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Cryptochrome 1b: a possible inducer of visual lateralization in pigeons?

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Abstract

The visual system of adult pigeons shows a lateralization of object discrimination with a left hemispheric dominance on the behavioural, physiological and anatomical levels. The crucial trigger for the establishment of this asymmetry is the position of the embryo inside the egg, which exposes the right eye to light falling through the egg shell. As a result, the right-sided retina is more strongly stimulated with light during embryonic development. However, it is unknown how this embryonic light stimulation is transduced to the brain as rods and cones are not yet functional. A possible solution could be the blue-light-sensitive molecule cryptochrome 1 (Cry1), which is expressed in retinal ganglion cells (RGCs) of several mammalian and avian species. RGCs have been shown to be functional during the time of induction of asymmetry and possess projections to primary visual areas. Therefore, Cry1-containing RGCs could be responsible for induction of asymmetry. The aim of this study was to identify the expression pattern of the Cry1 subtype Cry1b in the retina of embryonic, post-hatch and adult pigeons by immunohistochemical staining and to show whether Cry1b-containing RGCs project to the optic tectum. Cry1b-positive cells were indeed mainly found in the RGC layer and to lesser extent in the inner nuclear layer at all ages, including the embryonic stage. Tracing in adult animals revealed that at least a subset of Cry1b-containing RGCs project to the optic tectum. Thus, Cry1b-containing RGCs within the embryonic retina could be involved in the induction of asymmetries in the visual system of pigeons.

Introduction

Brain asymmetries can be found in a multitude of vertebrate species and affect various functions such as limb use, vocalization and object discrimination (Güntürkün, 2002; Ocklenburg *et al.*, 2013a; Ströckens *et al.*, 2013). The last decade has seen a surge in insights into the phylogeny and ontogeny of brain asymmetries (Vallortigara & Rogers, 2005; Schaafsma *et al.*, 2009; Roussigne *et al.*, 2012). However, core questions about the critical mechanisms that trigger such asymmetries during ontogeny are still unanswered. Amongst others, genetic (Domenichini *et al.*, 2011; Ocklenburg *et al.*, 2013b) and environmental (Rogers & Deng, 1999; Ocklenburg *et al.*, 2010) factors seem to play an important role in asymmetry formation. An excellent model organism in which to study the induction of neuronal lateralization by environmental factors is the pigeon, which exhibits a lateralization of its visual system. In this system, the right eye and left hemisphere show a distinct dominance for various discrimination tasks (Güntürkün, 1985; Yamazaki *et al.*, 2007), based on anatomical asymmetries in the tectofugal visual pathway (Güntürkün *et al.*, 1998). This lateralization is induced during embryonic development by asymmetrical light stimulation (Skiba *et al.*, 2002). Pigeon embryos take an asymmetrical position inside the egg, with the right eye pointing towards the eggshell while the left eye is occluded by the body (Fig. 1; also Kuo, 1932).

Every time the brooding hen stands up, light falls on the egg and the right eye is stimulated to a higher degree than the left, triggering visual asymmetries (Prior *et al.*, 2004; Buschmann *et al.*, 2006). There is, however, a major problem in this argumentation: During the early phase of induction of asymmetry, between embryonic days (E)14 and 17, the classic photoreceptors (rods and cones) are not yet functional (Bagnoli *et al.*, 1985; Rojas *et al.*, 2007). As pigeons hatch on E17, light input cannot be a critical factor as the embryos should be blind. What would be needed instead is a second group of photosensitive molecules located outside rods and cones that could mediate the induction of visual asymmetries. Such non-image-forming photoreceptors have been identified in the retinae of several mammalian and avian species and are assumed to subserve a role in circadian rhythm (Sancar, 2000; Van Gelder, 2003, 2005). One subgroup of these molecules is the cryptochrome 1 (Cry1) family. These molecules are blue-light photoreceptors localized in cones and retinal ganglion cells (RGCs; Miyamoto & Sancar, 1998; Mouritsen *et al.*, 2004). As RGCs mature early during embryonic development and establish first connections to the optic tectum at E16 (Manns & Güntürkün, 1997), it is possible that cryptochromes expressed in

