Orientation in migratory birds: Orientation mechanisms and their underlying neurobiological background

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Contents

Publications

I. Mouritsen H, Janssen-Bienhold U, Liedvogel M, Feenders G, Stalleicken J, Dirks P & Weiler R (2004): Cryptochrome and activity markers co-localize in bird retina during magnetic orientation. PNAS 101:14294-14299

II. Mouritsen H, Feenders G, Liedvogel M & Kropp W (2004): Migratory birds use head scans to detect the direction of the Earth's magnetic field. Curr Biol 14: 1946-1949

III. Mouritsen H, Feenders G, Liedvogel M, Wada K & Jarvis ED (2005): A night vision brain area in migratory songbirds. PNAS 102:8339-8344

IV. Feenders G, Liedvogel M, Wada K, Mouritsen H & Jarvis ED (2005): Movement-driven gene expression patterns implicate origin of brain areas for vocal learning. Submitted*

V. Feenders G, Liedvogel M, Wada K, Jarvis ED & Mouritsen H: Cluster N activity under changed light and magnetic field conditions during the night in *Sylvia* warblers. Manuscript

* This manuscript was submitted and recently returned with referee comments. We are working on the modifications and will resubmit the manuscript.

Summary of the Ph. D. thesis

Every year, people in Europe are awaiting the arrival of swallows and other birds that announce spring. Many bird species overwinter in warmer regions - the Mediterranean, and down to southern Africa - and they come back in spring to breed in our temperate climate zones. It has been a puzzle for many decades how the birds are capable of finding their way.

In autumn, thousands of young birds start their first journey alone, without any experience of the route or the final destination. They have to rely on a global compass system in combination with some internal information to choose the correct direction. Birds are known to be able to extract compass information from the sun, the stars, and the earth's magnetic field. However, until today, the underlying physiological and neurobiological mechanisms remain unclear.

During my PhD project I aimed at achieving a better understanding of the molecular and physiological mechanisms that are involved in detecting and processing directional cues during orientation behaviour in birds with special focus on the magnetic compass of night-migratory birds.

Before initiating any experiments, the orientation behaviour of the test birds, mainly garden warblers (*Sylvia borin*), was tested. This was done with the bird being placed into a round, transparent Plexiglas cage inside a three-dimensional Helmholtz-coil system. The latter allowed for precise manipulation of the ambient magnetic field. While observing the birds, we noticed that the birds performed a very stereotype sideways movement of their head. Analysis of these head scans during migratory restlessness behaviour revealed that they occur up to three times as often if the bird was exposed to a true-zero magnetic field (no magnetic information available) compared to the natural field environment. Furthermore, during the 10 minutes before initiating migratory restlessness behaviour at night, birds performed head scans with increasing frequency. In addition, birds chose to move into the correct direction significantly more often after they performed a head scan in a natural magnetic field compared to a true-zero magnetic field. These data lead to the suggestion that head scans are involved in the detection of the reference direction of the magnetic field.

If the head scans are indeed used for detecting any directional information of the ambient magnetic field the magnetoreceptor must be located in the bird's head. One theory currently under discussion about how birds may perceive magnetic field information is

based on a light-induced radical-pair process located in the bird's eye(s). The only currently known class of photoreceptor molecules that fulfil the requirements for being a primary receptor in such a mechanism in vertebrates are cryptochromes. The cryptochromes have been found in various tissues including the eyes of chicken and quail. In order to potentially act as magnetoreceptive molecules, cryptochromes have to exist also in the retina of migratory birds. Using immunohistochemical staining techniques, we could show that cryptochromes are expressed in specific cells of garden warbler retinas. The cryptochrome-containing cells furthermore showed neuronal activity during night-time, that is when the birds oriented according to the magnetic field. Such an expression pattern could not be found in non-migratory zebra finches (*Taeniopygia guttata*). These results support the idea of cryptochromes as a putative magnetoreceptor in (night-) migratory birds.

If retinal cryptochromes serve as magnetoreceptors, then a light-dependent signal will be sent to the brain. Thus, we expect one or more brain areas to be involved in processing the information. Such brain area(s) should be activated during magnetic orientation tasks. We applied behavioural molecular mapping in order to reveal active brain areas during magnetic compass orientation. This molecular-genetic technique visualizes immediate early genes (IEGs) that are expressed in neurons upon activation. Any behaviour may lead to a specific expression pattern that can be detected within the subsequent 10 - 60 minutes. Therefore, a careful control of the bird's behaviour is crucial. The analysis of the expression pattern of night-migratory birds (garden warblers and European robins, *Erithacus rubecula*) which sat still but awake during night-time in our orientation cage revealed a very distinct area in the anterior forebrain with pronounced IEG expression. This area, which we termed "cluster N" ("N" for night activation), did not show activity during the day. In contrast, in non-migratory zebra finches and canaries (*Serinus canaria*), cluster N showed activity neither during the day nor during the night. Thus, cluster N seems to be specifically activated at night-time in night-migratory species. Because this area is located either adjacent to or being part of the visual Wulst, i.e. a known integration area for visual information, cluster N may be related to visual/light input. Blocking the light input by covering up the eyes resulted in a significant reduction of IEG expression almost to baseline level. This result is specifically interesting in respect to light-dependent magnetoreception. Based on these data, we conclude that cluster N is involved in visual processing at night that seems to only occur in night-migratory birds and that cluster N

may thus be involved in integrating vision-mediated star and/or magnetic field information in night-migratory birds.

The brain activity patterns described so far are based on birds that were sitting still but awake during the day or night. In the last part of my thesis work, I compared these IEG expression patterns with those of birds that were moving around during the day or showing migratory restlessness at night. Cluster N showed pronounced gene expression during the night irrespective of migratory restlessness behaviour. But the comparison revealed a striking difference with 11 brain regions showing distinct activation upon motor activity. A detailed analysis of gene expression in those regions revealed a significantly positive correlation with the amount of movement (measured as wing beats, flights, and head scans) that could not be found in control areas known to be involved in other behaviours. Corresponding movement-induced regions were also present in the zebra finch suggesting that this is a general motor pathway in the avian forebrain. Interestingly, 7 of those regions are adjacent to or embedding the vocal nuclei that are known to be involved in song learning and production. Thus, the motor pathway seems strikingly similar to the vocal pathway. This supports the hypothesis that the vocal pathway may have evolved out of a pre-existing motor circuit. This conclusion will have important implications for the evolution of the avian brain as it may explain why three distantly related bird families are found to have highly similar brain structures involved in vocal learning and song production.

In summary, this Ph. D. thesis presents evidence for a light-dependent magnetoreceptor in the eyes of night-migratory birds: the birds seem to use head scans to detect the reference direction of the magnetic field, potential receptor molecules are expressed in the retina, and a distinct forebrain area is highly active processing light-dependent information at night under dim light. Furthermore, 11 brain regions could be identified to form the avian brain pathway controlling motor activity, and its location also seems to have important implications for the evolution of vocal learning pathways.

Zusammenfassung der Dissertation

Alljährlich wird in Mitteleuropa die Rückkehr der Schwalben erwartet, die den Frühling ankündigen. Viele Vogelarten überwintern in den warmen Regionen der Erde – bis ins südliche Afrika – und kehren im Frühjahr zum Brüten in die gemäßigten Breiten zurück. Seit vielen Jahrzehnten rätseln wir, wie die Vögel dabei den Weg finden.

Jeden Herbst starten Jungvögel ihre erste Reise, allein und ohne Vorkenntnisse über die Route oder das Ziel. Hierfür müssen sie globale Kompassysteme nutzen und diese mit angeborenen Richtungsinformationen verbinden. Es ist bekannt, daß Vögel die Sonne, die Sterne und das Erdmagnetfeld als visuelle Richtungsweiser nutzen können. Bis heute ist jedoch ungeklärt, welche physiologischen und neurobiologischen Mechanismen dem Orientierungsverhalten zugrunde liegen.

Im Rahmen meiner Promotion habe ich die molekularen und physiologischen Mechanismen untersucht, die bei nachtziehenden Singvögeln der Wahrnehmung von Richtungsinformationen, insbesondere des Erdmagnetfeldes, und damit verbundenen Verarbeitungsprozessen dienen.

Zu Beginn eines jeden Experiments wurde die Orientierung der Testvögel, i. d. R. Gartengrasmücken (*Sylvia borin*), während der Zugunruhe getestet. Hierfür wurde der Vogel in einen zylindrischen, transparenten Kunststoffkäfig innerhalb eines dreidimensionalen Helmholtz-Spulensystems gesetzt, welches eine kontrollierte Manipulation des umgebenden Magnetfeldes erlaubt. Während der genauen Verhaltensbeobachtung bemerkten wir, daß die Vögel den Kopf auf eine sehr stereotype Weise seitwärts drehen. Die Quantifizierung dieser spezifischen Kopfdrehungen, *head scans* genannt, ergab, daß diese dreimal so häufig auftreten, wenn der Vogel einem kompensierten Magnetfeld ausgesetzt ist (d. h. ohne jegliche Magnetfeldinformation), verglichen mit dem natürlichem Magnetfeld. Weiterhin konnten wir einen signifikanten Anstieg der *head-scan*-Frequenz in den Nächten direkt vor Einsetzen der Zugunruhe beobachten. Zudem ließ sich zeigen, daß die Richtungswahl der einem *head scan* folgenden Unrast-Bewegung im natürlichen Magnetfeld signifikant häufiger auf die korrekte Richtung fällt als in einem kompensierten Magnetfeld. Diese Befunde lassen vermuten, daß *head scans* zur Bestimmung einer Referenzrichtung des Magnetfeldes genutzt werden.

Wenn *head scans* tatsächlich der Richtungsbestimmung im Magnetfeld dienen, dann muß ein Magnetsensor im Kopf des Vogels vorhanden sein. Eine aktuelle Hypothese zur

Magnetfeld-Wahrnehmung beruht auf einem lichtinduzierten Radikalpaar-Mechanismus. Die einzige derzeit bekannte Molekülklasse in Vertebraten, welche die Anforderungen für einen primären Rezeptor in einem derartigen Mechanismus erfüllt, sind die Cryptochrome. Cryptochrome wurden bereits in verschiedenen Gewebetypen nachgewiesen, darunter im Auge von Huhn und Wachtel. Um als potentieller Magnetrezeptor in Frage zu kommen, müssen Cryptochrome auch in den Retinen von Zugvögeln vorhanden sein. Mit Hilfe von immunhistochemischen Färbemethoden konnten wir zeigen, daß Cryptochrome in spezifischen retinalen Zellen der Gartengrasmücke exprimiert werden. Die Cryptochrom-enthaltenden Zellen weisen zudem neuronale Aktivität während der Nacht auf, sprich zu einer Zeit, in der sich die Vögel am Magnetfeld orientiert haben. Ein derartiges Expressionsmuster war bei der Standvogel-Art Zebrafink (*Taeniopygia guttata*) nicht nachweisbar. Diese Ergebnisse unterstützen somit die Vermutung, daß Cryptochrome als mögliche Magnetfeldrezeptoren in (nacht-) ziehenden Vogelarten dienen.

Fungieren Cryptochrome als Magnetfeldrezeptoren, so sollten sie lichtabhängige neuronale Signale an das Gehirn weiterleiten. Hirnareale, die diese Information verarbeiten, sollten entsprechend bei der Magnetkompaß-Orientierung des Vogels neuronale Aktivität aufweisen. Die resultierenden Erregungsmuster im Gehirn lassen sich experimentell durch *behavioural molecular mapping* nachweisen: Diese molekulargenetische Methode ermöglicht die Visualisierung sogenannter *immediate early genes* (IEGs), d. h. Gene, deren Expression durch neuronale Aktivität angeschaltet wird. Jegliches Verhalten kann zu einem spezifischen Expressionsmuster führen, welches in den nachfolgenden 10 - 60 Minuten mit dieser Methode detektierbar ist. Aufgrund dieser Charakteristik ist eine präzise Verhaltenskontrolle des Vogels notwendig. Nach einer genauen Analyse der Hirnschnitte von nachtziehenden Vögeln (Gartengrasmücken und Rotkehlchen, *Erithacus rubecula*), welche in der Nacht ruhig und wach saßen, konnten wir im Vorderhirn ein spezifisches Areal mit ausgeprägter Aktivität identifizieren. Dieses Areal wies keine erhöhte Aktivität am Tage auf und wurde als „Cluster N“ benannt („N“ wie Nacht). Bei Zebrafinken und Kanarienvögeln (*Serinus canaria*) hingegen war das Cluster N sowohl tagsüber als auch nachts kaum aktiviert. Demnach scheint die Aktivität in Cluster N spezifisch während der Nacht in nachtziehenden Arten aufzutreten. Da dieses Areal benachbart zu oder eventuell ein Teil des visuellen Wulstes ist, d.h. einer der visuellen Integration dienenden Hirnstruktur, ist eine Verbindung von Cluster N zum visuellen System denkbar. Tatsächlich wurde die Aktivität im Cluster N durch lichtundurchlässige Augenklappen fast vollständig unterbunden. Dieses Ergebnis ist

besonders im Zusammenhang mit einem lichtabhängigen Magnetfeldrezeptor interessant. Aufgrund dieser Daten ist anzunehmen, daß das Cluster N spezifisch bei nachtziehenden Vogelarten an der Verarbeitung visueller Informationen in der Nacht beteiligt ist. Daraus folgend ist zu vermuten, daß das Cluster N der neuronalen Integration visuell basierter Sternen- und/oder Magnetfeldinformationen in nachtziehenden Vogelarten dient.

Die bisher präsentierten neuronalen Aktivitätsmuster wurden von Vögeln gewonnen, welche ruhig im Käfig saßen. Im letzten Teil meiner Arbeit verglich ich diese Expressionsmuster mit denen von Vögeln, welche sich im Käfig bewegten. Cluster N zeigt unabhängig vom Zugunruheverhalten des Vogels erhöhte Aktivität während der Nacht. Hingegen wies der Vergleich prägnante Unterschiede in elf Hirnregionen auf, die erhöhte IEG-Expression aufgrund von motorischer Aktivität zeigten. Eine genaue Analyse der motorisch aktivierten Regionen führte zu signifikant positiven Korrelationen zwischen der Höhe der IEG-Expression und dem Umfang motorischer Aktivität (Anzahl der Flügelschläge, Flüge, *head scans*). Derartige Korrelationen wurden in keiner der Kontrollregionen gefunden. Hingegen ließen sich bei Zebrafinken korrespondierende bewegungsabhängige Areale nachweisen. Es ist deshalb anzunehmen, daß im Vorderhirn von Singvögeln spezifische motorische Areale vorhanden und durch motorische Bahnen verbunden sind. Interessanterweise grenzen sieben dieser Regionen an die bekannten Gesangskerne (beteiligt am Erlernen und Produzieren von Gesang) bzw. umschließen diese Kerne. Somit scheint das motorische System dem Gesangssystems äußerst ähnlich, entsprechend der Hypothese, daß sich die Gesangsbahnen aus einem bereits vorhandenen motorischen Kreislauf entwickelt haben könnten. Diese Schlußfolgerung hat einen großen Einfluß auf die Erklärung der Evolution des Vogelhirns, da sich hiermit begründen läßt, daß in drei entfernt verwandten Vogelfamilien auffällig ähnliche Hirnstrukturen dem Gesangssystem funktionell zugeordnet sind.

Zusammengefaßt lassen die in dieser Dissertation dargestellten Ergebnisse vermuten, daß nachtziehende Singvogelarten einen lichtabhängigen Magnetrezeptor im Auge besitzen: Die Vögel benutzen *head scans*, um eine Referenzrichtung des Erdmagnetfeldes zu detektieren, potentielle Magnetrezeptor-Moleküle werden in der Retina exprimiert und ein spezifisches Areal im Vorderhirn weist erhöhte neuronale Aktivität während der Nacht auf, sofern Licht auf das Auge trifft. Weiterhin wurden elf Hirnareale identifiziert, welche durch motorische Aktivität stimuliert werden. Die Struktur dieses motorischen Systems läßt Rückschlüsse auf die Evolution des Gesangssystems zu.

Structure of the project

In this section I will give a brief overview of the structure of my Ph. D. project. The thesis comprises one introductory chapter and 5 manuscripts, three of them published (Paper I – III), one submitted* (Paper IV), and one as a manuscript (Paper V).

Scope of the Ph. D. project

The scope of my Ph. D. project was to examine the neurobiological and neurophysiological mechanisms underlying orientation behaviour of night-migratory birds with special focus on the magnetic compass orientation. In particular, the whole thesis can be split into three main parts, each of them addressing one specific question:

1. Do cryptochromes as potential magnetoreceptive molecules exist in the retina of night-migratory birds? (Paper I)

2. Can the behaviour during magnetic compass orientation tell us something about the mechanisms of orientation? (Paper II)

3. Can we identify one or more brain regions that are involved in orientation behaviour and/or in processing orientational information? (Paper III – V)

My main focus was directed on the third part.

General experimental design

The general study concept of how to link orientation behaviour and brain activity of night-migratory birds was initiated by H. Mouritsen and E. D. Jarvis (at Duke University, USA). The study design also allowed for an analysis of the retina on the molecular level. Thus, we could simultaneously extract behaviour data along with molecular and physiological data

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from the birds' brain and retina which allowed us to detect relations between the different components.

As this study was the very first to be conducted in the workgroup, one of my main responsibilities was to develop and improve the first experimental set-up designed to link orientation behaviour to processes in the brain. During this process, I critically evaluated the different aspects of the paradigm and consecutively optimized and modified the set-up and procedures to meet the upcoming demands.

For all experimental series that I conducted, it was necessary to start with a careful and continuous observation of the test bird. Therefore, I built a novel behavioural set-up consisting of a cylindrical transparent plexiglass cage with infrared-sensitive cameras mounted on top and at the side. This allowed for a continuous observation and recording and a consecutive detailed analysis of the bird's behaviour. I was the main responsible person for the evaluation of the behaviour in experiments with garden warblers, Sardinian warblers and zebra finches. The final analyses of the orientation and the behaviour were done together with M. Liedvogel.

Ensuring that the birds performed highly consistent behaviour that could be accurately quantified is a crucial prerequisite for all subsequent analyses, and thus a fundamental component of all papers being part of this thesis.

Based on this meticulously controlled behavioural paradigm, it was now possible to search for answers to the above mentioned questions.

1. Are cryptochromes as potential magnetoreceptive molecules existing in the retina of night-migratory birds?

If the birds' magnetic compass sense is light-dependent, cryptochromes are the most likely primary sensory molecules involved in sensing the magnetic field. Consequently, cryptochromes have to exist in the retina of migratory birds. Thus, the idea was to visualize cryptochrome proteins by use of immunohistochemistry. For this, we used the same birds as for the brain studies (cf. Paper III-V).

My contribution to this project consisted of critically observing and evaluating the behaviour of the birds and determining the point where tissue samples were collected. In addition to this, I was also involved in establishing a protocol for the immunohistochemical staining with antibodies against cryptochromes and immediate early genes (ZENK and c-

fos), so it was optimized for retina sections of garden warblers and zebra finches, respectively.

With this study, we could show that cryptochrome1 exists in the retina of night-migratory birds, and that the cryptochrome is located in the ganglion cells and the displaced ganglion cells (Paper I). The cryptochrome1 level is high during night time when the birds perform migratory orientation, whereas in non-migratory zebra finches the crptochrome1 level is low during the night time. Even more interesting, the cryptochrome1 expression pattern of garden warblers is colocalized with neuronal activity dependent immediate early genes. Thus, the cryptochrome1-containing cells also show neuronal activity during night time. This leads to the suggestion that cryptochrome1 is involved in the process of magnetic sensing (Paper I).

2. Can the behaviour during magnetic compass orientation tell us something about the mechanisms of orientation?

When observing the birds' orientation behaviour during the fundamental behavioural experiments, H. Mouritsen and I noticed highly stereotype head movements that we called "head scans" (Paper II). While M. Liedvogel, H. Mouritsen and W. Kropp worked on different aspects of the head scans during the actual migratory restlessness behaviour (comparing different magnetic field conditions, directional choice after head scans, analysing the position of the head), I suggested and performed an examination of the head scan frequency just before onset of migratory restlessness and compared it with the periods of constant migratory restlessness. It turned out that the head scan frequency increases just before onset of migratory restlessness suggesting that birds first carefully determine the reference direction of the ambient magnetic field prior to starting their orientation behaviour.

Additional analyses showed that the birds' head scan frequencies triple in a fully compensated zero magnetic field, and that the very first directional choice after a head scan in a natural magnetic field leads to an above-chance level of correct directional choices compared to an at-chance level in a zero magnetic field. Considering all data together, we conclude that head scan behaviour is used to detect the reference direction of the Earth's magnetic field (Paper II).

3. Can we identify one or more brain regions that are involved in orientation behaviour and/or in processing orientational information?

In an approach completely novel to bird orientation research, I examined the whole brain of migratory garden warblers and non-migratory zebra finches to detect one or more possible brain regions that are involved in orientation behaviour. I. e. brain region(s) that either play a role in receiving and processing orientational information, or that are related to the orientation behaviour itself (migratory restlessness). After the first experimental series and its preliminary results, I refined the experimental design in order to optimize the quality of the outcomes and to meet upcoming demands (Paper III, IV, V). I also independently designed the test series "red light" and "Sardinian warbler" to further elucidate the specificity of the identified activity patterns (Paper V).

Brain activity patterns that are based on behavioural molecular mapping are depending on the behaviour. Thus, I carefully observed and critically evaluated the birds' behaviour and sacrificed the birds after they had performed the wanted behaviour for a sufficiently long time interval. Consecutively, I dissected the brain and shock-froze the hemispheres. In the next step, I sectioned the hemispheres on a cryostat and used *in-situ* hybridization to visualize the neuronal activity of the brain by means of immediate early genes (mainly ZENK). The cDNAs of the immediate early genes were provided by the Jarvis lab at Duke University, USA. I established the behavioural molecular mapping method in garden warblers – a species on which such experiments had not previously been performed. In the final step, I processed the brain sections for permanent staining (autoradiography emulsion, Nissl staining). I applied the whole behavioural molecular mapping procedure, from behavioural observation to the permanent sections, on the garden warblers, Sardinian warblers, and the zebra finches, with assistance regarding the workload from M. Liedvogel.

After the permanent sections were completed, I did the first qualitative analyses of the garden warbler and zebra finch sections (Paper III, IV, V), and of the Sardinian warblers (Paper V), under supervision of E. D. Jarvis. For Paper III and V (garden warblers) M. Liedvogel and I collaborated on performing preliminary quantitative analyses. We improved the method of quantification, and for the final analysis H. Mouritsen and I performed independent quantifications to confirm the repeatability and objectiveness of the results. During this work, we focused on the brain area "cluster N" that we newly identified in our garden warblers. I could show that cluster N is not active in

non-migratory zebra finches (Paper III), whereas the area is highly active in the Sardinian warblers as a fairly sedentary relative of the garden warblers (Paper V). By comparing the garden warbler and zebra finch data with the data from European robins, which were obtained and processed by M. Liedvogel, M. Liedvogel and I could show that cluster N activity requires light input through the eyes (Paper III). Finally, I suggested an additional condition in which garden warblers are exposed to red light, a wavelengths range where night-migratory birds seem to be impaired in orientation. Performing this study, I measured equally high activity levels in cluster N as under white light exposure, a result that is discussed in detail in Paper V.

While analysing the brain sections, we realized that there were prominent differences in immediate early gene expression patterns between the garden warblers that were sitting still and those that were moving around in the cage. In order to measure these apparent differences, I quantified the immediate early gene expression level in the brain regions of interest. The expression level was then related to the amount of movement behaviour as expressed by the number of wing beats, flights, and head scans (quantified by M. Liedvogel, W. Kropp, and myself). For the specific brain regions I obtained positive correlations of the gene expression levels and the amount of movements (Paper IV). A closer examination of these regions and the anatomical findings of previous studies of other work groups led me to the conclusion that the identified motor areas are part of a general motor circuit that is running in parallel to the well-known song system circuits thus supporting the hypothesis that the song system may have evolved out of a pre-existing motor pathway (Paper IV).

Introduction: Orientation in Migratory Birds – Orientation Mechanisms and their underlying Neurobiological Background

Since many decades, people are fascinated by the phenomenon of bird orientation. In small scale it is known from homing pigeons but even more astonishing is the large scale migration of many passerine species each spring and autumn.

Why do some bird species migrate? Due to seasonal changes it is not always possible to survive at the same place during the whole period of a year. At temperate latitudes in Europe, Asia, and North America, the summer with its rich food sources is very good for breeding and feeding offspring. But in winter, many bird species could not survive at these latitudes due to low temperature, moist weather, and low food abundance (especially for species feeding on invertebrates). So the migrating behaviour evolved to optimise exploitation of resources and survival around the world: in spring birds spread out to their breeding areas and in winter they gather in warmer regions.

But how do birds find their way often covering thousands of kilometres?

1. Orientation strategies

When examining the orientation capabilities of animals, we have to separate two classes of orientation behaviour: 1. compass orientation and 2. goal orientation or true navigation. For compass orientation, only a more or less simple compass sense is required. I.e. the animal obtains directional information without knowledge of where exactly it is and without the necessity of having been at the goal site before. In contrast, orienting by true navigation is defined as orienting towards a specific goal. For this, a compass sense is necessary but not sufficient. The animal also has to obtain information about where it is (i.e. have a map sense) before it can use a compass sense to reach the goal. Keeping this classification in mind, several potential strategies exist that birds could use to orient.

1.1. Guiding

The easiest way to get from one place to another, e.g. from summer to winter areas, is by following experienced conspecifics. This does not require any skill in orientation or

navigation but just the instinct to follow adult individuals during a specific time window (Schütz 1943, 1951; Hochbaum 1955).

1.2. Compass orientation

However, many small birds are known to travel alone. Covering long and unknown distances as a single individual is possible by choosing a compass direction (Mayr 1952; Schmidt-König 1965; Berthold 1991; Mouritsen & Mouritsen 2000). This compass orientation is the simplest way of navigation in its broadest sense. The animal has to obtain information about the direction it has to choose and the ability to orient in that direction. Therefore, the animal needs innate directional information and a sensory system to perceive at least one reference direction. The compass direction can be connected to an internal clock controlling flight/migration duration and allowing for a change in direction at a certain time point. This so-called clock-and-compass strategy can lead to a relatively fine-tuned route without involving any map sense or requiring pre-information on the route or goal. It is furthermore sufficient to explain the geographical distribution of free-flying birds on their first autumn migration (Mouritsen 1998a; Mouritsen & Mouritsen 2000).

1.3. Path integration or route reversal

When desert ants leave their nest they run zigzagging across the field but return in a straight line when finding e.g. a food source (Wehner 1982; Wehner & Wehner 1986). This can simply be done by vector integration: describing each movement with a vector consisting of three components for a 3D-desert ground makes it possible to add every vector of each single movement, leading to one final vector at the goal/return point. Reversing this vector leads straight back home. However, this strategy is exclusive to homing and cannot be used for an outbound-journey to an unknown location.

1.4. True coordinate navigation

Birds (and other animals) may also orient by extracting coordinate information, i.e. a minimum of two global coordinates for the goal in respect to the starting point (Kramer 1953; Rabøl 1978, 1994; Mouritsen 2001).

1.5. Landmark orientation and piloting

Piloting (Griffin 1952; Schmidt-König 1965) suggests that animals use familiar landscape features, odour gradients, predictable sounds, etc. to find their way (home). This simple

way of orientation does not necessarily require an internal map of the area. It can be based on information like ‘turn 22° left when reaching the dead fir’. However, when the bird determines its position relative to the goal a landmark-based map is used. The bird then has to use a compass system in order to fly in the relevant direction and reach the goal.

2. Compass systems

Many bird species migrate individually and most remarkably even young birds on their first migration find their way correctly to the wintering grounds albeit having never travelled that route before. This means that the birds need to follow a certain compass direction that could for instance be provided by celestial cues as the sun during the day, the stars during the night, or by using the magnetic field of the earth as a reference system.

In order to reach the goal, birds need to ‘know’ which direction to choose for migration. This seems to be a particular problem for young birds travelling for their first time. However, Helbig, Berthold and colleagues could show that blackcaps (*Sylvia atricapilla*) posses innate compass information. They hand-raised blackcaps from eastern populations that migrate towards southeast through Europe (passing the Arabic peninsula) and blackcaps from western populations migrating towards southwest (passing Spain and the Street of Gibraltar). When tested for their orientation preferences during their first autumn, they chose different population-specific migratory directions with changes in orientation reflecting the natural migratory route of the free-flying conspecifics (Helbig et al 1989; Helbig 1994). In further experiments, Helbig performed cross-breeding experiments with blackcaps from the southeast and the southwest migrating populations. The offspring showed an intermediate migratory direction towards due south, strongly suggesting that their migratory direction is genetically inherited in a phenotypically intermediate mode (Helbig 1991). Another hint for a genetic base of migratory orientation was observed during the last decades: blackcaps started to migrate to the British Isles instead of heading towards the Mediterranean (Zink 1962; Schlenker 1981). Studies with hand-raised birds proved this direction to be genetically fixed (Berthold et al 1992).

The data show that blackcaps posses innate migratory direction information and that this genetically fixed information is flexible enough to react to external factors within a few generations. In recent years, the heritability of migratory behaviour has also been demonstrated in other species: for instance, cross-breeding experiments with black

redstarts (*Phoenicurus ochruros*) as a short-distance migrant and common redstarts (*Phoenicurus phoenicurus*) as a long-distance migrant resulted in offspring performing intermediate amounts of migratory restlessness (Berthold & Querner 1995).

In order to use this innate directional information for migration, a bird needs to extract compass information from one or more external reference cues. To test what compass system a bird is using, controlled orientation experiments have to be performed. Very conveniently, night-migratory birds, when placed in a cage during the migratory season, perform a behaviour called *Zugunruhe* or migratory restlessness: the birds show characteristic wing whirring and other motor activity during night-time and this activity is oriented in the migratory direction also chosen by free-flying conspecifics (Kramer 1949; Emlen & Emlen 1966; Mouritsen 1998b). In 1951, Kramer published his observations on directed activity of day-migratory starlings (*Sturnus vulgaris*) in a round cage: the activity was concentrated in the direction reflecting the migratory orientation known from wild starlings (Kramer 1951). In order to easily and time-efficiently measure the orientation of caged birds, Emlen developed a special cage, the so-called Emlen funnel (Emlen & Emlen 1966). This is a funnel-shaped cage that in its original form had an ink pad as the bottom of the cage and the cage walls covered with blotting paper. A bird, when placed into the cage, made ink-marks on the cage walls whenever it tried to fly in a specific direction. Later on, the ink-pad was removed and the walls were covered with typewriter correction paper (e.g. TippEx) on which the bird leaves scratches whenever it touches the cage wall (Rabøl 1979). This method is now used in most orientation studies with migratory passerines (e.g. Mouritsen 1998b; Wiltschko et al 2002a,b; Muheim & Akesson 2002; Giunchi & Baldaccini 2004) as it is easy to handle and rather friendly to the bird. The most important advantage of orientation cage studies is that it allows for a precise control of the cues that may affect the birds' orientation ability.

2.1. The sun compass

Using orientation cages, Kramer was the first to show that an environmental cue can provide a bird with compass information. He observed that migratory restlessness in starlings is correctly directed only when the sun is visible. When deflecting the sunlight with mirrors the birds showed the expected change in orientation preference (Kramer 1949, 1952). Thus, starlings use a sun compass to orient.

Not the sun's elevation but the azimuth of the sun, i.e. the sun's position reflected onto the horizon, is the relevant compass cue. Because the azimuth (and the elevation) of

the sun is changing during the course of the day, an internal clock is necessary to relate the sun's position with the time in order to keep a constant migratory direction. The easiest experiment to test this is to analyse the orientation of clock-shifted birds, i.e. birds kept under a shifted day-night cycle. If an internal clock is involved, the birds' orientation will change according to the time-shift. This could be shown for starlings (Hoffmann 1953a,b) and homing pigeons (*Columba livia*) (Schmidt-König 1958, 1961, 1965) in the early years of orientation experiments, and later in some other bird species like mallard (*Anas plathyrynchos*), scrub jays (*Aphelocoma coerulescens*), western meadow larks (*Sturnella neglecta*), and white-throated sparrows (*Zonotrichia albicollis*) (for review, see R Wiltschko & Wiltschko 1999). Furthermore, R. Wiltschko and Wiltschko (1981) could show that homing pigeons learn to use a sun compass when they are about three months old.

