocal fluorescence microscopy has been developed as a powerful technique for the investigation of chemical magnetic field effects.[9] The utility of the method was tested on a plant cryptochrome and a flavin-lysozyme model system, and used to detect temporally and spatially resolved magnetic field effects in flavin-doped single crystals of lysozyme. Anisotropic magnetic field effects were also reported for tetracene single crystals.

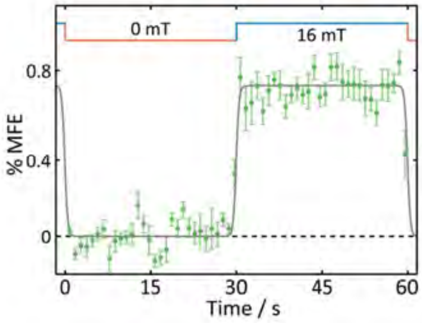

Figure 1. Average magnetic field effect on *At*Cry1. An applied magnetic field is switched between 0 and 16 mT every 30 s. The data show an average of four on-off steps. A step function was fitted to the data to guide the eye. From Ref. [9]

RADIOFREQUENCY (RF) ELECTROMAGNETIC FIELDS

Some of the strongest supporting evidence for a radical pair-based magnetic compass in migratory songbirds has come from behavioural experiments in which birds exposed to weak time-dependent magnetic fields lose their ability to orient in the geomagnetic field.[10-12] However, conflicting results and scepticism about the requirement for abnormally long quantum coherence lifetimes have cast a shroud of uncertainty over these potentially pivotal studies.

In an attempt to get some insight into these effects, we performed new behavioural experiments with night-migratory songbirds exposed to RF fields of different frequencies and developed new theoretical concepts and tools for predicting and understanding such effects.

Behavioural experiments

In earlier work we had shown that broadband RF fields in the range 0.1-10 MHz disturb the magnetic orientation of night-migratory European robins.[11] During the AFOSR funding period, we showed that such broadband RF-fields are much more disruptive than strong narrow-band fields including those at the Larmor frequency.[12] Our double-blinded experiments also demonstrated that previously claimed highly specific effects of RF-disturbances at the Larmor-frequency could not be replicated.[12]

A crucial property of a radical pair compass is the lifetime of the quantum spin coherence, because it essentially determines the sensitivity to weak external magnetic fields. We believe that the spin coherence lifetime can be estimated by discovering the frequency cut-off below which magnetic orientation is no longer disrupted by time-dependent magnetic fields. The idea here is that a weak (<< 50 μT) magnetic field oscillating so slowly that its intensity hardly changes during the coherence lifetime will be perceived as static and have a negligible effect on the spin dynamics. We therefore tested the orientation capabilities of European robins in an electromagnetically silent environment, under the specific influence of broadband electromagnetic noise covering the range from ~0.1 kHz to ~100 kHz in our unique, purpose-built, DARPA-funded laboratory.

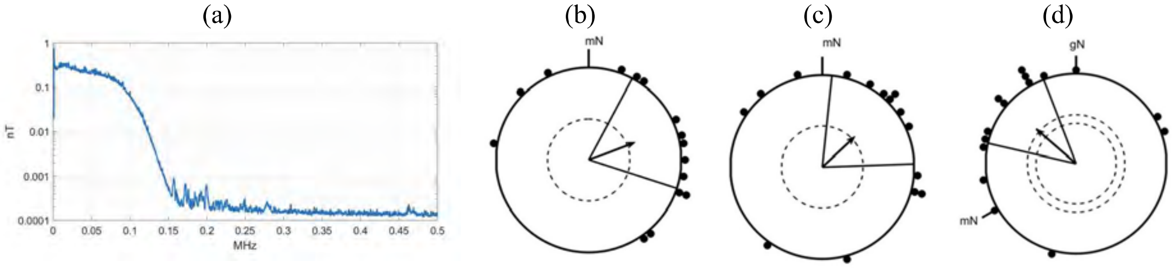

Figure 2. Electromagnetic noise in the range of 0.1-100 kHz ((a) max-hold measurement, 100 Hz measuring bandwidth) does not disrupt the magnetic compass orientation capabilities of a night-migratory songbird. (b) Spring orientation of blackcaps in the Earth's magnetic field condition without electromagnetic noise (mean = 68°, n = 15, p = 0.02). (c) and (d) Spring orientation of blackcaps exposed to electromagnetic noise covering the range from ~0.1 kHz to ~100 kHz. The birds were significantly oriented in the normal static magnetic field and shifted their orientation ca. 100° ((c) NMF-RF 48°, p = 0.03; (d) CMF-RF 311°, p = 0.004) as expected when the static field was rotated ~120°. From Ref. [13]

We already knew that robins are sensitive to time-dependent fields in the range 50-450 kHz,[11] which suggests that the spin coherence lasts for at least ~2 μs (i.e. 1/(450 kHz)). If the 0.1-100 kHz field would prevent the birds from orienting, it would suggest that the spin coherence lasts for more than ~10 μs (i.e. 1/(100 kHz). We therefore tested the effect of broadband electromagnetic noise in the range 0.1-100 kHz (Figure 2) on the magnetic compass orientation capabilities of Eurasian blackcaps (*Sylvia atricapilla*).

Thus, we observed no effects of 0.1-100 kHz electromagnetic noise[13] which suggests that the spin-coherence time is less than ~10 μs. Considering these results together with the results of Engels *et al.*,[11] the behavioural experiments suggest that the spin correlation time is between 2 and 10 μs.

Theoretical calculations

We have developed a new method for calculating the product yield of a radical pair recombination reaction in the presence of a weak time-dependent magnetic field.[14] This method successfully circumvents the computational difficulties presented by a direct solution of the Liouville-von Neumann equation for a long-lived radical pair containing many hyperfine-coupled nuclear spins. Using a modified formulation of Floquet theory, treating the time-dependent magnetic field as a perturbation, and exploiting the slow radical pair recombination, we showed that one can obtain a good approximation to the product yield by considering only nearly degenerate sub-spaces of the Floquet space. Within a significant parameter range, the resulting method was found to give product yields in good agreement with exact quantum mechanical results for a variety of simple model radical pairs. Moreover, it is considerably more efficient than the exact calculation, and it can be applied to radical pairs containing significantly more nuclear spins.

We have used this approach to explore the effects of various radiofrequency magnetic fields on biologically plausible radical pairs.[15] We conclude that the current model of radical-pair magnetoreception is qualitatively but not quantitatively able to explain the behavioural results. Assuming that an unknown mechanism amplifies the predicted effects, we suggested experimental conditions that have the potential to distinguish convincingly between the two types of radical pairs currently postulated as magnetic compass sensors.[16] In addition, these calculations have led to recommendations for experimental protocols that should increase the chance that future experiments can be independently replicated. Some of the predictions of this study have already been tested on European robins and blackcaps.[12,13] Tests at other frequencies are underway in Oldenburg.

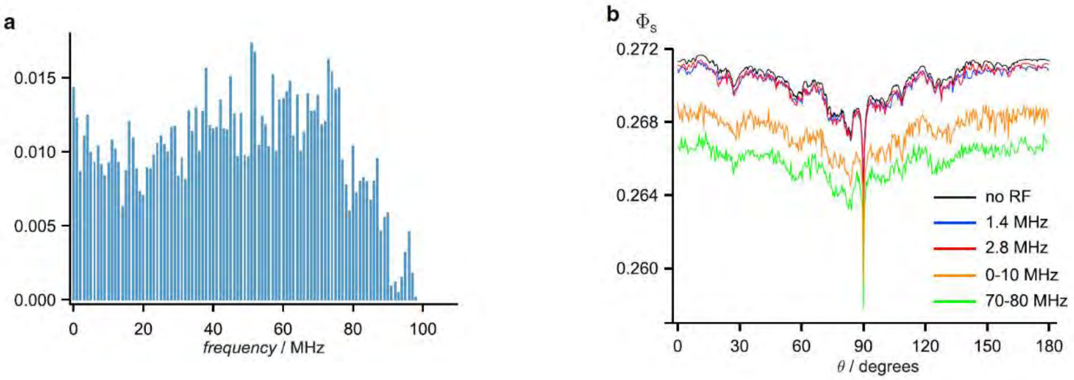

Figure 3. FAD-Trp radical pair with no exchange or dipolar interactions. (a) Action-spectrum histogram containing frequencies spread roughly uniformly over the range 0-100 MHz (bin width, 1 MHz). (b) The anisotropic singlet yield in the presence of 1.4 and 2.8 MHz single-frequency fields and 0–10 and 70–80 MHz broadband fields. The 1.4 MHz (blue) and 2.8 MHz (red) traces are very similar to the singlet yield in the absence of a radiofrequency field (black). From Ref. [15]

MODEL SYSTEMS

Throughout the period of the grant we have studied a number of model radical pair systems to learn more about the factors that control the responses of radical pair reactions to weak magnetic fields and hence the conditions for cryptochrome-based radical pairs *in vivo* to respond sensitively to the direction of the geomagnetic field.

Flavin-ascorbic acid reaction

The radical derived from ascorbic acid (vitamin C) is a potential component of a flavin-containing radical pair in cryptochrome.[16] To investigate this possibility, we have used the fluorescence-detection technique outlined above[8] to study the effects of sub-millitesla magnetic fields on the photoreaction of flavin mononucleotide with ascorbic acid.[17] Direct control of the reaction pathway was achieved by varying the rate of electron transfer from ascorbic acid to the photo-excited flavin. The data agree well with model calculations based on a Green's function approach that allows multinuclear spin systems to be treated including the diffusive motion of the radicals, their spin-selective recombination reactions, and the effects of the inter-radical exchange interaction. The protonation states of the radicals were uniquely determined from the form of the magnetic field-dependence. At pH 3.0, the effects of two chemically distinct radical pair complexes combine to produce a pronounced response to ~500 μT magnetic fields.

Flavomaquettes

To understand the functional requirements for radical pair compass magnetoreception, we have created a family of simplified, adaptable model proteins – maquettes – that contain a single tryptophan residue at different distances from a covalently bound flavin. Despite the complete absence of structural resemblance to the native cryptochrome fold or sequence, the maquettes exhibit strong magnetic field effects that rival those observed in the natural proteins *in vitro*. These novel maquette designs offer unprecedented flexibility to explore the basic requirements for magnetic sensing in a protein environment.

Magnetically sensitive flavin-tryptophan radical pairs (RP1) are formed in all three Trp-containing maquettes. The magnetic sensitivity arises, as it does in cryptochromes, from a competition between singlet recombination of RP1 and (de)protonation reactions that produce the stabilized RP2 state. We observe clear trends in the behaviour of the three maquettes that correlate with the expected distance between the Trp residue and the flavin.[18-20]

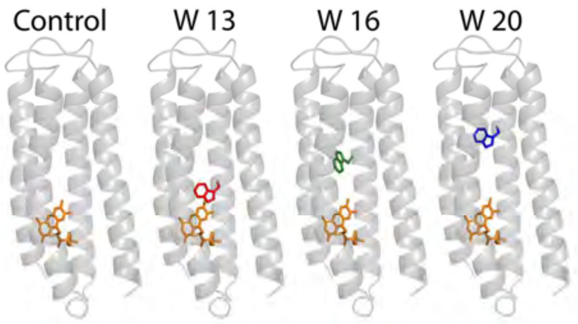

Figure 4. Flavomaquette designs based on α-helical bundles with a riboflavin chromophore (orange) covalently attached to a core cysteine residue: a control, which does not contain an electron donor, and three maquettes with a single tryptophan at position 13, 16, or 20 on helix 2 at increasing distances from the flavin. W13, W16, and W20 can undergo photoinduced electron transfer akin to that observed in cryptochromes. From Ref. [19]

However, comparing the maquettes with cryptochromes, there are also differences in behaviour. The most obvious is the presence in the maquette of just a single tryptophan residue thereby removing the possibility of sequential electron transfer. The maquettes have much greater conformational heterogeneity leading to a distribution of timescales for radical pair formation not seen for the natural proteins. Maquettes are also considerably more dynamic: radical pairs are probably formed most efficiently for conformations that bring the tryptophan close to the flavin. Nevertheless, charge separation is unable to compete effectively with intersystem crossing with the result that the magnetically sensitive radical pair in the maquettes is created in a triplet state rather than a singlet state and exhibits magnetic field effects of opposite phase to those found for natural cryptochromes.[18-20] This project was a collaboration with Les Dutton and Chris Moser at the University of Pennsylvania.