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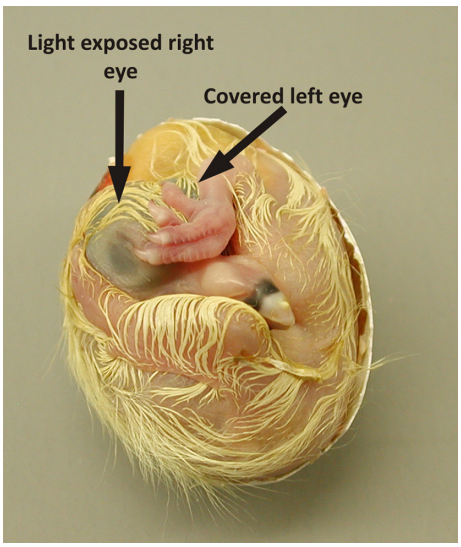

FIG. 1. Position of a pigeon embryo in the egg at E16, 1 day before hatching. The eggshell, chorion and amniotic sac that overlay the embryo were removed. Note the exposed right eye next to the eggshell and the left eye that is occluded by the body.

these cells mediate the induction of asymmetry. In birds, two cryptochrome subtypes, Cry1a and Cry1b, have been identified (Möller *et al.*, 2004). While Cry1a is only expressed in cones (Niessner *et al.*, 2011), which are not present in the asymmetry induction phase, Cry1b could be responsible for induction of asymmetry. To check whether Cry1b is a candidate we performed a series of experiments to show: (i) whether Cry1b is in general expressed in the pigeon's retina; (ii) whether it is expressed during embryonic and/or post-hatch development; and (iii) whether it is expressed in RGCs projecting to the asymmetrically organized tectofugal system.

Materials and methods

Labelling of Cry1b in adult animals

To map the general distribution of Cry1b in the retina of pigeons, 12 adult pigeons (*Columba livia*) of unknown sex were purchased from local breeders and placed in a dark box for 1 h to induce a retraction of rod and cone outer segments from the pigment epithelium. Animals were then deeply anesthetized with pentobarbital and transcardially perfused with a 4% paraformaldehyde (PFA) solution in phosphate-buffered saline, pH 7.4 (PBS). During perfusion, eyes were additionally injected with 4% PFA to ensure proper fixation of the retina. Following perfusion, eyes were removed and placed in a postfixation solution (4% PFA and 30% sucrose in PBS) for 1 h. After overnight incubation in PBS, retinae were dissected and put into a bleaching reagent (0.9% NaCl and 7.5% H_2O_2 in 10 mL distilled water with 50 µL of 25% NH_3 directly before incubation) for 30 min to bleach the pigment epithelium, covering outer segments of rods and cones. Retinae were then rinsed 3 × 10 min in PBS followed by removal of pigment epithelial pieces which were detached during bleaching. After incubation in a 30% sucrose solution overnight for cryoprotection, retinae were embedded in Tissue Tek© (Sakura Finetek Germany GmbH, Staufen, Germany), frozen in liquid nitrogen and stored at −80 °C for further usage. Slices 16 µm thick were cut using a cryostat (Leica Microsystems, Wetzlar, Germany) and directly mounted on microscope slides. For immunohistochemical staining, sections from the central retina were chosen. Staining against Cry1b was performed on slides as follows: slides were briefly washed in PBS to remove remaining Tissue Tek© and then incubated for 30 min in 0.3% H_2O_2. Afterwards, slides were rinsed 3 × 5 min in PBS followed by 1 h serum incubation (10% normal goat serum and 0.3% Triton X in PBS). Then slides were rinsed for another 3 × 5 min and incubated for 48 h in 1 : 1000 rabbit-anti-Cry1b primary antibody in PBS with 0.3% Triton X. The Cry1b antibody was custom-made by Genovac GmbH (Freiburg, Germany) and kindly provided by Christine Niessner (University Frankfurt, Germany). The antibody was directed against amino acidd 577–587 of chicken Cry1b (amino acid sequence: CNYGKPDKTSK). Specificity of the antibody was controlled by an ELISA performed by Genovac. As a control, antibody solution was blocked with 1 : 100 Cry1b peptide (Genovac GmbH). Antibody visualization was achieved with an ABC-DAB labelling with cobalt–nickel intensification (Manns & Güntürkün, 2003).

Labelling of Cry1b in post-hatch and embryonic pigeons

For five hatchlings of post-hatch days 1–2 and of unknown sex raised in our lab, the same method was used as for adults though bleaching or removal of pigment epithelium was skipped to avoid damage to the retina. For pigeon embryos a slight modification of the method was necessary. Two pigeon embryos at E16, bred in our lab, were removed from their eggs and decapitated. Eyes were removed and fixed overnight in postfixation solution. All further steps were performed as stated above.

