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The Visual System of Palaeognathous Birds: The Case of the Chilean Tinamou (*Nothoprocta perdicaria*)

Quirin Krabichler

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Vorsitzender:	Prof. Dr. Michael Schemann
Prüfer der Dissertation:	1. Prof. Dr. Harald Luksch
	2. Priv.-Doz. Dr. Mario Wullimann
	3. Prof. Dr. Manfred Gahr

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Nature is a harmonious mechanism where all parts, including those appearing to play a secondary role, cooperate in the functional whole. In contemplating this mechanism, thoughtless men arbitrarily divide its elements into essential and secondary, whereas the cautious thinker is content with classifying them as understood and poorly understood, regardless of their size and immediate usefulness. No one can predict their importance in the future.

—Santiago Ramón y Cajal, *Advice for a Young Investigator*, 1897

In loving memory of my grandparents
Marianne and Josef Wiggering.

Table of contents

List of figures

List of abbreviations

AOS	accessory optic system
BDA	biotinylated dextran amine
CTB	Cholera toxin B subunit
CVS	centrifugal visual system
GCL	ganglion cell layer
GLd	nucleus geniculatus lateralis, pars dorsalis
GLv	nucleus geniculatus lateralis, pars ventralis
GT	tectal gray
IPL	inner plexiform layer
INL	inner nuclear layer
IOC	isthmo-optic complex
ION	isthmo-optic nucleus
DTN	dorsal terminal nucleus
LM	nucleus lentiformis mesencephali
LTN/MTN	lateral and medial terminal nuclei
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
nBOR	nucleus of the basal optic root
NOT	nucleus of the optic tract
OPL	outer plexiform layer
ONL	outer nuclear layer
PHAL	Phaseolus vulgaris leucoagglutinin
PND	posterior nodal distance
RGC	retinal ganglion cell
RITC	Rhodamine-B-isothiocyanate
SRP	spatial resolving power
vSCN	visual suprachiasmatic nucleus

Short abstract / Kurzzusammenfassung

English:

Birds are highly visual animals and have been widely studied as models for the vertebrate visual system and its evolution. However, research has centered on only one of the two grand phylogenetic lineages of birds, Neognathae, while Palaeognathae have been mostly ignored. In my dissertation, I have conducted a comprehensive study of important elements of the visual system of a palaeognathous bird, the Chilean Tinamou (*Nothoprocta perdicaria*). These elements are the visual field, the retinal ganglion cell topography, the visual acuity, the neuroanatomy of the retinofugal projections to the brain, and the centrifugal visual system which projects from the brain back to the retina.

German:

Vögel sind außerordentlich visuelle Tiere und sind bereits intensiv als Modelle für das visuelle System der Wirbeltiere und seiner Evolution untersucht worden. Jedoch hat sich die Forschung nur auf eine der beiden großen phylogenetischen Linien der Vögel konzentriert, Neognathae, während Palaeognathae weitestgehend unbeachtet geblieben sind. In meiner Dissertation habe ich eine umfassende Studie wichtiger Elemente des visuellen Systems eines palaeognathen Vogels, des chilenischen Steißhuhns (*Nothoprocta perdicaria*) durchgeführt. Diese Elemente umfassen das visuelle Feld, die retinale Ganglionzell-Topographie, die Sehschärfe, die Neuroanatomie der retinofugalen Projektionen ins Gehirn, und das centrifugale visuelle System, welches vom Gehirn zurück zur Retina projiziert.

1. Introduction

1.1 Preface: Vision in vertebrates

Vision is one of the most complex senses in the animal kingdom (Werner and Chalupa 2013). It is based on the ability of photosensitive sensory neurons in an organism to absorb a distinct spectrum of the sun's electromagnetic radiation, and generate signals which are propagated to the central nervous system. There, complex neural operations integrate these activity patterns with patterns from other sensory systems and the brain's internal state. The result is what may be called visual perception and enables the organism to adapt its behavior according to visual stimuli in its environment. Vision is a phylogenetically ancient sense, and complex visual systems are found in representatives of both vertebrates and invertebrates (Sanes and Zipursky 2010). Because vertebrates and invertebrates possess remarkably similar genes and gene expression patterns for photoreceptors as well as eye and brain development, it is assumed that their common bilaterian ancestors in the late Precambrian had already possessed a visual system with a more primitive but fundamentally similar functional organization (Lamb, Collin, and Pugh 2007; Kaas 2009, 71, 140; Sanes and Zipursky 2010; Lamb 2013; Zhao et al. 2013).

Vertebrates[1] have evolved a particularly sophisticated visual system. One of the principal foundations of this course of history can be found in the principal architecture of their eyes. Their general function is to convey the optical projection

[1] Here and subsequently, „vertebrates" refers to jawed vertebrates (Gnathostomata) and lampreys (Hyperoartia), but not hagfishes (Myxini), whose visual system is quite exceptional (Lamb 2013; Lamb, Collin, and Pugh 2007).

of viewed objects as focused images onto a two-dimensional “screen” in the back of the eye, the retina. This is achieved by means of a pupil through which light rays from environmental objects pass similar as in a pin-hole camera, and a dioptric apparatus consisting of cornea and lens which focus these light rays and can actively change their refractive power for accommodation (Hughes 1977; Schaeffel, Murphy, and Howland 1999; Chung and Marshall 2014; Glasser, Troilo, and Howland 1994). The eye’s optical properties depend on the structure and organization of these ocular elements but also on the size of the eye, because larger eyes generally produce bigger images on their retina which allows for higher visual resolution or acuity. A further foundation of the sophistication of the visual system of vertebrates lies in their retina, which is far more than only a photographic film or image sensor. It is a neural interface consisting of millions of neurons that are inter-connected through complex circuitries (Lamb 2013). Thus, the signals which ultimately leave the retina towards the brain have already been transformed by the inter-retinal neural operations of these circuits. Furthermore, there is not only one single ‘output channel’ from the retina to the brain. Vertebrates had already very early in their evolution developed functionally segregated parallel visual pathways projecting from the retina to particular brain regions (Joselevitch and Kamermans 2009; Stone 1983). Finally, the primary visual centers receiving the retinofugal terminals are interconnected among themselves and with other visual areas of the brain. The neuroanatomy of these so-called retinofugal projections and their recipient areas in the brain are important aspects of the functional organization of the vertebrate visual system.

Thus, the visual system of vertebrates is composed of an array of elements or subsystems, including the eye and its optic apparatus, the retina, the retinofugal pathways and the central visual pathways. They are all in some way interconnected or interdependent and all of them play their parts in the visual function, ecology and behavior of animals. Importantly, these elements have to a certain degree remained relatively similar among different species, while evolution has

at the same time also produced much diversity which often corresponds to functional adaptations to particular life-styles. In order to understand the vertebrate visual system it is therefore necessary to comparatively study its elements in different species in the context of their phylogenetic relations as well as their particular life-styles.

1.2 Birds as models for studying the visual system

Much of the research investigating the vertebrate visual system has focused on mammalian model organisms such as mice, cats, and primates. The many other groups of vertebrates have generally been payed less attention. This is obviously so because we humans are foremost interested in understanding how vision works in ourselves, so we preferably study our mammalian relatives. However, it is equally important to also study non-mammalian vertebrates, for two main reasons: (1) by its own merit, since learning about how their diverse visual systems work helps us to better understand their ecology and behavior; and (2) to establish the fundamental principles underlying the functional organization and evolution of the visual system in vertebrates, which ultimately provides an indispensable context for understanding our own.

In both respects, birds are an exceptionally well-suited model for studying the visual system. Notably mammals are derived from nocturnal animals which had been – figuratively speaking – living in the shadow of the dinosaurs for many millions of years. This had led to a remarkable reduction of the visual system and the loss of some of its capacities such as tetrachromatic color vision (which is common in non-mammalian vertebrates). Most mammals including Rodentia (e.g. mice and rats) and Carnivora (e.g. cats) are only dichromatic, and solely the ancestors of today's primates managed to re-evolve at least trichromatic vision (D. M. Hunt et al. 2009). Birds on the other hand have always been mostly diurnal and can be arguably regarded as the most visual of all extant vertebrates (Butler 2012): Their eyes are huge relative to their brains size (Martin 1993), they have

tetrachromatic retinae with high densities of visual neurons, and vast parts of their highly developed brains (Olkowicz et al. 2016) are directly or indirectly related to vision (Cook 2000, 2001; Güntürkün 2000; Hodos 1993; D. M. Hunt et al. 2009; Martin 1993; Qadri and Cook 2015; Shimizu, Patton, and Husband 2010). Therefore, birds have been very valuable models in the field of visual neuroscience (Shimizu, Patton, and Husband 2010; Soto and Wasserman 2012), and also constitute the objects of study for the present work.

1.3 Studying the visual system of birds: Different points of view

Given that the visual system is that complex and consists of that many different elements, it is not possible for a single study to cover all aspects of it. For the present thesis, five elements were selected which represent highly relevant aspects of the visual system of birds and are therefore well suited for its comprehensive description in a species of interest: the visual field, the retina and its topography, the visual acuity, the retinofugal projections to the brain, and an important central visual pathway of birds, the centrifugal visual system, which is a feed-back projection from the brain to the retina. On the following pages, I will give introductions to each of these topics.

1.3.1 Visual fields, their implications and functions

The visual field is the portion of the three-dimensional space surrounding an animal's head, which is projected through the eyes onto the retinae at any time (Martin 2007). It is thus defined by the directions in which the eyes are facing (Heesy 2008; Heesy and Hall 2010), which in turn depends mainly on the anatomy of the eye sockets (orbita) in the skull. If the eyes' optic axes (optic axis = line through the centers of curvature of the lens and cornea; Walls 1942) point in

opposite directions, we speak of lateral eyes; if they point forwards, of frontal eyes (Figure 1). Birds, and vertebrates in general, possess a high diversity of visual field topographies, which have considerable relevance for the visual ecology of species.

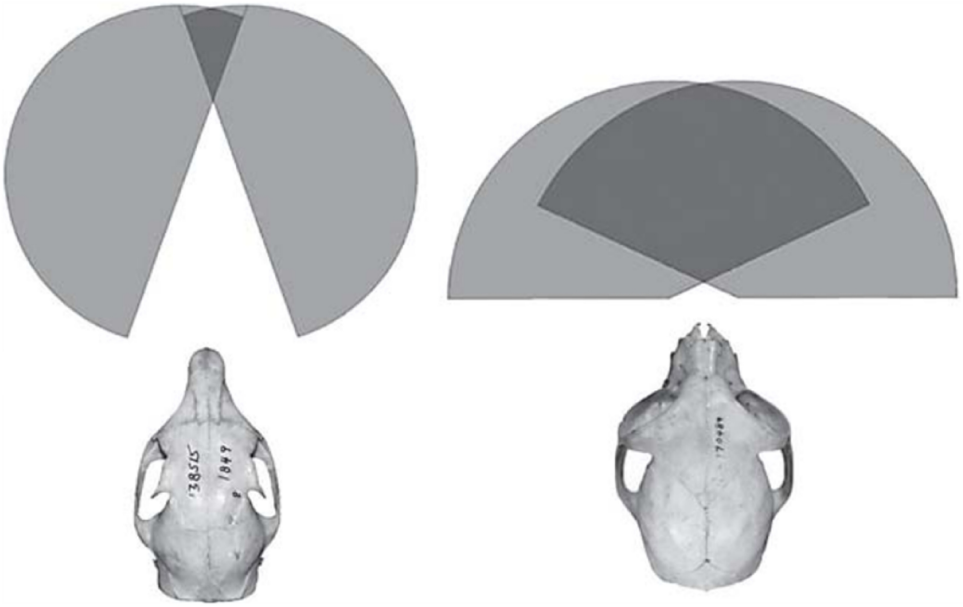

Figure 1: Dependence of the visual fields on orbit convergence in the skull. Laterally facing orbits and eyes produce lateral visual fields (left), while frontalized (convergent) orbits produce more frontal visual fields. Reproduced from Heesy 2008, with permission from Karger publishers (licence# 4124160733290).

In scientific studies of the visual field, the most relevant parameters are the cyclopean field, the binocular overlap, and the blind sector (Figure 2) (Martin 2009, 2014). The cyclopean field represents the total angular width of an animal's surrounding world, which is surveilled by the retinae of its both eyes together (Hughes 1977). The binocular sector results from frontalization of the eyes, which in turn depends on the convergent orientation of the orbita of the skull (Heesy 2004). This makes the monocular fields of both eyes overlap: the more frontally the eyes are directed, the bigger is the binocular overlap (Walls 1942) (see also Figure 1 & Figure 2). The blind sector is the part of the space around an animal's head that cannot be observed at all by the retina of either eye. It can depend on

the morphology of the head and the eyes, but similar as the binocular overlap it mostly depends on the degree of orbit convergence: the more frontalized the eyes, the bigger the blind sector (Figure 2). With respect to the binocular as well as blind sectors, both their angular dimensions and their positions relative to the animal's head can be relevant parameters in the study of visual fields (Martin 2014).

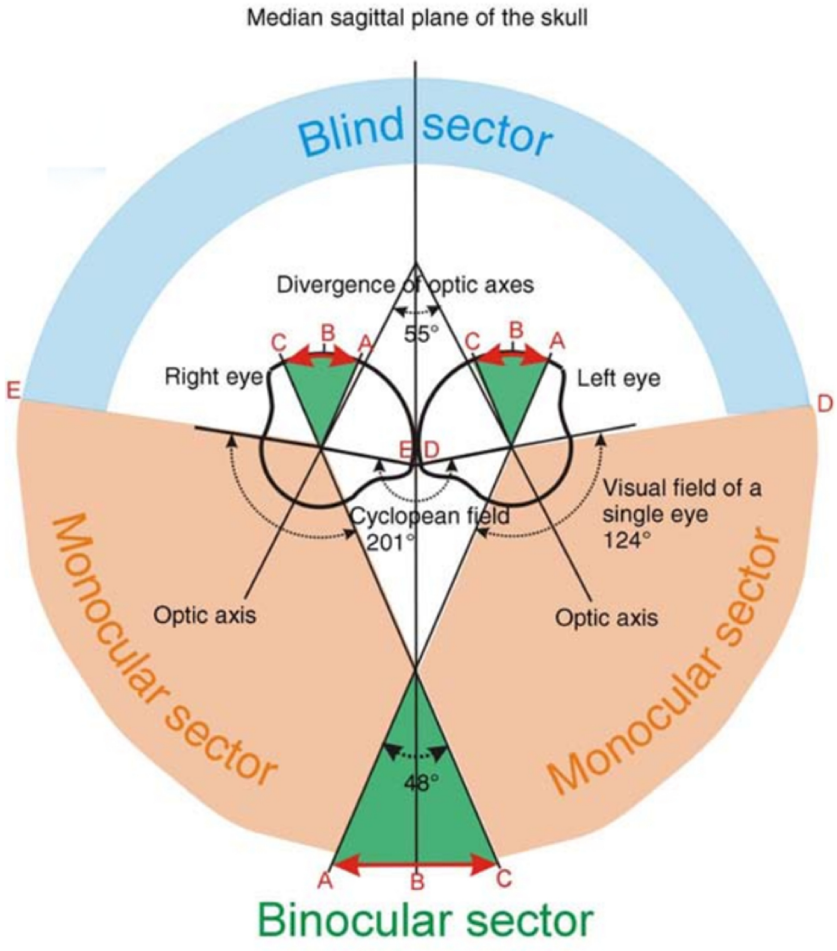

Figure 2: Different aspects of the visual field. The schematic represents a view from above onto a horizontal section through the skull of a tawny owl at the level of the eyes (frontal is downwards). Each eye has a visual field of 124°. The two eyes have a divergence angle of 55° relative to each other. This results in a binocular overlap of 48°, while most of the combined cyclopean visual field of 201° is monocular. Since the eyes are not lateral but quite frontal, a relatively large blind sector behind the head results. Reproduced from Martin 2009, with kind permission from the Association for Research in Vision and Ophthalmology (ARVO).

As already indicated, different vertebrate species possess different visual field topographies. Since the visual field has important consequences for how an animal sees the world and thus for its ecology and behavior (Hughes 1977; Martin 2012), its layout reflects in many ways the animal's life style and evolutionary history (Martin 2014). We humans as well as our primate relatives are animals with highly frontalized eyes, whose optical axes are directed forward almost in parallel (Heesy 2009; Hughes 1977). Also many non-primate vertebrate species have evolved frontalized eyes to various degrees. Eye frontalization (or orbit convergence) can be seen as a trade-off between beneficial and detrimental effects (Fernández-Juricic, Erichsen, and Kacelnik 2004; Fernández-Juricic et al. 2008; Martin 2009, 2014). On the downside, converging the eyes forward means decreasing the cyclopean visual field and increasing the blind sector behind the animal. This complicates surveillance of the environment surrounding the animal, making head movements necessary in order to compensate for the blind sector (Fernández-Juricic 2012). On the upside, eye frontalization results in a binocular overlap between the visual fields of both eyes, which opens a window to entirely new visual possibilities. A binocular overlap can have different functions. At least three distinct potential visual advantages can be distinguished, whose relevance may however depend on the specific visual behavior and life style of a species: Enhanced light sensitivity, enhanced contrast discrimination (both of which can be grouped under the term binocular summation), and real stereopsis by binocular fusion (Heesy 2009).

The function which we most commonly associate with binocular vision is the latter, stereopsis. By viewing the same object from the different vantage points of the two eyes at the same time, the two retinal images contain a spatial disparity of the fixed object relative to the background (Heesy 2009). When both images are combined in the brain, these disparities are interpreted to generate a powerful depth perception, which permits judgement of relative distances (Hughes 1977). However, while true stereopsis has been demonstrated in various mammals (Hughes 1977), especially in primates (Heesy 2009), it has been shown in only very few non-mammalian vertebrates conclusively (Hughes 1977;

Harmening and Wagner 2011; van der Willigen 2011; Nieder and Wagner 2000; Wagner and Frost 1993). Thus, although stereopsis appears to be a useful ability, it is questionable whether it is an important driver in the evolution of binocularity in general. One principal argument against it is that the ability to perceive stereopsis first requires more than only orbit convergence, in particular the neural circuits in the brain necessary for performing binocular fusion. It is therefore probable that other visual operations are the primary drivers of the evolution of binocularity, such as binocular summation which is advantageous for animals with a visually active nocturnal life style since it enhances signal-to-noise ratio, light sensitivity, and contrast sensitivity (Heesy and Hall 2010; Vega-Zuniga et al. 2013; Heesy 2009).

In birds, the story appears to be altogether a bit different. Most birds possess lateral eyes with relatively little orbit convergence (Heesy and Hall 2010) and consequently a small binocular overlap (Walls 1942; Fernández-Juricic, Erichsen, and Kacelnik 2004; Martin 2007). Some species do have increased binocular overlap, but with the exceptions of owls (Martin 1984) and various passerines (Baumhardt et al. 2014; Fernández-Juricic et al. 2008; Fernández-Juricic, Gall, et al. 2011; Moore et al. 2013, 2015), their binocular field usually does not exceed 30° in width (Martin 2009). It has been shown that birds use their frontal field for other tasks than their lateral fields: Slow-moving or stationary objects are fixated by adopting a frontal gaze, while fast-moving objects are fixated by a lateral gaze (Maldonado, Maturana, and Varela 1988). While this might give them the appearance of utilizing stereoptic vision, only very few birds seem to actually possess the ability of true stereopsis (Casini, Fontanesi, and Bagnoli 1993; Tyrrell and Fernández-Juricic 2017b), which has been neurophysiologically and behaviorally confirmed only in owls (Pettigrew and Konishi 1976; van der Willigen 2011; Wagner and Frost 1993; Nieder and Wagner 2000; Harmening and Wagner 2011) and falcons (Fox, Lehmkuhle, and Bush 1977). Apart from these few exceptions, the low degree of binocularity in most birds appears to be related neither to stereopsis nor to binocular summation for improved nocturnal vision, but rather to the

ability of controling the bill and its contents during visually guided feeding behaviors, nest construction and chick provisioning, or tool use in case of New Caledonian crows (Martin 2009; Troscianko et al. 2012). This means that for most birds it is more about being able to see the bill tip with each eye independently than about having a substrate for binocular fusion (Martin 2009). This has recently been given additional support by the finding that the degree of binocularity in birds with visually guided foraging modes is inversely correlated with their bill length, i.e. the *longer* the bill the *smaller* the binocular overlap (Tyrrell and Fernández-Juricic 2017b).

Moreover, the fact that most birds maintain a high degree of lateralization of the eyes indicates that lateral eyes are of particular ecological importance to them: They allow them to continuously monitor much of their surroundings without having to move their heads. Accordingly the most lateral eyes are generally found in species exposed to high predatory pressure, since they grant them optimized anti-predator vigilance (Hughes 1977). In an extreme case, a bird could have completely lateral eyes and full panoramic vision without any blind areas. In reality, full panoramic vision is seldom reached even in highly laterally-eyed species, because the average vertebrate eye has a monocular visual field of only approximately 170° (Walls 1942) which theoretically limits the maximum possible cyclopean vision to 340° (i.e. 20° short of panoramic vision), but a few bird species possessing full panoramic vision indeed exist (Martin 2012), including several species of ducks (Guillemain, Martin, and Fritz 2002; Martin 1986; Martin, Jarrett, and Williams 2007) and woodcocks (Martin 1994). All of these birds have in common that they do not forage under visual guidance and thus do not require to view their bill tips. In birds that are visual foragers and need to view their bill tips (e.g. during pecking), and are in addition exposed to high predatory pressures, a tradeoff appears to exist between on the one hand maximizing panoramic vision for vigilance, and on the other hand maintaining a binocular overlap that is just sufficiently big for visually controlling the bill.

1.3.2 Retina and retinal topography

The vertebrate retina is an extension of the brain, and represents a complex multi-layered neural tissue (Figure 3). While its cellular composition, and the densities, topographical distributions and local circuitries of the respective cell-types can be quite diverse between different vertebrate species, the general architecture is mostly the same. There are three cellular layers, which are separated from each other by two plexiform layers. The cellular layers are represented by a layer of ciliary photoreceptor cells called outer nuclear layer (ONL), a layer of bipolar, horizontal and amacrine cells called inner nuclear layer (INL), and a ganglion cell layer (GCL) containing the output neurons to the brain (Lamb 2013). The neurons in these three layers are sequentially interconnected via a sophisticated neural network whose synapses are mostly found in the intercalated plexiform layers. Photoreceptor cells are connected to cells of the INL via the outer plexiform layer (OPL), and INL cells to the ganglion cells via the inner plexiform layer (IPL). In order to illustrate the immense level of complexity it can be said that only 0.001 mm^3 of mouse retina including the IPL contain approximately 500,000 synaptic contacts (Helmstaedter et al. 2013).

The perception of a visual stimulus begins with the photoreceptor cells in the ONL, the outermost layer of the retina. Vertebrates possess two major types, rods and cones (cf. Figure 3), the former more sensitive ones serving for scotopic (= low light) vision, and the latter for photopic and color vision. Photoreceptor cells contain pigments that are photosensitive to specific bands of the light spectrum (Walls 1942), and various sub-types of cones exist which have different spectral sensitivities and thus enable color vision (D. M. Hunt et al. 2009). When a light stimulus is absorbed, an intracellular transduction cascade is generated which leads to hyperpolarization of the photoreceptor cell membrane (Lamb and Pugh 2006). This leads to the activation of bipolar cells in the INL which propagate their signals to the retinal ganglion cells (RGCs), while horizontal and amacrine cells in the INL convey modulatory lateral connections (Luo 2015). RGCs are the actual projection neurons of the retina, whose axons travel through the optic nerve and optic chiasma towards the visual centers of the brain.

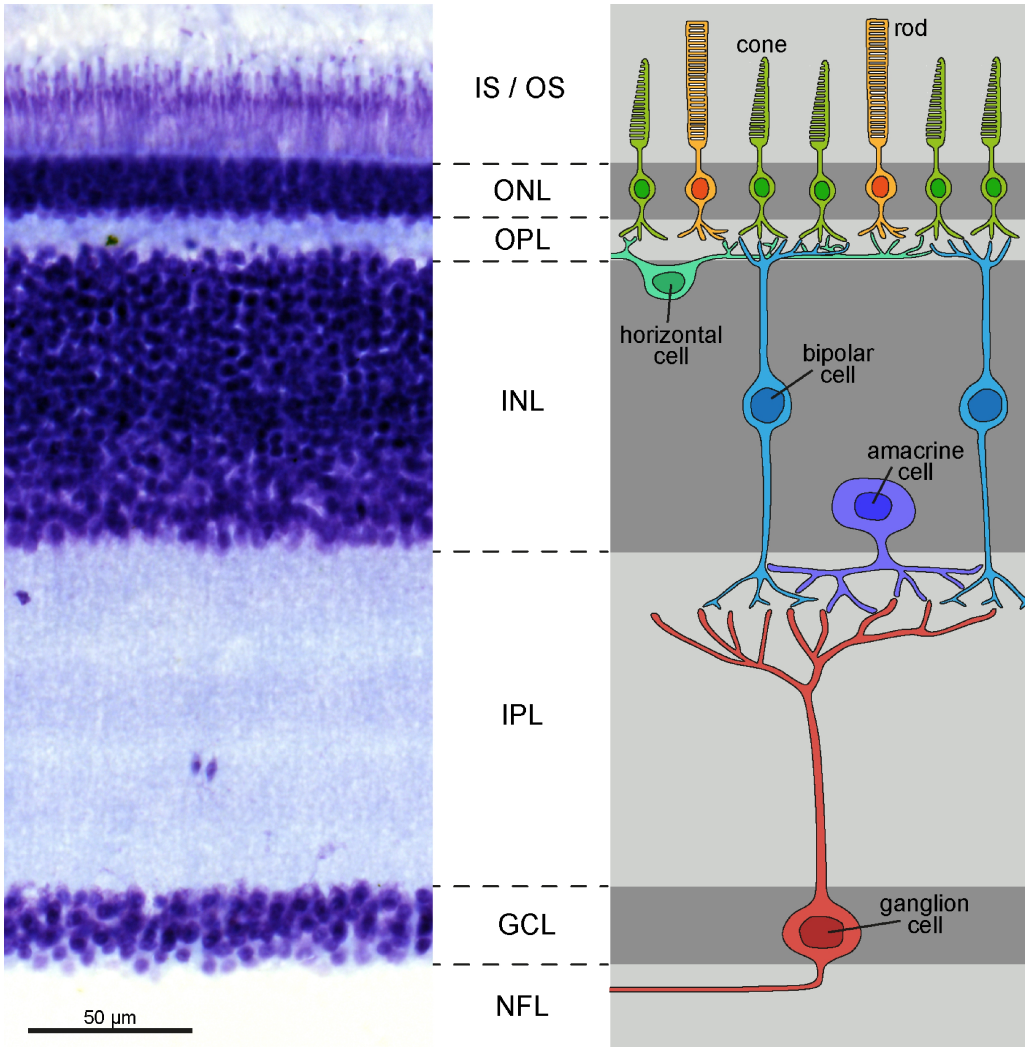

Figure 3: Structural organization of the vertebrate retina. Left: Photomicrograph of a perpendicular section through the retina of a bird, the Chilean Tinamou (*Nothoprocta perdicaria*), stained with Nissl. The typical layers of the vertebrate retina can be distinguished: inner and outer segments of the photoreceptors (IS/OS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL). Scalebar = 50µm. Right: Schematic representation of the basic cell types and their circuitry in the different retinal layers. Note that the direction of light falling onto the retina is from below.

Especially in animals with a visually active life style such as birds, the distribution of RGCs in retinal space is not homogeneous but strongly varies across the retina, leading to characteristic retinal topographies. The RGC topography can exhibit different functionally relevant specializations, i.e. high density areas which correspond to high-acuity regions of the visual fields (Figure 4; see also Krabichler et al. 2015). In many species, there is a central retinal spot of highest RGC density called *area centralis*, or in case of primates, *macula lutea*. Often,

but not always, the area centralis also features a retinal invagination called fovea, which is assumed to further enhance visual acuity (Marmor et al. 2008; Moore et al. 2017; Walls 1942).

Laterally-eyed birds that have a behaviorally relevant binocular overlap for visually guided behavior with their bill, tend to possess an dorso-temporal area in addition to the area centralis; this is an area of increased RGC density in the dorso-temporal retina, which subtends the frontal visual field around the bill (Budnik et al. 1984; Martinoya, Rey, and Bloch 1981). It thus provides for increased visual acuity in this part of the visual field (Bloch and Martinoya 1983; Querubin et al. 2009) and most likely serves for locating food (Coimbra, Collin, and Hart 2014a; Querubin et al. 2009) or for distance measurements during foraging (Nalbach, Wolf-Oberhollenzer, and Kirschfeld 1990).

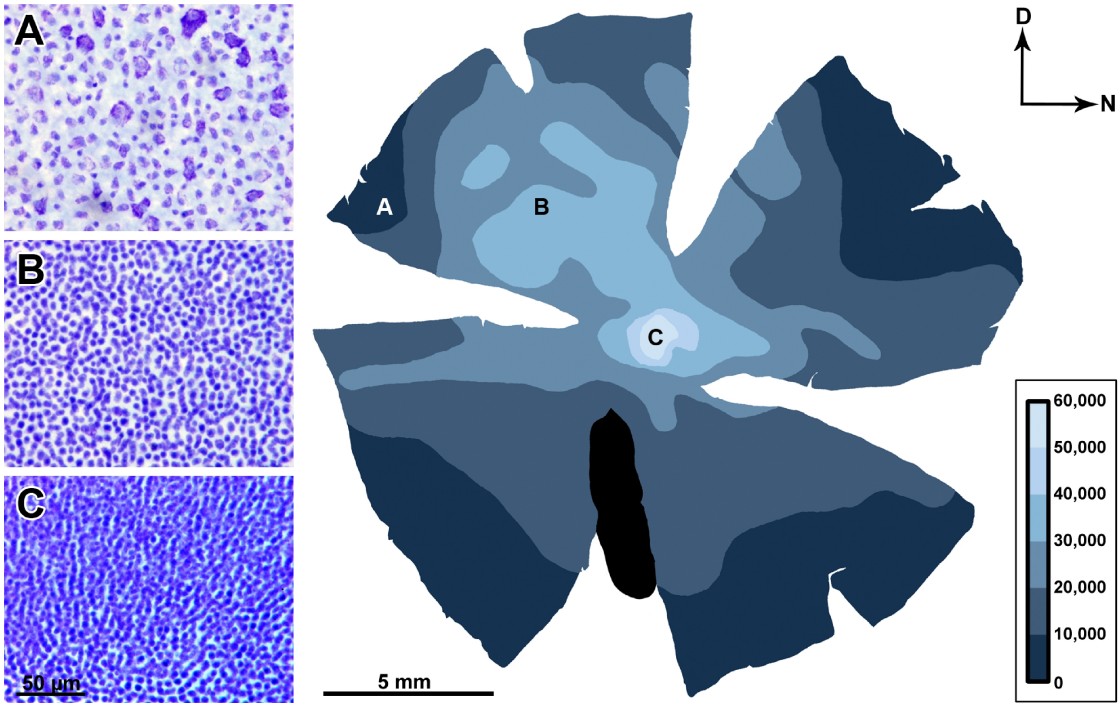

Figure 4: Retinal ganglion cell (RGC) topography. Distribution of RGCs in a retinal wholemount of the Chilean Tinamou (right eye). Right: Reconstructed isodensity map; darker contours represent lower, lighter ones higher RGC densities (numbers in color scale at the right indicate RGC numbers per mm²). Three specializations are present: A high-density area centralis, an area dorsalis that probably looks towards the bill, and a faint horizontal visual streak. Left: Photomicrographs of Nissl-stained RGC layer in the peripheral retina (A), dorso-temporal area (B) and area centralis (C). Scalebars: 50 µm (in C, for A–C); 5 mm (in topography map). Reproduced from Krabichler, Vega-Zuniga, Morales, Luksch, & Marín 2015, with permission from John Wiley and Sons (licence# 4123740906958).

