

## REVIEW

# Cryptochromes—a potential magnetoreceptor: what do we know and what do we want to know?

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Cryptochromes have been suggested to be the primary magnetoreceptor molecules underlying light-dependent magnetic compass detection in migratory birds. Here we review and evaluate (i) what is known about these candidate magnetoreceptor molecules, (ii) what characteristics cryptochrome molecules must fulfil to possibly underlie light-dependent, radical pair based magnetoreception, (iii) what evidence supports the involvement of cryptochromes in magnetoreception, and (iv) what needs to be addressed in future research. The review focuses primarily on our knowledge of cryptochromes in the context of magnetoreception.

**Keywords:** orientation and navigation; magnetic compass; magnetic sense; bird migration; night-migratory birds

## 1. INTRODUCTION

A wealth of behavioural data indicate that the magnetic compass of migratory birds is light-dependent and that the eye is somehow involved in sensing the reference compass direction provided by the geomagnetic field (see box 1). Theoretical considerations have suggested that cryptochromes are likely to be the primary sensory molecules in this light-dependent magnetodetection mechanism, which has been suggested to be radical pair based. The radical pair model with a focus on the physical chemistry of the primary reception mechanism has recently been reviewed in detail by Rodgers & Hore (2009). Here, we aim to review (i) what is generally known about different types of cryptochromes in different taxa, (ii) what is currently known about the biochemical and physical properties of the photolyase/cryptochrome family, (iii) what functional roles of photolyases/cryptochromes have been documented or assumed, and finally (iv) what we still need to know about cryptochromes in order to determine whether cryptochromes act as magnetoreceptor in birds.

## 2. LIGHT-DEPENDENT RADICAL PAIR BASED MAGNETORECEPTION

In 1978, Schulten proposed that magnetic compass sensing might have spin chemical origins

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One contribution of 13 to a Theme Supplement 'Magnetoreception'.

(Schulten *et al.* 1978; Schulten 1982). He suggested that the yield of a biochemical reaction proceeding via a radical pair might be sensitive to the orientation of an external magnetic field. Earth-strength magnetic fields influence correlated spin states of paired radicals because of anisotropy in the hyperfine interactions (i.e. magnetic coupling between an (unpaired) electron spin and a nuclear spin, e.g. <sup>1</sup>H, <sup>14</sup>N; Schulten 1982; Ritz *et al.* 2000; Weaver *et al.* 2000). Electron spins are not strongly coupled to the thermal bath (i.e. translational, rotational and vibrational motions of molecules) and therefore represent one of only a few molecular features that might plausibly be influenced by the Earth's magnetic field (Edmonds 2001). The suggested mechanism involves a light-induced electron transfer between two molecules. This electron transfer must result in the generation of a radical pair intermediate that can either exist in a singlet or a triplet excited state (the state depends on the spin correlation of the unpaired electrons being either antiparallel ( $\uparrow\downarrow$ ) in the singlet state or parallel ( $\uparrow\uparrow$ ) in the case of a triplet excited state). In a radical pair mechanism the magnetic field can alter the rates and product yields of these two naturally interconverting radical intermediate states depending on the orientation of the radical within the field and thus influence the proportion of biochemically different singlet or triplet end products (Ritz *et al.* 2000).

Both theoretical calculations (Eichwald & Walleczek 1996; Ritz *et al.* 2000) and *in vitro* experiments

Box 1. Behavioural evidence—the magnetic compass in birds.

- In all bird species studied so far, the magnetic compass was shown to be an **inclination compass** (Wiltschko & Wiltschko 1972). An inclination compass has been documented for short- and long-distance migrants, birds of both hemispheres, and night, twilight and day migrants (Wiltschko & Wiltschko 1996).
- The birds' magnetic compass seems to be **narrowly tuned to the total intensity** of the ambient magnetic field (Wiltschko & Wiltschko 1972, 1978, 2001), but **adaptable to changes** in field strength exceeding those experienced during natural migration (Wiltschko & Wiltschko 1972; Wiltschko *et al.* 2006).
- Migratory birds seem to use **head scans** to detect the reference direction provided by the Earth's magnetic field during magnetic orientation (Mouritsen *et al.* 2004*b*).
- Experiments on free flying *Catharus* thrushes suggest that, in free flight, the magnetic compass is **calibrated daily by sunset cues during twilight** (Cochran *et al.* 2004).
- Magnetic orientation performance under low-intensity monochromatic light is **wavelength-dependent**: birds orient well under blue, turquoise and green, but are challenged under yellow or red light (Wiltschko *et al.* 1993, 2001; Wiltschko & Wiltschko 1995*a*, 1999, 2001, 2002; Munro *et al.* 1997; Rappl *et al.* 2000; Muheim *et al.* 2002).
- Experiments testing birds under **higher intensities** or a **mixture of monochromatic light** colours as well as experiments where birds had time to adapt to yellow or red light suggest that a rather complex relationship between receptors is involved in magnetoreception, since birds in some cases appear to show oriented responses that were not in line with the expected migratory direction and in some cases bipolar responses (Wiltschko *et al.* 2000, 2003, 2004*a,b*).
- Experiments with oscillating magnetic fields in the low radio-frequency range have been shown to disrupt magnetic orientation behaviour of migratory birds (Ritz *et al.* 2004, 2009; Thalau *et al.* 2005). These results provide the strongest, albeit indirect, evidence that the biophysical mechanism underlying the magnetic compass of birds involves a **radical pair** reaction.
- The **pineal gland** of birds is at least **not crucial** for magnetic compass orientation: birds with their pineal gland removed are still able to use their magnetic compass to orient when receiving injections of melatonin (Schneider *et al.* 1994).
- It is important to keep in mind that the magnetic compass interacts with celestial compass cues and that the **magnetic compass is not essential for orientation**. Birds are still able to orient if celestial but no magnetic cues are available (e.g. Mouritsen 1998).

(Harkins & Grissom 1994, 1995; Brocklehurst 2002; Timmel & Henbest 2004; Henbest *et al.* 2008; Maeda *et al.* 2008) showed that the ratio between singlet and triplet products from radical pair reactions can be modulated by an Earth-strength magnetic field, thereby potentially providing the basis for a magnetic compass.

The effect does not necessarily affect differences in singlet–triplet yields, a simple ‘delay’ in the reaction kinetics (i.e. a variation of the lifetime of the radical pair intermediate) would be sufficient to specifically alter the reaction depending on the ambient magnetic field. This proposal has been revived by Ritz *et al.* (2000), who proposed that the retina with its almost perfect half-ball shape is well suited as an ordered structure, and that the radical pair intermediate is involved in some kind of visual reception system. According to this theory, the reaction yield anisotropy of the receptor radical pair governs the directional response. For further details on the radical-based magnetoreception hypothesis, see Ritz *et al.* (2010).

