

## Research



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# Lidocaine is a placebo treatment for trigeminally mediated magnetic orientation in birds

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Even though previously described iron-containing structures in the upper beak of pigeons were almost certainly macrophages, not magnetosensitive neurons, behavioural and neurobiological evidence still supports the involvement of the ophthalmic branch of the trigeminal nerve (V1) in magnetoreception. In previous behavioural studies, inactivation of putative V1-associated magnetoreceptors involved either application of the surface anaesthetic lidocaine to the upper beak or sectioning of V1. Here, we compared the effects of lidocaine treatment, V1 ablations and sham ablations on magnetic field-driven neuronal activation in V1-recipient brain regions in European robins. V1 sectioning led to significantly fewer Egr-1-expressing neurons in the trigeminal brainstem than in the sham-ablated birds, whereas lidocaine treatment had no effect on neuronal activation. Furthermore, Prussian blue staining showed that nearly all iron-containing cells in the subepidermal layer of the upper beak are nucleated and are thus not part of the trigeminal nerve, and iron-containing cells appeared in highly variable numbers at inconsistent locations between individual robins and showed no systematic colocalization with a neuronal marker. Our data suggest that lidocaine treatment has been a placebo to the birds and a placebo for the experimenters. Currently, the nature and location of any V1-associated magnetosensor remains elusive.

## 1. Introduction

The Earth's magnetic field represents an omnipresent navigational cue for birds during migration and could theoretically provide both directional (i.e. a 'compass') and positional (i.e. a 'map') information [1–5]. A large body of evidence suggests that birds possess a magnetic inclination 'compass' [1,2,6], which is embedded in their visual system [7–10]. The underlying magnetic compass sensor is hypothesized to be cryptochrome based [11–14], located in both of the birds' eyes [15,16], and the biophysical mechanism most likely relies on radical-pair-based spin-chemical reactions (for a comprehensive review, see [9]).

In addition to the light-based magnetic compass in the birds' eyes, two additional locations of magnetic sensors have been suggested to exist in birds. First, putative sensors in the vestibular system [17,18], which could be based on iron accumulations within the inner ear lagena [19,20] but whose biological function currently remains elusive. Second, iron-containing structures located in the upper beak within nerve endings of the ophthalmic branch of the trigeminal nerve (V1) of various bird species [21–23] have been suggested to provide positional information based on geomagnetic cues [24–26].

These putative trigeminal nerve-associated 'map' sensors were described as membrane-bound magnetite spherules arranged around a vesicle and interconnected with maghemite platelets. These structures were claimed to be located in six specifically restricted clusters along the lateral edges of the stratum laxum of the subepidermis in strict bilateral and spatial symmetry [21–23].

Since the discovery of these putative beak-based receptors, numerous behavioural studies have been performed in order to assess their role in magnetoreception. In most of them, the commonly available surface anaesthetic lidocaine was externally applied to the upper beak in order to temporarily inactivate the proposed magnetosensors. This treatment repeatedly led to the disruption of the so-called fixed-direction responses in European robins and Australian silvereyes [27–38]. A 'fixed-direction' response (e.g. [31,35,36]) is defined as a bird's change in migratory direction, which (i) is different from its season-specific migratory direction, (ii) does not change between seasons, and (iii) appears either in complete darkness [27], under red light [29,32], in unnaturally high light intensities [28,37,38] or under a bichromatic light regime [28,31,33,34]. As 'fixed-direction' responses were described as polar responses, i.e. the orientation of a bird by the polarity, not the inclination [1] of the magnetic field, the previously described iron-containing beak-based magnetosensors [21–23] were claimed to form the underlying sensory basis.

However, is lidocaine anaesthesia a reliable way to temporarily 'knock down' any putative upper beak magnetosensors in a typical orientation experiment?

Independent studies seriously question the ability of lidocaine to target the functionality of a specific sensor: Wallraff [39] reported highly variable and non-standardizable effects after intranasal application of lidocaine in order to deprive pigeons of olfaction. By contrast, bilateral sectioning of the olfactory nerve reliably and replicably eliminated olfactory perception. Schlund [40] reported systemic side effects which could influence navigational performance, because lidocaine not only might target the tissue of interest but also affects adjacent tissues through diffusion and blood stream transport [41] and because lidocaine passes the blood–brain barrier [42]. More specifically, Dornfeldt & Bilo [43] showed that lidocaine applied to the nasal cavity caused severe deficits in integrating visual and vestibular input, reflected in suppression of both vestibular and optokinetic nystagmus, due to the drugs' uptake via the olfactory mucous membranes. Other studies report severe effects of lidocaine, but not of olfactory nerve sectioning on the tonic immobility response of pigeons [43,44]. Thus, lidocaine treatment is known to have severe side effects on brain functioning and thus on behaviour.

Moreover, the intended anaesthetic effect of lidocaine is known to wear off after 10–15 min on human mucous skin, and it cannot be prolonged even with repeated applications [45]. The time course of any anaesthetic effect on European robin skin is unknown and any side effects can last for shorter or longer than the reported anaesthetic effect on human skin. The previously performed behavioural experiments, where lidocaine was used, typically lasted up to 75 min [32,34].

Surprisingly, the above-mentioned issues seem to have been completely neglected when disrupted orientation responses in birds after lidocaine treatment were interpreted as proof of the described iron-containing structures being indeed magnetoreceptors.