Carotenoid-porphyrin-fullerene triad

The first demonstration that a radical pair reaction could respond to a magnetic field as weak at 50 μT used a carotenoid-porphyrin-fullerene model compound chosen for its favourable photochemistry rather than any resemblance to cryptochrome.[21] We showed that the yield of the photo-induced radical pair could be affected by changing the direction of an applied magnetic field, although the signal-to-noise did not allow fields weaker than ~3 mT to be studied. We have now extended these measurements by using transient absorption spectroscopy to demonstrate a pronounced orientation-dependence of the magnetic field effects at field strengths relevant to magnetoreception (~50 μT).[22]

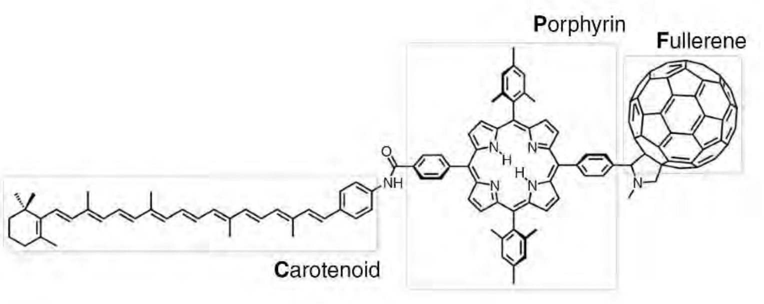

Figure 5. Structure of the triad molecule used to demonstrate anisotropic effects of a ~50 μT magnetic field. From Ref. [22]

CRYPTOCHROMES AS MAGNETORECEPTORS

In vertebrates, cryptochromes are the only class of proteins that form radical pairs upon photo-excitation. For this reason they are currently the only candidate proteins for light-dependent magnetoreception in higher animals. Cryptochrome 4 (Cry4) is particularly interesting because it has only been found in vertebrates that use a magnetic compass.

Magnetic sensitivity of *Drosophila* cryptochrome

Although, as outlined below, we have serious doubts about whether fruit flies can sense weak magnetic fields, the purified cryptochrome from *Drosophila melanogaster* (*Dm*Cry) does show clear magnetic field effects on its photochemistry.[23] The flavin-tryptophan radical pair state of this protein is magnetically sensitive, as found previously for *Arabidopsis thaliana* cryptochrome 1 (*At*Cry1)[24], *E. coli* photolyase (*Ec*PL)[24,25], and *Xenopus laevis* cryptochrome (*Xl*Cry, unpublished work). However, the kinetics of radical formation and decay are markedly different, reflecting the involvement of a fourth tryptophan (Trp) electron donor beyond the end of the "Trp-triad" that mediates radical pair formation in *At*Cry1, *Ec*PL and *Xl*Cry. The expected gain in magnetic sensitivity associated with the larger radical-radical separation, and hence smaller exchange and dipolar interactions, was found to be offset by the reduction in the back electron transfer rate within the flavin-tryptophan radical pair. Although the magnetic field effect on purified *Dm*Cry is not large (2-4% at 22 mT), it is not unreasonable to think that the magnetic sensitivity *in vivo* could be very different from that of the isolated protein *in vitro*.

Anatomical location of Cry4 within the retina of a migratory bird

To understand cryptochrome function and to study light-dependent magnetoreception at the physiological level, it is essential to know where Cry4 is located within the avian retina. When the AFOSR funding period started this was entirely unknown.

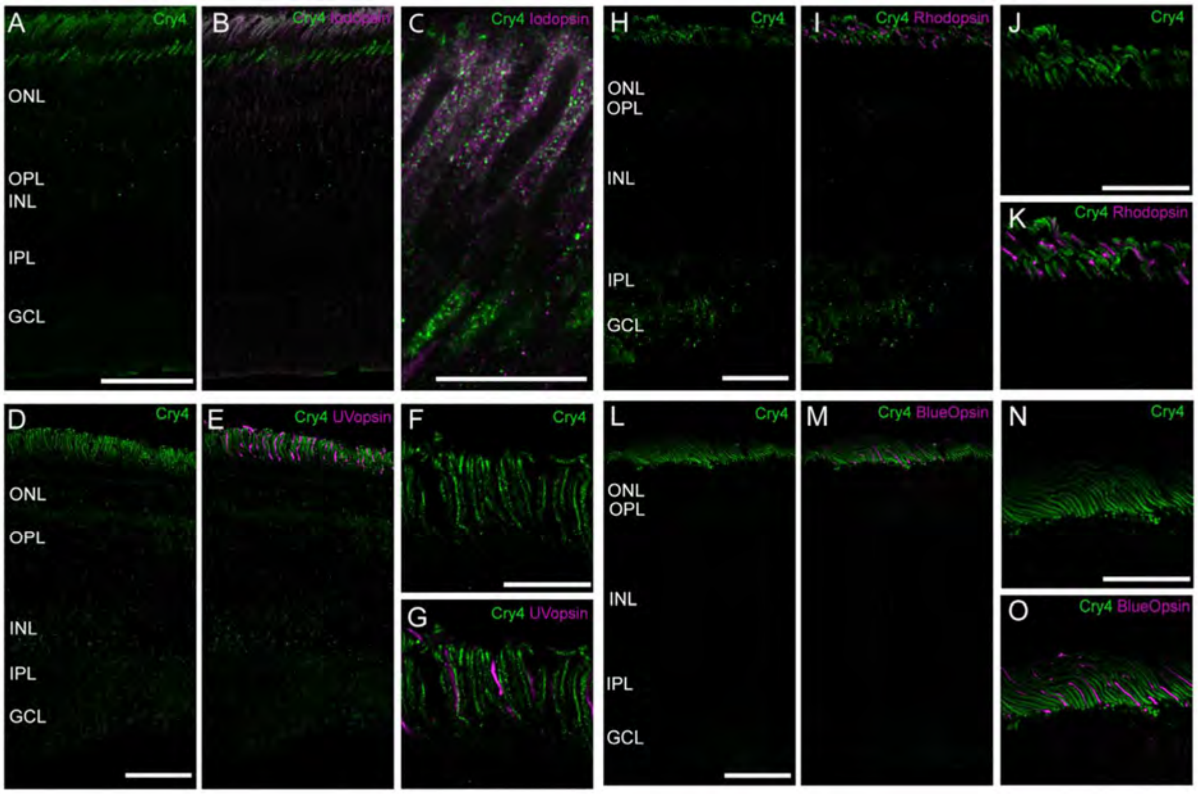

Figure 6. Cry4 is expressed in the outer segments of double cones and long wavelength single cones in the European robin retina. Vertical slices of European robin retinae labeled with the polyclonal Cry4 antibody (green) showed a strong expression of Cry4 protein in the outer segments of some but not all photoreceptor cells. Iodopsin (B and C), UVopsin (E and G), rhodopsin (I and K), and blue opsin antibody (M and O) were expressed in outer segments and inner segments of double and long-wavelength single cones, UV cones, rods, and blue cones, respectively. A closer look at the Cry4 labeling (C, green) together with a iodopsin antibody (C, magenta) showed that Cry4 is expressed in the same outer segments as iodopsin. A closer look at the double stainings of the polyclonal Cry4 antibody with UVopsin (G, magenta), rhodopsin (K, magenta), and blue opsin antibody (O, magenta) showed that the signals are not localized in the same cells. For illustration purposes, all images were enhanced in brightness. Scale bars in (A), (D), (H), and (L), 50 mm, in (C), 10 mm, and in (F), (J), and (N), 25 mm. (C), (F), (G), (K), (N), and (O) are enlargements of parts of the corresponding whole-retinal images. From Ref. [26].

We have shown that Cry4 is expressed in the outer segments of specific photoreceptors (Figure 6). We were able to exclude the UV cones, the blue cones, and the rods, and to show that, within the retinas of migratory European robins and domestic chicken (*Gallus gallus*), Cry4 seems to be expressed only in the double-cone and long-wavelength single-cone photoreceptor cells.[26] These are the two photoreceptor types that contain the long-wavelength opsin called iodopsin. The double-cone localization of Cry4 is particularly interesting because we suggested in our 2016 review[2] that the double cones would be the ideal location for light-dependent magnetosensitive molecules in birds. It is also striking that the functional significance of the cellular architecture of double cones has remained unclear even though they constitute the most common cone type in many birds. The cytosolic expression of Cry4 that we found[26] and its lack of a nuclear localization signal suggest that Cry4 is not involved in the regulation of circadian rhythmicity. Günther *et al*.[26] also included a suggested structure for European robin Cry4 based on homology modelling in collaboration with Ilia Solov'yov (Odense University, now Oldenburg University).

Furthermore, we compared the relative expression levels of all the different cryptochromes known at the time (Cry1a, Cry1b, Cry2, and Cry4) in migratory European robins (*Erithacus rubecula*) and in non-migratory chickens. Whereas there were no strong differences in the seasonal expression of any of the four cryptochromes in chicken retinas, we observed strongly increased expression of Cry4 in the retinas of European robins during the migratory seasons compared to the non-migratory seasons (Figure 7).