A third frequently observed topographical specialization is called horizontal visual streak. This is represented by a horizontal band of increased RGC density across the retina, from temporal to nasal. It is most often found in species which live in open habitat such as savannas, grassland, or the ocean. It was proposed by Hughes (1975, 1977) that visual streaks evolve in such species for constantly scanning the horizon, a notion known as the “terrain hypothesis”. Although there are exceptions to this rule, in which the sheer habitat type that a species lives in does not reliably predict the orientation or presence of a visual streak (e.g. Tyrrell et al. 2013), for many mammals and birds inhabiting open landscapes the terrain hypothesis holds true (Fernández-Juricic, Moore, et al. 2011; and references therein).

1.3.3 Visual acuity

Visual acuity means the quality of vision that a vertebrate eye can produce. It can be described by various definitions, but one of the more common ones is its formulation as the capability of resolving a grating pattern at a certain minimal spatial frequency or grating size (Kalloniatis and Luu 1995). A grating is a repetitive sequence of parallel and equally thick black and white bars. The spatial frequency is the number of repetitions (or “cycles”) of the basic unit “one black and one white bar” within a defined space, for example one degree of visual angle. One way of measuring the visual acuity threshold of an animal is by means of behavioral experiments. Animals are trained to react in a certain way (e.g. push a lever) if they can distinguish the gratings, which are presented to them at different spatial frequencies in order to determine the minimal spatial frequency the animals can resolve. However, this approach requires animal keeping and time-consuming training, which may not even be possible in many species.

An alternative approach is the mathematical approximation of the theoretical maximal spatial resolving power (SRP) from measurable parameters. Visual acuity mainly depends on the neural elements of the retina, but also on other factors. Of high importance is the eye size, which is actually one of the main

limiting factors of visual acuity (Hall and Ross 2007; Kiltie 2000). The size of the eye, or more specifically its focal length (also called posterior nodal distance or PND; Pettigrew et al. 1988), determines the size of the image that is projected onto the retina. Larger eyes produce bigger retinal images from each degree of angle of the eye's visual field. Since a bigger retinal image means more and spatially better separated details per area, bigger eyes have the potential for higher visual acuities than smaller eyes (Hughes 1977; Miller 1979; Pettigrew et al. 1988; Collin and Pettigrew 1989; Martin 1993; Garamszegi, Møller, and Erritzøe 2002; Ullmann et al. 2012).

The second limiting parameter on which visual acuity depends is of course the density of the "sampling elements" that perceive the retinal image and construct a neural representation of it (Miller 1979; Martin 1993). Primarily, these are the retinal photoreceptor cells which convert the light rays of the image that is projected onto the retina into neural signals (Hall and Ross 2007). Thus, their density provides the first-order limitation of the theoretically possible 'resolution' of the neural image representation (Hughes 1977). However, it is usually not the spatial resolution of the photoreceptors but rather of the RGCs that is propagated to the brain, because in most parts of the retina many photoreceptors converge onto fewer RGCs which thus represent the retinal 'output bottleneck' (Goodchild, Ghosh, and Martin 1996; Yamada et al. 2001; Völgyi et al. 2004; Joselevitch and Kamermans 2009; Werner and Chalupa 2013; Querubin et al. 2009; Hughes 1977). And in fact, behavioral experiments have corroborated that RGC density reliably predicts visual acuity thresholds, especially within the area centralis where the ratio of photoreceptor-to-RGC convergence is relatively low (Collin and Pettigrew 1989; Harman et al. 1986; Hodos, Miller, and Fite 1991; Pettigrew et al. 1988; Querubin et al. 2009; Kiltie 2000).

Thus, the SRP can be estimated from the RGC density in the area centralis and from the PND of the eye (Collin and Pettigrew 1989). The PND cannot be easily directly measured, but based on the known relatively constant PND-to-axial-

length ratio across vertebrates (Hughes 1977, pp. 651-661; Schaeffel and Howland 1988), it can be approximated from the eye's axial length as measured with a caliper (Martin 1993; Ullmann et al. 2012). From the PND, the retinal length subtending one degree of visual angle is calculated. Then, on basis of the RGC density, the number of RGCs present along this retinal length is calculated. According to the sampling theorem (Hughes 1977), which works on the premise that two neighboring RGCs are necessary to distinguish ("sample") one cycle of black-and-white grating, we can finally calculate the theoretical SRP limit as resolvable cycles of grating per degree of visual angle.

The method is simple and feasible, because only histology of retinal tissue and measurement of the axial length of the eye ball have to be performed, without the need of taking into account complicated optical or physiological parameters of the eye and retina (for example, refractive power of the eye is irrelevant, since the eye is assumed to be in its accommodated state when viewing natural scenes so that image resolution is not affected by it). Also it has been shown that the approximated SRP values correspond quite well with behaviorally determined visual acuity thresholds (see above). The necessary data can be easily and rapidly acquired in many different species with very simple measurements, and is thus an excellent tool for studying the visual ecology of animals.

Some examples of avian SRP values estimated by means of this method found in the literature are: Passeriformes such as the European starling *Sturnus vulgaris*, the house sparrow *Passer domesticus* and the house finch *Carpodacus mexicanus,* between 4.4 – 5.9 cycles/degree (Dolan and Fernández-Juricic 2010); Columbiformes such as the mourning dove *Zenaida macroura,* 6.5 cycles/degree (Dolan and Fernández-Juricic 2010); the barn owl *Tyto alba*, 8.4 cycles/degree (Wathey and Pettigrew 1989); phasianid Galliformes such as the Japanese quail *Coturnix japonica* and the grey partridge *Perdix perdix,* between 10 – 13 cycles/degree (Lisney et al. 2012); the meadowlark *Sturnella magna,* 10.3 cycles/degree (Tyrrell et al. 2013); the ostrich *Struthio camelus,* 19.3

cycles/degree (Boire et al. 2001); and the sacred kingfisher *Halcyon sancta,* 41 cycles/degree (Moroney and Pettigrew 1987).

1.3.4 Retinal projections and their visual pathways in the brain of birds and vertebrates

The retinal projections from RGCs to the brain represent parallel primary visual pathways (Casagrande and Xu 2003), which can be categorized according to their RGC class of origin (Callaway 2005; Schiller 2010; Chen and Naito 2009; Stone 1983) as well as by their target regions and related functions (Ebbesson 1970; Rodieck 1979; Zeigler and Bischof 1993; Major et al. 2003; Dacey 2004; Butler and Hodos 2005; Wylie et al. 2009; Shimizu, Patton, and Husband 2010; Zhaoping 2016). There are six major neuroanatomically distinct pathways (Figure 5), which are highly conserved among vertebrates: The midbrain optic tectum, the dorsal thalamus, the accessory optic system, the pretectum, the ventral thalamus, and the hypothalamus (Krabichler et al. 2015; Major et al. 2003; Vega-Zuniga et al. 2016).

The tectofugal pathway projects to the optic tectum (called superior colliculus in mammals), which is in birds particularly striking because it represents big lobes bulging out from each mesencephalic hemisphere (Luksch 2003; Wylie et al. 2009). The optic tectum contains a topographical representation of the whole retinal space (Hamdi and Whitteridge 1954; Cowan, Adamson, and Powell 1961; S. P. Hunt and Webster 1975; Clarke and Whitteridge 1976; Remy and Güntürkün 1991; Shimizu et al. 1994; Güntürkün 2000; Letelier et al. 2004; Wylie et al. 2009). Its major ascending relay is the thalamic nucleus rotundus (Lateral Posterior/Pulvinar complex in mammals; Karten 1979; Karten and Shimizu 1989; Reiner, Yamamoto, and Karten 2005) which subsequently projects to the entopallium in the telencephalon (extrastriate cortex in mammals; Ahumada-Galleguillos et al. 2015; Butler 1994; Engelage and Bischof 1993; Karten and Shimizu 1989; Mpodozis et al. 1996; Nguyen et al. 2004).

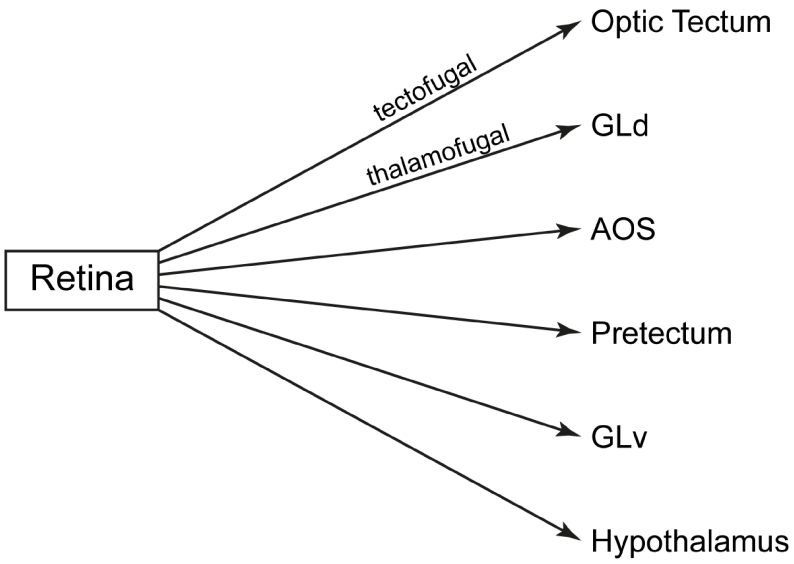

Figure 5: Overview of the six major retinorecipient target regions in the vertebrate brain. GLd = dorsal lateral geniculate (dorsal thalamus); AOS = accessory optic system; GLv = ventral lateral geniculate (ventral thalamus).

The thalamofugal pathway directly innervates the nucleus geniculatus lateralis, pars dorsalis (GLd; also known as dorsal lateral geniculate) in the dorsal thalamus, which in birds and reptiles projects to the visual hyperpallium or Wulst (Bravo and Pettigrew 1981; Güntürkün, Miceli, and Watanabe 1993; Karten et al. 1973; Miceli et al. 2008, 2006; Remy and Güntürkün 1991; Shimizu and Karten 1993; Butler 1994). Since in mammals the GLd directly projects to the primary visual cortex V1, it has been suggested that Wulst and V1 are homologous (Butler, Reiner, and Karten 2011; Karten 1979; Medina and Reiner 2000; Nguyen et al. 2004; Reiner, Yamamoto, and Karten 2005). This view is also supported by neurophysiological data (Bischof et al. 2016).

All vertebrates appear to possess both the tectofugal and thalamofugal pathway, but their relative proportions can differ considerably (Butler and Hodos 2005; Shimizu, Patton, and Husband 2010). In mammals the thalamofugal pathway predominates (Shimizu and Bowers 1999; Van Hooser and Nelson 2006) and is particularly hypertrophied in primates including humans (Livingstone and Hubel 1988; Merigan and Maunsell 1993), although many visually active/diurnal

mammals are also known to possess a well-developed tectofugal visual pathway (Fredes et al. 2012; Lane, Allman, and Kaas 1971; Major et al. 2003; Van Hooser and Nelson 2006; Vega-Zuniga et al. 2013, 2017). By contrast, in non-mammalian vertebrates such as birds the tectofugal pathway is the major route (Karten 1969; Karten and Shimizu 1989; Nguyen et al. 2004). In fact, in birds as many as 75-90% of the RGCs project onto the optic tectum (Bravo and Pettigrew 1981; Remy and Güntürkün 1991; Güntürkün 2000; Luksch 2003), and only a minor portion onto the thalamic dorsal lateral geniculate (Mpodozis et al. 1995; Bischof and Watanabe 1997). Only birds with a high degree of binocular overlap such as owls appear to possess a relatively enlarged thalamofugal visual pathway (Engelage and Bischof 1993), though the ratio of thalamo- to tectofugal projections is still lower than in mammals (Bravo and Pettigrew 1981).

A third important retinofugal visual pathway projects to the Accessory Optic System (AOS), which is highly conserved in vertebrates (Simpson 1984; Giolli, Blanks, and Lui 2006). The main retinorecipient nucleus of the AOS in birds is called Nucleus of the Basal Optic Root (nBOR), which appears to be homologous to the mammalian lateral and medial terminal nuclei (LTN/MTN) (Giolli, Blanks, and Lui 2006; McKenna and Wallman 1985; Simpson 1984). The avian nBOR (but not the mammalian LTN/MTN; Giolli, Blanks, and Lui 2006) receives most of its retinal afferents from displaced retinal ganglion cells, which are located in the INL instead of the ganglion cell layer (Fite et al. 1981; Karten, Fite, and Brecha 1977; Wylie et al. 2014). Functionally, the AOS is implicated in visual operations related to self-motion (‘optic flow’) (Frost, Wylie, and Wang 1990), optokinetic nystagmus (compensatory eye movements due to self-motion) and fine-tuning of eye movements (Giolli, Blanks, and Lui 2006).

The pretectum receives the fourth pathway of retinal projections. In birds, one projection innervates the Area pretectalis (Shimizu et al. 1994), which is homologous to the mammalian olivary pretectal nucleus since both project to the Edinger-Westphal nucleus and are in similar manners implicated in the pupillary constriction reflex (Butler and Hodos 2005). One of the largest pretectal retinorecipient structures in birds is the Nucleus lentiformis mesencephali (LM)

(Wild 1989; Wylie et al. 2014), which is probably homologous to both the nucleus of the optic tract (NOT) and the dorsal terminal nucleus (DTN) in mammals (Giolli, Blanks, and Lui 2006; McKenna and Wallman 1985; Simpson 1984). The avian LM receives topographical retinal projections from various classes of ganglion cells (Bodnarenko, Rojas, and McKenna 1988; Ehrlich and Mark 1984b; Gamlin and Cohen 1988b, 1988a), and apparently also from displaced ganglion cells (Wylie et al. 2014). It is highly interconnected with the accessory optic nBOR, and interestingly both have various descending projections in common, including to the pons, inferior olive and cerebellum (Pakan et al. 2006; Pakan and Wylie 2006; Wylie 2001; Wylie, Linkenhoker, and Lau 1997; Wylie et al. 2007). As these anatomical parallels indicate, the avian LM and mammalian NOT/DTN are also functionally closely related with the AOS, and take part in optic flow perception and optokinetic nystagmus generation (Giolli, Blanks, and Lui 2006; Iwaniuk and Wylie 2007; Simpson 1984; Simpson, Leonard, and Soodak 1988; Wylie 2013).

Apart from the LM or NOT/DTN, another structure classically regarded as pretectal receives a prominent retinotopically organized retinal projection. It is known as griseum tectale or tectal gray (GT) in both birds (Gamlin and Cohen 1988b) and mammals (in the latter it was formerly also known as “posterior pretectal nucleus”; Puelles 2016). Although the GT is now regarded as mesencephalic and thus not anymore part of the diencephalic pretectum (Puelles 2016), I will adhere to the original categorization here. The GT lies adjacent to the optic tectum at its anterior border and embryologically develops in a similar laminated manner which is partly retained in the adult (García-Calero, Martínez-de-la-Torre, and Puelles 2002). In birds, it is strongly interconnected with the optic tectum, LM, and nucleus geniculatus lateralis, pars ventralis (GLv; also known as ventral lateral geniculate) (Gamlin and Cohen 1988a; Vega-Zuniga et al. 2016), and is probably involved in orienting visuomotor and optic flow operations that are associated with this broader network (see below).

The fifth parallel retinofugal pathway innervates the ventral thalamus, most notably the GLv (Güntürkün and Karten 1991; Jones 1985). The GLv of birds has

a retinotopic topography (Crossland and Uchwat 1979; Ehrlich and Mark 1984a) similar to the optic tectum, LM and GT with which it is heavily interconnected (Crossland and Uchwat 1979; S. P. Hunt and Künzle 1976; Vega-Zuniga et al. 2014, 2016). Apart from these, it receives afferents from the telencephalic visual Wulst (primary visual striate cortex V1 in mammals) (Karten et al. 1973), and projects to the pons and the cerebellum, among other targets (Gamlin and Cohen 1988a). Functionally, the GLv has been proposed to be involved in optokinetic reflexes (Gioanni et al. 1991), because it responds intensely to visual motion stimuli (Pateromichelakis 1979). Evidence is now accumulating that the GLv is part of a sophisticated visuomotor network which includes the optic tectum, pretectum (in particular LM and GT), pons and cerebellum (Vega-Zuniga et al. 2016). This network may be the principal player in concerting gaze control and orienting movements of eyes and head (du Lac and Knudsen 1990; Pateromichelakis 1979; Vega-Zuniga et al. 2016).

The sixth pathway of retinal projections innervates the hypothalamus. The retinorecipient structure is called visual suprachiasmatic nucleus (vSCN). It is highly conserved in all tetrapod vertebrates (Cassone and Moore 1987; Derobert et al. 1999; Kaas 2009; Shimizu et al. 1994) and forms part of the circadian clock system (Brandstätter and Abraham 2003; Cantwell and Cassone 2006; Cassone 2015; Kaas 2009; Yoshimura et al. 2001).

In summary, six main parallel retinofugal pathways can be distinguished: The two major ascending visual pathways tectofugal to the optic tectum or superior colliculus and thalamofugal to the GLd, and four additional pathways to the AOS, the pretectum, the ventral thalamus, and to the hypothalamic vSCN. The general layout of this remarkable neuroanatomical segregation of primary retinal projections into parallel functional channels which already arise within the retina (Schiller 2010), is phylogenetically conserved. It can therefore be assumed to be essential for the functioning of the vertebrate visual system. By studying the patterns of retinal projections in different species we can learn about the variability of the organization and relative proportions of the parallel visual pathways, and how this may correlate with visual function and ecology.

1.3.5 Centrifugal visual system

In the previous chapter I have described the parallel retinofugal (i.e. exiting from the retina) projections from the retina to the brain. Curiously, vertebrates also possess a *centrifugal* pathway that goes in the opposite direction, from the central brain towards the retina. Centrifugal visual neurons in the brain send axons which traverse the optic nerve alongside the optic fibers and finally terminate in specific zones in the retina. Such a centrifugal visual system (CVS) has been found in all classes of vertebrates, but its neuroanatomy is highly variable between different taxa (Repérant et al. 2006, 2007). Depending on the species, the centrifugal visual neurons can be found in very different regions throughout the neuraxis from the forebrain to the rhombencephalon (Repérant et al. 2007). For example, in Chondrichthyes such as sharks, centrifugally projecting neurons are found in the optic tectum, while in many Osteichthyes (bony fish) they are found in the area of the terminal nerve (telencephalon) and in the dorsal isthmic region (between mes- and rhombencephalon). In frogs (Anura) such cells lie in the area of the terminal nerve, in the preoptic area and in the septal region. Mammals have variable distribution patterns of centrifugal visual neurons, such as in the preoptic area, hypothalamus, dorsal thalamus, pretectum, optic tectum, tegmentum, ventral isthmic region, and dorso-anterior rhombencephalon. In most reptiles (except snakes), i.e. lacertilia, chelonia and crocodylia, as well as birds, the cells are found in the tegmentum, dorsal isthmic region and dorso-anterior rhombencephalon (Repérant et al. 2007).

Also the numbers of centrifugal visual neurons in the brain are very variable, from only a handful in primates such as humans (Gastinger et al. 2006; Repérant and Gallego 1976) to several thousand in certain teleost fishes (Ito et al. 1984; Uchiyama 1989; Uchiyama and Ito 1984; Uchiyama, Sakamoto, and Ito 1981) as well as in some birds (Cowan and Powell 1963; Cowan 1970). Indeed, even within these latter two groups, numbers can be highly heterogeneous: In birds for example, raptors and on-the-wing feeding birds have a few hundred to a few thousand centrifugal visual neurons (Feyerabend, Malz, and Meyer 1994; Weidner et al. 1987), while on the other hand granivorous birds such as the

pigeon (Cowan and Powell 1963; Cowan 1970) or the chicken (Cowan and Clarke 1976) can have up to 12,000.

Further conspicuous variability is observed in the efferent terminals of the centrifugal visual neurons in the retina. While their general termination zone almost always lies at (or close to) the border between IPL and INL, the terminals can have very different morphologies. In most vertebrates, the centrifugal visual terminals are 'divergent', which means that they widely arborize and can cover a large area of the retina (Repérant et al. 2007). Examples of especially wide divergent terminals are found in goldfish (Kawamata, Ohtsuka, and Stell 1990; Ohtsuka, Kawamata, and Stell 1989), macaque monkeys (Gastinger et al. 1999, 2006) and humans (Repérant and Gallego 1976). The other type of centrifugal visual terminal is called 'convergent', because the centrifugal fibers do not ramify, or do so only in a very confined area. Thus, each efferent fiber forms synaptic contacts onto very few or even single target cells (Uchiyama and Stell 2005; Wilson and Lindstrom 2011). Convergent terminals have only been found in birds[2], where they coexist with divergent terminals (Repérant et al. 2007).

Birds have been the best studied models for the centrifugal visual system. This goes back almost 130 years, when centrifugal fibers were for the first time described in the avian retina by Santiago Ramón y Cajal, the illustrious father of modern neuroscience, using the Golgi impregnation method (S. Ramón y Cajal 1889, 1893). The retina of commonly studied birds such as chickens, quails, pigeons and passerines, contains both divergent and convergent centrifugal fibers (Chmielewski et al. 1988; Dogiel 1895; Fritzsch, de Caprona, and Clarke 1990; Lindstrom et al. 2009; Maturana and Frenk 1965; Woodson et al. 1995). Convergent terminals form dense pericellular nests around association amacrine cells in the IPL (Dowling and Cowan 1966; Lindstrom et al. 2009; Uchiyama and Ito 1993; Uchiyama, Ito, and Tauchi 1995). These cells are remarkable, because they are in fact axon-bearing neurons that project to other targets within the same retina (Uchiyama and Stell 2005; Uchiyama et al. 2004). Moreover, they

[2] with the single notable example of dogs (S. Ramón y Cajal 1893, 1909, 1911; Repérant et al. 2007)

are easily identified in many birds by immunohistochemistry against Parvalbumin (Fischer and Stell 1999; Lindstrom et al. 2009; Sanna et al. 1992; Uchiyama and Stell 2005), while both the association amacrine cells and the convergent terminals have a high activity of the enzyme NADPH diaphorase which can be used to mark them (Fischer and Stell 1999; Lindstrom et al. 2009; Morgan, Miethke, and Li 1994; Nickla et al. 1994). Divergent terminals on the other hand have not received much attention (see e.g. Lindstrom et al. 2009), and their targets continue to be unknown.

Few years after Ramón y Cajal's discoveries of the centrifugal terminals in the retina, their source in birds was for the first time identified by studying axonal degenerations after lesion experiments (Wallenberg 1898). They originate from the nucleus isthmo-opticus (ION) in the dorsal isthmic region (Cowan and Powell 1963). In many neognathous birds (Gutiérrez-Ibáñez et al. 2012), the ION neurons form a convoluted lamina around a central neuropil containing their stout and polarized dendritic trees (Clarke and Kraftsik 1996; Cowan 1970; Güntürkün 1987). The ION receives afferents mainly from the ipsilateral optic tectum (Cowan and Powell 1963; Crossland and Hughes 1978; Wallenberg 1898), more specifically from a population of neurons in layer 9-10 (Figure 6 C; cf. Crossland and Hughes 1978; Miceli et al. 1993; Uchiyama and Watanabe 1985; Uchiyama, Yamamoto, and Ito 1996; Woodson et al. 1991). In addition, also various possible extra-tectal afferents have been described (Miceli et al. 1997, 2002). The massive tectal projection to the ION forms part of a conspicuous circuitry, a "feedback loop" from ION to the retina to the optic tectum and again to the ION (Miceli et al. 1999; Repérant et al. 2006; Uchiyama and Stell 2005; Wilson and Lindstrom 2011). It is topographically organized (McGill, Powell, and Cowan 1966a, 1966b) in a homotopic manner (Li et al. 1998).

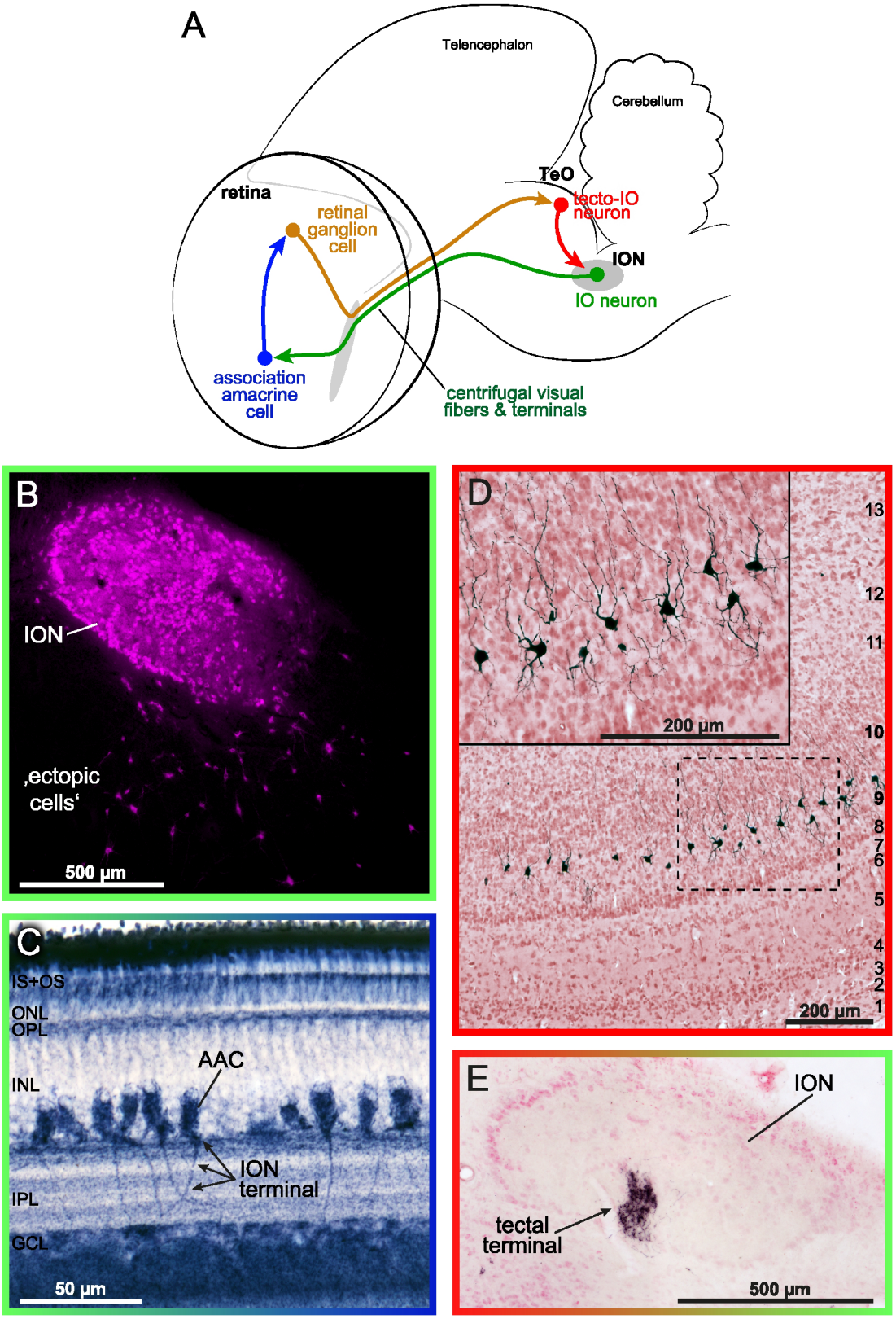

Figure 6: The centrifugal visual system in neognathous birds. (Continued on the following page.)

(Continued caption of Figure 6.) A: Schematic. B–E: Elements of the CVS in the chicken (Gallus gallus). B: The isthmo-optic nucleus (ION) retrogradely labeled from the eye by the tracer Rhodamine-B-isothiocyanate (RITC). Note the dense nucleus and the dispersed 'ectopic cells' ventral to it. C: The convergent centrifugal terminals from the ION synapsing on association amacrine cells (AACs), both labeled by NADPH diaphorase histochemistry (cf. Fischer and Stell 1999; Krabichler et al. 2017). D: The tecto-ION neurons in layer 9-10 of the optic tectum, retrogradely labeled from an injection of the tracer biotinylated dextran amine (BDA) into the ION. E: Terminals in the ION after injection of BDA into the optic tectum. [B, D & E: Coronal brain sections (left=lateral, down=ventral); D & E: Counterstained with Neutral Red; C: transverse retina section. C with permission from C. Gutiérrez-Ibáñez; E with permission from H. Luksch.]

Importantly, in addition to the ION neurons a second group of centrifugal visual neurons exist. They are less numerous and lie dispersed in areas outside the ION, and were therefore named "ectopic cells" (Clarke and Cowan 1975, 1976; Hayes and Webster 1981; O'Leary and Cowan 1982). Their multipolar morphology with wide dendrites as well as their bigger somata distinguish them clearly from proper ION neurons (O'Leary and Cowan 1982). Curiously, until the present day the afferents that innervate the ectopic cells have remained unknown. There is only weak evidence suggesting that they might receive a tectal projection comparable to their ION counterparts (Clarke 1985; LaVail and LaVail 1974). In addition, it has been conclusively demonstrated that the centrifugal axons from ectopic and ION proper cells give origin to different kinds of terminals in the retina. Based on several lines of evidence, Fritzsch et al. (1990) had suggested for the first time that the convergent centrifugal terminals originated from proper ION neurons, while the divergent terminals – whose presence for that matter had been widely ignored if not denied for many years – stemmed from the ectopic centrifugal neurons. This hypothesis was finally also confirmed by neuroanatomical tracings from the region of ectopic cells to the retina (Woodson et al. 1995).

The existence of a system conveying central feed-back from the brain to the retina has caused much curiosity regarding its possible functional significance, even more so due to its high degree of variability among vertebrates. While it is generally assumed that the CVS mediates some sort of central feed-back control or modulation of the retina (Uchiyama 1989), conclusive demonstrations of specific functions have remained elusive, despite over a century of investigation

and an abundance of hypotheses (reviewed in Repérant et al. 2006, and 1989). Debate has been especially livid regarding the possible functions of the conspicuous and relatively well-studied CVS of neognathous birds. Hypotheses have generally been centered on the ION-related convergent pathway. Lesion studies have hereby suggested that the ION plays a role in target selection (Rogers and Miles 1972; Uchiyama, Ohno, and Kodama 2012), while hypotheses deduced from the neuroanatomy and physiology of the circuitry have proposed various functions such as covert visual spatial attention without eye or head movements (Ohno and Uchiyama 2009), early detection of aerial predators (Wilson and Lindstrom 2011) – though this has already been refuted (Gutiérrez-Ibáñez et al. 2012) – and attention switching between emmetropic and myopic parts of the retina (Gutiérrez-Ibáñez et al. 2012).