Last but not least, elaborate attempts [46–48] failed to replicate previous findings related to the putative magnetoreceptors in the upper beak of pigeons. Iron-rich cells were found in highly variable numbers at inconsistent locations in the beaks of a large cohort of pigeons and the iron-rich cells colocalized with macrophage-specific markers rather than with neuronal markers [46,47]. Mouritsen [49] inspected parts of the raw data from Falkenberg *et al.* [23] and Treiber *et al.* [46] and came to the conclusion that the structures studied were indeed at least partly the same. Thus, the previously suggested candidate magnetoreceptive structures associated with V1 are almost certainly macrophages rather than magnetosensitive neurons.

Consequently, not only is the specific anaesthetic effect of lidocaine in robins questionable over the typical duration of a behavioural experiment, the targeted structures might not even exist. We therefore wondered why non-documented, putative anaesthesia of the upper beak, where the targeted structures seem to be macrophages rather than magnetosensors, would affect magnetic orientation behaviour [27–38].

A different technique, which has been used to investigate potential functional aspects of a beak-based magnetoreceptor, is surgical sectioning of V1. V1 is the only non-olfactory nerve which innervates the upper beak, parts of the facial skin and the nasal cavity. Studies using surgical ablation of V1 reported clear behavioural effects on the birds' ability to detect and/or to react to magnetic field changes [24,50]. Moreover, a significant decrease in magnetically induced neuronal activation has been observed in the primary V1-recipient hindbrain regions PrV (principal sensory nucleus of the trigeminal nerve) and SpV (spinal trigeminal nucleus) after V1 sectioning in two migratory bird species [51,52] and homing pigeons [53]. Finally, it was shown that V1 sectioning had no unspecific effects on the birds' ability to show migratory behaviour and to use their magnetic compass, because V1-lesioned birds oriented as well as controls in magnetic compass orientation experiments [8,24,54,55]. Based on the above considerations, we have repeatedly argued that V1 sectioning is the only sure way to prevent trigeminal magnetic information being transmitted to the brain via V1 [9,10,24,52,53,55,56].

We used the replicable observation of V1-dependent magnetic activation in PrV and SpV [17,51–53] as a diagnostic tool to directly test whether lidocaine treatment affects trigeminal-based magnetic activation in the hindbrain. Therefore, the first aim of this study was to analyse magnetic field-induced neuronal activation (reflected as nuclear expression of Egr-1) in order to assess the effect of lidocaine on putative beak-based magnetoreceptors. To do this, we compared the number of Egr-1-expressing neurons following magnetic stimulation in the trigeminal brainstem nuclei PrV and SpV in three experimental groups of European robins, in which either (i) V1 was sham sectioned, (ii) lidocaine was applied to the upper beak, or (iii) V1 was bilaterally sectioned.

The second aim of the present study was to test whether the iron-containing structures reported in the upper beak of European robins [23] indeed appear at the six claimed specific locations and if they are contained in neuronal fibre terminals. To do so, we performed histological analyses of European robins' beaks in order to assess the existence and distribution of iron-containing cells and to investigate if they colocalize with the general neuronal marker TUBB3.

**Table 1.** Number of Egr-1-expressing neurons counted in compartments of the V1-recipient regions in the trigeminal brainstem, and the statistical results of the group comparisons. PrV, principal sensory nucleus of the trigeminal nerve; SpV, spinal trigeminal nucleus.

	PrV	SpV
Sham		
mean	746	790
s.d.	192	219
Lido		
mean	582	857
s.d.	151	408
Sect		
mean	236	233
s.d.	117	122
ANOVA ( <i>p</i> )	0.001	0.002
multiple comparison (Bonferroni corrected) ( <i>p</i> )		
Sham versus Lido	0.246	0.988
Sham versus Sect	0.001	0.005
Lido versus Sect	0.005	0.003

## 2. Results

### 2.1. Egr-1 expression in the trigeminal brainstem

We determined and statistically compared the total number of Egr-1-expressing neurons in the trigeminal brainstem of three groups of European robins, all of which were exposed to a magnetic field stimulus containing frequent changes in all magnetic parameters (horizontal direction, inclination and intensity) for the duration of 90 min (for details, see the Material and methods section) after (i) sham sectioning of V1 (i.e. left intact; 'Sham';  $N = 7$ ), (ii) lidocaine application to the upper beak ('Lido';  $N = 6$ ), or (iii) surgical ablation of V1 ('Sect';  $N = 6$ ). The results are summarized in table 1 and shown in figure 1. In 'Sham' birds, we counted  $746 \pm 192$  (s.d.) Egr-1-expressing neurons in PrV (figure 1a,g) and  $790 \pm 219$  (s.d.) Egr-1-expressing neurons in SpV (figure 1b,g). 'Lido' birds showed an average of  $582 \pm 151$  (s.d.) Egr-1-expressing neurons in PrV (figure 1c,g) and an average of  $857 \pm 408$  (s.d.) Egr-1-expressing neurons in SpV (figure 1d,g). 'Sect' birds displayed  $236 \pm 117$  (s.d.) and  $233 \pm 122$  (s.d.) Egr-1-positive neurons in PrV (figure 1e,g) and SpV (figure 1f,g), respectively. This equals a 68% (PrV) and 71% (SpV) decrease in Egr-1-expressing neurons compared with the 'Sham' group and a 59% (PrV) and 73% (SpV) decrease compared with the 'Lido' group, respectively.