2.2. The star compass

Many birds migrate at night, when no sun information is available, so they need other cues to orient. Sauer (1957) was the first who tested birds – garden warblers (*Sylvia borin*) – under an artificial starry sky. The birds were well oriented, thus they were able to extract information from the stars. Ten years later Emlen conducted experiments with indigo buntings (*Passerina cyanea*) (Emlen 1967a,b). When he turned the horizontal axis of the planetarium sky by 180°, the birds changed their direction accordingly. Emlen (1970) further showed that the crucial information is the centre of rotation of the starry sky: When he shifted the rotational centre from the polar star (the rotation centre on the northern hemisphere) to Betelgeuse young birds raised under this condition acted as if Betelgeuse was located at the position of the polar star, i.e. north. Emlen suggested that birds may detect the rotational axis of the sky by extracting the north-south axis from the different linear velocities of the stars because stars close to the rotation centre have a lower linear velocity than the stars further away (despite same angular velocity). To date, there is no clear evidence of how birds may detect and learn the apparent rotation of the sky.

Emlen sought for a possible role of experiencing the starry sky in order to develop a star compass. Therefore, he hand-raised birds and exposed them to different star information. 2 out of 3 birds, that had not seen the starry sky before onset of migration, did not orient. Even subsequent exposure to the natural sky for 4 weeks did not evoke star-based orientation (Emlen 1969). Birds that were kept under an artificial sky with a shifted centre of rotation determined their star compass accordingly and kept to this pattern even

in the following migration seasons (Emlen 1972). With these studies Emlen could show that birds learn and determine the star compass within a crucial time period after that the star compass mechanism seems to be no longer adjustable.

Another question arising is whether birds need the complex information of the whole starry sky in order to establish a functioning star compass. W. Wiltschko & Wiltschko (1976) tested garden warblers with a very simplified starry sky: 16 pairs of little diodes illuminating small holes in the 'sky' imitated starlight. The birds were able to calibrate this artificial star pattern according to the geomagnetic field. Consecutively, they oriented correctly in relation to the simplified star pattern even when no magnetic direction information was available. Thus, the star pattern itself is not important for a star compass. In further studies, Wiltschko and colleagues could show that the crucial information to establish the star compass is the rotation of the starry sky: only birds that experienced a rotating sky, but not when being raised under a stationary sky, were later on able to use the starry sky for orientation (Wiltschko et al 1987). Remarkably, after establishing the star compass, even a stationary starry sky served sufficiently as a directional cue. This means that birds can extract directional information from the star constellation itself. The use of stars for orientation was further shown for blackcaps (Viehmann 1982), savannah sparrows (*Passerculus sandwichensis*, Bingman 1983), pied flycatchers (*Ficedula hypoleuca*, Bingman 1984), and European redstarts (Mouritsen 1998b). Moreover, Mouritsen & Larsen (2001) found evidence that the star compass of pied flycatchers and blackcaps is time-independent. These species extract compass- but not map-information from the star constellation itself.

In summary, the development of a functioning star compass requires that the bird experiences celestial rotation within a certain time period during ontogeny. Afterwards, the star pattern itself provides sufficient information for a compass reference direction.

2.3. The magnetic compass

Both during day and during night, birds need a compass cue that is available under cloud cover so migration/orientation is not blocked when the sun and stars are not visible. Already since 1882 it has been suggested (Viguier 1882) that birds use the magnetic field of the earth to orient. The earth can be seen as a huge magnet with field lines pointing out of the earth's surface at the magnetic south pole at perpendicular angle. The angle between the magnetic field lines and the earth's surface decreases with decreasing distance to the equator. At the equator, the field lines are oriented horizontally to the surface. The angle

increases again, but with an opposite vertical direction, so that at the magnetic north pole the field lines point into the earth's surface at perpendicular angle (Figure 1).

Merkel and Wiltschko (Merkel & Wiltschko 1965; Wiltschko 1967) were the first to show that European robins (*Erithacus rubecula*), when tested in orientation cages, change their direction in response to a change of the magnetic field. A variety of species have meanwhile been shown to use a magnetic compass, e.g. pied flycatcher, different *Sylvia* warblers, yellow-faced honeyeater (*Lichenostomus chrysops*) and snow buntings (*Plectrophenax nivalis*) (for an overview, see W Wiltschko & Wiltschko 1996, Table 1). During the following years researchers undertook a variety of behavioural studies to characterize the magnetic compass as described in the following paragraphs.

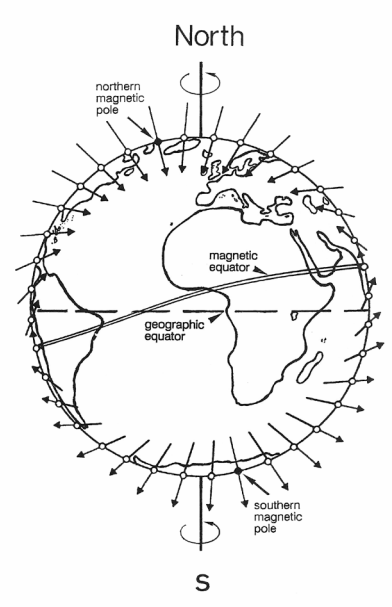

Figure 1: The earth and the geomagnetic field. The length and the angle of the arrows indicate the strength and the inclination of the magnetic field lines. (From R Wiltschko & Wiltschko 1995)

2.3.1. *The magnetic compass is an inclination compass*

The geomagnetic field can be described by three characteristics: The polarity of the field lines, the inclination (the angle between the field line and the gravity/earth's surface) and the intensity. The intensity is highest at the magnetic poles and weakest at the equator. The inclination changes from +90° at the magnetic south pole to -90° at the magnetic north pole, with 0° at the magnetic equator. The polarity is always the same, directed from magnetic south to north.

Humans make use of the polarity by using a horizontal compass needle to orient. In contrast, W. Wiltschko & Wiltschko (1972) showed that European robins possess a

magnetic compass that is insensitive to the polarity but instead based on the inclination. In their experiment they changed the direction of the vertical and/or the horizontal component of the magnetic field vector. By this, they observed a change in the birds' orientation whenever only one of the components was inverted, but not when both components were inverted which is equivalent to a change in polarity (Figure 2). Thus, the birds detect magnetic inclination implying that they can distinguish between polewards and equatorwards but not between North and South. At the equator the information is ambiguous due to the parallel orientation of the magnetic field lines, hence requiring the use of additional cues like stars or the sun for birds migrating across the equator in order to orient correctly.

To address the problem arising from the characteristics of the magnetic equator, W. Wiltschko & Wiltschko (1992) kept garden warblers for 2 days in a horizontal magnetic field during autumn migration. After this treatment, the birds reversed their orientation direction suggesting that the horizontal field lines serve as a trigger to switch the directional choice. Cochran and colleagues (Cochran et al 2004) showed that free-flying thrushes recalibrate their magnetic compass by means of sunset cues. Thus, it is well possible that birds when crossing the (magnetic) equator use a variety of cues, such as stars and sunset cues, to recalibrate their orientation system in order to keep the correct direction.

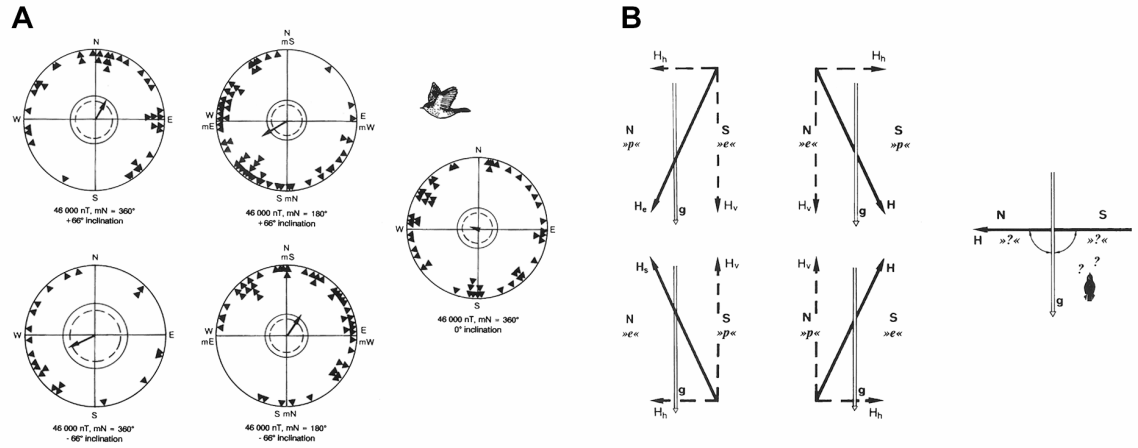

Figure 2: *A*: Magnetic compass orientation behaviour of European robins under different inclination angles. *Triangles*: headings of individual birds; *Arrows*: vector of the mean orientation of the group, the length representing the length of the r-vector as an indication of the concentration. *Circles*: significance border of the Rayleigh test ($p<0.05$ and $p<0.01$). *B*: Components of the magnetic field vectors used in the experiments as shown in *A*. *Upper left diagram* depicts the local geomagnetic field. Reversing only the horizontal (*upper central diagram*) or the vertical (*lower left diagram*) component leads to a reversion of orientation. Reversing both components (*lower central diagram*) results in a change of polarity that does not affect the magnetic compass orientation of the birds. The *right diagram* shows the situation at the magnetic equator where the field lines run horizontally. (From R. Wiltschko & Wiltschko 1995)

2.3.2. *The magnetic compass has an intensity window*

Most orientation experiments take place at one location, where the birds are housed and tested. This means, that the total intensity of the magnetic field is constant. W. Wiltschko (1972, 1978) tested whether birds are able to orient in magnetic fields of different total intensities. The results suggested correct orientation directions within an intensity frame of about ± 20 %. When he exposed the birds to intensities deviating more than 20 % from the local intensity, the birds were disoriented. But when the birds had three days to adapt to the new intensity window, they were well-oriented. Thus, the birds were able to shift the functional range according to the new local conditions. Additionally, they still showed correct orientation under the previous intensity range. Interestingly, the birds were also able to adapt to intensity values well out of the natural range. But they were not able to interpolate and orient under intensities they had not experienced before, even if those were of intermediate values with respect to the experienced ones.

2.3.3. *The magnetic compass is wavelength dependent*

Another characteristic of the magnetic compass investigated in behavioural experiments during the last years is its wavelengths dependency: birds are disoriented under light of wavelengths in the yellow-red part of the spectrum, whereas they are able to correctly orient under monochromatic blue or green light (European robins: Wiltschko et al 1993; W Wiltschko & Wiltschko 1995, 1999; Muheim et al 2002; Australian silvereyes (*Zosterops lateralis*): Munro et al 1997a; garden warblers: Rappl et al 2000) (Figure 3). Muheim et al (2002) tested European robins under monochromatic light with a narrower wavelengths peak than those that have been used in previous studies. By this they could narrow the wavelength to 560.5 nm where European robins are still well-oriented and 567.5 nm where the birds are abruptly disoriented. These results are hard to explain without the visual system being involved in magnetosensing. It seems very likely that the magnetic field is perceived via the eyes by non-visual photopigments like the blue-light receptor cryptochrome (see next paragraph; Cashmore 1997). However, the situation is not that simple since Wiltschko et al (2004a) could show that, when adding monochromatic yellow light to green or blue, the magnetic orientation of robins became fixed to one direction, irrelevant of the migratory season. The authors suggest a minimum of two receptors responsible for magnetosensing that interact in an antagonistic fashion (W Wiltschko & Wiltschko 2001; Wiltschko et al 2004a,b). But the data are too inconclusive to allow for a definitive interpretation. An explanation becomes even more difficult

because the orientation of migratory birds is additionally sensitive to the intensity of the monochromatic light. European robins and Australian silvereyes showed seasonally appropriate directions under monochromatic light of low intensities and of wavelengths in the green-yellow range of the spectrum, whereas the orientation became fixed (irrespective of the season) or even disrupted when increasing the intensity (Wiltschko et al 2000; W Wiltschko & Wiltschko 2001; Muheim et al 2002; Wiltschko et al 2003a). This intensity-effect is dependent on the wavelength and the species. Wiltschko et al (2003a) showed for Australian silvereyes, that the fixed direction under bright green light is not based on the inclination of the magnetic field vector as the direction did not change after inverting the vertical component of the magnetic field. Thus, it remains unclear how the different wavelengths and intensities affect a light-dependent magnetoreceptor.

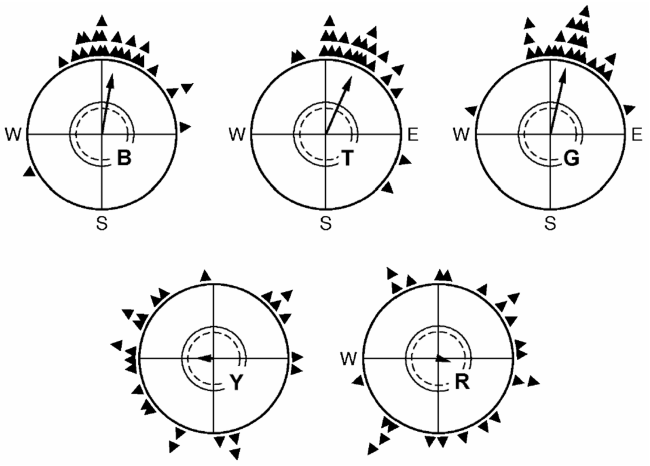

Figure 3: Magnetic compass orientation behaviour of European robins under different wavelengths. The birds were correctly oriented under monochromatic light of the blue (*B*), turquoise (*T*), and green (*G*) spectral range, whereas they were disoriented under monochromatic yellow (*Y*) or red (*R*) light. Symbols as in Figure 2. (From W Wiltschko & Wiltschko 2002)

2.3.4. *Is the magnetic compass based on magnetite or light-mediated?*

To explain how birds may perceive the magnetic field, two main theories are currently under discussion. The first one is based on small single domain, paramagnetic or superparamagnetic particles – e.g. magnetite – embedded in tissue. Single domain particles act like compass needles and could evoke neuronal signals for instance by mechanical deformation of the cell and/or by changing the biophysical characteristics of ion channels leading to a change in membrane potential (e.g. Kirschvink et al 2001). Other processes based on mechanical deformations are assumed for para- or superparamagnetic particles because these particles only pertain a permanent magnetic moment in the presence of an external magnetic field (Shcherbakov & Winklhofer 1999; Fleissner et al 2003). Fleissner et al (2003) could show that superparamagnetic magnetite is embedded as clusters in the

tissue of the upper beak of homing pigeons. The pattern of occurrence is highly ordered, making up three groups at a fixed distance on each margin of the inner beak. Each group contains 10 to 15 clusters of magnetite crystals and is embedded in a dendrite ending of an un-myelinated neuron. This arrangement could directly evoke a neuronal signal. Non-crystalline platelets, seemingly of iron phosphate, in the same cells may act as a magnetic enhancer to increase sensitivity to the rather weak magnetic field lines of the geomagnetic field. Thereby, the detection threshold of the nanocrystalline magnetite may be improved. There are other studies suggesting a magnetoreceptor based on single domain magnetite: such a magnetoreceptor should be affected by a strong magnetic pulse that will alter the magnetization of the single-domain magnetite crystals. Beason and Semm (1996) tested the magnetic orientation of bobolinks (*Dolichonyx oryzivorus*) before and after treatment with a strong magnetic pulse and observed a shift in direction after the pulse-treatment which can be explained by an involvement of single-domain magnetite. This shift disappeared when the ophthalmic branch of the trigeminal nerve was anaesthetized, suggesting that this nerve is involved in magnetite-based magnetosensing (see below). However, because the anaesthetized birds were oriented correctly, another magnetoreceptor system must be responsible for the birds' magnetic compass orientation. This is in line with findings from Munro et al (1997b) who treated Australian silvereyes with a strong magnetic pulse but separated juvenile, inexperienced birds from adult birds that had migrated before. The results suggest that the pulse does affect adult birds but not the juvenile ones. This means that only the magnetic orientation of the adults seem to involve single domain magnetite. This magnetite-based system may therefore be used to detect changes in intensity, irrespective of the direction of the bird's head within the magnetic field, thus being part of a magnetic map sense rather than a global magnetic compass sense (Munro et al 1997b; Fleissner et al 2003; Mouritsen & Ritz 2005). In summary, the young silvereyes as well as the bobolinks of Beason and Semm (Beason & Semm 1996) must have used an alternative magnetic compass receptor system.

The second theory proposes a light-mediated magnetoreceptor. First described by Schulten et al (1978) and further developed by Ritz et al (2000), it suggests that migratory birds could sense the compass direction of the earth's magnetic field through radical-pair processes in differently oriented, light sensitive molecules of the retina and thus perceive the magnetic field as visual patterns. In more detail, the transduction process starts with molecules that form radical-pairs upon photoexcitation. These radical-pairs can exist in singlet and triplet state with each state known, at least in some radical-pair reactions

(Cintolesi et al 2003), to result in different end-products with differing biochemical properties (Figure 4a). The interconversion between the singlet and triplet state is affected by magnetic fields of very weak strength – like the geomagnetic field. Depending on the ratio of singlet to triplet state the product yields differ and hence influence the biophysical characteristics of the cell accordingly. Subsequently, an amplification process is necessary to enhance the neuronal signal. This could be possible by a direct connection of the radical-pair system with a neurotransmitter so that the modulation of the radical-pair reaction will change the amount of neurotransmitters being released in the cell and consequently alter the neuronal signal. Or, alternatively, the radical-pair system may exploit the transduction process of an existing sensory system like vision by modulating e.g. the sensitivity of the photoreceptors. A connection to the visual system may bear an additional advantage: the radical-pair forming molecules need to be fixed in an array in order to provide the directional dependence; such an arrangement could be provided by highly ordered retinal structures. If the visual system of the bird, i.e. the eye, indeed contains a primary magnetoreceptor, one can imagine that the bird may see the magnetic field as a pattern (e.g. an intensity gradient, Figure 4b) superimposed on the original picture generated by the retina (Ritz et al 2000).

At present, the only known potential candidate molecules for a light-dependent, radical-pair-based magnetoreceptor in the vertebrate eye are cryptochromes. This multigene family has first been discovered as blue-light photoreceptors in plants (Ahmad & Cashmore 1993; Lin et al 1995). They are also ubiquitous present in mammalian tissue, including the eye where they seem to play a role in circadian rhythm regulation (reviews: Cashmore et al 1999; Sancar 2004; mouse: Miyamoto & Sancar 1998; humans: Todo et al 1996; Hsu et al 1996). Similar results were obtained from chicken and quail (Haque et al 2002; Fu et al 2002). Cryptochromes are closely related to a class of photolyases and possess likewise the catalytic chromophore flavin-adenine dinucleotide (FAD) and a second chromophore acting in light-harvesting. These functional structures make the cryptochromes suitable for a radical-pair-based magnetoreception system.

If cryptochromes are the radical-pair forming molecules according to the Ritz et al model, then these molecules should exist in the retina of migratory birds. Thus, we searched for cryptochromes in the retina by use of immunohistochemistry. As shown in our paper (Mouritsen et al 2004a, paper I), we found cryptochrome 1a (Cry1a) to be expressed in the ganglion cell layer, in large, displaced ganglion cells in the inner nuclear layer, and to a

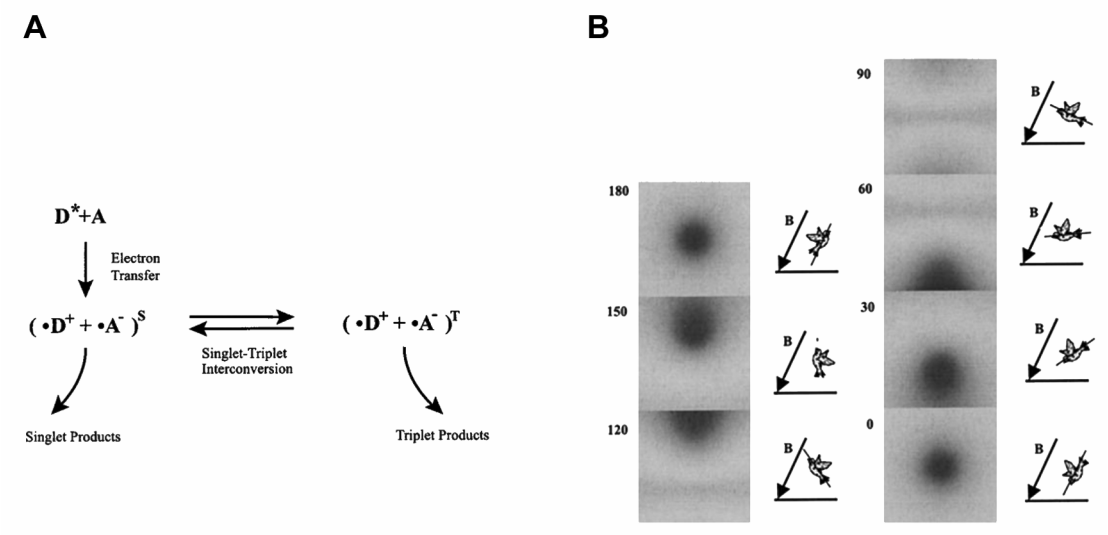

Figure 4: The radical-pair process. *A*: Reaction scheme of the radical-pair process starting with a photoexcitation-induced electron transfer from a donor molecule (*D*) to an acceptor molecule (*A*). The singlet triplet interconversion depends on the alignment of the molecules in the ambient magnetic field. *B*: Visual modulation patterns of how birds may see the magnetic field when flying at various angles in respect to the magnetic field vector. (From Ritz et al 2000)

smaller degree in the photoreceptor layer. In migratory garden warblers, the expression level of Cry1a was slightly higher during night, when the birds performed magnetic orientation tasks, than during the day, when the birds showed regular movements in the cage (Figure 5). Furthermore, the cryptochrome-containing cells showed neuronal activity during night. In contrast, in non-migratory zebra finches (*Taeniopygia guttata*), cryptochrome expression was most prominent during the day which is in line with the general circadian expression pattern known from other species (cf. references cited above). At night, cell types that showed high cryptochrome expression and neuronal activity in garden warblers had little or no cryptochrome expression and reduced neuronal activity in the zebra finches (Mouritsen et al 2004a). Of special interest is the specific expression of cryptochromes in displaced ganglion cells in migratory birds, as those cells are known to project to the midbrain area nBOR (nucleus of the basal optic root), where cells have been claimed to be sensitive to magnetic fields (Semm et al 1984; Semm & Demaine 1986). Thus, our results show that molecules, fulfilling the biophysical properties required for the Ritz et al model, exist in the eyes of migratory birds and that they are found in highly active cells sending information to the brain during magnetic orientation behaviour. Therefore, our results, supported by the study of Möller et al (2004) showing cryptochrome expression in the retina of European robins, are in line with the idea that the eye is the

magnetosensitive organ with the cryptochromes as possible magnetosensitive photopigments.

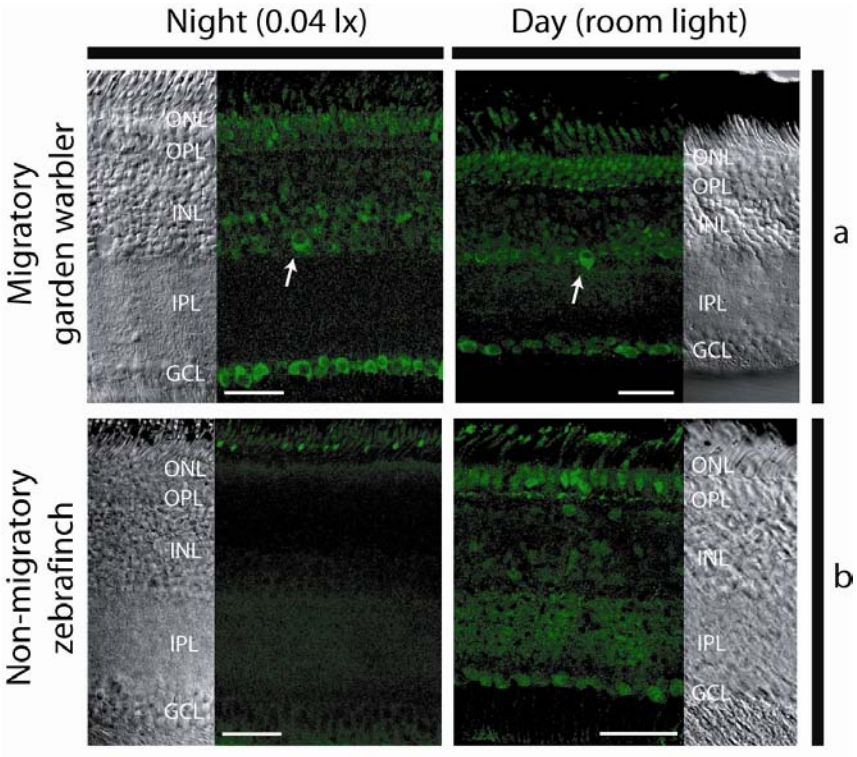

Figure 5: Immunohistochemical staining of CRY protein expression in the retina (see paper I). Sections of migratory garden warblers (*a*) and non-migratory zebra finches (*b*). Retina sections were taken at night under dim light (*left column*) and at day (*right column*), respectively. The *white arrows* mark displaced ganglion cells. *ONL*: outer nuclear layer; *OPL*: outer plexiform layer; *INL*: inner nuclear layer; *IPL*: inner plexiform layer; *GCL*: ganglion cell layer. Scale bar: 40 µm.

Other efforts were undertaken to test on the behavioural level whether the magnetic compass orientation of migratory birds is based on radical-pair forming molecules. Biophysical considerations led to the conclusion that weak oscillating magnetic fields of high frequency should influence the spin states of the radical-pair forming molecules and hence distort the direction-encoding of the molecules resulting in disorientation behaviour (Cintolesi et al 2003, Ritz et al 2004). Ritz, Wiltschko and colleagues tested the magnetic orientation of European robins under such weak, high-frequency oscillating fields and found that the magnetic orientation abilities of the birds are indeed affected (Ritz et al 2004; Thalau et al 2005). This supports the idea of a radical-pair mechanism underlying the magnetic compass.

2.3.5. *The magnetic compass seems to be lateralized*

Lateralization is a common phenomenon in birds and other animals, with specific functions being restricted to or dominated by one hemisphere. Chicks (*Gallus domesticus*), for example, are dominated by the left hyperpallium, a forebrain structure, during visual discrimination tasks (Deng & Rogers 1997). Food storing tits need the right eye to retain memory information of the location of previously cached food items (Clayton 1993; Clayton & Krebs 1993). In general, the visual system of birds seems to be separated in a left-eye-system responding to detailed properties of a stimulus and a right-eye-system to categorize stimuli (Rogers 1996).

In 2002, Wiltschko et al published a study with European robins that had one of their eyes covered with a light-tight eye cap during magnetic orientation tasks. They could show that robins having their left eye covered are well oriented, whereas an eye cover on the right eye resulted in disoriented behaviour (Wiltschko et al 2002a). Equivalent tests in Australian silvereyes led to the same result (Wiltschko et al 2003b). These studies support the idea of a magnetosensor in the eye and point to a dominance of the right eye over the left eye during magnetic orientation tasks. Preliminary data on robins in our workgroup showed that the birds performed more head scans (Mouritsen et al 2004b) with their right eye occluded by light-tight eye-covers than when they had their left eye covered. However, these data are currently based on a pilot study with only 6 individuals and a proper experimental series has to be performed to support the hypothesis of a lateralized magnetosensor in the eye.

With the methods we applied to visualize neuronal activity patterns in the retina and the brain (compare with paper I and III), we were not able to detect any differences between the left and right retina and brain hemispheres, respectively. This may be because our technique is not sensitive enough to detect small differences.

2.4. Interaction of different cues

In the previous parts I have described a variety of cues birds can use for orientation. Under natural conditions the birds have often more than one cue at their disposal, e.g. at night the stars and the magnetic field, during day the sun and the magnetic field. Studies aiming at understanding how birds prioritize the different cues led to ambiguous and controversial results. Able and Able demonstrated that in young and possibly also adult savannah sparrows the magnetic compass is calibrated by celestial cues during day and/or night (Able & Able 1996, 1999). Weindler et al (1998) could show the same in young pied

flycatchers. In contrast, in the studies of Sandberg and colleagues (Sandberg et al 2000) as well as Akesson and colleagues (Akesson et al 2002) the birds apparently calibrate the celestial cues by means of the magnetic field. Muheim et al (2004) point out that there may be two different periods to distinguish in respect of orientation: a pre-migratory and a migratory period. Each period apparently has its own hierarchy of orientation cues. Recently, Cochran et al (2004) tracked the orientation of free-flying *Catharus* thrushes throughout the night after the birds had been exposed to a shifted magnetic field during the sunset hours in an outdoor cage. The researchers could show that the birds calibrate their magnetic compass according to sunset cues (e.g. the polarized skylight or the sun itself) and then follow the magnetic direction throughout the night irrespective of conflicting star information. The next evening the thrushes recalibrated the compass direction before departure. Thus, it seems that birds use a variety of available directional cues. The relevance of those cues is most likely to be specific to the species, the age and the actual situation the bird is facing (e.g. geography, weather). Further studies have to be carried out carefully in order to shed light on the interrelation of the different compass cues.

3. Behaviour and Brain

This far, I presented many behavioural studies trying to understand how birds are able to find their way, especially when flying alone the first time over long distances. These experiments not only help us to understand the orientation behaviour of birds but also provide hints about the possible underlying physiological mechanisms. Some early studies focused on the physiology itself, revealing special structures such as magnetite (Walcott et al 1979; Beason & Brennan 1986) or neuronal systems like the trigeminal nerve and the nBOR (Semm & Demaine 1986; Semm & Beason 1990) that may at least partly be involved in magnetosensing. These older results are still heavily debated.

There is another approach, however, that may bring us an important step forward in understanding the avian orientation system. This is to investigate what brain areas are involved in integrating and processing information for orientation and navigation. Only the interplay between the sensory system receiving the information, and a neuronal network integrating and processing the information will enable the bird to solve those tasks. If we know what brain areas are involved in this task and combine this information with known

functional and connectivity data, we may derive valuable information on what physiological mechanism(s) support the birds' orientation system.

What neuronal pathways may be responsible for orientation and navigation in birds? Can the orientation tasks be solved based on known networks? Or do birds possess specialized systems that enable them to find their way?

Many years ago, iron deposits in the head and neck region of birds and particularly in the beak region were detected and discussed as the possible magnetoreceptor (Walcott et al 1979; Presti & Pettigrew 1980; Beason & Nichols 1984; Beason & Brennan 1986). The trigeminal nerve and more specifically the ophthalmic branch of this nerve is located close to those iron deposits so researchers started to perform electrophysiological recordings from the trigeminal nerve and specifically from the ophthalmic branch searching for responses to magnetic stimuli. Semm and Beason recorded some positive responses of the ophthalmic nerve and the trigeminal ganglion to magnetic stimuli (Beason & Semm 1987, 1996; Semm & Beason 1990). But, as mentioned above, these results are still controversially discussed, since several research groups were unable to replicate these findings. Recently, Mora and colleagues could show that the ophthalmic branch of the trigeminal nerve is necessary in homing pigeons to succeed in a magnetic anomaly discrimination task, whereas the close-by located olfactory nerve is not involved (Mora et al 2004). This supports the idea of the ophthalmic nerve to be involved in magnetic sensing, at least in relation to a magnetic map sense.

Not only in the peripheral nerve system but also in the brain, magnetosensitive cells were reported: from the nBOR (nucleus of the basal optic root), the optic tectum and the pineal (Semm et al 1984; Semm & Demaine 1986; Beason & Semm 1987). But the results are not clear-cut and have proven difficult to reproduce; in fact nobody has ever been able to reproduce any of these findings. Another brain area of potential interest is the hippocampal formation, an area known to be involved in spatial memory, e.g. caching and retrieving food items (Sherry & Vaccarino 1989; Hampton & Shettleworth 1996; Smulders & deVoogd 2000). Despite intensive studies on the hippocampus and its possible role in orientation no clear evidence could be brought up to support this idea. However, recent studies point towards an involvement of the hippocampus in the map sense but not in a compass sense (Bingman et al 1999; Gagliardo et al 2001; for review, see Bingman & Able 2002). Thus, brain areas relevant for compass-orientation remain to be identified.

3.1. Behavioural molecular mapping

Because no clear hints exist about what brain areas may play a role, one should examine the whole brain in order to detect the areas in question. This can best be done by use of a special technique, called *behavioural molecular mapping*. The method is well-known from studies on the song-system in songbirds but had not previously been used in bird orientation research (except one recent study on homing in pigeons, Shimizu et al 2004). It is based on the visualization of neuronal activity dependent genes to detect brain areas being active during specific behavioural tasks. In more detail, when an animal – in our case a bird – is performing any kind of behaviour, specific areas in the brain are activated. Those areas receive sensory input and process the information to an output signal. This signal in turn is either transferred to further processing and/or integration areas or it is sent to the periphery as a motor signal or sensory loop.