There are only two studies which have also taken the role of the ectopic cell pathway into account (Dillingham, Guggenheim, and Erichsen 2013, 2017). Dillingham et al. (2013) showed in chicken that electrolytic lesions of the ION, the region of ectopic cells, and their combined isthmo-optic tract which runs to the retina, all led to a significant axial length shortening (hyperopia) of the contralateral eye, which was however mostly reversed after 21 days. In a follow-up paper (Dillingham, Guggenheim, and Erichsen 2017) they showed that chicks raised under constant light developed similar effects, with the addition that hyperopia in the ipsilateral eye (which only receives efferents from ipsilateral ectopic cells but not ipsilateral ION cells) persisted after 21 days. Thus, in addition to possible other functions, the CVS including the ectopic cells may be involved in eye development and homeostasis. However, this hypothesis will have to be tested in further species, and in any case it may be doubted to be the only function of the avian CVS, which has evolved such variability among birds, and such a high degree of specialization in some species (Gutiérrez-Ibáñez et al. 2012).

In order to reach a more thorough understanding of the CVS, we arguably first need to better understand its phylogenetic plasticity which has generated the high degree of diversity that we observe. For example, at some point during the

evolution of birds, an ION must have first started to emerge together with its peculiar connectivity, since no other group of vertebrates possesses such a nucleus. It is therefore important to study the organization of the CVS and its circuitry in birds which reportedly do not possess any distinct ION. This is the case for some neognathous birds, which may have secondarily reduced the ION (Gutiérrez-Ibáñez et al. 2012), but especially in basal birds belonging to the infraclass Palaeognathae, which have been repeatedly reported to lack an ION (Gutiérrez-Ibáñez et al. 2012; Craigie 1930; Verhaart 1971).

1.4 Motivation for this thesis: The palaeognathous knowledge gap in avian visual neuroscience

Research into the avian visual system has been conducted in an array of species mostly belonging to the orders Galliformes (most notably the chicken, e.g. Luksch, Khanbabaie, and Wessel 2004), Columbiformes (pigeons, e.g. Wylie 2013), Passeriformes (songbirds such as crows and zebra finches; e.g. Wagener and Nieder 2017; Keary et al. 2010) and Strigiformes (such as the barn owl, e.g. Dutta, Wagner, and Gutfreund 2016). Importantly, all of these are members of only one of the two great avian infraclasses, Neognathae (Prum et al. 2015). The other one, Palaeognathae, has so far been mostly ignored. It contains relatively few species: the flightless ‘ratites’ (Ostriches, Emus, Nandus, Cassowaries and Kiwis) and the volant Tinamous of South and Middle America (Yonezawa et al. 2017; Bertelli 2016; Harshman et al. 2008). They are separated from the Neognathae by over 100 million years of divergent evolution (Yonezawa et al. 2017; Mayr 2014) and are indeed regarded as phylogenetically “primitive” or basal, since they conserve various reptilian features not anymore present in Neognathae, most distinctly their blood proteins and their rigid palate (an upper jaw bone) which morphologically resembles the dinosaurian palate such as in *Tyrannosaurus sp.* (Cabot 1992). The Palaeognathae thus bridge the gap between Neognathae and the next living relatives of birds, crocodiles, which in

phylogenetic terms lie approximately 250 million years apart (Brusatte, O'Connor, and Jarvis 2015; Yonezawa et al. 2017). This taxonomic position makes Palaeognathae highly interesting objects for the study of the visual system, with the potential to yield important new insights into the visual system and its evolution in birds.

1.5 Aims of this thesis

The aim of my dissertation has been to conduct a comprehensive investigation of the visual system of a novel palaeognathous model species, the Chilean Tinamou (*Nothoprocta perdicaria*). As elaborated in the Introduction, this task was approached from five different angles, each representing an integral part of the visual system: visual field, retinal ganglion cell topography, visual acuity, retinofugal projections and centrifugal visual system.

Two subsequent studies were conceived, of which the first one covered the first four topics and the second one the last topic. The goals of the first study thus were:

- to measure the visual field;
- to analyze the retinal ganglion cell topography;
- to estimate the theoretical limits of visual acuity based on eye morphology parameters and maximum retinal ganglion cell density;
- to reveal the patterns of retinofugal projections to the brain by means of injections of the neural tracer Cholera Toxin B subunit (CTB) into the eye.

The second study aimed at achieving an in-depth investigation of the the centrifugal visual system of the Chilean Tinamou by means of *in vivo* and *in vitro* neural tracing experiments with single- and double-tracings, immunohistochemistry, and immunofluorescence. Its specific goals were:

- to analyze the neuroanatomy of the Chilean Tinamou's centrifugal visual neurons by means of intra-ocular tracing experiments with CTB or Rhodamine-B-isothiocyanate (RITC) as tracers, using different section planes of the brain (transverse, horizontal and sagittal);
- to reveal the presumed tectal afferents (including their termination patterns) to those centrifugal visual neurons, by means of double-tracer experiments with intra-tectal tracer injections into the intermediate layers (layers 9-10) using either biotinylated dextran amine (BDA) or Phaseolus vulgaris leucoagglutinin (PHAL), and intra-ocular RITC injections retrogradely labeling the centrifugal visual neurons;
- to identify and characterize the tectal neurons that project to the centrifugal visual neurons, by retrogradely labeling them from tracer injections into the region of the centrifugal visual neurons;
- to identify the centrifugal visual terminals in the retina, either *in vivo* by means of anterograde tracing after CTB injections into the region of the centrifugal visual neurons, or *in vitro* by anterograde tracings from the optic nerve head in the isolated retina using fluorescent biocytin or dextran tracers;
- to identify the retinal target cells of the centrifugal visual fibers by means of NADPH diaphorase histochemistry and immunofluorescence against known markers of the association amacrine cells in Neognathae.

2. Methods

This doctoral thesis has been conducted within the framework of a bilateral international collaboration between the Chair of Zoology (Prof. Harald Luksch), TUM School of Life Sciences Weihenstephan, Technische Universität München (TUM) in Germany, and the El Rayo Lab (Prof. Jorge Mpodozis), Departamento de Biología, Facultad de Ciencias, Universidad de Chile in Santiago de Chile. The *in vivo* and *in vitro* experiments with Chilean Tinamous (*Nothoprocta perdicaria*) were conducted by the author at the El Rayo lab in Chile during several research stays, under the supervision of Dr. Gonzalo Marín who also provided funding for purchasing the birds and materials associated with the experiments. Histological processing, data analysis and compilation for publication were conducted in the Chair of Zoology at TUM, and overall funding and supervision of the study was provided by Prof. Harald Luksch.

All Chilean Tinamous specimens were acquired from a Chilean breeder (Tinamou Chile, Los Ángeles, Región del Biobío, Chile) and kept at the faculty's animal keeping facility. Keeping and experiments were conducted according to the permission and approval from the bioethics committee of the Facultad de Ciencias of the Universidad de Chile, and all treatments were in compliance with the guidelines of the National Institute of Health (NIH) on the use of animals in experimental research. A total of 31 Chilean Tinamous were used during the course of this thesis. *In vivo* techniques used were neural tracer injections under deep anesthesia into the eye (intraocular), optic tectum, or dorsal isthmic region ('IOC region'). All these methods are in detail described in the two included publications (Krabichler et al. 2015, 2017). *In vitro* experiments represented extracellular neural tracings in an *in vitro* preparation of retinal tissue from euthanized Chilean Tinamou specimens, which was maintained in a physiologically active state by immersion in Ames' ringer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The method is in detail described in Krabichler et al. (2017).

Furthermore, this thesis is supplemented by hitherto unpublished data from an experiment, whose methods are not found in the included articles. Here we performed an intraocular injection of kainate two weeks before a normal intraocular CTB injection. Kainate, a glutamate analog, is known for its cytotoxic effects on many retinal neurons (Tung, Morgan, and Ehrlich 1990; Ehrlich, Teuchert, and Morgan 1987) and has been used to selectively trace the unaffected centrifugal visual system from the eye, while inhibiting orthograde retinofugal tracer transport by killing off RGCs (Miceli et al. 1993). The injection procedure of kainate was generally analogous to intraocular CTB injections as described in the included articles. 80 µl of a 1% solution of kainate (product# K0250 from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 0.1M phosphate buffer pH7.4 was intraocularly injected into one eye of a Chilean Tinamou under full anesthesia (ketamin/xylacine induction and isoflurane maintainance as decribed in Krabichler et al. 2017). After recovery, the bird was blind on one eye but other than that showed normal behavior, normal food and water uptake and no weight loss. The intraocular CTB-injection into the kainate-treated eye as well as all following procedures were identical as described in Krabichler et al. (2015, 2017).

The fixed tissue resulting from the experiments was generally transferred to Prof. Luksch's lab at the TUM in Germany and processed as well as analyzed there, while a minor part was processed at the El Rayo lab in Chile. The neural tracings were revealed by immunohistochemistry or immunofluorescence using antibodies against the tracer substances. Further tissue processing methods included histochemistry and immunofluorescence against intrinsic markers. All histological methods including the histochemical, immunohistochemical and immunofluorescence protocols are detailed in the two publications (Krabichler et al. 2015, 2017).

3. Main part: Thesis by publication

This thesis by publication consists of two first-author papers published in peer-reviewed international scientific journals. Both papers were published in the Journal of Comparative Neurology, the first one in 2015, the second one in 2017. Together, they have successfully covered all aims of this thesis as outlined in section 1.5 (“Aims of this thesis”, pp. 28f.). In the present section, for each article a summary is provided and the role of the author of this dissertation in the study is detailed. In Appendix A and B, both original articles are included as published in the journal, with permission from the rightsholder (see below).

3.1 Krabichler et al. (2015) J. Comp. Neurol.

Full citation: Krabichler, Quirin, Tomas Vega-Zuniga, Cristian Morales, Harald Luksch, and Gonzalo J. Marín. 2015. “The Visual System of a Palaeognathous Bird: Visual Field, Retinal Topography and Retino-Central Connections in the Chilean Tinamou (*Nothoprocta Perdicaria*).” *Journal of Comparative Neurology* 523 (2): 226–50. doi:10.1002/cne.23676.

3.1.1 Summary

This paper provided a comprehensive description of important elements of the visual system in the Chilean Tinamou as outlined in chapter 1.4 (p. 28). Analyzing the visual field of this bird revealed that it had quite lateral-standing eyes with a relatively small frontal binocular overlap of 20°, a wide cyclopean field of 300°, and an accordingly relatively small blind sector of 60°. The retinal structure and ganglion cell (RGC) topography was studied in retinal wholemounts as well as in histological sections. Various topographical specialization were present in the Tinamou retina: A faint horizontal visual streak, an area dorsotemporalis

presumably viewing towards the beak and thus the frontal binocular overlap, and a pronounced area centralis. The maximum RGC density in the area centralis was conspicuously high, 61,900 cells/mm². This number was corroborated by means of stereological cell countings in transverse retinal sections, where it was further found that the RGC layer was strikingly thick, with 5-6 layers of cells stacked over one another. Also, a shallow foveal depression was present in the area centralis. By means of the eye measurements and the maximum RGC density the Chilean Tinamou's maximum visual spatial acuity was estimated to reach 14.0 cycles per degree. This relatively high value despite this bird's small eyes resulted from the high RGC density, which may represent an adaptation for providing optimized visual acuity within the given anatomical restrictions of small eyes. Finally, the pattern of retinofugal projections to the brain was studied by intraocular tracer experiments with Cholera toxin B subunit (CTB). The retino-recipient visual brain centers were well-developed, while the general pattern of retinal projections was relatively similar as described in neognathous birds. Major projections were found to the optic tectum, lateral dorsal geniculate (GLd) in the dorsal thalamus, pretectum, accessory optic system, lateral ventral geniculate (GLv) and in the visual hypothalamus (visual suprachiasmatic nucleus). A striking finding was a direct retinal projection to deep layers of the optic tectum, which normally receives retinal fibers only to its superficial layers. Such deep tectal retinal projection had before only been described in embryonic chickens and could deserve further investigation in the future (cf. Discussion 4.2.3, pp. 45 ff.; and Figure 7, p. 48). The intraocular CTB injections also retrogradely labeled a substantial number of centrifugal visual neurons, despite the absence of a proper isthmo-optic nucleus (ION) with clearly marked boundaries as found in most Neognathae. This was the first time that such a layout of centrifugal visual neurons was shown in any bird, and the first time that centrifugal visual neurons were revealed in a palaeognathous species.

3.1.2 Role of the author

The contributions of the authors were clarified in the article: „All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: QK, GM, HL, TVZ. Acquisition of data: QK, CM, GM. Analysis and interpretation of data: QK, HL, TVZ, GM. Drafting of the manuscript: QK, GM, TVZ. Critical revision of the manuscript for important intellectual content: HL, GM, TVZ. Statistical analysis: QK. Obtained funding: GM, HL. Study supervision: HL and GM.“ (Krabichler et al. 2015; QK = Quirin Krabichler, GM = Gonzalo Marín, HL = Harald Luksch, TVZ = Tomas Vega-Zuniga, CM = Cristián Morales).

To specify this information, the author of this dissertation had a leading role at all stages of this project, while continuous supervision and guidance was given by the author's mentor Dr. Tomás Vega-Zuniga, the Chilean project supervisor Dr. Gonzalo Marín, and the author's doctoral supervisor Prof. Harald Luksch. The manuscript was drafted by the author, with the aid and revisions from his mentor and supervisors. Apart from a few supplementary experiments, all *in vivo* experiments were conducted by the author, as well as all histological procedures and subsequent analysis.

3.1.3 Reproduction of the original article

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3.2 Krabichler et al. (2017) J. Comp. Neurol.

Full citation: Krabichler, Quirin, Tomas Vega-Zuniga, Denisse Carrasco, Maximo Fernandez, Cristián Gutiérrez-Ibáñez, Gonzalo Marín, and Harald Luksch. 2017. "The Centrifugal Visual System of a Palaeognathous Bird, the Chilean Tinamou (*Nothoprocta Perdicaria*)." *Journal of Comparative Neurology* 525 (11): 2514–34. doi:10.1002/cne.24195.

3.2.1 Summary

This paper represents an in-depth neuroanatomical study of the centrifugal visual system (CVS) of the Chilean Tinamou, as outlined in chapter 1.4 (p. 29). Originally, the study was motivated by a previous paper which had reported on the basis of normal counterstained brain sections that the Chilean Tinamou in contrast to most Neognathae did not possess an isthmo-optic nucleus (ION; Gutiérrez-Ibáñez et al. 2012). In contrast to this however, we showed that intraocular CTB-injections retrogradelly labeled a substantial cluster of centrifugal neurons residing in the same cerebral region as the neognathous ION (Krabichler et al. 2015). Interestingly, this cluster which was named isthmo-optic complex (IOC) did not possess any clearly defined nuclear boundaries as the ION, and its constituent neurons morphologically resembled the 'ectopic cells' of Neognathae (cf. section 1.3.5, pp. 21ff; Krabichler et al. 2015). In order to investigate how the circuitry of the non-ION CVS of Palaeognathae is organized, we conceived a comprehensive study to analyze the main components and connectivity of this system by means of a series of different neural tracing experiments. First, new intraocular CTB injection experiments were performed to examine the neuroanatomy of the Tinamou's IOC in different section planes (transverse, sagittal and horizontal). This served in particular as preparation for the subsequent complicated IOC tracer injection experiments by providing orientation and stereotaxic landmarks, but it also revealed new neuroanatomical details of the IOC. For example, although the IOC consists of multipolar neurons

comparable to neognathous ‘ectopic cells’, it is not uniform but possesses a densely packed core region and a surrounding region of dispersed centrifugal visual neurons with wide dendritic arbors. In order to study the IOC’s afferents, double-tracer experiments into the optic tectum and eye were performed. This showed that the IOC receives afferents from the tectum similar as the ION of Neognathae. However, these fibers form delicate and wide-spread ramifications within the IOC region, which stands in striking contrast to the dense tecto-ION terminals in Neognathae (cf. Figure 6 E, p. 25). Therefore, tracer injections into the IOC region were made to reveal the tectal neurons of origin of those fibers. Retrogradely labeled cells were found in layer 10a of the optic tectum, comparable to the neognathous tecto-ION neurons, and identified as the Tinamou’s tecto-IOC neurons. These were much more sparsely distributed than in Neognathae such as the chicken (cf. Figure 6 D, p. 25), but possessed wider dendritic trees which spanned the spaces between neighboring cells. Finally, the centrifugal terminals in the retina were analyzed by means of *in vitro* and *in vivo* tracings. Both types of experiments showed congruently that all centrifugal visual terminals from the Tinamou’s IOC are of the divergent type, ramifying widely at the border of IPL and INL. Thus they resembled the terminals from neognathous ‘ectopic cells’, while no convergent terminals as from ION cells were present. Curiously, no target cells resembling the association amacrine cells of Neognathae were found by any of the established methods such as NADPH-diaphorase histochemistry (cf. Figure 6 C, p. 25). Together, the sparse distribution of tecto-IOC neurons and the divergent, wide-spread projections from the optic tectum to the IOC as well as from the IOC to the retina suggested that the CVS of the Tinamou has a lower spatial resolution than the CVS of Neognathae such as the chicken. The implications of these findings for understanding the evolution of the avian CVS were discussed. It was concluded that the general circuitry of a tectal cell population projecting to a dorsal isthmic population of centrifugal visual neurons which in turn project to the IPL of the retina appears to be an ancestral characteristic conserved in both Palaeognathae and Neognathae, while the configuration of the system’s constituent elements is prone to evolutionary change.

3.2.2 Role of the author

The contributions of the authors are clarified in the article: “All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: QK, TVZ, GM, HL. Acquisition of data: All *in vivo* and *in vitro* experiments were conducted at the Universidad de Chile. Most of them were done by QK during several research visits, while a few complementary experiments were done by MF, DC, TVZ and GM. Histology and histochemistry were performed by QK at the Universidad de Chile and at the Technische Universität München in Germany. Analysis and interpretation of data: QK, TVZ, CG, HL, GM. Drafting of the manuscript: QK. Critical revision of the manuscript for important intellectual content: TVZ, CG, HL, GM. Obtained funding: GM, HL. Study supervision: HL and GM.” (Krabichler et al. 2017; QK = Quirin Krabichler, TVZ = Tomas Vega-Zuniga, GM = Gonzalo Marín, HL = Harald Luksch, DC = Denisse Carrasco, MF = Maximo Fernandez, CG = Cristián Gutiérrez-Ibáñez)

To specify this information, the author of this dissertation had a leading role at all stages of this project, while continuous supervision and guidance was provided by the author’s mentor Dr. Tomás Vega-Zuniga, the Chilean project supervisor Dr. Gonzalo Marín, and the author’s doctoral supervisor Prof. Harald Luksch. The manuscript was mainly drafted by the author, with corrections and revisions from his mentor and supervisors. All *in vivo* experiments were conducted by the author, except one which had to be performed in Chile in his absence. Also all *in vitro* experiments, tissue processing and analysis were performed by the author.

3.2.3 Reproduction of the original article

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4. Discussion

4.1 The Chilean Tinamou as a palaeognathous model for experimental studies

The research articles included in the present doctoral thesis (Krabichler et al. 2015, 2017) represent the most comprehensive experimental study of the visual system of a palaeognathous bird that has to date been conducted, while previously only isolated studies on photoreceptors of various Palaeognathae (Braekevelt 1998; Sillman et al. 1981; Wright and Bowmaker 2001; Hart et al. 2016) and one on the visual field of the ostrich (Martin and Katzir 1995) had existed. The reasons for the paucity of previous experimental research in Palaeognathae may be found both in their lack of accessibility (many of them being exotic species rarely bred in captivity) and difficulties of manageability. For example, birds such as ostriches, emus and cassowaries, are difficult to keep and handle in a research environment due to their size and aggressiveness. Kiwis in turn are endangered species protected under law in New Zealand (Martin et al. 2007).

Tinamous in turn stick out as promising palaeognathous model candidates. The family Tinamidae contains 47 extant species endemic to the Neotropics of South and Middle America (Bertelli 2016). They are exceptional Palaeognathae, not only because they are the only ones which have retained their ability to fly (albeit somewhat crudely), while all of their ratite relatives had lost flight on independent evolutionary events (Baker et al. 2014), but also because they are smaller than most other palaeognathous representatives, having about the size of old-world partridges (Cabot 1992). In fact they bear some resemblance to the latter, which must have led early colonists to name Tinamous “perdices” (Old-world Spanish for partridges), in spite of the lack of any close relationship. Their small size suggests that they may be the Palaeognathae best suitable for experimental

studies, because that makes them relatively easy to keep, handle and also anesthetize.

The only problem concerning the use of Tinamous as laboratory animals is getting access to them in sufficient numbers. Until little more than two decades ago no commercial-scale poultry breeding of Tinamous had yet been successfully undertaken anywhere in the world (Kermode 1997), and therefore scientific projects that in any way require frequent supply of living specimens had been impossible. Since that time however, efforts to breed various species of Tinamous in captivity have been undertaken in different places in South America and also in Canada, and after progress had initially been slow (cf. for example Cromberg et al. 2007; Garitano-Zavala et al. 2004), a few species of Tinamous have been gradually becoming established as new domesticated poultry (cf. for example Queiroz et al. 2013; Martínez and Marini 2007). Nowadays, the Chilean Tinamou (*Nothoprocta perdicaria*), is an especially well-suited species, because living birds have since some years been readily available for purchase from a commercial breeder in Chile (Tinamou Chile, Los Ángeles, Región del Biobío, Chile).

This easy availability made it possible for us to conduct the systematic experimental studies of the Chilean Tinamou's visual system that constitute the present doctoral thesis. Subsequently, we managed to successfully establish protocols for performing neurobiological *in vivo* experiments under full anesthesia in these still not very domesticated animals. The present doctoral thesis and its included research articles bear testimony to this accomplishment and furthermore suggest that the Chilean Tinamou has excellent potential to become a suitable and versatile new model species for experimental studies of Palaeognathae.

4.2 The Chilean Tinamou's visual system

The present dissertation has studied the visual system of the palaeognathous Chilean Tinamou from various angles. The first study (Krabichler et al. 2015) focused on the visual field, retinal topography, visual acuity and retinofugal pathways to the brain. The second one investigated in detail the organization of this bird's centrifugal visual system. In this chapter, the major findings from both studies are recapitulated and discussed in a comparative, evolutionary context. In order to make this context more accessable however, it is suitable to first explain some important principles generally associated with the evolution of the brain and sensory systems, as well as any other organ.

When contemplating how the sensory and nervous system has evolved in different species, we often notice that there are systems or parts of systems which have changed relatively litte, while other systems or parts of systems have changed a lot. These two phenomena are generally called phylogenetic conservation and phylogenetic plasticity. If a characteristic has remained mostly unchanged between different species, we speak of phylogenetic conservation, if it has evolved variations, of phylogenetic plasticity. Since evolution is a highly complex and multi-facetted process, precise mechanisms that underlie either of the two phenomena are epistemologically difficult if not impossible to disentangle. Among possible causes of conservation in sensory and nervous systems might be the following: (1) Structural restriction; highly integrated biological systems such as established brain circuits may have a restricted evolvability because of the complex interconnectedness and resulting interdependance of their elements, which makes it more improbable for each element to change without compromising the functional integrity of the system (Katz 2011; Tierney 1995). (2) Behavioral restriction; structures and neural systems that are functionally related to specific behaviors or life-styles are

conserved through phylogeny, as long as that behavior and/or life-style is also maintained (Vega-Zuniga et al. 2013, 2017).

Plasticity, on the other hand, is especially pronounced in species that have adopted a radically different behavioral ecology as compared to their relatives, and therefore evolved derived features specialized for specific behavioral functions. Notable examples include highly gustatory cyprinid fishes such as the Goldfish, which has evolved a hypertrophied vagal lobe with complex lamination that is somewhat reminiscent of the avian optic tectum (Morita and Finger 1985), or South-American electric fish, which as part of their electric lateral line system evolved a laminated enlarged dorsal part of the torus semicircularis (homologous to part of the inferior colliculus in mammals) (Carr et al. 1981; for reviews see Wullimann and Grothe 2013; and Nieuwenhuys, ten Donkelaar, and Nicholson 1998).

The patterns of conservation and plasticity shaping the visual system of birds can strongly and unpredictably vary from species to species as well as from trait to trait, since variations in one element of the visual system can be expected to have implications for other elements. Owls for example possess very frontalized eyes with big binocular overlaps (Iwaniuk and Wylie 2006; Martin 1984, 2014), and in association with this also developed a relative increase of the thalamofugal visual pathway with a more complex GLd and bigger visual Wulst (Karten and Nauta 1968; Iwaniuk et al. 2008). The wulst of owls contains neurons which respond to binocular disparity and thus probably allows true stereovision (Pettigrew and Konishi 1976). Together, these traits may be associated with the life-style as nocturnally active hunters (Martin 2014; Wylie, Gutiérrez-Ibáñez, and Iwaniuk 2015). Among Palaeognathae, Kiwis are highly curious cases, because they went nocturnal and almost completely gave up on vision while in turn specializing on auditory, olfactory and tactile senses (Martin et al. 2007). Their visual system is highly reduced on all levels: they possess small eyes and despite their nocturnality almost no binocular overlap (Martin et al. 2007), their retina is thin with reduced laminae (Corfield et al. 2015), and they have strongly reduced

visual brain centers such as the optic tectum and pretectum (Corfield et al. 2016; Martin et al. 2007; Craigie 1930).

Keeping the ideas of phylogenetic conservation and plasticity in mind, we will now turn back to the Chilean Tinamou's visual system.

4.2.1 Visual fields

As we have seen, the Chilean Tinamou has lateral eyes with a big cyclopean field and a relatively small binocular overlap of 20° (Krabichler et al. 2015). Altogether, this bird has a "type I visual field" according to the categories of Martin 2007 (i.e. narrow and vertically elongated binocular overlap of 20-30°), similar as a great variety of neognathous as well as palaeognathous species with diurnal and ground-feeding life-styles (Martin 2007). Its small binocular field may serve the bird for visual guidance of the bill during feeding. However, it is clear from the small binocular overlap that binocularity is not of major importance for the Tinamou's way of living. Rather, since it is a prey species (Cabot 1992; hunted by birds of prey, cf. Jiménez and Jaksić 1989; and Figueroa and Corales 1999; as well as carnivora, cf. Pearson and Pearson 1955), the relatively large cyclopean field which results from the lateral eyes assumedly is more relevant to the Tinamou.

The evolution of visual fields is governed by a combination of phylogenetic conservation and plasticity. In birds there is a clear phylogenetic determinant which means that closely related species often have similar visual field layouts. On the other hand, visual fields can also be strikingly plastic and evolve relatively quickly depending on visuoecological needs of a species (Martin 2014; Wylie, Gutiérrez-Ibáñez, and Iwaniuk 2015). For example, relatively large binocular overlaps are conserved in almost all passerines (reviewed in Martin 2014; cf. Moore et al. 2015), whereas the adoption of drastically different life-styles in some passerines has led to the evolution of more laterally placed eyes such as in the case of swallows, which are aerial insect-catchers (Tyrrell and Fernández-Juricic 2017a). As a second example, most ducks (Anatidae) possess very little

or even no binocular overlap at all, because they are non-visually-guided tactile and filter feeders (Martin 1986; Guillemain, Martin, and Fritz 2002), whereas in contrast a closely related duck species, the blue duck *Hymenolaimus malacorhynchos*, shows a visually active feeding behavior and has evolved a substantial binocular overlap of 34° (Martin, Jarrett, and Williams 2007).

The Chilean Tinamou's visual field can be compared with the ostrich *Struthio camelus* (Martin and Katzir 1995). In fact, their visual fields have almost identical dimensions, with respect to their binocular overlaps (Tinamou: 20°, ostrich 20°), their cyclopean fields (Tinamou 300°, ostrich 290°) and their blind fields (Tinamou 60°, ostrich 70°). Thus, although about 80 million years of divergent evolution separate the lineages of the ostrich and the Chilean Tinamou (Yonezawa et al. 2017), it is possible that their visual field topographies represent a case of phylogenetic conservation from their common ancestor. Both species have similar visuoecological needs, most importantly scanning their environment for predators in open terrain since both are inhabitants of steppe and savannah terrain. This life-style may have maintained the balance between relatively lateral eyes for scanning and a small binocular overlap for visual guidance of the bill.

4.2.2 RGC topography and visual acuity

Un unexpected finding was the high number and density of retinal ganglion cells (RGCs) in the Tinamou's retina, which was also reflected in the bird's relatively high visual acuity as estimated according to the sampling theorem (cf. Introduction 1.3.3, pp. 13ff.; Krabichler et al. 2015). This indicates that the Chilean Tinamou has very good visual capacities despite its relatively small size as compared to other Palaeognathae such as the ostrich or emu. Also, the RGC topography revealed three different specializations, a pronounced area centralis with a shallow fovea, a dorso-temporal area (or area dorsalis), and a faint horizontal visual streak. The area centralis provides high-acuity vision, also for viewing distant objects. The streak provides some help in permanent surveillance of the horizontal surroundings. The dorso-temporal area assumedly

looks toward the frontal lower visual field, where the binocular overlap around the bill is, and thus suggests that the Tinamou might use his bill under binocular visual guidance during foraging and other behaviors. These retinal characteristics together with the visual field data (see above) indicate that this bird is a visual “generalist”, with specializations for various tasks such as surveillance, visual guidance of the bill, and high-acuity foveal vision for fixating near and far objects.

Retinal topography is phylogenetically relatively plastic and changes over time depending on the visual ecology of species. Therefore a high diversity of the RGC topography is found in the animal kingdom. However, changes still occur more or less slowly such that within closely related groups the topographical pattern of RGCs remains mostly conserved. For example, the closely related species of the waterfowl family Anatidae have acquired very different ecologies, especially with respect to foraging modes such as diving, dabbling near the surface and grazing on land (Guillemain, Martin, and Fritz 2002). While one would expect their RGC topographies to have changed according to the different demands on vision during foraging, all have very similar RGC topographies (Lisney et al. 2013). A similar example is represented by Australian oscine passerines of the superfamily Meliphagoidea which despite having evolved a diverse adaptive radiation to different feeding behaviors such as nectarivory, insectivory and frugivory as well as generalist combinations of various modes, have retained the same basic pattern of RGC topography (Coimbra, Collin, and Hart 2014b). Analogous findings have also been made in suboscine passerines, the tyrant flycatchers of the family Tyrannidae (Coimbra et al. 2006, 2009). Given a bit more time of evolutionary divergence however, RGC topographies change at last. For example, various genera of cockatoos (Psittaciformes), whose lineages had diverged from each other between 22-14 million years ago, today exhibit notable differences in their RGC topographies reflecting their different life-styles and foraging modes (Coimbra, Collin, and Hart 2014a).