Tectoretinal tracing

To reveal whether possible Cry1b-containing cells project to the optic tectum, four adult pigeons were injected with either the retrograde tracer choleratoxin subunit B (CtB; Sigma, Deisenhofen, Germany) or Texas Red® dextran amine 10 000 MW (TDA; Invitrogen Life Technologies GmbH, Darmstadt, Germany) into the optic tectum. For tracer application, animals were deeply anesthetized with ketamine and xylazine (0.12 mL/100 g body weight) and fixed in a stereotactic apparatus (Karten & Hodos, 1967). Feathers, skin and bones overlying the optic tectum were removed, while care was taken not to damage any muscles. A glass micropipette (inner tip diameter 12–15 µm) mounted on a nanolitre injector (World Precision Instruments, Sarasota, FL, USA) was used to inject a total of 1380 nL CtB as a 1% solution (w/v) in distilled water or 1104 nL TDA as a 12.5% solution (w/v) in distilled water with 3% DMSO into the optic tectum. Ninety-two nanolitres of tracer was injected at 15 (CtB) or 12 (TDA) injection sides at dorsal, medial and ventral positions between A1.50 and A5.50, according to the atlas of Karten & Hodos (1967), at a depth of 1 mm. Most ventral and dorsal parts were not injected to prevent leakage of tracer into outer liquor space or into diencephalic visual areas. After 72 h survival time, pigeons were transcardially perfused and retinae processed as stated above. For the CtB–Cry1b double labelling, a goat anti-CtB antibody (Calbiochem, Merck, Darmstadt, Germany, cat. no. 227040) at a dilution of 1 : 5000 and the rabbit-anti-Cry1b antibody at a dilution of 1 : 250 were applied, using the same protocol as stated above without H_2O_2 incubation. For visualization, slides were incubated in 1 : 1000 Alexa 488-coupled donkey anti-rabbit (Invitrogen; cat. no. A-11034) and 1 : 1000 Alexa 594-coupled donkey anti-goat antibody (Invitrogen; cat. no. A-11058) in PBS with 0.3% Triton X for 1 h. Confirmation of CtB injection side within the brain was performed on 40-µm-thick brain slices with the goat anti-CtB antibody

in a dilution of 1 : 5000 using the ABC-DAB labelling method described above (Manns & Güntürkün, 2003). Double labelling of TDA and Cry1b was achieved using the same protocol but only staining for Cry1b as TDA already shows fluorescence in the red spectrum. A part of the fluorescence-labelled slices were counterstained with 0.2% DAPI (4′,6-diamidino-2-phenylindole; Serva Electrophoresis GmbH, Heidelberg, Germany) in distilled water to alleviate identification of cell layers. Slices were analyzed using a Zeiss Axio Imager M1 Microscope (Carl Zeiss MicroImaging, Göttingen, Germany), with a 40 × objective (numerical aperture 0.75). All experiments were performed in compliance with the guidelines of the National Institutes of Health for the care and use of laboratory animals and were approved by a national committee (North Rhine-Westphalia, Germany).

Results

Staining for Cry1b in adult retinae revealed strong labelling of cells in the ganglion cell layer (GCL) with a majority (though not all) GCL cells being labelled. Furthermore, labelling could be observed in few scattered cells within the outer border of the inner nuclear layer (INL) next to the inner plexiform layer (Fig. 2A). Cry1b-positive cells were only labelled in their soma, sparing the nucleus. A weak Cry1b labelling of fibres exiting the GCL was observable in fluorescence staining; however, signal strength of this labelling was so low that it could represent a nonspecific binding of the antibody. In post-hatch retinae, the labelling pattern was similar to adult animals; however, fewer labelled cells were found in the INL (Fig. 2C). In embryonic retinae, labelled cells were also present within the GCL although with lower signal intensity than in adult and post-hatch animals. A very weak labelling in the INL was observable in embryos but signal intensity of this labelling was too low to confirm specific staining (Fig. 2D). Furthermore, cytoarchitecture in embryonic and post-hatch retinae was distinctively different from the adult, with the photoreceptor layer and the outer nuclear layer being less structured and more diffuse, confirming the immature state of these layers during the last stages of embryonic development in pigeons. A control experiment in which the Cry1b antibody was blocked by incubation with Cry1b peptide delivered no labelling at all (Fig. 2B).