The statistical analysis revealed no significant differences between the number of Egr-1-expressing neurons in the 'Lido' group compared with the 'Sham' group. By contrast, the 'Sect' group showed significantly lower numbers of Egr-1-expressing neurons than both the 'Sham' group and the 'Lido' group (PrV: ANOVA  $F_{2,16} = 17.04$ ,  $p < 0.001$ , followed by a pairwise multiple comparison (Bonferroni corrected): 'Lido' versus 'Sham'  $p = 0.246$ ; 'Sect' versus 'Sham',  $p < 0.001$ ; 'Sect' versus 'Lido',  $p = 0.005$ ; SpV: ANOVA  $F_{2,16} = 9.93$ ,  $p = 0.002$ , followed by a pairwise multiple comparison (Bonferroni corrected): 'Lido' versus 'Sham',

$p = 0.988$ ; 'Sect' versus 'Sham',  $p = 0.005$ ; 'Sect' versus 'Lido',  $p = 0.003$ ) (figure 1g and table 1).

### 2.2. Iron-containing cells in the upper beak

To ascertain whether a six loci magnetite-based magnetic sense system exists in the upper beak of robins, we performed Prussian blue (PB) staining on serial sections coupled with anatomical mapping. We constructed a model beak of the European robin identifying five distinct landmarks (figure 2) to enable accurate mapping along the rostro-caudal axis. Quantification revealed a very high degree of variation in the number ( $1639 \pm 3461$  (s.d.)) and the distribution of PB-positive cells ( $n = 8$  birds), with total numbers varying between 12 and 9984 (figure 3). Neither a bilateral distribution nor a patchy concentration in six spots was observed, which largely corroborates previous observations of a rather inconsistent distribution of PB-positive cells in the upper beak of pigeons [46,47].

As has been previously reported, the PB signal within cells of the subepidermis appeared as punctate spheres as well as light blue cytoplasmic staining (figure 4a–c). Counter-stainings with nuclear fast red showed that most, if not all, iron-rich cells are nucleated (figure 4d–f). Neuronal immunolabelling using an antibody against TUBB3 revealed that less than 0.6% of PB-positive cells colocalized with TUBB3 ( $n = 735$  cells in eight birds) (figure 5a,b).

## 3. Discussion

### 3.1. General observations

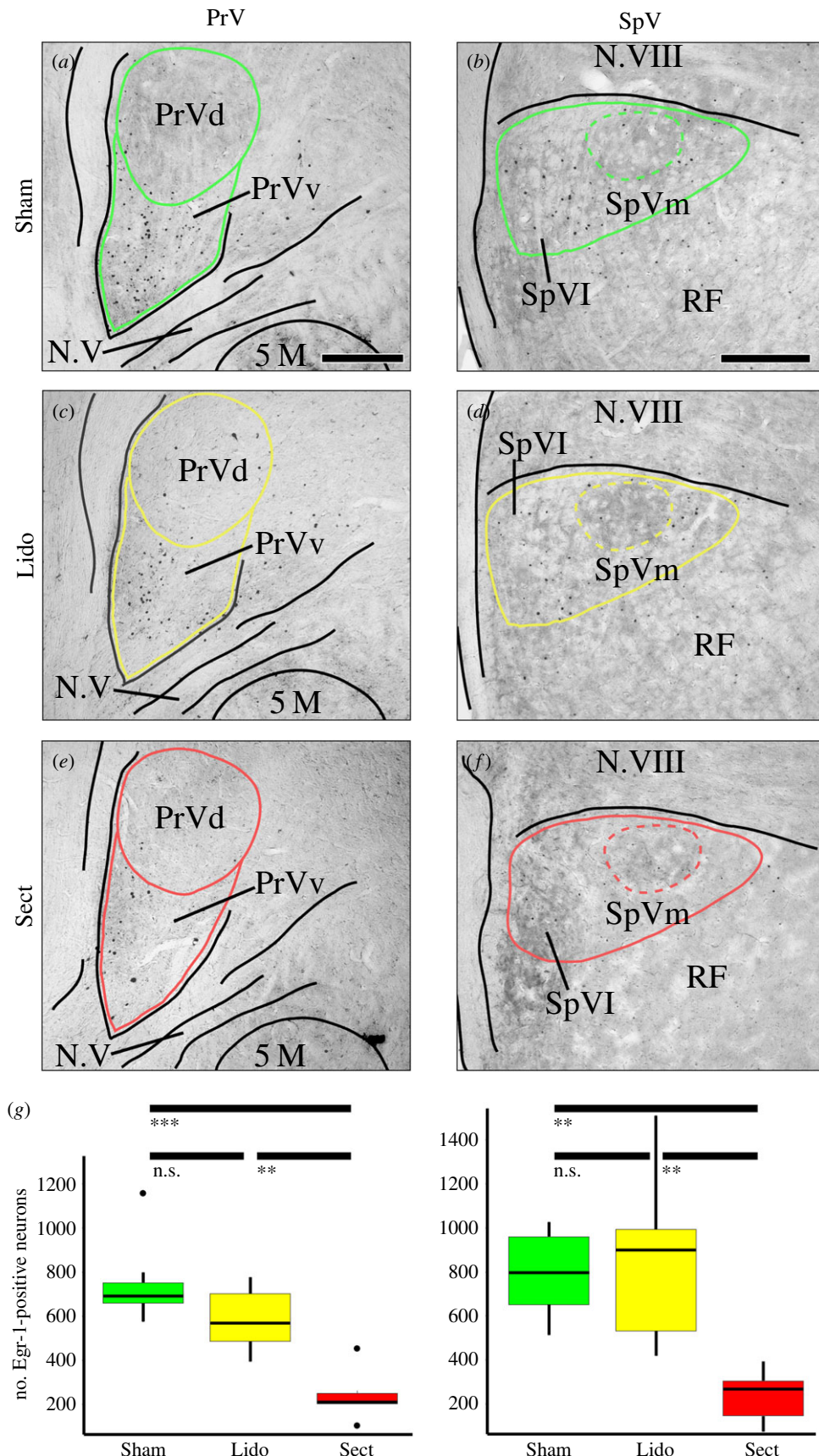
Anaesthesia with lidocaine applied to the mucous skin of the upper beak, as has been used in numerous studies in order to temporarily 'inactivate' a beak-based magnetic sensor, did not significantly affect the magnetic activation of trigemino-recipient brain structures PrV and SpV in migratory European robins, whereas sectioning of V1 led to a significant decrease in magnetic field-induced neuronal activation (figure 1g). Previous studies replicably showed that the observed activation is mediated by V1 and that it is very likely to be induced by magnetic stimuli, because down-regulation of Egr-1 was achieved both by V1 sectioning and by compensation of the ambient magnetic field [51–53].