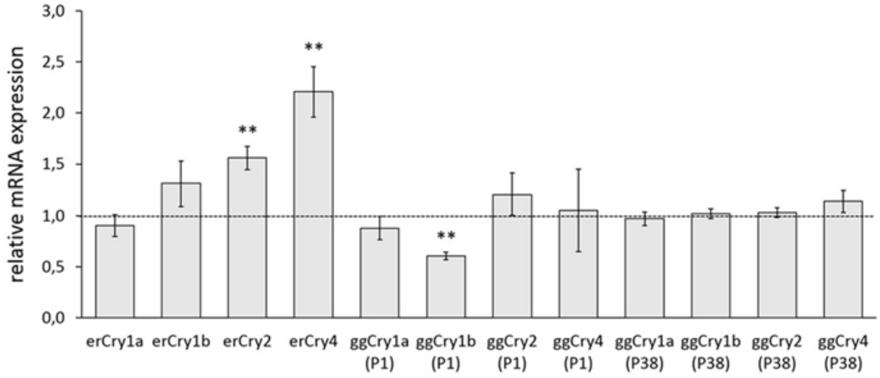

Figure 7. Relative expression of the cryptochromes of European robins and chicken in retinas collected during the migratory autumn/spring seasons relative to the expression during the non-migratory winter/summer seasons. A value of 1.0 indicates the same level of expression in both seasons (n = 8 + 8 for European robins, 6 + 6 for chickens (P1), and 4 + 4 for chickens (P38); mean values ±SEM are shown). A value $x > 1$ indicates an x-fold higher expression level during the migratory season. Significance was tested using the Mann-Whitney-Wilcoxon test, ** $p < 0.01$. From Ref. [26]

In a very recent paper, we discovered that there exist two isoforms of Cry4: Cry4a (the previously known Cry4) and Cry4b.[27]

Structure and photochemistry of pigeon cryptochrome-4

A variety of evidence suggests that of the four known avian cryptochromes, Cry4 is most likely to act as a magnetic compass sensor. Validation of a cryptochrome-based mechanism of magnetoreception has been hampered by the absence of structures of vertebrate cryptochromes that have functional photochemistry. In collaboration with Joseph Takahashi (University of Texas Southwestern) we have determined crystal structures of *Columba livia* (pigeon) cryptochrome-4 (*Cl*Cry4) that confirm the extension of the Trp-triad sequence of tryptophan residues to include a fourth tryptophan (W369) and possibly a tyrosine (Y319) residue at the protein surface that impart an unusually high quantum yield of photoreduction.[28]

Magnetic sensitivity of European robin Cry4

One of the most significant achievements of our AFOSR project has been a collaborative study of magnetic field effects on European robin Cry4. A manuscript has been under consideration by a high profile journal for more than a year now.[29] We have just completed what we hope will be the experiments that will finally convince the Editor (if not all of the referees) that it should be accepted for publication. In summary:

- We have expressed and purified the first cryptochrome with the essential FAD chromophore incorporated from any truly migratory animal, and generated the associated mutants eliminating in turn each of the four tryptophans involved in the electron transport chain.
- We have continuously developed a suite of highly sensitive spectroscopic techniques specifically for measuring the effects of weak magnetic fields on cryptochrome photochemistry (CRDS, BBCEAS, TA). Several optimisations of these methods are used in this study for the first time.

- Such techniques provide information on timescales from picoseconds to hours with spectral resolution throughout the visible range of wavelengths.
- We report the first measurements of magnetic field effects on a cryptochrome (Cry4) from a migratory bird.
- Magnetic field effects on *Er*Cry4 are 1-2 orders of magnitude stronger than those on Cry4 from non-migratory chicken and pigeon.
- EPR experiments clearly establish that *Er*Cry4 has a functioning Trp-tetrad.
- Measurement of the relative magnetic sensitivities of the 3rd (C) and 4th (D) flavin-tryptophan radical pairs, suggesting that Trp$_{\mathrm{C}}$ in *Er*Cry4 does most of the magnetic sensing whereas Trp$_{\mathrm{D}}$ could be mainly responsible for initiating biochemical signalling.
- Our data suggest that RP$_{\mathrm{C}}$ and RP$_{\mathrm{D}}$ may be in rapid dynamic equilibrium leading to a range of new interpretation possibilities that have not been considered for any previously studied cryptochrome from any organism.
- *In vitro* measurement of the times required for photochemically reduced states of bird cryptochromes to revert to the fully oxidised state demonstrate that there are conditions under which bird Cry4s recycle rapidly enough that they could be suitable as *in vivo* magnetoreceptors.
- Estimates of the rates of the relevant electron transfer steps in *Er*Cry4 have been obtained.

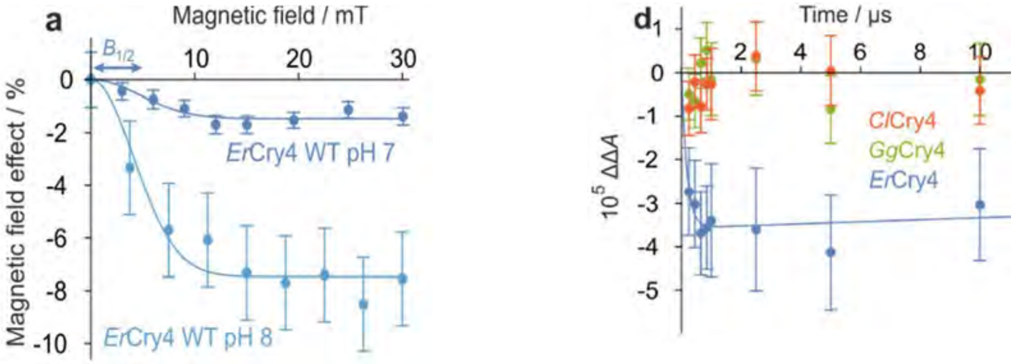

Figure 8. Magnetic field effects on the yields of photo-induced radicals in Cry4s. **Left**: Percentage magnetic field effect on the optical absorbance of photo-induced radicals 2 μs after a 450 nm laser pulse. Data were measured by CRDS at 530 nm. **Right**: Change in the photo-induced optical absorbance of three avian Cry4s caused by a 30 mT magnetic field, measured by CRDS at 530 nm. Within the signal-to-noise, the magnetic field effects on the Cry4s from non-migratory pigeon and chicken could not be distinguished from zero. The error bars represent one SEM and the line is a smooth curve to guide the eye. From Ref. [29]

Light-induced conformational changes

Little is known about how a magnetically induced change in the quantum yield of radicals in cryptochrome could lead to neuronal signals. By analogy with plants, in which the blue-light signalling functions of cryptochromes are reasonably well understood, it is presumed that photo-reduction of the FAD in cryptochrome produces a conformational change in the C-terminal domain that alters the protein's ability to bind to other proteins. This, in turn, is imagined to lead to a biochemical signalling and amplification cascade and hence changes in the rate and/or relative timing of action potentials and/or the rate of release of neurotransmitters.

We have performed preliminary hydrogen-deuterium exchange (HDX) mass spectrometry (MS) experiments on *Drosophila* cryptochrome (the first such measurements on any cryptochrome).[30] We believe they reveal the primary event in the magnetic signal transduction pathway: a blue light-dependent conformational change in the structure of the C-terminal region of the protein detected as an increase in the uptake of deuterium from deuterated buffer solution by residues in the C-terminal peptide.

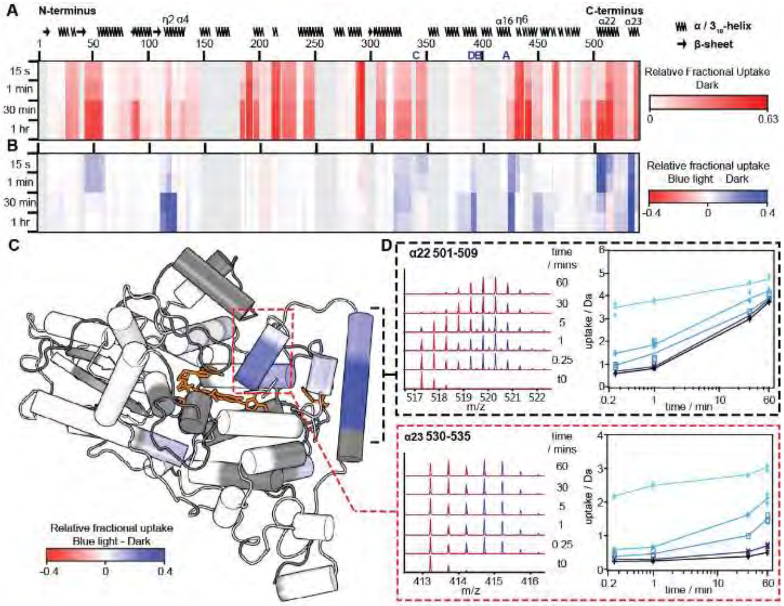

Figure 9. Conformational dynamics of wild-type *Dm*Cry observed by HDX mass spectrometry. (**A**) The degree of deuterium incorporation expressed as an uptake heat-map under dark conditions. HDX-MS was performed on *Dm*Cry after exposure to a deuterated buffer solution for 0.25, 1, 30 and 60 minute incubation periods. Estimates of the uptake at each residue are shown having been normalised to the theoretical maximum and correspond to values between 0 % (yellow) and 63 % (red) as indicated in the colour bar. A sequence coverage of 80.5% was observed with regions of no coverage shown in grey. Secondary structure elements, α -helices, β-sheets, 310-helices (η), are depicted above the sequence. (**B**) The change in deuterium uptake for WT *Dm*Cry upon the continuous exposure to blue light displayed as a differential heat-map. A positive change (blue) corresponds to greater uptake for the blue light environment. Regions which undergo no change in deuterium incorporation are shown in white and some residues undergo minor protection and are shown in red. (**C**) Uptake difference after 15 s of labelling mapped onto the *Dm*Cry crystal structure (PDB: 4GU5). (**D**) Representative mass spectra are shown for 2 peptides, 501-509 and 530-535, in the C-terminal tail, α-23 and α-22 helices, which both show a large blue-light-induced increase in deuterium uptake. Dark spectra are shown in red with those exposed to blue light in coloured blue over the time course 0.25 - 60 min. Undeuterated time zero, t_0, spectra are shown on the bottom for reference. White regions indicate no coverage and the FAD/Trp-tetrad residues are shown in orange. The corresponding centroid extractions are plotted as a function of time. Reduced blue-light intensity uptake curves are also shown as darker shades of blue. From Ref. [30]

Comparison of the extent of H-D exchange in the wild-type protein and in mutants W342F and W394F in which either the third (Trp$_C$) or fourth (Trp$_D$) tryptophan in the electron transfer chain was replaced by phenylalanine seem to be consistent with [FAD$^{\bullet-}$ TrpH$_C^{\bullet+}$] being the magnetic sensor and [FAD$^{\bullet-}$ TrpH$_D^{\bullet+}$] being responsible for signalling.

Chemical amplification of magnetic field effects

Fluorescence-detection methods have been used to discover a new and potentially important amplification phenomenon. We have demonstrated that the primary magnetic field effect on flavin photoreactions can be chemically amplified by slow radical termination reactions under conditions of continuous photoexcitation.[31] The nature and origin of the amplification were revealed by studies of the intermolecular flavin-tryptophan and flavin-ascorbic acid photocycles and the closely related intramolecular flavin-tryptophan radical pair in cryptochrome. Amplification factors of up to 5.6 have been observed for magnetic fields weaker than 1 mT. We believe that substantial chemical amplification could have a significant impact on the viability of a cryptochrome-based magnetic compass sensor.

DROSOPHILA BEHAVIOUR IN MAGNETIC FIELDS

Frustratingly, much of the evidence for a radical-pair-based magnetic compass sense in migratory songbirds is circumstantial, largely due to the challenges posed by genetic modification of wild birds. For this reason *Drosophila* has been recruited as a model organism and there have been several reports of cryptochrome-mediated magnetic effects on fly behaviour that have been widely interpreted as support for a radical pair sensor in birds. Although it is not clear why flies should have evolved a magnetic sense, a broadly reproducible behavioural paradigm in *Drosophila* would greatly facilitate the search for the sensory molecules, genetic basis, and the neuronal pathways. We therefore decided to implement two of the published *Drosophila* behavioural assays in our own laboratories with the intention of extending them to include exposure to radiofrequency electromagnetic stimuli.

The first experiment is a binary-choice T-shaped maze assay developed by Gegear *et al.*[32-34] using an exact replica of the original apparatus. A ~500 μT magnetic field was applied in one arm of the maze, and no field in the other, by flowing identical currents parallel and antiparallel, respectively, through identical sets of double-wrapped coils to ensure that any minor heating effects would be the same in the two arms. The apparatus, together with white strip-lights, was contained in a wooden box placed inside an electromagnetically shielded chamber (4 m × 5 m × 2.5 m) in a wooden building in Oldenburg that attenuated background radiofrequency fields by a factor of 10^6 (Ref. [12]). Flies were tested in groups of ~100.