Tectal injections of CtB or TDA in adult animals labelled a large number of cells in the GCL. For CtB, whole cell bodies sparing the nucleus were stained while TDA injections delivered only granular staining within the cytoplasm (Fig. 3). Control stains against CtB of brain slices of injected animals confirmed that all injections were placed correctly in the optic tectum, with no spread of tracer in other retinorecipient areas such as the nucleus geniculatus lateralis pars dorsalis. Double labelling of tracers and Cry1b showed an overlap of the two stainings though not all Cry1b-positive cells showed tracer labelling and vice versa. Counterstains with DAPI confirmed the positioning of both labellings within the GCL, demonstrating that the labelled cells were indeed RGCs (compare Fig. 3).

Discussion

This study shows for the first time that Cry1b is present in the pigeon retina. In embryonic, post-hatch and adult pigeons, Cry1b is expressed strongly in the majority of GCL cells and to a lesser extent in cells of the INL at the border with the inner plexiform layer. Furthermore, we could show by injections of retrograde tracers into the optic tectum that at least a subpopulation of Cry1b-containing RGCs project to this primary visual area in adult pigeons. Thus, Cry1b-positive RGCs could be the missing link between pre-hatch visual stimulation and the onset of asymmetries in the pigeon's visual system.

The discovery of Cry1b-positive ganglion cells is in line with similar data from chicken, garden warblers and mice. Haque *et al.* (2002) analyzed the distribution of Cry1 mRNA (not distinguishing between subtypes) in retinae of adult chicken and found the highest levels in the photoreceptor layer and the GCL, while the expression in the INL was lower. Mouritsen *et al.* (2004) performed immunohistochemical staining against Cry1 (again not distinguishing between subtypes) in the retinae of garden warblers and described that 95–100% of GCL and 10–15% INL cells were labelled, while photoreceptors evinced a very high expression pattern. As Niessner *et al.* (2011) showed that Cry1a peptide in chicken is expressed primarily in ultraviolet cones of the photoreceptor layer, it is likely that the Cry1 mRNA found by Haque *et al.* (2002) and the Cry1 peptide found by Mouritsen *et al.* (2004) in the GCL and INL is Cry1b. This further strengthens our results as we found Cry1b-labelled cells in pigeons only in the GCL and INL but not in the photoreceptor layer. In mice, Cry1 mRNA was restricted to GCL and INL, again supporting our findings (Miyamoto & Sancar, 1998). The fact that Cry1b in pigeons is present during the last stages of embryonic and post-hatch development gives for the first time evidence that the embryonic pigeon retina can transmit light-induced activity patterns towards central structures, thereby bypassing the still non-functional rods and cones. As discussed below, this could be a crucial missing link for the induction of visual object discrimination asymmetries in this species.

The injections of the retrograde tracers CtB and TDA into the optic tectum resulted in a large number of labelled cells in the GCL which were at least partly double-labelled with Cry1b. Thus, at least some of the RGCs are Cry1b-positive and project to the optic tectum, the largest primary visual areas in birds (Güntürkün, 2000). It is extremely likely that an even greater number of Cry1b-positive RGCs project to primary visual areas but were not labelled by our tracing. The reasons for this are that, first, our CtB and TDA injections did not cover the whole optic tectum and therefore some tectal-projecting RGCs were missed. Second, other subpopulations of RGCs are known to project to other primary visual structures of the mesencephalon and diencephalon (Güntürkün, 2000), and third, a very small proportion of RGCs project to the ipsilateral optic tectum (Weidner *et al.*, 1985), which was not covered by our tracing.