How long does it take for the plasticity of the RGC topography to manifest itself in Palaeognathae? As only very few species have been studied with respect to

RGC topography, it is not yet possible to answer this question. Our study in the Chilean Tinamou (Krabichler et al., 2015) represents the first report in a tinamiform species. The only other studied Palaeognath is the ostrich *Struthio camelus*. Its retina features a very different RGC topography than the Chilean Tinamou, with a pronounced horizontal visual streak, a not very marked area centralis, and a very little pronounced dorso-temporal area (Boire et al. 2001). The phylogenetic split of the ostrich from all other Palaeognathae occurred about 80 million years ago (Yonezawa et al. 2017), so both lineages have had a relatively long time for evolving different retinal traits. More palaeognathous species will have to be studied with respect to their retinal topography. For example, with respect to Tinamiformes it would be interesting to study representatives of the subfamily of the "forest tinamous" (Tinaminae), which in contrast to the "steppe tinamous" (Rhynchotinae; including the Chilean Tinamou) live in tropical and subtropical forests of South America (Cabot 1992). The two groups diverged at least 17 million years ago (Bertelli, Chiappe, and Mayr 2014; Bertelli 2016), and it would be interesting to study in how far the different, more visually obstructed habitats of forest tinamous have influenced the evolution of RGC topography.

4.2.3 Retinofugal projections

The retinal projections and retinorecipient brain centers of the Chilean Tinamou are well-developed, in agreement with its assumedly good visual capacities as indicated by the RGC topography. The general pattern of projections was found to be quite similar to the known scheme of Neognathae with roughly similar life-styles (diurnal and ground-feeding), such as chickens or pigeons (Krabichler et al. 2015, and references therein). This shows that these elements of the visual system are relatively conserved with little change on a macro- and mesoscopic scale, despite the rather long evolutionary divergence between Palaeognathae and Neognathae of over 100 million years (cf. chapter 1.3.2; Yonezawa et al. 2017; Mayr 2014). Our data therefore suggested that the last common ancestor

of Neognathae and Palaeognathae probably already possessed a very similar organization of the retinal projections and visual brain centers, which may stem from even further back in the evolutionary lineage leading towards modern birds.

However, the retinorecipient brain centers of the Chilean Tinamou held a surprise which represented a striking variation from the visual system of Neognathae, the finding of direct retinal projections to the deep tectal layers 11-13. In our 2015 article, we discussed this finding in light of what was then known. Given that such a tracing had previously been described in embryonic chicken but was claimed to disappear before hatching (Omi, Harada, and Nakamura 2011), and that on the other hand also a transient projection from the ION to the optic tectum during embryonic stages of the chicken had been reported (Wizenmann and Thanos 1990), we reasoned that in the Tinamou each of these scenarios represented a possible expalantion for the presence of deep tectal terminals labeled after intraocular tracer injections (in the case that the isthmo-optic neurons were the source, the tracer would have had to travel retrogradely from the eye to the isthmo-optic neurons and from there again anterogradely to the terminals in the optic tectum). In both scenarios we reasoned that, due to the reported absence in adult Neognathae, the deep tectal terminals in the adult Tinamou could represent a paedomorphy (the evolutionarily fixed retention of an originally juvenile trait in the adult bird).

With the aim to clarify the source of the deep tectal terminals in the Tinamou, we injected kainate into the eye two weeks before injecting CTB, in order to extinguish the orthograde retinofugal projections while leaving intact the retrograde pathway of the centrifugal visual system (cf. Methods, pp. 30f.; Miceli et al. 1993). The hitherto unpublished results are shown in Figure 7. Clearly, while a normal CTB injection without kainate treatment labeled conspicuous varicosities in tectal layers 11-13 (Figure 7 A, B), the kainate-pretreated case (Figure 7 C, D) showed a strong reduction or in fact almost complete absence of deep tectal terminals. Meanwhile, the IOC was strongly labeled with no sign of impairment by the kainate treatment (data not shown). This demonstrates that the deep tectal terminals in the Chilean Tinamou are in fact deep tectal *retinal* terminals

and represent a retinofugal pathway that originates from some type of RGC. The identity of the RGCs of origin as well as the functional significance of this projection should be studied in-depth in the future. Furthermore, the retinofugal projections should be studied in more palaeognathous species, as well as revisited in Neognathae in order to determine if they possess a similar projection in adults that has hitherto been overlooked. This would also help to clarify if the deep tectal retinal pathway represents a characteristic conserved among all Aves, or if it is specific to Palaeognathae.

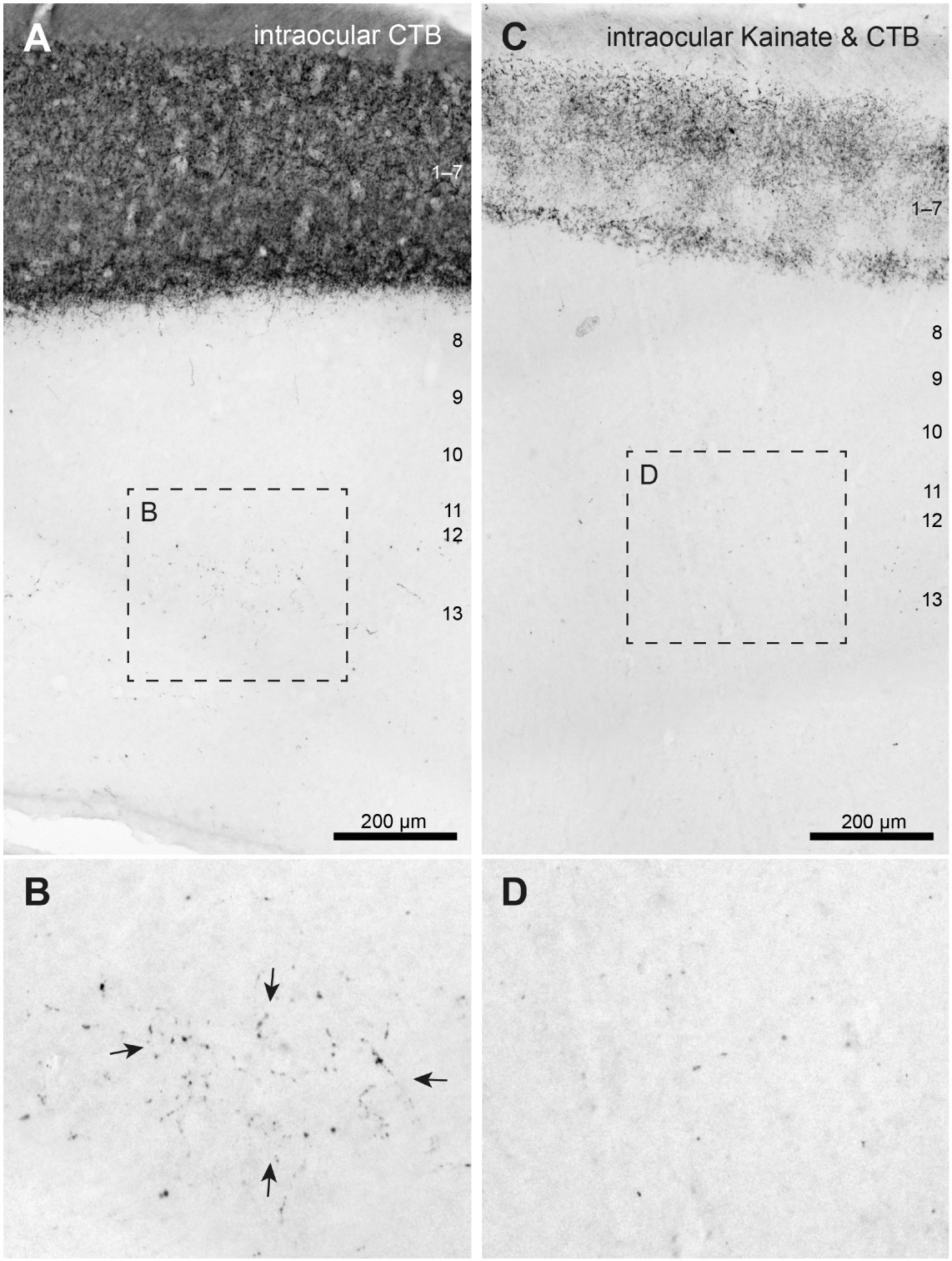

Figure 7: The deep tectal terminals in the Chilean Tinamou represent retinofugal projections. A: Normal CTB injection into the eye labeled in addition to the well-known retinofugal terminals in layers 1-7 of the optic tectum also terminals in deep layers 11-13. B: Enlargement of inset marked in A, with arrows indicating CTB-labeled terminal varicosities. C: When most of the retinal ganglion cells were destroyed by intraocular injection of kainate, the subsequent CTB injections resulted in a strong reduction of the projection to the main retinorecipient tectal layers 1-7, and importantly to an almost complete degeneration of the deep tectal terminals. D: Enlargement of inset marked in C, indicating absence of varicosities.

4.2.4 Centrifugal visual system

Perhaps the most significant, and certainly the most spectacular results of our investigation of the Chilean Tinamou's visual system relate to its CVS. The first important finding was our discovery of the presence of a substantial but non-nuclearly organized group of centrifugal visual neurons, which are completely indistinguishable in normal counter-stained sections (Gutiérrez-Ibáñez et al. 2012; Krabichler et al. 2015). Subsequently, when we studied this system in detail focusing on its connectivity, we found that the general circuitry is conserved among Neognathae and Palaeognathae, while the configuration and neuroarchitecture of the system's elements are highly diverse in the two groups (Krabichler et al. 2017), which is indicative of a high level of phylogenetic plasticity of these parts of the CVS. Basically, in many Neognathae the ION has a conspicuous laminar organization, and convergent tectal afferents as well as retinopetal efferents which are both organized in a finely resolved topography. The IOC of the Chilean Tinamou in turn represents a loosely organized population of neurons which receive divergent, widely ramifying afferents from relatively sparsely distributed tectal neurons and sent divergent, widely ramifying fibers to the retina.

Is the CVS of the Tinamou "more primitive" than the neognathous one? Certainly the impressively laminated ION and its circuitry of certain granivorous Neognathae appears to be more specialized. For example, such a organization could convey a higher spatial resolution than the diffuse organization in the Tinamou. However it should be kept in mind that while some Neognathae such as the chicken or pigeon possess a very big ION with up to 12,000 cells, other Neognathae have very small ION whose numbers range from a few thousand to only several hundred, and which even lack any conspicuous laminated organization (cf. chapter 1.3.5, pp. 21f.; Gutiérrez-Ibáñez et al. 2012). The Chilean Tinamou's IOC possesses approximately 4400 neurons (approx. 4100 contralaterally and 300 ipsilaterally projecting; Krabichler et al. 2015), which is more than the ION of many Neognathae. Biased value judgements should therefore be avoided. Rather, our study of the Tinamou's CVS corroborates the

importance of investigating the connectivity of seemingly unspecialized neural circuits. The high diversity of ION sizes and complexities even within Neognathae indicates that the CVS components and their configuration have a high plasticity across all Aves, Palaeognathae and Neognathae alike. Systematic studies of the circuitries in more bird species with different ION organizations may help us to learn about the general mechanisms behind the evolution of the avian CVS.

In any case, the remarkable number of IOC neurons in the Tinamou and their similar general connectivity as in Neognathae suggest that its CVS must be in some ways functionally and behaviorally relevant for the Tinamou's life-style. However, which this function precisely is, is not yet known. In fact not even in well-studied neognathous species such as the chicken and the pigeon has any clear picture emerged with respect to the function of their CVS. Recent authors have suggested that the complex neognathous ION serves for switching attention between emmetropic upper/horizontal and myopic lower visual fields, and correlated this with feeding behavior, stating that species which feed close to the substrate and thus need to regularly switch to a myopic lower visual field tend to possess larger and more complex ION than others (Gutiérrez-Ibáñez et al. 2012; Wylie, Gutiérrez-Ibáñez, and Iwaniuk 2015). The palaeognathous Tinamou also feeds close to the substrate and its RGC topography furthermore features a dorso-temporal area which presumably looks towards the lower and frontal visual field during visually guided feeding. However, it has not evolved a highly complex or even very large ION. Importantly, those authors reached their conclusions based on volumetry/allometry studies of normal counter-stained material from a broad range of different species. This completely ignores the presence of any loosely organized centrifugal visual neurons such as in the Chilean Tinamou (which those authors had claimed not to possess an ION). As our studies have shown, mere volumetry/allometry on the basis of gross histological landmarks such as nuclear boundaries may not be sufficient for reaching functional conclusions regarding the CVS. Rather it is necessary to study connectivity in more species by intraocular tracer injections. In the future,

the microconnectomics of the CVS of the Tinamou, as well as of Neognathae with small or seemingly absent ION, should be further studied in detail.

The Tinamou may specifically also serve for further studies regarding the identity and evolutionary origin of the association amacrine cells. Since these cells are conspicuous and relatively easy to identify in Neognathae with big ION (see for example Figure 6 C, p. 24), it is striking that the same methods have not yielded results in the Tinamou retina (Krabichler et al. 2017). Either association amacrine cells are an evolutionary novelty of Neognathae, or equivalent cells do exist in the Tinamou retina but have a different phenotype (and gene expression), possibly because the specific phenotype of the neognathous ones emerged alongside with the evolution of convergent centrifugal terminals. This problem could be addressed by comparative single-cell transcriptomics of avian and reptilian retinae.

Lastly, studies of the amniote CVS should also be extended to include crocodiles, the closest living relatives of birds (Brusatte, O'Connor, and Jarvis 2015; Yonezawa et al. 2017). It is already known that crocodiles possess several thousand centrifugal visual neurons in the area of the dorsal isthmus similar as birds, however their distribution is still more diffuse than in the Chilean Tinamou (Derobert et al. 1999; Ferguson, Mulvanny, and Brauth 1978; Médina et al. 2004). The connectivity of these neurons in crocodiles is so far unknown. A study of the CVS connectomics in crocodiles analogous to our study in the Chilean Tinamou could yield relevant results for better understanding the evolution of the CVS in amniotes. As in the Tinamou, the existence and identity of association amacrine cells in the crocodile retina would also be an important subject to study.

4.3 A tribute to comparative neurobiology

The present thesis has studied the visual system of the Chilean Tinamou, a hitherto unstudied species from a hitherto little investigated basal branch of the avian phylogenetic tree, the Palaeognathae. One might ask: Why does this kind of research matter? What if anything can such studies teach us about how the brain, in particular our own one, works? After all, nowadays most research in the neurosciences focuses on 'big models' such as the mouse *Mus musculus* (Van Hooser and Nelson 2006; DeFelipe 2011; Brenowitz and Zakon 2015), which offer benefits such as easy keeping and breeding in captivity, a broad range of sophisticated tools including viral vector tracing, optogenetics, knock-out and transgenic lines, and the synergy resulting from big scientific communities studying the same species and developing new model-specific tools. Such benefits make 'big models' powerful objects for scientific research and have enabled neuroscience to rapidly advance in its course towards understanding the brain.

However, it must also be asked if there are potential drawbacks to the 'big model' approach. It turns out that there are: In the long run, focusing on only few model species neglects the vast biological diversity of animals and creates a knowledge bottleneck with the dangerous tendency to overgeneralize results from few species to all other animals including us humans. These overgeneralizations remain undetected for a long time, because if only isolated model species have been studied, phenomena that are species-specific variations cannot be distinguished from those which may indeed be common to many different species (Brenowitz and Zakon 2015). Even more importantly, it can be argued that by studying only few species, scientific knowledge will sooner or later hit a wall which hinders further progress, since this approach

disregards the essential necessity of historical (i.e. phylogenetic) context for understanding the functional organization of the nervous system of animals. Such context is essential because it teaches us how neural structures evolved alongside the evolution of the species, and how changes in their life-styles are reflected in their brains.

This directly leads us to the alternative approach in contrast to 'big model' neuroscience. This neuroscientific discipline, called comparative neurobiology, investigates the different brain architectures in the animal kingdom in light of their phylogenetic history. It can actuallly be regarded as the original discipline upon which the whole field of neuroscience has been founded (Striedter 2005). Many of the first ground-breaking neuroscientific discorveries were made by the great fathers of neuroscience such as Santiago Ramón y Cajal while comparing brain architecture in different vertebrates (S. Ramón y Cajal 1888, 1889, 1893; P. Ramón y Cajal 1898; S. Ramón y Cajal 1909, 1911), and there are today still various renowned scientific journals such as the Journal of Comparative Neurology (since 1891) which are completely dedicated to this field. The comparative approach takes into account that all species originally diverged from common ancestors and that their nervous systems represent mosaics resulting from a complex interplay between phylogenetic conservation which maintains existing structures and plasticity which allows for the emergence of novelties and specialized variations (Northcutt 1984). Brain structures do not come into existence from nothing, they generally evolve from pre-existing structures of some form and thus display patterns of similarities and variations that reflect their evolutionary history and phylogenetic relationships (Figure 8; Kaas 2009; Naumann et al. 2015; Nieuwenhuys, ten Donkelaar, and Nicholson 1998).

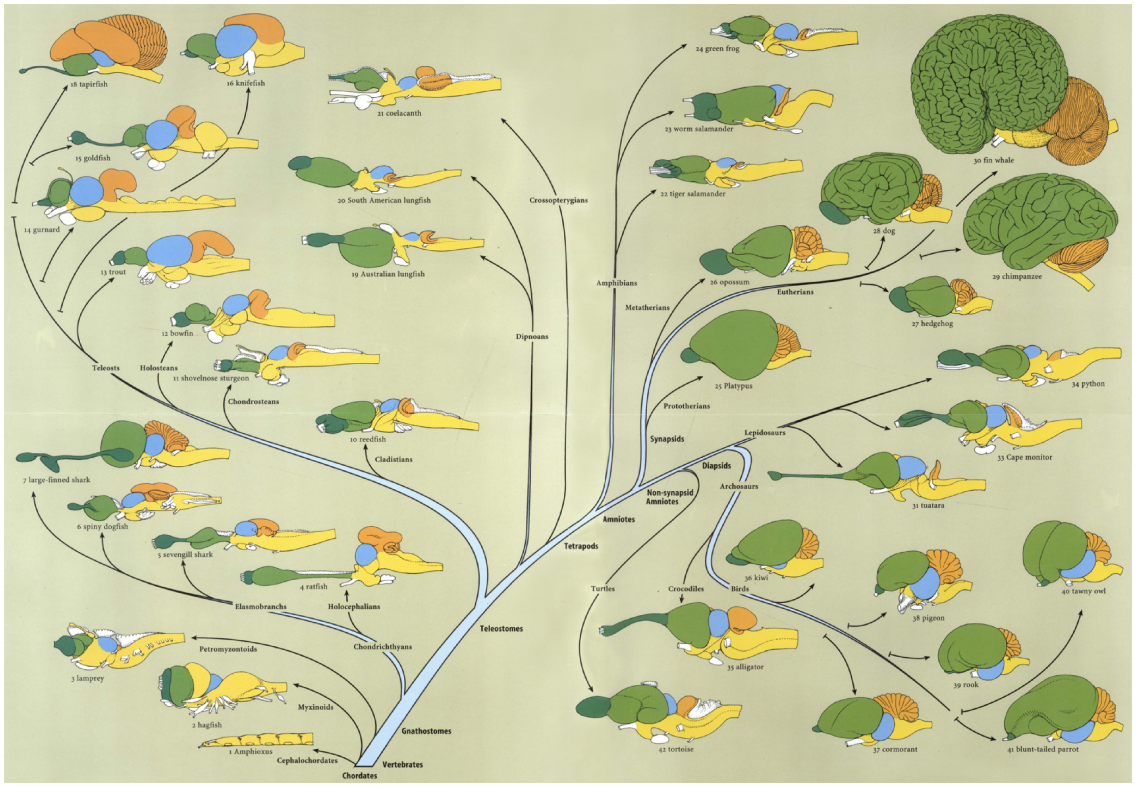

Figure 8: Diversity of vertebrate brains. Dark green - olfactory bulb; light green - telencephalon; blue - midbrain tectum; orange - cerebellum; yellow - remaining parts. Reproduced from Nieuwenhuys et al. 1998, with permission of SPRINGER in the format Thesis/Dissertation via Copyright Clearance Center (licence# 4124180240060).

The essential power of the comparative approach is thus that knowledge gained from studying the brain of any vertebrate species has transferable implications for understanding neurobiological phenomena in the brains of other species and ultimately yields universal neurobiological principles (Vecera 2012; Brenowitz and Zakon 2015; Naumann et al. 2015; Niven and Chittka 2016). By comparison of different species, evolutionarily conserved and divergent characteristics can be distinguished, which can enable us to better understand the course of evolution of brains and possible principles that have guided it (Kaas 2009). This in turn explains how structural variations in brains are related to different functions and behaviors, such as they have emerged during evolution (Edelman, Baars, and Seth 2005; Iwaniuk, Lefebvre, and Wylie 2009; Kaas 2009; Petkov and Jarvis 2012; Cauchoix and Chaine 2016; Niven and Chittka 2016). Thus, despite the current prevalence of the ‘big model’ approach, the history of

neuroscience is replete with examples of ground-breaking findings of universal neurobiological importance, which were made and could only have been made in ‘exotic’ species (Manger et al. 2008; Brenowitz and Zakon 2015).

The precedent discourse should not be misinterpreted as arguing that ‘big model’ neuroscience is without merits and should be given up, on the contrary. However, it outlines some very important points in favor of comparative neurobiology and argues for its continuing and lasting relevance for advancing neuroscience. This dissertation on the Chilean Tinamou visual system represents an examplary case of comparative neurobiology and bears some witness to its power. It has shown that the neuroscientific study of ‘exotic’ species, especially in case of relatively distant outgroups to more commonly studied species, can open up interesting and relevant new perspectives regarding the organization and evolution of the brain and sensory systems. The ‘deep tectal’ retinal terminals and the curious organization of the palaeognathous CVS both stick out as findings with a potential to have future impact on our understanding of the function and evolution of the avian visual system, and thus visual systems in general. Therefore, the studies contained in the present dissertation pave the way for future comparative research on the avian visual system and suggest to continue to investigate Palaeognathae as an outgroup to neognathous birds.

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Appendix A – Krabichler et al. 2015 (full article)

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The Visual System of a Palaeognathous Bird: Visual Field, Retinal Topography and Retino-Central Connections in the Chilean Tinamou (*Nothoprocta perdicaria*)

Quirin Krabichler,[1]* Tomas Vega-Zuniga,[1] Cristian Morales,[2] Harald Luksch,[1]* and Gonzalo J. Marín[2,3]*

[1]Chair of Zoology, Technische Universität München, Freising-Weihenstephan, Germany
[2]Laboratorio de Neurobiología y Biología del Conocer, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago de Chile, Chile
[3]Facultad de Medicina, Universidad Finis Terrae, Santiago de Chile, Chile

ABSTRACT

Most systematic studies of the avian visual system have focused on Neognathous species, leaving virtually unexplored the Palaeognathae, comprised of the flightless ratites and the South American tinamous. We investigated the visual field, the retinal topography, and the pattern of retinal and centrifugal projections in the Chilean tinamou, a small Palaeognath of the family Tinamidae. The tinamou has a panoramic visual field with a small frontal binocular overlap of 20°. The retina possesses three distinct topographic specializations: a horizontal visual streak, a dorsotemporal area, and an area centralis with a shallow fovea. The maximum ganglion cell density is 61,900/ mm^2, comparable to Falconiformes. This would provide a maximal visual acuity of 14.0 cycles/degree, in spite of relatively small eyes. The central retinal projections generally conform to the characteristic arrangement observed in Neognathae, with well-differentiated contralateral targets and very few ipsilateral fibers. The centrifugal visual system is composed of a considerable number of multipolar centrifugal neurons, resembling the "ectopic" neurons described in Neognathae. They form a diffuse nuclear structure, which may correspond to the ancestral condition shared with other sauropsids. A notable feature is the presence of terminals in deep tectal layers 11–13. These fibers may represent either a novel retinotectal pathway or collateral branches from centrifugal neurons projecting to the retina. Both types of connections have been described in chicken embryos. Our results widen the basis for comparative studies of the vertebrate visual system, stressing the conserved character of the visual projections' pattern within the avian clade. J. Comp. Neurol. 523:226–250, 2015.

INDEXING TERMS: retinal ganglion cells; retinal projections; centrifugal visual system; isthmo-optic nucleus; optic tectum; avian; deep tectal pathway; RRID: AB_10013220

As a group, birds rank among the most visual vertebrates that ever lived on earth. Their reliance on vision is manifested in very large eyes and a highly differentiated visual system, in which the visual pathways and nuclei, conforming to a common vertebrate neural *Bauplan*, are particularly distinct and well developed (Güntürkün, 2000; Karten, 1969).

However, in spite of large-scale comparative studies exploring the allometric variations of specific brain structures (Corfield et al., 2012; Iwaniuk et al., 2005, 2010), systematic anatomical and electrophysiological investigation of the avian visual system has focused on only few species: the chicken (*Gallus gallus*; Ehrlich and Mark, 1984a,b; Koshiba et al., 2005; Luksch et al., 2001; Verhaal and Luksch, 2013; Wang et al., 2004, 2006), the

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*CORRESPONDENCE TO: Quirin Krabichler, Lehrstuhl für Zoologie, Technische Universität München, Liesel-Beckmann Strasse 4, 85354 Freising-Weihenstephan, Germany. E-mail: quirin.krabichler@tum.de; Harald Luksch, Lehrstuhl für Zoologie, Technische Universität München, Liesel-Beckmann Strasse 4, 85354 Freising-Weihenstephan, Germany. E-mail: harald.luksch@wzw.tum.de; and Gonzalo J. Marín, Laboratorio de Neurobiología y Biología del Conocer, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Casilla 653, Chile. E-mail: gmarin@uchile.cl

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rock pigeon (*Columba livia*; Benowitz and Karten, 1976; Binggeli and Paule, 1969; Karten et al., 1973, 1997; Letelier et al., 2004; Marín et al., 2003, 2012; Mpodozis et al., 1995; Remy and Güntürkün, 1991; Shimizu et al., 1994), the quail *(Coturnix coturnix*; Budnik et al., 1984; Ikushima et al., 1986; Maturana and Varela, 1982; Norgren and Silver, 1989a), the barn owl (*Tyto alba*; Bravo and Pettigrew, 1981; Gutfreund, 2012; Gutfreund et al., 2002; Harmening and Wagner, 2011; Knudsen, 2002; Pettigrew and Konishi, 1976; Wathey and Pettigrew, 1989), and the zebra finch (*Taeniopygia guttata*; Bischof, 1988; Faunes et al., 2013; Keary et al., 2010; Schmidt and Bischof, 2001; Schmidt et al., 1999); all of them pertaining to the Neognathae, the grand clade to which most extant bird species belong.

Modern birds, or Neornithes, however, include a second extant clade, the Palaeognathae (Hackett et al., 2008), encompassing six living families: Struthionidae (ostrich), Dromaiidae (emu), Casuariidae (cassowaries), Apterygidae (kiwi), Rheidae (rheas), and Tinamidae (tinamous) (Harshman et al., 2008). Surprisingly, apart from a few studies (e.g., on the retinal topography of the ostrich [Boire et al., 2001; Rahman et al., 2010], on the photoreceptors of ostrich and rhea [Wright and Bowmaker, 2001], or on the sensory systems of the kiwi [Martin et al., 2007]), the Palaeognathae have been vastly ignored by comparative neurobiologists, even though their considerable phylogenetic distance from the commonly studied Neognathae — 120–130 million years (Brown et al., 2008; Haddrath and Baker, 2012) — makes them a very interesting subject for gaining insights into the evolution of the avian visual system and the scale of the phylogenetic plasticity of its constituent elements.

Undoubtedly, the lack of attention toward palaeognathous birds can be largely explained by their scarcity and, not least, by their difficult manageability: most Palaeognaths are rather large, fierce animals, such as the ostrich or the emu, whereas the smaller kiwis exhibit highly derived characteristics with a greatly reduced visual system (Martin et al., 2007).

However, there is one palaeognathous group without such drawbacks: The Tinamiformes, consisting of the sole family Tinamidae, represent 47 species in nine genera (Bertelli and Porzecanski, 2004; Bertelli et al., 2014), which are endemic to the Neotropics of South and Middle America (Cabot, 1992). They are diurnal birds, and are generally medium-sized (the largest about the size of a pheasant). Intriguingly, they are the only living Palaeognathae that can fly. Even so, they are ground-dwelling birds and make use of their short but strong wings only to escape from immediate danger or to reach their roost (Cabot, 1992; Conover, 1924; Pearson and Pearson, 1955). This remarkable lifestyle suggests well-developed sensory capacities, particularly in the visual system, and especially in those tinamous inhabiting open terrains, the Steppe tinamous (subfamily Nothurinae; Bertelli et al., 2014).

Figure 1. The Chilean tinamou (*Nothoprocta perdicaria*) in the wild. Lateral view and frontal portrait (inset). Photography by Sergio Bitran M. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In the present study, as a first step in an overall investigation of the visual system of a Steppe tinamou, the Chilean tinamou (*Nothoprocta perdicaria*; Fig. 1), we mapped the extent of the visual field, examined the topography of the retinal ganglion cell layer (GCL) and, by injecting cholera toxin subunit B (CTB) into the eye, traced the retinal connections to the central targets in the brain.

MATERIALS AND METHODS

Seven adult Chilean tinamou (*Nothoprocta perdicaria*) were used in this study. They were acquired from a Chilean breeder (Tinamou Chile, Los Ángeles, Chile). The animals were kept in cages with food and water *ad libitum*. All efforts were made to minimize animal suffering, and experiments were conducted in compliance with the guidelines of the U.S. National Institutes of Health on the use of animals in experimental research, with the approval of the bioethics committee of the Facultad de Ciencias of the Universidad de Chile.

Measurement of the visual field

The visual field measurements were conducted by the methods described in Vega-Zuniga et al. (2013). Four animals were anesthetized with a mixture of ketamine (120 mg/kg IP) and xylazine (4 mg/kg IP) and mounted in a stereotaxic head holder in the center of a custom-built campimeter. The head was positioned so that the palpebral fissures were aligned with the campimeter's equator (analysis of photographs of

relaxed birds showed that the normal posture of the head is inclined downward by approximately 10° relative to this position). During the experiment, the eyelids of the birds were held open with thin strips of masking tape, and the eyes were kept constantly moist by applying sterile NaCl solution every few minutes. We then used an ophthalmoscopic reflex technique to measure the visual fields of both eyes of each bird, determining the nasal and temporal limits of the retinal reflections and noting the angles in a conventional latitude/longitude coordinate system.

Retinal whole-mounts

For analysis of the retinal whole-mounts, we followed the methods described by Ullmann et al. (2012). The eyes of three animals were enucleated from their sockets after phosphate-buffered saline (PBS) perfusion of the animals (see below), their axial length was measured with digital calipers, and they were hemisected close to the ora serrata. The vitreous body was removed from each retina, which was then dissected from the sclera, ending with the excision of the optic nerve head and pecten. With forceps and fine paintbrushes, the retina was cleared from the pigment epithelium and, after flattening with four radial incisions, was whole-mounted on gelatin-coated slides, left to dry and firmly attach to the gelatin, and fixed overnight with paraformaldehyde (PFA) vapors at 60°C. Afterward, the retina was Nissl-stained, dehydrated in ascending alcohols followed by clearing in xylene, and coverslipped with DPX (Sigma-Aldrich Chemie, Steinheim, Germany). No assessment was made of possible areal shrinkage of the retina, which is reportedly minimal in whole-mounted retinas affixed to gelatin-coated slides (Wässle et al., 1975).