Moreover, the previously described distribution of intracellular iron accumulations in the upper beak of European robins [23] does not seem to be conserved, as the studied animals showed extremely high variability in the number and distribution of PB signal in the subepidermal layer of the upper beak (figure 3).

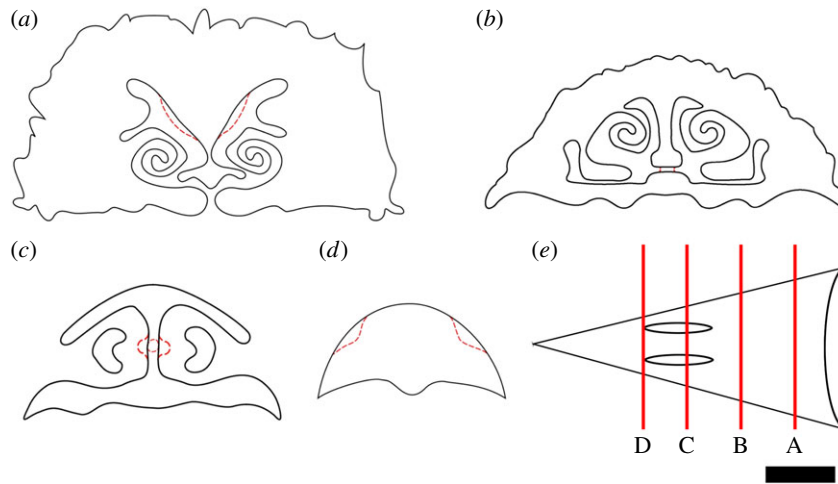
Finally, iron-containing structures in the subepidermal layer of European robin beaks generally do not seem to be contained in neurons, as less than 0.6% of PB-positive colocalized with the neuronal marker TUBB3 (figure 5a,b) and the vast majority of them were clearly nucleated (figure 4d–f). The fact that all somata of the neurons forming the trigeminal nerve are located in the trigeminal/gasserian ganglion at the level of the rostral brainstem [57] means that any nucleated cell in the beak cannot be a part of V1 and thus excludes the possibility that the observed iron-containing structures are contained in V1 fibre terminations.

While it remains possible that a small percentage of cells in the upper beak of European robins might have evaded PB





**Figure 1.** Magnetic field-induced expression of Egr-1 in the trigeminal brainstem. (a,c,e) Frontal sections through PrV in 'Sham' (a), 'Lido' (c) and 'Sect' (e) birds. (b,d,f) Frontal sections through SpV in 'Sham' (b), 'Lido' (d) and 'Sect' (f) birds. Rostral is up, lateral is left. Note the strongly increased nuclear Egr-1 expression under 'Sham' (a,b; green) conditions. Egr-1 expression in PrV is confined to the crescent-shaped ventral PrVv part. (g) Quantitative analysis of Egr-1 expression. Boxes represent the upper/lower quartile, black line depicts the median; whiskers show a greatest value excluding outliers; outliers (black dots), defined as a value deviating more than 3/2 times the upper quartile. The number of Egr-1-expressing nuclei in PrV and SpV is significantly decreased only when the ophthalmic branch of the trigeminal nerve (V1) was cut (e,f; red) but is not significantly affected after lidocaine application to the upper beak (c,d; yellow). N.V, trigeminal nerve; N.VIII, vestibulo-cochlear nerve; PrVd, principal sensory nucleus of the trigeminal nerve; PrVv, ventral part of the nucleus of the trigeminal nerve; RF, reticular formation; SpVI, lateral part of the spinal trigeminal nucleus; SpVm, medial part of the spinal trigeminal nucleus; SM, motor nucleus of the trigeminal nerve. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Scale bars: 200  $\mu\text{m}$  (a, for a,c,e; b, for b,d,f).



**Figure 2.** Anatomical landmarks for the robin beak. (a–d) Coronal views of the four anatomical landmarks employed to normalize the spatial distribution of PB-positive cell counts between birds. Landmark 1 (a) was defined as the region where the central septum of the olfactory epithelium changed from a 'U' to a 'V' shape. Landmark 2 (b) was defined as the region where the central septum joined the ventral nasal cavity. Landmark 3 (c) was defined as the location where the small lateral buds of the nasal cavity disappear. Landmark 4 (d) was defined as the position where the nasal cavity is no longer visible. The fifth landmark (not shown) marks the rostral end of the beak. (e) Schematic top view of a beak showing the approximate locations of landmarks (a–d). Scale bar: 300  $\mu\text{m}$  in (e).

labelling and/or very few cells, which both contain iron (as depicted by PB labelling) and seem to be positive for the neuronal marker TUBB3 (less than 0.6%, see Results), are actually magnetoreceptors, we consider this option as highly unlikely. A much more likely interpretation, supported by a recent study which failed to identify any intracellular structures consistent with biogenically derived magnetite [48], is that the previously described ferric structures are macrophages [46,47].