The presence of Cry1b during the critical phase of asymmetry induction could explain how visual asymmetries in pigeons are induced. Pigeons regularly leave their nests during breeding, allowing light to fall on their eggs for ~ 1 min each time. Though short, these phases of illumination are sufficient to establish asymmetries in the visual system which persist throughout the life of the animal (Buschmann *et al.*, 2006). It is likely that during these phases light transverses the eggshell, reaches the retina and causes an activation of Cry1b in RGCs. Indeed, Rogers (2006) showed that ~ 8% of the ambient light intensity can be detected within the egg. As the left eye is occluded by the embryo's body (see Fig. 1), the right eye receives a higher light stimulation and thus a stronger activation of Cry1b cells. We showed that Cry1b-containing RGCs project to the optic tectum and previous studies have reported that retinotectal projections are functional from E16 on (Manns & Güntürkün, 1997). Therefore, it is reasonable to assume that this imbalance of activation between left and right eye is also transferred to the optic tectum. Although it is likely that Cry1b-driven activation does not carry any image-forming information, the mere activity differences reaching the optic tectum could shape image-forming processing in

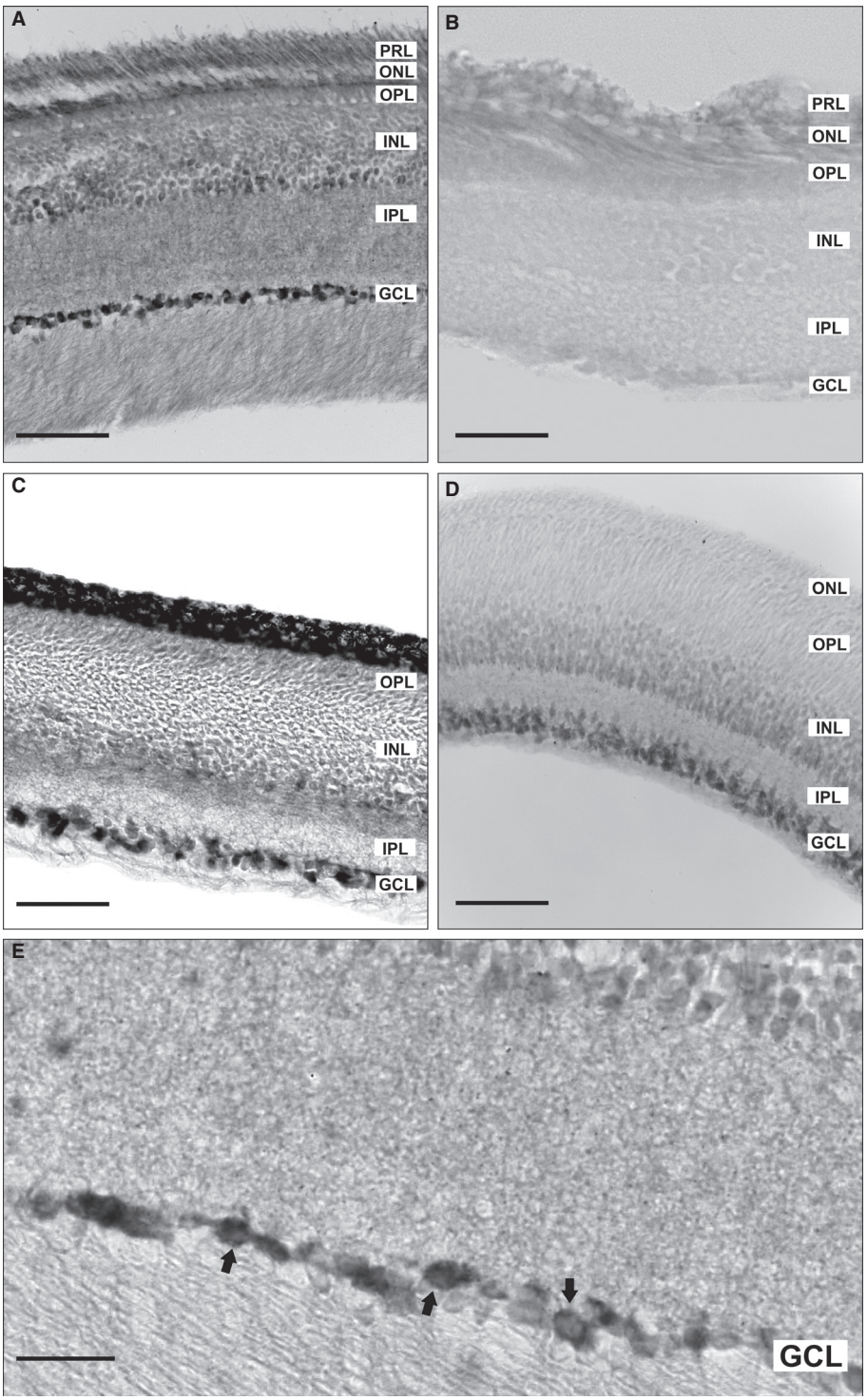

FIG. 2. (A) Cry1b-labelled cells in the GCL of adult pigeons. A small number of labelled cells were also visible in the INL. (B) Blocking of antibodies with Cry1b peptide prevented the staining. (C and D) Staining against Cry1b in (C) post-hatch and (D) embryonic pigeons revealed labelling in the GCL with few weakly labelled cells in the INL. Note that the strong black labelling in (C) covering the photoreceptors is the pigment epithelium which was not removed in this animal and has a natural black colour. (E) Higher magnification of Cry1b-labelled cells within the GCL of adult pigeons. Arrows indicate cells showing that Cry1b is restricted to the soma, leaving the nucleus blank. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer. Scale bars, 50 μm (A–D), 20 μm (E).

this structure. Blockade of right eye activity around hatch indeed prevents the establishment of left hemispheric asymmetry in object discrimination (Prior *et al.*, 2004). Such activity differences could for example trigger different expression patterns of neurotrophic factors in the left and right optic tecta. It has been shown that the neurotrophic factors BDNF and NT-3 as well as their corresponding high-affinity receptors TrkB and TrkC are already expressed in the optic tectum before hatching (Theiss & Güntürkün, 2001). Further-