The second experiment was an attempt to replicate and extend observations (Fedele *et al.*[35]) of cryptochrome-dependent magnetic field effects on the ability of fruit flies to climb vertical surfaces (negative geotaxis). Two sets of equipment were constructed. In both, flies were monitored as they climbed inside transparent cylindrical tubes after being sucked (using a vacuum system) or knocked (using a mechanical shock) to the base of the tubes. In the former experiment (a modified version of that used by Fedele *et al.*), flies were tested individually. In the latter (closer in design to Fedele *et al.*), 10 insects were observed simultaneously. All measurements were performed in the magnetically shielded research facility in Oldenburg under meticulously controlled, double-blinded conditions.

In total, 64,241 flies were tested in the two-arm maze and 9,160 in the negative geotaxis apparatus. No statistically significant magnetic field effects were found. The conclusion of this extensive study is that *Drosophila* cannot be trained to associate a sucrose reward with a magnetic field using the Gegear *et al.*[32-34] paradigm and that *Drosophila* negative geotaxis is not affected by external magnetic fields. As a result, we seriously doubt whether *Drosophila* can sense magnetic fields at all and thus strongly suggest that night-migratory songbirds remain the organism of choice for elucidating the mechanism of light-dependent magnetoreception.

A manuscript has been written and will be submitted to a high profile journal as soon as some final control experiments have been completed.[36]

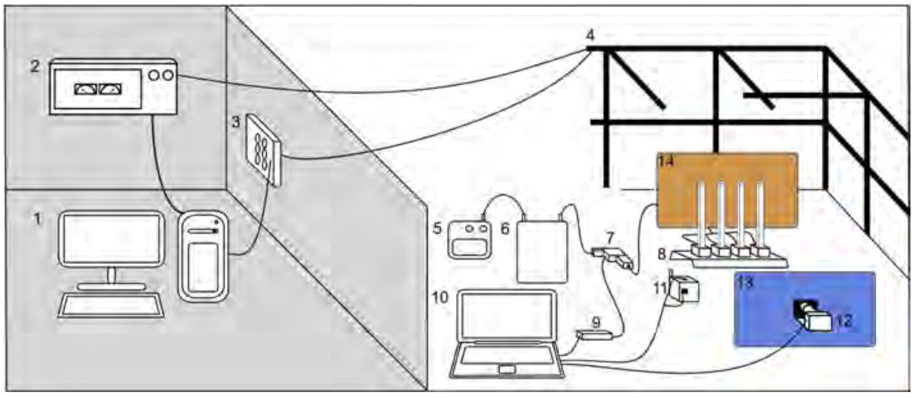

Figure 10. Schematic of the "FlyVac" apparatus for studying *Drosophila* negative geotaxis. The currents in the coils were controlled by an external computer (1) via two data acquisition (DAQ) cards. The first connected the computer to the power supplies (2) and controlled the magnitudes of the currents in the coils. The second controlled the switching box (3) responsible for the directions of the currents (parallel or antiparallel) through the windings of each coil (4). In the schematic, most of the 12 coils were omitted for clarity. A picture of the chamber and the coils can be found in Ref.[12] A MATLAB script controlled the two DAQ cards (a) to provide an analogue output to the power supply and (b) to switch the direction of the currents via a digital output. Other components (described above): vacuum pump (5), buffer tank (6), solenoid valve (7), FlyVac apparatus (8), relay (9), and a laptop (10) that controlled the valve, sensors (11) and video camera (12). One LED plate (13) illuminated the tubes; the other (14) provided an infrared background for the video camera. The gravity set-up, the LEDs plates, the camera, the sensors and the valve were placed on a table in the middle of the coils as far away from them as possible. From Ref. [36]

COMPUTATIONAL STUDIES

Throughout the project, experimental work has been supported and complemented by theoretical and computational studies of the spin dynamics of cryptochrome-based radical pairs.

Electron spin relaxation

A crucial aspect of the cryptochrome hypothesis is that the electron spin coherence with which the radical pairs are created persists for long enough to allow the geomagnetic field to affect the singlet-triplet interconversion. At the time the AFOSR grant was awarded, very little was known about the mechanisms and rates of electron spin relaxation — the process that restores the spins to thermal equilibrium — in radical pair states of cryptochromes.

We started by combining all-atom molecular dynamics simulations, Bloch-Redfield relaxation theory and spin dynamics calculations to assess the effects of spin relaxation on the performance of cryptochrome as a compass sensor.[37] Both flavin-tryptophan and flavin-$Z^\bullet$ radical pairs were studied ($Z^\bullet$ is a radical with no hyperfine interactions). Relaxation was considered to arise from modulation of hyperfine interactions by librational motions of the radicals and fluctuations in certain dihedral angles. For *Arabidopsis thaliana* cryptochrome 1 (*At*Cry1) we found that spin relaxation implies optimal radical pair lifetimes of the order of microseconds, and that flavin-$Z^\bullet$ pairs are less affected by relaxation than flavin-tryptophan pairs. Our results also demonstrate that spin relaxation in isolated *At*Cry1 is incompatible with the long coherence times that have been postulated to explain the disruption of the avian magnetic compass sense by weak radiofrequency magnetic fields. We conclude that a cryptochrome sensor *in vivo* would have to differ dynamically, if not structurally, from isolated *At*Cry1. Our results clearly mark the limits of the current cryptochrome hypothesis and lead to a better understanding of the operation of radical pair magnetic sensors in noisy biological environments.

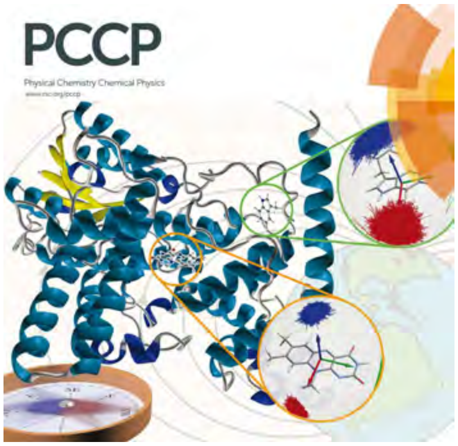

Figure 11. *Phys. Chem. Chem. Phys.* cover illustration[37] showing representations of the orientational phase space sampled by small-angle fluctuations of the positions of the aromatic rings of the flavin and Trp_C radicals in *At*Cry1. Motions such as these cause loss of spin coherence in the flavin-Trp_C radical pair.

Loss of coherence, resulting from interactions of the spins with their fluctuating environment, is generally assumed to degrade the sensitivity of a radical pair compass to the direction of the Earth's magnetic field. It is also possible that certain spin relaxation mechanisms can enhance its performance. Focussing on the flavin–tryptophan radical pair in *At*Cry1, correlation functions for fluctuations in the distance between the two radicals were obtained from molecular dynamics (MD) simulations and used to calculate the spin relaxation caused by modulation of the exchange and dipolar interactions. We found that intermediate spin relaxation rates afforded substantial enhancements in the sensitivity of the reaction yields to an Earth-strength magnetic field. Supported by calculations using toy radical pair models, we argue that these enhancements could be consistent with the molecular dynamics and magnetic interactions in avian cryptochromes.[38]

In a related study,[39] we combined Redfield relaxation theory with analytical solutions of a rotational diffusion equation to assess the impact of restricted rotational motion of the radicals on the operation of the cryptochrome compass. We concluded (a) that relaxation is slow when rotational motion of the radicals within the protein is fast and highly constrained; (b) that in a regime of slow relaxation, the motional averaging of hyperfine interactions has the potential to improve the sensitivity of the compass; and (c) that consideration of motional effects can significantly alter the design criteria for an optimal compass. In addition, we showed that motion of the flavin radical is likely to be compatible with its role as a component of a functioning radical-pair compass, whereas the motion of the tryptophan radical seems less ideal, unless it is particularly fast.

The magnetically sensitive species in cryptochrome *in vivo* is commonly assumed to be $[\mathrm{FAD}^{\bullet-}\ \mathrm{TrpH}^{\bullet+}]$, formed by sequential light-induced intraprotein electron transfers from a chain of tryptophan residues to the flavin adenine dinucleotide chromophore. However, some evidence points to superoxide, $O_2^{\bullet-}$, as an alternative partner for the flavin radical. The absence of hyperfine interactions in $O_2^{\bullet-}$ could lead to a more sensitive magnetic compass, but only if the electron spin relaxation of the $O_2^{\bullet-}$ radical is much slower than normally expected for a small mobile radical with an orbitally degenerate electronic ground state. In a recently published study we used spin dynamics simulations to model the sensitivity of a flavin-superoxide radical pair to the direction of a 50 μT magnetic field.[40] By varying parameters that characterize the local environment and molecular dynamics of the radicals, we identified the highly restrictive conditions under which a $O_2^{\bullet-}$-containing radical pair could form the basis of a geomagnetic compass sensor. We concluded that the involvement of superoxide in compass magnetoreception must remain highly speculative until further experimental evidence is forthcoming.

Amplification of magnetic field effects by radical scavengers

We have proposed a new version of the current model of radical pair magnetoreception in which spin-selective radical recombination reactions are not essential.[41] One of the two radicals is imagined to react with a paramagnetic scavenger via spin-selective electron transfer. By means of simulations of the spin dynamics of cryptochrome-inspired radical pairs, we showed that the new scheme offers two clear and important benefits. The sensitivity to a 50 μT magnetic field is greatly enhanced and, unlike the current model, the radicals can be more than 2 nm apart in the magnetoreceptor protein. The latter means that animal cryptochromes that have a tetrad, rather than a triad, of tryptophan electron donors can still be expected to be viable as magnetic compass sensors. Lifting the restriction on the rate of the spin-selective recombination reaction also means that the detrimental effects of inter-radical exchange and dipolar interactions can be minimised by placing the radicals much further apart than would be possible in the current model.

Subsequently, we have shown that this model is more resilient to spin relaxation than the conventional radical pair mechanism.[42] Surprisingly, the scavenging reaction can protect the spin system against the effects of fast spin relaxation in one of the radicals, thereby lending credibility to the suggestion that radical pairs, such as $[\mathrm{FADH}^{\bullet}\ \mathrm{O}_2^{\bullet-}]$ could be involved in radical-pair based magnetoreception. This finding may help explain behavioural observations that appear to be incompatible with the more conventional $[\mathrm{FAD}^{\bullet-}\ \mathrm{TrpH}^{\bullet+}]$ radical pair.

A "quantum needle"

Previous theoretical descriptions of radical pairs have been unable to account for the high precision with which birds are able to detect the direction of the Earth's magnetic field. Suspecting that this might stem from the use of highly simplified models of the hyperfine interactions within the radicals, we set out to simulate the behaviour of more realistic cryptochrome-based radical pairs. We found that when the spin coherence persists for longer than a few microseconds, the output of the sensor contains a sharp feature, referred to as a spike.[43] The spike arises from avoided crossings of the quantum mechanical spin energy-levels of radicals formed in cryptochromes. Such a feature could deliver a heading precision sufficient to explain the navigational behaviour of migratory birds in the wild. Our results (i) afford new insights into radical pair magnetoreception, (ii) suggest ways in which the performance of the compass could have been optimized by evolution, (iii) may provide the beginnings of an explanation for the magnetic disorientation of migratory birds exposed to anthropogenic electromagnetic noise, and (iv) suggest that radical pair magnetoreception may be more of a quantum biology phenomenon than previously realized. This work, published in *Proc. Natl. Acad. Sci. USA* under the title *The quantum needle of the avian magnetic compass*, was highlighted on the front cover of the journal and has been cited 73 times.[43] It also attracted a certain amount of media interest.