Retinal cross sections

Two Chilean tinamou eyes were removed immediately after perfusion of the animal (see below), hemisected at the ora serrata (see Fig. 4A), and postfixed for 6 hours in 4% PFA. The eyecups were then transferred into a 30% sucrose/PBS (0.1 M: 0.023 mM NaH_2PO_4 and 0.08 mM Na_2HPO4, pH 7.4; with NaCl 0.75%) solution until they sank. A gelatin embedding solution was produced by adding 10 g sucrose and 12 g gelatin type A (Sigma-Aldrich Chemie) to 100 ml $H_2O_{dest.}$ and heating it to 55°C to dissolve the gelatin. Both the eyecups in sucrose solution and the gelatin solution were put into an oven at 37°C until they reached the same temperature. Then the vitreous bodies were removed from the eyecups, which were subsequently embedded in gelatin. The gelatin–eyecup blocks were trimmed, and put into 4% PFA for postfixation for 2–5 hours and afterward into 30% sucrose/PBS for cryoprotection until they sank. They were sectioned with a cryostat (Kryostat 1720, Leica, Wetzlar, Germany) at 30 μm in both the transversal and horizontal planes, and the sections were mounted on gelatin-coated slides, Nissl-stained, rapidly dehydrated in ascending alcohols followed by clearing in xylene, and cover-slipped with DPX.

Visual acuity estimation of the eye

The maximal spatial resolving power (SRP) was approximated using the sampling theorem (Hughes, 1977). This is a way to estimate the theoretical maximal visual acuity from the eye's posterior nodal distance (PND) and the peak density of retinal ganglion cells (RGCs) (Collin and Pettigrew, 1989; Pettigrew et al., 1988; Ullmann et al., 2012). The inclusion of non-ganglionic cell populations (i.e., displaced amacrine cells) in the estimation is negligible because of the

Abbreviations

AC	area centralis
AOS	accessory optic system
AP	area pretectalis
APd	area pretectalis, pars dorsalis
CO	optic chiasm
cpd	cycles per degree
CTB	cholera toxin subunit B
DAB	diaminobenzidine
DLAmc	n. dorsolateralis anterior thalami, pars magnocellularis
DLL	n. dorsolateralis anterior thalami, pars lateralis
DTA	dorsotemporal area
EC	ectopic cell
ECR	ectopic cell region
GCL	retinal ganglion cell layer
GLv	n. geniculatus lateralis, pars ventralis
GLd	n. geniculatus lateralis, pars dorsalis
GT	tectal gray
IGL	intergeniculate leaflet
ION	isthmo-optic nucleus
LA	n. lateralis anterior thalami
LdOPT	n. lateralis dorsalis optici principalis thalami
LM	n. lentiformis mesencephali
LMl	n. lentiformis mesencephali, pars lateralis
LMm	n. lentiformis mesencephali, pars medialis
nBOR	n. of the basal optic root
nIV	nucleus nervi trochlearis
nMOT	n. marginalis tractus optici
OT	optic tract
PBS	Phosphate-buffered saline
PFA	paraformaldehyde
PND	posterior nodal distance
PT	n. pretectalis
RGC	retinal ganglion cell
ROI	region of interest
Rt	n. rotundus
SO	stratum opticum
SPC	n. superficialis parvocellularis
SpRt	n. suprarotundus
SRP	spatial resolving power
Tdp	deep tectal pathway
TeO	optic tectum
TIO	isthmo-optic tract
vSCN	visual suprachiasmatic nucleus
VLT	n. ventrolateralis thalami

relatively very small ratio of such cells in high-density retinal areas (Hayes and Holden, 1983). Because no direct measurement of the PND was made, the known approximate PND to axial length ratio of 0.60 in diurnal birds was used as described in the literature (Boire et al., 2001; Hughes, 1977; Martin, 1993; Ullmann et al., 2012): PND=0.60 × axial length. The angle covering 1 mm on the retina is then: $\alpha = \arctan \frac{1\ mm}{PND}$. Spatial resolution is estimated by calculating the number of cells covered by 1 degree of visual arc in the area centralis (AC). Because the cell density is given in cells/mm^2, the square root is applied to convert it to cells/mm. The number of cells per degree is: *cells per degree* $= \frac{density\ at\ area\ of\ peak\ cell\ distribution}{\alpha}$. Finally, the result has to be divided by 2, because at least two cells are necessary for one cycle of grating (one light and one dark bar in one degree of visual angle). Thus, the SRP is given in *cycles per degree* (cpd): $SRP\ [cpd] = \frac{cells\ per\ degree}{2}$.

Neuronal tracing experiments

For the intraocular tracer injection experiments, five birds were sedated and anesthetized with a mixture of 4% halothane and oxygen, delivered at a constant flow of 1 L/min using a customized mask placed around the bill.

The skin dorsal to the eye socket was incised with a scalpel to expose the eyeball. A small cut was made in the dorsal sclera, through which CTB (20 µl of ~0.83% in PBS with 2% dimethylsulfoxide [DMSO]; List Biological, Campbell, CA) was injected into the eye's vitreous body with a Hamilton (Reno, NV) syringe. After the procedure, the skin wound was closed with instant adhesive and treated with antiseptic povidone–iodine solution.

The birds were then allowed to recover. After survival periods of 5–7 days, the animals were deeply anesthetized and perfused intracardially with PBS and subsequently 4% PFA (in PBS). The brains were dissected from the skull, postfixed in 4% PFA, and transferred into a 30% sucrose/PBS solution until they sank.

The brains were sectioned in the transversal plane with a cryostat or a freezing microtome at a section thickness of 50 µm, collected in PBS, and alternately separated into three or four series for subsequent anti-CTB immunohistochemistry. The sections were immersed in 90% methanol/3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity, and incubated overnight with a primary polyclonal anti-CTB antibody raised in goat (List Biological, cat. no. 703, RRID: AB_10013220; diluted 1:40,000 in PBS/0.3% Triton X-100/5% normal rabbit serum). After a subsequent 1-hour incubation with a secondary biotinylated anti-goat IgG (H+L) antibody raised in rabbit (Vector, Burlingame, CA; diluted 1:1,500 in PBS/0.3% Triton X-100), ABC solution (avidin–biotin peroxidase complex; Vectastain Elite ABC Kit, Vector) was added to bind to the biotinylated secondary antibodies. In a final step, the ABC peroxidase activity was used for diaminobenzidine (DAB) precipitation by incubating the sections for 6 minutes in a 0.025% DAB/0.0025% H_2O_2 solution (using DAB-buffer tablets for microscopy; Merck, Darmstadt, Germany) in imidazole–acetate buffer/1% $NiSO_4$ for intensification and contrast enhancement (Green et al., 1989).

Processed sections were mounted on gelatin-coated slides, counterstained according to standard Nissl or Giemsa protocols or left clear ("CTB plain"), and coverslipped with DPX after dehydration in ascending alcohol series and clearing in xylene.

Stereology

Retinal whole-mounts

Microscopic examination and photographing of the histological material were performed under an Olympus BX63 microscope with an attached DP26 digital color camera (Olympus, Tokyo, Japan).

Four retinal whole-mounts (two right eyes and two left eyes) were analyzed. The Nissl-stained ganglion cells were counted live by using the microscope software CellSens Dimension v1.7 (Olympus Soft Imaging Solutions, Münster, Germany). Using a ×60 water immersion objective, cell counting was performed according to the fractionator principle (Gundersen, 1977) in regions of interest (ROIs) sampled at regular intervals, while using the focus control in order to better differentiate cells from one another.

To define the ROIs and draw the retinal GCL isodensity maps, we took photomicrographs of the entire Nissl-stained retinal whole-mounts (stitched together by the microscope software), projected them onto the wall with a beamer, and drew their contours onto graph paper at a scale of 20:1. The ROI positions were defined by a 2 × 2-cm grid on the graph paper, which thus corresponded to a 1 × 1-mm grid on the true-scale retinal whole-mount. The respective coordinates of each grid point were targeted with the motorized microscope stage, and at each position an ROI of 100 × 100 µm was defined in the software as an unbiased counting frame (Gundersen et al., 1988b). According to this principle, we only counted neurons within the ROI or touching the ROI frame at two out of four sides (the other two being the adjacent "exclusion edges"). RGCs could be easily distinguished from the small, spindle-shaped glial cells (Wathey and Pettigrew,

1989), which were disregarded in the counting, but distinction from displaced amacrine cells by cytological criteria (Ehrlich, 1981) would only have been feasible in areas of low cell densities. Therefore, we decided not to distinguish between RGCs and displaced amacrine cells, and all our data presented here include displaced amacrine cells, but not glial cells.

Cell counts were filled into the hand-drawn retina map, which was then digitalized with a scanner. In Photoshop CS5 (Adobe Systems, San Jose, CA), isodensity contours were drawn to visualize the cell distribution of the GCL across the retina. Furthermore, the total cell number in the GCL was estimated by assuming mean cell densities for the isodensity areas and multiplying those values by the respective areas in mm^2, according to the following model (Vega-Zuniga et al., 2013):

$$N_{total} = \sum_{i=1}^{n} A_i \; \bar{d}_i \quad \begin{cases} \bar{d}_i = \left(\dfrac{d_{inner} + d_{outer}}{2} \right), & i \geq 2 \\ \bar{d}_i = d, & i = 1 \end{cases}$$

where A_i are the isodensity areas, $\bar{d}_i$ the respective mean densities, and d_{inner}, d_{outer} the cell densities for the isodensity contours confining each area, respectively.

Retinal cross sections

Because of the high density of neurons in the GCL, a modified optical disector method (Hatton and Von Bartheld, 1999) was applied to remedy the problem of bias due to differential shrinkage in frozen nervous tissue sections (Carlo and Stevens, 2011). Under the microscope using a ×60 water immersion objective and differential interference contrast (DIC), RGCs were counted in 30-μm-thick retinal cross sections across the whole section thickness in a 33.3-μm-long (x-axis; parallel to the GCL) counting frame with an exclusion edge on one side (Gundersen, 1977; Gundersen et al., 1988a,b). In the y-axis no exclusion edge was necessary, because the GCL was counted in its full width (see Fig. 4). An exclusion surface was defined in the uppermost focal plane of the section by only counting Nissl-stained perikarya coming into best focus below it. By these rules, counting was performed at 13 random positions around and within the foveal depression in three adjacent sections containing the AC. The numbers thus acquired resembled the numbers of cells/999 μm^2 of retinal surface (30 × 33.3 μm), respectively, and their mean was converted to cells per 1 mm^2 by multiplication with 1,001.

Estimation of centrifugal neurons

The total number of centrifugal neurons in the dorsal isthmic region was estimated by using an unbiased optical fractionator stereology approach (West, 1999; West et al., 1991), similar to the method previously described (Gutiérrez-Ibáñez et al., 2012). In the histological material of one tinamou, all sections of one out of four series (i.e., every fourth section) that contained retrogradely labeled neurons were analyzed by randomly superimposing a 0.01-mm^2 square grid, and defining an unbiased counting frame (Gundersen, 1977) of 0.05 × 0.05 mm^2 at each grid node. At each counting frame position, the section thickness was measured with the microscope focus, and guard zones were established at the upper and lower surface to account for sectioning irregularities. The guard zones were defined so that the z-space in between them had a known fraction of the section thickness (about 2/3), such that a cuboid was formed under the counting frame. This counting cuboid was unbiased in that three adjacent sides of it served as "exclusion edges" and the other three as "inclusion edges" (Gundersen et al., 1988a). Neurons were counted when their perikarya came into focus residing inside the cuboid or touching one of the inclusion sides and not touching any of the exclusion sides. Furthermore, the mean diameters of all counted cell profiles ($n = 180$ contralateral, $n = 14$ ipsilateral) were measured in the microscope software.

Coefficients of error (CEs) for the retinal cross section as well as the centrifugal neurons counts were calculated with Scheaffer's equation (Schmitz and Hof, 2000).

RESULTS

Visual field measurements

Figure 2 depicts the results from the ophthalmoscopic visual field analysis. Because the results from all eight eyes measured were highly similar (with the standard deviations at each coordinate mostly far below 10°, and in the frontal binocular visual field always below 4°), we show only one representative case. The Chilean tinamou possesses a maximum frontal binocular overlap of 20° (Fig. 2A,B), which is located about 13° above the line connecting the pupil with the tip of the bill (Fig. 2A). The overlap extends some 80° from above to below, with its biggest (and generally broader) field above the bill tip. The bill's projection falls amid the binocular field. Within the horizontal plane (Fig. 2B), the tinamou has, in addition to the binocular overlap, a monocular field of 140° (thus, each eye has a field of 160°). The blind sector to its rear measures 60°. Altogether, the bird has a panoramic visual field of 300°.

Eye morphology, retinal topography, and regional specializations

Five enucleated eyes were measured with a digital caliper. The axial length (AL) was 10.68 ± 0.43 mm, the

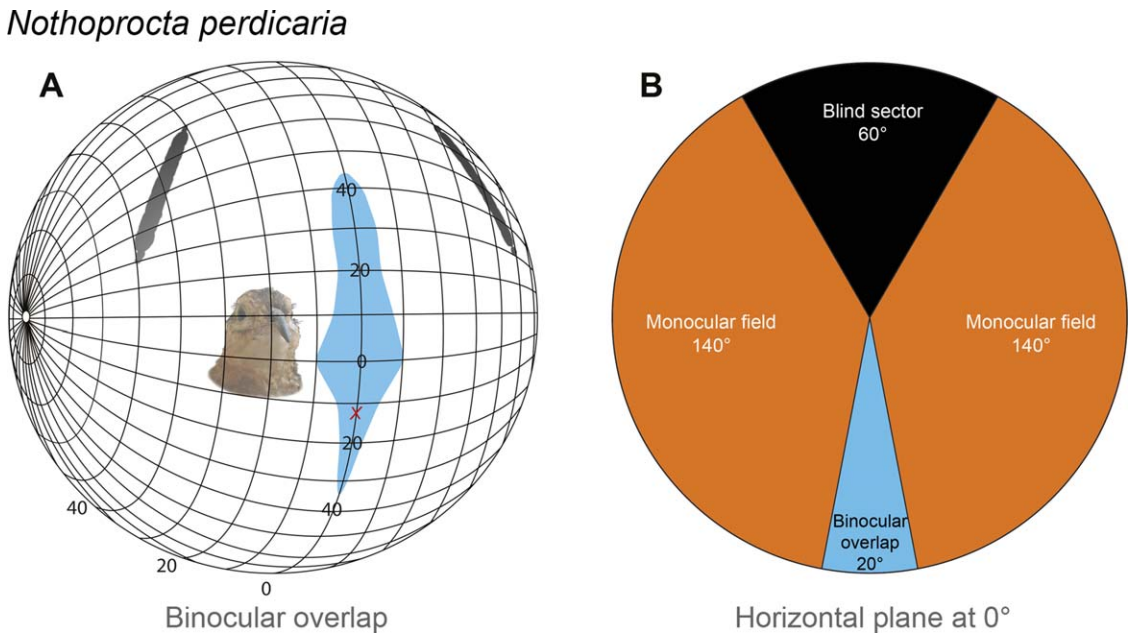

Figure 2. Visual field and binocular overlap. **A:** Perspective view of an orthographic projection of the tinamou's frontal binocular visual field and the pectens. The maximum binocular overlap is approximately 20° azimuth (conventional latitude/longitude system). The tip of the bill points toward −13° latitude (cross), and is completely encompassed by the binocular overlap. **B:** Plan view of the azimuthal plane through the visual field along 0° latitude. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

transverse diameter 14.79 ± 0.25 mm, and the corneal diameter (CD) 6.26 ± 0.41 mm. The "eye shape," the $\log_{10}$ of the CD:AL ratio (Hall and Ross, 2007), was −0.232. The three flat-mounted retinal whole-mounts analyzed had an average area of 257.1 ± 4.3 mm^2. Stereological analysis of the Nissl-stained GCL allowed us to estimate the quantity of neurons in the GCL and reveal the topographical specializations of the Chilean tinamou retina. The total number of neurons in the GCL was estimated at 4.3 ± 0.2 × 10^6. The average neuron density across the entire retinal surface is thus 16.8 ± 0.8 × 10^3 neurons/mm^2.

Drawing isodensity contours with predefined thresholds revealed three types of retinal topographical specializations. Because all three retinal topography maps were very congruent, we show only one representative map (Fig. 3). Close to the center lies a high-density area centralis (AC; Fig. 3C), slightly nasally to the optic disk and pecten oculi. The maximum RGC density estimated in this region is 61.9 ± 2.3 × 10^3 RGCs/mm^2, more than 3.5 times the average neuron density in the retina. Dorsally and slightly temporally to this area, there is a broad dorsotemporal area (DTA; Fig. 3B) of high neuron density between 30 and 40 × 10^3 neurons/mm^2, which is segregated from the AC by a narrow part of lower neuron density. A horizontal visual streak extends nasally and temporally from the AC, dorsal to the pecten. It is of slightly lower neuron density than the DTA, ranging from 20 to 30 × 10^3 neurons/mm^2. Insets in Figure 3 illustrate the scope of variation in GCL neuron density and RGC morphology, which occurs across different topographical areas of the retina. In the outer, low-density periphery (Fig. 3A), the RGCs tend to be larger and fewer than in the high-density areas (e.g., AC or DTA).

Retinal cross-section structure

We made retinal cross-sections for two distinct reasons. First, microscopy of the whole-mounts suggested that in high-density areas the RGCs were stacked over one another, which compromised the achievement of confident cell counts in such regions. We reasoned that we could test our results by applying optical dissector stereology to cross sections. Second, in the whole-mounts it was not possible to ascertain whether the AC of the Chilean tinamou retina contained a true fovea or not. Freshly dissected retinae appeared to have a moderate depression at this position with a slightly different color, visible under a stereomicroscope (Fig. 4A). Therefore, we sectioned two retinae at 30 μm, one transversally and one horizontally, and studied the central region in more detail.

Figure 4B depicts a transverse section at the level of the AC, which is located dorsal to the nasal portion of the optic nerve head (compare Fig. 3). Because we had prepared the complete section series, and another one in

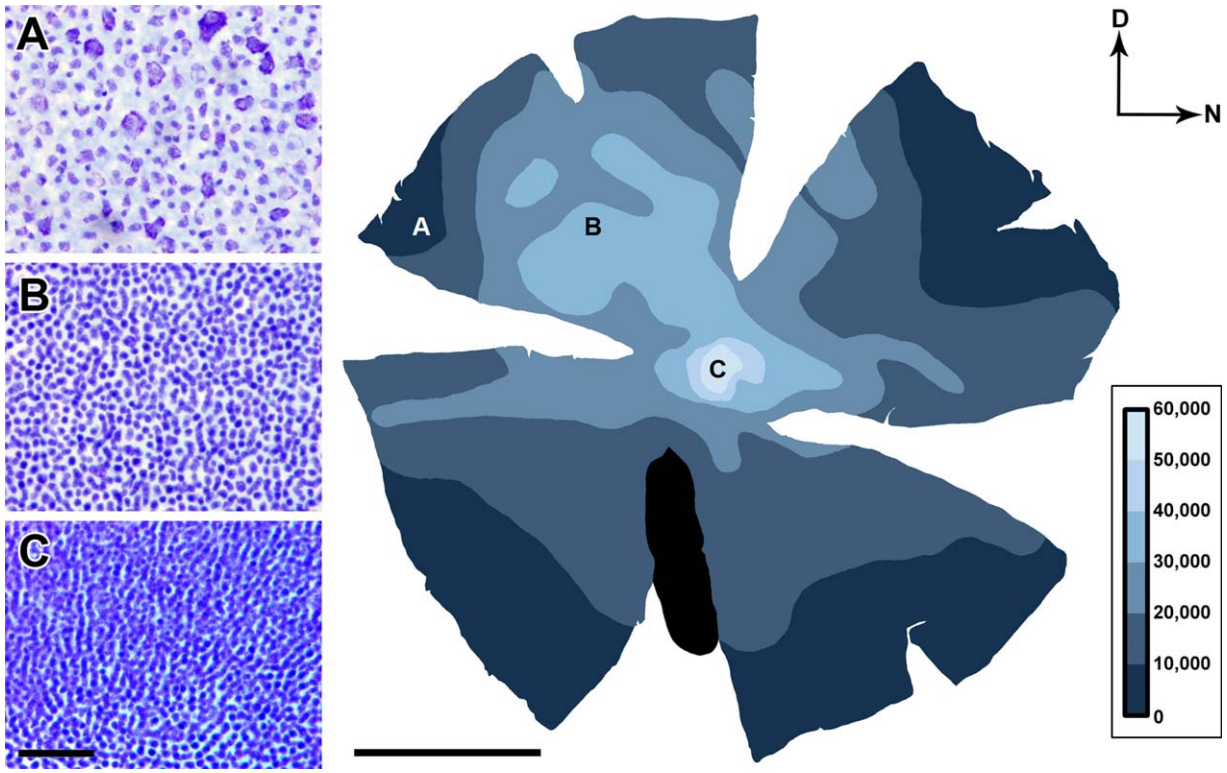

Figure 3. Topographical distribution of neurons in the retinal ganglion cell layer (GCL).The shaded scale on the right indicates the neural density within the respective isodensity contours (in cells/mm^2). Insets on the left show photomicrographs of Nissl-stained regions at representative positions: near the border (**A**), in the dorsotemporal area (**B**), and in the area centralis (**C**), demonstrating the substantial density differences within the GCL. The black patch marks the position of the pecten. Dorsal is up and nasal is to the right (as indicated by arrows). Scale bar = 50 μm in C (applies to A–C); 5 mm in topography map to the right. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the horizontal plane, we could ascertain that the section shown passes through the very center of the AC, showing the clearest representation of the depression. As the inset of the AC (Fig. 4C) shows, the depression can be distinguished in the GCL and all subsequent layers down to the outer nuclear layer (ONL), except for the inner and outer segments of the photoreceptors (IS + OS). Thus, the Chilean tinamou retina appears to possess a concaviclivate fovea, although shallow and little pronounced.

In the AC, the GCL is approximately 25–30 μm thick and contains five to six stacked layers of RGCs, which appear to be organized in a gross columnar fashion. A similar organization can be seen in the inner nuclear layer (INL), which contains densely packed bipolar, amacrine and horizontal cells. It has a pronounced thickness, ranging from 100 to 125 μm in the perifoveal region. In regions of lower cell densities, the stacking decreases and the columnar organization vanishes (Fig. 4C–E). Accordingly, the other retinal layers (INL, ONL, and the photoreceptor segments [IS + OS]) are less thick in regions of lower RGC density (Fig. 4D,E), with the exception of the inner plexiform layer (IPL), which in the DTA is even thicker than in the AC (100–105 vs. 60–80 μm).

Our stereological analysis of the AC in the GCL cross sections (see Materials and Methods) yielded 58.1 ± 2.3 × 10^3 RGCs/mm^2 of retinal surface (CE = 0.0109). If only samples in the center of the foveal depression were taken into account, the estimation was slightly lower (57.6 ± 2.4 × 10^3 RGCs/mm^2 of retinal surface; CE = 0.0337); in the case of all samples except those in the fovea, it was slightly higher (58.4 ± 2.5 × 10^3 RGCs/mm^2 of retinal surface; CE = 0.0081).

SRP estimation

The theoretical maximum of visual acuity (i.e., the SRP) was estimated from the eye's axial length and RGC density in the AC (see Materials and Methods). Because the focal length of the tinamou eye was not directly measured, the evaluation is partly based on the assumption that there is a constant PND to axial length ratio of 0.6 in birds (Hughes, 1977; Martin, 1993; Ullmann et al., 2012). The focal length was thus estimated at 6.41 mm. As described above, two different values

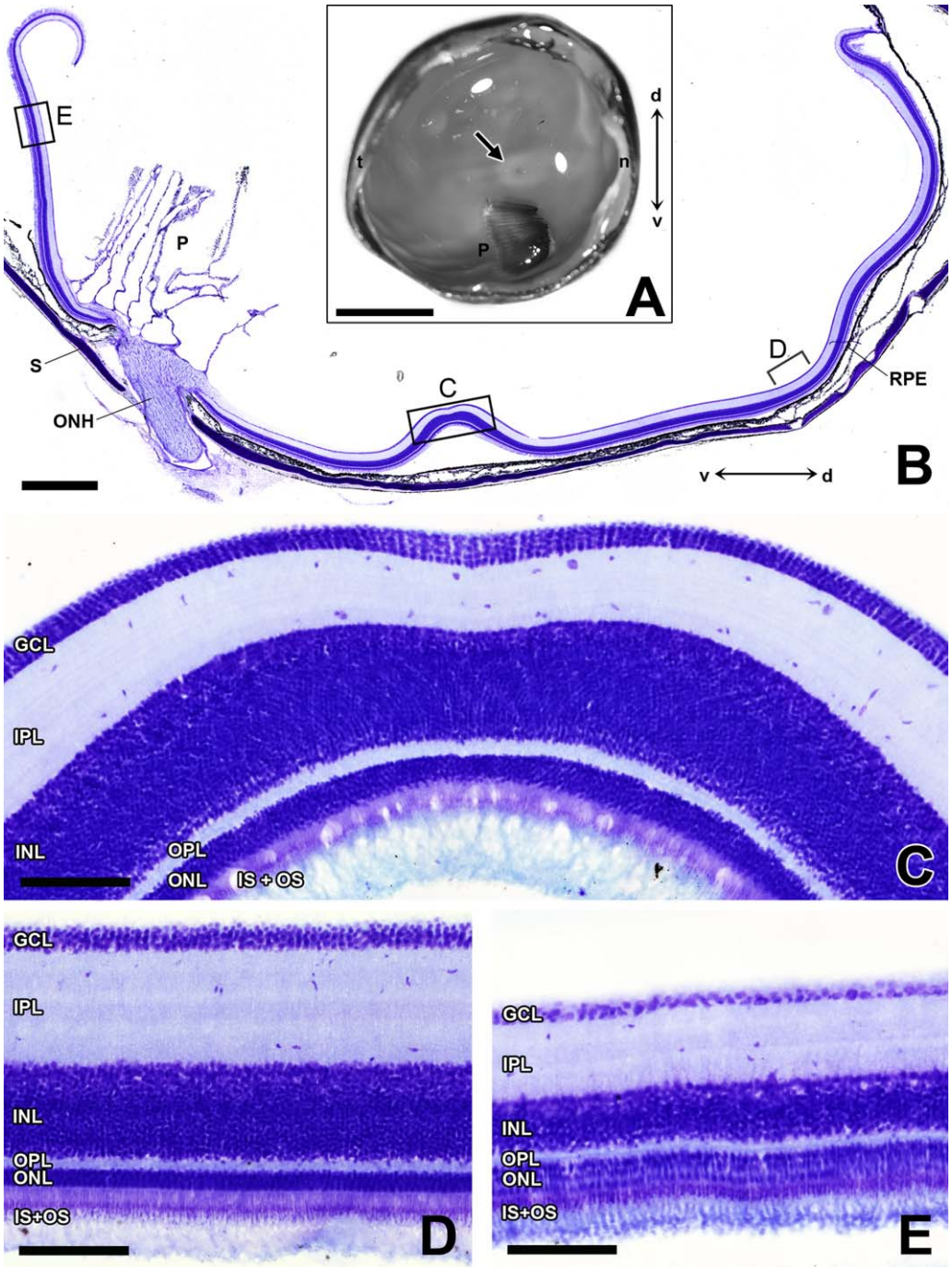

Figure 4. The retinal structure and the shallow fovea of the tinamou. **A:** Photo of a hemisected eyecup (same orientation as in Figure 3; n = nasal, t = temporal). The central fovea (arrow) is clearly visible as a small depression located dorsal to the pecten. **B–E:** Nissl-stained transverse sections (30 μm) of the retina displaying the retinal laminae; from inner to outer: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and the inner (IS) and outer segments (OS) of the photoreceptors. **B:** Overview of a section through the area centralis and the optic nerve head (ONH) with the pecten (P) attached. Sclera (S) and retinal pigment epithelium (RPE). **C:** Enlarged view of a section through the middle of the area centralis (as marked in B). Note the shallow fovea manifested as a small depression in the GCL and INL, and the pronounced thickness of the retinal layers. The ganglion cells form stacks of about five to six cells. The elevation of the retina in the central aspect is an artefact due to a wrinkle in the retina formed by detachment from the retinal pigment epithelium (RPE) during fixation. **D:** Detail of dorsotemporal area. The layers are generally thinner than in the area centralis (except for the IPL, which is thicker), and the GCL contains notably fewer neurons. **E:** Detail of the GCL near the ventral border of the retina. Note that most layers are thinner, the photoreceptor segments are much shorter, and the ganglion cells are scarcer and larger. Scale bar = 5 mm in A; 1 mm in B; 100 μm in C–E. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the maximum RGC density in the AC were obtained: The retinal whole-mount analysis yielded $61.9 \pm 2.3 \times 10^3$, and the cross-section three-dimensional stereology $58.3 \pm 1.3 \times 10^3$ RGCs/mm^2. Using both values resulted in SRP estimations of 14.0 and 13.6 cycles/degree, respectively.

The Chilean tinamou brain

The dissected brain of the adult Chilean tinamou (Fig. 5) measures approximately 2 cm in length from the tip of the olfactory bulb to the posterior end of the medulla. The three birds used for the tracer experiments weighed between 386 and 540 g (442 ± 85 g), and their brains weighed 1.93 ± 0.12 g after perfusion and postfixation. These values lie amid those of related tinamou species, and also the allometric relation of body weight to brain weight falls in line with other Tinamidae (Corfield et al., 2008). The Chilean tinamou brain's shape is roughly similar to a pigeon or chicken brain. The visual Wulst of the telencephalon is fairly conspicuous from the outside, and the lobe of the optic tectum (TeO) is well developed and relatively large.

Primary visual projections

Transverse section series with various counterstaining procedures (Nissl, CTB Nissl, CTB Giemsa) or with plain anti-CTB immunohistochemistry were produced of the five available Chilean tinamou brains with intraocular injections of CTB. Retinal terminals were found in all retinorecipient areas known from neognathous birds: in the dorsal and the ventral thalamus, the hypothalamus, the pretectum, the tectum, and the accessory optic system (Figs. 6–9). The vast majority of retinal afferents made a complete decussation at the chiasma opticum (Figs. 6, 7) and were therefore confined to the contralateral hemisphere (with respect to the eye that had received the tracer injection). Careful scrutiny also revealed sparse ipsilateral fibers and terminals, which were found in some dorsal thalamic, pretectal, and AOS structures (see below), but not at all in the TeO.

Dorsal thalamus

The well-known components of the avian dorsolateral geniculate (GLd) complex (classically also called the *nucleus opticus principalis thalami* [OPT]) receive a substantial retinal input (Figs. 7C,D, 8A). In the *n. dorsolateralis anterior thalami, pars lateralis* (DLL), the largest nucleus of the GLd complex, the retinal terminals were distributed exclusively into its ventral portion (Figs. 7C,D, 8A). The *n. dorsolateralis anterior thalami, pars magnocellularis* (DLAmc), which could be delimited from the laterally adjoining DLL by its slightly larger cells, received very few retinal fibers, mostly confined to its anterior ventral part (Fig. 8A). The *n. lateralis dorsalis optici principalis thalami* (LdOPT) appeared heavily innervated by retinal fibers, where they formed large terminal clusters, very distinct from other retinorecipient zones (Fig. 8A). Although this nucleus was difficult to distinguish from the adjacent DLL in plain Nissl material, it appeared as a very well-defined nucleus when the retinal projections were visualized. Another dorsal thalamic structure clearly receiving retinal terminals was the *n. suprarotundus* (SpRt; Fig. 8A). Retinal fibers without terminals were further seen in the *n. superficialis parvocellularis* (SPC; data not shown).