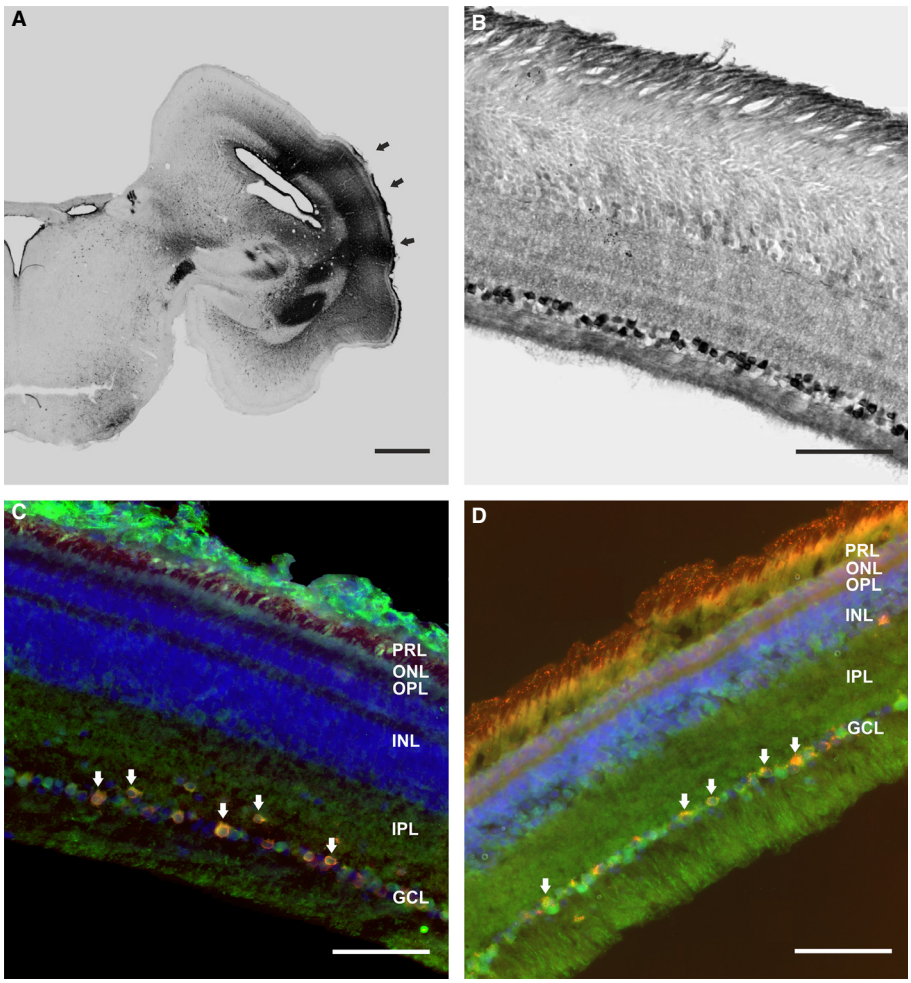

FIG. 3. (A) Immunohistochemical labelling of the injection sides of the tracer CtB into the optic tectum. Arrows indicate three separate injection sides. (B) Immunostaining against the tracer in retinal slices led to strongly labelled cells within the GCL. Injection of the tracer TDA at the same location led to an identical staining pattern in the retina (not shown). (C and D) Fluorescent double-labelling using immunostaining against Cry1b and the tracers (C) CtB or (D) TDA led to Cry1b-positive cells in the GCL and to few labelled cells in the INL (green labelling). Immunostaining of the tracers led again to labelled cells in the GCL (red labelling). The majority of traced cells also contained Cry1b, which led to yellow labelling (indicated by arrows). Retinae in (C) and (D) were counterstained with the cell marker DAPI (blue labelling). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer. Scale bars, 1000 μm (A), 50 μm (B–D).

more, Manns *et al.* (2005) demonstrated a transient asymmetric TrkB/Ras activity in the tectum of pigeon embryos, indicating a lateralized and light-input driven effect of BDNF. As the authors suggested, this could influence the development of structural asymmetries of the tectum (Güntürkün, 1997; Manns & Güntürkün, 1999, 2003) and of tectal projection patterns (Güntürkün *et al.*, 1998) in the tectofugal pathway, resulting in behavioural asymmetries of the adult animal.

When trying to identify a possible trigger for the induction of visual asymmetries in pigeons, one should consider other non-rod- or cone-based photoreceptive molecules in the avian retina besides Cry1b. Within the cryptochrome family, Cry1a and Cry2 are also expressed in the avian retina. However, as stated above, Cry1a has been shown to only be expressed in cones (Niessner *et al.*, 2011), which are not functional at the time of induction of asymmetry (Bagnoli *et al.*, 1985; Rojas *et al.*, 2007). Also, Cry2 is possibly not involved in the initiation of visual asymmetry: While there is a slight expression of Cry2 mRNA in the GCL in adult chickens, its strongest retinal expression is in the PRL and INL (Bailey *et al.*, 2002). Furthermore, Möller *et al.* (2004) have shown that the Cry2 sequence contains a nuclear localization signal and concluded that the nucleus is a rather unlikely position for a functional photoreceptor. It is therefore more likely that Cry2 plays a role in retinal clock functions, as has been shown by Liu *et al.