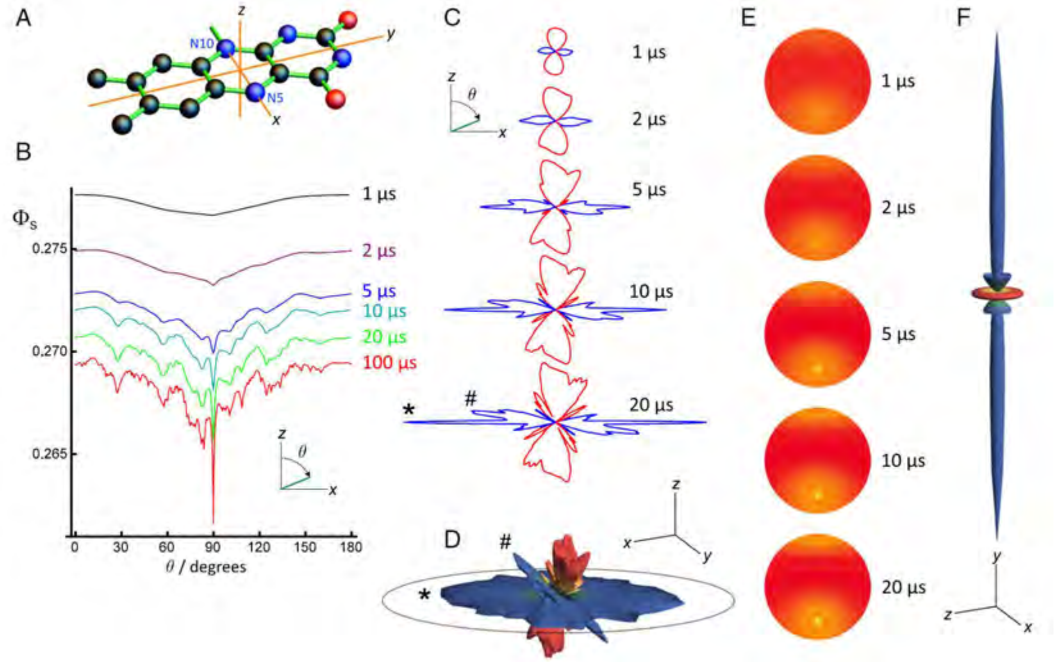

Figure 12. Reaction yields of a [FAD$^{\bullet-}$ TrpH$^{\bullet+}$] radical pair. (**A**) The axis system used in the simulations superimposed on the tricyclic flavin ring system. (**B**) The variation of the singlet yield, Φ_S, with the field direction, θ, for radical pairs with lifetimes between 1 and 100 μs. For clarity, two of the traces have been offset vertically: by –0.001 (light green) and –0.002 (red). θ specifies the direction of the magnetic field in the *zx* plane of the flavin. (**C**) The same data as in B (1- to 20-μs lifetimes) presented as 2D polar plots. In each case, only the anisotropic part of Φ_S is shown, with red and blue indicating values, respectively, larger and smaller than the isotropic value. The five plots are drawn on the same scale. The blue features at θ = ±90° (labeled * in the 20-μs plot) are the spikes. (**D**) The anisotropic part of Φ_S (10-μs lifetime) presented as a 3D polar plot. A circle in the *xy* plane (θ = 90°) is included as a guide to the eye. The blue disk in the *xy* plane (labeled *) gives rise to the spike. The smaller blue disk, labeled # (also in C), angled at ∼40° to the *xy* plane, comes principally from the N1 indole nitrogen of TrpH$^{\bullet+}$. Its tilt reflects the orientation of the indole group of the tryptophan relative to the flavin. (**E**) Visual modulation patterns calculated from Φ_S (1- to 20-μs lifetimes) representing the directional information available from an array of cryptochrome-containing magnetoreceptor cells distributed around the retina. The bright spot in the lower half of the pattern arises from the spike. (**F**) 3D polar plot of Φ_S (10-μs lifetime) averaged over a 360° rotation around an axis in the *xy* plane. This object has been rotated by 90° relative to D and scaled up by a factor of 2.1. The patterns in E were calculated using the same averaging procedure. From Ref. [43]

Separating the effects of light and magnetic stimuli

A potentially serious but largely ignored complication in the radical pair model is how directional information derived from the Earth's magnetic field can be separated from the much stronger variations in the intensity and polarization of the incident light. We have proposed a simple solution to this problem in which these extraneous effects are cancelled by taking the ratio of the signals from two neighbouring populations of magnetoreceptors.[44] Geometric and biological arguments were used to derive a set of conditions that make this possible. We argued that a likely location of the magnetoreceptor molecules would be in association with ordered opsin dimers in the membrane discs of the outer segments of double-cone photoreceptor cells.

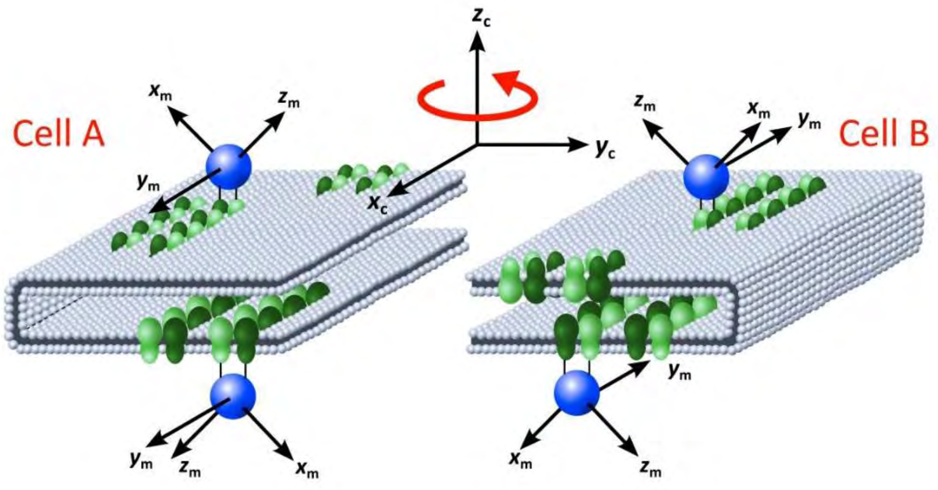

Figure 13. Schematic picture of the hypothetical interaction of cryptochrome magnetoreceptors (blue spheres) with aligned tracks of rhodopsin dimers (green) embedded in the membrane discs in the outer segments of photoreceptor cells. The figure shows a small section of one of the discs in two neighbouring cells, A and B, related by a 180° rotation around the cell axis, zc, which coincides with the direction of the incoming light. The cryptochrome molecule is imagined to bind identically to the top and bottom layers of the discs. Consequently, binding to rhodopsin tracks in the top and bottom of the membrane discs could fix the cryptochrome molecules into one of two mirror image orientations in each cell. The probability of absorbing a photon is the same for all the cryptochromes in both cells. However, cells A and B will respond differently to an external magnetic field due to the differing orientations of the cryptochrome z-axes. Thus, taking the ratio of the outputs from the two cells will give a signal that depends solely on the magnetic field conditions. From Ref. [44]

Superparamagnetic nanoparticles as a test for cryptochrome-based magnetoreception

Although there is evidence that cryptochromes are involved in the magnetosensory pathway, these experiments do not prove that they are the magnetic sensors: they could, instead, play a non-magnetic role as signal transducers downstream of a different the primary sensor. We have proposed an experiment with the potential to distinguish these functions. The principle is to use superparamagnetic nanoparticles to disable any magnetic sensing role by enhancing the electron spin relaxation of the radicals so as to destroy their spin correlation. We used spin dynamics simulations to show that magnetoferritin, a synthetic, protein-based nanoparticle, has the required properties.[45] If cryptochrome is the primary sensor, then it should be inactivated by a magnetoferritin particle placed 12-16 nm away. This would prevent a bird from using its magnetic compass in behavioural tests and abolish magnetically sensitive neuronal firing in the retina. The key advantage of such an experiment is that any signal transduction role should be completely unaffected by the tiny magnetic interactions ($<< k_B T$) required to enhance the spin relaxation of the radical pair.

How quantum is radical pair magnetoreception?

Radical pair magnetoreception has frequently been touted as an example of "quantum biology" on the basis that the electrons must exist in a coherent spin state. We were therefore interested to see whether one can *only* account for the coherent spin dynamics using quantum mechanics. We found that semiclassical approximations to the spin dynamics of radical pairs only provide a satisfactory description of the anisotropic product yields when there is no electron spin–spin coupling, a situation unlikely to be consistent with a magnetic sensing function.[46,47] Although semiclassical methods perform reasonably well for shorter-lived radical pairs with stronger electron-spin coupling, for which it is valid to treat the hyperfine interactions as a perturbation, the accurate simulation of anisotropic magnetic field effects relevant to magnetoreception seems to require full quantum mechanical calculations. We therefore conclude that radical pair avian magnetoreception may indeed have a place in "quantum biology".

Information theory assessment of the precision of a radical pair compass

It is an open question whether a mechanism based on photo-induced radical pairs in cryptochromes could be sufficiently sensitive given the low-light levels experienced by nocturnal migrants. The scarcity of available photons results in significant uncertainty in the signal generated by the magnetoreceptors distributed around the retina. We have used results from Information Theory to obtain a lower bound estimate of the precision with which a bird could orient itself using only geomagnetic cues.[48] Our approach bypasses the current lack of knowledge about magnetic signal transduction and processing *in vivo* by computing the best-case compass precision under conditions where photons are in short supply. The method was used to assess the performance of three plausible cryptochrome-derived flavin-containing radical pairs as potential magnetoreceptors. Two main conclusions emerged. First, the low photon flux on a clear moonless night appears to pose a significant sensitivity challenge for a light-dependent magnetoreceptor, whatever the identity of the radical pair. Second, our analysis suggests that the flavin-tryptophan radical pair that accounts for the magnetic sensitivity of purified cryptochromes is less well suited as a sensor than a radical pair in which the tryptophan radical has been replaced by one containing no or very few hyperfine couplings.

Ascorbic acid as an electron donor

We have previously suggested that ascorbate ions could interact with surface-exposed tryptophan radicals in photo-excited cryptochromes leading to the formation of a radical pair comprised of the reduced form of the flavin adenine dinucleotide cofactor, $\mathrm{FAD}^{\bullet-}$, and the ascorbate radical, $\mathrm{Asc}^{\bullet-}$.[16] The idea is that this species could provide a more sensitive compass than a $[\mathrm{FAD}^{\bullet-}\ \mathrm{TrpH}^{\bullet+}]$ radical pair. We have used molecular dynamics simulations of *Dm*Cry and *Er*Cry1a, to characterize the transient encounters of ascorbate ions with tryptophan radicals in cryptochrome in order to assess the likelihood of the $[\mathrm{FAD}^{\bullet-}\mathrm{Asc}^{\bullet-}]$ pathway.[49] We showed that ascorbate ions are expected to bind near the tryptophan radicals for periods of a few nanoseconds. The rate at which these encounters happen is low, and it is therefore concluded that ascorbate ions are unlikely to be involved in magnetoreception if the ascorbate concentration is ≤ 1 mM.