As has been mentioned before, the vast majority of retinal projections to the GLd were confined to the

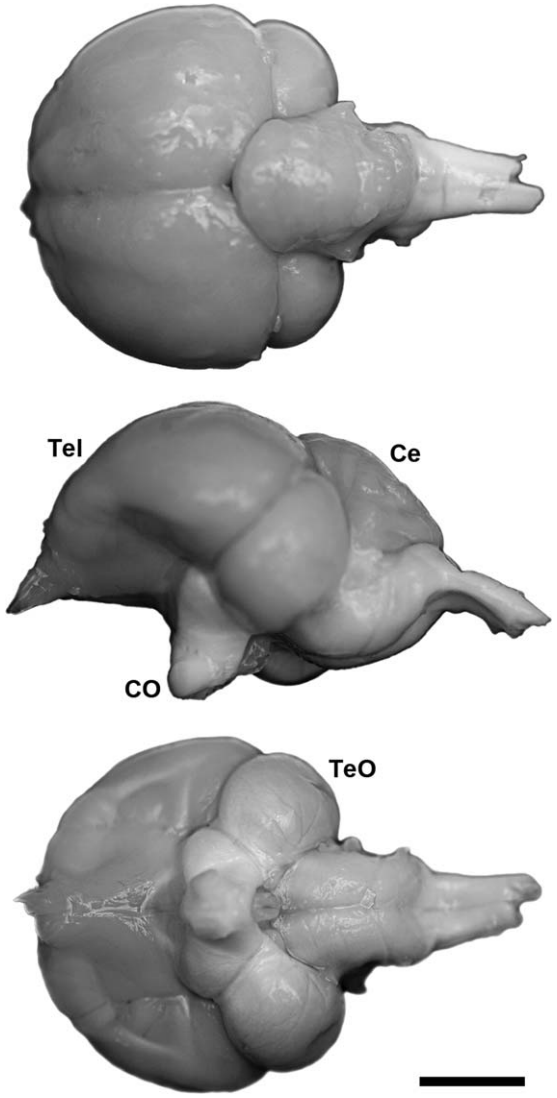

Figure 5. Photographs of the dissected brain. From dorsal (top), lateral (middle), and ventral (bottom). Ce, cerebellum; CO, chiasma opticum; Tel, telencephalon; TeO, optic tectum. Scale bar = 5 mm.

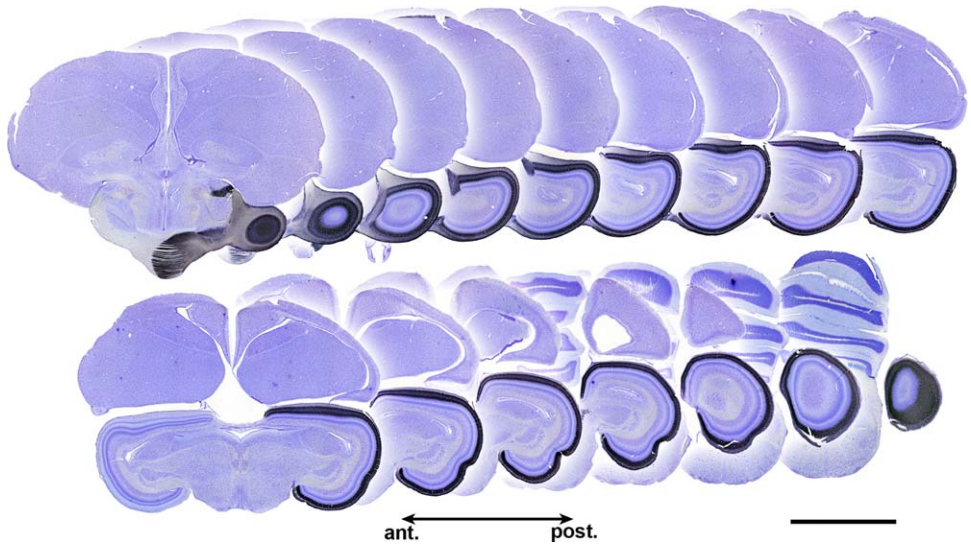

Figure 6. Projection pattern of retinal terminals in the contralateral optic tectum. Series of coronal Nissl-stained sections across the brain, demonstrating the contralateral retinal afferents to the TeO. The sections stem from one complete CTB-reacted series, presented at anteroposterior intervals of 400 µm. Note that the entire TeO is labeled, illustrating that the tracer was taken up by the whole extent of the retina. Scale bar = 5 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

contralateral hemisphere, but sparse terminals were also found in two ipsilateral GLd subunits: the DLL and the LdOPT (data not shown).

Ventral thalamus

As in all birds, the ventral thalamus of the Chilean tinamou is dominated by the *n. geniculatus lateralis, pars ventralis* (GLv; Figs. 7B–E, 8C). The GLv shows a laminated structure (Guiloff et al., 1987), with two clearly visible laminae: the *lamina interna* (GLv-li) with tightly packed somas receiving very sparse retinal afferents, and a neuropil layer (GLv-ne) with dense retinal terminals (Vega-Zuniga et al., 2014). Another nucleus of the avian ventral thalamus is the *n. lateralis anterior thalami* (LA), which showed a high density of retinal terminals (Figs. 7A,B, 8B). This nucleus appears very large in the tinamou compared with the pigeon, for example (Güntürkün and Karten, 1991). In addition, we found a low density of fibers and terminals in the *n. marginalis tractus optici* (nMOT; Figs. 7B–D, 8B) which, as in other birds, first appears at the rostral margin of the thalamus and continues to form an envelope around the LA (Güntürkün and Karten, 1991), and more caudally around the *n. rotundus* (Rt) just below the DLL. In the *n. ventrolateralis thalami* (VLT), which lies between the GLv and Rt and is a known retinorecipient region in birds (Schulte et al., 2006), we found only a few sparse terminals (Fig. 7D).

In terms of ipsilateral retinal projections in the ventral thalamus, we found only a few scattered terminals in the anterior portion of the LA (data not shown).

Hypothalamus

Retinal afferents to the hypothalamus were not very dense and terminated in a diffuse region at the dorsal border of the anterior optic tract (Figs. 7A,B, 9A). We could not differentiate between a lateral and a medial part as described in the pigeon (Shimizu et al., 1994). Instead, the projection pattern we found seemed to conform only to the lateral structure described there. Following the nomenclature put forward by Cantwell and Cassone (2006), we call it the visual suprachiasmatic nucleus (vSCN).

Pretectum and AOS

Several pretectal structures showed innervation from the retina (Figs. 7D,E, 9B): The *n. lentiformis mesencephali* (LM), which is divided into a medial (LMm) and a lateral (LMl) lamina (following the nomenclature by Gamlin and Cohen, 1988a,b; Pakan and Wylie, 2006; Pakan et al., 2006; Sorenson et al., 1989) juxtaposed between the ventral and dorsal strata optica medial to the TeO, showed very dense retinal innervation. Immediately lateral to the LM, a broad sheet with similarly dense retinal projections constitutes the tectal gray (GT). Other retinorecipient structures are found dorsally to the *n. pretectalis* (PT): Following the nomenclature of Gamlin and Cohen (1988a), these are the *area*

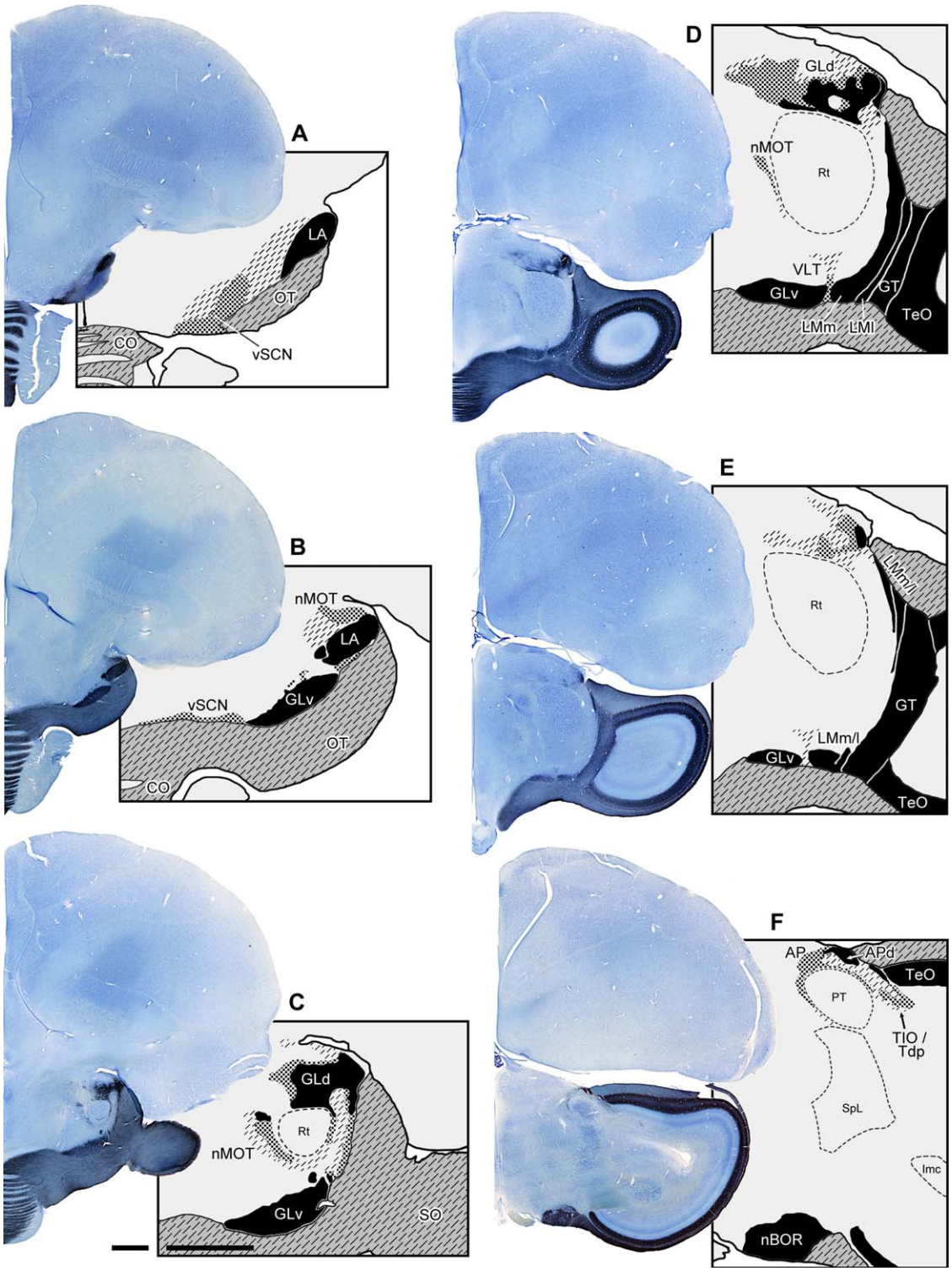

Figure 7. Overview of the retinal projections to central targets in the brain. **A–F:** Each panel displays a coronal section, counterstained with Giemsa, from rostral (A) to caudal (F), along with a corresponding schematic of the CTB-labeled retinal terminal fields. All typical target areas receiving contralateral retinal input are well-developed. They are observed in the hypothalamus (vSCN; A,B), the thalamic ventrolateral geniculate complex (LA, GLv; A–E) and adjoining regions (nMOT, VLT; B–D), the dorsolateral geniculate complex (GLd; C,D), the TeO (D–F), the pretectum (LMm, LMl, GT, AP, APd; D–F), and the accessory optic system (nBOR; F). Also visible is the centrifugal isthmo-optic tract (TIO; F), which includes the tract of the deep tectal pathway (Tdp; compare Fig. 11). For abbreviations, see list. Scale bars = 1 mm in C (apply to A–F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

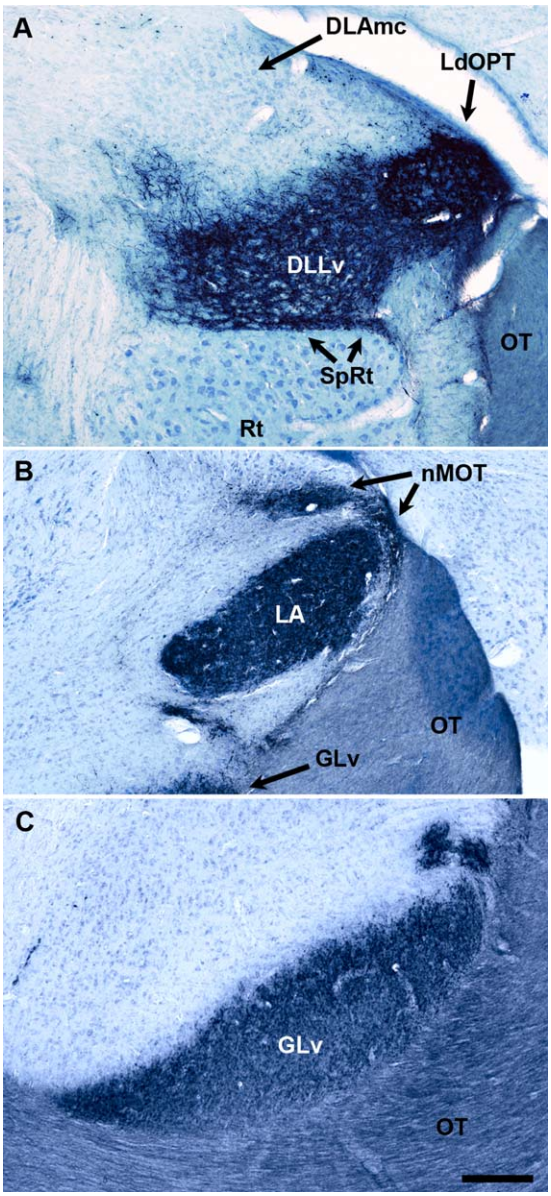

Figure 8. Detailed view of the retinal projection pattern to the contralateral thalamus. **A:** Photomicrographs of a coronal section through the anterior thalamus showing the retinorecipient substructures of the GLd complex. The strongest input is found in the DLLv, SpRt, and LdOPT, the latter appearing very distinct due to the strongly labeled dense terminals. The DLAmc receives very little or no retinal input. **B:** Terminal fields in the LA and the surrounding nMOT. **C:** Dense terminals in the lamina externa of the GLv in the ventral thalamus. Counterstained with Giemsa. For abbreviations, see list. Scale bar = 200 μm in C (applies to A–C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

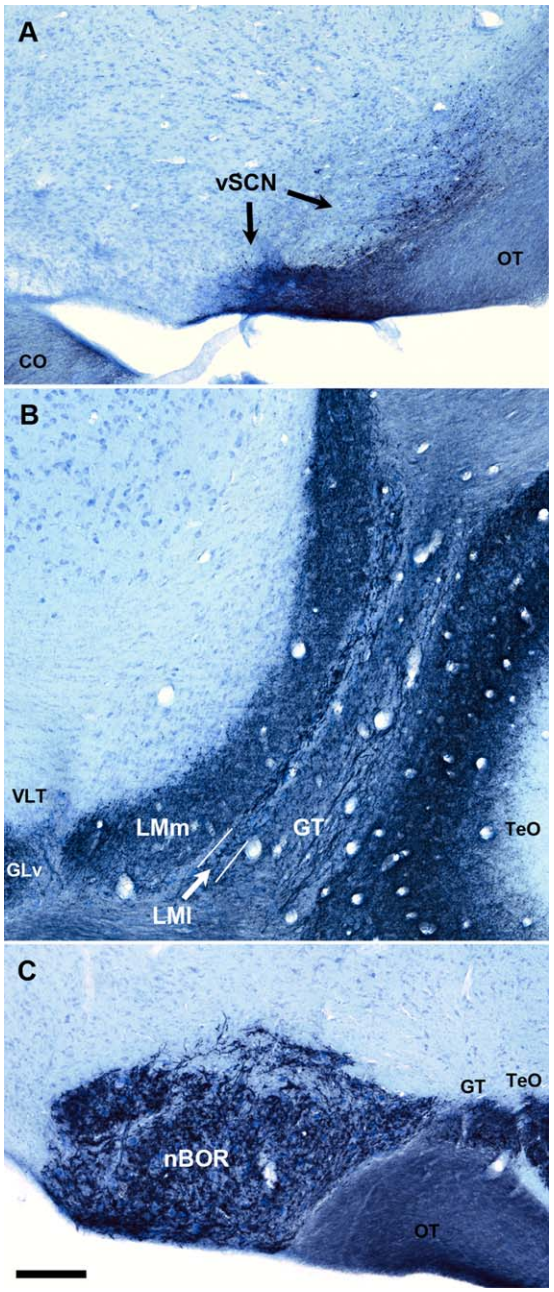

Figure 9. Detailed view of retinal projections to the hypothalamus, pretectum, and accessory optic system. **A:** Photomicrograph of a coronal section through the hypothalamus showing scattered terminal fields in the vSCN. **B:** In the pretectum, dense terminal fields are found in the GT and the two substructures of the LM (LMl and LMm). **C:** The GT continues toward the posterior until adjoining the nBOR. Counterstained with Giemsa. For abbreviations, see list. Scale bar = 200 μm in C (applies to A–C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pretectalis (AP) and especially its dorsal subdivision, the *area pretectalis pars dorsalis* (APd), which was strongly labeled (Fig. 7F). In all these structures (the GT, LM, AP, and APd), very sparse ipsilateral retinal terminals were also found (data not shown). At the posterior margin of the optic tract, we found dense retinal terminals in the nucleus of the basal optic root (nBOR; Figs. 7F, 9C), which forms part of the accessory optic system

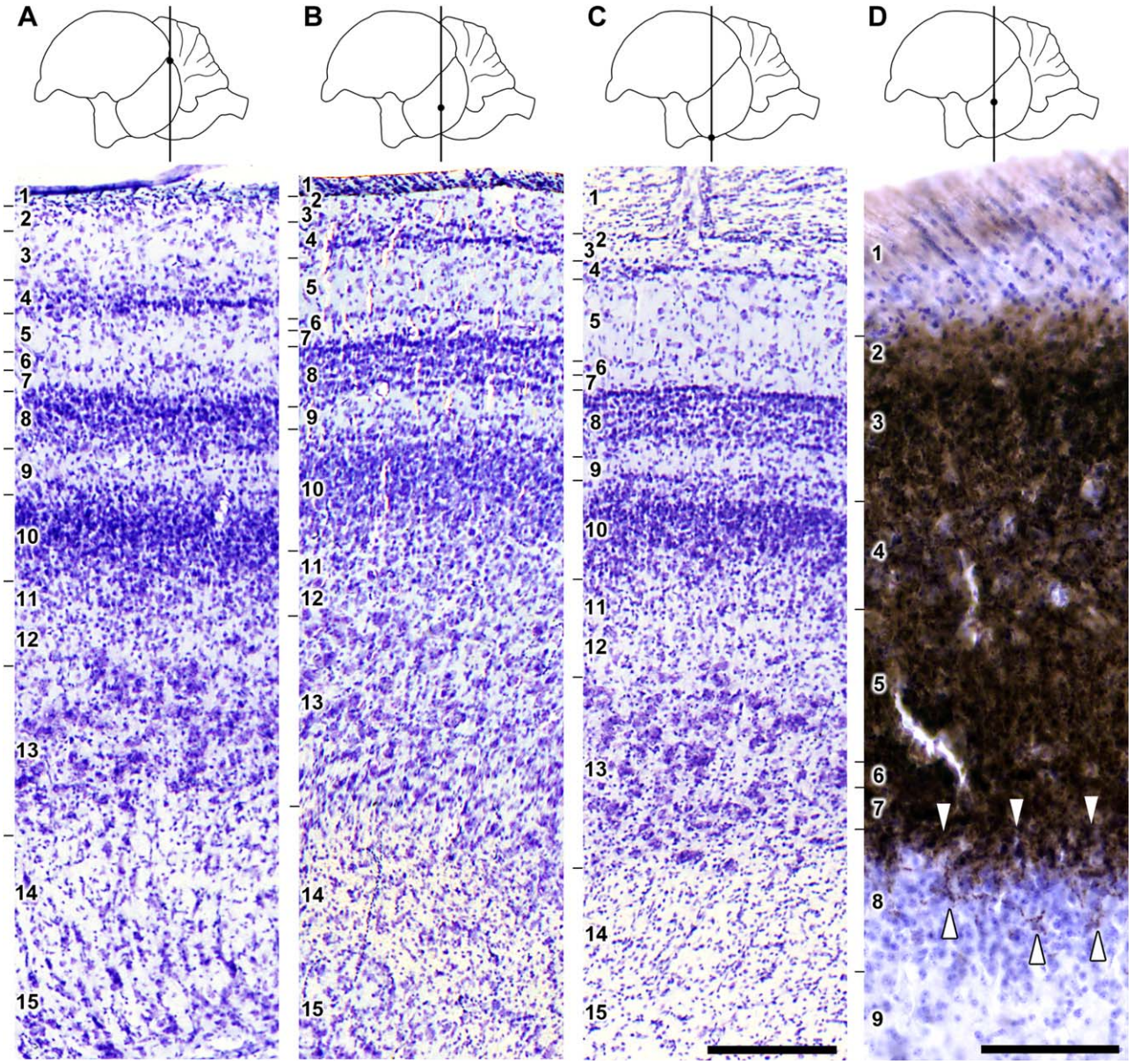

Figure 10. Morphology of the tectal layers. Lamination pattern in the dorsal (**A**), lateral (**B**), and ventral (**C**) TeO, and enlarged view of the CTB-reacted retinorecipient layers (**D**). Relative widths of tectal layers vary considerably from dorsal to ventral. The retinorecipient layer L5 increases from dorsal to ventral, whereas L2 and L3, and also L4, diminish. L6 and L7, in contrast, have a relatively constant width. Layer L8 is very conspicuous compared with other birds, not only because of its thickness, but most notably because it contains retinal terminals (arrowheads in D). All sections are stained with Nissl. Scale bar = 200 μm in C (applies to A–C); 100 μm in D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(AOS). Sparse terminals were also found on the ipsilateral side (data not shown).

Optic tectum

The whole anteroposterior and dorsoventral extent of the TeO was labeled by anti-CTB immunohistochemistry (Fig. 6), showing that the intraocularly injected tracer had been taken up uniformly across the entire retina. All retinal projections were exclusive to the contralateral TeO. Dense terminals were found in the superficial layers (L2–L7) of the *stratum griseum et fibrosum superficiale* (SGFS). The layers that receive retinal afferents vary considerably in thickness along the dorsoventral axis of the TeO (Fig. 10). Whereas in the dorsal aspect, L3 and L4 cover more than half the width of all retinorecipient layers taken together, in the lateral aspect they cover little more than a third and in the ventral aspect less than a third. By contrast, L5 gains in width

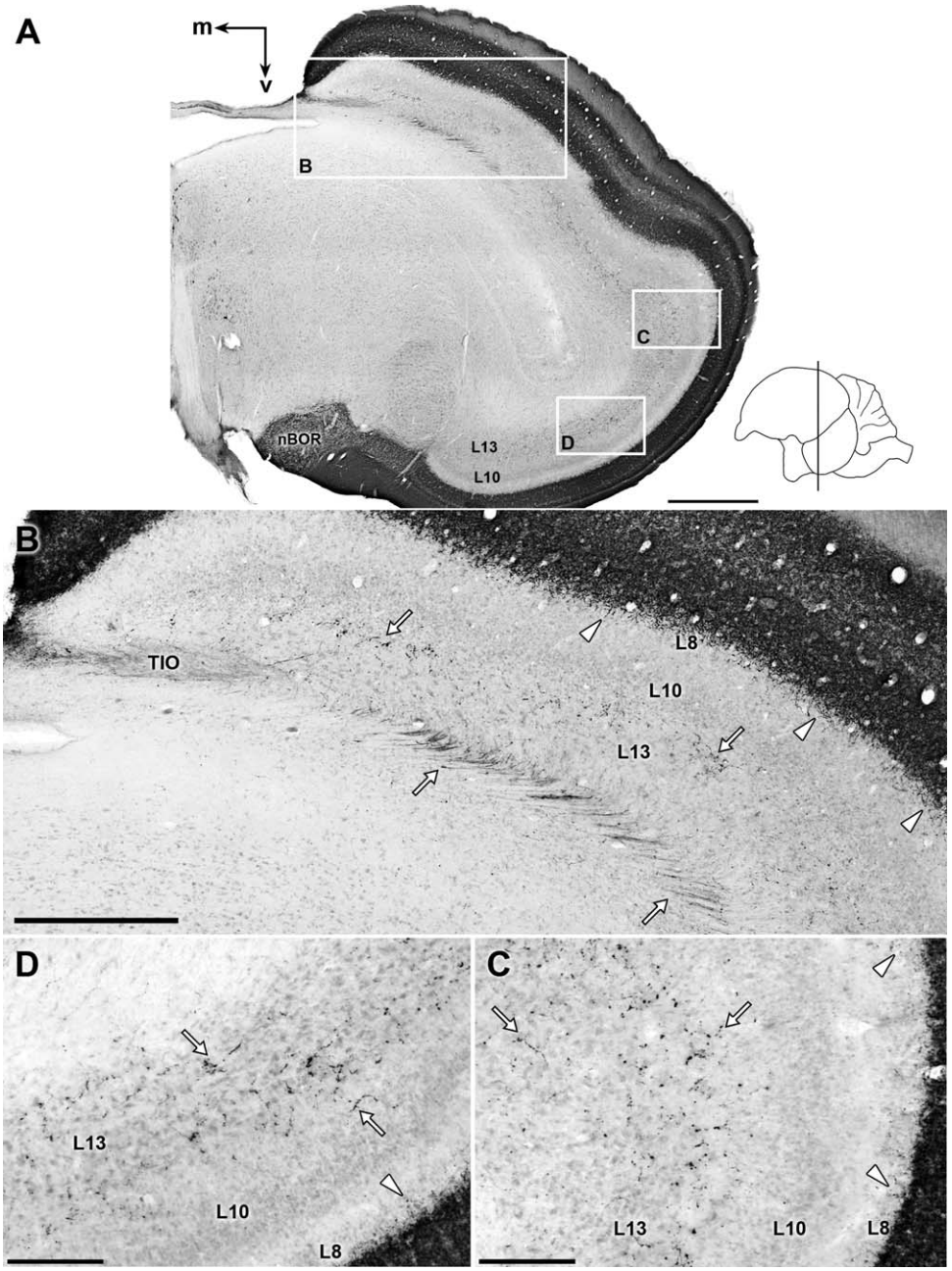

Figure 11. Deep tectal terminals after intraocular CTB injection. **A:** Overview of a coronal section through the contralateral TeO, with the indication of subsequent insets (BD). **B:** Origin of the deep pathway and terminals in L11–13. A fiber tract enters the TeO laterally (left arrow), branching off from the isthmo-optic tract (TIO). The fibers run along the periventricular zone (upward arrows) and turn radially outward to reach their target areas (downward arrows). Note also that retinal terminals exceeding the classical retinorecipient layers 1–7 and entering L8 can be distinguished (arrowheads; compare Fig. 10). **C,D:** Detailed photomicrographs of deep tectal varicosities (arrows) in the dorsal (B), lateral (C), and ventral (D) parts of the TeO, demonstrating their ubiquity. As in B, retinal terminals in L8 are very conspicuous (arrowheads). Counterstained with Giemsa. Scale bar = 1 mm in A; 500 μm in B; 200 μm in C,D.

from dorsal to ventral, occupying little over a quarter of the total thickness dorsally, to almost a half laterally and more than a half ventrally. Layers L2, L6, and L7 do not change notably in width, although L6 contains a substantially lower density of neurons in the ventral aspect than in the lateral and dorsal aspects.

In addition to the classical tectal retinorecipient layers 1–7, a considerable amount of retinal terminals surpassed L7 and entered L8 (Figs. 10, 11). Here they formed sparse ramifications, mostly in the outer two-thirds of the lamina, but sometimes throughout its extent. L9 did not contain any terminals or fibers.

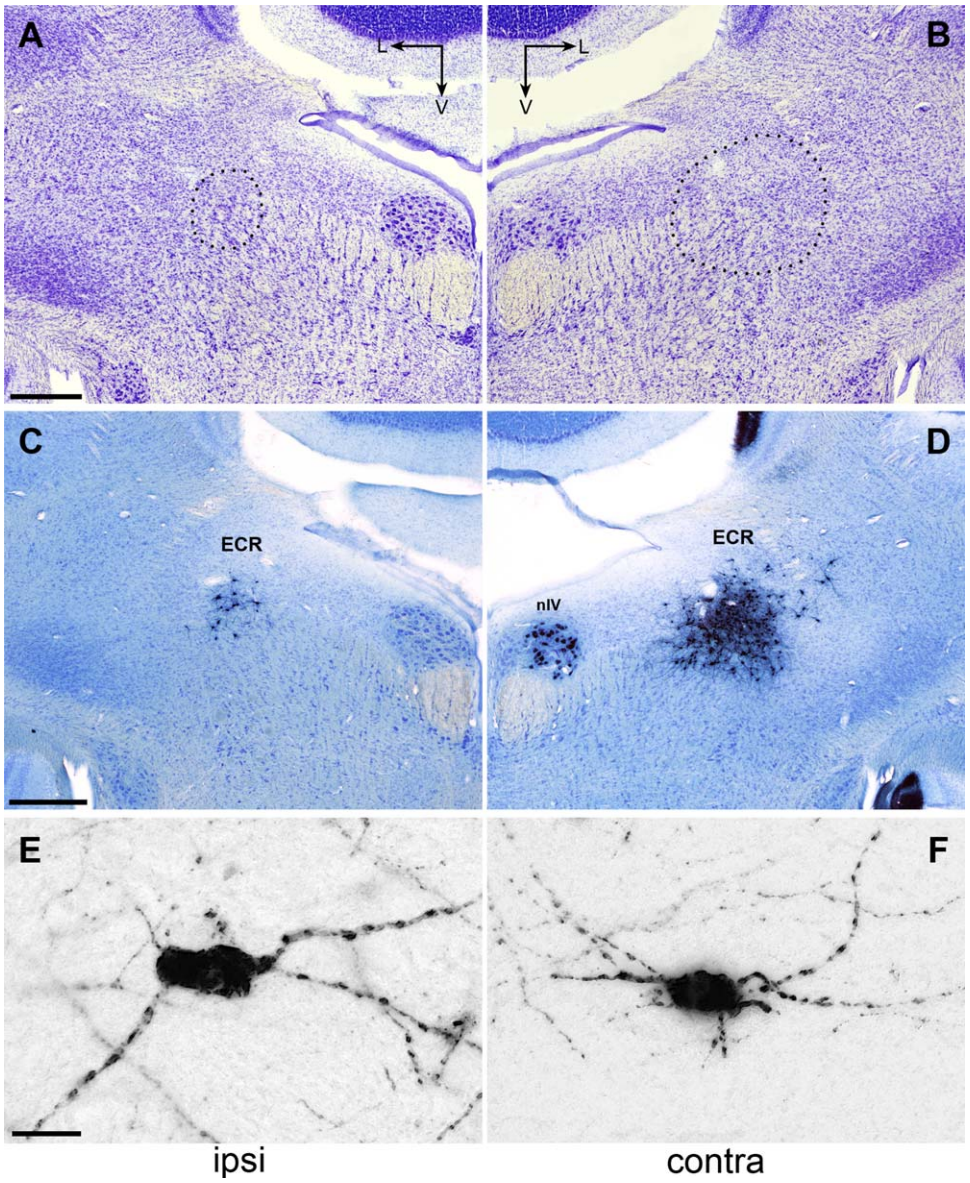

Figure 12. The isthmo-optic region of the Chilean tinamou, demonstrated by an intraocular CTB-injection. Coronal sections through the isthmic region ipsilateral (A,C E) and contralateral (B,D,F) to the injected eye. Note that although no structured isthmo-optic nucleus (ION) is distinguishable in Nissl-stained sections (**A,B**), anti-CTB reaction reveals a large number of contralateral, and a lower number of ipsilateral, retrogradely labeled centrifugal neurons (**C,D**). The great majority of these neurons are large (>20 μm) and multipolar (**E,F**), resembling the ectopic cells surrounding the ION of Neognathous birds. A and C as well as B and D, respectively, are consecutive sections from two series of the same brain, thus representing almost identical positions. Orientations given in A and B apply for all panels of their respective columns. On the contralateral side, the oculomotor nucleus trochlearis (nIV) also contains some retrogradely labeled neurons (see D), presumably from tracer spill into the periocular space. C and D are counterstained with Giemsa. E and F are extended focal imaging (EFI) extractions of z-stacks. ECR, ectopic cell region. Scale bar 500 μm in A (applies to A,B) and C (applies to C,D); 20 μm in E (applies to E,F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Notably, in all intraocular injections, we found a sparse but evident amount of fibers and terminals forming a conspicuous band from layers L11 through L13 (Fig. 11). The density and distribution of these deep tectal terminals was fairly uniform across the entire TeO from anterior to posterior, but was more concentrated in the dorsal than in the ventral TeO (Fig. 11B–D). These "deep terminals" do not correspond to retinal fibers coursing radially from layer 7 toward the deep tectal layers. Instead, they represent terminals of axons that branch off

TABLE 1.
Primary Antibody Used in This Study

Antigen	Immunogen	Details	Dilution
CTB (cholera toxin subunit B)	Purified choleragenoid (cholera toxin subunit B aggregate)	List Biological, goat polyclonal, cat. no. 703, RRID:AB_10013220	1:40,000

from the isthmo-optic tract (TIO; Fig. 11A,B) and then proceed laterally into the TeO, running along L15 and the tectal ventricle. Thereafter, they bend off to cross radially through layers L14 and L13 toward their terminal location (Fig. 11A,B). The terminals have a striking morphology, with large bulbous-like varicosities that are distributed in layers L11 and L12, and more densely in L13 (Fig. 11C,D). L10 is almost completely free of such terminals.