### 3. REQUIRED CHARACTERISTICS OF THE PUTATIVE PRIMARY MAGNETIC SENSORY MOLECULES

For a spin chemical receptor mechanism located in the bird's eye to detect the direction of magnetic fields as weak as that of the Earth's, several stringent conditions must be met (Grissom 1995; Adair 2000; Ritz *et al.* 2000; Weaver *et al.* 2000; reviewed in detail in Rodgers & Hore 2009): (i) any suggested candidate molecule must exist in the retina of migratory birds, (ii) the cells containing the candidate molecule have to be active at night, when the bird performs magnetic orientation, (iii) motion of the radical pair forming molecule has to be restricted, i.e. the molecule must somehow be at least partly fixed in the cell, (iv) the molecule has to be excited by light, (v) the absorption spectrum has to match the range that has been shown to elicit magnetic orientation performance in behavioural experiments (Wiltschko *et al.* 1993, 2000, 2001, 2003, 2004*a,b*; Wiltschko & Wiltschko 1995*a*, 1999, 2001, 2002; Munro *et al.* 1997; Rappl *et al.* 2000; Muheim *et al.* 2002), (vi) the initial electron transfer must not randomize the original parallel or opposite spin relationship of the two electrons, i.e. conservation of spin angular momentum is crucial, which is not true for all electron transfer processes but is often the case when the transfer is induced by photo-excitation (Ritz *et al.* 2000), (vii) the half-life of the light-excited radical pair intermediates including the spin correlation of the two electrons has to persist long enough (more than 1  $\mu$ s) to allow the Earth's magnetic field to modulate the singlet triplet interconversion of the radical pair, (viii) because the influence of the Earth's magnetic field on the putative chemical processes is rather weak, the effect must presumably be integrated over a large area, and (ix) a suitably sensitive transduction of the changing product yields is necessary (e.g. one reaction product might be a neurotransmitter, the other might not; or one end-product interfering with the sensitivity of a visual receptor, the other not).

Considering that the putative light sensitive magnetoreceptor should be located in the eye, the visual system might be used for transduction. This allows for the distinct possibility that birds may ‘see’ the Earth's magnetic field (Ritz *et al.* 2000;

Mouritsen *et al.* 2005; Heyers *et al.* 2007; Liedvogel *et al.* 2007a; Zapka *et al.* 2009).

#### 4. CRYPTOCHROME

In their key theoretical paper, Ritz *et al.* (2000) suggested that the blue-light receptor cryptochrome would be a promising candidate for a photo-magnetoreceptor for the following reasons: cryptochromes which have been discovered in plants, birds and other animals are the only known class of photoreceptor molecules found in vertebrates that have been shown to be able to form a radical pair upon photoexcitation, at least in some organisms (e.g. Liedvogel *et al.* 2007a; Biskup *et al.* 2009).

Cryptochromes were first discovered as blue-light and ultraviolet (UV-A) photoreceptors in plants (Ahmad & Cashmore 1993). Cryptochromes share moderate sequence (but significant structural) similarity to photolyases, which are flavin containing proteins that catalyse the repair of UV light-damaged DNA via electron transfer mechanisms, but cryptochromes do not have photolyase activity (Hsu *et al.* 1996; Öztürk *et al.* 2007). It is widely accepted that cryptochromes are probably the evolutionary descendants of DNA photolyases (Cashmore *et al.* 1999).

The first cryptochrome encoding gene to be identified was *Arabidopsis* HY4 (Ahmad & Cashmore 1993). The gene product (a protein of 681 amino acids) was named Cry1 from 'cryptochrome' previously proposed for 'cryptic' blue-light receptor (Gressel 1979). Cryptochromes have now been found in other plants (e.g. Small *et al.* 1995; Cashmore 1997; Imaizumi *et al.* 2000, 2002; Xu *et al.* 2009) and in various animal lineages, including cubozoa (Brinkman & Burnell 2007), insects (e.g. Todo *et al.* 1996; Emery *et al.* 1998; Egan *et al.* 1999; Rosato *et al.* 2001; Zhu *et al.* 2005, 2008), fish (e.g. Kobayashi *et al.* 2000; Levy *et al.* 2007), amphibians (e.g. Zhu & Green 2001), birds (e.g. Bailey *et al.* 2002; Mouritsen *et al.* 2004a; Möller *et al.* 2004; Helfer *et al.* 2006) and mammals (e.g. Hsu *et al.* 1996; Todo *et al.* 1996; Kobayashi *et al.* 1998; Griffin *et al.* 1999; Kume *et al.* 1999; van der Horst *et al.* 1999).

Cryptochromes are implicated in multiple blue-light-dependent signalling pathways in plants and animals (extensively reviewed in Cashmore *et al.* 1999; Deisenhofer 2000; Sancar 2000, 2003, 2004; Ahmad 2003; Bouly *et al.* 2003; Lin & Todo 2005). In plants, cryptochromes control different aspects of growth and development, i.e. involvement in de-etiolation responses such as inhibition of hypocotyl growth (Ahmad & Cashmore 1993; Lin *et al.* 1995, 1998; Lin 2002), anthocyanin accumulation (Ahmad *et al.* 1995) in leaf and cotyledon expansion (Cashmore *et al.* 1999; Lin 2002; Lin & Shalitin 2003), transitions to flowering (El-Din El-Assal *et al.* 2003) or regulation of blue-light regulated genes (Jiao *et al.* 2003).

In animals, cryptochromes have been shown to play a direct role in circadian rhythms as components of the circadian pacemakers (Miyamoto & Sancar 1998; van der Horst *et al.* 1999; Sancar 2000, 2004; Shearman

*et al.* 2000; Zhu *et al.* 2008). In *Drosophila*, cryptochromes seem to be more indirectly involved by feeding light information into the circadian clock (Stanewsky 2002; Busza *et al.* 2004) and recent experiments have suggested a cryptochrome-dependent magnetic sensitivity of the *Drosophila* circadian clock (Yoshii *et al.* 2009), which may stimulate future research in the field of light-dependent magnetoreception. In monarch butterflies *Danaus plexippus* vertebrate-like cryptochrome (Cry2; in addition to *Drosophila*-like cryptochrome (Cry1) that probably functions as a blue-light photoreceptor for circadian clock entrainment) was suggested to function as a core clock element. In the monarch butterfly's brain, Cry2 may even have an additional role as an output that regulates circadian activity in the central complex (a topographically ordered neuropil structure in the centre of the insect brain), the putative location of the sun compass (Zhu *et al.* 2008). In migratory birds, cryptochrome is mainly discussed as putative receptor molecule for light-dependent magnetic compass orientation. Most research in the field of migratory bird orientation is carried out on the two model organisms European robin *Erithacus rubecula* and garden warbler *Sylvia borin* (figure 1a). Today four different members of the cryptochrome family have been identified in migratory birds (Möller *et al.* 2004; Mouritsen *et al.* 2004a; see paragraph on the identification of cryptochromes in the retina of migratory birds), but the functional role of migratory bird cryptochromes still remains to be determined with any certainty. A further type of cryptochrome, distinct from 'classic' plant (*Arabidopsis* HY4) or animal (*Drosophila* or *Homo sapiens*) cryptochromes, though more closely resembling the latter (Brudler *et al.* 2003), was first identified in cyanobacteria *Synechocystis* sp. (Hitomi *et al.* 2000). On the basis of sequence alignment these DASH-cryptochromes ('DASH' to indicate the relationship with cryptochromes found in *Drosophila*, *Arabidopsis*, *Synechocystis* and *Homo*) were grouped together with the animal cryptochromes and (6-4) photolyases, but the perception of this class of proteins is not well established yet, and is currently undergoing a shift, as recent data show that DASH-cryptochromes are single strand DNA photolyases (Kleine *et al.* 2003; Selby & Sancar 2006; Pokorny *et al.* 2008). Recent results indicate that DASH-cryptochromes could work as transcriptional regulators (Brudler *et al.* 2003; Daiyasu *et al.* 2004), DNA repair enzymes for single-stranded DNA (Selby & Sancar 2006), and they have further been suggested to participate in circadian input pathways (Facella *et al.* 2006; Brunelle *et al.* 2007).