Whenever a forebrain area is activated, a special class of genes is expressed: the so-called immediate early genes (IEGs). Examples are ZENK (acronyms zif-268, Egr-1, NGF-1A, Krox-24), c-fos and arc. The mRNA of these genes can be detected from about 10 minutes after neuronal activity occurred and remain for about 60 minutes before degradation. A peak occurs after 30 minutes of neuronal activation (Jarvis & Nottebohm 1997). IEGs are mainly transcription factors, but their exact function is not known, yet. However, ZENK and c-fos are not activated by neuronal activity in all brain cell types. These exceptions in birds are some thalamic neurons, telencephalic neurons receiving primary sensory input from the thalamus, and globus pallidus neurons (Mello & Clayton 1995). By using the characteristics of these genes – expression upon neuronal activity and subsequent degradation after one hour – one can map the mRNA expression pattern by means of in-situ hybridization with e.g. a radioactive probe. The riboprobe labels those brain areas that were active at some point within the last 60 minutes, mainly within the last 30 minutes of the bird's life.

Since any sensory or motor stimulation may be reflected in the brain by IEGs, it is important to observe and record exactly what the bird was doing during the last ~60 minutes of its life in order to interpret the results correctly. The experimenter has to control as many variables as possible to extract the target information.

3.2. Brain and orientation behaviour

For the main part of my Ph. D. project I aimed at identifying brain areas possibly involved in orientation behaviour of night-migratory songbirds. Since orientation behaviour involves

both a sensory and a motor component, I studied both aspects and designed experiments to separate the two components. I applied the technique of behavioural molecular mapping to analyse two different orientation systems: the star compass and the magnetic compass.

I started with a pilot study to examine star compass learning in young birds that had not experienced any star information before. When presenting a starry sky the very first time, any brain area that is involved in detecting the sky rotation and/or learning the star pattern may be activated. For this study, we had 11 garden warbler nestlings caught from the wild at an age of 5 – 13 days and hand-raised indoors without star information available. In early August, the birds were exposed for 60 minutes to a simplified artificial starry sky built after the model used by the Wiltschkos (W Wiltschko & Wiltschko 1976). The results from the star compass experiment so far did neither give sufficiently detailed information on the learning process of a star compass nor on brain areas possibly involved in star compass orientation. This project was the first of a series based on behavioural molecular mapping, and as we gained more experience with the method, it became clear that the unclear results of the first experiments were due to insufficient control of the parameters. Particularly, it was not possible to distinguish brain activation due to sensory, learning, and motor activity. For instance, the birds were placed in regular bird cages, resulting in very unspecific movement behaviour that in turn led to pronounced motor-induced gene expression (compare with Feenders et al 2005, paper IV). In addition, the birds had a natural magnetic field available that they may have used for orientation. The analysis of the brain expression pattern revealed activity in a distinct forebrain area likewise what we found in the main experimental series. This area is possibly involved in (specialised) night-time vision and/or orientation information processing (see below; Mouritsen et al 2005). Another complicating factor in the star compass study is that it is not known at what age exactly the birds learn the star compass, and whether the learning process takes place within a few hours of 'watching' the starry sky or whether the compass has to be established after experiencing several nights under a starry sky. With hindsight, it is not too surprising that this pilot study did not lead to clear-cut results.

Much more promising seemed to be an investigation of the magnetic compass orientation behaviour because extensive knowledge exists about the factors that influence this orientation system. Thus, we started to search for brain areas possibly involved in integrating and/or processing information relevant for the magnetic compass. In order to keep gene expression noise due to variable motor behaviour low, we designed a novel orientation cage consisting of a cylindrical transparent Plexiglas cage fitted with a circular

perch. In this orientation cage, our test birds tend to show very stereotype migratory restlessness behaviour by performing wing whirring while perched, or they were sitting quietly on the perch. This behaviour is less erratic than what we experienced from tests in Emlen funnels where the birds tend to jump up the wall and slide back to the bottom. Furthermore, infrared cameras allowed us to observe the test birds at day and night without disturbance.

When observing the birds in this orientation cage during migratory restlessness, we noticed a very stereotype behaviour besides the well-known migratory restlessness behaviour: the birds turned their head sideways and back to the longitudinal body axis, often followed by a turn towards the opposite side (Mouritsen et al 2004b, paper II). The frequency of those head scans was tripled when the birds were exposed to a true-zero magnetic field compared to a natural magnetic field. The frequency also increased significantly directly before onset of migratory restlessness each night. Furthermore, we could show that birds, tested in a natural magnetic field, choose more often to move into the correct migratory direction than into the wrong direction after performing a head scan, whereas their movement direction was random following a head scan in a true-zero magnetic field. These findings led to the conclusion that night-migratory birds seem to use head scans to detect the reference direction provided by the ambient magnetic field.

The use of head scans would also mean that a magnetoreceptor is located in the bird's head, which is in line with the strong evidence for a light-based magnetoreceptor in the birds' eyes. Thus, we compared brain activity patterns of a group of birds experiencing day-light at day-time with birds kept under dim light at night-time. Furthermore, we compared groups exposed to different magnetic field conditions in order to probe for an effect of the ambient magnetic field on the activity patterns. A third factor we had to control for was the behaviour of the birds: when birds show migratory restlessness they move around in the cage which will lead to motor-induced gene-expression. This expression may mask magnetosensing-induced brain activity. Thus, we compared birds that were showing motor activity with birds that were sitting still but awake in the cage for at least 45 minutes between bouts of migratory restlessness behaviour.

Based on the results of night-migratory garden warblers and European robins we identified a brain area, which we named "cluster N". Cluster N showed high ZENK expression during night-time, but not during day-time (Figure 6). In non-migratory zebra finches and canaries (*Serinus canaria*), cluster N is not present or at least not active. Thus, cluster N seems to be specific to night-migratory birds at night-time, i.e. dim light. Because

cluster N is localized in a forebrain area close to or possibly being part of a known visual area, the visual Wulst, we wanted to know whether cluster N activity is somehow related to visual input. This would be of high relevance for a possible light-mediated magnetoreceptor. We therefore covered up the eyes of European robins in order to block any light from reaching the eye. This led to a significant decrease (to near baseline level) of ZENK expression in cluster N. The data further showed that the increased activity in cluster N as observed in night-migrating birds is not due to circadian effects, because samples were collected from birds which had their eyes covered at the same time points as we collected samples showing high cluster N activity in garden warblers with no eye covers.

Thus, the data suggest that cluster N is a brain area specific for night-migratory songbirds and that it is highly activated during night-time when sufficient light is available to process visual information.

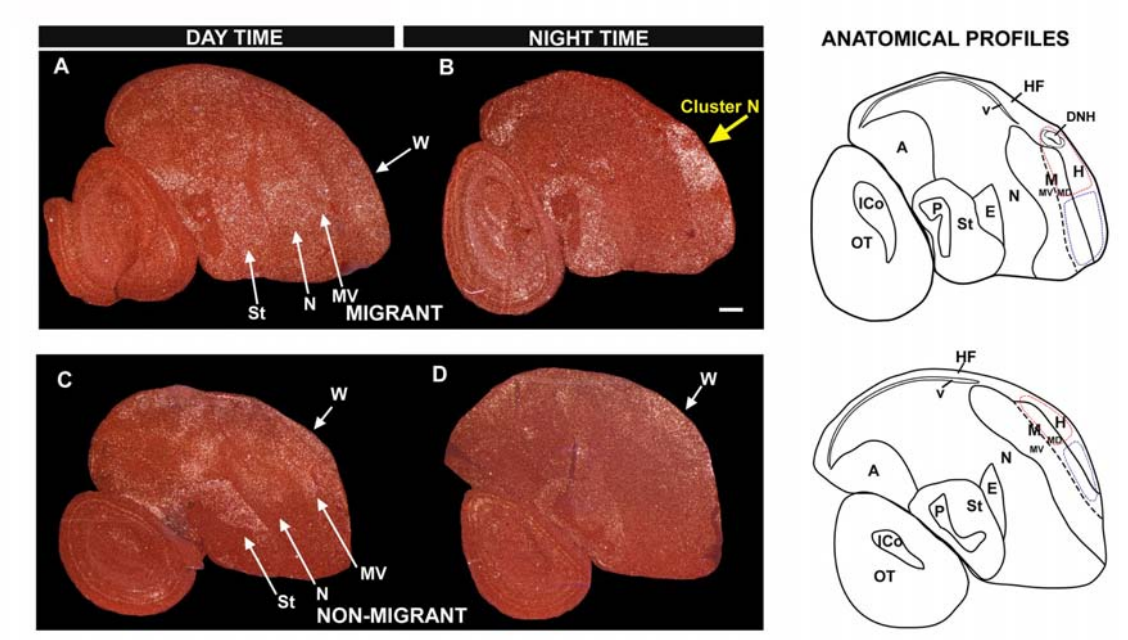

Figure 6: Darkfield images of brain ZENK expression patterns (see paper III). Parasagittal sections of night-migratory garden warblers (upper panel, *A* & *B*) and non-migratory zebra finches (lower panel, *C* & *D*). *A*, *C*: expression pattern during the day. *B*, *D*: expression pattern during the night. Dorsal is up; anterior is right. White-silver grains: ZENK expression; red: Nissl staining. *Right*: camera lucida drawings; the red and blue lines mark the areas used for quantification. *A*: arcopallium; *DNH*: dorsal nucleus of the hyperpallium; *E*: entopallium; *H*: hyperpallium; *HF*: hippocampal formation; *ICo*: intercollicular complex; *IHA*: interstitial region of the hyperpallium apicale (*HA*); *M*: mesopallium with ventral (*MV*) and dorsal (*MD*) part; *N*: nidopallium; *OT*: optic tectum; *P*: pallidum; *St*: striatum; *v*: ventricle; *W*: visual Wulst. Scale bar: 0.5 mm.

3.3. Cluster N – a brain region involved in sensing or processing magnetic information?

To further investigate the function of cluster N, we tested the activity pattern under different magnetic field conditions (described in the manuscript). Before interpreting the results, we have to realize that, if the magnetic field is perceived via the eyes as a modulation of a primary light-induced signal, following the theory by Ritz et al (2000), a basic signal will always occur as soon as there is light available to initiate the process. This first step is independent of the ambient magnetic field, so the brain regions connected to the visual receptors will receive a neuronal signal and process this signal irrespective of the magnetic field the bird is experiencing. Hence, magnetic modulations would only be detectable if they are so strong that they lead to large and consistent enough changes in overall neuronal activity that can be detected by behavioural molecular mapping.

We did not detect any significant effect of different magnetic field conditions on the cluster N activation pattern. Thus, the method of behavioural molecular mapping is likely to be too insensitive to detect the magnetic field modulations of the primary visual signal. Another possibility is that magnetic modulations may increase the firing rate in some neurons while other neurons are depressed. Such changes would not be detectable by behavioural molecular mapping, since ZENK expression reflects the overall integration of neuronal firing that occurred within the preceding ~60 min. Electrophysiological recordings will be necessary to measure a potential direct response of the neurons and detect possible modulations in the neuronal signals. However, if no light is available no primary light signal can be produced and the brain would therefore have no signal to process. This is the situation in birds wearing eye covers.

Besides in complete darkness or a completely compensated magnetic field, night-migratory songbirds are also known to be disoriented under monochromatic light of the orange-red part of the spectrum (see above). Although Wiltschko et al (2004b) could show that robins are able to orient under red light after pre-exposure to this light, monochromatic red light does in most cases disturb the immediate orientation ability of birds. Therefore, I exposed garden warblers to red light (peak at 650 nm) for 45 minutes and afterwards quantified the ZENK expression in cluster N. The results showed no differences between birds exposed to red light and the control birds exposed to white light (see manuscript). It may be that 45 minutes of red light exposure are long enough to re-establish the orientation ability. However, a continuous observation of the birds' orientation during red-light exposure will be necessary to more precisely elucidate the characteristics of this adaptation

process, especially the length of exposure that is needed to possibly establish correct directedness.

This far, the study on cluster N focused on night-migratory bird species. Another question about cluster N is whether it is specialized in migratory birds or constrained to certain species. In the first part of the project we also examined zebra finches and canaries, both non-migratory songbird species, and they did not show cluster N activation at any time of the day or night. However, these species are relatively distantly related to warblers (garden warbler) or thrushes (European robin). Thus, I tested Sardinian warblers (*Sylvia melanocephala*) as a close relative of garden warblers and as a species that is fairly sedentary and has previously been used for comparison as a non-migratory *Sylvia*-species (Healy et al 1996; Mettke-Hofmann & Gwinner 2003). Interestingly, the Sardinian warblers also showed high gene expression in cluster N, but the results have to be interpreted carefully. It is known from field observations, that Sardinian warblers undertake directed movements in Spain: in autumn, especially the northern populations tend to move south or down from the mountains, and they return in spring (Cramp 1998). Even some of our individuals performed migratory restlessness in the orientation cages. Thus, Sardinian warblers seem to inhere some migratory behaviour. This is consistent with recent findings that natural populations of blackcaps, and possibly most other species, comprise individuals with a higher and individuals with a lesser degree of migratory behaviour and that the degree of migratory behaviour can shift easily, influenced by the genes (Pulido et al 1996). Previously, Berthold et al (1990, 1994) have shown that the degree of migratory behaviour can evolve rapidly in a microevolution process. In the case of Sardinian warblers, there is clear evidence for some directed movement behaviour and for this the birds may very well possess the ability to orient by means of a magnetic compass at night. However, I did not find any data of orientation tests in this species. So we cannot say definitely whether Sardinian warblers are able to orient according to a magnetic field or not. This will be an interesting project that has best to be carried out in Spain.

3.4. Movement-induced activity pattern and its implication for the evolution of neuronal pathways

In search of brain areas involved in magnetic orientation we examined brains of birds that were sitting still and compared the activity patterns with those of birds that were moving around in the cage during the day or showing migratory restlessness at night. A

comparison of the activity patterns revealed striking differences: While the brains of sitting birds show very little IEG expression except in cluster N at night-time and in day-vision areas during the day (Mouritsen et al 2005, paper III), a distinct and highly consistent activity pattern can be found in active birds at any time of the day. As described in Feenders et al (2005, paper IV), a close analysis revealed that this movement-induced activity pattern is an inverse picture compared to that known from vocal-learning bird species (e.g. songbirds) during song learning and production. Areas known to be involved in song learning and production were silent in a moving bird, whereas the brain tissue surrounding these seven song nuclei showed high activity. In comparison, birds that are singing show high IEG expression in the song nuclei but only little to no expression in the brain areas surrounding the song nuclei. In addition to the seven regions neighbouring the song nuclei, we could identify three additional forebrain regions and parts of the cerebellum to be movement-related. We found similar patterns in zebra finches that do not perform migratory restlessness but instead regular movements in the cage. The results suggest that the 11 identified brain regions are a general feature of songbirds if not of all birds.

The detection of movement-related brain regions adjacent to or embedding the song nuclei are of special interest because it has been shown in previous studies that those regions or parts of them are interconnected by neuronal pathways in a similar fashion as the song nuclei are connected by the vocal pathways (Margoliash et al 1994; Durand et al 1997; Iyengar et al 1999; Farries 2001; Diekamp & Güntürkün 2003). Those connectivity results led to the idea of general neuronal pathways in the avian brain. Our findings, in turn, support the idea of a neuronal circuit existing in parallel to the vocal pathways and that this pathway is involved in motor control (Figure 7). Moreover, the data give further evidence that the song system may have developed out of this motor pathway. This conclusion seems a logical consequence for singing behaviour: When birds sing they need to control muscles of the beak, the tongue, the syrinx and the lungs. Therefore, learning new vocalizations requires a highly fine-tuned control of those muscles in order to create new sounds or imitate heard songs. Exploiting pre-existing motor-pathways for the development of a system specialised for singing seems most suitable.

The identification of motor-related brain areas is of further interest as it will support future studies using behavioural molecular mapping. Because most behavioural tests include a motor component, researchers will now be able to distinguish between movement-induced gene expression and activity that is based on other parameters.

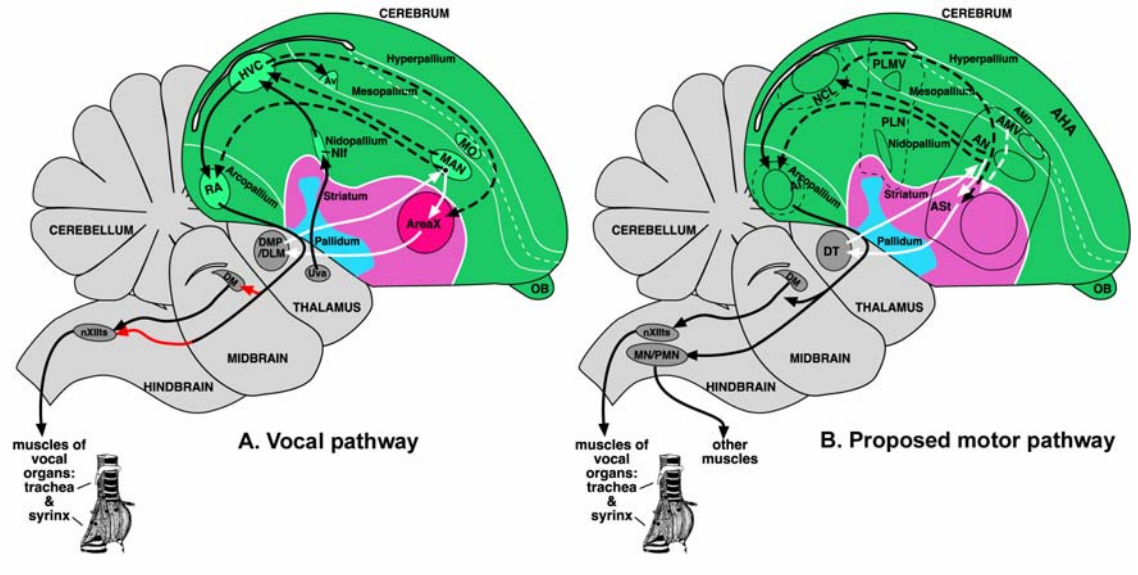

Figure 7: Schematic drawing of the known vocal pathway (*left*) and the hypothesized motor pathway (*right*) (see paper IV). *Left diagram*: The white arrows indicate known connections of the anterior vocal pathway nuclei that are responsible for song learning. The black arrows indicate known connections of the posterior vocal pathway nuclei involved in hearing song. Dashed lines mark proposed connections between the anterior and the posterior pathway. *Right diagram*: motor activated areas are highlighted by black lines, and dashed lines for areas localized more lateral than the present view. Black, white, and dashed arrows mark connections of the motor activated regions that are similar to the vocal pathway connections as shown in the left diagram. *AHA*: anterior hyperpallium apicale; *AI*: intermediate arcopallium; *AMV/AMD*: anterior ventral/dorsal mesopallium; *AN*: anterior nidopallium; *ASt*: anterior striatum; *Av*: avalanche; *DLM*: medial nucleus of dorsolateral thalamus; *DM*: dorsal medial nucleus of the midbrain; *DMP*: dorsal medial nucleus of the posterior thalamus; *HVC*: HCV song nucleus; *MAN*: magnocellular nucleus of the anterior nidopallium; *MO*: oval nucleus of the mesopallium; *NCL*: caudolateral nidopallium; *NIf*: interfacial nucleus of the nidopallium; *PLMV*: posterior lateral ventral mesopallium; *PLN*: posterior lateral nidopallium; *RA*: robust nucleus of the arcopallium; *Uva*: nucleus uvaeformis

In summary, this thesis presents evidence in support of a light-mediated magnetoreceptor from behavioural studies, immunohistochemical analysis of the retina and a detailed examination of neuronal activity patterns of the brain during magnetically guided migratory restlessness behaviour in caged songbirds. The data obtained from the brain analyses are also interesting from an evolutionary point of view as they strongly support the hypothesis of a general motor pathway, existing in the songbird brain, which forms the basis for the vocal pathways that are found to be highly similar in all three distantly related groups of vocal learning birds.

4. Outlook

With the data presented in this thesis, many more questions arise to be answered in future experiments. In this paragraph I will mention some of the most important issues that the follow-up studies will need to examine (many of these are also summarized in Mouritsen & Ritz 2005).

A very important basic question is to know whether cryptochromes are indeed sensitive to the magnetic field and in which way. In-vitro expression of the proteins and biophysical measurements are needed to examine the actual properties of these molecules in respect to magnetic forces. Furthermore, in order to function as a magnetoreceptor, the molecules have to be fixed in the tissue, thus an analysis of the structure of cryptochrome-containing cells in the retina will be necessary.

If a signal coding for magnetic field information is evoked in the retina and sent to the brain, the question is, whether cluster N is really the projection area. If cluster N is involved in integrating and processing magnetic field information electrophysiological recordings in the eye and in cluster N should lead to some specific neuronal responses being influenced by magnetic stimuli. Otherwise, the area may e.g. respond to changes in light-level. An alternative experiment that should be carried out is to lesion cluster N and test the orientation of the birds afterwards. If cluster N is involved in magnetic compass orientation behaviour, birds with cluster N lesioned should no longer be able to orient correctly. Furthermore, it will be interesting to know whether day-migrants show enhanced cluster N activity during orientation.

The study on motor-induced brain areas in songbirds need to be replicated in the other vocal-learning groups (hummingbirds and parrots) as well as in some vocal non-learning species in order to test its generality. From an evolutionary point of view, it will also be of high importance to test in more detail whether specific motor areas are related to specific body parts, i.e. muscle groups, as found in the mammalian motor cortex.

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Abbreviations

A	arcopallium
AAc	central nucleus of the anterior arcopallium
ACM	caudal medial arcopallium
AHA	anterior hyperpallium apicale
AI	intermediate arcopallium
ALMV	anterior lateral ventral mesopallium
ALN	anterior lateral nidopallium
AMD	anterior dorsal mesopallium
AMP	anterior motor pathway
AMV	anterior ventral mesopallium
AN	anterior nidopallium
Area X	area X
ASt	anterior striatum
Av	avalanche
AVP	anterior vocal pathway
Cb	cerebellum
CMM	caudal medial mesopallium
CSt	caudal striatum
DLM	medial nucleus of the dorsolateral thalamus
DLN	dorsal lateral nidopallium
DM	dorsal medial nucleus of the midbrain
DMm	magnocellular nucleus of the dorsomedial thalamus
DMP	dorsal medial nucleus of the posterior thalamus
DNH	dorsal nucleus of the hyperpallium
E	entopallium
Field L (1,2,3)	field L (1,2,3)
GP	globus pallidus
H	hyperpallium
HA	hyperpallium apicale
HF/Hp	hippocampal formation/hippocampus
HVC	HCV
ICo	inferior colliculus
IEGs	immediate early genes
IHA	intercalated hyperpallium apicale
LAM	lateral nucleus of the anterior mesopallium
LAN	lateral nucleus of the anterior nidopallium
LMAN	lateral MAN
M	mesopallium
MAN	magnocellular nucleus of the anterior nidopallium
MD	dorsal mesopallium
MLd	dorsal part of the lateral mesencephalic nucleus
MMSt	magnocellular nucleus of the medial striatum
MO	oval nucleus of the mesopallium
MR	migratory restlessness behaviour
MV	ventral mesopallium
N	nidopallium
nXIIts	12^{th} nucleus tracheosyringeal part

NAOc	oval nucleus complex of the anterior nidopallium
nBOR	nucleus of the basal optic root
NCL	caudal lateral nidopallium
NCM	caudal medial nidopallium
NIf	interfacial nucleus of the nidopallium
NLc	central nucleus of the lateral nidopallium
OT	optic tectum
P	pallidum
PLMV	posterior lateral ventral mesopallium
PLN	posterior lateral nidopallium
PLSt	posterior lateral striatum
PMP	posterior motor pathway
PVP	posterior vocal pathway
RA	robust nucleus of the arcopallium
S	septum
St	striatum
Uva	nucleus uvaeformis
v	ventricle
VA	vocal nucleus of the arcopallium
VAM	vocal nucleus of the anterior mesopallium
VAN	vocal nucleus of the anterior nidopallium
VASt	vocal nucleus of the anterior striatum
VLN	vocal nucleus of the lateral nidopallium
W	visual Wulst

Curriculum Vitae

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Paper I

Mouritsen H, Janssen-Bienhold U, Liedvogel M, Feenders G, Stalleicken J, Dirks P & Weiler R:

> Cryptochrome and activity markers co-localize in bird retina during magnetic orientation.

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Cryptochromes and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation

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Migratory birds can use a magnetic compass for orientation during their migratory journeys covering thousands of kilometers. But how do they sense the reference direction provided by the Earth's magnetic field? Behavioral evidence and theoretical considerations have suggested that radical-pair processes in differently oriented, light-sensitive molecules of the retina could enable migratory birds to perceive the magnetic field as visual patterns. The cryptochromes (CRYs) have been suggested as the most likely candidate class of molecules, but do CRYs exist in the retina of migratory birds? Here, we show that at least one CRY1 and one CRY2 exist in the retina of migratory garden warblers and that garden-warbler CRY1 (gwCRY1) is cytosolic. We also show that gwCRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, which also showed high levels of neuronal activity at night, when our garden warblers performed magnetic orientation. In addition, there seem to be striking differences in CRY1 expression between migratory and nonmigratory songbirds at night. The difference in CRY1 expression between migrants and nonmigrants is particularly pronounced in the large displaced ganglion cells known to project exclusively to a brain area where magnetically sensitive neurons have been reported. Consequently, cytosolic gwCRY1 is well placed to possibly be the primary magnetic-sensory molecule required for light-mediated magnetoreception.

Since the description of animal magnetosensory capabilities in the 1960s (1–3), it has been convincingly shown that songbirds can use a magnetic compass for orientation during their migratory journeys (3–6), but the physiological mechanisms enabling migratory birds to sense the reference direction provided by the Earth's magnetic field still remain unknown. Two types of potential magnetoreception mechanisms have been suggested over the past decades: one mechanism that is based on magnetite particles and one mechanism that is based on photoreceptors forming radical-pair intermediates (for summary, see ref. 7). Although no direct physiological or molecular evidence has been reported, numerous orientation cage experiments with captive migratory songbirds have revealed several important characteristics of their magnetic compass.

The magnetic compass of migratory songbirds is an inclination compass; that is, it detects the axis but not the polarity of the magnetic field lines (4–5). Furthermore, magnetic orientation in migratory songbirds depends on the wavelength of the ambient light (8–11). Migratory songbirds are active and orient magnetically under dim blue and green light, whereas they are active but disoriented under dim red light (8–10). These findings strongly suggest that photoreceptor molecules in the eye are involved in magnetoreception and that these photoreceptor molecules should absorb in the blue and green range of the spectrum. The involvement of photoreceptors in the eye is further supported by the finding that birds with their right eye covered seem unable to perform magnetic orientation (12). A recent behavioral experiment (13) testing the magnetic orientation responses of European robins, *Erithacus rubecula* (a night-migrating songbird), exposed to oscillating magnetic fields provided strong indirect evidence that the magnetic-inclination compass of night-migrating songbirds is based on a radical-pair mechanism (7, 13).

Photoreceptor-based radical-pair mechanisms were suggested by Schulten *et al.* (14) and strongly elaborated on by Ritz *et al.* (7). They are based on the fact that radical-pair reactions will be modulated differently depending on the direction of the Earth's magnetic field relative to the orientation of the radical-pair-forming molecule (7, 15, 16). In short, the current hypothesis (7) further suggests that light in the blue-green range will excite photoreceptors forming radical pairs upon photoexcitation in the retina of the migratory bird. Because of the shape of the retina (half ball) and the presumed fixed orientation of the radical-pair-forming photoreceptors inside the cells, the magnetic field would modulate the radical-pair reaction and, thereby, the light sensitivity differently in different parts of the retinas, leading to perception of the magnetic field as visual patterns (7). Radical-pair-mediated magnetoreception would not be able to detect the polarity of the field lines, but only their axis, which is in line with the inclination-based nature of the songbird magnetic compass (4, 10).

Based on these theoretical considerations and behavioral evidence, the primary magnetic-sensory molecule in the retina of migratory songbirds should be a photopigment that is excited by light in the blue-green range and forms radical pairs upon photoexcitation. The cryptochromes (CRYs) (17–22) have been suggested as the most likely candidate class of molecules (7) because they are blue-green photoreceptors in plants (17, 19, 22) and because closely related 6,4-photolyases have been shown to form radical pairs upon photoexcitation (23). Other classes of photoreceptors, such as phototropsins (24) and chlorophylls (25), found in plants can also undergo radical-pair reactions. Rhodopsins should not be able to form radical pairs because photoexcitation leads to cis–trans isomerization of retinal rather than an electron transfer (e.g., ref. 26). Thus, CRYs are the only currently known class of molecules found in the retina of vertebrates that are likely to fulfill the physical and chemical characteristics that are required for function as the primary magnetic sensor (7).

Therefore, the aims of the present article are to (*i*) test whether CRYs exist in the retina of migratory birds performing a magnetic orientation task at night, (*ii*) elucidate their cellular location within the retina, (*iii*) test whether the CRY-containing cells show neuronal activity when migratory birds perform night-time magnetic orientation behavior, and (*iv*) compare the

Abbreviations: CRY, cryptochrome; gwCRY, garden-warbler CRY; INL, inner-nuclear layer.

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results of the first three aims in migratory songbirds with a nonmigratory songbird.

Materials and Methods

The migratory behavior of night-migratory songbirds can be studied in caged birds because they are so eager to migrate that they will show migratory-restlessness behavior (wing-flapping and jumping on the perch) that is oriented in the direction in which they want to fly (27–29). In the present study, we tested 30 migratory garden warblers, *Sylvia borin*, and 10 nonmigratory zebra finches, *Taeniopygia guttata*, in orientation cages. The birds were kept indoors in a wooden laboratory under the natural light–dark cycle. At approximately noon, birds that were to be tested were marked with a small stripe of reflective tape on the head and transferred to a circular orientation cage. The timed lights went off at local sunset, reducing the light level in the room to 0.04 lux, originating from four diffused white light bulbs that were not directly visible to the birds. The behavior of the birds inside the orientation cage was monitored by an infrared (840 nm) video camera. The orientation of the birds was tested in the undisturbed geomagnetic field (field strength, 48,300 nT; inclination, 67°) or in an equivalent magnetic field (field strength, 48,300 ± 200 nT; inclination, 67° ± 0.2°) turned 120° ± 0.2° in the horizontal plane by 3D ($2 \times 2 \times 2$ m) Helmholtz coils (28).

During these orientation experiments, we carefully observed the birds on a video screen in real time and collected the retinas of 11 garden warblers immediately after they had shown at least 1 h of consistent migratory-restlessness behavior. All birds spent ≥100 min in the orientation cage after the lights went off before being killed. We also collected retinas from four garden warblers in room-light conditions in which they do not show migratory behavior. As nonmigratory controls, we collected the retinas of 10 zebra finches (five during the day and five at night).

When a given bird had shown ≥1 h of migratory-restlessness behavior (garden warblers at night; garden warblers during the day and all zebra finches just had to stay awake), it was killed according to the German legislation regulating the use of animals in research. Actual collection times varied between 22:23 and 00:40, equivalent to between 1 h and 40 min and 4 h after the lights went off. The day birds were collected between 19:48 and 20:30 having spent the preceding 12–14 h under constant room-light (275 lux) conditions. In all cases, eyecups were prepared within 6–13 min of the death of the bird under dim red light and were fixed in 2% paraformaldehyde, embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and frozen to −80°C.