Centrifugal neurons (ION)

In the dorsocaudal isthmus of the midbrain, a large quantity of retrogradely labeled neurons was found on the contralateral side (Fig. 12D), and a minor quantity on the ipsilateral side (Fig. 12C). These retinopetal (centrifugal) neurons were scattered over a considerable area within the neuroanatomical region of the avian isthmo-optic nucleus (ION) and its ectopic cell region (ECR). However, in Nissl-stained sections a clear nuclear organization as observed in most birds was not recognizable (Fig. 12A,B; see also Gutiérrez-Ibáñez et al., 2012).

Our stereological estimation of the number of retrogradely labeled centrifugal neurons yielded 4,120 cells (CE = 0.0658) and 323 cells (CE = 0.0963) on the contralateral and the ipsilateral side, respectively. Mean diameters of contralateral profiles varied from 8.2 to 24.5 μm, with an average of 16.4 ± 3.1 μm. Those of ipsilateral profiles varied from 12.6 to 22.2 μm, with an average of 17.6 ± 2.7 μm. Note that the neurons' orientations could not be taken into account for the measurements. Morphologically, the neurons were mostly large and multipolar (Fig. 12E,F), whereas smaller monopolar and fusiform neurons resembling typical avian isthmo-optic neurons were scarce.

DISCUSSION

In this study, we provide the first results of a systematic investigation of the visual pathways of a Palaeognathae representative, the Chilean tinamou (*Nothoprocta perdicaria*). We show that the retina of the tinamou possesses an elevated number of ganglion cells arranged in three distinct topographical specializations: an area centralis (AC) with a shallow fovea, a horizontal visual streak, and a dorsotemporal area (DTA). Accordingly, the visual field is highly panoramic, with a restricted frontal binocular overlap. As can be seen in our neuronal tracer data, the normal avian pattern of retinal central projections is well-developed and differentiated. However, we also found a remarkable projection to the deep layers of the TeO labeled after intraocular CTB injection. Similar projections have previously been described in embryonic chickens but are absent in adult animals (Wizenmann and Thanos, 1990; Omi et al., 2011).

Although no clear ION (Repérant et al., 2006) is distinguishable (Fig. 12A,B; Gutiérrez-Ibáñez et al., 2012), we found a high number of retrogradely labeled centrifugal neurons in the dorsal isthmic region, some of them projecting to the ipsilateral retina (Fig. 12C–F). Because tinamous represent a "basal" avian group, their centrifugal visual system may represent the link between the well-defined ION of most neognathous birds and the centrifugal visual system of the closest living relatives to birds, crocodiles (Müller and Reisz, 2005), who like the Chilean tinamou, also show a diffuse arrangement of the isthmo-optic neurons (Médina et al., 2004).

Visual field

Visual field measurements can tell us a great deal about animals' ecology and behavior (Martin, 2007). The most interesting aspects are the size and position of the frontal binocular overlap, the general extent of the lateral monocular fields, and the size of the blind area behind the bird. With respect to the binocular field, Martin (2007) distinguishes three main types in birds: Type 1 fields, with a binocular overlap between 20° and 30°, the bill's projection falling centrally or slightly below the center, and with a blind area behind the head; type 2 fields with ≤10° overlap, the bill at its periphery or outside, and no blind area to the rear; and type 3 fields, with large overlaps and large blind areas behind (owls). According to this schematic, the Chilean tinamou barely has a type 1 field (Fig. 2), which is mostly found in birds that forage by visual guidance of the bill (e.g., pecking), and/or that care for their chicks by feeding them (Martin et al., 2005; Martin, 2007). Tinamous do forage by pecking and by using their bill to dig in the ground for food (Cabot, 1992). In comparison with the other Palaeognaths studied, the binocular field of the Chilean tinamou appears to be similar to that of

the ostrich (Martin and Katzir, 1995), and larger than that of the kiwi, which is a nocturnal bird with a specialized olfactory sense (Martin et al., 2007).

The binocular field of the Chilean tinamou is presumably rather restricted, but with the aid of convergent eye movements it could become larger and include the retinal DTAs (especially around the bill). This could provide increased spatial resolution, and perhaps stereopsis. It may also provide functions for optic flow-field integration, which seems to be an important function of binocularity in birds (Martin and Katzir, 1999; Martin, 2007).

RGC density and visual acuity

The Chilean tinamou shows a variety of traits and specializations, which indicate a strong reliance on its visual sense. The "eye shape" value of −0.232 is typical of a diurnal bird (Hall and Ross, 2007; Lisney et al., 2012a). In the retina, we found a high overall quantity of approximately 4.3 million neurons. We could not quantify the ratio of the displaced amacrine cell population included in our data, because a distinction by morphological criteria (Ehrlich, 1981) was not practicable in retinal areas of high neuron densities (Collin and Pettigrew, 1988; Lisney and Collin, 2008; Lisney et al., 2012b; Wathey and Pettigrew, 1989). In various neognathous birds, displaced amacrine cells have been reported to constitute varying portions of the GCL neurons, for instance 30–35% (Ehrlich, 1981) or 32% (Chen and Naito, 1999) in the chicken, 11% (Hayes, 1984) or 40% (Binggeli and Paule, 1969) in the pigeon, or 20–30% in the quail (Muchnick and Hibbard, 1980). Arguably, we could have applied one of those ratios to our data, but given the considerable variation among Neognathae, we did not see a benefit in doing so.

Despite this caveat, the overall GCL count found in the tinamou is high compared with similar counts estimated for many other birds, such as Galliformes (Budnik et al., 1984; Ehrlich, 1981; Ikushima et al., 1986; Lisney et al., 2012b), Anseriformes (Fernández-Juricic et al., 2011; Lisney et al., 2013; Rahman et al., 2007a), Columbiformes (Binggeli and Paule, 1969), Passeriformes (Coimbra et al., 2006, 2009; Rahman et al., 2006, 2007b), various Strigiformes (barn owl, northern saw-whet owl, short-eared owl; Lisney et al., 2012a; Wathey and Pettigrew, 1989), Procellariiformes (Hayes and Brooke, 1990), Sphenisciformes (Coimbra et al., 2012), and Struthioniformes (ostrich; Boire et al., 2001). Of all the avian species studied so far, the Chilean tinamou is only surpassed by some particularly visually-specialized ones, for instance some owls (snowy owl, great horned owl, great gray owl, barred owl, and northern hawk owl; Lisney et al., 2012a), probably kingfishers (Moroney and Pettigrew, 1987), and Falconiformes (Inzunza et al., 1991), although in the latter two cases no total RGC number quantifications have been provided by the authors.

With respect to the maximal GCL neuron density, the Chilean tinamou also ranks high among birds, if not vertebrates. In Neognathae, the displaced amacrine cell density is reportedly uniform across the entire retina (Ehrlich, 1981) and of a negligible magnitude for RGC estimations in high-density areas (Bravo and Pettigrew, 1981; Collin and Pettigrew, 1988). Therefore, our estimation of 61.9×10^3 neurons/mm^2 in the AC probably corresponds to true RGCs (see above), almost reaching the values obtained in eagles and hawks, who possess 65 and 62×10^3 cells/mm^2 in the foveal region of their GCL, respectively (Inzunza et al., 1991).

However, visual acuity is not only limited by the density of RGCs, but also by the eye's focal length, which is proportional to its axial length (Hall and Ross, 2007; Martin, 1993; Walls, 1942). The theoretical SRP can be estimated from the eye's focal length and the maximal RGC density under the assumption that one cycle of grating can be resolved by two adjacent ganglion cells (Collin and Pettigrew, 1989; Pettigrew et al., 1988; Ullmann et al., 2012). The Chilean tinamou's relatively high SRP value of 13.6–14.0 cycles/degree, higher than, for example, phasianid Galliformes such as the chicken (6.5–8.6 cycles/degree; Gover et al., 2009; Schmid and Wildsoet, 1998) or the quail (4.3–4.9 cycles/degree; Lee et al., 1997), reflects the relatively small eyes of this bird, for which the high RGC density can only partly compensate. In contrast, the ostrich, despite its relatively low maximal RGC density of approximately 9,000 cells/mm^2, has a high estimated SRP of between 17.0 and 22.5 cycles/degree (Boire et al., 2001) because of its large eyes (axial length 39 mm; Martin and Katzir, 1995). Thus, the high number and density of RGCs in the Chilean tinamou retina can be seen as a way to increase visual acuity within the anatomical constraint of a relatively small eye size.

Retinal topography

Topographical specializations in the retinal cell distribution have long been recognized to be of importance for eco-behavioral functioning of vertebrate vision (Hughes, 1977). Three distinct types of *areae* (AC, horizontal visual streak, and DTA) characterized by elevated retinal cell densities are frequently found in birds (Güntürkün, 2000), and all of them are present in the Chilean tinamou (Figs. 3, 4). The AC, which subserves the bird's lateral visual field, contains in addition to the already-discussed high RGC density a shallow concaviclivate fovea (Fig. 4A,B). This type of fovea, in contrast to the deep convexiclivate type (Walls, 1963), covers a wider retinal area and has

been proposed to accomplish a better functionality in vigilance behavior (Fernández-Juricic, 2012). In comparison, the most basal Neognathae and thus closest neognathous relatives, Galliformes, generally do not possess a fovea in their retina (Lisney et al., 2012b), although the quail has been reported to have a shallow one (Ikushima et al., 1986). It has to be noted, however, that the tinamou specimens used in this study were acquired from a breeder. Thus, the shallowness of the fovea could be the result of domestication, which has been reported to alter the fundus oculi considerably (Walls, 1942; Wood, 1917), and wild tinamous might possess a more pronounced fovea than described here.

Distinct from the AC, a large DTA covers almost a quadrant of the Chilean tinamou retina (Fig. 3). The presence of a DTA (or area dorsalis) is an often-found retinal feature of granivorous birds (Budnik et al., 1984; Güntürkün, 2000), because it covers the anteroventral aspect of the visual field and thus aids in object (food) recognition and pecking behavior (Martin, 2007; Nalbach et al., 1990). Fittingly, the Chilean tinamou's diet, which consists mostly of seeds and sometimes insects, is gathered by pecking and digging with the beak (Cabot, 1992; Conover, 1924). Interestingly, in contrast to this idea, a number of phasianid Galliformes reportedly lack a DTA, even though they are ground-foragers (Lisney et al., 2012b). Thus, other factors may contribute to the presence or absence of a DTA in a bird species, and it is definitely curious that the basal tinamou possesses this feature whereas many Galliformes do not.

Engulfing the AC, but distinct from the DTA, the tinamou retina also features a horizontal visual streak (Fig. 3). According to the *Terrain Hypothesis* (Hughes, 1977), this specialization frequently evolves in animals living in open or semi-open habitats without dense arboreal vegetation, because it provides them with improved visual capacities for scanning the horizon, e.g., for predators. Quite a number of studies support this proposition, such as in the red kangaroo *Macropus rufus* (Hughes, 1975), the giraffe *Giraffa camelopardalis* (Coimbra et al., 2013), anatid ducks (Lisney et al., 2013), the Canada goose *Branta canadensis* (Fernández-Juricic et al., 2011), seabirds (Hayes and Brooke, 1990), and non-nocturnal owls living in open habitats (Lisney et al., 2012a), and even in such distant species as nonvertebrate crabs (Zeil et al., 1986) or coleoid cephalopods (Talbot and Marshall, 2011). Also, another palaeognathous bird species, the ostrich *Struthio camelus* (Boire et al., 2001), which lives in the savannas and Sahel of Africa, possesses a pronounced horizontal visual streak. The Chilean tinamou conforms well to this hypothesis, because it lives exclusively in open habitats (Cabot, 1992; Conover, 1924).

Central retinal projections

The overall pattern of retinal projections in the Chilean tinamou is mostly consistent with the pattern found in Neognathous birds, implying that this shared organization of the avian visual system was fully present in the last common ancestors of Palaeognathae and Neognathae over 120 million years ago, and has in both groups remained highly conserved during this long time span of separate evolution.

Dorsal thalamus

Representing the first stage of the thalamofugal pathway, the dorsal lateral geniculate (GLd) of the tinamou receives considerable input (Figs. 7C,D, 8A), although clearly not as much as the TeO. Similar to the pigeon (Güntürkün and Karten, 1991; Güntürkün et al., 1993; Miceli et al., 1975, 2008) and the quail (Watanabe, 1987), the strongest retinorecipient GLd elements are the ventral portion of the DLL (= DLLv of Miceli et al., 2008), its most ventral subdivision, the SpRt, and the LdOPT (we adhere to the nomenclature of Güntürkün and Karten, 1991, whereas others have identified it as the DLAlr [Ehrlich and Mark, 1984a; Watanabe, 1987], or as a portion of the DLLd [Miceli et al., 1975, 2008]). The high density and defined pattern of retinal input in the LdOPT suggest that it is an important relay of the tinamou's thalamofugal pathway, similar to what is assumed in neognathous birds (Ehrlich and Mark, 1984a; Watanabe, 1987). In addition, it contains conspicuously large retinal terminals (Fig. 8A), analogous to what has been noted in the pigeon (Güntürkün and Karten, 1991).

Ventral thalamus

The ventral thalamus of the tinamou appears to be very similar compared with other birds. The LA and GLv are well developed (Figs. 7A–E, 8B,C), and the GLv-ne of the latter is densely innervated by retinal terminals. The nMOT (Figs. 7B–D, 8B), which may be the homologue of the mammalian intergeniculate leaflet (IGL; Güntürkün and Karten, 1991; Harrington, 1997), has rather scarce retinal innervation compared with the pigeon (Güntürkün and Karten, 1991); however, its general neuroanatomical organization is very similar.

Hypothalamus

Retinal input to the avian hypothalamus is mainly confined to a small lateral portion (Cantwell and Cassone, 2006; Cassone and Moore, 1987; Cooper et al., 1983; Gamlin et al., 1982; Norgren and Silver, 1989b; Shimizu et al., 1994). Some studies also report scarce retinal afferents to a second, medial hypothalamic division, e.g., in the pigeon (Shimizu et al., 1994) and in the chicken (Cantwell

and Cassone, 2006). In the palaeognathous tinamou we could not find any retinal terminals or fibers in the medial hypothalamic region; however, we found input to the lateral portion (Figs. 7A, 9A), which we call the vSCN, following the nomenclature of Cantwell and Cassone (2006). Interestingly, in the closest extant relatives of birds, the crocodiles, retinohypothalamic projections to both a lateral and a medial portion of the hypothalamus have been described (Derobert et al., 1999).

Pretectum and AOS

We generally found the typical avian retinal projection pattern to the pretectum and AOS (Figs. 7D–F, 9B,C) that, for example, has been described in the chicken (Ehrlich and Mark, 1984a), the quail (Norgren and Silver, 1989a), and the pigeon (Gamlin and Cohen, 1988a). On the contralateral side, the projections comprise a large and densely labeled GT, LMm, and LMl, as well as a substantial nBOR of the AOS. Furthermore, the AP and especially its dorsal subdivision (APd) were labeled from retinal input, similar to the description by Gamlin and Cohen (1988a) and Shimizu et al. (1994) in the pigeon.

Optic tectum

The tinamou's TeO, which in birds generally receives the majority of retinal fibers (Benowitz and Karten, 1976; Luksch, 2003; Mpodozis et al., 1995; Ramón y Cajal, 1909; Wylie et al., 2009), is particularly prominent (Fig. 6). Its retinorecipient layers 1–7 receive dense afferents (Fig. 10D), suggesting a tectofugal pathway of considerable proportions. The dominance of the tectofugal pathway appears to be a common trait in Tinamiformes, because two other species of this family are reported to possess large tectofugal components relative to brain volume (Bee de Speroni and Carezzano, 1995; Iwaniuk et al., 2010).

The general organization of the Chilean tinamou TeO is similar to that of neognathous birds. Altogether, its lamination appears to be more complex than in the chicken TeO (Karten, 2007), but not as complex as a passerine TeO (Faunes et al., 2013; Karten et al., 2013). The relative width changes of the various tectal layers from dorsal to ventral (especially L5; see Results; Fig. 10) are generally similar to findings in the pigeon (Karten et al., 1997). In the pigeon, however, the change in L5 is more dramatic (compare Fig. 6 of Karten et al., 1997) and whereas in the pigeon L4 is almost nonexistent in the ventral TeO, in the tinamou it remains a thin but distinct lamina (Fig. 10C).

We found that layer 8 is relatively prominent in the tinamou, and, interestingly, although classically considered non-retinorecipient, it receives retinal terminals throughout the TeO (Fig. 10D; arrowheads in Fig. 11). To our knowledge, this has not been reported in an adult bird. However, an even denser L8 projection appears to be present in a neognathous Caprimulgiform, the band-winged nightjar *Caprimulgus (Systellura) longirostris* (Juan E. Salazar and Jorge Mpodozis, manuscript in preparation, personal communication). It has been shown in chicken that during embryonic development, retinal fibers pervade the classical retinorecipient layers 1–7 and intrude into L8 and L9; a few even intrude into L10. This transient projection progresses until E14, begins to degenerate at E16, and is almost gone by E17 (Omi et al., 2011). Possibly, this embryonic projection is maintained in some birds such as the Chilean tinamou and the band-winged nightjar. Because both birds possess an enlarged L8, this retinal projection may have to do with a functional specialization of this lamina.

We regard the finding of fibers and terminals in the deep tectal layers 11–13 (Fig. 11) as a very significant result. Because they were labeled by intraocular tracer injections, they either represent a projection from retinal neurons or collateral branches from ION neurons projecting to the retina.

In embryonic chickens, a very similar pathway has been described by Omi et al. (2011), which first appears at E8–E9, degenerates from E14 onward, and entirely disappears after hatching. These authors assumed that this projection originated in the retina, stating that the "retinal fibers. . .run [dorsally] along the medial edge of the TeO after invading the tectum and turn toward the lateral side." The fibers that seem to give rise to the deep tectal terminals in the tinamou fit rather well with this description in that they seem to enter the tectum at its dorsomedial margin and then turn laterally. However, when these fiber bundles are followed along the transverse section series from anterior to posterior, they surprisingly form a continuum with the TIO (Fig. 11B; compare Fig. 7F). Thus, they may be either retinal fibers running along the TIO, or they may even be bifurcating side branches of the TIO providing a feedback from the centrifugal system to the TeO. In fact, Wizenmann and Thanos (1990) traced a transient projection from centrifugal ION neurons to the tectum between E9 and E16, which was corroborated by double-labeling experiments. It is therefore probable that the results reported by Omi et al. (2011) represent the same transient projection from the ION to the TeO, rather than a retinal projection. At present, we cannot decide between either possibilities and further experiments will be needed to clarify the source of these terminals. Whatever the case, the deep tectal pathway of the adult tinamou would be equivalent to pathways transiently expressed in Neognaths such as the chicken.

Centrifugal visual system

The centrifugal visual system of birds generally consists of two components, the organized ION and a surrounding region of "ectopic cells" (EC) (Clarke and Cowan, 1975; Hayes and Webster, 1981; Miceli et al., 1999; Wilson and Lindstrom, 2011), both respectively projecting to the retina in a characteristic fashion (Nickla et al., 1994; Uchiyama et al., 2004). Although most birds examined to date possess a well-defined ION, a recent large-scale comparative study in which the authors examined Nissl material of several dozen bird species could not find any distinguishable ION in the Chilean tinamou (Gutiérrez-Ibáñez et al., 2012). Similarly, two other palaeognathous birds were previously reported to lack an ION—the brown kiwi (Craigie, 1930) and the ostrich (Verhaart, 1971).

Retrograde labeling from our intraocular tracer experiments has now revealed that the Chilean tinamou possesses a considerable population of centrifugal neurons (Fig. 12). These cells appear to correspond mostly to ECs, for several reasons: First, they are not organized in a distinctive nuclear structure as is the typical neognathous ION. Second, we were unable to identify tufted monopolar neurons resembling "true" ION neurons of Neognathae (Cowan, 1970; Miceli et al., 1995). Instead, all of the tinamou's centrifugal neurons appear to be large and multipolar (Fig. 12E,F), like the ECs of neognathous birds (Cowan and Clarke, 1976). Third, a portion of the cells project to the ipsilateral retina (Fig. 12C,E), a common characteristic of avian ECs (Repérant et al., 2006).

Intriguingly, the tinamou isthmo-optic system bears a striking resemblance to the centrifugal visual system of crocodilians, the closest extant relatives of birds (Müller and Reisz, 2005). The homology of the centrifugal visual system in the Archosauria is stressed by the finding that the large majority of centrifugal neurons of both *Crocodylus niloticus* (Médina et al., 2004) and *Caiman crocodilus* (Ferguson et al., 1978) reside in an isthmic region with the same embryological origin as the avian isthmo-optic system (rhombomere 0) (Clarke and Cowan, 1976; Cowan and Clarke, 1976; Médina et al., 2004; O'Leary and Cowan, 1982; Repérant et al., 2007).

Like avian ECs (and tinamou isthmo-optic neurons), the crocodilian isthmo-optic system does not possess any clearly organized nuclear structure (Médina et al., 2004). In addition, morphologically the crocodilian centrifugal neurons closely resemble the ECs of birds, because in both the crocodile and the caiman most of these neurons are multipolar or fusiform. Although the existence of a few monopolar neurons resembling neognathous ION cells was reported in the crocodile, they do not project exclusively to the contralateral retina like neognathous "true" ION cells (Médina et al., 2004), and furthermore, the caiman completely lacks such cells (Ferguson et al., 1978).

Therefore, it is possible that the "true" isthmo-optic nucleus is a synapomorphy of Neognathae, and some characteristics of the basal "crocodilian" condition (e.g., only "ectopic" centrifugal neurons) are maintained in palaeognathous birds. The alternative possibility would be that a "true" ION was present in the last common ancestor of Palaeognathae and Neognathae, but was secondarily reduced in the tinamou. In fact, this may have occurred in some nonbasal neognathous species of the order Procellariiformes and the closely related pelicans (Gutiérrez-Ibáñez et al., 2012). Unfortunately, no retrograde tracing studies have been conducted in these species, so the existence of a perhaps small but "true" ION cannot be ruled out. A well-organized ION is such a widespread condition in Neognathae that it seems likely that a palaeognathous centrifugal visual system composed of ectopic cells as in the tinamou is indeed an ancestral condition and unique among birds. On the grounds of these points, the centrifugal visual system of Palaeognathae such as the Chilean tinamou may represent an intermediate stage between crocodiles and neognathous birds, filling a gap of approximately 250 million years since the crocodile-bird split (Müller and Reisz, 2005).

CONCLUSIONS: WHY STUDY TINAMOUS?

The present study provides for the first time a comprehensive description of the visual system of a palaeognathous bird, including its visual field, retinal topography, and retinal connections. Although it is clear that in general the visual system is highly conserved across the Amniote phylum, the comparative study of a basal bird may help to elucidate important aspects of its evolution in finer detail. Because of the long evolutionary divergence between the Neognathae and Palaeognathae, both similarities and differences between these clades are of interest. The similarities (conserved characteristics) may represent the basal avian conditions that existed in their common ancestors over 120 million years ago, whereas the differences illustrate which elements of the avian visual system have been subjected to evolutionary change.

At the level of retino-central connectivity of the tinamou, two elements have emerged as interesting differences to Neognathae and deserve further investigation: First, the adult deep tectal terminals, which in Neognathae have only been reported at embryonic stages; and second, the centrifugal visual system, which appears to resemble more closely the crocodilian than the neognathous avian condition.

Future research should also investigate the tinamou's higher visual projections, as well as the central organization of other sensory pathways. Qualitative observations of Nissl-stained material (as is shown in Fig. 6) reveal interesting cytoarchitectonics in field L, the n. basalis, the arcopallium, and the Wulst. A better understanding of the tinamou's pallial circuits would contribute to widening the basis for comparative studies across vertebrates, providing new insights about the evolution of the pallium and of the brain organization as a whole.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: QK, GM, HL, TVZ. Acquisition of data: QK, CM, GM. Analysis and interpretation of data: QK, HL, TVZ, GM. Drafting of the manuscript: QK, GM, TVZ. Critical revision of the manuscript for important intellectual content: HL, GM, TVZ. Statistical analysis: QK. Obtained funding: GM, HL. Study supervision: HL and GM.

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Appendix B – Krabichler et al. 2017 (full article)

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RESEARCH ARTICLE

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The Centrifugal visual system of a palaeognathous bird, the Chilean Tinamou (*Nothoprocta perdicaria*)

Quirin Krabichler[1] | Tomas Vega-Zuniga[1] | Denisse Carrasco[2] | Maximo Fernandez[2] | Cristián Gutiérrez-Ibáñez[1] | Gonzalo Marín[2,3*] | Harald Luksch[1*]

[1]Lehrstuhl für Zoologie, Technische Universität München, Freising-Weihenstephan, Germany

[2]Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

[3]Facultad de Medicina, Universidad Finis Terrae, Santiago, Chile

Correspondence: Quirin Krabichler, Lehrstuhl für Zoologie, Technische Universität München, Liesel-Beckmann Strasse 4, 85354 Freising-Weihenstephan, Germany.
E-mail: quirin.krabichler@tum.de

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Abstract

The avian centrifugal visual system, which projects from the brain to the retina, has been intensively studied in several Neognathous birds that have a distinct isthmo-optic nucleus (ION). However, birds of the order Palaeognathae seem to lack a proper ION in histologically stained brain sections. We had previously reported in the palaeognathous Chilean Tinamou (*Nothoprocta perdicaria*) that intraocular injections of Cholera Toxin B subunit retrogradely label a considerable number of neurons, which form a diffuse isthmo-optic complex (IOC). In order to better understand how this IOC-based centrifugal visual system is organized, we have studied its major components by means of in vivo and in vitro tracing experiments. Our results show that the IOC, though structurally less organized than an ION, possesses a dense core region consisting of multipolar neurons. It receives afferents from neurons in L10a of the optic tectum, which are distributed with a wider interneuronal spacing than in Neognathae. The tecto-IOC terminals are delicate and divergent, unlike the prominent convergent tecto-ION terminals in Neognathae. The centrifugal IOC terminals in the retina are exclusively divergent, resembling the terminals from "ectopic" centrifugal neurons in Neognathae. We conclude that the Tinamou's IOC participates in a comparable general IOC-retina-TeO-IOC circuitry as the neognathous ION. However, the connections between the components are structurally different and their divergent character suggests a lower spatial resolution. Our findings call for further comparative studies in a broad range of species for advancing our understanding of the evolution, plasticity and functional roles of the avian centrifugal visual system.

KEYWORDS

isthmo-optic nucleus, ectopic centrifugal neurons, retinopetal, divergent terminals, optic tectum, tecto-isthmal, association amacrine cells, tyramide signal amplification, RRID:AB_10013220, RRID:AB_2315144, RRID:AB_477329, RRID:AB_2336126, RRID:AB_2534088

1 | INTRODUCTION

The visual system is generally thought of in terms of feed-forward pathways from the retina to the brain. However, after Santiago Ramón y Cajal first demonstrated terminal endings in the retina of birds (Ramón y Cajal, 1889), a brain-to-retina feed-back projection called centrifugal visual system has been found in all major vertebrate taxa. While the neuroanatomical location of the centrifugal visual neurons can be surprisingly diverse among different vertebrates (reviewed in Repérant, Miceli, Vesselkin, & Molotchnikoff, 1989; Repérant et al.

Abbreviations: AAC, association amacrine cell; BDA, biotinylated dextran amine, 3 kDa; CTB, Cholera toxin B subunit; DAB, diaminobenzidine; FRL, formatio reticularis lateralis; Imc, nucleus isthmi, pars magnocellularis; INL, inner nuclear layer; IO, isthmo-optic; IOC, isthmo-optic complex; ION, isthmo-optic nucleus; IV, trochlear nucleus; Ipc, nucleus isthmi, pars parvocellularis; IPL, inner plexiform layer; LLD, dorsal nucleus of the lateral lemniscus; MLd, nucleus mesencephalicus lateralis, pars dorsalis; nIV, trochlear nerve; PHAL, *Phaseolus vulgaris* leucoagglutinin; RITC, Rhodamine B isothiocyanate; RT, room temperature; tecto-IO, tecto-isthmo-optic; TeO, optic tectum; TIO, isthmo-optic tract; TSA, tyramide signal amplification.

*Gonzalo Marín and Harald Luksch are co-senior authors.