### 3.2. The questionable use of lidocaine in orientation experiments

Lidocaine treatment was reported to disrupt so-called fixed-direction responses in a large number of studies [27–38]. This specific behaviour was defined as a bird's change in migratory direction, which is different from its season-specific direction irrespective of season and appearing under various artificial light regimes (see Introduction). Notably, the birds oriented by the polarity but not the inclination of the magnetic field, which led to the above-mentioned authors' interpretation that birds, under certain light conditions, 'switch' from a vision-mediated, quantum-chemical inclination compass to a fundamentally different, trigeminally mediated and iron-mineral-based polarity compass mechanism, thereby pointing towards 'complex interactions between magnetoreceptors in the eye, the upper beak and the visual system' [58]. Irrespective of the validity of this interpretation, a dysfunctional magnetic polarity orientation response after lidocaine treatment is hard to explain based on the non-existent effect of lidocaine treatment on magnetosensitive neurons in the trigeminal system reported in this study.

## 4. Conclusion

Lidocaine treatment does not significantly affect magnetic field-driven Egr-1 expression in the trigeminal brainstem of European robins. Thus, any effects of lidocaine treatment are not due to a selective temporary inactivation of putative beak-based magnetosensors. Owing to the reported side effects of lidocaine treatment, it cannot be excluded that the reported

disorientational responses [27–38] are caused by unspecific, non-trigeminal-based disrupted course control, altered general well-being, fear and/or motivation to orient. Thus, lidocaine should not be used to inactivate putative beak-based magnetosensors in any future experiments. Furthermore, we show that the distribution of iron accumulations in the upper beak of European robins is neither conserved nor does a reasonable number of iron-containing cells colocalize with the neuronal marker TUBB3, thereby giving strong evidence that the previously described iron clusters in the upper beak of European robins are not contained in V1 fibre terminals and thus are highly unlikely to function as magnetosensors. Currently, V1 sectioning does seem to remain the only valid diagnostic tool to assess trigeminal-based magnetoreception to date.

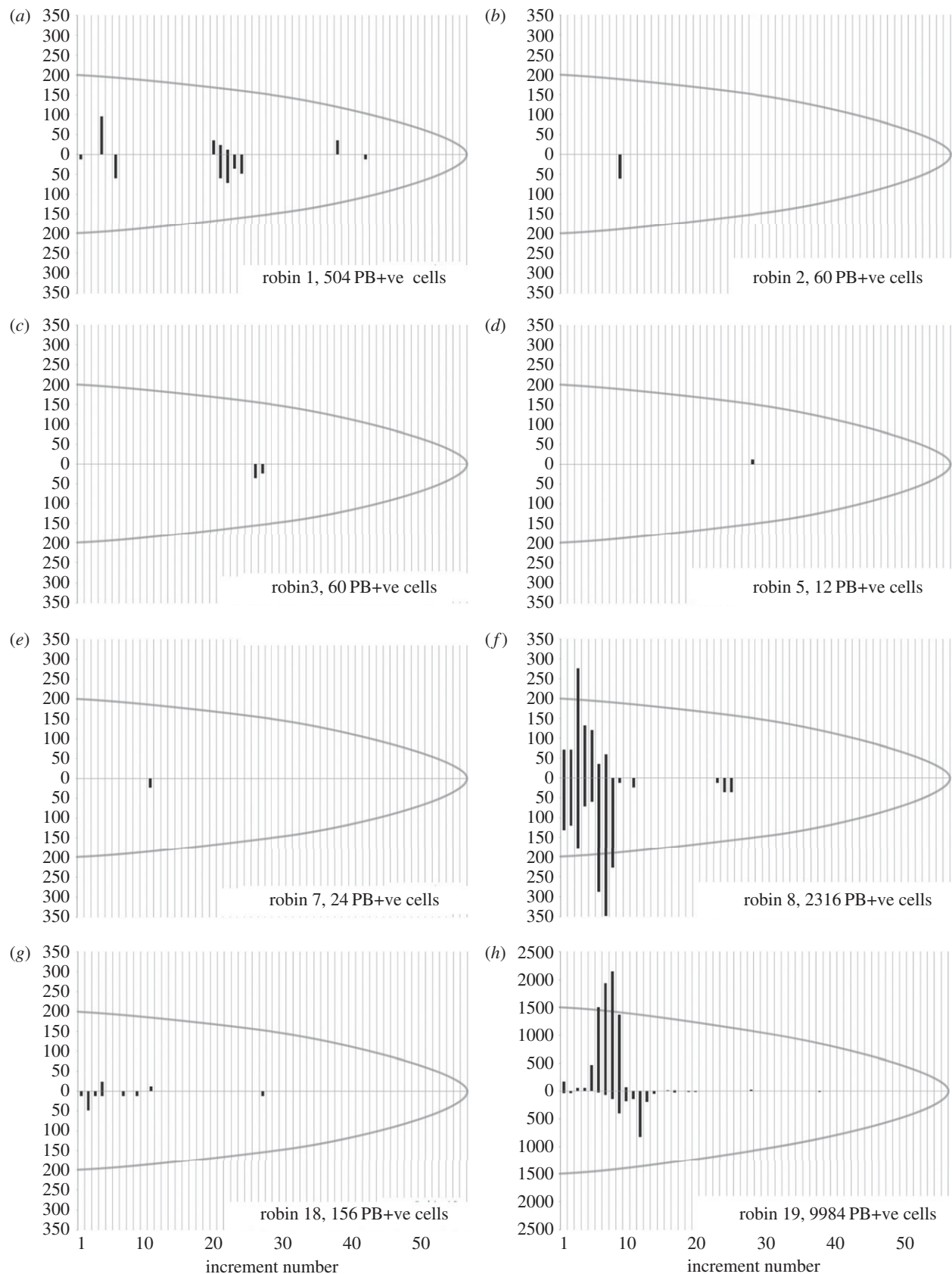
## 5. Material and methods

### 5.1. Animals

European robins were caught at the campus of the University of Oldenburg, Germany, and were housed indoors under the local and circannual photoperiod.