* (2012). It should also be noted that, although Cry1 subtypes are in general believed to be photosensitive in birds (Bailey *et al.*, 2002; Möller *et al.*, 2004; Mouritsen *et al.*, 2004; Niessner *et al.*, 2011), the photoreceptive properties of cryptochromes have not yet been proven in this class. However, studies with human cryptochrome subtypes indicate that vertebrate cryptochrome can be photoreceptive (Foley *et al.*, 2011), which makes it very likely that this is, as assumed, also the case in birds.

In contrast to Cry1a and 2, melanopsin, an opsin-based photoreceptor identified in avian RGCs (Tomonari *et al.*, 2005), could play a role in the induction of visual asymmetries. Melanopsin is expressed in RGCs of neonatal mice and embryonic chicken (Johnson *et al.*, 2010; Verra *et al.*, 2011) and these RGCs project to primary visual areas (Van Gelder, 2003; Ecker *et al.*, 2010). Regarding the fact that there are two potential candidates for a photoreceptive substance to trigger visual asymmetries in pigeons, one might argue

that one of them should be sufficient to solve this task. However, this is not necessarily the case. Van Gelder (2003, 2005) suggested that co-expressed melanopsin and cryptochrome could function in collaborative ways. Melanopsin serves as the primary photoreceptor while cryptochrome, as a supplementary photoreceptor, is needed to reach the full amplitude of excitation. However, neither melanopsin expression in pigeons nor a possible synergy effect of Cry1b and melanopsin has been proven yet.

Taken together this study provides for the first time evidence for a rod- and cone-independent potential light sensitivity in the retina of embryonic pigeons. We furthermore showed that this sensitivity is based in RGCs, which project to primary visual areas. Though we currently have no direct evidence that Cry1b-containing RGCs are indeed activated by light during the critical phase, they remain a viable candidate for induction of asymmetry.

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Abbreviations

Cry1, cryptochrome 1; CtB, choleratoxin subunit B; E, embryonic day; GCL, ganglion cell layer; INL, inner nuclear layer; PBS, phosphate-buffered saline, pH 7.4; RGC, retinal ganglion cell; TDA, Texas Red® dextran amine, 10 000 MW.

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