#### 5. STRUCTURAL CHARACTERISTICS OF CRYPTOCHROMES

The state of the art on structural biology of photolyases and cryptochromes has recently been reviewed by Müller & Carell (2009), and will therefore not be covered in detail here. In brief, several structures of photolyases (Park *et al.* 1995; Tamada *et al.* 1997; Komori *et al.* 2001; Kort *et al.* 2004; Mees *et al.* 2004;

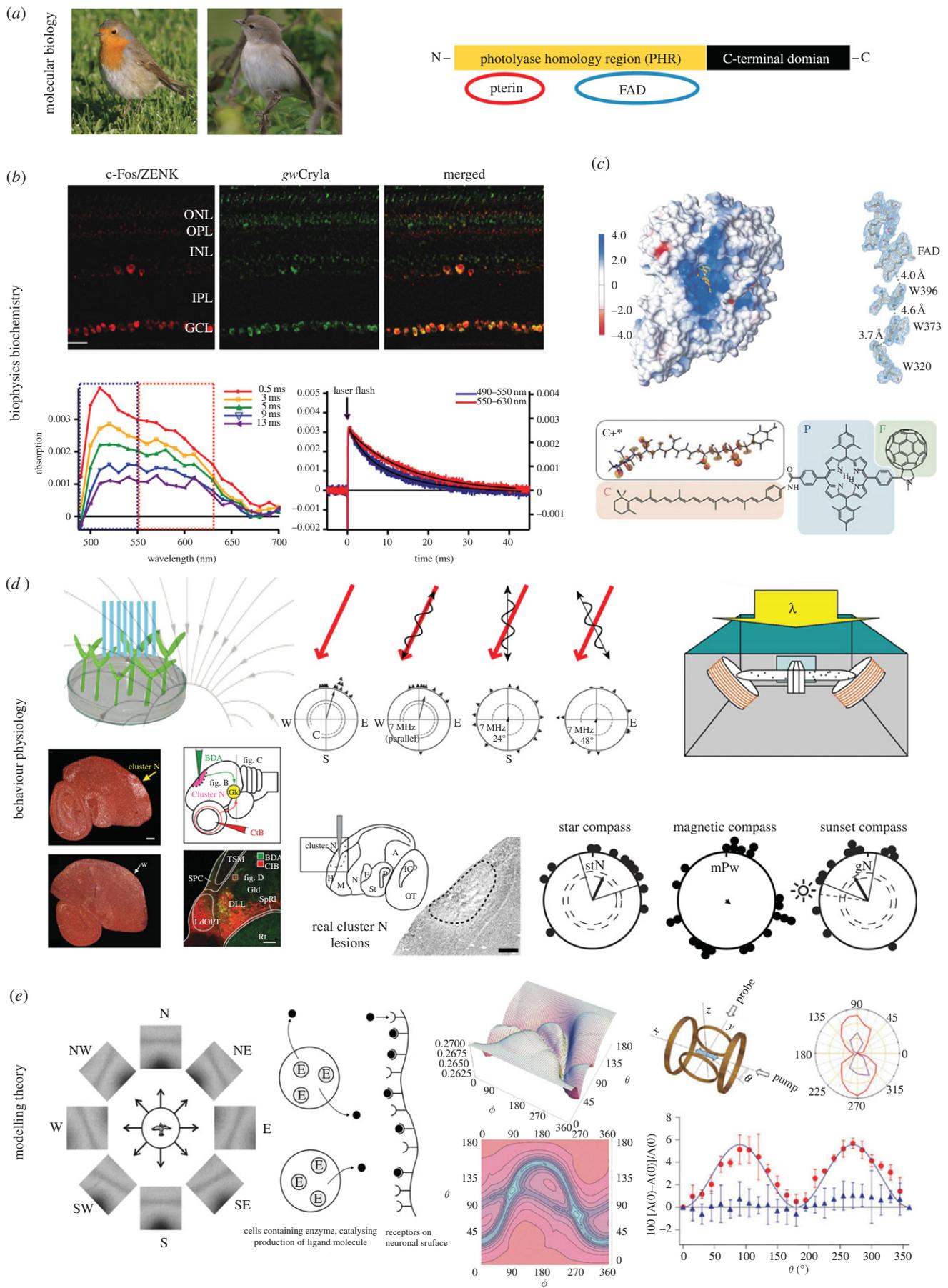


Figure 1. (Caption opposite.)