For the detection of CRY1 transcripts, total RNA was extracted from the retinas by means of a NucleoSpin RNA II Kit according to the manufacturers protocol (Macherey & Nagel), which included treatment with DNase I to exclude contamination with genomic DNA. cDNA synthesis was performed from either 2 μg of RNA with SuperScript II Rnase H^- reverse transcriptase (Invitrogen) or from ≈700 ng of RNA with Revert AID H^- First-Strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocols. First-round hot-start PCRs (total volume, 25 μl) included 2 μl of cDNA/1× reaction buffer (Promega)/1,25 mM $MgCl_2$/0.2 mM each dNTP (Roche Diagnostics, Mannheim, Germany)/0.8 μM each primer (MWG Biotech, Ebersberg, Germany)/1 unit of *Taq* polymerase (Promega). cDNA synthesis and contamination with genomic DNA was controlled by using intron-spanning β-actin-specific primers [USP, 5′-GGCATGTGCAAGGCCGGCTTC-3′ (exon 2); and DSP, 5′-GGATGGCATGAGGGAGCGCGT-3′ (exon 4)] with the following conditions: 2 min at 95°C; 35 cycles of 30 s at 95°C, 1 min at 64°C, and 1.5 min at 72°C; and 15 min at 72°C). For the detection of the 642-bp CRY1 transcripts in the left and right retina, degenerate primers [USP, 5′-TTGTCGACAATGCTGGAAG(CT)TGGA-3′; and DSP, 5′-TTGAATTCTTCTTC(CT)(GT)GA(CT)T(AT)G-G(AG)CG-3′] were used with the following conditions: 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 59°C, and 1 min at 72°C; and 2 min at 72°C). The entire coding region of garden-warbler CRY1 (gwCRY1) cDNA was obtained by means of long PCR (3 min at 94°C; and 40 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C) with 2 units of Taq plus 0.04 plaque-forming units of polymerase (Stratagene) and the two following additional sets of specific primers: USP^N, 5′-AGCAAGGTCTCCTTTCATCCTCTCAATATTCAGA-3′; DSP^N, 5′-AGCACGGTCTCCCATGGGGGTGAACGCCGTGCACTGGTT-3′ (amplification of the 5′ end); USP^C, 5′-AGCAAGCGGCCGCTTATCAATTTGTGCTCTGCCGCTGGACTTT-3′; and DSP^C, 5′-AGCACGGTCTCCGAAACAGATCTACCAGCAGCTT-3′ (amplification of the 3′ end). The latter primers were designed to get overlapping fragments encoding the N- and C-terminal regions of CRY1, which were then used to reconstruct the full-length gwCRY1 coding sequence. The same procedures were applied to detect a 237-bp fragment of CRY2 by using the following primer pair: USP, 5′-ACGAAGAATTCGACGAAAGCCACATCCAG-3′; and DSP, 5′-AGCACGTCGACGAACCCCATCTGCATCCA-3′. cDNA encoding the C-terminal part of CRY2 (777 bp) was obtained by means of nested PCR using the following primer pairs: USP, 5′-TTATGAGGCCCCCGATTTTCTGTTG-3′; DSP, 5′-AATCCCCTGAGACAGGAACCCTGAAGCC-3′; USP^{nest}, 5′-GTTCCTGCTCCTCTGGTCACCTCTT-3′; and DSP^{nest}, 5′-AGGAACCCTGAAGCCTTGGCAAAG-3′. All cloned fragments were subjected to nucleotide sequencing, and DNA similarities and identity scores were analyzed by FASTA and WU-BLAST2 algorithms.

Frozen cryostat sections (12–15 μm) of the retinas, which were mounted on gelatin-coated slides, were used for immunohistochemical labeling with CRY1 antibodies directed against the near-C-terminal part of the CRY1-protein by following standard protocols (30). Primary-antibody incubation was carried out overnight at 4°C for CRY1/sc-5953 (diluted 1:100), Egr-1/sc-189 (diluted 1:500), and c-Fos/sc-253 (diluted 1:200/1:500). All antibodies were purchased from Santa Cruz Biotechnology and diluted in PBS/0.3% TX-100. Immunoreactivity was visualized by indirect immunofluorescence using FITC-conjugated donkey anti-goat IgG for CRY1 detection and Cy3-conjugated donkey anti-rabbit IgG for Egr-1 and c-Fos detection. In control experiments, primary antibodies were omitted or preabsorbed with the appropriate inhibitory peptide (sc-5953 P, sc-189 P, or sc-253 P), which was diluted 1:50 in the incubation buffer. In double-labeling experiments, incubations with either both primary or both secondary antibodies were carried out at the same time. Labeled sections were analyzed by using a TSC confocal microscope (Leica, Nussloch, Germany), and brightness and contrast of the images were adjusted to the same background level by using PHOTOSHOP software (Adobe Systems, San Jose, CA). The immunosignal intensity was quantified relative to background intensity at the confocal microscope by encircling relevant areas on retina slices from different experimental groups incubated on the same slide, such that the intensity index = {[2× immunostaining intensity (ISI) in ganglion cells/(ISI in inner plexiform + ISI in GCaxon layer)] −1}. Specificity of the antibodies was tested by means of immunoblotting using standard protocols (30). Immunoreactive proteins were detected by using the chemiluminescence method (ECL-system; Amersham Pharmacia Buchler, Braunschweig, Germany). The Western blot analyses were replicated four times with different birds and positive rat-brain controls. The strengths of the bands were in line with the immunosignal quantifications. In control experiments, the primary antibody (diluted 1:500 in PBS) was preadsorbed by its inhibitory peptide (diluted 1:50).

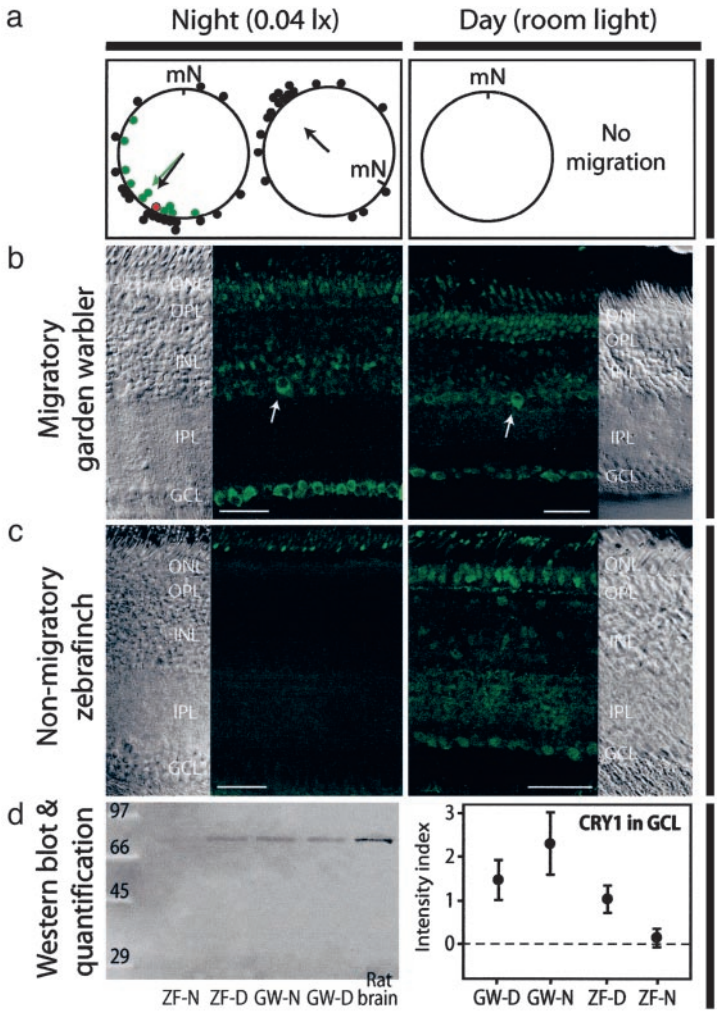

Fig. 1. CRY protein expression in the retina of migratory garden warblers and nonmigratory zebra finches. (*a*) Magnetic orientation of garden warblers. Each data point indicates the mean orientation of one individual. The arrows indicate group mean vector lengths (*r*). The orientation of the night-birds (Night) from which retinas were collected (green symbols) were highly oriented in the normal migratory direction ($n = 11$, mean = 223°, $r = 0.77$, and $P < 0.001$). The red dot represents the night bird whose retina is shown in *b*. During the day, the birds do not show orientation behavior (Day). mN, Magnetic North. (*b*) Immunohistochemical staining of CRY1 protein during nocturnal magnetic orientation (*Left*) and during the day (*Right*) in migratory garden warblers (images from the same slide taken with identical settings). Large displaced ganglion cells marked with arrow. (*c*) Immunohistochemical staining of CRY1 protein during the night (*Left*) and day (*Right*) in nonmigratory zebra finches (images from same slide taken with identical settings). Labeling of photoreceptor outer segments and Müller cell end-feet in *b* and *c* is unspecific. (*d*) Example of Western blot analysis confirming specificity of the antibody and quantification of CRY1 expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. (Scale bar, 40 μm.)

Results

The magnetic-orientation responses of 30 migratory garden warblers were tested at night in circular orientation cages inside a wooden laboratory. The birds showed clear magnetic orientation (Fig. 1*a*) in their species-specific migratory direction (normal field: mean = 215°, $n = 30$, $r = 0.62$, and $P < 0.001$), and they significantly (Mardia–Watson–Wheeler test; $n = 48$, $\chi^2 = 19.6$, and $P < 0.001$) changed their orientation as predicted (mean = 316°, $n = 18$, $r = 0.51$, and $P < 0.01$) in a turned magnetic field (magnetic North = 120°).

CRY expression at the mRNA level was first analyzed by RT-PCR. First, a gwCRY1 fragment of ≈642 bp (Fig. 2), including the region encoding the FAD-binding region thought to be involved in the radical-pair reaction (18, 23), was amplified with degenerated CRY1 primers. Subsequently, the complete coding sequence was reconstructed (GenBank accession no. AJ632120), and FASTA and WU-Blast2 analysis of the gwCRY1 nucleotide sequence revealed the highest homology (93%) with chicken CRY1 (GenBank accession no. AY034432). We also amplified a 777-bp fragment encoding the C terminus of gwCRY2 (GenBank accession no. AY739908), which showed highest homology (89%) with chicken CRY2 (GenBank accession no. AY034433). Both gwCRYs belong to the animal CRY family (31). Subsequent immunohistochemistry revealed that gwCRY1 was located in the cytosol, whereas gwCRY2 was located in the nucleus. Because a CRY working as the primary magnetoreceptor must be oriented in the cells and, therefore, is most likely linked to cytosolic skeleton proteins, a nucleic location is very unlikely. For these reasons, we focused subsequent experiments on gwCRY1 only.

The specificity of the CRY1 antibodies was confirmed by four independent sets of immunoblots [all showing only one immunoreactive protein at the appropriate molecular mass of ≈70 kDa (Fig. 1*d*)], which disappeared when the antibody was preabsorbed with the corresponding peptide. In migratory garden warblers, immunolabeling for CRY1 was found in specific cell populations across the retina. Invariably, 95–100% of garden-warbler ganglion cells showed high levels of cytosolic CRY1 with a tendency toward higher expression in night birds than in day birds (t test, df = 8, $t = 2.06$, $P = 0.07$; Fig. 1 *b* and *d*). All large, displaced ganglion cells were also strongly labeled for CRY1 in garden warblers in both day and night. The photoreceptor layer of garden warblers showed high expression of CRY1 particularly in day birds. In the inner-nuclear layer (INL) of garden warblers, 10–15% of the cells were CRY1-positive, and night garden warblers tended to show higher levels of CRY1 in a cell population with elongated somata. In nonmigratory zebra finches, the expression in day birds closely resembles that found in garden warblers during the day, except that the large displaced ganglion cells were always CRY1-negative in zebra finches (Fig. 1*c*). However, during the night, CRY1 expression in nonmigratory zebra finches dropped dramatically to nonspecific background level (night mean of intensity index [(signal/background) − 1] = 0.14 ± 0.21 (SD); day mean of intensity index = 1.04 ± 0.31;

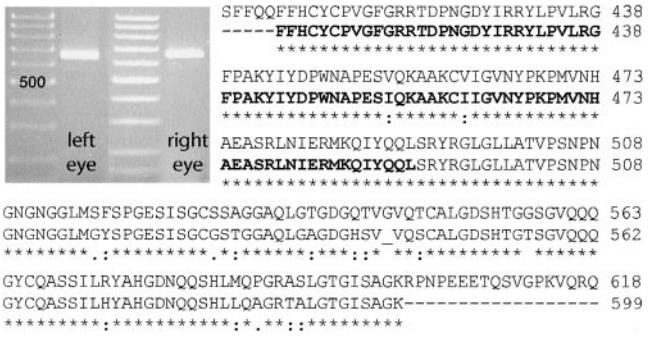

Fig. 2. Detection of a CRY1 transcript with the expected size of ≈642 bp in the left and right retinas of garden warbler (lower sequence). The deduced amino acid sequence for this fragment reveals 91% identity with the specific C-terminal region of chicken CRY1 (upper sequence). The FAD-binding domain thought to be involved in radical-pair reactions is shown in bold. Upper left, 5′ end; lower right, 3′ end.

t test, df = 8, $t = 5{,}35$, and $P < 0.001$; Fig. 1 *c* and *d*). This decrease in CRY1 expression is in line with the normal circadian oscillation found previously in CRY1 mRNA in nonmigratory domestic chicken and quail (20, 21) but strikingly different from the unusually high expression levels [mean intensity index = 2.31 ± 0.72 (SD)] found in migratory garden warblers at night (Fig. 1*d*). There were no obvious differences in CRY1 patterns between the right and left eye (12).

To be potentially relevant for magnetoperception, the CRY-containing retinal cells of garden warblers must be active at night when the birds perform magnetic orientation. We tested the neuronal-activity pattern of retinal cells by using the markers c-Fos and ZENK (*zif*268, *E*gr-1, *N*GF-1A, *K*rox-24). The transcription factors (immediate early genes) c-Fos and ZENK are known to show vision-dependent expression in the retina of many animals (32–35), including chicken (32), and their expression requires neuronal activity (36–37). The c-Fos and ZENK proteins were detectable in a cell 15–20 min after neuronal activity occurred, and peak protein expression was detected after 45–60 min (38). Thus, c-Fos and/or ZENK protein detected immunohistochemically in a cell was produced by neuronal activity 15–90 min before the tissue was fixed, which matches the time frame in which our birds performed magnetic orientation. Consequently, colocalization of c-Fos and ZENK protein in garden-warbler retinal cells showing high CRY levels would show that these cells were sending neuronal information to the brain while our birds oriented to the geomagnetic field at low night light levels (0.04 lux).

As expected, immunostaining and Western blot analysis revealed higher c-Fos expression in both zebra finches (*t* test, df = 8, $t = 7.18$, and $P < 0.001$) and garden warblers (*t* test, df = 8, $t = 3.25$, and $P < 0.02$) during the day, when vision is more active, than during the night (Fig. 3*a*). But whereas c-Fos expression during the day was similar in garden warblers and zebra finches, at night it was significantly higher (*t* test, df = 8, $t = 4.474$, and $P < 0.01$) in the migratory garden warblers. In these night garden warblers, c-Fos is strongly expressed in ganglion cells and in ≈5% of the cells within the INL (Fig. 3*b*). ZENK was strongly expressed in the nuclei of garden-warbler ganglion cells (Fig. 3*c*). The c-Fos and ZENK signals colocalized with CRY1 in all garden-warbler ganglion cells and large displaced ganglion cells but only sporadically in other cell types at night during magnetic orientation (Fig. 3 *b* and *c*).

In their theoretical article, Ritz *et al.* (7) specifically suggested that the large, morphologically distinct (39, 40), and displaced ganglion cells are likely to be a location for magnetoreception because they project exclusively to the nucleus of the basal optic root (39, 40), where magnetically sensitive neurons have been reported (41) and visual flow-fields arising from self-motion are processed (42). The scarce, large CRY1-positive cells found in the INL of the garden warbler (Figs. 3 and 4) share the features reported for displaced ganglion cells in birds (39, 40) (in particular, their large size, disk-shaped nuclei, and large cytosolic space) and, thus, are easy to locate by Nomarski optics. Thy1, a general marker for ganglion cells, also in birds (43), further confirmed their identification as displaced ganglion cells. We analyzed >100 of these large cells in the garden warbler and >30 in the zebra finch, and we found striking differences in CRY1 and c-Fos expression. In the migratory garden warbler (day and night), the large displaced ganglion cells always contained strong CRY1 immunolabeling and a strong label for c-Fos (Fig. 4). In contrast, the large displaced ganglion cells of nonmigratory zebra finches (day and night) were always CRY1-negative (Fig. 4). Furthermore, all of the >20 large displaced ganglion cells that were analyzed in night zebra finches were c-Fos-negative. In zebra finches during the day, as expected because of normal visual processes, both c-Fos-positive (active) and c-Fos-negative (inactive) large displaced ganglion cells were found, but even when active, they never expressed CRY1.

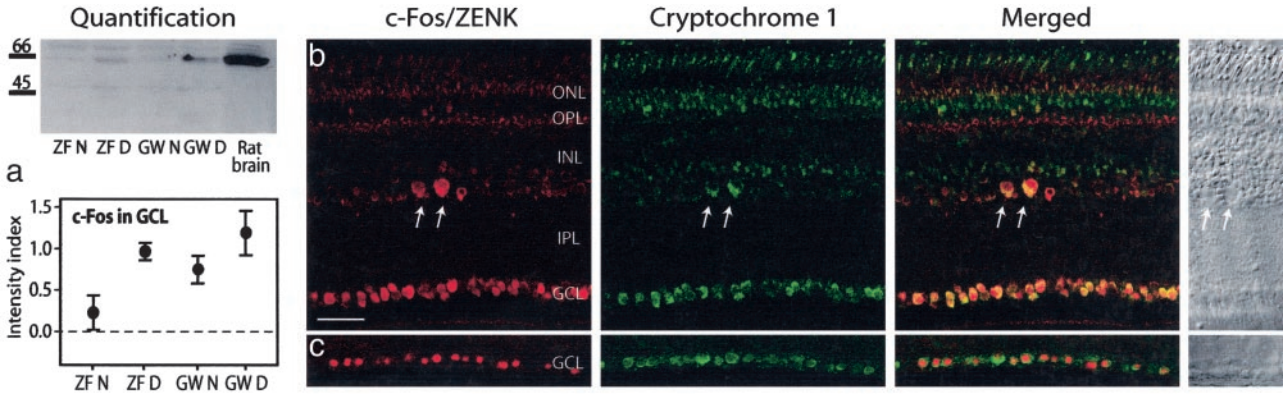

Fig. 3. Colocalization of CRY1 and neuronal-activity markers in the same 300-nm-thick retina slice from a magnetically orienting garden warbler. (*a*) Example of Western blot analysis confirming specificity of the antibody and quantification of c-Fos expression in the ganglion cell layer. For definition of intensity index, see *Materials and Methods*. Dashed line indicates unspecific background level of expression. (*b* and *c*) Immunohistochemical double labeling of CRY1 (cytosolic) with the neuronal-activity markers c-Fos (*b*; cytosolic and nucleic) and ZENK (*c*; exclusively nucleic; only the ganglion cell layer is shown) reveal that CRYs are found in night-active ganglion cells and in large displaced ganglion cells of the INL (arrows). (Scale bar, 40 μm.) GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

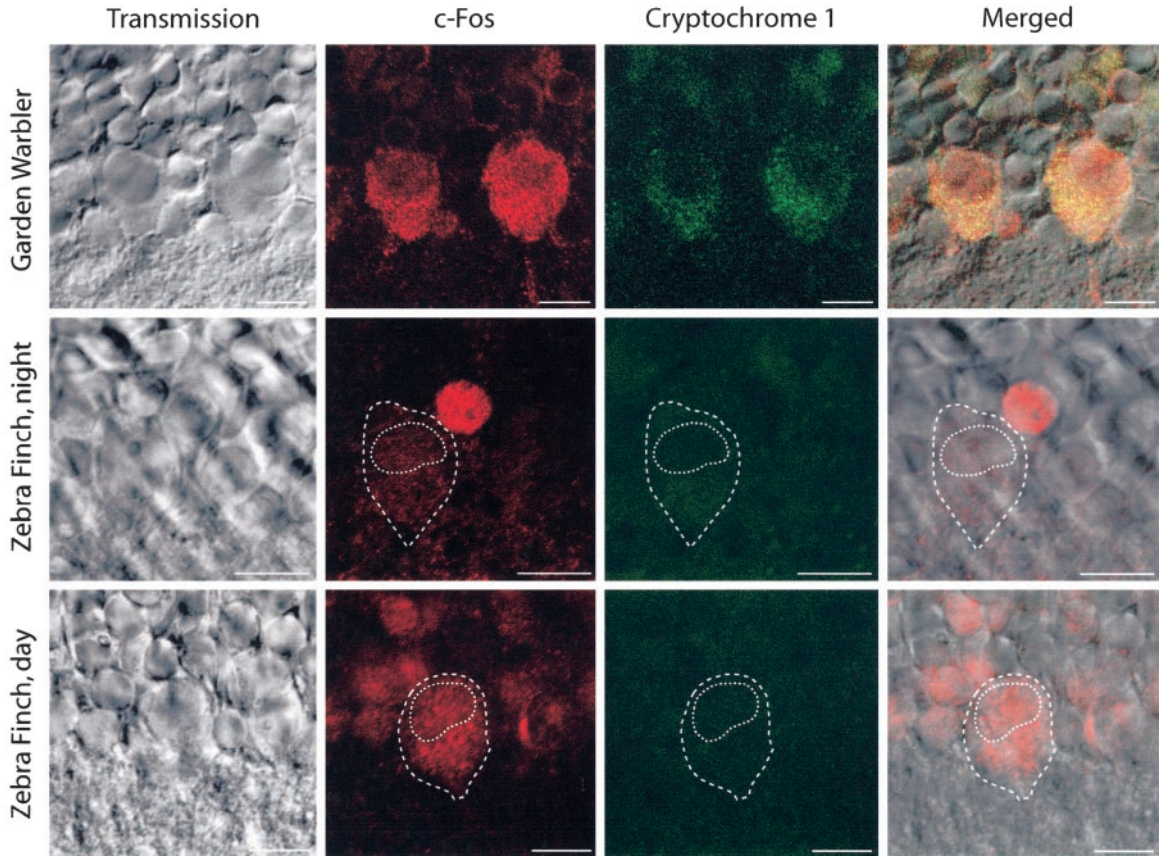

Fig. 4. High magnification confocal images confirm the colocalization of CRY1 with the neuronal-activity marker c-Fos in large displaced ganglion cells in the garden warbler [during both night (shown) and day (data not shown)]. In contrast, the large displaced ganglion cells never express CRY1 in zebra finches, neither during the night nor during the day, when both active (c-Fos-positive, as shown) and inactive (c-Fos-negative) large displaced ganglion cells occur in zebra finches.

Discussion

Although no direct link between CRY-containing cells and magnetoreception is currently known, we present here a number of strong correlations supporting such a link. We have shown that at least one CRY1 exists in the retina of migratory birds and that this gwCRY1 is apparently predominantly or even exclusively located in the cytosol. We have shown that CRY1 is concentrated in specific cells, particularly in ganglion cells and in large displaced ganglion cells, and we have shown that these cells also show high levels of neuronal activity at night during magnetic orientation in migratory garden warblers. That is, the CRY-containing cells in the eye send information to the brain at night when garden warblers orient to the geomagnetic field. Consequently, cytosolic CRY1 is well placed to possibly take over the role of the required substrate for magnetoreception according to the Ritz *et al.* model (7). We also show that there seem to be striking differences in CRY expression between migratory and nonmigratory birds at night. As in domestic chicken, there seems to be virtually no CRY1 in the retina of nonmigratory zebra finches at night, whereas it is strongly expressed in migratory garden warblers at night. The differences in CRY1 expression between migrants and nonmigrants are particularly pronounced in the large displaced ganglion cells that are known to project exclusively to the nucleus of the basal optic root, where magnetically sensitive neurons have been reported (41). Furthermore, the neuronal activity of the ganglion cells is significantly lower at night in nonmigratory zebra finches than in migratory garden warblers, suggesting that the ganglion cells send more information to the brain at night in migratory birds. Because CRYs are found in the retina of both night-migratory and nonmigratory birds during the day, our data do not exclude that a CRY-mediated magnetic compass could be used also during the day, when wavelength-dependent magnetic-compass orientation has also been observed (10).

CRYs in other vertebrates have been shown to be involved in the inhibitory branch of the autoregulatory transcriptional loop controlling the circadian clock (19), whereas the presumed photoreceptor function of vertebrate CRYs is still debated (17, 19). Our data do not exclude a clock function of the gwCRYs, and the high CRY1-expression in night-migratory garden warblers could be a result of their round-the-clock activity during the migratory season. However, garden warblers taken on nights when they do not show migratory restlessness also show high levels of CRY1-expression (H.M., U.J.-B., M.L., G.F., and R.W., unpublished data), and the cytosolic localization of CRY1 that we found in the garden warbler does not seem to favor a role in the clock transcriptional loop because these processes occur in the nucleus (17).

In conclusion, our results support the hypothesis that CRYs, in addition to their circadian functions (17–22), could indeed play a role in magnetic field reception. Having made this statement, we stress that there are still many questions that must be answered before we can state conclusively that the CRYs are the primary magnetic sensor in migratory birds. Although it has recently been shown that the radical pair of 6,4-photolyase, which is highly homologous to the CRYs, live long enough (>5 μs) for geomagnetic field effects to take place (44), it, for instance, still needs to be shown how the orientation of the CRYs is fixed in cells so that light-induced signals will be modulated

differently by the magnetic field depending on location in the retina. However, the location of gwCRY1 in the cytosol means that it could be fixed to the cytoskeleton. In any case, having identified the CRY-containing cells in the retina opens a number of experimental possibilities, which we believe will ultimately lead to an understanding of the molecular and physiological mechanisms underlying magnetic-compass orientation in migratory birds.

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Paper II

Mouritsen H, Feenders G, Liedvogel M & Kropp W:

Migratory birds use head scans to detect the direction of the earth's magnetic field.

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Migratory Birds Use Head Scans to Detect the Direction of the Earth's Magnetic Field

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Summary

Night-migratory songbirds are known to use a magnetic compass [1–3], but how do they detect the reference direction provided by the geomagnetic field, and where is the sensory organ located? The most prominent characteristic of geomagnetic sensory input, whether based on visual patterns [4–7] or magnetite-mediated forces [8, 9], is the predicted symmetry around the north-south or east-west magnetic axis. Here, we show that caged migratory garden warblers perform head-scanning behavior well suited to detect this magnetic symmetry plane. In the natural geomagnetic field, birds move toward their migratory direction after head scanning. In a zero-magnetic field [10], where no symmetry plane exists, the birds almost triple their head-scanning frequency, and the movement direction after a head scan becomes random. Thus, the magnetic sensory organ is located in the bird's head, and head scans are used to locate the reference direction provided by the geomagnetic field.

Results and Discussion

We observed and recorded the behavior of 35 night-migratory garden warblers, *Sylvia borin*, placed individually in a cylindrical orientation cage (Figure 1A), directly to hard disk and/or to video tape by two infrared (840 nm) video cameras (top and side view, inbuilt IR light sources) at night (indoors, light level 0.04 lux from four diffused white light bulbs not directly visible to the birds) or during the day (indoors, room light level 275 lux). Each bird had a thin line of infrared-reflective tape glued to its head, and they were tested inside a windowless wooden house. Seven birds were tested during the day in the natural geomagnetic field. The other birds were tested while showing migratory restlessness at night in the natural geomagnetic field ($n = 11$), a zero magnetic field [10] ($n = 11$) or a changing magnetic field switching 120° every 5 min ($n = 6$). The birds tested at night in the natural magnetic field (NMF) showed magnetic orientation directed in the normal migratory direction (Figure 1B), whereas the birds tested in the zero-magnetic field (ZMF) oriented randomly (Figure 1C). How did the NMF birds detect the compass direction of the geomagnetic field?

The video recordings suggested that birds, in addition to their migratory restlessness behavior, perform repeated head-scanning behavior (see movies in the Supplemental Data available with this article online). A naïve observer (W.K.) counted the number of head scans performed by each individual bird during a 1 hr period. We defined a head scan as the turn of the bird's head from the body axis position to an angle turned clearly more than 60° to the left or right, followed by the subsequent return of its head to the straight-ahead position while the bird remained at the same spot (Figure 1D). By requiring that the bird must return its head to the body axis position before moving in the cage, we avoided counting head turns, which always precede movement in a new direction. The side-view camera showed that the head-scanning behavior was performed in the horizontal plane. Sometimes a bird makes a head scan to one side only; other times, one head scan is immediately followed by another head scan in the opposite direction. Four pieces of evidence strongly suggest that head-scanning behavior is directly involved in the process of sensing the geomagnetic reference direction needed for magnetic compass orientation.

First, garden warblers exposed to a zero-magnetic field (ZMF) made 141 ± 33 (SD) head scans in 60 min, whereas birds tested under any other magnetic condition only made 52 ± 35 (SD) head scans in 60 min (see Figure 1E). The increased head-scanning frequency observed in the ZMF birds is highly significant (one-way ANOVA followed by Tukey all pair-wise comparison method: the ZMF group differs significantly [$p < 0.001$] from all other groups, whereas all other differences between groups are non-significant [$0.22 < p < 0.99$]). On average, the birds, irrespective of magnetic condition, performed an equal number of head scans to the left and to the right (mean = 50% ± 18% [SD]). We also quantified the number of flights and wing beats performed by each bird during the same 60 min period, but we found no significant differences depending on the magnetic field condition (flights: one-way ANOVA on ranks, $p = 0.99$, wing beats: one-way ANOVA, $p = 0.77$). Thus, differences in head scan frequency are not due to differences in activity level between NMF and ZMF birds.

Second, garden warblers strongly increased their head-scanning frequency before initiating their first migratory restlessness behavior (repeated jumping and wing flapping on the perch). Birds observed in the orientation cages typically sat still for 10–60 min after the lights went off. During the last 10 min before initiating their first migratory restlessness behavior that night, all birds made many repeated head scans. Within this period, the average head scan frequency increased gradually from ~2/min to about ~6/min (Figure 2A). This strong increase in head-scanning frequency suggests that the birds carefully determined the reference direction of the geomagnetic field before starting their orientation behavior. Once migratory restlessness behavior was initiated, birds experiencing the NMF made less than one head scan per minute on average, whereas birds experiencing the ZMF performed two to three head scans

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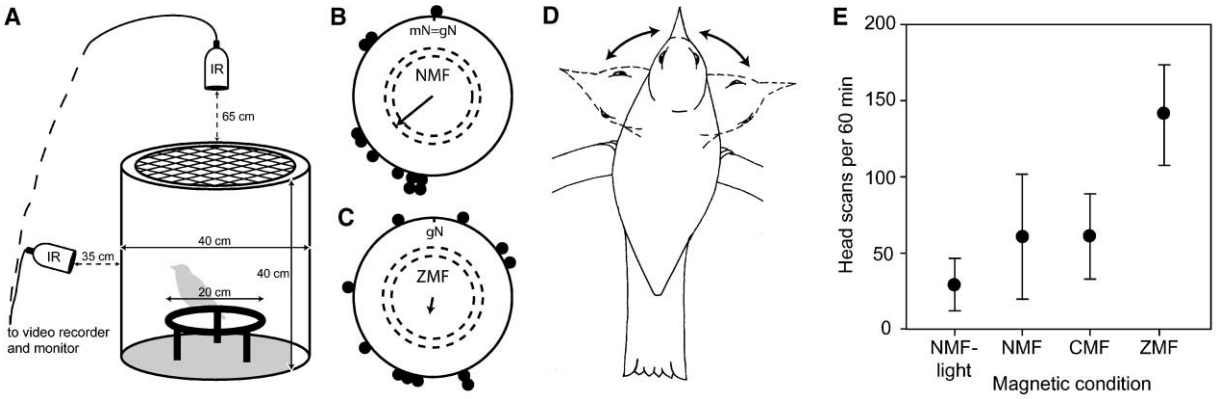

Figure 1. Caged Garden Warblers Perform Head Scan Behavior during Magnetic Orientation

(A) Design of our orientation cage.

(B) Garden warblers tested in the natural magnetic field oriented in their southwesterly migratory direction ($\alpha = 231°$, $r = 0.60$, $p < 0.02$). Each dot indicates the mean orientation of one individual garden warbler (measured as its head's location relative to the center of the cage determined 5 times per s during 45–60 min of constant migratory restlessness behavior). The arrow indicates the group's mean vector length (r). The inner- and outer-dashed circles indicate the length of the group's mean vector needed for significance ($p < 0.05$ and $p < 0.01$, respectively), according to the Rayleigh test. mN = magnetic North; gN = geographic North.

(C) Garden warblers tested in a zero-magnetic field oriented randomly ($\alpha = 187°$, $r = 0.22$, $p = 0.60$).

(D) Schematic drawing of head-scanning behavior.

(E) Number of head scans performed by garden warblers within 60 min under four different conditions: during the day in the natural magnetic field (NMF-light) and while showing migratory restlessness at night in the natural magnetic field (NMF), a changing magnetic field (CMF), or a zero magnetic field (ZMF).

per minute (Figures 1E and 2B). The fact that the birds continued to perform regular head scans throughout the night, even in the natural magnetic field, suggests that they have not transferred magnetic information to other cues in their cage or surroundings.