CRYPTOCHROME INTERACTION PARTNERS

Refining the identification of putative Cry4 interaction partners

The aim of this part of the project was to identify *de novo* potential Cry interacting proteins in the avian retina focussing primarily on European robin Cry4. We used the yeast-two-hybrid system (Y2H)[50] to screen avian cDNA libraries without relying on any preconceived prediction about the nature or characteristics of the *Er*Cry4 interacting proteins. In the initial phase of the project, we compared the UAS-GAL4 system with the split-ubiquitin system, which would have been a reasonable choice as it can be applied for membrane associated protein-protein interaction processes. However, the split-ubiquitin system proved to be less valid and was therefore not applied in our screening efforts.[51] For validation of the screening systems, we tested first whether yeast cells transfected separately by each single Y2H vector construct could express the expected bait or prey proteins. Such expression is essential for the Y2H approach to work. Furthermore, we tested for any background signals due to auto-activation by comparing the immunoblot results of empty Y2H vectors with *Er*Cry4 containing vectors (Figure 14). The UAS-GAL4 system showed strong expression with both plasmids (bait and prey proteins). Further, a serial drop test (see Figure 1B in Wu *et al.*, 2020)[51] for any background interaction of *Er*Cry4 with empty Y2H vectors and thus with any unspecific proteins present in the yeast cells was also applied. In conclusion, the GAL4 system showed no background interaction of *Er*Cry4 under all tested conditions. Thus, the UAS-GAL4 system turned out to be a valid choice for Y2H screening of *Er*Cry4 interaction partners.

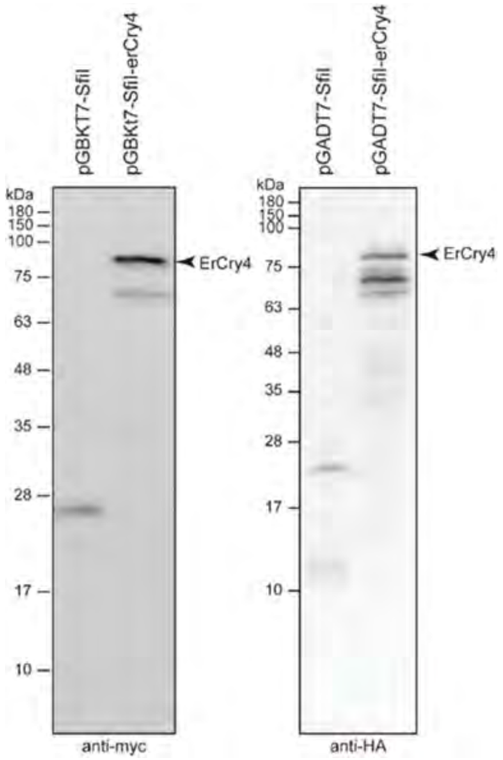

Figure 14. Testing the usability of the UAS-GAL4 Y2H system in screening for *Er*Cry4 interaction partners. Western blots demonstrate the expression of *Er*Cry4 (arrow) using the pGBK7-SfiI (left panel) or the pGADt7-SfiI vector (right panel). No expression was seen in the empty control vector (left lanes in both panels). The HA antibody (1:500) was used to detect pGADT7-SfiI expressed proteins and the myc antibody (1:500) was used to detect pGBKT7 expressed proteins.[51]

An overview of the workflow featuring the steps of transformation, screening, background control and validation of the Y2H screening approach is shown in Figure 15 and will be described in more detail below.

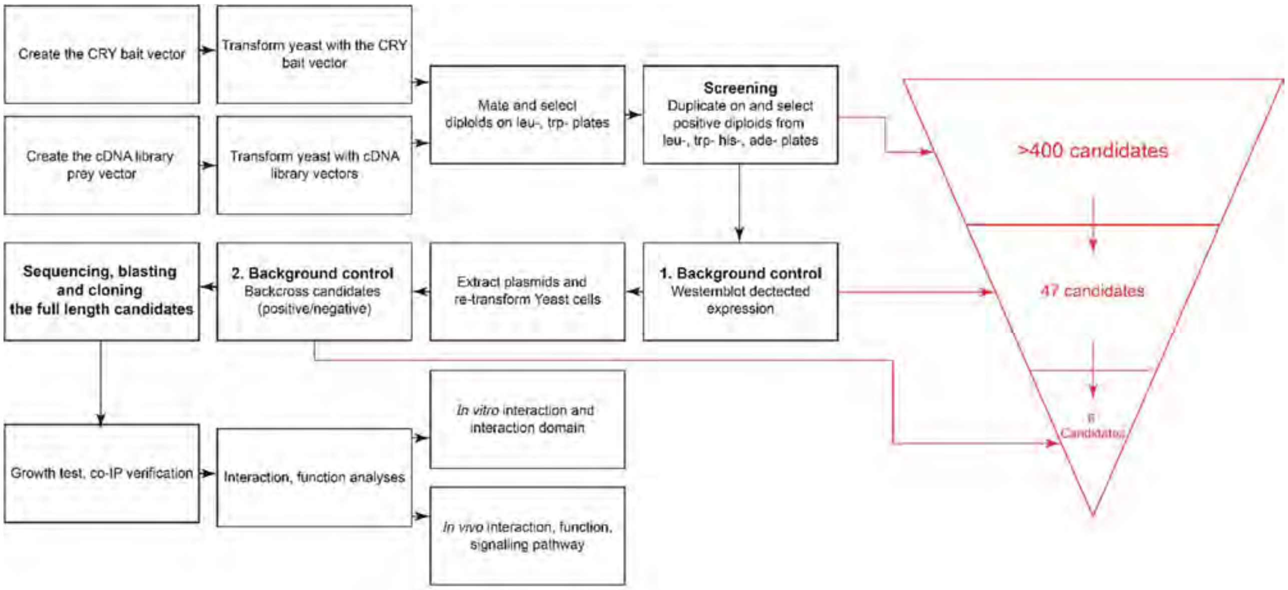

Figure 15. Work flow chart of the screening approach that was used for the Y2H system.

False positive results as background are common in Y2H screening; they are, however reducible. We followed several steps to avoid potential false positive results. RNAs smaller than 1kb have been removed from total RNA; expression of putative candidates have been verified through immunoblotting and polypeptides smaller than 10 kDa were not considered further; self-activation of potential candidates leading to signals with an empty vector have been removed from the list. Indeed, a large number of candidates (more than 400) showed up at early stages of the Y2H procedure. However, applying these rigorous selection procedures, we narrowed down the number of potential candidates to six. These six proteins appeared as highly specific candidates for interacting with *Er*Cry4. Representative results are shown in Figure 16 in the form of a drop test. Cells were diluted to the concentration of 10^6 cells/mL in sterilized H_2O and 10 µL cell suspension has been used for each drop.

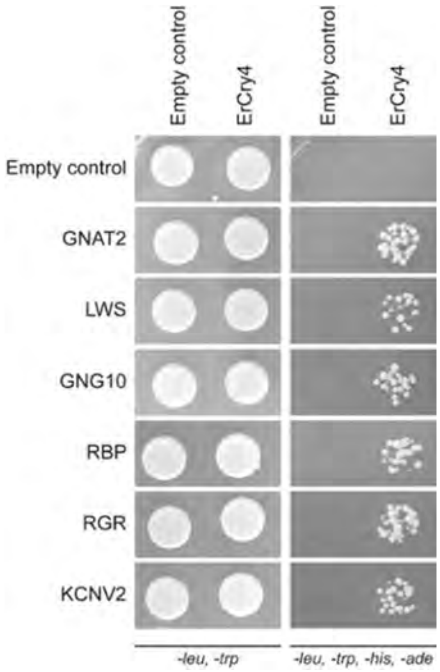

Figure 16. Testing of the six candidates selected as potential interaction partners of *Er*Cry4. (A) Full length genes of the candidates were cloned into the pGADT7-SfiI vector. Left: All yeast strains grew on the control plates as expected. Right: All the six full-length candidates interacted with *Er*Cry4 but not with the empty vectors or when grown on plates lacking leucine, histidine, tryptophan and adenine, demonstrating a specific interaction (the two vectors support growing of yeast cells on plates lacking leucine and histidine due to their selective markers). A positive interaction triggers the reporter gene expression leading to a growth on the plates lacking tryptophan and adenine. Different plates are displayed based on the candidate genes that were selected from more than 400, which were found at early stages of the screening efforts.

Blast alignments (NCBI) of the sequences of these candidates revealed their phylogenetic homology to other genes of close bird species. Genes code for the following proteins (Table 1): guanine nucleotide-binding protein G(t) subunit alpha-2 (GNAT2), long-wavelength opsin (LWS), guanine nucleotide-binding protein subunit gamma 10 (GNG10), potassium voltage-gated channel subfamily V member 2 (KCNV2), retinol binding protein 1 (RBP1) and retinal G protein-coupled receptor (RGR).

Table 1. Potential *Er*Cry4 interaction candidates resulted from current screening

Candidate	Most similar ORF	Species	Identity	Gene	NCBI Reference
1	GNAT2	*Ficedula albicollis*	97.18%	G protein subunit alpha transducin 2	XM_005059369.1
2	LWS	*Taeniopygia guttata*	93.63%	long wavelength sensitive opsin	NM_001076702.1
3	GNG10	*Numida meleagris*	98.07%	G protein subunit gamma 10	XM_021379609.1
4	RBP1	*Sturnus vulgaris*	97.79%	retinol binding protein 1	XM_014870256.1
5	RGR	*Ficedula albicollis*	96.28%	retinal G protein coupled receptor	XM_005048136.2
6	KCNV2	*Ficedula albicollis*	98.72%	Potassium voltage-gated channel subfamily V member 2	XP_016160160.1

In addition, an interaction of *Er*Cry4 was also identified for two additional candidates assigned as RLBP1 and KCNG4. These genes code for retinaldehyde-binding protein 1 and a voltage-gated potassium channel of the G subfamily. Currently, we lack the full-length gene information of these candidates. Thus, we focussed subsequent work on the six candidates in Table 1 and tested which interact more specifically with the C-terminus of *Er*Cry4 using the same drop test as in Figure 16. Watari *et al.*[52] predicted the C-terminus of chicken Cry4 to be a structurally flexible target region, which could be the case for the robin orthologue as well. Our results showed that the C-terminus of *Er*Cry4 might specifically interact with proteins coded by genes LWS, RGR and KCNV2 (Figure 17).

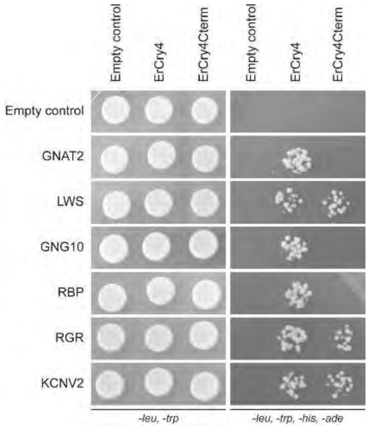

Figure 17. Drop tests to exam the interaction between full lengths *Er*Cry4 or the C-terminus of *Er*Cry4 (*Er*Cry4 468-527) with the potential interaction partners.