Figure 1. (*Opposite.*) Key evidence suggesting that cryptochromes could function as magnetoreceptors. (a) Photos (©Henrik Mouritsen) show the two migratory model bird species from which, so far, four different members of the cryptochrome multigene family have been detected in the retina (Möller *et al.* 2004; Mouritsen *et al.* 2004a; and box 2 of this paper). The schematic illustration indicates the structure of a typical cryptochrome protein (redrawn after Lin & Todo 2005). (b) Cryptochrome has been shown to be expressed in retinal cells that are neuronally active when the birds perform magnetic orientation. The upper panel shows immunohistochemistry staining of a cross section of a garden warbler retina, indicating from left: the expression pattern of neuronal activity marker proteins (c-Fos/ZENK, see main text for details on the use of immediate early genes as neuronal activity markers) in red, the cryptochrome protein expression pattern (which probably included signal from several cryptochromes and not just from *gwCry1a* as originally thought) in green, and the merged picture which shows colocalization (yellow) of both proteins (reprinted from Mouritsen *et al.* (2004a). Copyright (2004) National Academy of Sciences, USA). The two graphs show the transient absorption spectra (bottom left) and absorption time profiles (right) of garden warbler cryptochrome 1a. The radical decay rates (blue and red curve, bottom right) indicate that garden warbler cryptochromes form long lived radical species (half-life approx. 10 ms) (reprinted from Liedvogel *et al.* (2007a)). (c) The first cryptochrome structure presented was cryptochrome-DASH of *Synechocystis* sp. (reprinted from Brudler *et al.* (2003). Copyright (2003) with permission from Elsevier (copyright-hyperlink for version published electronically: <http://www.cell.com/molecular-cell>)); the conserved electron transfer chain is shown at the right. The structure of a migratory bird (or any other animal) cryptochrome remains to be solved. The lower part of (c) shows the structure of the carotenoid–porphyrin–fullerene model system that was designed to demonstrate the feasibility of chemical magnetoreception (reprinted from Maeda *et al.* (2008). Copyright (2008) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)). (d) Top left: observations of light-dependent, cryptochrome mediated magnetic field effects on plant growth have been claimed (Ahmad *et al.* 2007), but unfortunately these findings could not be replicated in a blinded study (Harris *et al.* 2009; figure redrawn after Ahmad *et al.* 2007). Top centre: further evidence supporting a radical pair based magnetic compass comes from the observation that weak radiofrequency magnetic fields seem to disrupt the ability of birds to orient by the Earth's magnetic field (Ritz *et al.* 2004, 2009, 2010; Thalau *et al.* 2005). The red arrow indicates the orientation of the Earth's magnetic field. The wavy lines indicate the orientation of the oscillating field applied. The circular diagrams show the magnetic orientation of European robins in the magnetic field scenario indicated above the diagram. Each triangle at the circle periphery indicates the mean orientation of an individual bird based on three tests under the given magnetic condition. Arrows indicate the group mean vectors. Inner and outer dashed circles indicate the radii of the group mean vectors needed for directional significance according to the Rayleigh test (inner,  $p < 0.05$ ; outer,  $p < 0.01$ ) (redrawn after Ritz *et al.* (2004). Copyright (2004) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)). Top right: conditioning experiments on *Drosophila* fruit flies (setup shown) have recently suggested that a cryptochrome-based magnetosensitive system may also exist in this animal (reprinted from Gegear *et al.* (2008). Copyright (2008) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)). Bottom left: in migratory birds, a forebrain area (cluster N) has been identified, which shows light-dependent, movement-independent, neuronal activation at night time when migratory birds performed magnetic orientation under dim light (sagittal brain section through centre of cluster N showing neuronal activation pattern as indicated by ZENK mRNA expression (white signal) at night in a migratory (upper photo) and non-migratory bird (lower photo); Mouritsen *et al.* 2005; Liedvogel *et al.* 2007b; Feenders *et al.* 2008; reprinted from Mouritsen *et al.* (2005). Copyright (2005) National Academy of Sciences, USA). Bottom centre: a tracing study documented a functional connection between the retinal ganglion cells and cluster N via the thalamofugal visual pathway (schematic drawing). The photo shows that in the visual thalamus, red axons projecting from the eye meet with green backfilled neurons projecting from the thalamus to cluster N (reprinted from Heyers *et al.* (2007)). Bottom right: the photo shows an example part of a sagittally cut brain section through the centre of cluster N from a cluster N lesioned European robin, stained with a neuroanatomical marker. Notice that the tissue where cluster N should be located is destroyed due to the lesion. The circular diagrams show that cluster N lesioned birds tested in a planetarium simulating the local starry sky (stN, star north) still orient towards the typical north-northeast spring migratory direction, that birds with cluster N lesions could not orient using their magnetic compass (mPw, magnetic poleward), and that birds with cluster N lesions could also orient during sunset, presumably using their sun compass (gN, geographical north). Radial lines indicate the 95% confidence intervals (figure redrawn after Zapka *et al.* (2009). Copyright (2009) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)). (e) Theoretical work has shown that, in principle, a radical pair based mechanism could form the basis of magnetic compass orientation (e.g. Ritz *et al.* 2000; Weaver *et al.* 2000; Cintolesi *et al.* 2003; Solov'yov *et al.* 2007). Left: theoretical visual modulation patterns by the Earth's magnetic field for a bird flying parallel to the horizon looking towards different directions, assuming radical pair receptors oriented perpendicularly to the retina's surface in the bird's eye (reprinted from Ritz *et al.* (2000). Copyright (2000), with permission from Elsevier (copyright-hyperlink for version published electronically: <http://www.cell.com/biophysj>)). Centre left: a model for an enzyme (E)-catalysed magnetic field sensitive radical pair reaction. The catalytic reaction produces a ligand (black circles) that leaves the cell and binds to a receptor attached to neural tissue. Subsequent signal transduction depends on the equilibrium between free and unbound ligand (reprinted from Weaver *et al.* (2000). Copyright (2000) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)). Centre right: theoretical calculations of radical pair reaction product yields: the picture shows a simulation of the orientation dependence of singlet recombination probability for a flavin–tryptophan radical pair under Earth-strength magnetic fields ( $B_0 = 50 \mu\text{T}$ , the lifetime of the radical pair is  $5 \mu\text{s}$ ); orientation dependence is shown both as a three-dimensional representation (top) and as a contour plot (bottom). The direction of the applied magnetic field with respect to the radical pair is defined in terms of the polar angles  $\phi$  and  $\theta$  (reprinted from Cintolesi *et al.* (2003). Copyright (2003) with permission from Elsevier (copyright-hyperlink for version published electronically: <http://www.elsevier.com/locate/chemphys>)). Right: illustration of the chemical compass model (also see c, bottom) providing the proof of principle that a light-induced, radical pair process in a chemical molecule can be sensitive to Earth-strength magnetic fields (Maeda *et al.* 2008). Right, top left: the experimental setup used to measure the anisotropy of the magnetic field effect: the direction of the applied magnetic field is generated by two orthogonal pairs of Helmholtz coils; the sample alignment along the  $x$ -axis is shown by the blue arrow;  $\phi$  is the angle between the magnetic field vector and the  $x$ -axis; continuous probe light and pump laser pulses spread along the  $x$  and  $y$  axes. Top right: a polar plot of the anisotropy of the magnetic field effect as a function of  $\phi$  is plotted for an aligned sample (purple), and (red) by photoselection (i.e. orientation-selective excitation of chromophores by photon absorption). Right, bottom: absorption data from the photoselection measurements (red dots) are plotted as a function of the direction of the applied magnetic field  $\phi$ . As a control, the blue dots confirm that no angular dependence is present in a scenario when the polarization axis of the probe light is vertical (i.e. along  $z$ -axis; reprinted from Maeda *et al.* (2008). Copyright (2008) with permission from Nature (copyright-hyperlink for version published electronically: <http://www.nature.com>)).

Table 1. Functional and structural evidence on photolyase/cryptochrome proteins. The table lists all photolyase and cryptochrome proteins from different species where functional information with respect to a putative role of cryptochromes as magnetoreceptor and/or structural information are available. Experimental data in the context of magnetosensitive responses and/or the detection of radical pair reactions both *in vitro* and *in vivo* are indicated. CPD = cyclobutane pyrimidine dimer; PHR = photolyase homology region.

species	protein	structure resolved?	radical pair mechanism/ magnetic field effect?	reference
<i>Anacystis nidulans</i>	AnCPD-PHR	+		Tamada <i>et al.</i> (1997) Mees <i>et al.</i> (2004) Kort <i>et al.</i> (2004)
<i>Arabidopsis thaliana</i>	At(6-4)-PHR	+		Hitomi <i>et al.</i> (2009)
	AtCry1	+	physiological cry-mediated magnetosensitive response (500 $\mu$ T)	Giovani <i>et al.</i> (2003) Brautigam <i>et al.</i> (2004) Ahmad <i>et al.</i> (2007) Harris <i>et al.</i> (2009)
	AtCry2			Banerjee <i>et al.</i> (2007)
	AtCry3	+		Song <i>et al.</i> (2006) Huang <i>et al.</i> (2006) Klar <i>et al.</i> (2007)
<i>Drosophila melanogaster</i>	d(6-4)-PHR	+		Maul <i>et al.</i> (2008) Glas <i>et al.</i> (2009)
	dCry		behavioural cry-based magnetosensitive response (500 $\mu$ T)	Berndt <i>et al.</i> (2007) Gegear <i>et al.</i> (2008)
<i>Escherichia coli</i>	EcCPD-PHR	+	+ (39 mT)	Li <i>et al.</i> (1991) Park <i>et al.</i> (1995) Aubert <i>et al.</i> (2000) Henbest <i>et al.</i> (2008)
<i>Sulfolobus tokodaii</i>	StCPD-PHR	+		Fujihashi <i>et al.</i> (2007)
<i>Synechocystis</i> sp.	Cry DASH	+		Brudler <i>et al.</i> (2003)
<i>Thermus thermophilus</i>	TtCPD-PHR	+		Komori <i>et al.</i> (2001) Klar <i>et al.</i> (2006)
<i>Xenopus laevis</i>	XlCry-DASH			Biskup <i>et al.</i> (2009)
<i>Sylvia borin</i>	gwCry1a		radical pair formation (transient absorption)	Liedvogel <i>et al.</i> (2007a)
model chemical compass	carotenoid–porphyrin–fullerene model system		+ ( $\leq 50 \mu$ T)	Maeda <i>et al.</i> (2008)