Third, during migratory restlessness behavior, most garden warblers made occasional flights to the top of the cage followed by fluttering around and landing on the bottom of the cage. After sitting at the bottom of the cage for a few seconds, most birds returned to the perch, where they usually sat still for 10–60 s before reinitiating migratory restlessness behavior. During this period of sitting still after a flight, the birds seem to reorient themselves before continuing their migratory restlessness behavior. This is evidenced by a highly significant 2-fold increase in head-scanning frequency during the first minute following a flight off the perch compared with any other 1 min period (176 head scans observed in 93 1 min periods immediately after a flight compared to 225 head scans observed in 238 other 1 min periods; chi-square test: $df = 1$, $\chi^2 = 49.5$, $p < 0.001$).

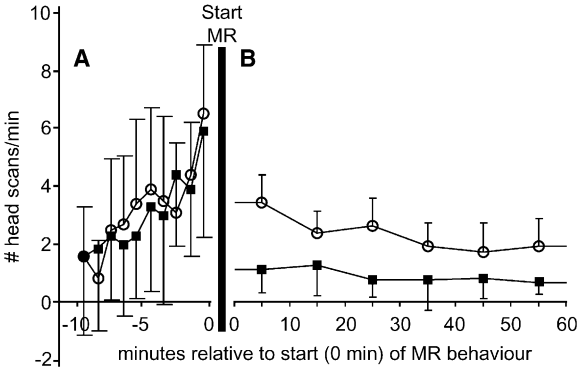

Figure 2. Number of Head Scans Performed per Minute Just before and during Migratory Restlessness Behavior

(A) Just before initiation of their first migratory restlessness behavior at night, garden warblers strongly increase their head-scanning frequency. On the x axis, "0" indicates the time when a bird performed its first migratory restlessness behavior. The symbols are as follows: ■: NMF birds; ○: ZMF birds.

(B) During migratory restlessness behavior, birds experiencing a ZMF continue to show a relatively high head-scanning frequency (two to three head scans per minute), whereas birds experiencing NMF conditions only make about one head scan per minute on average. Error bars indicate (symmetrical) standard deviations.

Fourth, if head scans indeed help garden warblers detect the reference compass direction provided by the geomagnetic field, one should expect that the birds in the natural magnetic field move more toward than away from their mean migratory direction after performing a head scan, whereas the direction of movement after a head scan in a zero-magnetic field should be close to random. We tested this by observing the garden warblers' very first move immediately after they performed a head scan. This was done by placing an arrow on the TV monitor pointing in the overall mean direction of the individual bird. Then, if a bird with a mean orientation of 205°, for example, performs a head scan while sitting on the circular perch at 140°, a clockwise move along the perch would be toward the "correct" direction, whereas a counter-clockwise move would be counted as a move in the "wrong" direction. If it performs a head scan at 265°, a counter-clockwise move would be correct and a clockwise move would be wrong. For both the NMF and ZMF condition, we only analyzed birds that showed an overall mean direction oriented in the appropriate, southwesterly mean migratory direction (215° ± 60°) characteristic for garden warblers during autumn migration. Otherwise, no clear correct migratory direction

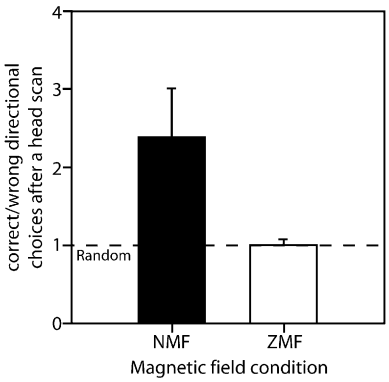

Figure 3. Orientation of NMF and ZMF Birds Immediately Following a Head Scan

Birds experiencing a natural magnetic field move 2.39 ± 0.63 times as often toward their correct migratory direction than away from it after performing a head scan. In contrast, birds experiencing a zero-magnetic field move equally often toward and away from their mean migratory direction following a head scan. Error bars indicate standard deviations.

could be defined. Furthermore, to be reliably analyzed, a bird must have performed most of its migratory restlessness behavior on the perch, where one can clearly determine if the next movement following a head scan is in the correct or wrong direction. All the garden warblers performing migratory restlessness behavior on the perch in the natural magnetic field moved significantly more toward their mean migratory direction after performing a head scan than away from it (number of correct moves/number of wrong moves = 2.39 ± 0.63, Figure 3), whereas the direction of movement following a head scan in a zero-magnetic field was random (number of correct moves/number of wrong moves = 1.01 ± 0.06; difference between NMF and ZMF birds: Mann-Whitney U-test, $p < 0.01$; Figure 3). This difference cannot be explained by a difference in individual directedness (r values) between the ZMF (0.19 ± 0.12) and NMF (0.24 ± 0.18) birds because this difference was nonsignificant (t test, $p = 0.67$).

Based on these converging pieces of evidence, we suggest that caged garden warblers, and possibly night-migratory birds in general, use head movements to detect the reference compass direction of the earth's magnetic field. If magnetoreception in birds is magnetite mediated [8, 9], we suggest that the head movements are designed to scan for the maximum or minimum magnetic field strength direction. If magnetoreception is vision dependent [4–7], we suggest that the purpose of the head scans is to detect the symmetry axis of the magnetically modulated visual patterns that characterize the magnetic-field axis [5] and/or to improve detection of these gradually changing patterns by the visual system, which is more sensitive to them when they move. In fact, due to the predicted graded nature of the magnetically modulated, virtual visual patterns, they may very well be undetectable unless the bird moves its eye relative to the pattern [11]. We also suggest that the strongly increased head-scanning frequency observed in ZMF-birds during migratory restlessness behavior is a result of their repeated, unsuccessful attempts to find a symmetry plane or pattern that does not exist.

The fact that head scanning seems to be used by garden warblers to detect the compass direction of the geomagnetic field confirms that their magnetic sensor must be located in the head. If head scanning is performed by all birds, this will remove an important uncertainty in our search for the avian magnetic sensor and would provide crucial knowledge when designing magnetic-manipulation devices for free-flying birds [12]. Furthermore, virtually all psychophysical experiments designed to elucidate the functional characteristics of the avian magnetic compass have been unsuccessful [13]. We suggest that head scan counts can be used as a much-needed psychophysical measure to determine many unknown functional characteristics of the avian magnetic compass.

Supplemental Data

Supplemental Data including two movies are available at http://www.current-biology.com/cgi/content/full/14/21/1946/DC1/.

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Paper III

Mouritsen H, Feenders G, Liedvogel M, Wada K & Jarvis ED:

A night vision brain area in migratory songbirds.

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Night-vision brain area in migratory songbirds

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Twice each year, millions of night-migratory songbirds migrate thousands of kilometers. To find their way, they must process and integrate spatiotemporal information from a variety of cues including the Earth's magnetic field and the night-time starry sky. By using sensory-driven gene expression, we discovered that night-migratory songbirds possess a tight cluster of brain regions highly active only during night vision. This cluster, here named ''cluster N,'' is located at the dorsal surface of the brain and is adjacent to a known visual pathway. In contrast, neuronal activation of cluster N was not increased in nonmigratory birds during the night, and it disappeared in migrants when both eyes were covered. We suggest that in night-migratory songbirds cluster N is involved in enhanced night vision, and that it could be integrating vision-mediated magnetic and/or star compass information for night-time navigation. Our findings thus represent an anatomical and functional demonstration of a specific night-vision brain area.

behavioral molecular mapping | bird orientation | cognition | magnetic sense | ZENK (zif268, Egr-1, NGF-1A, and Krox-24)

Night-migratory songbirds use both a geomagnetic and a star compass to orient during migration (1–6) but the underlying brain circuits are unknown. Star-compass orientation requires vision in dim light for processing constellations of the night-time starry sky. Surprisingly, magnetic-compass orientation also seems to require night vision: magnetic-compass orientation is dependent on the wavelength of the dim ambient light (4, 7), birds with their right eye covered seem unable to perform magnetic compass orientation (8), and birds can still perform magnetic compass orientation with their pineal gland removed (9). Current evidence suggests that visual sensing of the magnetic field occurs through the eyes by means of a light-activated, radical-pair based magnetodetector (4, 7, 10–13). Another type of magnetodetector based on magnetite seems to be predominantly involved in detecting changes in magnetic intensity and/or inclination (4, 14–16). Together, these findings predict that processing of both magnetic- and star-compass information during night-time migration requires specialized night-time visual processing in night-migratory songbirds. We tested this hypothesis by using sensory-driven gene expression (17) to identify brain regions involved in night- and day-time vision in migratory and nonmigratory songbirds. Our results suggest that night-migratory songbirds do indeed possess a brain area specialized for night vision.

Methods

Test Subjects. We examined two distantly related species of wild-caught night-migratory songbirds [garden warblers (GWs), *Sylvia borin*; and European robins (ERs), *Erithacus rubecula*] and two distantly related species of nonmigratory songbirds [zebra finches (ZFs), *Taeniopygia guttata*; and canaries (CNs), *Serinus canaria*]. The GWs, ERs, and ZFs were kept inside a wooden building under the local photoperiod for at least 5 days before the experiment. Behavioral tests were performed during the GW and ER migratory seasons from August 22 to October 18, 2002 and 2003 and April 15 to May 25, 2003, except for three GWs tested July 17 to August 3, 2003. The canaries were taken from the Jarvis laboratory collection of birds when awake at night and day to confirm consistency between nonmigrants. All animal procedures were approved by the Animal Care and Use Committees of Bezirksregierung Weser-Ems (Oldenburg, Germany) and/or Duke University Medical Center (Durham, NC).

Test Procedures. On testing day, our night-group birds (n = 12 GWs, 4 ERs, and 5 ZFs) were individually put into a custom-designed cylindrical, transparent Plexiglas cage fitted with a circular perch placed 8.5 cm above the ground in the center of the cage (18) (the two CNs were placed in sound isolation boxes). The birds were allowed to get used to the cage or soundbox for 3–12 h. At dusk (local photoperiod), the room lights were turned off except for four small, diffused light bulbs simulating a natural moonlit night (0.04 lux). This light intensity is typically used in behavioral orientation tests with night-migrants (2, 6, 7, 10). Our day-group birds (n = 5 GWs, 5 ZFs, and 5 CNs) were tested in full room light (≈275 lux). Each bird's behavior was continuously observed in real time by two infrared (840 nm) video cameras (top-view and side-view) connected to a split-screen surveillance monitor and in parallel recorded to videotape (25 frames per sec). No night-bird was collected earlier than 100 min after the lights went off, thereby ensuring that any possible brain activity induced by the day/night transition had decreased to its baseline level by the time the bird was collected. We also took care that no external acoustic signals reached the bird. To minimize brain activity because of movement and other factors, we collected birds only after they had been sitting relatively still but awake (eyes open) for at least 45 min but mostly for 60 min or more, while a minimum of other behaviors occurred (0–16 flights [most performed 0 flights] and occasional walking on the perch or floor; exception: ZFs tested during the day did not sit still). After the birds were killed, their brains were rapidly dissected, embedded in Tissue-Tek O.C.T. (Sakura Finetek, Zoeterwoude, The Netherlands), and quick-frozen in a dry ice/ethanol bath at −80°C.

Eye Capping. The ERs (n = 4) night-time group with eyes open was compared with another group of ERs (n = 4) fitted with light-tight eye caps to prevent any visual stimulus from reaching the eyes. The eye caps were built of small black plastic–velour cylinders (diameter = 10 mm) enlarged with Leucoplast tape (Beiersdorf AG, Hamburg, Germany) and light-tight black tape. Three to 5 h before onset of darkness, the eye caps were glued onto the bird's head to cover the eyes completely without any direct contact to the eyes themselves. The bird was put into the cage, observed continuously, and collected as described.

Gene Expression Analyses. We measured expression of ZENK [acronym for zif268, Egr-1, NGF-1A, and Krox-24 (19)] and c-fos immediate early genes in the brain. Expression of ZENK and c-fos mRNA in the brain is driven by neuronal activity and can

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Abbreviations: GW, garden warbler (*Sylvia borin*); ER, European robin (*Erithacus rubecula*); ZF, zebra finch (*Taeniopygia guttata*); CN, canary (*Serinus canaria*); ZENK, zif268, Egr-1, NGF-1A, and Krox-24; MD, dorsal mesopallium; DNH, dorsal nucleus of the hyperpallium; GluR1, glutamate receptor 1 subunit.

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be detected in neurons ≈5 min after onset of neural firing with peak expression after 30–45 min (17, 20). Therefore, increased cumulative mRNA expression marks brain regions that were active during the last 45–60 min of sensory stimulation or behavior. However, ZENK and c-fos are not activated by neural activity in all brain cell types. These exceptions in birds are some thalamic neurons, telencephalic neurons receiving primary sensory input from the thalamus, and globus pallidus neurons (21). It has been suggested that these neurons do not express the specific neurotransmitter receptors necessary to induce ZENK and c-fos expression in response to neuronal activity (20). In all other neuron types, which constitute roughly two-thirds of the avian brain, expression of ZENK and/or c-fos follows neuronal firing.

For one bird per group, 12-μm frozen sections were cut throughout the entire brain (left hemisphere in the sagittal plane and the right hemisphere in the coronal plane). We observed no differences in gene expression between left and right hemispheres and therefore continued cutting only sagittal sections of the left hemisphere for all remaining birds. We chose sagittal sections because cluster N was best seen in this plane. Corresponding sections of all birds were fixed in 4% paraformaldehyde and processed by *in situ* hybridization with antisense 5′-[α-[^{35}S]thio]UTP-labeled riboprobes of a ZF ZENK (K.W. and E.D.J. clone), c-fos (K.W. and E.D.J., unpublished data), or glutamate receptor 1 subunit (GluR1) of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor cDNAs (22) by following described procedures (22). We used GluR1 as an anatomical marker in combination with Nissl staining to identify anatomical boundaries of cerebral subdivisions. The hybridized sections were first exposed to x-ray films (Biomax MR, Kodak) for 1–4 days, dipped into an autoradiographic emulsion (NTB2, Kodak), incubated for 3–4 weeks at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl violet acetate solution (Sigma). X-ray film brain images were digitally scanned from a dissecting microscope connected to a SPOT III charge-coupled device camera with SPOTadvanced imaging software. For quantification, a person naïve to the experimental conditions used PHOTOSHOP 6.0 (Adobe Systems, San Jose, CA) to measure the mean pixel intensities in the brain regions of interest on a 256-level gray scale pixel range. We measured ZENK expression by calculating the difference in mean pixel density between the relevant brain region and a comparably sized region anterior to it in the same brain subdivision(s) [e.g., cluster N − (anterior hyperpallium + anterior dorsal mesopallium)]. We use the term "region" to refer to a specific part of a brain subdivision and the term "area" to refer to a cluster of regions. To test for significant differences between individual groups, the SIGMASTAT software package was used to perform one-way ANOVA followed by a Student–Newman–Keuls multicomparison.

Results

Both species of night-migratory songbirds when awake at night showed a striking pattern of high ZENK expression confined to the dorsal part of the cerebrum (Fig. 1*A* II, yellow arrow). ZENK expression in this area was not affected by movement behavior (unpublished work) and only medium to low levels of ZENK expression were found throughout the rest of the brain. To anatomically define this cerebral area showing very high expression, in addition to examining Nissl stains, we hybridized adjacent brain sections to GluR1, which distinguishes the boundaries between the avian cerebral brain subdivisions (22, 23). The GluR1- and Nissl-stained pattern revealed that this area consists of five brain regions (Fig. 1*B*): (*i*) a portion of the hyperpallium apicale, (*ii*) a portion of the interstitial region of the hyperpallium apicale, (*iii*) a portion of the dorsal mesopallium (*iv*) a nucleus embedded within this portion of the hyperpallium apicale that we named dorsal nucleus of the hyperpallium (DNH), and (*v*) a shell of cells around DNH that we named the DNH-shell. We designated these regions according to the recent anatomical nomenclature of the avian brain (23). The DNH had higher GluR1 and lower ZENK expression relative to the surrounding hyperpallium, indicating that it behaves differently from the surrounding hyperpallium. The DNH-shell had slightly lower ZENK expression and was characteristically different from the other parts of the hyperpallium (Fig. 1*B*; and Dominik Heyers, personal communication). In contrast to the strong night-time activation, this cluster of regions showed no significant ZENK induction during the day (Fig. 1 *A*I and *C*). c-fos, another activity-dependent gene, like ZENK, also showed strikingly high expression in this cluster of regions at night (Fig. 2). As expected, GluR1 did not show a difference between day and night (data not shown). We therefore named this group of five regions "cluster N" (N for night-activation). Cluster N is fairly large, taking up ≈40% of the hyperpallium and dorsal mesopallium in the sagittal slice where cluster N activation is most prominent. It extends ≈1 mm rostrocaudal, ≈1.5 mm mediolateral, and ≈1.5 mm dorsoventral.

The strong night-time activation in cluster N observed in the two migratory songbird species was not found in the two nonmigratory species (Fig. 1 *A* and *C*). Although, in nonmigrants, expression in the entire hyperpallium and MD (posterior to anterior) in the equivalent sagittal plane was slightly higher than in other brain regions at night, there was a decreasing tendency in absolute ZENK expression levels at night [mean intensity index: nonmigrants day = 95 ± 13 (SD), nonmigrants night = 76 ± 20; *t* test $P = 0.15$].

The hyperpallium is also known as the "Wulst" (meaning bulge) with its central–dorsal part functioning as the "visual Wulst" (24). Because no defined anatomical boundaries are known of the visual Wulst, we cannot prove whether cluster N is a part of the visual Wulst (being either a specialization or having evolved out of it) or whether it is located adjacent to it. However, a relationship to the visual Wulst is very likely because the anterior part of cluster N is localized in the mediodorsal part of the hyperpallium, which corresponds to the described posterior extensions of the visual Wulst (24). Based on this knowledge and on the predicted visual nature of magnetic- and star-compass input to the brain in combination with the fact that cluster N activation seems specific to night-migrants, we suspected that cluster N could be processing visual information at night. To test this hypothesis, we performed ZENK *in situ* hybridization on brains from night-migrants that had both eyes covered by light-tight eye caps during the dim-light night. After becoming used to the eye caps, these birds sat relatively still for extended periods of time, as did the birds without eye cover. Blocking visual input resulted in strong reduction of dim-light, night-time-induced cluster N ZENK expression (Fig. 3 *A*–*C*). The DNH-shell did not show reduction of ZENK expression, and thus these cells are still active in complete darkness (Fig. 3*B*). We conclude that the majority of cluster N activation requires dim-light, visual input.

To check for possible circadian effects, we compared cluster N expression levels in birds at multiple time points after the onset of night-time, dim-light levels (within a 6-h range) and found that the expression levels in cluster N in birds with both eyes open or closed did not correlate with the time of brain collection relative to the onset of dim-light levels (Fig. 3*D*). Furthermore, birds with covered eyes showed low ZENK expression at times when other birds sitting in dim-light show high ZENK expression in cluster N (Fig. 3*D*). Thus, cluster N activation cannot simply be because of circadian rhythms.

To test for a possible seasonal variable, we examined three GWs during their nonmigratory breeding season in July and early August, when they did not show evidence of migratory

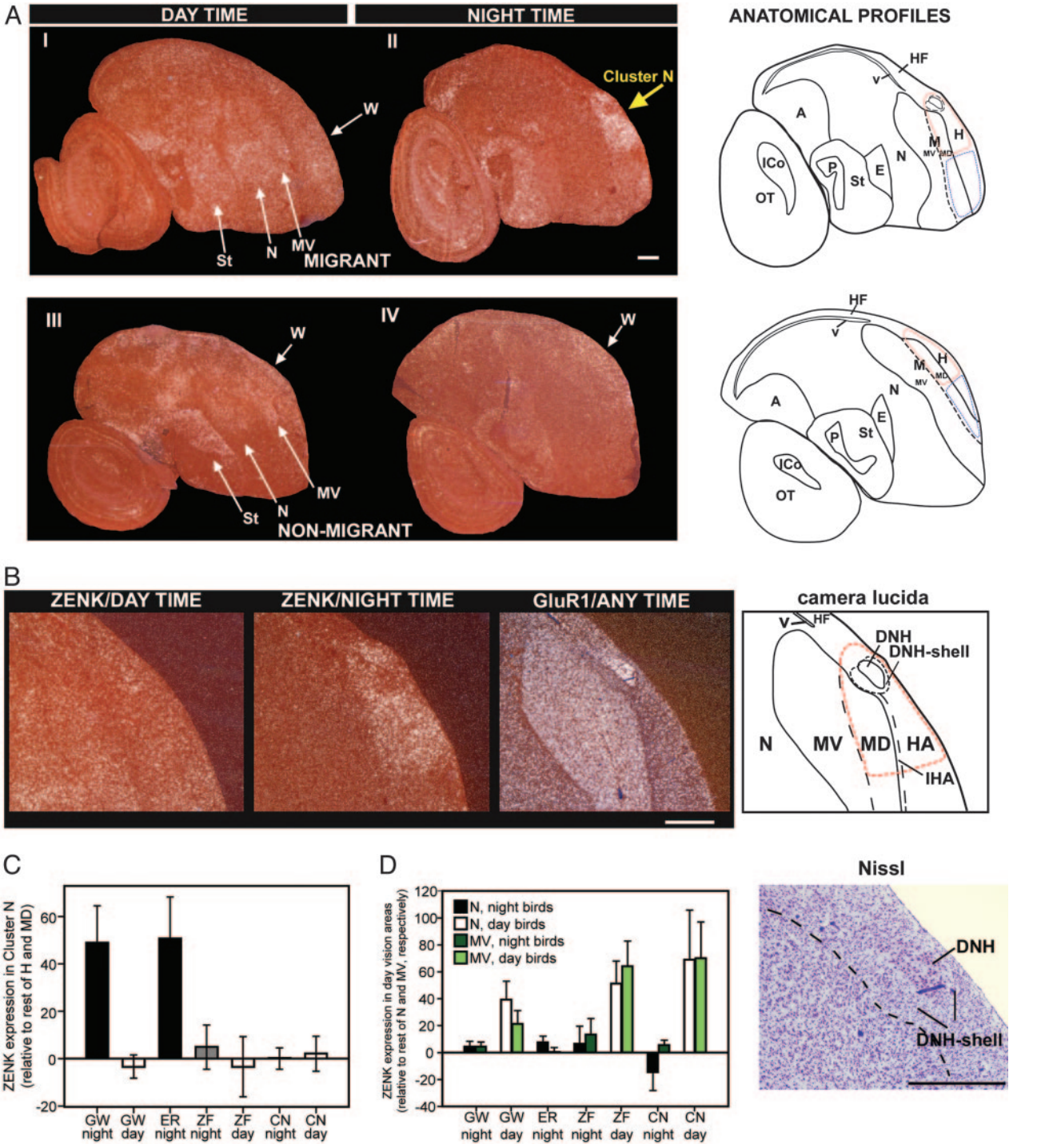

Fig. 1. ZENK brain activation patterns. (*A*) Day- and night-time visual activation in night-migratory GWs (I and II) and nonmigratory ZFs (III and IV). Parasagittal brain sections are shown. (Dorsal is up; anterior is right; white-silver grains, ZENK expression; red, Nissl stain; white arrows in I and III, regions activated during day-time vision; yellow arrow in II, regions activated in migrants during night-vision.) Arrows are drawn at different angles for each species to reflect that the relative orientation of the GW cerebrum is naturally angled more forward than the ZF cerebrum. The red and blue highlighted regions in the anatomical drawing indicate the regions quantified from for *C*. (*B*) Anatomical characterization of cluster N in GWs. The red-dashed line highlights the cluster N area of ZENK expression. (*C*) Differences in cluster N ZENK expression among groups were highly significant {cluster N, i.e., [posterior hyperpallium (H) + dorsal mesopallium (MD)] − [anterior H + MD]; by one-way ANOVA, df = 6; $df_{residual}$ = 29; $F = 22.4$; $P < 0.001$}. Relative cluster N ZENK expression in GWs and ERs at night was significantly higher compared with all other groups (Student–Newman–Keuls multicomparison test: $P < 0.001$ for all individual comparisons). Relative cluster N ZENK expression in GWs during the day and in the comparable region in nonmigrants (ZFs; CNs) during the day or night was not significantly different among each other (Student–Newman–Keuls multicomparison test: $P > 0.73$ for all comparisons) nor from anterior H + MD (all 95% confidence intervals include 0, which indicates identical expression in cluster N and the rest of H + MD.) The *y* axis shows relative pixel density on a 256 gray scale. (*D*) ZENK induction in known day-vision regions was significantly higher during the day (one-way ANOVA: $P < 0.001$ for all within-species comparisons between day and night birds except the mesopallium ventrale (MV) comparison for GWs where $P = 0.06$). Error bars = standard deviations. A, arcopallium; P, pallidum; E, entopallium; St, striatum; N, nidopallium; M, mesopallium; H, hyperpallium; ICo, intercollicular complex; v, ventricle; OT, optic tectum; HF, hippocampal formation; IHA, interstitial region of the hyperpallium apicale (HA); DNH, dorsal nucleus of the hyperpallium; W, visual Wulst). (Scale bars, 0.5 mm.) A high-resolution PDF version of Fig. 1 is available in the supporting information on the PNAS web site.

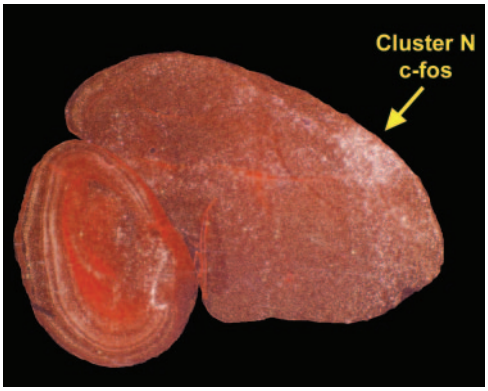

Fig. 2. Example of cluster N c-fos induction at night in night-migrants (GW). The sagittal section is more lateral than that in Fig. 1, where the DNH nucleus is no longer present. A high-resolution PDF version of Fig. 2 is available in the supporting information on the PNAS web site.

activity behavior at night. These animals showed increased ZENK expression in cluster N during dim-light, night-time conditions and at expression levels that did not differ from those seen in birds tested during the migration season (*t* test, $P = 0.37$). Thus, cluster N night-time activation is not purely due to the increase of night-time activity during the migratory season in the migrants but appears to be specific to night-migratory species at all times of the year.

In contrast to the night-time activation in cluster N, during day-light hours consistent increases in expression occurred in a set of regions surrounding the entopallium in both night-migratory and nonmigratory birds (Fig. 1 *A*I, 1*A*III, and 1*D*). The entopallium receives thalamic visual input, and like other primary sensory telencephalic neurons it does not show prominent ZENK expression (21). The day-time activated regions around the entopallium included a portion of the nidopallium and a portion of the ventral mesopallium, forming a ventral to dorsal column of brain activation. We suggest that these regions are involved in day-time vision. Support for this suggestion comes from visual pathway connectivity and vision-activated electrophysiological neural firing in these brain regions in ZFs and other bird species (25, 26).

Discussion

Our results identify a brain area, cluster N, active specifically in night-vision. We suggest that cluster N is evolutionarily related to the visual Wulst. It appears to be localized either posterior to or as a specialized part of the visual Wulst, a visual area comparable to the mammalian striate visual cortex V1 (27). The visual Wulst is part of the thalamofugal pathway (striate visual

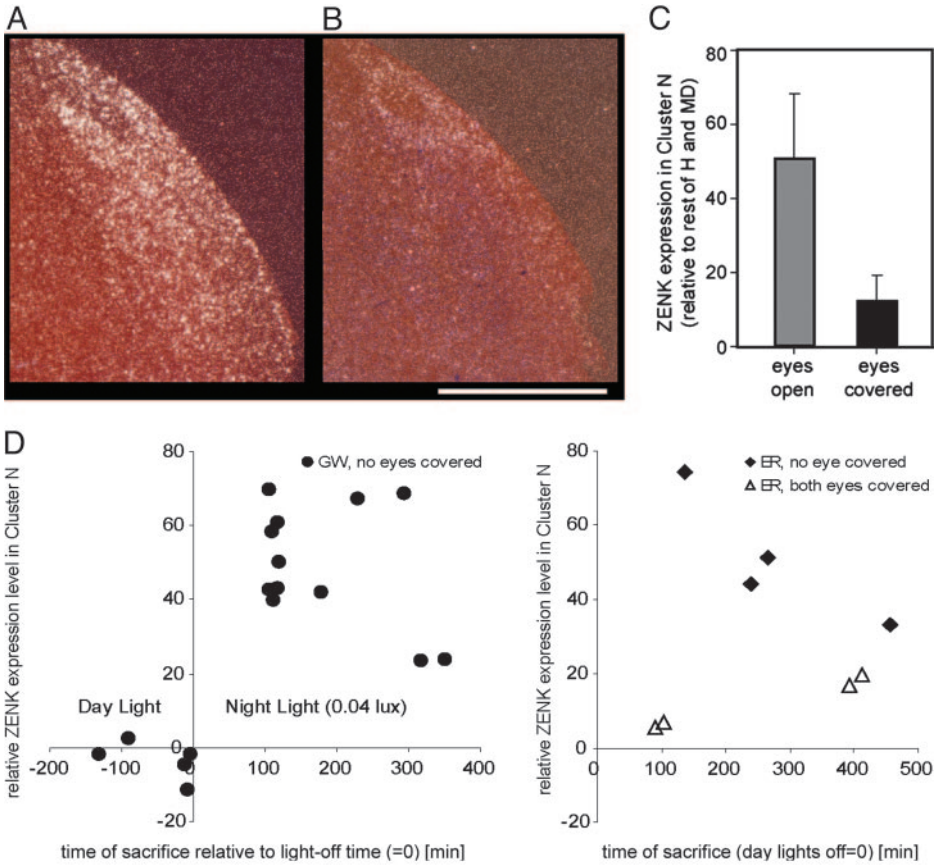

Fig. 3. Cluster N ZENK induction at night in night-migrants is visually driven. (*A* and *B*) ZENK expression in cluster N in a night-migrant, ER, with eyes open (*A*) or with both eyes covered by light-tight eye-caps (*B*). (Scale bar, 1 mm.) (*C*) Covering the birds' eyes led to a significant reduction in cluster N ZENK expression (*t* test, $t = 4.080$; df = 6; $P < 0.01$; $n = 4$ per group). The expression in the covered-eye birds is still above zero because the cluster N area quantified included the shell of high expression still present around the DNH nucleus (refer to *B*). (*D*) Cluster N ZENK expression as a function of time for all migratory birds used in this study. (*Left*) GWs with both eyes open. Along the *x* axis, time point 0 is when the day-lights were turned off and the dim night-time lights were turned on. The amount of time the lights were off did not affect increased cluster N ZENK expression (linear regression of night-time values, $F = 1.55$, $P = 0.24$). (*Right*) Birds with covered eyes show low ZENK expression at times when other birds sitting in dim-light show high ZENK expression throughout cluster N. A high-resolution PDF version of Fig. 3 is available in the supporting information on the PNAS web site.

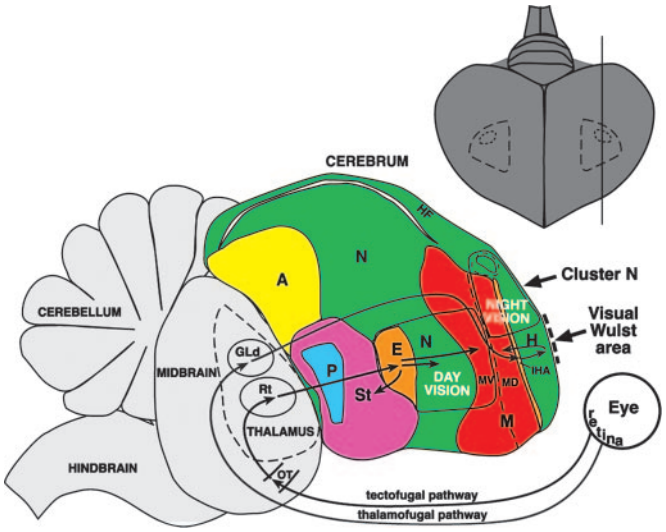

Fig. 4. Schematic drawing of a brain showing the relative locations of the day- and night-vision (cluster N) activated brain regions in night-migrant songbirds. The thamalofugal and tectofugal visual pathways have been determined in other bird species. Upper right, the extent of cluster N seen from the dorsal surface of the brain, determined from serial parasagittal and coronal sections hybridized to ZENK. GLd, lateral geniculate nucleus, dorsal part; Rt, nucleus rotundus; additional abbreviations are as in legend of Fig. 1.

pathway in mammals) that transfers information directly from the retina to the thalamus, then to the interstitial region of the hyperpallium apicale, and finally to hyperpallium apicale (Fig. 4). The thalamofugal pathway is separate from another visual system called the tectofugal pathway (extrastriate visual pathway in mammals). The avian tectofugal pathway transfers information from the retina to the tectum of the midbrain and, from there, via the thalamus to the entopallium, surrounding nidopallium, and ventral mesopallium (Fig. 4) (25). In nonmigrants, we found that both cerebral visual pathways showed day-time activation. At night, only the visual Wulst area of the thalamofugal pathway still showed some activation, albeit decreased in relation to the day-time level. However, in migrants cluster N showed very high activation at night. These findings cannot prove whether nonmigrants actually do not possess an area comparable to cluster N or whether they do have such an area that does not show enhanced activation during the night. However, because the visual part of the Wulst is typically more centrally located (posterior–anterior) in the hyperpallium, and cluster N is located at the posterior end of hyperpallium, the results suggest that in night-migratory birds, cluster N evolved out of the preexisting thalamofugal visual pathway as a specialized posterior part of or an attachment to the visual Wulst.