### 5.2. Magnetic stimulation

At dusk, single birds were placed in a cylindrical cage fitted with a round perch and covered with a nylon netting [59]. The cage was placed in a double-wrapped Merrit four-coil system [60,61] inside a wooden hut shielded with aluminium [62]. The set-up was illuminated with incandescent light bulbs (spectrum given in the electronic supplementary material of [8] with an intensity of approximately  $2.3 \text{ mW m}^{-2}$ ). We provided highly variable magnetic stimuli in order to prevent sensory adaptation. The stimulus was identical to the one that has previously been shown to successfully activate the trigeminal brainstem of several bird species [51–53]. This stimulus was computer generated using a custom-written script (Matlab + Data Acquisition toolbox; Mathworks, Natick, MS, USA). It consisted of two alternating 5 min magnetic stimuli blocks. During the first 5 min block, an Earth-strength magnetic field was rotated approximately  $90^\circ$  every 30 s around the horizontal axis while leaving inclination ( $67.6 \pm 0.8^\circ$ ) and intensity ( $48800 \pm 400 \text{ nT}$ ) at roughly local geomagnetic field levels in Oldenburg. During the second 5 min block, the birds were exposed

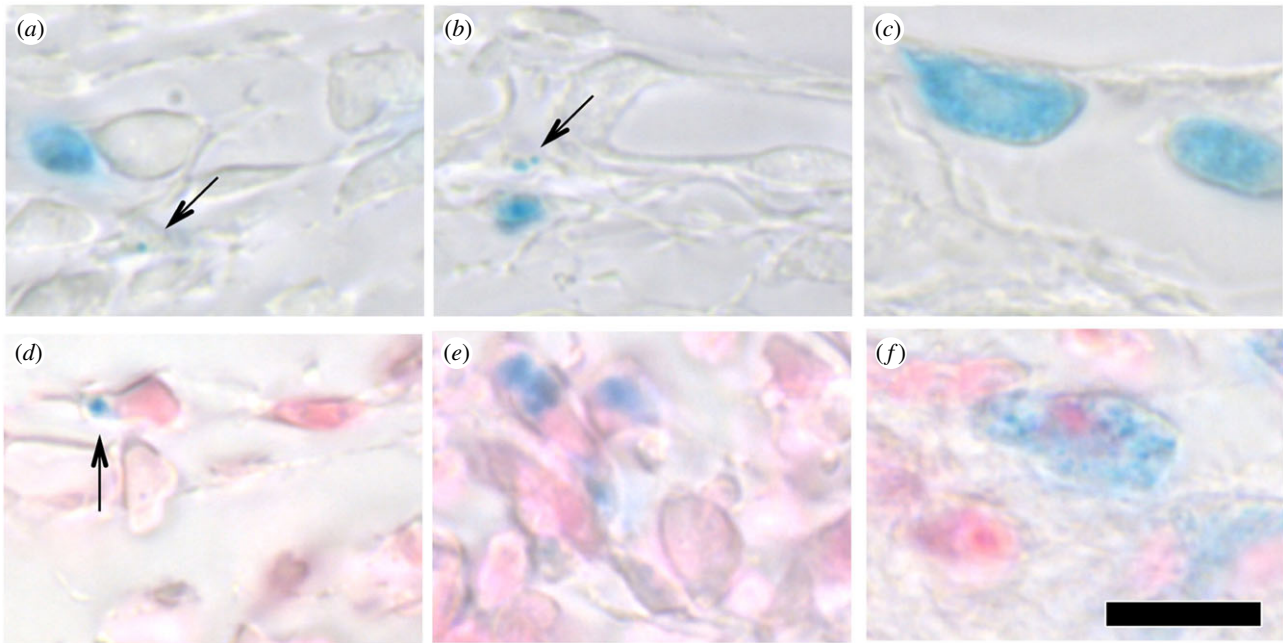


**Figure 3.** Distribution of PB-positive cells. (a–h) Graph showing the distribution of PB-positive cells along the rostral-caudal axis of the beak for eight individual European robins in 240  $\mu\text{m}$  increments. Approximate total cell counts are shown in the bottom right-hand corner, along with the bird number. Note the wide variation in the distribution and number of PB-positive cells per bird, and the different y-axis, e.g. for robin 19 (h).

to random and independent variations of each of the three components of the magnetic field (horizontal direction, inclination and intensity). Every 30 s, a value between  $-70\,000$  and  $+70\,000$  nT was randomly chosen for each of the three magnetic axes. The randomized aspects of the stimulus were newly

generated for each 5 min period. This resulted in magnetic field stimuli that strongly varied in field intensity (18 500–111 000 nT), horizontal direction ( $0$ – $359^\circ$ ) and inclination ( $-84.9$  to  $+76.6^\circ$ ). Both stimulus blocks repeatedly alternated every 5 min. As excess motor behaviour [63] and/or sensory, ‘non-magnetic’ mechano-





**Figure 4.** Iron-rich cells in the ventral subepidermis of robins. (*a–c*) Representative images of PB-positive cells found in the ventral subepidermis in European robins. Note the presence of punctate spheres (highlighted with arrows), as well as light blue cytoplasmic staining. (*d–f*) Representative images of PB-positive cells stained with nuclear fast red, showing that iron-rich cells in the ventral subepidermis are nucleated. Scale bar: 10  $\mu\text{m}$  (*f*, for *a–f*).

thermo- and nociceptors in the upper beak, palate and nasal cavity [64] can lead to trigeminal activation, we carefully monitored the behaviour of each bird in the test arena in real time using infrared cameras (840 nm). The magnetic exposure lasted 90 min and thus approximately reflects the duration of a typical behavioural orientation experiment [27–36] when considering the time window of approximately 15 min before a sensory stimulus induces detectable Egr-1 expression on the protein level [65,66].