Interaction of candidate *Er*Cry4 interaction partners with other cryptochromes

To test whether the identified Cry4 interaction partner candidates also interact with any of the other cryptochromes known to be expressed in the European robin retina, we tested whether *Er*Cry1a, *Er*Cry1b and *Er*Cry2 interacted with each of these six candidates. Drop tests as described above and like the one shown in Figure 18 revealed no specific interaction of *Er*Cry1a and *Er*Cry2 with any of the six candidates (Figure 17). Interaction of *Er*Cry1b was inconclusive as it showed an interaction with all six candidates, but also with the empty control vector, suggesting a general unspecific interaction generated by *Er*Cry1b itself. This suggests that *Er*Cry4 differs from the other Cry forms in European robin, and that *Er*Cry4 interacts with its own set of specific interaction partners.

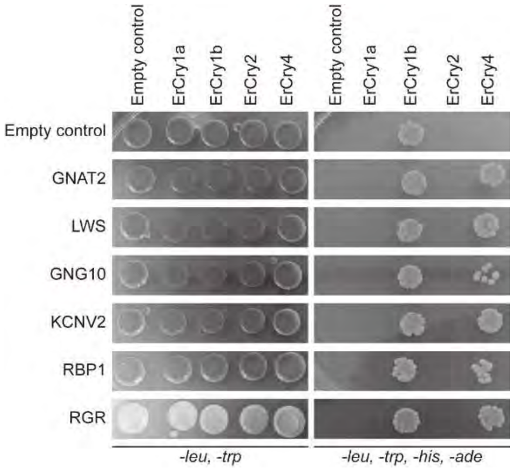

Figure 18. Testing for putative interactions between various Cry forms and putative interaction partners of *Er*Cry4. Experimental details are described in Wu *et al*.[51] Yeast strains containing the corresponding plasmids mated and 10^4 of the formed diploid cells were dropped on to SD-leu-trp and SD-leu-trp-his-ade plates. pGBKT7-SfiI and pGADT7-SfiI empty plasmids were used as negative controls. Although interactions were observed only between *Er*Cry1b and *Er*Cry4 with the putative interaction partners, there is also an interaction visible between *Er*Cry1b and the empty control vector.

Interaction of *Er*Cry4 with IscA1 and clock proteins

A current controversial issue in the field of magnetoreception is the role of the iron-sulphur protein IscA1 that was suggested to interact with pigeon Cry4. However, whether this interaction also exists in European robin is yet unknown. Using our Y2H system we tested whether different Crys and IscA1 proteins can form a complex (Figure 19). Putative interactions between Cry and IscA1 variants were probed by a drop-test as described above indicating an interaction between *Dm*Cry and IscA1 from *Drosophila* and robin, respectively (Figure 19). However, we found no evidence for an interaction between Cry4 and IscA1 from both European robin and chicken. The binding of murine p53 with the SV40 large T-antigen served as positive control.

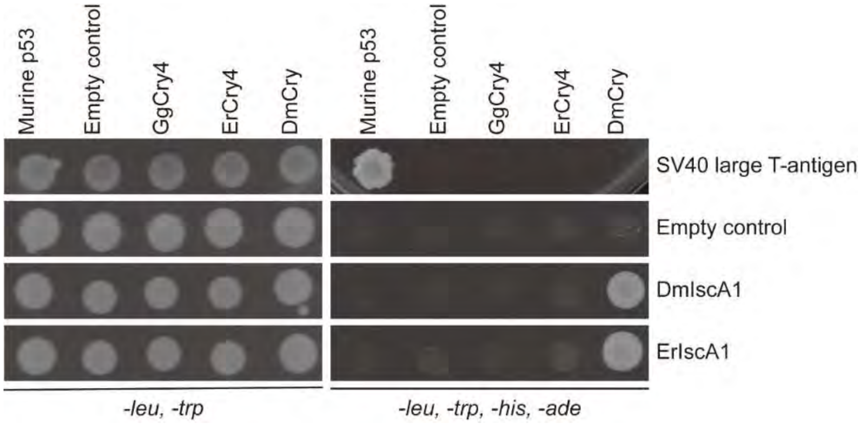

Figure 19. Putative interactions between Cry and IscA1 variants.

Moreover, circadian and seasonal expression profiles of *Er*Cry isoforms showed that *Er*Cry4 expression would not be circadian-rhythm-based, in contrast to Cry1a, Cry1b and Cry2.[26] To test this conclusion by an independent approach, we used the Y2H assay to identify the interaction between a clock protein (period protein 2, Per2) and *Er*Cry1a and *Er*Cry4. The Y2H test showed an interaction between Per2 and Cry1a, but not with Cry4 (Figure 20), strongly supporting the previous conclusion that Cry4 might be involved in magnetic sensing rather than regulation of circadian rhythm.

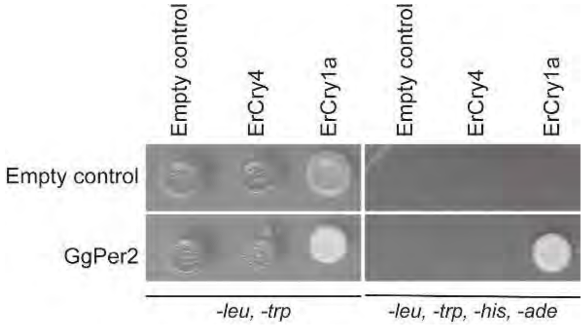

Figure 20. Test of interaction between Per2 and *Er*Crys.

Heterologous expression and purification of candidates for functional analysis

All six genes listed in Table 1 have been cloned into variable carrier vectors, for expression either in prokaryotic or eukaryotic cells. Three candidate genes (GNAT2, GNG10, RBP1) are predicted to code for non-membrane proteins. For these genes, a prokaryotic expression system has been applied and genes have been cloned into the pCold vector. SBL21 cells have been used for protein expression under the induction of low temperature (15 °C). However, expression of G(t) subunit alpha-2 in bacteria resulted in an insoluble pellet, which is typically observed for the vertebrate photoreceptor G protein transducin, but every other type of G protein alpha-subunit can be expressed in *E. coli* in soluble and active form. This problem had been solved in the past by constructing a chimeric G protein alpha-subunit, in which the amino acid sequences of the regions causing aggregation were exchanged.[53] We performed the corresponding cloning for the European robin G(t) subunit alpha-2 using sequences from an inhibitory G protein alpha subunit. The chimeric protein was to a large extent soluble after expression in *E. coli* and could be purified (Figure 19). It undergoes a conformational change triggered by binding of the transition state analogue $\text{GDP}\cdot\text{AlF}_4^-$, which we could detect by tryptophan fluorescence (data not shown). Thus, the protein was suitable for subsequent protein-protein interaction studies.

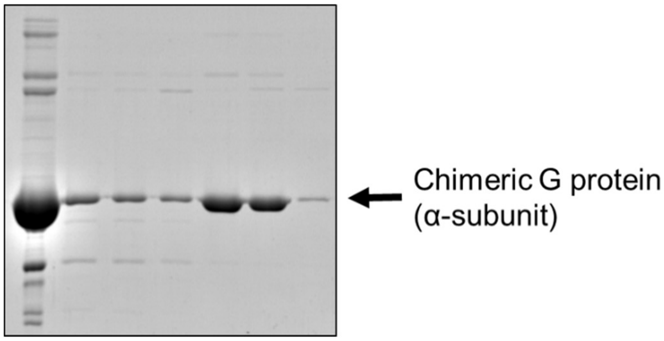

Figure 21. Chimeric G protein fractions obtained after purification using column chromatography. Samples were analysed by polyacrylamide gel electrophoresis and Coomassie blue staining.

Protein-protein interaction studies of Cry4 and putative binding partners

For interaction studies we used surface plasmon resonance (SPR), a biosensor based technique that allows to monitor binding events in real-time by recording association and dissociation rates of a binding process. For this purpose, one binding partner (e.g. G protein alpha subunit) is immobilized via amine-coupling chemistry on the sensor chip surface that contains activated carboxy-groups (details of the general procedure are described in Koch[54]). The second binding partner (e.g. purified Cry4) is applied in capillary flow system and flushed over the surface (Figure 22). Any binding event is monitored as a change in resonance units (RU), dissociation of the formed complex is triggered by flushing the surface with the running buffer solution (Figure 23). Preliminary results as shown in Figure 23 showed a binding process between the chimeric G protein alpha-subunit 2 and *Er*Cry4 of moderate affinity (apparent K_D-value was in the lower micromolar range).

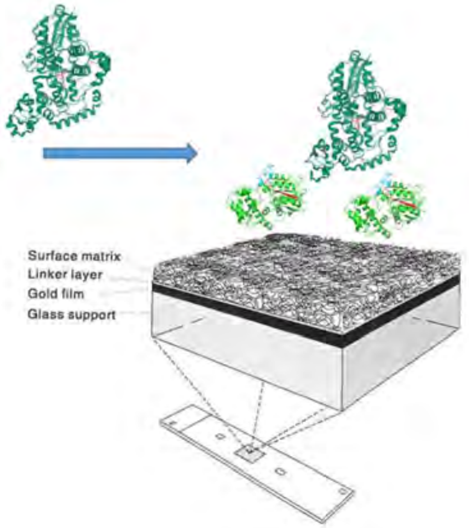

Figure 22. SPR sensor chip containing glass support, a gold film and a carboxy-modified dextran layer as surface matrix. The chimeric G protein alpha subunit-2 was immobilized and purified *Er*Cry4 was applied in the analyte phase.

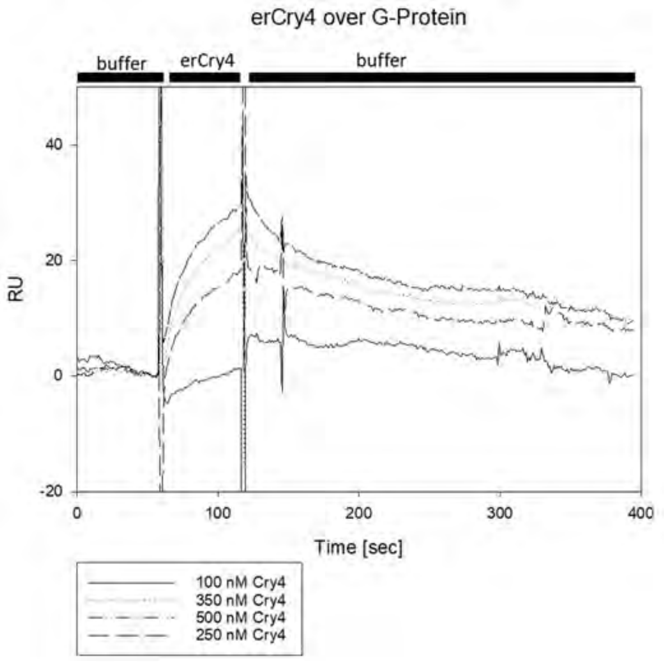

Figure 23. Sensorgrams showing interaction of *Er*Cry4 with chimeric G protein alpha subunit-2 in the analyte phase. Black bars indicate buffer flow or injection of *Er*Cry4 at concentrations shown below the sensorgram image.

For membrane proteins listed in Table 1, we use a eukaryotic expression system. Corresponding genes will be cloned into the following plasmids for transfection: pIRES:GFP, pcDNA3.1 and pcDNA3.1-HA. Plasmids contain an additional CACC ribosome entry site to enhance their expression. These plasmids have been used for transient expression in HEK cells or to create HEK cell lines with stable expression of those genes. A HEK cell line expressing LWS in a pcDNA3.1-HA vector is in progress, but the amount of the red cone opsin protein (gene LWS in Table 1) so far was not sufficient for protein-protein interaction studies using biophysical methods (e.g. SPR). While these expression trials are still in progress, we used instead synthetic peptides that cover the four cytoplasmic loops of European robin red cone opsin (Figure 24) for interaction studies. In a test series involving all cone opsin derived peptides and a peptide with a scrambled sequence as control, we detected only an interaction of peptide no. 2 (yellow loop in Figure 24 named LWO-2) with Cry4 (Figure 25). All other peptides showed no binding event. Interestingly, Cry4 from European robin and from pigeon showed similar changes in RU indicating a similar binding process (Figure 25).