The pattern of night-time and day-time activation suggests another view of the functional organization of the avian cerebrum (Fig. 4). The dorsal mesopallium and the hyperpallium above it are activated as a columnar unit (i.e., cluster N), whereas the ventral mesopallium and the nidopallium ventral to it are activated as another columnar unit (day-time visual activation). This pattern of activation suggests that the hyperpallium is functionally associated with the dorsal mesopallium, whereas the nidopallium is functionally associated with the ventral mesopallium, and that perhaps cluster N with its five subregions represents a functional cerebral system.

The increased ZENK and c-fos expression in cluster N and the fact that this increased expression disappears when the birds' eyes were covered suggest that cluster N increased neural firing during night vision. The consequence of induction of ZENK and other immediate early genes in cluster N of night-migratory songbirds is presumably the same as that proposed for other brain areas that express ZENK (17, 20) (i.e., it regulates the transcription of other genes to either replace proteins that get metabolized when the involved neurons become highly active or to stabilize circuits involved in new information processing).

Why would night-migrants need to evolve a distinct night-vision system, when all songbird species are able to see at night? We suggest that night-migrants may require specialized development of a cerebral system such as cluster N for seeing better at night and/or for visual night-time navigation. The navigation signals sensed and the information processed could be star-light constellations and/or the Earth's magnetic field. The latter possibility would be in line with theoretical (11), molecular (13), and behavioral (4, 7, 8, 10, 12, 18) evidence suggesting that the Earth's magnetic field modulates the light sensitivity of specialized receptor molecules differently in various parts of the retina, leading to perception of the magnetic field as visual patterns (11, 13). As a consequence, the brain regions ultimately extracting the reference direction provided by the geomagnetic field should process and compare purely visual input from the retina. This prediction is in line with ZENK and c-fos expression patterns in the eyes of migratory GWs. Retinal ganglion cells showed high neuronal activity levels at night during magnetic orientation, and these active cells contained high concentrations of cryptochromes, a class of photoreceptor molecules suggested to be involved in magnetodetection (13). In contrast, in nonmigratory ZFs retinal ganglion cells showed lower ZENK expression at night when very little cryptochrome was found in the eyes (13).

The discovery of a distinct night-vision brain area in night-migratory songbirds will allow investigators to probe a specific site in the central nervous system of vertebrates for biological mechanisms of night vision and navigation. Verifying these suggestions will require future studies with electrophysiological recordings and lesions of cluster N.

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Paper IV

Feenders G, Liedvogel M, Wada K, Mouritsen H & Jarvis ED:

Movement-driven gene expression patterns implicate origin of brain areas for vocal learning.

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Movement-driven gene expression patterns implicates origin of brain areas for vocal learning

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Running Title: Movement in the brain

Brain structures and pathways that control vocal learning in birds are strikingly similar among distantly related groups, even though they are not found in their closely related vocal non-learning relatives. This led to the hypothesis that vocal learning brain pathways evolved independently in the distantly related groups but under pre-existing constraints. Here, we show what this constraint may have been. We studied movement-driven gene expression in migratory songbirds displaying migratory restlessness behavior and in non-migratory songbirds displaying general movements. We discovered that in songbirds, migratory or not, vocal learning brain nuclei active during vocalizing are embedded in or adjacent to brain areas active during head, wing, and body movements. We also found high activation in the anterior medial hyperpallium apicale and subdivisions of the cerebellar cortex, consistent with known somatosensory pathway inputs. These movement activated brain areas were also present in female songbirds that do not have vocal nuclei nor learn vocalizations. These findings present the first global mapping of avian brain motor areas and suggest that vocal learning brain systems evolved out of a pre-existing motor pathway that controls movement.

Key words: vocal pathway, language, migration, migratory restlessness, flight, ZENK

Introduction

Vocal learning, the behavioral substrate for human language, is a rare trait to date found in three distantly related groups of birds: songbirds, hummingbirds, and parrots (**Fig. 1A**). In these groups, vocalizing-driven immediately early gene (IEG) expression has revealed that each, remarkably, possess exactly seven cerebral vocal nuclei active in the production of learned vocalizations (**Fig. 1B**;Jarvis et al., 1998; Jarvis and Mello, 2000; Jarvis et al., 2000). Three of the nuclei form a column within the anterior vocal pathway (**AVP; Fig. 1B, red**), a pathway that in songbirds is necessary for vocal learning (Scharff and Nottebohm, 1991). The other four nuclei form a posterior vocal pathway (**PVP; Fig. 1B, yellow**), a pathway that in songbirds is necessary for the production of the learned vocalizations (Nottebohm et al., 1976; Simpson and Vicario, 1990). None of these seven cerebral vocal nuclei have been found in vocal non-learners (Kroodsma and Konishi, 1991; Haesler et al., 2004; Wada et al., 2004). Yet, both vocal learners and non-learners have brainstem vocal nuclei DM and nXIIts (**Fig. 1B**) that are responsible for production of innate vocalizations (Wild, 1997a; Jarvis et al., 2000). Therefore, the implications are remarkable: either vocal learning evolved multiple independent times and each time, it did with seven similar cerebral brain structures; or vocal learning was present in a common avian ancestor ~65 million years ago and then was lost multiple independent times (Jarvis et al., 2000).

In either case, the vocal learning brain pathways in birds share notable similarities with motor brain pathways in mammals (Perkel and Farries, 2000; Jarvis, 2004a; Jarvis, 2004b; Doupe et al., 2005). The AVP (**Fig. 1B, white arrows**) is similar in connectivity and function to mammalian pre-motor cortical-basal-ganglia-thalamic-cortical loops. The PVP (**Fig. 1B, black arrows**) is similar in connectivity and function to the mammalian primary motor pathway where the motor cortex sends projections to midbrain, hindbrain,

and spinal cord non-vocal motor neurons. The PVP sends a major projection from the arcopallium (RA in songbirds) to midbrain (DM) and hindbrain (nXIIts) vocal neurons, the latter controlling muscles of the avian vocal organ - the syrinx. For both pathways, neuron populations adjacent to vocal nuclei, as well as in comparable locations in vocal non-learning birds have been noted to form similar connections as the vocal nuclei (Margoliash et al., 1994; Iyengar et al., 1999; Farries, 2001; Diekamp and Gunturkun, 2003; Jarvis, 2004b; Doupe et al., 2005; Farries et al., 2005). However, the functions of most of these adjacent regions are not known. The above findings taken together have been used to suggest there may have been a pre-existing constraint on the evolution of vocal learning to develop out of a conserved network, as one possibility among several to explain evolution of brain pathways for vocal learning (Farries, 2001; Jarvis, 2004b). This study shows that the vocal learning brain nuclei of songbirds are embedded within or adjacent to brain areas that are specifically active in the generation of movement, and thus vocal learning pathways seem to have evolved out of a pre-existing motor system. This is a logical way to evolve vocal learning, because the production of new vocalizations requires precise control of syringial muscles.

Materials and Methods

Behavior apparatus. General behavior of birds includes a variety of motor activities that are difficult to quantify and not always consistently performed. Thus, in order to use behavioral molecular mapping to identify motor activated brain areas, as done for singing (Jarvis and Nottebohm, 1997), it is necessary to find a behavioral paradigm that allows consistent and repetitive motor behavior by the animal suitable for quantification. One such highly stereotyped behavior is migratory restlessness (MR), performed by migratory

songbirds at night. During their migratory season, caged birds perform wing whirring and other consistent movements in a preferred direction (Kramer, 1949; Emlen and Emlen, 1966) corresponding to the migratory orientation of their free-flying conspecifics (Mouritsen, 1998; Mouritsen and Larsen, 1998). To reliably record and quantify MR and non-migratory movement behavior in songbirds and relate this behavior to activity-dependent gene regulation, we designed a behavior apparatus consisting of a cylindrical, transparent Plexiglas-cage (height 40 cm, diameter 40 cm) with a circular perch (diameter 20 cm) placed 8.5 cm above the ground in the center of the cage (**Fig. 2**; Mouritsen et al., 2004a; Mouritsen et al., 2005). This apparatus allowed us to carefully observe the bird's behavior in real time. We found that migratory songbirds during dim light either sit in this orientation cage for extended periods of time or perform MR behavior more consistently and stereotypically than they do in the Emlen funnels (Emlen and Emlen, 1966) normally used for orientation experiments. We also found that the migratory birds use head scans apparently to detect the direction of the Earth's magnetic field (Mouritsen et al., 2004a; Mouritsen et al., 2004b). Zebra finches, a non-migratory songbird species, would mostly run around in the cage in day light conditions, or just sit in dim light conditions.

Animal groups. A total of 38 garden warblers (*Sylvia borin*; 29 males and 9 females; migratory songbirds) and 10 zebra finches (*Taeniopygia guttata*, 9 males and 1 female, non-migratory songbirds) were used for this study. The garden warblers were caught on Helgoland and around Oldenburg, Germany, in May-September, 2002/2003, and acclimated to captivity for a minimum of 5 days. Zebra finches were purchased from a local breeder. Behavioral tests were performed during the autumn migratory season for warblers, between August 22 and October 18, 2002 and 2003, and during the non-migratory season between July 17 and August 3, 2003. On the day of testing, a thin stripe

of IR-reflective tape (Retroreflective tape, 3M) was glued to the top of the bird's head. The IR tape was used to track and record the bird's movements with an infrared-sensitive video camera in dim light. Tracking movement behavior in dim light versus day light allowed us to separate out movement-induced from day light-induced gene expression. For the dim light conditions, the birds were placed into the cylindrical cage before 13:30h and food was removed 90 minutes before onset of darkness (simulated local photoperiod). Some birds were exposed to an artificially changed magnetic field, but these manipulations did not affect the IEG expression in motor activated areas, and the results of these conditions will therefore be described elsewhere.

To separate sensory (visual) from motor components of movement behavior we collected birds from four groups: 1) animals that sat relatively still in the cage during the day time, showing a minimum of movement behavior (e.g. less than 5 flights, 100 wing beats, and/or 50 head scans in 1h); 2) animals that remained awake and relatively still during the night in dim light conditions (0.04 lux) for an extended period of time; 3) animals that displayed general motor activity during the day (e.g. several hundred to several thousand defined movement events); and 4) animals that displayed MR motor behavior during the night. In addition, we analyzed two groups of non-migratory zebra finches to control for migration-specific activation: one group displaying regular motor activity during the day and one group sitting still but awake at night. All animal procedures were approved by the Bezirksregierung Weser-Ems and/or the Duke University Institutional Animal Care and Use Committee.

Behavior observation and recording. The behavior of all birds was recorded by the infrared video cameras to videotape (25 frames/sec). In parallel, we carefully observed the birds' behavior in real-time on a split-screen surveillance monitor from 1h before the start

of an experiment and until the experiment was finished. The 1h waiting period allowed the decay of any gene expression induced either by behaviors before the observation period or by the light-dark transition (Jarvis and Nottebohm, 1997). We only collected a bird after it produced repetitive movement behavior or was sitting still but awake while a minimum of other behaviors occurred for at least 45 min (mostly for 1h or more), the peak time of ZENK mRNA expression (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). To avoid hearing-driven gene expression (Mello et al., 1992; Jarvis and Nottebohm, 1997) due to noise, we made sure that no sounds from the experimenters or from the outside reached the birds during the critical hour. In case of accidental noise (from indoors or outdoors), we waited at least 1h before starting the experiment again. When at least 45 min of the required highly consistent behavior was observed, we sacrificed the bird, rapidly dissected its brain, separated the two hemispheres along the mid-sagittal plane, embedded them in TissueTek O.C.T. (Sakura Finetek, NL), and quick-froze them to -80°C in a dry ice/ethanol bath within 6-12 min after taking the bird out of the cage.

Gene expression analyses. 12μm frozen sections were cut almost throughout most of the left hemisphere, using sagittal sections to maximize the amount of brain tissue per section. Corresponding sections of all birds were fixed in 4% paraformaldehyde and processed by in-situ hybridization with antisense S^{35}-UTP labeled riboprobes of a zebra finch ZENK (acronym for zif268, Egr-1, NGF-1A, and Krox-24) or GluR1 (glutamate receptor 1) cDNA following previously described procedures (Wada et al., 2004). We used GluR1 as an anatomical marker in combination with Nissl staining to identify anatomical boundaries of brain structures. Expression of ZENK mRNA in the brain is driven by neuronal activity - except in primary thalamic recipient neurons of the cerebrum (L2, entopallium, and basorostralis), the globus pallidus, and parts of the thalamus (Mello and Clayton, 1995;

Jarvis, 2004a). ZENK can be detected in neurons about 10 min after onset of neuronal activity with peak expression after 30-45 min (Jarvis and Nottebohm, 1997), and therefore increased cumulative mRNA expression marks brain areas that were active during the last 45-60 min of the birds behavior. The hybridized sections were first exposed to X-ray film (Biomax MR, Kodak) for 1-4 days, then dipped into autoradiographic emulsion (NTB2, Kodak), incubated for 3 to 4 weeks at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl-violet acetate solution (Sigma). Sections of one in-situ hybridization experiment were treated identically (exposure time & staining) to allow for direct comparisons. X-ray film brain images were digitally scanned from a dissecting microscope connected to a SPOT III CCD camera with SPOT Advanced imaging software (Diagnostic Instruments, Inc.). For quantification, a person naïve to the experimental conditions used Adobe Photoshop 6.0 to measure the mean pixel intensities in the brain areas of interest on a 256 grey scale. To test for significant differences between individual groups, the SigmaStat software (SPSS, Inc.) package was used to perform ANOVAs followed by a Holm-Sidak multi-comparison, or regression tests. We excluded two garden warblers from the analysis, as the in-situ signal on those sections was very weak and not reliable.

Behavior analysis. We defined and quantified the most prominent types of movements. Some specific movement behaviors of the two species in the cylindrical cage were different:

Number of wing-beats (garden warbler and zebra finch): During the dim light conditions, when garden warblers perform constant MR in our apparatus, they whirred their wings rapidly while perched. During the day, they will flap their wings while perched at a much lower rate and often in preparation to fly off the perch. Zebra finches only

occasionally flapped their wings while perched. We consider wing whirring and flapping all as wing beats. The amount of wing beats during flapping was relatively simple to quantify manually, as the birds performed it at a slow rate. The amount during whirring was not. Thus, we first measured the mean wing beat frequency during constant MR for several birds by watching the video frame-by-frame. This mean frequency (11 wing-beats/sec) was then transferred to the keys of a PC keyboard so that when a key assigned to a Matlab program was pressed continuously, the output signal was identical to the wing-beat frequency. Thereafter, the observer watched all video tapes in real-time and either held the key down for the time the bird performed constant wing-whirring or flying around in the cage or made individual key-strokes for isolated wing flaps while perched. The time and number of each button-press event were analyzed using a custom-written Matlab program.

Number of flights (garden warbler): We reviewed the same videos again and instead of counting the number of wing beats, we counted each time the bird flew off the perch or off the bottom of the cage. Zebra finches were excluded from this quantification as they hardly ever performed flights in the cylindrical cage.

Number of head scans (garden warbler): We reviewed the same videos again and counted head scans. Garden warblers perform head scan behavior, particularly during MR in dim light, apparently to sense the magnetic field (Mouritsen et al., 2004a). The birds typically turn their head to the left or the right, sometimes alternating. We defined one head scan as a head turn of more than 60° to the left or right and approximately back to the longitudinal body axis (sometimes immediately followed by a head scan to the opposite side) within a few seconds while the bird remained at the same position in the cage. We did not observe such consistent head scan behavior in zebra finches.

Running (zebra finches): Zebra finches tended to run around along the perimeter of our cylindrical apparatus. Because this was a persistent behavior, we found it easier to measure by subtracting the time periods the bird was sitting still for 5 sec or more from the 1h total observation interval.

Number of perch jumps (zebra finches): Instead of making flights, zebra finches tended to hop from one spot of the round perch to another. We counted every time the bird hopped across the perch covering more than 45 ° or on/off the perch.

Results

Gene activation as a result of movement behavior

We found that relative to birds that were still, garden warblers that performed non-vocal movement behavior, during the day or night, had induced ZENK gene expression within 10 cerebral regions and in parts of the cerebellum (**Figs. 3 and 4A, anterior and posterior regions; statistics in Table 1A**). Five of the 10 cerebral regions spanned the *anterior* portions of the five brain subdivisions that make up the medial half of the cerebrum and surrounded the AVP vocal nuclei in males. The other five cerebral regions made up a *posterior* forebrain pattern of activation, all laterally adjacent to the four PVP vocal nuclei in males and associated auditory fields (**Figs. 3 and 4A**).

Anterior Regions:

1. The anterior striatum (ASt) surrounding the vocal nucleus Area X (**Fig. 3Ac,d**)
2. The anterior nidopallium (AN) surrounding the vocal nucleus MAN (**Fig. 3Ac,d**)
3. The anterior ventral mesopallium (AMV) surrounding the vocal nucleus MO (**Fig. 3Ac,d**)
4. The anterior dorsal mesopallium (AMD; **Fig. 3Ac,d**)
5. The anterior hyperpallium apicale (AHA; **Fig. 3Ac,d**)

Posterior Regions:

6. The posterior lateral striatum (PLSt) lateral to the auditory field of CSt (**Fig. 3Ag,h**)

7. The posterior lateral nidopallium (PLN) lateral to vocal nucleus NIf and adjacent auditory fields L1 and L3 (**Fig. 3Ag,h**)

8. The posterior lateral ventral mesopallium (PLMV) lateral to vocal nucleus Av and auditory field CMM (**Fig. 3Ag,h**)

9. The dorsal lateral nidopallium (DLN) lateral and posteriorly adjacent to the vocal nucleus HVC and auditory HVC shelf (**Fig. 3Ag,h; 5A-C**)

10. The intermediate arcopallium (AI) laterally adjacent to song nucleus RA and the auditory RA cup (**Fig. 3Ag,h; 5D-F**).

The anterior activated regions (ASt, AN, AMV, AMD, and AHA) were located medially in the frontal half of the brain (**Fig. 3Ac,d**); they began with the AVP vocal nuclei medially and ended with them further laterally. We note here that MO is situated in the MV part of the mesopallium, as revealed by comparisons of GluR1 and ZENK expression (**Fig. 3Ad vs 3Ba**; also described further below for the zebra finch). For the posterior regions, the extent of the activation began with the lateral part of the PVP nuclei HVC and RA, and ended further laterally. DLN abutted the lateral-posterior part of vocal nucleus HVC and became larger further laterally, where HVC was not present (**Fig. 5A-C**). The activated region within AI also abutted the lateral part of vocal nucleus RA (**Fig. 5D-F**). PLN surrounded a strip of cells of no ZENK expression lateral to auditory L2, which also has no ZENK expression (**Fig. 3g,h vs 3c,d;** Mello and Clayton, 1994)). None of the four major vocal nuclei, AreaX, MAN, HVC, or RA, showed ZENK activation relative to birds sitting still (**Figs. 3 and 5**). As for the smaller vocal nuclei MO, NIf, and Av, they were more

difficult to locate due to their small size, but in the animals where they could be clearly identified, we did not note appreciable ZENK expression (**Fig. 3Ad** for MO; Supplementary **Fig. S1A** for NIf and Av). This is in contrast to the ZENK levels seen in these nuclei after singing (see below and Supplementary **Fig. S1B**; Jarvis and Nottebohm, 1997).

The exact pattern of gene expression varied between individuals and groups, as did the pattern of motor behavior. During the day time, garden warblers made more flights (549.4±135.8 [std. error] for day; 113.1±32.2 for night; t-test, $p<0.001$, $t=-4.659$) and in these birds, the posterior areas had higher activation relative to animals that performed MR during the night (**Fig. 4A, Table 1A**), and the anterior pattern of activation was more uniform throughout the respective brain subdivisions (**Fig. 3Ac vs 3Ad**). In the birds that performed MR, the part of AN directly caudal to the vocal nucleus MAN had the highest expression of all activated areas (**Fig. 3Ad & 4A**). For the cerebellum, in birds that performed MR, high ZENK activation was specific to lobules I-VI (**Fig. 6B**). For those that performed more flights, activation was more evenly distributed across lobules I-X (**Fig. 6C**) albeit a persisting difference between lobules VIb and IXa ($p<0.012$, $t=3.216$, Mann-Whitney). In general, the activation patterns in the cerebrum had a semi-columnar organization and of two types with parallel rostral and caudal boundaries: columns defined by St, N, and MV, and columns defined by HA and MD.

Correlation with the amount of movement

In all vocal learning birds, the amount of IEG activation in vocal nuclei is linearly proportional to the amount of vocalizations produced per 30-60 minutes and this has been a critical test to determine if the gene expression is motor-driven (Jarvis and Nottebohm, 1997; Jarvis et al., 1998; Jarvis and Mello, 2000; Jarvis et al., 2000). To test for this

property in the defined regions of this study, we performed correlation analyses on the amount of gene expression with the amount of movement. We plotted the level of gene expression against the number of wing beats, as wing beats made up the main part of garden warbler motor activity. The wing beat count includes both those performed while perched and in flight. They ranged in different birds from 0 to 7409 in 60 minutes, including day time and night time groups. This analysis resulted in statistically significant polynomial 2nd order regression relationships between wing beats and gene expression for all 11 brain areas (**Fig. 4B, Table 1B**). ZENK expression levels reached a maximum at around 6000 wing beats and then saturated, and may have even started to decrease (or perhaps habituate; Mello et al., 1995) in some birds. The correlations were strongest for the regions of the anterior forebrain column ASt-AN-AMV and the cerebellum.

Although the expression relationships we measured were significantly correlated with wing beats, there was still obvious scatter of expression values. Thus, we asked whether the relationships were any tighter with the number of flights or with the number of head scans. Regressions with flights or head scans alone were significant but weaker than with wing beats. Of these two categories, the strongest relationship with flights was found in AHA ($r=0.700$; $p<0.001$) followed by AMD ($r=0.681$; $p<0.001$; **Table 1B**) – consistent with the more uniform pattern of expression in AHA of birds that flew a lot (**Fig. 3Ac**). The strongest relationship with head scans was found in AN directly caudal to MAN ($r=0.664$; $p<0.001$) followed by ASt caudal to AreaX ($r=0.602$; $p=0.002$) – consistent with AN being the area of highest activation in birds performing MR in the night in connection with head scan movements (other values for head scans and quantifications in other parts of ASt and AN not shown). Thus, the areas we identified may be preferentially activated during different movement types and/or movements of different body parts, and this could create the scatter of values.

Non-motor related sensory behaviors

In these 11 brain regions, there was no induction due to sensory stimulation by day- or dim-light (**Fig. 4A, compare sitting day still and night still birds, Table 1A**). To determine whether the movement related induction was specific to these brain regions or reflected a general increase in brain activation in moving birds, we analyzed ZENK expression in six regions known to show ZENK induction following various sensory tasks or stimuli: the hippocampus (Hp), which shows IEG activation induced by spatial tasks, e.g. food-hoarding (Smulders and DeVoogd, 2000); NCM, a higher order auditory processing region, activated upon hearing species-specific songs (Mello et al., 1992); L3, a lower order auditory region activated upon hearing (Theunissen et al., 2004); ALN and ALMV immediately anterior to the entopallium (E, a visual area), activated by day-light vision (Mouritsen et al., 2005); and cluster N (a HA-MD column), activated by dim-light, night-vision in migratory songbirds (Mouritsen et al., 2005).

None of these six brain regions showed the activation pattern seen in the 11 movement related brain regions (**Fig. 4A, other brain areas**). Instead, the Hp and NCM showed no differences among groups (**Figs. 3 and 4A, Table 1A**). L3 (as well as L1 examined qualitatively) showed high ZENK expression only during MR behavior (**Figs. 3Ad and 4A, night-time movement group**), and no induction during day time movement (**Figs 3Ac and 4A**). We surmise that L3 (and L1), which processes simple sounds, may be responding to the self-produced wing whirring sounds of MR behavior; in support of this idea, L3 expression had a significant correlation with the number of wing beats but not with the number of flights made (**Table 1B**). ALN and ALMV showed strong induced ZENK expression by day light (**Figs. 3Ae,g and 4A; Table 1A, sitting still birds**). There was a secondary weak relationship with movement (**Figs. 4A, Table 1A**). We surmise that

ALN and ALMV, which receives input from the entopallium that is involved in visual motion perception (Nguyen et al., 2004), may be responding to optic flow in both day and night moving birds; in support of this idea, ALN and ALMV had a significant correlation with the number of flights made but not with the number of wing beats (**Table 1B**); flights will cause optic flow, but wing whirring while sitting will not. Cluster N showed strong induced expression by dim light and had no noticeable relationship with movement (**Figs. 3Af,h and 4A**). We also examined the entopallium (E) as well as the globus pallidus (GP), two areas known not to express ZENK (Mello and Clayton, 1995); but GP is involved in motor behavior (Reiner et al., 2004a). We found a *very small* quantitative increase in the day time animals (**Fig. 4A, Table 1A**) suggesting that this region may in fact express some ZENK at a barely detectable level, but there was no overall difference in moving animals. We conclude that high ZENK activation in the 11 brain regions in moving animals is specific to these areas and not the result of a general increase in brain ZENK expression.

To verify the functional and anatomical relationships with an independent statistical measure, we performed principal component analysis (PCA) on all quantified brain regions together. The PCA nicely separated the brain regions and revealed additional information. Component 1 separated the movement-induced regions from all other regions (**Fig. 7**). Component 2 further split the movement activated regions into distinct clusters: an anterior cluster, tightest for ASt, AN, AMV (adjacent to AVP vocal nuclei) along with the Cb; a posterior cluster consisting of PLSt, PLN, and PLMV; and a posterior cluster consisting of DLN and AI (adjacent to HVC and RA, respectively). Component 3 separated the non-movement related areas among each other, but keeping ALN and ALMV, day activated regions, together as a cluster. This clustering of different regions together across adjacent brain subdivisions, as opposed to different regions from the same brain subdivision, supports the columnar organization noted earlier.

We noticed that some areas in the brains of our animals showed high ZENK expression not related to any specific group. These included, but were not limited to the lateral striatum, which had high expression in all groups, and the anterior and posterior parts of the arcopallium, which had high expression in some birds of all groups (**Fig 3Ae-h;** quantifications not shown). Perhaps activation in these areas is associated with being awake, or simply standing. Nevertheless, activation was not specific to the movement groups, lending further support that the activation pattern of the 11 brain regions is specific to movement.

A non-migratory songbird

We analyzed brains of zebra finches, a non-migratory songbird species, that were placed in our behavior apparatus. Zebra finches were highly mobile during the day where they ran and jumped around in the cage. The ZENK expression patterns in moving day time finches were similar to the ones found in the moving day time group of warblers, except that in zebra finches that mainly ran around in the cage, the activation of the anterior ASt-AN-AMV column was more narrowly focused around the AVP nuclei (**Fig. 6B,E**). Activation in the AMD-AHA column still spanned the caudal to rostral extent of these brain regions. There were scattered cells with high expression in some vocal nuclei (AreaX and MO; **Fig. 6B**), but this paled in comparison to the high activation induced in the vocal nuclei by singing (**Fig. 6C**). For the cerebellum, a gradient of higher expression was concentrated towards lobule VI as in garden warblers, but there was one interesting deviation: a zebra finch that constantly flipped its head side-to-side and up-and-down showed higher activation across lobules VII-VIII (**Fig. 6F**). There was not enough variation in running and hopping behavior of the zebra finches to test for correlations. Taken together, these

results show that movement associated ZENK activation within the described 11 brain regions is not specific to garden warblers or MR behavior, but is more general.

Females: vocal non-learners

Females of many songbird species do not have vocal learning behavior, i.e. song, and have atrophied vocal nuclei (except for LMAN) in their forebrain. This is the case for zebra finches (Nixdorf-Bergweiler, 1996) and for garden warblers (noted here for atrophied vocal nuclei). We separated out the females in the groups described above, and found that in the female zebra finch (n=1) and garden warblers (n=6) that performed movement behavior (running, wing beats, flights, and/or head scans) ZENK gene activation was present within the same 10 cerebral regions and in the cerebellum as seen in males (n=4 finches and 14 warblers), but without the presence of negative expression regions of vocal nuclei (**Fig. 9,** except for LMAN). Thus, these movement-activated brain regions appear to be present independent of the vocal nuclei.

Discussion

This is the first study that we are aware of to functionally identify non-vocal motor brain areas in birds. We found that cerebral areas that show motor-driven gene expression during movement behavior surround or are adjacent to the vocal nuclei that show motor-driven gene expression by singing. The anatomical extent of the non-vocal motor areas is much larger than that of the vocal nuclei, which is consistent with a much greater amount of musculature involved in controlling head, wing, and body movements compared to that necessary for controlling the syrinx. Our behavioral approach, however, was not specific enough to map a possible homunculus organization as seen in the mammalian motor cortex

(Penfield and Rasmussen, 1957), or motor versus premotor distinctions. Below we discuss the implications of our results for understanding motor pathways in birds and the evolution of brain pathways for vocal learning.

General implications for future studies using IEG expression

Since it is difficult to make birds sit absolutely still during a sensory task and obviously impossible during behavioral tasks, general motor activation has the potential to distract experimenters' attention from differences in more relevant brain areas. Thus, knowing the brain regions controlling motor behavior will be important for future studies using behavioral molecular mapping to identify brain areas involved in specific behaviors and/or sensory learning tasks. Future experimenters can now exclude the motor activated areas from their analyses or perform correlations of the gene expression with the amount of movement of their birds. Conversely, the discovery of the functional gene expression activation of these brain areas will now allow investigators to probe specific sites in the avian central nervous system for control of movement.

Motor Pathways in Birds

The activation patterns in the cerebrum were organized in a semi-columnar fashion, with parallel rostral and caudal boundaries. The anterior-medial St-N-MV column (ASt-AN-AM) mirrored a singing-driven gene expression column consisting of AreaX, MAN, MO, i.e. the AVP vocal nuclei. A posterior-lateral St-N-MV column consisted of PLSt-PLN-PLMV, situated lateral to the PVP vocal nuclei. The only non-columnar activation was found in DLN and AI, lateral to song nuclei HVC and RA that are also not organized as a column. Both the vocal and non-vocal motor patterns suggest that much like the functional columnar organization of mammalian cortical layers and associated striatum,

brain systems in the avian cerebrum may have a functional columnar organization sculpting out portions of each brain subdivision: striatum, nidopallium, mesopallium, hyperpallium, and arcopallium.

Interestingly, the connectivity of the AVP vocal nuclei in songbirds (**Fig. 10A**; and in parrots) is comparable to the corresponding regions around them (Durand et al., 1997; Iyengar et al., 1999; Farries, 2001; Diekamp and Gunturkun, 2003). These corresponding regions are connected in a pallial-basal-ganglia-thalamic-pallial loop (**Fig. 10;** white arrows): anterior mesopallium (formerly hyperpallium ventrale) to anterior nidopallium (formerly neostriatum), these two regions to the striatum (formerly LPO), the striatum via pallidal-like neurons to the dorsal thalamus and the dorsal thalamus back to the anterior nidopallium (connectivity of MO or the surrounding MV is not known in songbirds, but in parrots projects to the striatum (Durand et al., 1997)). Thus, we suggest that the anterior-medial portion of the avian forebrain forms a general anterior motor pathway (AMP; or more literally a premotor pathway) for motor behavior in birds. Our results do not indicate whether this AMP is involved in motor learning, as is the AVP, but like the AVP it is active in production of motor behavior.

The connectivity of the PVP vocal nuclei may also be similar to the connectivity among several of the posterior movement activated brain areas (**Fig. 10; black arrows**). DLN is situated in the dorsal lateral nidopallium (NCL), which has connections similar to songbird HVC (Margoliash et al., 1994; Iyengar et al., 1999; Farries, 2001). The NCL receives input from the areas around and between LMAN and MMAN in songbirds, and from the comparable region in non-songbirds; it projects to the AI, which in turn projects to brainstem premotor and motor neurons. The shell around LMAN also projects to AI, in a similar manner as LMAN projects to RA (Iyengar et al., 1999). It is not possible to determine definitively whether the DLN and the part of AI we identified in this study are

exactly the regions that receive input from around MAN and connect with each other. However, since the general NCL region receives input from the anterior nidopallium and projects to the arcopallium, it is likely that the motor-activated DLN has the same connectivity. Taken together, the expression patterns found here and the connectivity known from other studies suggest a non-vocal posterior motor pathway (PMP) working in parallel to the PVP.