### 5.3. Application of lidocaine

Following the methodological procedures published previously [27–36], shortly before magnetic exposure, a cotton bud soaked with 2% xylocaine (lidocaine; Astra Zeneca, Wedel, Germany, PZN: 03839482) was gently rubbed along the mucous skin at the inner edges of the upper beaks of the experimental animals.

### 5.4. Nerve sectioning

Birds were fully anaesthetized through an intramuscular injection of ketamine/Domitor<sup>®</sup> and immobilized using a custom-built head holder. We gained access to V1 through an incision along the dorsal rim of each eye and gentle retraction of the eyeball. Approximately 5 mm of both V1s were removed to prevent refusion. After the surgery, all cuts were resealed with cyanoacrylate surgical glue. After at least 60 min, antisedan was used to antagonize anaesthesia. Supplemental analgesia (Metacam; Boehringer Ingelheim, Ingelheim, Germany) was orally provided up to 72 h post-surgery. Birds had at least one week to recover from the surgery before participating in any further experiments. For the sham-ablated birds, the entire surgical procedure was performed as described above except that the nerve was left intact.

### 5.5. Tissue processing

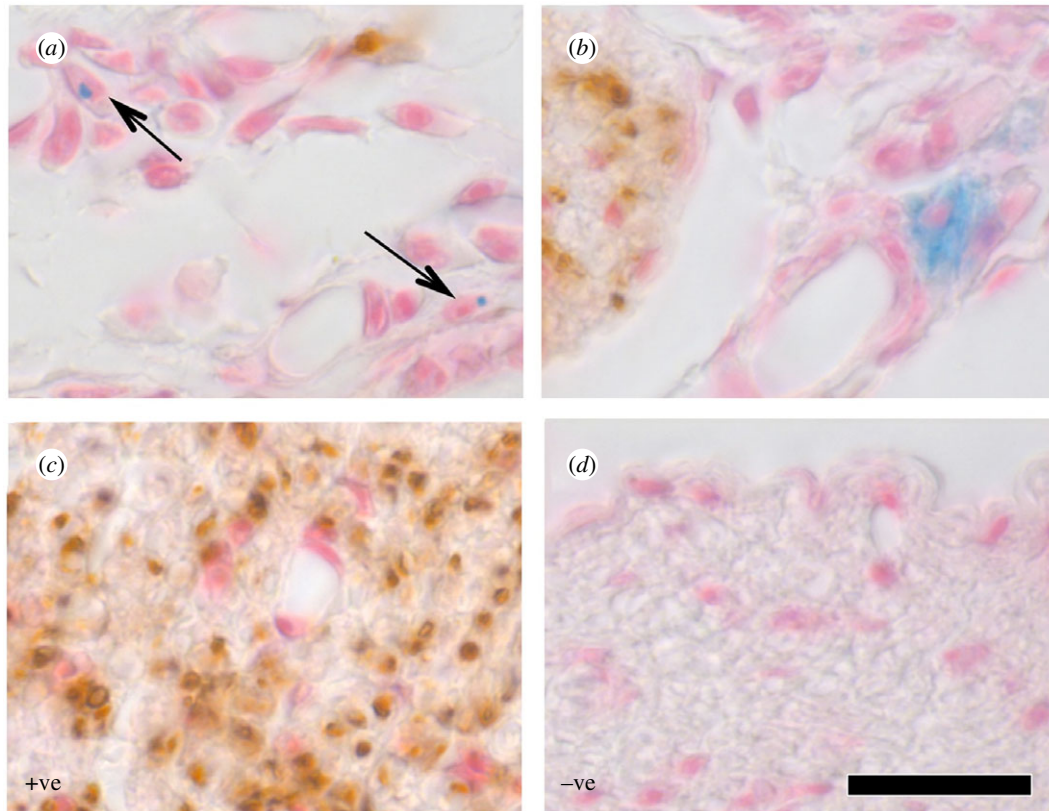
Immediately after the magnetic exposure, the birds were deeply anaesthetized with an overdose of ketamine and Domitor and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS).

The brains were extracted from the skull, post-fixed for 18 h, cryoprotected in 30% sucrose/PBS and cryo-sectioned into six parallel series of 40  $\mu\text{m}$  thick slices. Beaks were post-fixed for 18 h with 4% PFA/PBS, dehydrated in a graded series of ethanol (70%, 96% and 100%), embedded in paraffin, cut with ceramic-coated blades into 10  $\mu\text{m}$  sections and mounted on electrostatic slides.

### 5.6. Behavioural molecular mapping

Behavioural molecular mapping has successfully been used to link specific behaviour and magnetoreception to brain activity patterns in numerous studies (e.g. [51,67–70]). This method is based on the detection of immediate early genes, e.g. Egr-1 (ZENK, zif-268, NGFI-A and krox-24; [71]), which is expressed in most parts of the bird's brain [72] including the trigeminal brainstem complex [17,51–53]. Egr-1 protein reaches its expression peak after approximately 60 min and can be kept at high levels for several hours with a continuously variable stimulus [65,66].