Klar *et al.* 2006; Fujihashi *et al.* 2007; Maul *et al.* 2008; Glas *et al.* 2009; Hitomi *et al.* 2009) and cryptochromes (Brudler *et al.* 2003; Brautigam *et al.* 2004; Huang *et al.* 2006; Klar *et al.* 2007) are available today, but the structure of an animal cryptochrome remains to be solved (functional and structural data available for different photolyases/cryptochromes in the context of chemical magnetoreception are summarized in table 1). The defining characteristics of cryptochromes are N-terminal domains with marked sequence similarity to DNA photolyases (Deisenhofer 2000; Brautigam *et al.* 2004). Within the amino-terminal photolyase homology region (PHR), cryptochromes and photolyases have similar three-dimensional structures, characterized by an N-terminal  $\alpha/\beta$  domain and a C-terminal  $\alpha$ -helical domain. A further defining characteristic of both plant and animal cryptochromes are C-terminal extensions of varying size, not present in photolyases (see figure 1a for a schematic sketch of cryptochrome

domains). This extension is longer in most plant cryptochromes than in animal cryptochromes and is generally less conserved than the PHR region. The C-terminal domain is likely to be essential for a number of cryptochrome-specific functions, i.e. in *Arabidopsis* Cry1 it mediates the signalling mechanism of constitutive blue-light response (Yang *et al.* 2000). Cry-DASH proteins/ssDNA-photolyases lack this domain (Lin & Shalitin 2003).

The protein structure of cryptochromes includes two non-covalently bound cofactors: flavin adenine dinucleotide (FAD), which is essential for catalysis, and a second light-harvesting chromophore. Due to their sequence- and structural similarities to photolyases, the second chromophore of cryptochromes is assumed to be either an 8-hydroxy-5-deazariboflavin (8-HDF) or a 5,10-methenyltetrahydrofolate (MTHF; Malhotra *et al.* 1995; Hsu *et al.* 1996). In photolyases, the role of the second chromophore as an antenna, harvesting light to initiate the photoreaction process is well

established (e.g. Klar *et al.* 2007). Because there is spectral overlap between the absorption spectra of the flavin and antenna molecule fluorescence in this two-chromophore system, the antenna molecule can transfer excitatory energy to the catalytic cofactor (Jorns *et al.* 1990; Park *et al.* 1995). This Förster-type energy transfer occurs from the short-wavelength absorbing donor (the antenna molecule) to the long-wavelength absorbing catalytic cofactor. The energy transfer efficiency depends on the distance between donor and acceptor molecule and has been shown to be very high (70–100%; Payne & Sancar 1990; Kim *et al.* 1992) in photolyases. For *Arabidopsis* Cry3 (DASH type), it was calculated to be between 78 and 87 per cent (Song *et al.* 2006). The FAD-access cavity of the helical domain is the catalytic site of photolyases and is also predicted to be important in the mechanism of cryptochrome functions (for a review see Lin & Todo 2005). Electron transfer between the FAD cofactor and the cryptochrome molecule is an essential part of the suggested radical pair mechanism (Ritz *et al.* 2000). Even though cryptochromes bind similar cofactors to photolyases, they lack DNA repair activity (Lin *et al.* 1995; Malhotra *et al.* 1995) suggesting evolution of novel functions of the cryptochromes' role in signalling.

Despite high topological similarities and apparent functional analogy, plant and animal cryptochromes seem to have evolved independently from different ancestral photolyases, with animal cryptochromes more similar to type 6-4 photolyases and plant cryptochromes sharing greater sequence similarity with type I microbial photolyases (Kanai *et al.* 1997).

## 6. EXPERIMENTAL EVIDENCE SUPPORTING THE INVOLVEMENT OF CRYPTOCHROMES IN MAGNETIC SENSING

### 6.1. Identification of cryptochromes in the retina of migratory birds

Explanation and deeper understanding of the previously obtained behavioural results (see box 1) supporting an involvement of a light-induced radical pair magnetoreception mechanism inevitably require the presence of a radical pair forming photopigment in the eye of the migratory bird (*prerequisite* (i)). Therefore, the experimental demonstration of the presence of cryptochromes in the retina of night-migratory songbirds (Möller *et al.* 2004; Mouritsen *et al.* 2004a) was the key prerequisite and set the scene for all subsequent experiments. Today, four members of the cryptochrome family have been identified in migratory songbirds: Cry1a and Cry1b, Cry2 (Möller *et al.* 2004; Mouritsen *et al.* 2004a; Liedvogel *et al.* 2007a) and Cry4 (new sequence information presented here; see box 2 and figure 2).

### 6.2. Location of cryptochromes within the retina of birds

Immunohistochemical stainings have shown that garden warbler Cry1 (*gwCry1*) is predominantly

expressed in the cytosol of ganglion cells (the retinal cell class which communicates information from the eye to the brain), large displaced ganglion cells (retinal ganglion cells that are not located in the ganglion cell layer but in the inner half of the so-called inner nuclear layer; in pigeons, the displaced ganglion cells have been shown to project preferentially to the nucleus of the basal optic root, a part of the accessory optic system; Karten & Finger 1976; Karten & Fite 1977), and in photoreceptor cells (Mouritsen *et al.* 2004a). From the cell-anatomical point of view, the membrane structures of the photoreceptor cell outer segment discs and associated structures or the inner segments, which each provide a oriented cylindrical membrane would provide ideal substrates for the highly oriented ensemble of molecules required for a radical pair based magnetoreception mechanism. Therefore, we consider the cryptochromes located in the photoreceptor cells to be the most probable magnetoreceptor candidates. Another interesting finding is the high cytosolic/membrane immunoreactivity of *gwCry1* in ganglion cells and photoreceptor cells (Mouritsen *et al.* 2004a). This rather hints towards a putative role of these cryptochromes as magnetic compass detectors than towards a function as a control element of the internal circadian clock, since the cryptochromes would have to get to the nucleus in order to perform a regulatory clock function.

Also, the cryptochrome expression levels significantly differed between migratory and non-migratory birds at night (Fu *et al.* 2002; Haque *et al.* 2002; Mouritsen *et al.* 2004a,b): during the day, high levels of cryptochrome are found both in migratory and non-migratory species (Mouritsen *et al.* 2004a,b). During night time, cryptochrome levels in non-migratory zebra finches drop to background expression levels, whereas cryptochrome expression levels in migratory garden warblers at night are as high as or even higher than during the day (Mouritsen *et al.* 2004a). This finding of high cryptochrome expression during the night time seems to be a special adaptation in migratory birds.