We also found activation within three brain subdivisions that do not have vocal nuclei – the AHA, the AMD adjacent to it, and the cerebellum. Activation in these brain regions during movement may be somatosensory related. The AHA has been proposed to be somatosensory pallium, as it receives substantial somatosensory input and shows neural firing to somatosensory stimulation along the body and limbs (Wild, 1997b; Wild and Williams, 2000a). Some of the heaviest input into AHA comes from wing somatosensory pathways (Wild and Williams, 2000a), consistent with our finding of higher correlation of gene activation with flights. AHA also receives projections from AMD, but the source of inputs to AMD is not well known (Wild and Williams, 2000a). Interestingly, in songbirds, AHA sends some of its heaviest cerebral projections to the areas around the AVP vocal nuclei, to the NCL lateral to HVC, and AI (Wild and Williams, 1999) – all in a pattern strikingly similar to the movement related gene expression we found here in zebra finches (**Fig. 10**). In female songbirds, the AHA cerebral projection pattern is similar to the movement related gene expression pattern we found without vocal nuclei (Wild and Williams, 1999). This overlap of connectivity and gene expression patterns suggests that AHA may transmit somatosensory input into anterior and posterior motor pathways adjacent to vocal nuclei, and perhaps modulate their activity.

Like AHA, the cerebellum also receives somatosensory input to modulate motor neurons for fine coordination of movements (Wild and Williams, 2000b; Necker, 2001). It

was in the cerebellar granule layer where we found the high levels of movement-related ZENK expression. This granule layer projects to the molecular layer and from there onto the purkinje neurons, which then send out motor commands. We did not see activation in these other neuron populations. Presumably they do not have the necessary receptors for activity-induced ZENK expression (Jarvis, 2004a; Wada et al., 2004), and/or may be silenced by DNA methylation at cis-regulatory promoter elements. The granule layer of the cerebellum lobules in birds have two somatotopic body representations: one located in the anterior half of the cerebellum from lobules I-VI and the other located in the posterior half from lobules IX-X (Necker, 2001). Lobules VII and VIII receive input from the pallium, including AHA (Wild and Williams, 2000b). In the anterior half, lobules I-III receive input from the neck, III-V from the wing, and VI from the legs (with some input from the legs also to III-V) (Necker, 2001). This anterior half showed preferentially higher activation during MR movement, consistent with lobules III-VI responsible for wings and legs that are intensely used during MR. Activation was more uniform throughout the lobules when flights were preferentially made, consistent with the idea that flights involve the utilization of more body musculature than wing whirring while perched.

Given these findings, we hypothesize that during movement, a posterior and anterior motor system consisting of columns and components of St-N-MV and A control the production and sequencing of movements, and that a cerebral somatosensory system consisting of a medial HA-MD column provides somatosensory feedback to the anterior and posterior motor systems and the cerebellum. The more a movement is performed, the more the motor and also somatosensory activity causes increased neural activity-dependent gene expression in the controlling brain regions. The patchiness of the cerebral activation patterns in different animals performing predominantly different movements suggests that there are parallel motor pathways for either different movements or muscles groups. This

hypothesis and our related findings have implications for understanding the evolution of vocal learning.

Evolution of vocal learning

Seven of the 10 cerebral brain regions active in movement behavior are adjacent to or surround the well-known songbird PVP and AVP vocal nuclei. Thus, our functional results combined with connectivity findings (**Fig. 10**) suggest that the vocal learning system of songbirds (and perhaps of distantly related vocal learning birds) evolved out of a pre-existing motor system that controls learning and producing non-vocal motor behavior (Farries, 2001; Jarvis, 2004b). If true, then only one type of major connection is missing in vocal non-learners – from the arcopallium to the DM premotor and nXIIts vocal motor neurons of the brainstem (**Fig. 10A, red arrows**; Wild, 1997a; Jarvis, 2004b). If during evolution, such connections are formed through mutation then it may be possible to usurp a pre-existing motor cerebral pathway for the cerebral control of vocal motor behavior. The part of the pathway that could be exploited may determine the location of the AVP nuclei, which is quite similar among the different vocal learners (**Fig. 1**). This suggests an important anatomical constraint. This constraint may be the location of neurons that control head movements, as head scanning showed the highest activation relationship with the brain regions posteriorly adjacent to the AVP nuclei. Perhaps in order to evolve vocal learning, it is necessary to usurp the circuit synapsing onto muscles close to the syrinx – i.e. head and neck muscles. The location of the posterior motor activated areas lateral to auditory regions may also place them in an opportunistic position to take advantage of auditory input for evolving a vocal motor learning system, which requires auditory-related sensorimotor feedback.

Vocal learning also exists in some distantly related mammals (humans, bats, cetaceans, and elephants (Reiss and McCowan, 1993; Esser, 1994; Poole et al., 2005). For humans, recent analyses indicate that cerebral vocal regions are adjacent to or embedded in the motor and premotor brain areas (Lieberman, 2002; Jarvis, 2004b). This has been supported by recent findings showing that the premotor part of that system, Broca's area, controls oral-facial (but not laryngeal) movements in a non-human primate, macaques, a vocal non-learner (Petrides et al., 2005). Thus, the evolution of vocal learning brain systems out of a pre-existing motor system could well be a general feature of the vertebrate brain.

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Figure Legends

Figure 1. Phylogenetic relationships and brains of avian vocal learners. *(A)* One view of phylogenetic relationship of living birds (Sibley and Ahlquist, 1990), with vocal learners highlighted in red, possible independent gains of vocal learning highlighted with red dots, or possible independent losses highlighted with green dots. *(B)* Semi-3D view of seven cerebral vocal regions (yellow and red in cerebrum) found in each vocal learner, and of auditory regions (blue) found in all birds. Red-labeled vocal nuclei and white arrows: *anterior vocal pathway (AVP)*. Yellow-labeled vocal nuclei and black arrows: *posterior vocal pathway (PVP)*. Only a few connections in hummingbirds are known and that of songbird MO is not known. Scale bars: 1 mm. Figure modified from Jarvis et al. (2000) and Jarvis, (2004b). Brain area abbreviations are found in the legend of figure 3.

Figure 2. Experimental behavior apparatus. The apparatus is cylindrical, made of transparent Plexiglas on the sides and bottom, a removable net at the top, and a round black perch at the center of the floor. Infrared-emitting video-cameras (*IR*) attached to video monitors and recording equipment are used to observe and record the bird's behavior. Reproduced with permission from Mouritsen et al. (2004a).

Figure 3. Movement activated brain areas. (*Part A and B of the figure are to on separate adjacent pages) (A)* Representative darkfield images of ZENK expression in medial *(a-d)* and lateral *(e-h)* sagittal sections of the four main garden warbler groups studied. Dorsal is up, anterior is right. White silver grains: ZENK expression. Red: nissl stain. *(B)* GluR1 expression pattern used along with Nissl staining to localize brain subdivision boundaries. The GluR1 expression pattern shows that MD and MV are part of a single structure, the

mesopallium (Reiner et al., 2004b). Camera lucida drawings are to the right. Red outlines: extent of movement activated regions. Green outlines: day vision activated regions. Blue outlines: night vision activated regions. The abbreviations are placed in the center of quantified regions shown in figure 4. Scale bar, 2 mm.

Abbreviations:

A, arcopallium; AAc, central nucleus of the anterior arcopallium; ACM, caudal medial arcopallium; AHA, anterior hyperpallium apicale; AI, intermediate arcopallium; AMD, anterior dorsal mesopallium; AMV, anterior ventral mesopallium; AN, anterior nidopallium; ASt, anterior striatum; Av, avalanche; Cb, cerebellum with granular (*gr*), purkinje (*p*) and molecular (*mol*) cell layers; CMM, caudal medial mesopallium; CSt, caudal striatum; DLN, dorsal lateral nidopallium; DM, dorsal medial nucleus of the midbrain; DMm, magnocellular nucleus of the dorsomedial thalamus; E, entopallium; GP, globus pallidus; HA, hyperpallium apicale; Hp, hippocampus; ICo, inferior colliculus; IHA, intercalated hyperpallium apicale; LAN, lateral nucleus of the anterior nidopallium; LAM; lateral nucleus of the anterior mesopallium; M, mesopallium; MAN, magnocellular nucleus of the anterior nidopallium; MLd, dorsal part of the lateral mesencephalic nucleus; MMSt, magnocellular nucleus of the medial striatum; MO, oval nucleus of the mesopallium; MD; dorsal mesopallium; MV, ventral mesopallium; N, nidopallium; NAOc, oval nucleus complex of the anterior nidopallium; NIDL, dorsal lateral intermediate nidopallium, NIf, interfacial nucleus of the nidopallium; NLc, central nucleus of the lateral nidopallium; nXIIts, 12^{th} nucleus tracheosyringeal part; PLMV, posterior lateral ventral mesopallium; PLN, posterior lateral nidopallium; PLSt, posterior lateral striatum; RA, robust nucleus of A; S, septum; St, striatum; v, ventricle; VA, vocal nucleus of the arcopallium; VAM, vocal nucleus of the anterior mesopallium; VAN, vocal nucleus of the

anterior nidopallium; VASt, vocal nucleus of the anterior striatum; VLN, vocal nucleus of the lateral nidopallium.

Figure 4. Quantification of ZENK expression in garden warbler brains. *(A)* ZENK expression levels (image pixel density) in the four groups of birds, in 11 brain regions with movement-induced gene expression (*anterior, posterior*, and *Cb [lobule VI] regions*) and in other brain regions that do not show expression specifically related to movement (*other*). The absolute cluster N (ClN) values are different from our previous report (Mouritsen et al., 2005), because we did not use other brain regions as a normalization factor in the present study. Error bars: SE. *(B)* Example regression graphs for brain regions that show ZENK expression levels correlated (ASt, AN, AMV) and not correlated (Hp) with movement performed within the last 60 min. Statistics for *(A)* and *(B)* are in Table 1A and 1B, respectively. Brain area abbreviations are in the legend of figure 3.

Figure 5. Movement-driven induced gene expression adjacent to vocal nuclei HVC and RA. *(A-C)* Medial-to-lateral series, beginning lateral to HVC of Figure 3Ad, showing that region of DLN expression (white arrows) becomes larger the further lateral from HVC (black arrows); brain sections are from a garden warbler that performed MR behavior in the night. *(D-F)* Medial-to-lateral series showing AI expression (white arrows) lateral to song nucleus RA (black arrows); sections are from a garden warbler that performed flights during the day. The HVC shelf expression in *(A)* was not always seen further medially, and we do not know how much of this is movement (this study) or hearing related (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). The dark spot in DLN of panel *(C)* is torn tissue. The arcopallium (including RA; Jarvis and Nottebohm, 1997) generally has less

induced ZENK expression in its core region than do other brain subdivisions. Dashed line in E shows the boundary of the arcopallium with the nidopallium dorsal to it and striatum anterior to it. Sections are sagittal; dorsal is up, anterior is right. Scale bar, 0.5 mm.

Figure 6. Higher power view of ZENK expression in the cerebellum of garden warblers that *(A)* sat relatively still for 60 min during the night, making only one flight, *(B)* that performed MR behavior for 60 min during the night, and *(C)* that made flights and moved around in the cage during the day. Lobule numbering follows Necker (2001). Scale bar, 1 mm.

Figure 7. ZENK expression in zebra finch brains. Shown are brain sections of anterior regions of *(A)* a bird sitting still, *(B)* running around the cylindrical cage, and *(C)* singing; there is some self-hearing induced expression in L3 and L1 around L2 and in CMM posterior to AMV. *(D)* Quantification of ZENK expression levels in anterior regions (ASt, AN, AMV) around AVP vocal nuclei that shows movement induced expression and a control region (Hp). Error bars: SE. *(E)* Anatomical profile of the brain section shown in *(B)*. *(F)* High power image of the cerebellum of a zebra finch that repetitively flipped its head, resulting in exceptionally high ZENK expression in the transition zone of lobules VII to VIII. All in-situ images are sagittal sections; rostral right, dorsal up. Scale bar in *(A-C)* 2 mm; in *(F)* 0.5 mm. Brain area abbreviations are in the legend of figure 3.

Figure 8. Principal component analysis. Two views are shown: the left shows component 1 more clearly; the right shows component 2 more clearly. Values from each brain region are plotted according to the first three principal components. They are color-coded

according to clustering with nearest neighbors in the graph. Brain area abbreviations are in the legend of figure 3.

Figure 9. Movement-driven ZENK expression in males versus females. (*A)* Male and (*B)* female garden warbler sections, at a high power view of the anterior region of activation. The male AreaX and MAN have very low expression, and male MO and female MAN is slightly higher. A central column in this female's striatum has a gradient of lower expression, and the dorsal part of it we speculate could be remnants of AreaX or simply part of the pattern of movement-driven gene expression dependent upon the type of movements performed. Dorsal is up, anterior is right. Scale bar: 0.5mm. Brain area abbreviations are in the legend of figure 3.

Figure 10. Schematic drawing of vocal motor pathway *(A)* and the hypothesized non-vocal motor pathway *(B)*. Motor activated brain areas of the anterior forebrain surrounding the AVP vocal nuclei (AreaX, MAN, and MO) in *(B)* are defined at the minimum size seen in this study. Motor activated areas adjacent to PVP vocal nuclei (HVC, RA, NIf, and Av) in *(B)* are defined at their average size, and with dashed lines, as they are situated lateral to the plane of section shown. White arrows: connectivity of AVP pathway *(A)* and of brain areas immediately outside of the AVP vocal nuclei in songbirds *(B)*. Black arrows: connectivity of PVP pathway *(A)* and of brain areas outside of the PVP vocal nuclei of songbirds *(B)*. The compiled connectivity findings for the non-vocal areas are derived form the studies mostly in (Margoliash et al., 1994; Wild et al., 1997; Iyengar et al., 1999; Bottjer et al., 2000) and also compiled in (Farries, 2001; Jarvis, 2004b). The projection of AMV to the striatum is proposed based upon findings in parrot (Durand et al., 1997) and pigeon (Veenman et al., 1995). The connection from MAN to HVC and RA is generally

shown; specifically medial MAN projects to HVC and lateral MAN to RA. Abbreviations. DMP, dorsal medial nucleus of the posterior thalamus; MN, motor neurons; NCL, caudal lateral nidopallium; PMN; premotor neurons; UVa, nucleus uvaformis. Remaining abbreviations are in the legend of figure 3.

Supplementary Figure S1. Further assessment of motor-driven ZENK expression in and outside of vocal nuclei. *(A)* High power views of NIf and Av from a garden warbler that performed MR movement behavior (same animal as in Figure 3Ac). Note sharp columnar boundary of expression anterior to NIf that crosses brain subdivision boundaries of the striatum, nidopallium, and mesopallium. Scale bar: 0.5 mm. *(B)* Brain section of zebra finch that sang showing higher activation in HVC, NIf, Av, AreaX, MAN, and MO. Scale bar: 2 mm. Brain area abbreviations are in the legend of figure 3.

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Table 1: Statistical analysis. *(A)* Results of one-way ANOVA followed by Holm-Sidak all-pairwise multicomparison test for all brain regions quantified in Figure 4A. *(B)* Results of 2nd order polynomial regression, for all brains regions quantified, including for the graphs in Figure 4B. Significant differences are highlighted with red text.

brain area			ASt	AN	AMV	AMD	AHA	PLSt	PLN	PLMV	DLN	AI	Cb	Hp	NCM	L3	ALN	ALMV	Cluster N	E	GP
A. ANOVA		p-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.243	0.064	0.001	0.001	0.001	0.001	0.208	0.053
post-hoc test		day_{still} $night_{still}$	0.853	0.269	0.823	0.593	0.476	0.760	0.516	0.536	0.567	0.075	0.744			0.841	0.001	0.001	0.001		
		day_{still} day_{act}	0.001	0.001	0.007	0.019	0.007	0.003	0.002	0.002	0.001	0.029	0.029			0.852	0.022	0.035	0.314		
		$night_{still}$ $night_{act}$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.018	0.004	0.001	0.001			0.001	0.019	0.043	0.111		
		day_{act} $night_{act}$	0.995	0.347	0.990	0.790	0.579	0.152	0.052	0.006	0.001	0.065	0.763			0.006	0.001	0.001	0.001		
B. polynomial	wing beats	R=	0.821	0.834	0.712	0.738	0.723	0.641	0.654	0.560	0.638	0.618	0.777	0.076	0.111	0.498	0.337	0.305	0.244	0.049	0.344
		p<	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.911	0.826	0.012	0.136	0.210	0.363	0.961	0.125
	flights	R=	0.575	0.612	0.653	0.681	0.700	0.678	0.655	0.632	0.631	0.624	0.624	0.228	0.235	0.297	0.516	0.467	0.399	0.159	0.366
		p<	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.426	0.415	0.238	0.006	0.020	0.057	0.654	0.093

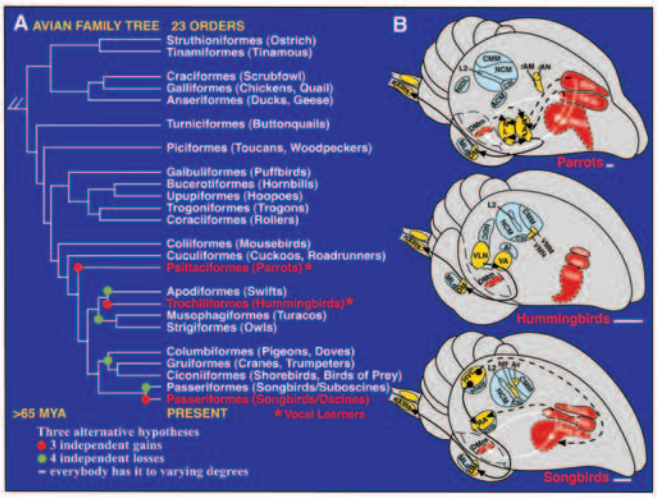

A AVIAN FAMILY TREE 23 ORDERS
Struthioniformes (Ostrich)
Tinamiformes (Tinamous)
Craciformes (Scrubfowl)
Galliformes (Chickens, Quail)
Anseriformes (Ducks, Geese)
Turniciformes (Buttonquails)
Piciformes (Toucans, Woodpeckers)
Galbuliformes (Puffbirds)
Bucerotiformes (Hornbills)
Upupiformes (Hoopoes)
Trogoniformes (Trogons)
Coraciformes (Rollers)
Coliiformes (Mousebirds)
Cuculiformes (Cuckoos, Roadrunners)
Psittaciformes (Parrots)*
Apodiformes (Swifts)
Trochiliformes (Hummingbirds)*
Musophagiformes (Turacos)
Strigiformes (Owls)
Columbiformes (Pigeons, Doves)
Gruiformes (Cranes, Trumpeters)
Ciconiiformes (Shorebirds, Birds of Prey)
Passeriformes (Songbirds/Suboscines)
Passeriformes (Songbirds/Oscines)
>65 MYA
PRESENT
* Vocal Learners
Three alternative hypotheses
3 independent gains
4 independent losses
= everybody has it to varying degrees
B
Parrots
Hummingbirds
Songbirds

Figure 1

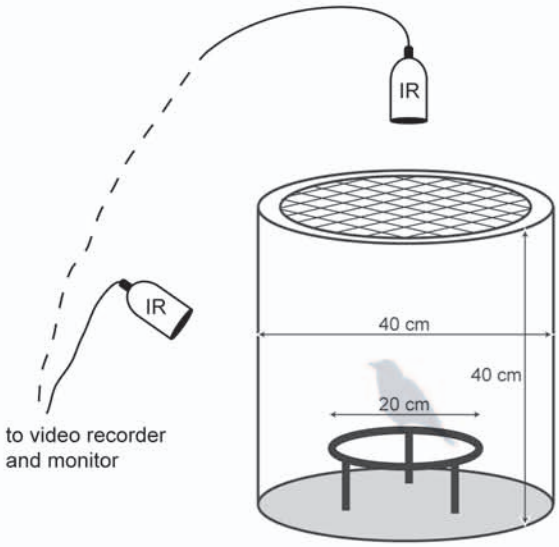


Figure 2

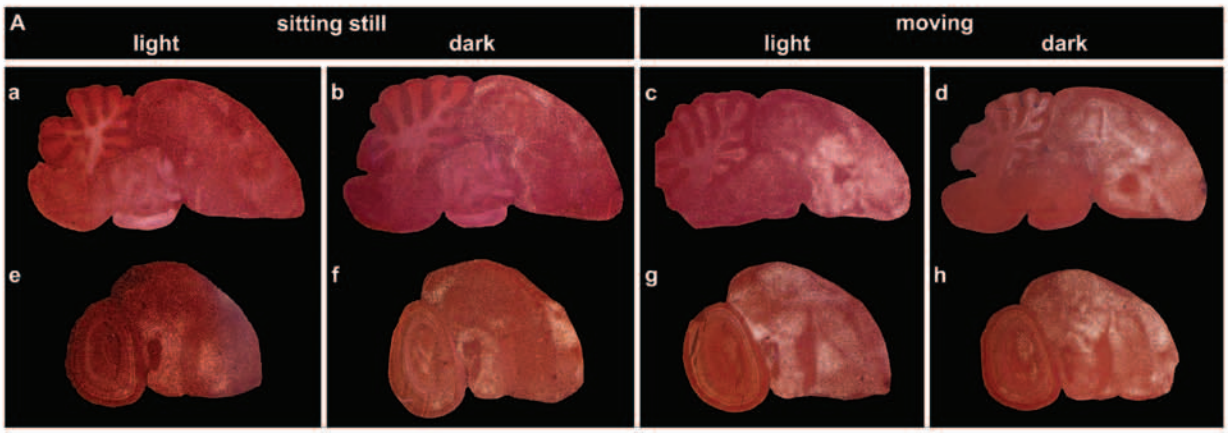
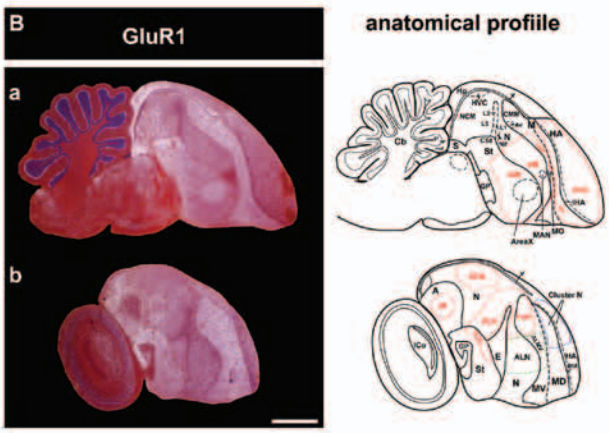


Figure 3A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GluR1
a
b
anatomical profiile
Cb
St
HA
MAN
MO
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E
ALN
MD
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N

Figure 3B

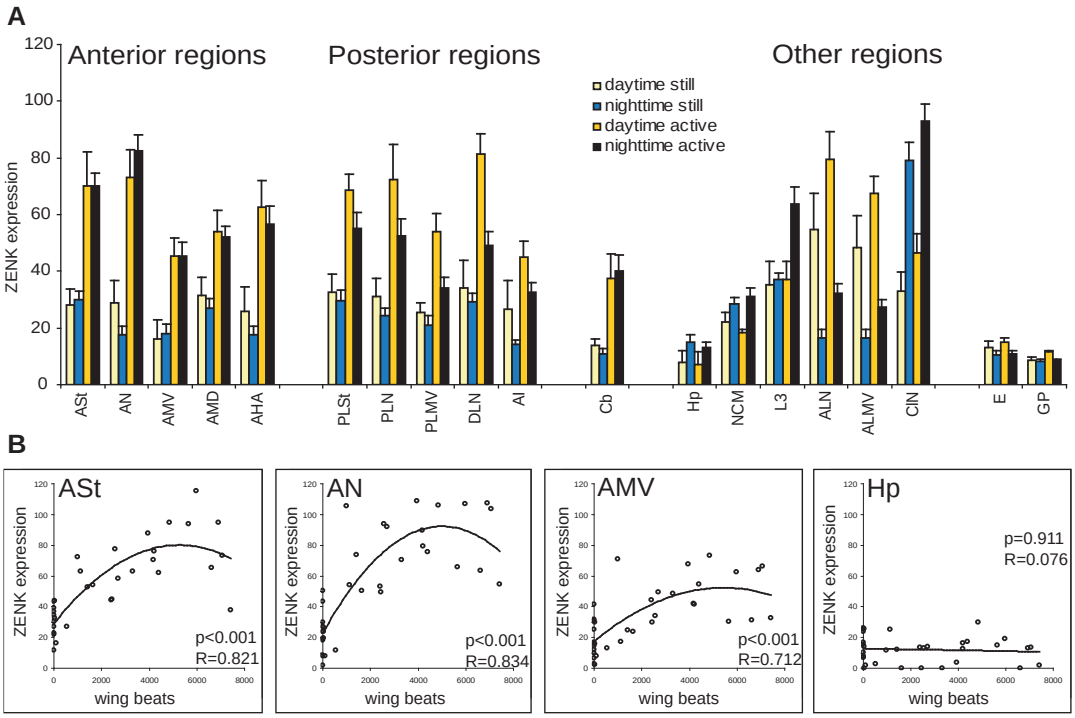

A
Anterior regions
Posterior regions
Other regions
daytime still
nighttime still
daytime active
nighttime active
ZENK expression
ASt
AN
AMV
AMD
AHA
PLSt
PLN
PLMV
DLN
AI
Cb
Hp
NCM
L3
ALN
ALMV
CIN
E
GP
B
ASt
p<0.001
R=0.821
AN
p<0.001
R=0.834
AMV
p<0.001
R=0.712
Hp
p=0.911
R=0.076
wing beats

Figure 4

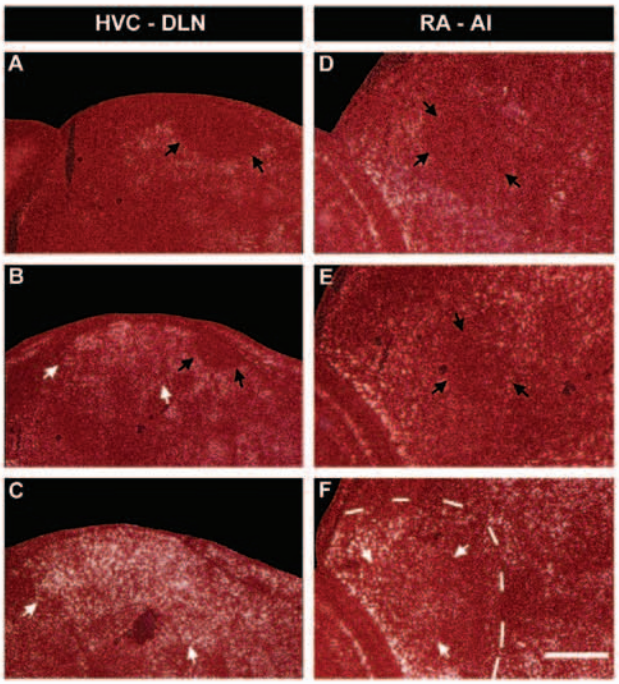


Figure 5

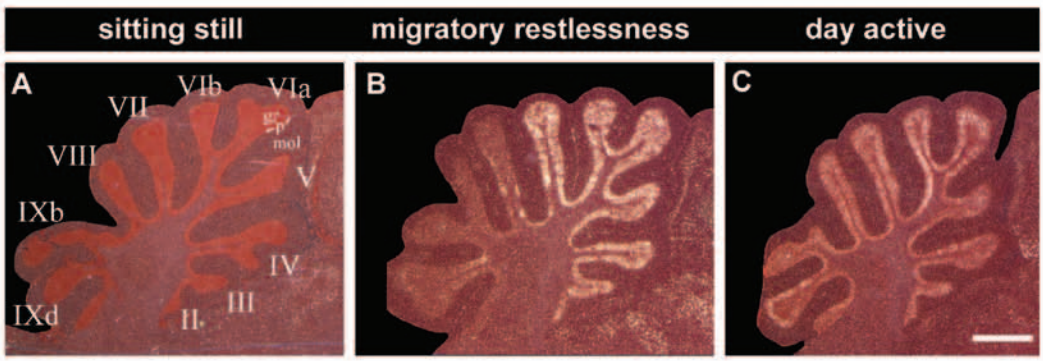

sitting still
migratory restlessness
day active
A
VII
VIb
VIa
gr
p
mol
VIII
V
IXb
IV
III
IXd
II
B
C

Figure 6

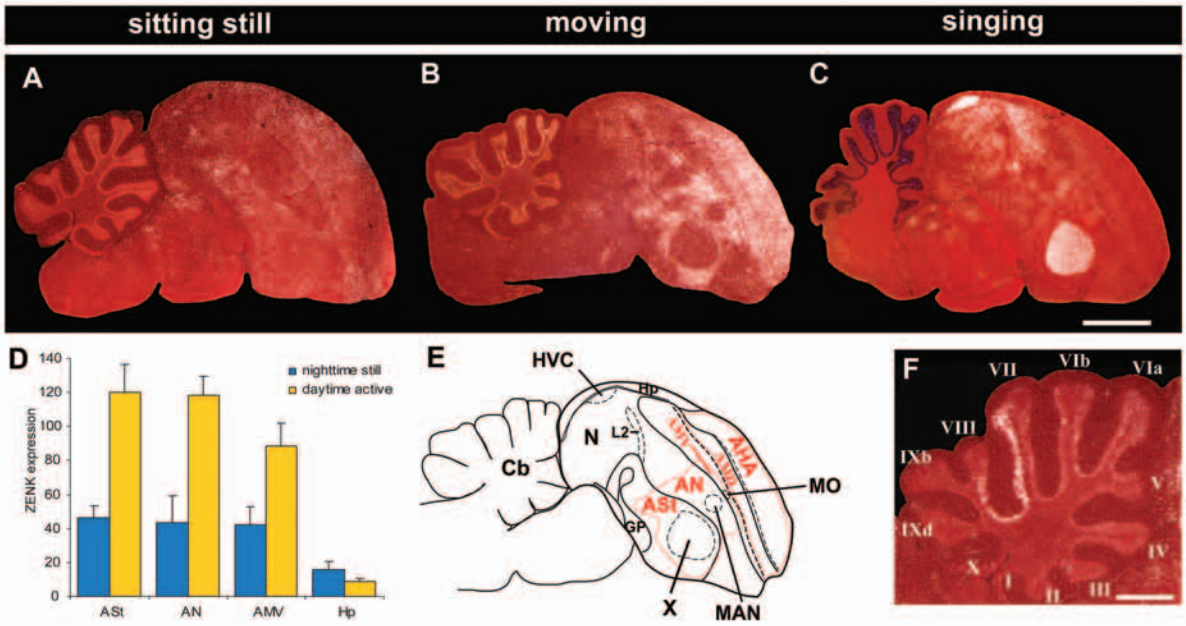
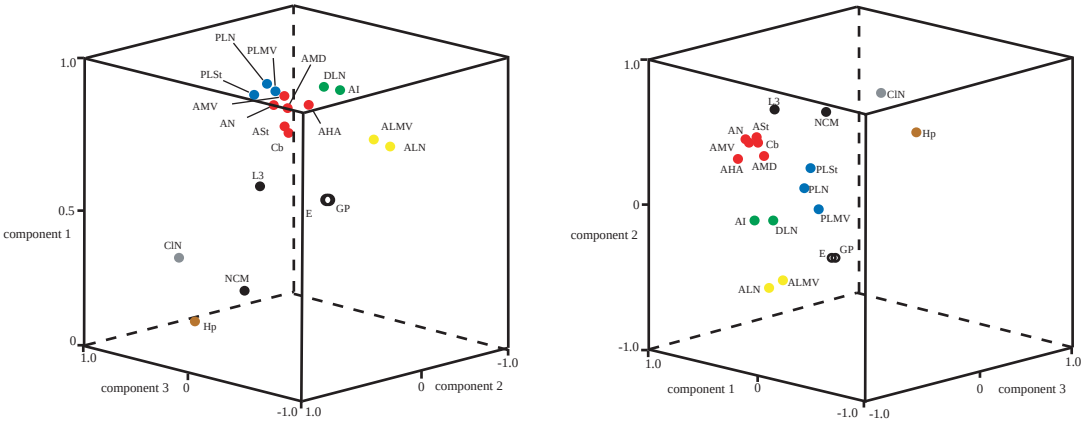

sitting still
moving
singing
A
B
C
D
ZENK expression
nighttime still
daytime active
ASt
AN
AMV
Hp
E
HVC
Hp
N
L2
Cb
AHA
AN
ASt
GP
MO
X
MAN
F
VII
VIb
VIa
VIII
IXb
V
IXd
IV
X
I
II
III