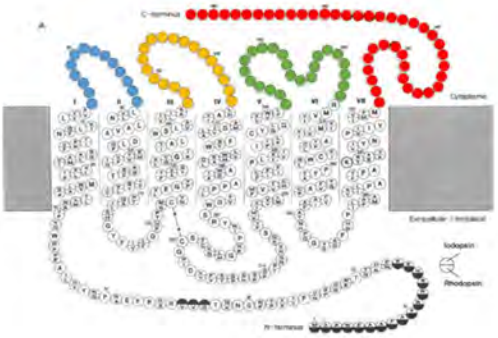

Figure 24. Schematic drawing of cone opsin topology. Putative cytoplasmic loops are shown in colours blue, yellow, green and red. Based on the amino acid sequence of European robin cone opsin synthetic peptides covering the cytoplasmic loop regions were used in interaction studies.

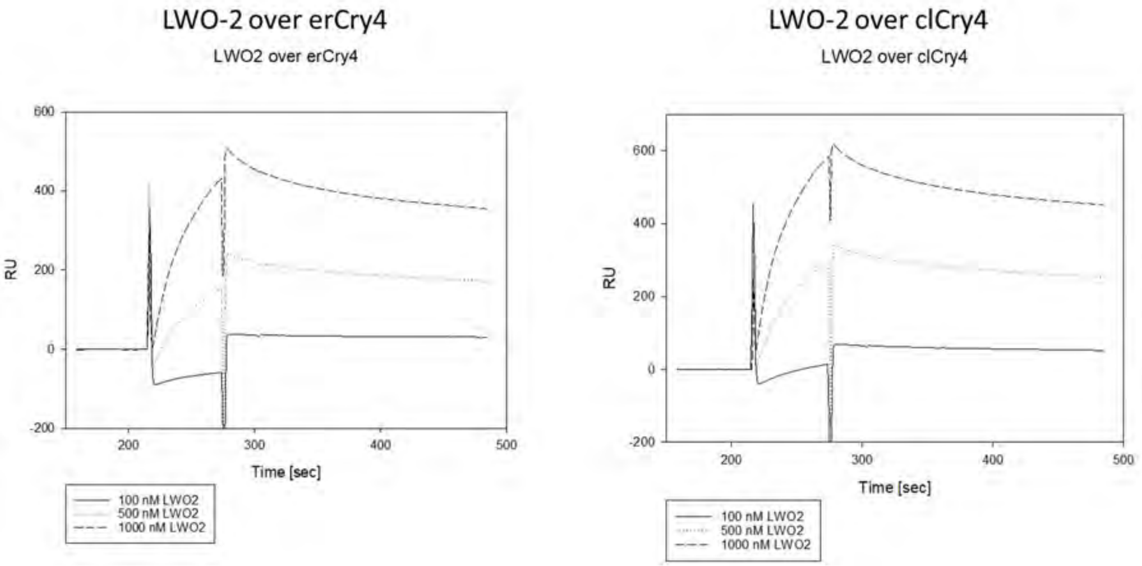

Figure 25. Sensorgrams showing interaction of cone opsin peptide no. 2 (LWO-2) with European robin and pigeon Cry4 (*Er*Cry4 and *Cl*Cry4). The peptide was supplied in the analyte phase in different concentrations as shown below the sensorgram image.

Summary

Four of the six candidates represent proteins that typically operate in signaling pathways. Long wavelength-sensitive opsin (red opsin) is the photoreceptor present in long wavelength sensitive cones. It is noteworthy that the interaction of *Er*Cry4 with red opsin matches the localization of *Er*Cry4 in long wavelength single cones.[26] Two subunits of heterotrimeric cone G protein transducin ($G_t\alpha$ and $G_t\gamma$) were also identified in the screening (Figure 26A). A further candidate gene listed in Table 1 is KCNV2 coding for the voltage-gated potassium channel $K_v8.2$, which is the modifying subunit in heteromeric delayed rectifier potassium channels forming complexes with the K_v2 channel family subunits (Figure 26B). The other two, retinal binding protein and retinal G protein coupled receptor, are involved in the transport and isomerization of the chromophore all-*trans*-retinal. Therefore, they are components of the visual cycle (Figure 26C).

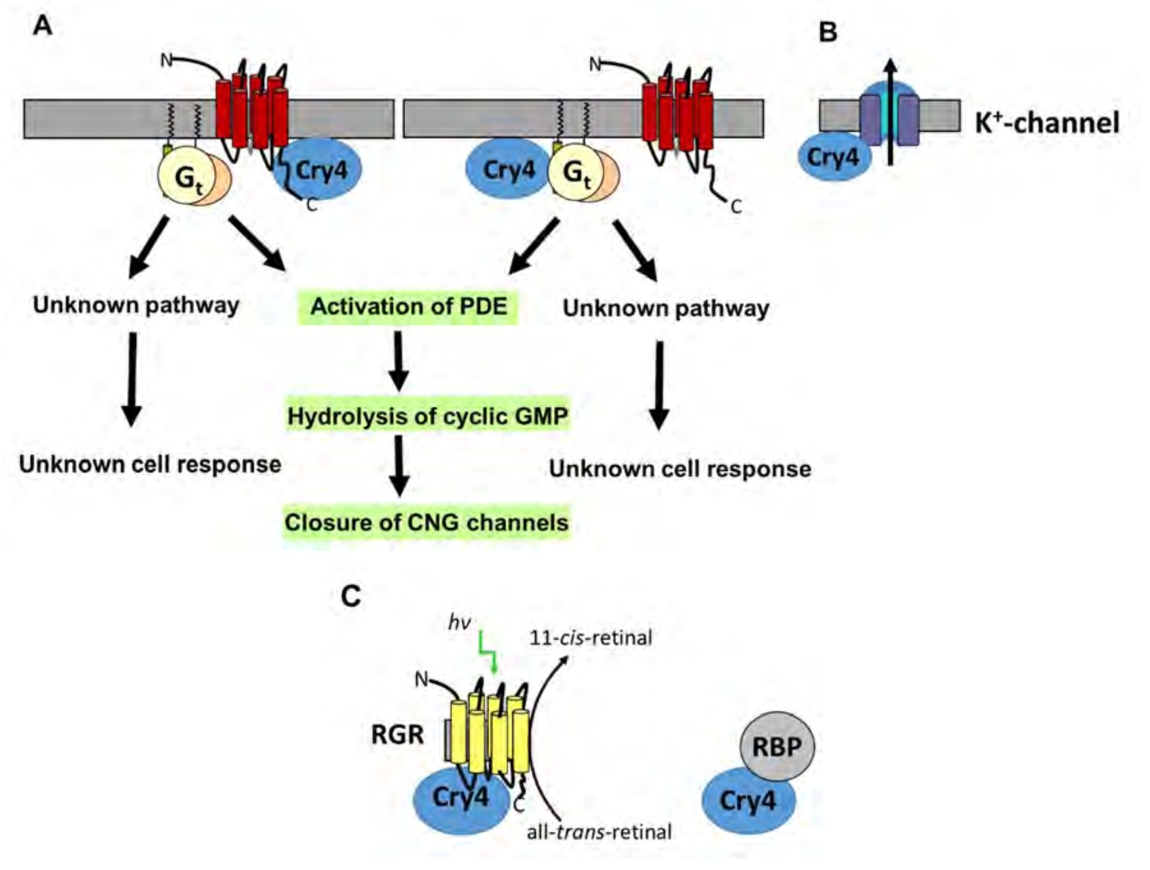

Figure 26. Potential signalling pathways in magnetoreception involving *Er*Cry4 and its hypothetical interaction partners. (A, left scheme) *Er*Cry4 might form a complex with long-wavelength-sensitive opsin triggering activation of G(t) subunit alpha-2 ($G_t\alpha$)-mediated phototransduction. Alternatively, interactions between *Er*Cry4 and $G_t\alpha$ could also lead to activation of downstream signalling proteins not involving opsin (A, right scheme). The downstream events could be part of the classical phototransduction pathway leading to closure of cyclic nucleotide-gated (CNG) channels via hydrolysis of cGMP. However, an unknown pathway leading to an unknown cell response could be involved. Both $G_t\alpha$ and guanine nucleotide-binding protein subunit gamma 10 ($G_t\gamma$) are attached to membranes by lipid anchors. Thus, they might anchor *Er*Cry4 to the surface of the disk membrane. The seven-transmembrane receptor opsin could also serve as a membrane anchor for *Er*Cry4. (B) A potential complex involving *Er*Cry4 and the voltage-gated potassium channel subunit $K_v8.2$, which is the modifying subunit in a heteromeric K^+-channel, could directly affect the membrane potential of the cell. (C) Putative interaction of *Er*Cry4 with RBP and the retinal photoisomerase RGR that catalyzes the transformation of all-*trans*-retinal to 11-*cis*-retinal.

The identity of *Er*Cry4 interacting proteins allows us to sketch different putative scenarios of a signaling pathway (Figure 26).[51] For any prove or disprove of these scenarios, we are working on protein-protein interaction studies using purified proteins in reconstituted systems.

MAGNETIC MAP SENSE OF BIRDS (Side project)

In addition to the light-dependent magnetic compass located in their eyes, the birds seem to have an additional magnetic sense associated with the ophthalmic branch of the trigeminal nerve (V1).

We demonstrated that magnetic map navigation in a migratory songbird (Eurasian reed warbler) requires trigeminal input and that the trigeminal brainstem area PrV, which receives its input primarily from V1, is also highly activated by magnetic stimuli.[55] We could also show that this magnetic information must originate from sensors associated with V1 in the northern wheatear, a long-distance night-migratory songbird.[56] Finally, we could show that geomagnetic map information can modulate nocturnal migratory restlessness but not fueling in a long-distance migratory songbird.[57,58]

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A total of 36 publications acknowledge AFOSR funding: numbers 2-4, 7-9, 12-15, 17-19, 22, 23, 26-28, 31, 37-46, 48, 49, 51, and 55-58 in the following list. A further manuscript is under consideration for publication by *Nature* (29) and two more are in preparation (30,36).

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LIST OF SYMBOLS, ABBREVIATIONS and ACRONYMS

*At*Cry	*Arabidopsis thaliana* (plant) cryptochrome
BBCEAS	Broadband cavity-enhanced absorbance spectroscopy
*Cl*Cry	*Columba livia* (pigeon) cryptochrome
CRDS	Cavity ring-down spectroscopy
Cry	Cryptochrome
cDNA	Complementary DNA
*Dm*Cry	*Drosophila melanogaster* (fruit fly) cryptochrome
*Ec*PL	*E. coli* photolyase
EPR	Electron paramagnetic resonance
*Er*Cry	*Erithacus rubecula* (European robin) cryptochrome
FAD	Flavin adenine dinucleotide
HDX	Hydrogen-deuterium exchange
IscA1	Iron-sulphur cluster assembly 1 protein
MS	Mass spectrometry
RF	Radiofrequency
RNAi	RNA interference
TA	Transient absorption
TrpH	Tryptophan
WT	Wild type
Y2H	Yeast-two-hybrid