To be potentially relevant for magnetoreception, it is important to show that at least some cryptochrome containing retinal cells are active at night when the garden warblers perform magnetic orientation (*prerequisite* (ii)). This could be demonstrated by mapping the neuronal activity pattern of retinal cells using neuronal activity marker genes ZENK (acronym for the gene known in other species as *zif-268*, *egr-1*, *NGF-IA* and *krox-2*) and c-Fos (Mello *et al.* 1992; Jarvis & Nottebohm 1997; Mouritsen *et al.* 2004a), both of which are known to show vision-dependent expression in the retina of many animals (Dragunow & Faull 1989; Yoshida *et al.* 1993; Araki & Hamassaki-Britto 1998; Fisher *et al.* 1999) including chicken (Fisher *et al.* 1999), and their expression requires neuronal activity (Worley *et al.* 1991; Chaudhuri *et al.* 1995; see figure 1b, upper panel).

In magnetic orientation experiments on garden warblers during night time, both activity marker genes co-localized with cryptochromes in most ganglion cells and large displaced ganglion cells, which are the

Box 2. Identification and cloning of a partial coding sequence of Cry4, a new potentially magnetosensitive cryptochrome candidate from the migratory garden warbler [GenBank accession no. GQ896539].

We identified a new member of the cryptochrome multigene family, which is expressed in the retina of garden warblers. This is the fourth type of cryptochrome molecule to be found in migratory birds; Cry1a, Cry1b and Cry2 have previously been identified in European robins (Möller *et al.* 2004) and garden warblers (Mouritsen *et al.* 2004a).

#### Methods

RNA isolation and cDNA synthesis from garden warbler retina was carried out according to Mouritsen *et al.* (2004a). To detect the additional and so far unknown Cry4 of the cryptochrome multigene family in garden warbler, we designed degenerate primers from publicly available sequence information of cryptochromes from several taxa using a cloning strategy similar to the one described earlier for identification of garden warbler Cry1a, Cry1b and Cry2 (Mouritsen *et al.* 2004a). Forward primer (0.8  $\mu$ l), 5'-AGCACGTCGACGAACCCCATCTGCATCCA-3' and reverse primer (0.8  $\mu$ l) 5'-ACGAA-GAATTCGACGAAAGCCACATCCAG-3' were used for polymerase chain reaction (PCR) amplification with conditions described in Mouritsen *et al.* (2004a); primer sequences were derived from a sequence alignment of cryptochrome 2 cDNAs from mouse [NM009963], human [NM021117], chicken [AY034433]. The same primer combination was used for detection of partially coding sequence for Cry2 [AY739908] (Mouritsen *et al.* 2004a). The additional amplification of Cry4 sequence reported here can be explained by cross-priming of the oligonucleotide pair at sequences conserved between Cry 2 and Cry 4. The resulting 237 bp long PCR product was cloned into pTBlue-3 by means of the Perfectly Blunt cloning kit (Novagen) and transformed into NovaBlue cells. Plasmid purification and sequencing was carried out as described in Mouritsen *et al.* (2004a). DNA similarities and identity scores were analysed by WU-BLAST2 and FASTA algorithms.

#### Results

The amplified partially coding sequence (see figure 2) was confirmed as being derived from garden warbler cryptochrome 4 (*gwCry4* [GQ896539]). WU-BLAST2 and FASTA algorithms reveal closest homology with three other avian cryptochrome 4 sequences published: the partial coding sequence of garden warbler Cry4 reveals 95% sequence identity score with cryptochrome 4 from the house sparrow *Passer domesticus* [AY494987] and the predicted Cry4 sequence of zebra finch *Taeniopygia guttata* [XM\_002198497], both belonging to the songbirds as does the garden warbler, and 89% identity with the established sequences of chicken [AY102068]. In comparison, the identity scores of the newly amplified cryptochrome fragment with garden warbler Cry1a [AJ632120], Cry1b [DQ838738] and Cry2 [AY739908] are 76% (in all cases) and 75%, respectively, for both Cry1a [AY585716] and Cry1b [AY585717] from European robins, which confirms the identity of the amplified fragment being cryptochrome 4 (*gwCry4*). This is the fourth type of cryptochrome molecule identified for the migratory garden warbler.

<i>S. borin</i>	<b>GATCTGCTGGTACAAGGATGCAGAGAGGCTCCACAAATGGAAAACGGCACAGACAGGGTT</b>	60
<i>T. guttata</i>	GATTTTCGTGGTACAAGGATGCAGAGAGGCTCCACAAATGGAAAACGGCAAAGACAGGGTT	60
<i>P. domesticus</i>	GATCTGCTGGTACAAGGATGCAGAGAGGCTCCACAAATGGAAAACGGCACAAACAGGGTT	60
<i>P. gallus</i>	---CCGTTGGTATGAGGATGCTGAGAGGCTCCACAAATGGAAAACGGCTCAGACGGGGTT	57
	*****	
<i>S. borin</i>	<b>CCCATGGATTGATGCCATTATGACCCAGCTGCGCCAGGAAGGTTGGATCCATCACCTGGC</b>	120
<i>T. guttata</i>	CCCATGGATCGATGCCATTATGACCCAGCTGCGCCAGGAAGGCTGGATCCATCACCTGGC	120
<i>P. domesticus</i>	CCCATGGATCGATGCCATTATGACCCAGCTGCGCCAGGAAGGCTGGATCCATCACCTAGC	120
<i>P. gallus</i>	CCCATGGATTGATGCCATCATGACCCAGCTGCGCCAGGAGGGCTGGATCCACCACCTTGC	117
	*****	
<i>S. borin</i>	<b>TCGGCATGCTGTGCGCTGCTTCTGACACGGGGGCACCTTTGGATCAGCTGGGAAGAGGG</b>	180
<i>T. guttata</i>	TCGGCATGCTGTGCGCTGCTTCTGACACGGGGGACCTTTGGATCAGCTGGGAAGAGGG	180
<i>P. domesticus</i>	TCGGCATGCTGTGCGCTGCTTCTGACTCGAGGGGACCTGTGG-----	163
<i>P. gallus</i>	CCGGCACGCTGCGCTGCTTCTGACCCGTTGGGACCTTTGGATCAGCTGGGAGGAGGG	177
	*****	
<i>S. borin</i>	<b>AATGAAGGTGTTTGAAGAGCTGCTCATAGATGCTGACTATAGCATCAATGCTGGGAA</b>	237
<i>T. guttata</i>	AATGAAGGTGTTTGAAGAGCTGCTTTTATAGATGCTGACTACAGCATCAACGCTGGGAA	237
<i>P. domesticus</i>	-----	
<i>P. gallus</i>	CATGAAGGTGTTTGAAGAGCTGCTTTTATAGATGCTGACTACAGCATCAACGCAGGGAA	234
	*****	

Figure 2. Partial sequence alignment of Cry4 partially coding DNA sequence of the migratory garden warbler *Sylvia borin* [GQ896539], house sparrow *Passer domesticus* [AY494987], chicken *Gallus gallus* [AY102068] and zebra finch *Taeniopygia guttata* [XM\_002198497]. The alignment shows that Cry 4 sequence is highly homologous across species, a common feature of members of the cryptochrome/photolyase family.

cell types sending visual information from the eye to the brain. This diagnostic tool for visualizing cells' activity status does not work in retinal photoreceptor cells as they do not communicate via axon potentials, but it is probable that at least some photoreceptor cells are active when the ganglion cells are highly active.