Every second section of the six parallel series of brain slices from each bird were reacted free-floating with the immuno-ABC-technique [73] using glucose oxidase instead of hydrogen peroxide [74]. Each incubation step was followed by rinsing the brain sections three times in PBS for 5 min each. Endogenous peroxidases were saturated by incubation with 0.3% hydrogen peroxide for 30 min, and unspecific binding sites were blocked with 10% normal goat serum (Kraeber, Ellerbek, Germany) dissolved in PBS containing 0.3% Tween-20 (PBS-T) for 30 min. Slices were incubated for 72 h with rabbit polyclonal Egr-1 antibody (sc-189; Santa Cruz, CA, USA; working dilution: 1 : 1000 in PBS-T) at 4°C with gentle agitation. Afterwards, sections were incubated for 60 min each with biotinylated secondary antibodies and avidin-coupled peroxidase complex (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was detected using a 3',3'-diaminobenzidine (Sigma, Deisenhofen, Germany) reaction. The substrate reaction was stopped in 0.1 M sodium acetate. Sections were mounted on glass slides, dehydrated in a graded series of ethanol (70%, 96% and 100%) and coverslipped. One series per bird was stained for acetylcholine esterase activity to facilitate the determination of anatomical boundaries in the trigeminal brainstem [51–53,75].



**Figure 5.** Neuronal immunostaining. (a,b) Representative images of the ventral subepidermis stained with PB, nuclear fast red (pink) and TUBB3 (brown). We found less than 0.6% of PB-positive cells colocalized with TUBB3 ( $n = 735$  cells in eight birds). Arrows in (a) highlight PB-positive cells. (c) A positive control, TUBB3 staining of the ophthalmic branch of the trigeminal nerve. (d) A negative control (no primary antibody) when staining the ophthalmic branch of the trigeminal nerve. Scale bar: 10  $\mu\text{m}$  (d, for a–d).

### 5.7. Quantification of magnetic field-induced Egr-1 expression

We counted the number of Egr-1-positive neurons in every second section through both PrV and SpV (at levels of the vestibulo-cochlear nerve (N.VIII)) on both sides of the brain. Thus, the reported results approximately reflect half of the total number of activated neurons in these brain areas. Countings from the two hemispheres were pooled, because we did not observe any significantly lateralized expression.

The following strict double-blind protocol was followed to exclude ‘wishful thinking’ artefacts from our analyses: blindness to the experimental conditions was achieved by mounting brain slices on blinded glass slides. The number of Egr-1-expressing neurons was counted independently by three experimenters who were unaware of the experimental conditions each bird underwent. Potential biases based on different staining intensities [51–53,76] were minimized by staining slices from birds belonging to each of the experimental groups simultaneously. Thus, three sets of brain slices from a given individual, placed on three different microscope slides, underwent the above-mentioned staining procedure and analyses three independent times. To limit the number of animals killed, 10 of the 19 individuals belonging to either the ‘Sham’ and/or ‘Sect’ group had already participated in a previous study [51], but they were independently and blindly re-quantified by experimenters who were not related to [51]. All additionally used individuals were tested in the same buildings with the same level of reticular formation screening in identical set-ups under identical conditions as described in [51].

### 5.8. Prussian blue staining

The PB staining followed the procedures described previously [46,47]. In short, every third beak slide was deparaffinized and

stained in 5% potassium hexacyanoferrate in 10% HCl for 20 min, before washing three times in distilled water, and counterstained with nuclear fast red (Sigma, Deisenhofen, Germany). Following dehydration and mounting, PB-positive cells in the ventral subepidermis were counted using a Zeiss Axioplan 2 light microscope. For each beak, the number of PB-positive cells was counted on every 12th section and multiplied by a factor of 12 to obtain estimated total cell counts.

### 5.9. Anatomical mapping

To determine the spatial and numerical distribution of PB-positive cells, we constructed a model beak by averaging the dimensions of all analysed beaks using the following five anatomical landmarks: (i) central septum of the olfactory epithelium changing from a ‘U’ to a ‘V’ shape; (ii) central septum joining the ventral nasal cavity; (iii) distal tip of the small lateral buds of the nasal cavity; (iv) distal tip of the nasal cavity; and (v) rostral end of the beak (figure 2). We normalized and displayed the total estimated PB-positive cell counts for every bird to this anatomical standard in 56 240  $\mu\text{m}$  thick increments along the rostro-caudal axis of the beak (figure 3).

### 5.10. TUBB3 immunohistochemistry

The TUBB3 gene encodes for Tubulin beta-3 protein and is constitutively expressed in neural tissues [77]. Slides adjacent to those with PB-positive cells were selected for TUBB3 staining, deparaffinized, washed in PBS (pH 7.4) and incubated with a monoclonal mouse TUBB3 antibody (MMS-435P; Covance, Princeton, NJ, USA; working dilution: 1:1000) overnight in 0.1% Triton PBS (pH 7.4) with 2% skimmed milk in a humidifying chamber. A mouse avidin–biotin complex kit (Vector Laboratories, Burlingame, CA, USA) coupled with the chromophore



DAB (3,5-diaminobenzidine) (Vector Laboratories) was used to visualize staining of the primary antibody. Afterwards, sections were washed three times in PBS and stained with nuclear fast red [46,47] or potassium hexacyanoferrate, as described in the 'Prussian blue staining' section.

**Ethics.** All animal procedures were performed in accordance with local and national guidelines for the use of animals in research and were approved by the governmental authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit/LAVES, Oldenburg, Germany, Az.: 33.12-42502-04-10/0423).

**Data accessibility.** Raw data are provided in the electronic supplementary material data accompanying this manuscript.

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