Figure 7


PLN
PLMV
AMD
PLSt
DLN
AI
AMV
AN
ASt
AHA
ALMV
Cb
ALN
L3
E
GP
ClN
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Hp
1.0
0.5
0
component 1
1.0
component 3
0
-1.0
1.0
0
component 2
-1.0
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Figure 8

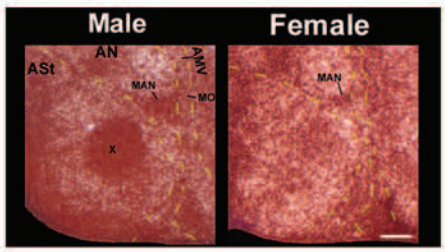

Figure 9

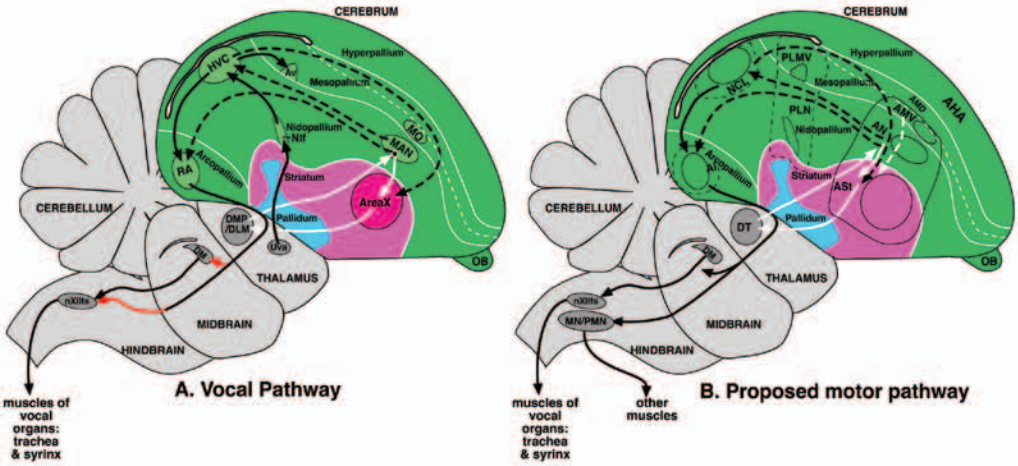
CEREBRUM
Hyperpallium
HVC
Mesopallium
Nidopallium
nNIf
MAN
MO
RA
Arcopallium
Striatum
AreaX
CEREBELLUM
DMP
/DLM
Pallidum
Uva
OB
THALAMUS
nXIIts
MIDBRAIN
HINDBRAIN
A. Vocal Pathway
muscles of
vocal
organs:
trachea
& syrinx
CEREBRUM
Hyperpallium
PLMV
NCL
Mesopallium
AMV
AHA
PLN
Nidopallium
AMV
AN
Arcopallium
Striatum
AS!
CEREBELLUM
DT
Pallidum
OB
THALAMUS
nXIIts
MN/PMN
MIDBRAIN
HINDBRAIN
muscles of
vocal
organs:
trachea
& syrinx
other
muscles
B. Proposed motor pathway

Figure 10

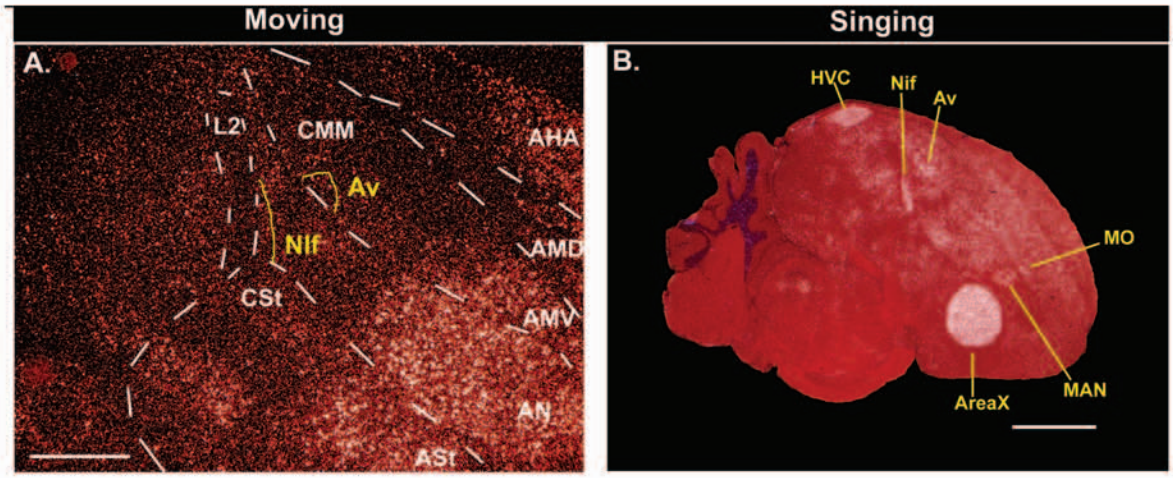
Moving
Singing
A.
L2
CMM
AHA
Av
Nif
AMD
CSt
AMV
AN
ASt
B.
HVC
Nif
Av
MO
AreaX
MAN

Figure S1

Paper V

Gesa Feenders, Miriam Liedvogel, Kazuhiro Wada, Erich Jarvis & Henrik Mouritsen:

Cluster N activity under changed light and magnetic field conditions during the night in *Sylvia* warblers.

Manuscript

Cluster N activity under changed light and magnetic field conditions during the night in *Sylvia* warblers

Gesa Feenders, Miriam Liedvogel, Kazuhiro Wada, Erich Jarvis & Henrik Mouritsen

Abstract

In our previous study we described cluster N, a forebrain area in songbirds that shows high neuronal activity at night-time in night-migratory birds but not in non-migratory species. Cluster N proved to be induced by light leading to the idea that it may function as a specialized night-vision area in night-migratory birds and may be involved in processing star and/or magnetic compass information. In the present study we aimed at a more detailed analysis of cluster N with respect to magnetic compass orientation. We examined cluster N activity in garden warblers (*Sylvia borin*) under different magnetic field scenarios and under monochromatic red light, conditions that are known to change or even disrupt the magnetic compass orientation behaviour. We could not detect any differences in cluster N activity. This may be because either the available light served as the primary signal for a light-based magnetoreceptor; or a second receptor type exists with some sensibility in the long wavelength part of the spectrum as concluded from previous adaptation experiments. We furthermore tested a fairly sedentary *Sylvia* warbler to analyse whether cluster N activity correlate with the degree of migratory behaviour expressed by different species within the same phylogenetic clade. Cluster N showed prominent ZENK expression in the Sardinian warbler (*Sylvia melanocephala*), similar to that observed in garden warblers. Thus, it seems that cluster N activity occur at night in all species within predominantly migratory groups of birds probably because such birds have the capability to switch fast between migratory and sedentary life styles.

Introduction

Migratory passerines are well known for their orientation skills, travelling every spring and autumn between breeding grounds and wintering areas that are often thousands of kilometres apart. Since many decades, it is known that passerines migrating at night can use the geomagnetic field to orient (e.g. Wiltschko 1967; species overview: W Wiltschko & Wiltschko 1996; Cochran et al 2004). Despite intensive experimental efforts, the underlying mechanism(s) of magnetoreception are still not understood. Currently, two hypotheses on how birds may sense the magnetic field are both supported by experimental evidence (review: Mouritsen & Ritz 2005). One is based on superparamagnetic particles that will align according to an external magnetic field leading to small distortions of the cell. Those distortions will hence e.g. affect ion channels leading in turn to a change in membrane potential (Kirschvink et al 2001; Fleissner et al 2003). This magnetite-hypothesis is mainly discussed with relevance to a magnetic map sense (Munro et al 1997a; Fleissner et al 2003). In contrast, for a magnetic compass sense, where the bird extracts global directional information, a light-dependent radical-pair mechanism is suggested (Ritz et al 2000). The Ritz et al theory suggests that light-sensitive molecules form radical-pairs upon photoexcitation and that these radical-pairs can exist in singlet and triplet states which interconverse. Under certain circumstances, this interconversion is affected by an external magnetic field so that the product yields of the singlet and triplet states are altered depending on the orientation of the molecule within the magnetic field. Because singlet and triplet products differ in their characteristics, a change in product ratio could alter the biophysical characteristics of the cell. This modulation can be used e.g. to alter the sensitivity of visual photoreceptors in the eye. Furthermore, the highly ordered structures of the retina may be of advantage, because the magnetosensitive molecules need to be arranged in a fixed array in order to provide directional information.

Cryptochromes are the most probable candidates as a radical-pair forming photoreceptor molecule in birds (Ritz et al 2000; Mouritsen et al 2004a; Möller et al 2004). The main assumptions derived from a cryptochrome-based radical-pair mechanism are met by recent behavioural findings: 1. The orientation of night-migratory birds is dependent on the wavelength spectrum of the available light (e.g. Wiltschko et al 1993; Wiltschko & Wiltschko 1999; Munro et al 1997b; Rappl et al 2000; Muheim et al 2002) and the results are in reasonable agreement with the absorption spectrum of plant cryptochromes (Lin et al 1995). 2. High-frequency oscillating magnetic fields disrupt magnetic orientation (Ritz et

al 2004; Thalau et al 2005) as such weak oscillating fields should directly affect the singlet-triplet transition (Cintolesi et al 2003; Ritz et al 2004). 3. Cryptochromes have been shown to be expressed in the retina of night-migratory birds (Mouritsen et al 2004a; Möller et al 2004); the expression occurred in cells that were highly active when birds performed magnetic orientation and clear differences in expression were observed between migratory and non-migratory species (Mouritsen et al 2004a).

If light-dependent processes in the eyes are involved in the process of magnetoperception, birds should possess a brain area that is specialized in processing visual information at night. In a previous study, we identified a forebrain area, named cluster N, which is specifically activated during the night in night-migratory songbirds (Mouritsen et al, 2005). Blocking visual input inhibited neuronal activity in cluster N. Thus, in night-migratory songbirds, cluster N seems to be a brain region specialized for night-time vision that could be used to sense the geomagnetic field and/or to perceive directional information from the stars.

To further elucidate the function of cluster N and its possible role in processing magnetic information we aimed to measure the expression of neuronal activity dependent immediate early genes (behavioural molecular mapping) in cluster N under various light- and magnetic-field-conditions that are known to alter the magnetic orientation behaviour of birds.

The aim of our first experimental series was to test garden warblers (*Sylvia borin*) under different magnetic field conditions including a completely compensated zero magnetic field (ZMF) where it was shown that redstarts (*Phoenicurus phoenicurus*) cannot orient (Mouritsen 1998). If cluster N – as a possibly higher integration area – is integrating magnetic field information, one could assume this area to show a distinctly different activity pattern because a true-zero magnetic field does not provide any magnetic information at all. But we have to remember that the assumed underlying receptive mechanism is light-based with the light itself being the primary signal. Hence, whenever light of a specific wavelength is available – as given in our magnetic field study presented here – radical-pairs should be formed upon photoexcitation. Although the singlet-triplet-interconversion is sensitive to the magnetic field, leading to signal modulations, the signal itself will be present in any case, even in a true-zero magnetic field. It is important to realize that methods based on immediate early gene expression only indicate the overall mean activity level in a brain area. Therefore, if the magnetic field up-regulates activity in some neurons while it down-regulates the activity of others, or if the modulations are

generally small, behavioural molecular mapping may not be sensitive enough to detect such modulations. In other words, a difference in immediate early gene expression between magnetic field conditions would be a strong indication of magnetic-field-processing taking place in cluster N, whereas a negative result (no differences) would not allow any clear interpretation.

The aim of our second experimental series was to expose birds to monochromatic red light of wavelength and intensity known to disrupt the magnetic compass orientation. This wavelength should be outside the absorption spectrum of the cryptochromes. However, Wiltschko et al (2004a) tested European robins after a 1h pre-exposure to red light and found the birds to be well-oriented. It is not known, yet, how long this adaptation period has to last in order to facilitate orientation under long-wavelength light. So if cluster N is involved in magnetic information processing, the red light exposure we apply may be long enough to either increase the sensitivity of the receptor to respond to red light or evoke a signal in an alternative receptor system (for discussion, see Wiltschko et al 2004a) both leading to neuronal activity in cluster N. So again, if activity disappeared under red light, it would be supporting an involvement of cluster N in magnetic-field-processing whereas activity in cluster N would not allow any clear interpretation.

Our third experimental series aimed at investigating whether immediate early gene expression in cluster N differs in closely related species showing different degrees of migratory behaviour. We know from our previous studies (Mouritsen et al 2005) that the two non-migratory species we tested, zebra finch (*Taeniopygia guttata*) and canary (*Serinus canaria*), showed strongly reduced activity compared to the two migratory species, garden warbler and European robin (*Erithacus rubecula*). Are such differences also found in closely related species? To test this, we investigated immediate early gene expression in cluster N of Sardinian warblers (*Sylvia melanocephala*), a fairly sedentary *Sylvia* species.

Materials and methods

Animal groups. A total of 43 garden warblers (GW, *Sylvia borin*) were caught on Helgoland and around Oldenburg, Germany, or in Rybachy, Russia, during autumn 2001-2002 and spring 2003. In august 2004, 5 Sardinian warblers (SW, *Sylvia melanocephala*) were caught in Navarra, Spain, and transported to Oldenburg, Germany. All birds were

housed indoors, under a simulated local photoperiod. They were kept at least 3 days in captivity before testing. Behavioural tests were performed with 35 GWs during autumn migratory season between August 22 and October 18, 2002 and 2003. Three GWs were tested during the non-migratory season between July 17 and August 3, 2003. 5 GWs were tested under white or monochromatic red light during non-migratory season between December 13 and 22, 2004. The 5 SWs were tested during autumn migration season between September 11 and October 8, 2004.

Behaviour apparatus. Behavioural molecular mapping aims at identifying functional units in the brain. Since everything an animal is doing requires brain processing, experimenters using behavioural molecular mapping must carefully observe, record and/or control the animal's behaviour during the last ~90 minutes of the animal's life. The more isolated and consistent the target behaviour is, the easier the data will be to interpret. Examining the orientation performance of migratory birds is based on their specific behaviour called *Zugunruhe* or migratory restlessness: during their migratory season, caged birds perform wing whirring and consistent movements in a preferred direction (Kramer 1949) corresponding to the migratory orientation of their free-flying conspecifics (Mouritsen 1998; Mouritsen & Larsen 1998). The direction can be measured in Emlen funnels, a standard technique (Emlen & Emlen 1966; Rabøl 1979). However, Emlen funnels are not well suited for our purpose, because birds show a variety of erratic movements in the funnels. Therefore, we developed a new orientation cage variation (Mouritsen et al, 2004a,b, 2005). This setup consists of a cylindrical, transparent Plexiglas-cage (height 40 cm, diameter 40 cm) with a circular perch (diameter 20 cm) placed 8.5 cm above the ground in the centre of the cage. The cage was placed inside a 2x2x2 m Helmholtz-coil system (described in detail in Mouritsen, 1998). This allowed for controlled manipulation of the magnetic field in any direction. 4 small light bulbs placed on the floor provided uniform dim illumination of ~1 mW m^2 intensity (equivalent to 0.04 lux, simulating a moon-lit night). Two infrared-sensitive cameras, providing top-view and side-view, were connected to a split-screen surveillance monitor to allow for real-time observation and recording to video (25 frames/sec) and PC (5 frames/sec) during day and night. Birds tested in this set-up performed very stereotype behaviour by either showing consistent migratory restlessness or sitting still and awake on the perch. Experiments took place in a wooden house providing access to an undisturbed natural magnetic field (67° ± 1° inclination, 47700 nT ± 600 nT total intensity).

Experimental groups.

Magnetic field conditions. In order to examine a possible effect of different magnetic field conditions on cluster N activation, we compared three different magnetic field scenarios:

a) natural magnetic field (NMF) during night-time; N = 5
b) changing magnetic field (CMF) during night-time: magnetic north switching every 5 minutes 120° back and forth; N = 13
c) zero magnetic field (ZMF) during night: a completely compensated magnetic field with no magnetic information available; N = 10

As a control group we tested GWs during day-time in a NMF (NMFday); N = 10.

Monochromatic red light. For our red light experiments, the white light bulbs generating the dim, diffuse light environment were replaced by an array of diodes emitting light between 600-700nm with a clear peak at 650nm. In previous studies the peak wavelength, leading to a disoriented behaviour, ranged from 617nm to 635nm (Wiltschko et al 1993; Rappl et al 2000; Muheim et al 2002; Wiltschko et al 2004a,b). 4 GWs were tested under red light (intensity 1.02 mW m^2), in an unaltered magnetic field (NMF). In order to compare the results with the data collected from birds under white light in the previous study (Mouritsen et al 2005), we tested one additional individual under the standard white light.

Sardinian warbler. Three SWs were tested under CMF condition during night-time in dim white light, and two SWs were tested in NMF at day-time in full room light. We chose CMF in order to allow for direct comparison of the results with previous data collected from garden warblers.

All birds were tested following the procedures described below.

Testing procedure. On the day of testing, a thin stripe of IR-reflective tape (Retroreflective tape, 3M) was glued to the top of the bird's head. The IR tape was used to track and analyse the bird's orientation on the digital videos using a custom-written MATLAB programme. This programme computes the position of the bird's had by screening each single frame for the white spot caused by the reflective tape and relating its position to the centre of the cage. The birds were placed into the cylindrical cage before 13:30h and food was removed 90 minutes before onset of darkness (between 20:00h and 20:30h). For the day-time control group, the birds were placed into the cage during day-time without food and observation started after approximately 1 hour of habituation to the cage. This habituation period was necessary to let any handling-induced immediate early gene

expression decrease to baseline. After a bird performed a minimum of 45 minutes of the desired constant behaviour, i.e. either showing migratory restlessness (or regular movements during the day) or sitting still but awake, the animal was sacrificed by decapitation and the brain was rapidly dissected. The two hemispheres were separated along the mid-sagittal plane, embedded in TissueTek O.C.T. (Sakura Finetek), and quick-frozen to -80°C in a dry ice/ethanol bath.

Gene expression analysis. For one bird per group (magnetic field condition) frozen sections (12µm) were cut throughout the whole brain: the left hemisphere in the sagittal plane, the right hemisphere in the frontal plane. As no differences were observed between the two hemispheres, we continued to cut the left hemisphere, only. We chose the sagittal plane as it provided a very distinct overview of the cluster N expression pattern. Corresponding sections were fixed in 4% paraformaldehyde and processed by *in-situ* hybridization with antisense S^{35}-UTP riboprobes of ZENK cDNA (zebra finch). ZENK is an immediate early gene that is expressed after a neuron gets activated. The mRNA can be detected in the tissue approximately 10-60 minutes after activation occurred, with a peak after circa 30-45 minutes (Jarvis & Nottebohm 1997). The hybridized sections were first exposed to x-ray films (Biomax, Kodak) and then dipped into autoradiography emulsion (NTB2, Kodak), incubated for 4-6 weeks at 4°C, developed using Kodak developer (D19) and fixer, Nissl stained with cresyl violet acetate (Sigma) and coverslipped with permount glue. For quantification, the x-ray film brain images were digitally scanned using a dissecting microscope and SPOT III CC camera. Using SPOT ADVANCED (Spot software, Diagnostics Instruments) and ADOBE PHOTOSHOP (Adobe Systems Inc.) software, the brain regions of interest were encircled and the mean pixel density was measured on a 256-level grey scale. Quantification of cluster N followed the same procedure as described before (Mouritsen et al, 2005): the relative pixel density of cluster N area, comprising posterior-dorsal parts of the brain regions hyperpallium apicale (HA), intercalated hyperpallium apicale (IHA), dorsal mesopallium (MD), the dorsal nucleus of the hyperpallium (DNH) and the DNH-shell, was subtracted by the values of the anterior-ventral part of HA, IHA and MD (compare with Figure 2A,B).

Results

Magnetic conditions

All GWs were tested for correct magnetic orientation prior to the ZENK expression analysis. The results show a clear directedness towards south-west in the unmanipulated natural MF, whereas the birds showed no significant direction under ZMF-conditions (Figure 1A); this confirmed that our birds did use the magnetic field for orientation. No celestial cues were available to the birds. For quantification of cluster N ZENK expression, we only analyzed the brains of birds that had been sitting still but awake for a minimum of 45 min prior to sacrifice. By doing this, we avoided gene expression due to motor behaviour (Feenders et al, submitted) that might mask small differences in expression level due to magnetic sensing. The expression level of ZENK in cluster N did not differ significantly between birds that were exposed to NMF, CMF or ZMF (Figure 1B; one-way ANOVA, followed by Holm-Sidak multicomparison test: $0.660>p>0.085$ for all NMF, CMF, ZMF comparisons). All three groups were significantly different from the day-time group (one-way ANOVA, followed by Holm-Sidak multicomparison test: $p<0.001$), thus confirming the previous results that during day-time cluster N activity decreases to baseline level.

Monochromatic red light

We exposed 4 GWs to monochromatic red light for 45 minutes and consecutively quantified ZENK expression in cluster N. We found high ZENK expression in cluster N that did not differ significantly from the pattern seen under white light in NMF at night but that was different to the expression level at day (Figure 2, one-way ANOVA followed by Holm-Sidak multicomparison test; red light versus white light: $p=0.342$; red light versus day: $p<0.001$).

Since we conducted the experiments during winter when GWs are not migrating provided further information on the consistent activity of cluster N throughout the year. As described previously, birds tested in non-migratory season during summer showed no seasonal difference in cluster N expression (Mouritsen et al 2005). The present data complete the annual circle to show that activity of cluster N is not affected by season.

Sardinian warblers

In the brains of SWs tested in CMF under white light, cluster N was highly active (Figure 3A) and the ZENK expression pattern was very similar to that seen in GWs. As observed previously in GWs, the Cluster N ZENK expression level during night-time was significantly higher than during day-time (Figure 3B, t-test, $p=0.005$, $t=-7.677$). However, in SWs during day-time, the shell around the DNH showed high ZENK expression especially in one of the two birds tested (not shown). This was never observed in the GWs. With the present data, the reason for this difference in expression pattern remains unclear.

Discussion

In our previous publication, we described cluster N as a brain area that is specifically activated during night time in night-migratory songbirds (Mouritsen et al 2005). We suggested this brain area to be involved in special night vision and possibly in information processing related to magnetic and/or star compass orientation. In the present study we performed a more detailed characterization of cluster N with the hope of gaining additional insight into its possible function.

The presented data show robust immediate early gene expression in cluster N under various magnetic field conditions, including a completely compensated magnetic field. As mentioned in the introduction, this finding is not surprising because in all our magnetic conditions the birds were exposed to dim white light. Under this light regime, the radical-pair mechanism is assumed to function correctly. A primary signal will then be sent to the brain irrespective of the ambient magnetic field, solely based on the light input. The direct connection of cluster N activity with light input was shown in our previous work (Mouritsen et al 2005) where blocking the light input by covering the eyes led to baseline activity in cluster N. It seems that any putative magnetic modulations are not strong enough and/or not unidirectional so analyses based on immediate early gene expression are not sensitive enough to detect a difference.

We further observed prominent ZENK expression in cluster N after the birds had been exposed 45 minutes to monochromatic red light. In theory, light of this wavelength is thought to be too low in energy to start the radical-pair process. However, recent findings lead to the assumption that at least two different receptor-types exist in the retina of birds (Wiltschko et al 2004a,b). Thus, it seems likely that a second receptor-type gets activated

under our red light condition and sends a signal to the brain as reflected by cluster N activity.

The third experiment with Sardinian warblers revealed significant neuronal activity in cluster N at night but not during the day, thus providing a similar pattern to that observed in garden warblers. Sardinian warblers are fairly sedentary in the Mediterranean and frequently used as a non-migratory *Sylvia*-warbler (e.g. Healy et al 1996; Mettke-Hofmann & Gwinner 2003). But it has been observed that individuals of this species undertake directed movements in autumn and spring (Cramp 1998). Although there is no evidence in the literature about how Sardinian warblers may orient, it would not be too surprising if this species has the ability to orient e.g. according to the magnetic field. In fact, non-migratory birds can also use a magnetic compass (homing pigeons, *Columba livia*: Keeton 1971; Wiltschko & Wiltschko 2001; chick, *Gallus domesticus*: Freire et al 2005a,b), but it is not yet clear if the mechanism is the same in migrants and non-migrants. Further evidence that at least a partial migratory behaviour is likely to exist in Sardinian warblers is provided by the studies of Pulido and colleagues (Pulido et al 1996). They show that within natural populations of blackcaps (*Sylvia atricapilla*) and probably many other species there exist individuals showing more migratory behaviour, less migratory behaviour, or no migratory behaviour at all, and that any population can evolve into a migratory or non-migratory state within few generations. Therefore, it is highly likely that all species in a predominantly migratory clade of birds, such as the *Sylvia* warblers, posses the ability to orient according to a magnetic compass because sedentary/migratory behaviour has probably evolved and re-evolved repeatedly over the last 10000 years (review: Berthold 1999; Piersma et al 2005).

In conclusion, all data that have been collected about cluster N until now do not answer whether cluster N is an integrative area for magnetic compass orientation, or whether it is 'just' a night vision area specialized in night-migratory species including possibly their close relatives. A specialized night vision area may be necessary to extract directional information from the starry sky, or it may trigger the bird's alertness to initiate night-migratory behaviour. Electrophysiological recordings and studies with lesions of cluster N are crucial to elucidate the ultimate function of cluster N. In addition, it will be interesting to test day-migratory birds during their migratory season for a possible existence of cluster N activity during day-time migration.

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Figure Legends

Figure 1: ZENK expression in garden warbler brains under different magnetic field conditions. *A* & *B*: Orientation of garden warblers in a natural magnetic field (*A*) and a compensated true-zero magnetic field (*B*). Each dot represents the mean orientation of one individual bird. The arrow indicates the mean orientation of the group, with its lengths representing the length of the r-vector as an indication of concentration. The dashed circles indicate the radius of the group mean vector needed for significance ($p<0.05$ and $p<0.01$) according to the Rayleigh Test of uniformity. *C*: Relative ZENK expression levels of birds exposed to natural (NMF), changing (CMF) or zero magnetic field (ZMF) compared with birds collected during day-time (day). Error bars: standard error.

Figure 2: ZENK expression in garden warbler brains. *A*: Darkfield image of cluster N ZENK expression under dim white light at night (*left*) and after 45 min exposure to monochromatic red light (*right*). Dorsal is up, rostral is right. *B*: Anatomical profile of the right image shown in *A*. *C*: Quantification of ZENK expression level in cluster N, comparing exposure to standard white light with red light. Error bars: standard error. Scale bar: 1 mm. A: arcopallium; E: entopallium; HF: hippocampal formation; H: hyperpallium; ICo: colliculus inferior; IHA: intercalated hyperpallium accessorium; M: mesopallium including ventral (MV) and dorsal (MD) part; N: nidopallium; OT: optic tectum; P: pallidum; St: striatum; v: ventricle; W: visual Wulst.

Figure 3: ZENK expression in Sardinian warblers. *A*: Darkfield image of ZENK expression in a sagittal section showing cluster N. Dorsal is up, rostral is right. *B*: Anatomical profile of the image shown in *A*. The red dashed line marks the boundary of cluster N. *C*: Quantification of ZENK expression level in cluster N, comparing day-time and night-time group. Error bars: standard error. Scale bar: 1 mm. For abbreviations see figure 1.

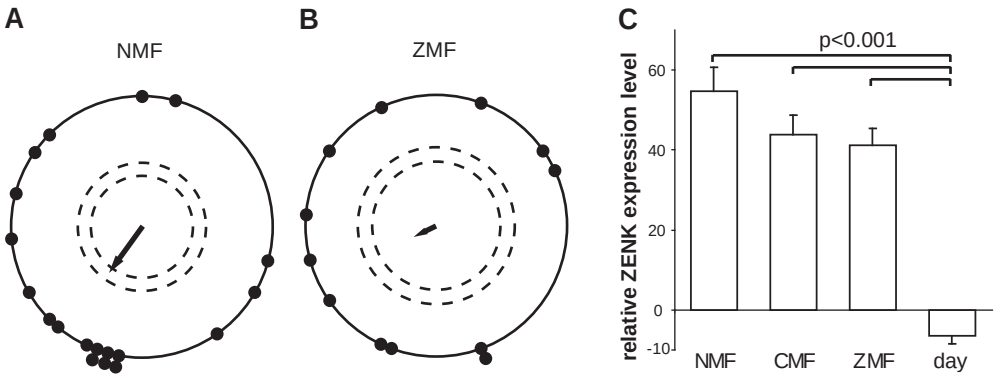
A
NMF
B
ZMF
C
p<0.001
relative ZENK expression level
60
40
20
0
-10
NMF
CMF
ZMF
day

Figure 1

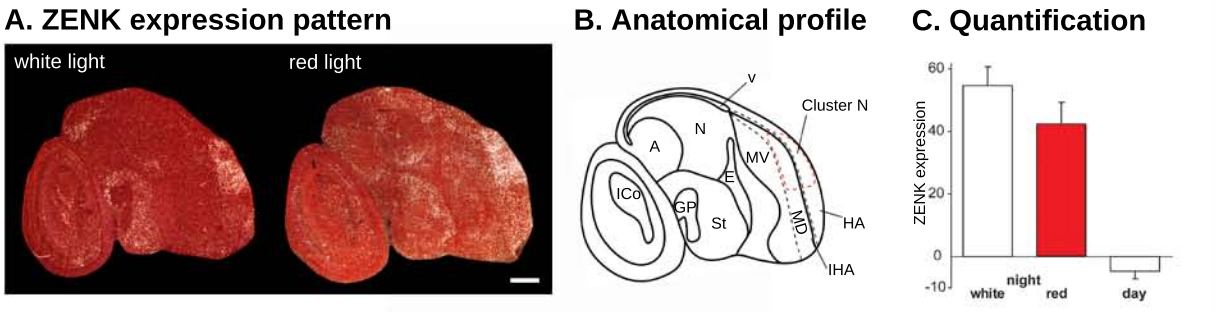
A. ZENK expression pattern
white light
red light
B. Anatomical profile
v
Cluster N
N
A
MV
E
ICo
GP
St
MD
HA
IHA
C. Quantification
ZENK expression
60
40
20
0
-10
night
white
red
day

Figure 2

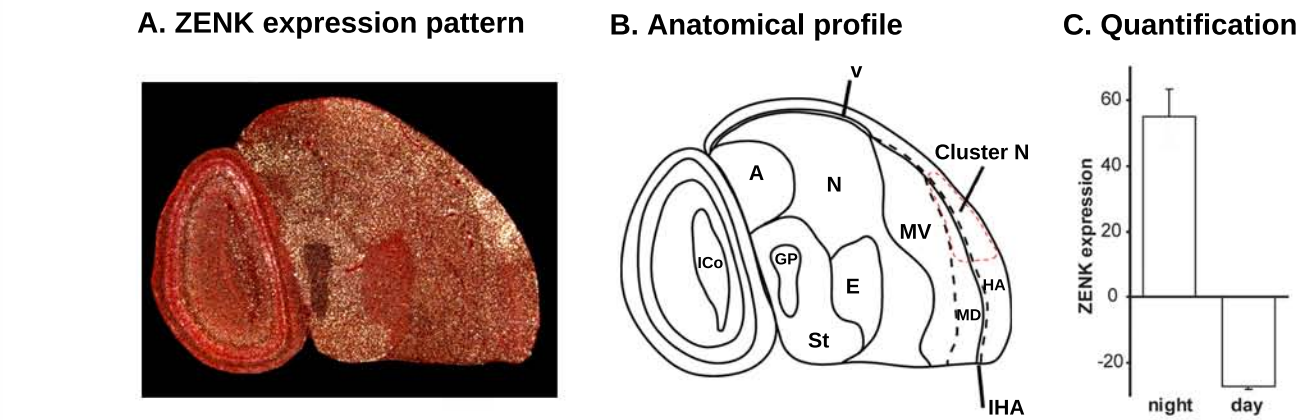
A. ZENK expression pattern
B. Anatomical profile
C. Quantification
V
Cluster N
A
N
MV
ICo
GP
E
HA
MD
St
IHA
60
40
20
0
-20
ZENK expression
night
day

Figure 3

Erklärung:

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbstständig angefertigt und nur die angegebenen Hilfsmittel verwendet habe.

Oldenburg, im März 2006

Gesa Feenders