Cryptochromes could be directly linked to the visual pathway and there is growing evidence showing that cryptochrome expressing retinal cells and 'cluster N', a vision-dependent forebrain region that shows high levels of neuronal activation during night time when birds perform magnetic orientation under dim light (Mouritsen *et al.* 2005; Feenders *et al.* 2008), are

connected via the thalamofugal pathway, which is one of the three well-known visual pathways in birds (Heyers *et al.* 2007). Cluster N shows characteristic light-dependent, movement-independent neuronal activation at night time in migratory birds (Mouritsen *et al.* 2005; Liedvogel *et al.* 2007b; Feenders *et al.* 2008), and lesioning of cluster N disrupts the magnetic compass orientation capabilities of migratory European robins (Zapka *et al.* 2009). The robins with lesioned cluster N could still perform star compass orientation and sun compass orientation showing that cluster N is required for magnetic compass orientation only (Zapka *et al.* 2009). Since cluster N is part of the visual system of birds, these findings strongly support the idea of a light-dependent, vision-mediated magnetoreception and is in line with an involvement of a photoreceptor molecule like cryptochrome in magnetic compass information detection (figure 1d).

### 6.3. Biophysical characterization of bird cryptochromes and related molecules

To investigate the putative involvement of cryptochrome in a light-mediated magnetic compass based on a radical pair reaction, the protein chemistry and biophysical properties of cryptochromes must be deciphered in order to understand the potential and suitability of cryptochrome as a magnetic field detector. Do cryptochromes form spin-correlated radical pairs with coherent singlet–triplet mixing that can be influenced by the Earth's magnetic field? Experiments focusing on these questions are based on chemical and biophysical characterization of recombinantly expressed proteins from various species. In photolyases, the light-induced reduction of the FAD cofactor (photoreactivation process) triggers subsequent electron transfer along a cascade of three uniformly conserved tryptophans followed by proton uptake (Aubert *et al.* 2000). This motif is conserved in all structurally characterized photolyases/cryptochromes and it is probable that this or a similar electron transfer also takes place in animal cryptochromes (see below), but this still needs to be experimentally validated. Compared with photolyases, we know relatively little about the photoreaction process in cryptochromes, particularly animal cryptochromes.

An alignment of Cry1a and Cry1b (Cry1a,b are alternative splicing products; Möller *et al.* 2004) of garden warbler and European robin with published sequences of the photolyase/cryptochrome family shows that the three tryptophane residues (Trp382, Trp359 and Trp306 in *Escherichia coli* photolyase) involved in the electron transfer pathway for photoreduction in *E. coli* photolyase (*Ec*PL; Aubert *et al.* 2000) and *Arabidopsis* Cry1 (*At*Cry1; Giovani *et al.* 2003; Zeugner *et al.* 2005) are also conserved in migratory bird cryptochromes (Liedvogel *et al.* 2007a; Solov'yov *et al.* 2007). However, at the current stage we cannot say anything about their potential function as an electron donor in a radical pair reaction in migratory bird cryptochromes. In *Drosophila* cryptochrome (*d*Cry), the conserved tryptophane residues do

not appear to act as electron donors (Froy *et al.* 2002; Song *et al.* 2007; Hoang *et al.* 2008; Öztürk *et al.* 2008).

One apparent difference between photolyases and cryptochromes is the redox form of their signalling state. Whereas in photolyases the excited state of the flavin chromophore is the fully reduced state (FADH<sup>-</sup>), both *in vitro* and *in vivo* studies of cryptochrome flavoproteins suggest that absorption of blue light (*prerequisite* (v), but note the limitations of this result as outlined below) leads to formation of protein bound flavosemiquinone radical (FAD<sup>•</sup>/FADH<sup>•</sup>; Giovani *et al.* 2003; Banerjee *et al.* 2007; Berndt *et al.* 2007; Liedvogel *et al.* 2007a). In analogy to photolyases (Aubert *et al.* 2000), it is currently assumed that in this reaction, photoexcited FAD (in its fully oxidized ground state) gets reduced to the flavosemiquinon radical via intraprotein electron transfer along a sequence of three conserved tryptophan residues (figure 1c). Each of these three radical pair states that are formed during the photoreduction process might interact with the Earth's magnetic field, but recent results along the tryptophan triad in *E. coli* photolyases suggest that the radical pair intermediate interacting with the magnetic field is only the radical species between the terminal tryptophan and the flavin cofactor (Lukacs *et al.* 2008). In both *Arabidopsis* and the migratory garden warbler (figure 1b) half-life times of the cryptochrome radical intermediate were optically determined and characterized to be on the time scale of several milliseconds (*prerequisite* (vii), but note current limitations outlined in the following; Giovani *et al.* 2003; Bouly *et al.* 2007; Liedvogel *et al.* 2007a). Life times of these orders of magnitude could be well suited to allow for a radical pair based magnetoreception mechanism, but it is important to realize that the radical pairs do not only have to be long lived, but the spin-correlation has also to persist for at least approximately 1  $\mu$ s to allow for any magnetic field effect. This issue can be addressed by time-resolved electron paramagnetic resonance (EPR) spectroscopy and measurements of the magnetic field-sensitivity of the radical pair lifetime. Using this approach, direct formation of radical pair intermediates was recently demonstrated for *Xenopus laevis* Cry DASH (*Xl*Cry-DASH); these data further suggest radical pair formation from a singlet state precursor (Biskup *et al.* 2009). Similar observations of long-lived (more than 10  $\mu$ s) radical species have been reported for photolyases (Gindt *et al.* 1999; Weber *et al.* 2002; reviewed in Weber 2005).

Another key prerequisite for cryptochrome-based magnetoreception is that the rate and yield of the radical pair process must depend on the orientation of the protein in the Earth's magnetic field. Magnetic field effects on the photochemical yield of a flavin–tryptophan radical pair have recently been demonstrated in *E. coli* photolyase (Henbest *et al.* 2008). This experiment provides the proof of principle that photolyases (and due to the close relatedness probably cryptochromes as well) possess the magnetic and kinetic properties that are essential to function as a magnetoreceptor. To date, garden warbler cryptochrome is the only migratory bird cryptochrome that

has been preliminarily characterized (Liedvogel *et al.* 2007a). In summary, spectral analyses on recombinantly expressed and purified garden warbler cryptochrome 1a could show that cryptochrome 1a of migratory garden warblers are excited by light (*prerequisite* (iv)) and that their absorption spectrum has a range that partly corresponds to the wavelength range known to elicit magnetic orientation performance in behavioural experiments (*prerequisite* (v)), but note the limitations of this result in the context of cryptochrome as a potential magnetoreceptor, as birds are also able to perform magnetic compass orientation under green light (centred around 565 nm), where the flavin cofactor of the cryptochromes does not absorb *in vitro*. Furthermore, garden warbler cryptochrome has been shown to form radical pair intermediates with millisecond lifetimes upon photoexcitation in the blue spectral range. The crucial model assumption (*prerequisite* (vii)) that garden warbler cryptochromes form long lived (approx. 10 ms) radical pair intermediates, and thus have the potential to be differentially affected depending on the direction of Earth-strength magnetic fields, could be optically (but not yet by means of EPR, see above) validated (Liedvogel *et al.* 2007a). Together these findings provide experimental evidence that cryptochromes possess all so far testable properties needed to act as a magnetic compass (Liedvogel *et al.* 2007a; Henbest *et al.* 2008; Biskup *et al.* 2009).

Further support for the possibility that a photochemical reaction can act as a magnetic compass sensor comes from a study on a carotenoid–porphyrin–fullerene molecule, which has been selected as a ‘model chemical compass’ (Maeda *et al.* 2008; see figure 1c for model compounds). The data obtained from this model chemical compass provide the general proof of principle that a radical pair based magnetic compass is feasible (figure 1e). The model chemical compass allowed a detectable response in Earth-strength magnetic fields (approx. 50  $\mu\text{T}$ ) at least at  $-20^\circ\text{C}$  and below. The experimental data also provide insight into structural features and kinetics *in vitro* (i.e. anisotropical response to an external magnetic field and favourable asymmetric distribution of hyperfine interactions) that are required for a magnetoreceptor to detect directional information from the Earth’s magnetic field, which is a crucial requirement to putatively be able to work as a magnetic compass. Efforts to further optimize the model chemical compass to work at physiological temperatures are on the way (Rodgers & Hore 2009).

#### 6.4. Magnetic responses in the model plant *Arabidopsis thaliana*

Experiments on *A. thaliana* have suggested that magnetic intensity affects cryptochrome-dependent growth responses (figure 1d; Ahmad *et al.* 2007). But these reported cryptochrome-mediated magnetic field effects on plant growth (Ahmad *et al.* 2007) could not be replicated in an independent study (Harris *et al.* 2009). These findings would be very important, if they turn out to exist and be independently replicable, since even though magnetic responses do not seem biologically relevant for the plant, they would show in principle that

biological tissue is sensitive to the magnetic field responses that are linked to cryptochrome-dependent signalling pathways and could thus confirm the ability of cryptochrome to mediate magnetic field responses. The claimed magnetosensitive responses can best be explained by the radical pair model, as *Arabidopsis* cryptochromes form radical pairs after photoexcitation (Giovani *et al.* 2003; Bouly *et al.* 2007) and these experiments might reflect common physical properties of photoexcited cryptochromes in both plants and animals. Assuming that the reported effects are real, one could further speculate that the magnetic field sensitivity of many organisms was not necessarily a huge step in evolution. Magnetic modulations of cryptochrome responses are likely to be an intrinsic property of the molecule, which was present by chance and might then have been selectively refined in birds to allow for vision-dependent magnetoreception/magnetic compass orientation. However, in the light of the fact that the effect found in the non-blinded experiments of Ahmad *et al.* (2007) could not be independently replicated in the seemingly more carefully controlled and blinded experiments of Harris *et al.* (2009), we want to emphasize here that any interpretation of these experiments should be done with great caution.

## 7. CONCLUSIONS AND FUTURE RESEARCH

Recent progress achieved through interdisciplinary studies of cryptochrome molecules and by studies on the magnetic compass mechanisms of migratory birds has provided a lot of correlative evidence supporting the role of one or more cryptochromes in magnetic compass information detection. Nevertheless, there is still no conclusive evidence demonstrating that cryptochromes are the primary sensory molecule underlying light-dependent magnetic compass orientation in birds. Many open questions remain (many of which are summarized in Mouritsen & Ritz (2005) and Rodgers & Hore (2009)).

For instance, it is essential that the motion of the radical pair forming molecule is restricted (*prerequisite* (iii)); otherwise no molecule can function as a primary magnetic compass detector providing accurate directional information. In photoreceptor cells, the highly oriented membrane structures would provide a well-suited substrate to allow for fixed orientations of cryptochromes. Other candidate anchor structures in other cell types are cytoskeleton proteins and cytosolically embedded membranes. However, at the current stage, the anchoring, spatial organization and exact position of cryptochromes within the cells have still not been demonstrated. Since cryptochrome is a globular protein, it cannot be directly embedded into any membrane itself, but would have to interact either directly or indirectly with a membrane protein. To investigate whether cryptochromes are motion-restricted in the retinal cells of any bird, further experiments determining the localization of the protein on an ultramicroscopic scale and protein–protein interaction studies are necessary.

As the primary magnetoreceptor molecule underlying light-dependent magnetoreception remains unknown, any cryptochrome or any combination of several members of this multigene family are equally probable candidates. But how many different members of the cryptochrome multigene family are expressed in the retina of migratory birds? What are their spectral sensitivities and can they explain the results of orientation experiments under various wavelengths? What are the signalling pathways and the three-dimensional crystal structure of migratory bird cryptochromes? Future experiments on identification, characterization and subsequent recombinant expression of all members of the cryptochrome multigene family expressed in the retina of migratory birds are necessary. In addition to extending the set of candidate molecules within species, we also need to investigate cryptochromes from additional bird species, including day-, night- and non-migratory birds.

Even though the molecular reaction mechanism for most photolyases is now well established, the mechanism of the closely related magnetic compass receptor candidate cryptochrome remains unclear. Understanding structure and function of animal cryptochromes in general and cryptochromes of migratory birds in particular in greater depth requires further investigation, such as crystallization studies and experiments focusing on understanding the catalytic reaction mechanism, signalling pathways and the role of the putative electron donor sites in the electron transfer pathway, e.g. by site directed mutagenesis of the putatively involved Trp-donor sites. As suggested in Maeda *et al.* (2008), spectroscopic measurements similar to those carried out on the model chemical compass should be transferred to experiments on isolated cryptochromes. These *in vitro* experiments will finally allow testing for the existence of magnetic field effects on cryptochrome radical pair reactions, one key untested requirement for potential cryptochrome function as primary receptor molecule in a magnetic compass.

Even if we answer all these questions, we are likely to still be stuck with important, but inherently correlative, indirect evidence. So, how could we conclusively demonstrate that cryptochromes are the primary sensory molecules underlying light-dependent magnetic sensing in an animal?

There are at least two obvious and very direct approaches, which could conclusively implicate cryptochromes in magnetodetection. One such approach would involve electrophysiological recordings from cryptochrome expressing cells in the retina and the second approach would need to combine a robust and easily replicable behavioural assay or conditioning paradigm with genetic manipulation of the tested animals.

Both of these approaches are extremely challenging in practice. Electrophysiological recording of neuronal responses to changes in the magnetic field has been hampered by a large number of irreproducible claims, probably because interferences between the magnetic stimuli and the recording equipment have been interpreted as real neural responses to magnetic fields. The second approach is hampered by the difficulty of finding a truly robust behavioural paradigm in a

genetic model animal such as *Drosophila*, the laboratory mouse *Mus musculus* or the zebrafish *Danio rerio*. Magnetic conditioning of birds to magnetic stimuli has proven incredibly difficult to replicate (Wiltschko & Wiltschko 1995*b*; in the laboratory of H.M., we have tried to replicate several conditioning paradigms which appeared straightforward according to the literature, including the chicken paradigm of Freire *et al.* (2005), but with no success). The only reasonably robust behavioural paradigm, where the biological relevance of the response is fully understood, is the magnetic compass response based on migratory restlessness behaviour in night-migratory birds in orientation cages such as Emlen funnels (Emlen & Emlen 1966). Here, the challenge is rather on the genetics side, since all migratory bird species are notoriously difficult to breed in captivity and, so far, no single transgenic (migratory) bird has been produced. Various temporary genetic interference techniques may be feasible in principle, but they would certainly also be very challenging to conduct in an extreme non-standard animal like a wild-caught migratory bird.

To sum up, much correlative evidence supports the hypothesis that cryptochromes may function as the primary magnetoreceptor molecule in a light-dependent magnetic compass, but it will be very challenging to provide a direct demonstration for this hypothesis. Considering the difficulty of independent replication in the past, any direct demonstration should only be generally accepted after it has been independently replicated using double-blind approaches.

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