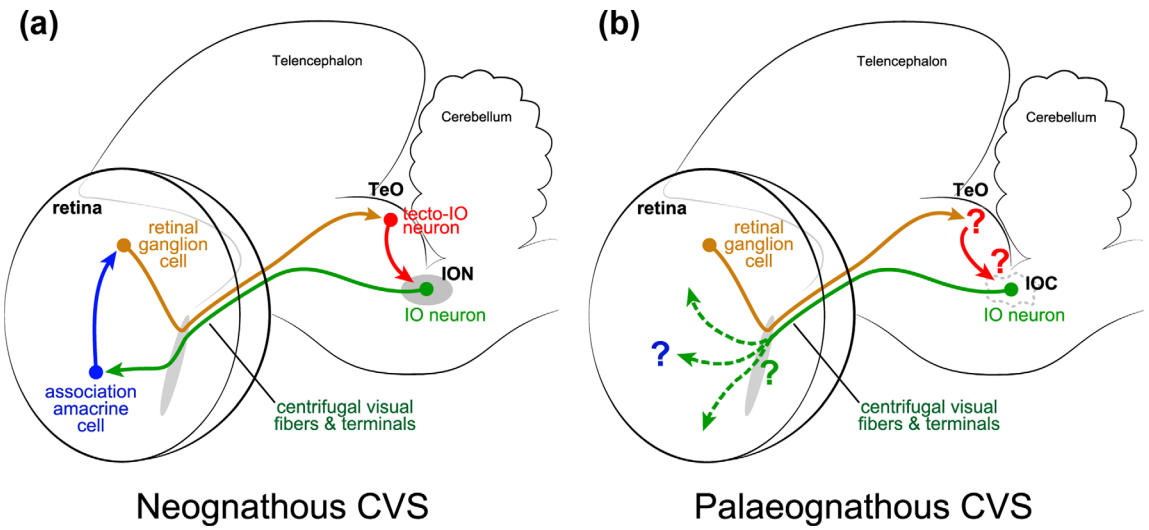

FIGURE 1 Schematic of the centrifugal visual system circuitry in birds. (a) In Neognathae, the isthmo-optic nucleus (ION) is an organized isthmic structure that receives a topographic projection from the optic tectum (TeO). The IO neurons project to the ventral half of the contralateral retina, where they synapse on specific target cells in the inner nuclear layer (INL), called "association amacrine cells" (AACs). These are axon-bearing and are thought to project to ganglion cells in other parts of the retina. Tectofugal ganglion cells send axons to the TeO, which contact the L9-neurons projecting to the ION. (b) In Palaeognathae, the IO neurons form a diffuse structure (isthmo-optic complex, IOC) at a comparable location as the Neognathous ION. The circuitry in which these neurons participate has so far not been studied. Schematic adapted from "Lesion of the isthmo-optic nucleus impairs target selection for visually guided reaching" by Uchiyama, Ohno, & Kodama, 2012, Behavioural Brain Research, 233, 359-366 [Color figure can be viewed at wileyonlinelibrary.com]

2006, 2007), in nonmammalian amniotes these cells are found in the isthmus, the junction between mid and hindbrain (Repérant et al., 2006).

The centrifugal visual system of neognathous birds (Figure 1a) generally contains the largest number of such cells and has therefore received much attention (Repérant et al., 1989; Uchiyama, 1989; Wilson & Lindstrom, 2011). Most of their centrifugal visual neurons constitute the isthmo-optic nucleus (ION; Cowan, Adamson, & Powell, 1961), which is conspicuously organized as a convoluted lamina of cells around a central dendritic neuropil (McGill, Powell, & Cowan, 1966a, 1966b). The main afferents to the ION originate from a distinct population of tecto-isthmo-optic (tecto-ION) neurons in L9-10 of the ipsilateral TeO, which project topographically upon the ION (Holden, 1968), and have been described in detail in the chicken *Gallus gallus* (Woodson, Reiner, Anderson, & Karten, 1991), the quail *Coturnix japonica* (Uchiyama, Yamamoto, & Ito, 1996; Uchiyama & Watanabe, 1985), and the pigeon *Columbia livia* (Miceli, Repérant, Bavikati, Rio, & Volle, 1997, Miceli, Repérant, Marchand, & Rio, 1993; Woodson, Reiner, Anderson, & Karten, 1991). In addition to the proper ION, a smaller number of morphologically different "ectopic centrifugal neurons" lie in surrounding areas (Clarke & Cowan, 1975). Barely any data exist on their afferents (Repérant et al., 2006), though they might also receive tectal projections (O'Leary & Cowan, 1982; Woodson et al., 1991). The axons of both ION and ectopic centrifugal neurons project to the retina where they terminate at the border of the inner plexiform layer (IPL) and the inner nuclear layer (INL) (Chmielewski, Dorado, Quesada, Geniz-Galvez, & Prada, 1988; Dogiel, 1895; Hayes & Holden, 1983). The axons of the ION neurons form "convergent" terminals (Fritzsch, de Caprona, & Clarke, 1990; Uchiyama & Ito, 1993), which are dense pericellular nests on "association amacrine cells" (AACs) in the INL (Fischer & Stell, 1999; Lindstrom et al., 2009; Nickla et al., 1994; Ramón y Cajal, 1911, 1893; Uchiyama & Ito, 1993; Uchiyama & Stell, 2005). The AACs have axons and project to other parts of the retina (Catsicas, Catsicas, & Clarke, 1987a; Lindstrom, Azizi, Weller, & Wilson, 2010; Uchiyama, Aoki, Yonezawa, Arimura, & Ohno, 2004; Uchiyama & Barlow, 1994). Axons from ectopic centrifugal neurons, however, form "divergent" terminals, which broadly ramify in the IPL (Fritzsch et al., 1990; Woodson et al., 1995). Little is known about their postsynaptic targets, though it has been reported that they might contact displaced ganglion cells (Dogiel, 1895; Hayes & Holden, 1983; Maturana & Frenk, 1965; Nickla et al., 1994) and "flat" amacrine cells (Dogiel, 1895; Maturana & Frenk, 1965).

In contrast to Neognathae, birds of the order Palaeognathae which comprises Struthioniformes (ostriches), Rheiformes (rheas), Tinamiformes (tinamous), Apterygiformes (kiwis), and Casuariiformes (cassowary and emu; Prum et al., 2015), seem to lack a proper ION (Craigie, 1930; Gutiérrez-Ibáñez, Iwaniuk, Lisney, Faunes, Marín, & Wylie, 2012; Verhaart, 1971). Nonetheless, as we previously demonstrated by neural tracing in the Chilean Tinamou (*Nothoprocta perdicaria*), they do possess a high number of centrifugal visual neurons, which form a loosely congregated complex in the same isthmic region where an ION would be expected (Krabichler, Vega-Zuniga, Morales, Luksch, & Marín, 2015). This different organization may implicate structural and functional differences of the centrifugal visual system of Palaeognathae compared with Neognathae; however, the circuitry of palaeognathous centrifugal visual system in the brain and retina is still completely unknown (Figure 1b). Since Palaeognathae diverged from their sister clade Neognathae ~100 million years ago (see Figure 2; Brusatte,

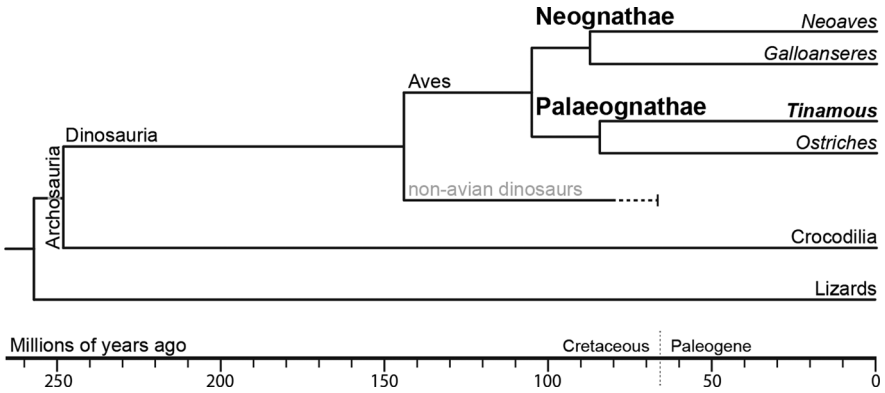

FIGURE 2 Abbreviated cladogram showing the position of Tinamous within the phylogenetic tree of the Archosauria. Phylogeny and divergence times are shown as inferred from a combination of nuclear and mitochondrial genome data and fossil markers (based on Brusatte et al., 2015, and Yonezawa et al., 2017). Note that the two grand extant clades of birds, Palaeognathae and Neognathae, are separated by ~100 million years of divergent evolution

O'Connor, & Jarvis, 2015; Claramunt & Cracraft, 2015; Jarvis et al., 2014), the comparative study of their differently organized centrifugal visual system may provide important insights into the evolution and behavioral significance of the avian centrifugal visual system, questions which continue to be unresolved in spite of many hypotheses put forward (e.g., Dillingham, Guggenheim, & Erichsen, 2013, 2017; Gutiérrez-Ibáñez et al., 2012; Marín, Letelier, & Wallman, 1990; Uchiyama, Ohno, & Kodama, 2012; Wilson & Lindstrom, 2011).

In the present study, we have combined in vivo and in vitro pathway tracing in the Chilean Tinamou, in order to reveal the principal elements and connectivity of the centrifugal visual system of a palaeognathous bird. We examine the morphology and neuroanatomy of the centrifugal neurons, their afferents from the TeO, the tecto-isthmo-optic neurons in the TeO, and the centrifugal terminals in the retina. We then compare these results with the well-studied neognathous centrifugal visual system and discuss possible implications for understanding the evolution and function of the avian centrifugal visual system.

2 | MATERIALS AND METHODS

Twenty-three adult Chilean Tinamous (*Nothoprocta perdicaria*) of both sexes (weight 398 ± 23 g [mean ± SD]), obtained from a Chilean breeder (Tinamou Chile, Los Ángeles, Chile) were used in this study. The animals were kept in the animals facilities of the Department of Biology at the Universidad de Chile, with food and water *ad libitum*. All efforts were made to minimize animal suffering, and the experiments were conducted in compliance with the guidelines of the NIH on the use of animals in experimental research, as well as with the approval of the bioethics committee of the Faculty of Sciences of the Universidad de Chile. For in vivo experiments, general anesthesia was induced by IM injection of a mixture of 40 mg/kg Ketamine (Ketamil, Troy Laboratories Pty Ltd, Glendenning, Australia; maximum total dose 120 mg/kg) and 2.5 mg/kg Xylazine (Xylavet, Alfasan International BV, Woerden, Holland; maximum total dose 7.5 mg/kg) and the bird was placed in a stereotaxic device immobilizing its head with ear bars and a beak holder. Body temperature was monitored via a cloacal thermometer and stabilized at 39–40°C by a heating pad connected to a feedback temperature control unit (Frederick Haer and Co., Brunswick, Maine, USA). Deep anesthesia during the experiments was maintained by 1–2% vaporized isoflurane delivered via a custom-built face mask at a constant flow of 200 ml/min oxygen, using a semiopen nonrebreathing Jackson-Rees circuit coupled to a gas anesthesia system (Matrx VIP 3000, Midmark, Dayton, OH).

2.1 | In vivo tracer injections

Three birds received intraocular injections of Cholera Toxin B subunit (CTB; #104, List Biological Laboratories Inc., Campbell, CA, USA). The skin covering the dorsal-most sclera of the closed eye was disinfected with 70% ethanol and diluted iodine. A Hamilton syringe (Reno, NV) with a sterile 30G cannula and loaded with 1% CTB in 0.1M phosphate buffered saline pH 7.4 (PBS; 0.75% NaCl) and 2% dimethyl sulfoxide (DMSO) was positioned using a micromanipulator. Skin and sclera were punctured and the further path of the cannula into the eye was ophthalmoscopically monitored. CTB (15 μl) were injected into the eye's vitreous body near the retina. After 10 min, the syringe was slowly retracted.

For the experiments with intracerebral injections of neuronal tracers, each bird first received an intraocular injection of 20 μl Rhodamine B isothiocyanate (RITC; Sigma-Aldrich Chemie GmbH, Steinheim, Germany; 10% in PB with 2% DMSO) into the eye contralateral to the experiment, analogous to the CTB injections, in order to retrogradely label the centrifugal visual neurons. Then, the head plumage was trimmed where necessary and the scalp was disinfected with 70% ethanol and diluted iodine. After exposing the skull by a longitudinal incision, craniotomy was performed to open a window overlying either the right TeO or the right telencephalic hemisphere. In the first set of experiments (n = 4), tracer was injected into the TeO. After exposing the tectal dura, a micropipette containing the neuronal tracer solution

was positioned perpendicular to the surface of the accessible TeO and inserted to depths between 600 and 800 μm. Either *Phaseolus vulgaris* leucoagglutinin (PHAL; Vector Laboratories Inc., Burlingame, CA, USA; 2.5% in PBS) or biotinylated dextran amine of 3 kDa (BDA, Thermo Fisher Scientific, Waltham, MA, USA; 10% in 0.1M phosphate buffer pH 7.4 [PB]) served as tracers. The PHAL injections were performed with an air-pressure system (Picospritzer II, General Valve, Fairfield, NJ) combined with iontophoresis (5 μA, 7s on/off pulse series, 10 min) using a Midgard Precision Current Source (Stoelting Co., Wood Dale, IL, USA). BDA was injected at volumes between 115 and 230 nl with a Nanoliter 2000 injector (World Precision Instruments, USA).

Another set of experiments (n = 12) aimed at injecting into the Tinamou's isthmo-optic complex (IOC). The heads of the birds were immobilized at a custom angle, in which the ear bars were in horizontal alignment with the beak commissure. In order to avoid cerebellar blood vessels, the IOC region was approached via the telencephalon, while the interhemispheric superior sagittal sinus (midline) served as orientation reference in the mediolateral dimension, and the transverse sinus of the cerebrocerebellar fissure in the anteroposterior dimension. After determining depth landmarks (such as the ventrocaudal telencephalic dura) by electrophysiological recordings with tungsten electrodes, a glass micropipette (tip diameter 30 μm) filled with CTB (1% in 0.1M PBS) was lowered to the stereotaxic coordinates of the IOC. Injections were performed with the Picospritzer combined with iontophoresis analogous to PHAL (see above). About 10 min after the injection in both sets of intracerebral experiments, the micropipette was slowly retracted, the craniotomy window was closed with the skull fragment and bone wax, and the skin wound was sutured.

After every experiment, sterile saline with an analgesic (Ketoprofen; Koralen 10%, Centrovet, Chile; 3 mg/kg) was administered SC and the animals were allowed to recover. After 5–10 days of survival, the birds were euthanized with an overdose of Ketamine/Xylazine IV and perfused through the arteriae carotides with 600 ml of warm saline followed by 300 ml of cold 4% paraformaldehyde (PFA) in PB. During the final minutes of the saline perfusion (before switching to PFA), the eyes were enucleated, hemisected at the ora serrata, freed from the vitreous body and fixed for 30 min in 4% PFA/PB. After PFA perfusion, the brain was dissected from the skull and postfixed in 4% PFA/PB overnight. Tissue was stored in PB containing 0.025% sodium azide. Brains and retinas were embedded in Gelatin (12% gelatin type A, 10% sucrose in H_2O at 37°C) and postfixed in 4% PFA/PB for 2 hr. After equilibration in a 30% sucrose/PB solution, tissue was cryosectioned on a sliding microtome at 45–60 μm.

2.2 | Retinal in vitro tracings

Four birds were euthanized by an overdose of Ketamine/Xylazine, their eyes immediately enucleated and hemisected, and the eye cups with the retinas (after removal of the vitreous with forceps) maintained in oxygenated (95% O_2/5% CO_2) Ames' Medium supplemented with 1.9 g/l sodium bicarbonate (AM; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), at room temperature (RT). A Vaseline ring was formed around the severed optic nerve. After performing a fresh cut of the optic nerve stump, a drop of distilled water was applied on the stump for 5 min. Then, the water was removed and 2 μl of a solution of 10% Dextran, Alexa Fluor 546, 10,000 MW (Dextran10K-Alexa546; Thermo Fisher Scientific, Waltham, MA, USA) in H_2Od were pipetted onto the transected nerve. The site was covered with Vaseline and the preparation was transferred into fresh AM, where it was incubated for 12–18 hr under continuous oxygenation at RT, in the dark. Finally, the Vaseline was removed and the eye cup was rinsed in PB, followed by fixation in 4% PFA/PB for 30 min. Tissue was stored in PB containing 0.025% sodium azide. The retinas were extracted and whole mounted (Ullmann, Moore, Temple, Fernández-Juricic, & Collin, 2012), embedded in Gelatin (see above), and cryosectioned in the horizontal (flattened) plane at 60 μm.

2.3 | NADPH-diaphorase histochemistry

Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry was conducted following the method described in Fischer & Stell (1999). Briefly, slides carrying retinal sections were washed three times in 0.1 M Tris-Buffer (pH 8.0) and then incubated in 0.1 M Tris-buffer with 1 mg/ml β-NADPH (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; Cat# N5130), 0.4 mg/ml nitrotetrazolium blue (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; Cat# N6876), 2 mg/ml $CaCl_2$ and 0.3% Triton X-100, for 60 min at 37°C.

2.4 | Immunohistochemistry

All antibodies used in this study are listed in Table 1, including their specifications, RRID, and dilutions. For anti-CTB and anti-PHAL immunohistochemistry, sections were first immersed in 90% methanol/3% H_2O_2 for 12 min to quench endogenous peroxidase activity. After rinsing in PBS, they were incubated overnight at RT in a solution with primary antibodies goat anti-CTB (RRID:AB_10013220) or goat anti-PHAL (RRID:AB_2315144) diluted in PBS/Triton X-100 (PBS-Tx) 0.3% with 5% normal rabbit serum, followed by rinsing in PBS and incubation with a secondary antibody (biotinylated rabbit anti-Goat [RRID:AB_2336126] diluted in PBS-Tx 0.3% for 2 hr at RT). After rinsing, avidin-biotin peroxidase complex (ABC; Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA; 3.2 μl/ml in PBS-Tx 0.5%/4% NaCl) was added. Finally, detection was accomplished by diaminobenzidine (DAB) precipitation in a solution of 0.025% DAB (DAB-buffer tablets for microscopy, Merck KGaA, Darmstadt, Germany), 0.0025% H_2O_2, 0.069% imidazole and 1% $NiSO_4$ in 0.175M acetate buffer, pH 6.0 (Green, Sviland, Malcolm, & Pearson, 1989). For fluorescence microscopy, sections were incubated for 2 hr with Streptavidin-Alexa488 (Streptavidin Alexa Fluor® 488 conjugate, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:600 in PBS-Tx 0.5%. In cases where BDA was used as a tracer, ABC/DAB or Streptavidin-Alexa488 was applied as above, but directly after the quenching step without primary/secondary antibodies. Retinal sections were reacted for immunofluorescence against Parvalbumin. Tissue was blocked in PBS-Tx 0.5% with 1% bovine serum albumin and 10% normal goat serum. It was incubated over night at RT in primary antibody mouse anti-Parvalbumin (RRID:AB_477329), diluted in PBS-Tx 0.5%. After 4x

TABLE 1 Antibodies used in this study.

Antibody	Antigen	Immunogen	Source, Cat. #, Host species, clonality, RRID	Dilution
anti-CTB	CTB (cholera toxin B subunit)	Purified choleragenoid (cholera toxin B subunit aggregate)	List Biological Laboratories Inc., Campbell, CA, USA; Cat# 703; Lot. 7031E; goat (polyclonal) RRID:AB_10013220	1: 40,000
anti-PHAL (biotinylated)	PHAL (*Phaseolus vulgaris* leucoagglutinin)	Purified *Phaseolus vulgaris* erythro/leucoagglutinin (E+L)	Vector Laboratories Inc., Burlingame, CA, USA; Cat# BA-0224; Lot. W1121; goat (polyclonal) RRID:AB_2315144	1: 2,000
anti-Parvalbumin	Parvalbumin	Purified frog muscle parvalbumin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany; Cat# P3088; Lot. 122M4774V; mouse (monoclonal) RRID:AB_477329	1: 2,000
biotinylated anti-Goat, made in rabbit	Goat IgG (H+L)	Purified goat IgG	Vector Laboratories Inc., Burlingame, CA, USA; Cat# BA-5000; rabbit RRID:AB_2336126	1: 1,500
Alexa Fluor 488 anti-Mouse, made in goat	mouse IgG (H+L)	mouse IgG (H+L)	Thermo Fisher Scientific, Rockford, IL, USA; Cat# A-11029; Lot. 1170049; goat (polyclonal) RRID:AB_2534088	1: 500

rinsing in PBS, secondary antibody Alexa Flour 488 goat anti-Mouse (RRID:AB_2534088; diluted in PBS-Tx 0.1%) was added for 6 hr at RT. The mouse anti-Parvalbumin generated against purified frog muscle Parvalbumin is highly specific and recognizes a single band of ~12 kDa in Western blots of lysed brain tissue, the molecular weight of Parvalbumin (Ramamurthy & Krubitzer, 2016).

2.5 | Tyramide Signal Amplification

In the present study, some tracings of thin neural connections had originally produced falsely negative results. Despite the high sensitivity of our tracer of choice CTB (Angelucci, Clascá, & Sur, 1996) in these cases, standard protocols failed to reveal relevant neuroanatomical details. This indicates that thin or sparse neural circuits may have such low tracer uptake and transport that the sensitivity of standard immunohistochemistry is insufficient to detect them. We found that these limitations could be overcome by tyramide signal amplification (TSA). This method, applied after the ABC step, strongly amplifies the immunohistochemical signal. In the presence of H_2O_2, the peroxidase activity causes the formation of short-lived radicals of biotin-labeled tyramide, which covalently bind to any nucleophilic residues nearby (Bobrow, Harris, Shaughnessy, & Litt, 1989) and potentiate the biotin-sites available for further detection. Although TSA has been widely used in e.g., in situ hybridization techniques, it has very seldom been combined with neuronal tracing (Adams, 1992; Papp & Palkovits, 2014). Our results suggest that by revealing fine neuroanatomical details which would otherwise remain hidden, TSA has the potential to upgrade classical in vivo tracing experiments to satisfy the elevated demands of modern microconnectomics (Swanson & Lichtman, 2016; Vega-Zuniga et al., 2016). For performing TSA, protocols up to the ABC step were identical to above. Then, brain- and retina-sections were incubated in 0.0001% biotin-tyramide (IRIS Biotech GmbH, Marktredwitz, Germany; Cat# LS-3500, Lot. 1407008) and 0.003% H_2O_2 in 0.05M borate buffer, pH 8.5, for 30 min. After washing, final detection was performed with either a second ABC step followed by DAB, or Streptavidin-Alexa488 (see above).

2.6 | Analysis

Sections were mounted on gelatin-coated slides, counterstained according to standard Nissl or Giemsa protocols or left clear, and cover-slipped with DPX Mountant (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; after dehydration/xylene clearing), or *n*-propyl gallate fluorescence mounting medium (Giloh & Sedat, 1982). Microscopy was performed with an Olympus BX63 microscope with attached digital cameras (DP26 color for brightfield, XM10 monochrome for epifluorescence), or an Olympus Fluoview FV1000/BX61 confocal laser scanning microscope (Olympus, Tokyo, Japan). Images were processed in the microscope software (Olympus Fluoview FV10-ASW v04.02.01.20, RRID:SCR_014215; or CellSens Dimension v1.7, RRID:SCR_014551) and the ImageJ distribution Fiji (Schindelin et al., 2012; RRID:SCR_002285).

3 | RESULTS

3.1 | Anatomy of the IOC

As previously reported (Gutiérrez-Ibáñez et al., 2012; Krabichler et al., 2015), the palaeognathous Chilean Tinamou appears to lack an ION in brain sections counter-stained with Nissl or Giemsa (Figure 3a,b). However, a CTB injection into the eye retrogradely labels a large number of centrifugal visual neurons in the contralateral isthmic region, as well as a smaller number on the ipsilateral side (Figure 3c; Krabichler et al., 2015). We suggest to call this cell group "isthmo-optic complex" in Palaeognathae, in order to distinguish it from the ION of neognathous birds. For the present study, we did several new intraocular tracing experiments with CTB and sectioned the brains in different planes (transverse and horizontal) in order to get a better picture of the IOC's neuroanatomy.

The isthmo-optic tract, which contains the centrifugal axons toward the retina, took the same trajectory as in neognathous birds (Galifret, Condé-Courtine, Repérant, & Servière, 1971), leaving the IOC dorsolaterally (Figure 4aa, b), then coursing rostrally along the dorsal rim of the TeO (Figure 4aa–ab; cf., figures 7f and 11b in Krabichler

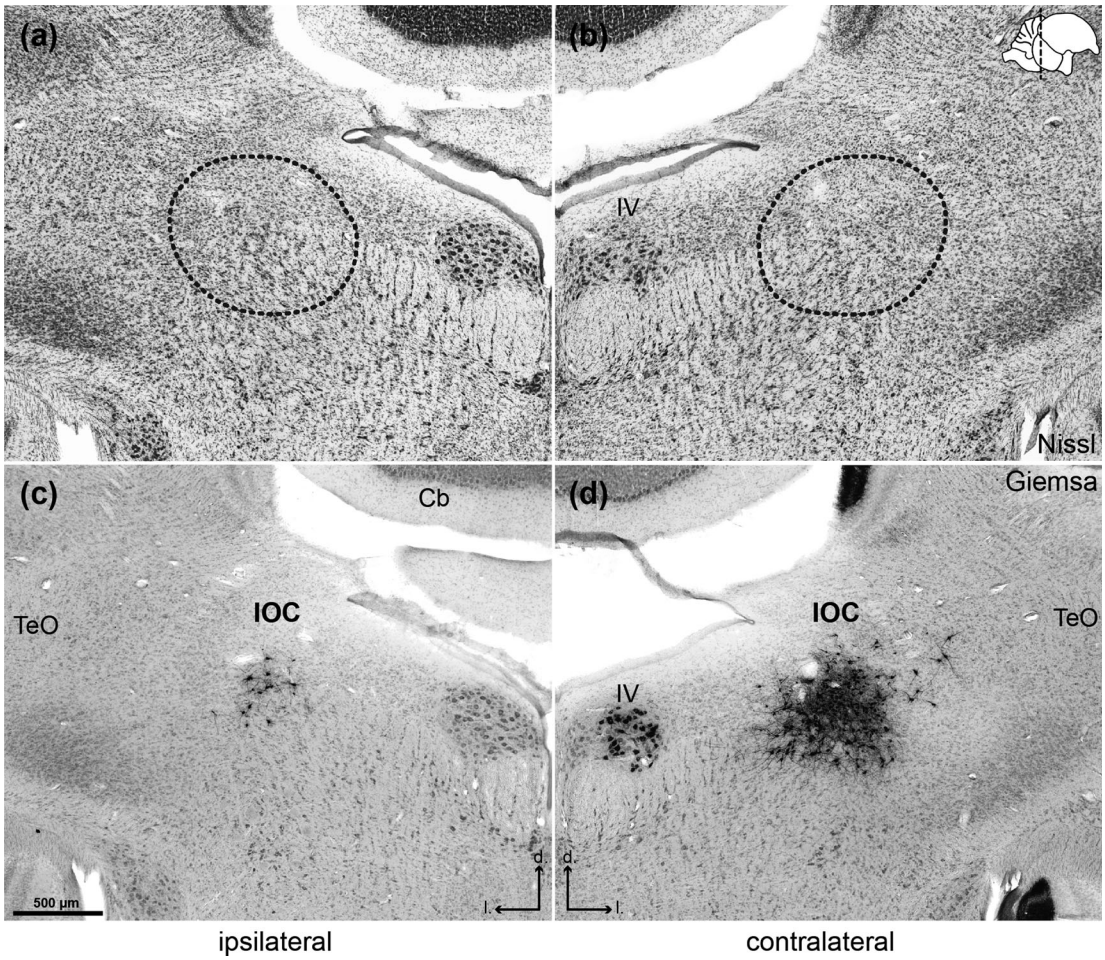

FIGURE 3 The isthmo-optic neurons in the Chilean Tinamou do not form a clear isthmo-optic nucleus (ION). (a and b) In Nissl-stained brain sections at the level of the isthmus, an ION is not distinguishable. (c and d) Adjacent sections from the same brain as in (a) and (b), but processed for immunohistochemical detection of intraocularly injected tracer Cholera Toxin B subunit (CTB) and counterstained with Giemsa. This reveals a diffuse but cell-rich cluster of retrogradely labeled IO neurons, the isthmo-optic complex (IOC). A large number of CTB-positive cells are found contralateral to the injected eye (d), while few are marked on the ipsilateral side (c).Orientation: *d*. dorsal; *l*. lateral. Abbreviations: TeO, optic tectum; Cb, cerebellum; IV, trochlear nucleus. Adapted from "The visual system of a Palaeognathous bird: Visual field, retinal topography and retino-central connections in the Chilean Tinamou (*Nothoprocta perdicaria*)" by Krabichler et al., 2015, Journal of Comparative Neurology, 523, 226–250

et al., 2015) and finally joining the rostroventral stratum opticum in direction of the optic nerve. The IOC was found in a topologically similar position as the neognathous ION (Figure 4aa–ad). It was located dorsally in the isthmic region and measured ~600–700 μm mediolaterally, 700 μm anteroposteriorly and 800–900 μm dorsoventrally. On its lateral side, it was bordered by the dorsal nucleus of the lateral lemniscus (LLD) and the formatio reticularis lateralis (FRL). Ventral to it lay the A8 dopaminergic cell group (formerly known as rostral locus coeruleus; Reiner et al., 2004), as indicated by a dense cluster of anti-tyrosine-hydroxylase-positive neurons in this region (data not shown). Dorsally and dorsomedially, the IOC was separated from the tegmental pia by a cell-rich region, which according to its cytoarchitecture might be part of the central gray.

In contrast to the ION of most Neognathae, which is a clearly demarcated, laminarly organized nucleus, the Tinamou's IOC was less well-structured. Nevertheless, most retinopetal (CTB-positive) neurons were located in a densely packed "core" region (Figure 4b,c,e), while a smaller number of "ectopic" cells was found further away at distances of as much as 500 μm or more (see arrows in Figure 4b,c). The ipsilaterally projecting neurons also mostly lay within the IOC core, and no bilaterally projecting IOC neurons were found after injecting different types of fluorescent CTB (CTB Alexa 488 and 546) into the eyes. Despite its density, the boundary of the IOC core region was not clearly circumscribed but merged into the surrounding neuronal areas. Apart from centrifugal (CTB-positive) neurons, the IOC also contained Nissl-stained CTB-negative neurons (Figure 4d), which might either be interneurons or cells from different structures lying interspersed with the IOC neurons.

All CTB-positive IOC neurons had a multipolar morphology with numerous primary dendrites splitting off in various directions (Figure 4e, f). Within the IOC core, the neurons were embedded in a dense neuropil from their dendrites (Figure 4e, f). They showed no sign of laminar

orientation or any other kind of regular organization, as can be seen in both the transverse (Figure 4b) and the horizontal plane (Figure 4c,e). The diffuse boundary of the IOC core was indicated by a notable decrease in density of the neuropil (Figure 4e). Interestingly, beyond the core region a number of dendrites extended radially away and deep into surrounding tissue (arrowheads in Figure 4b,c). While some of them may have belonged to peripheral IOC neurons, many could indeed be observed to originate from neurons within the core (see arrowheads in Figure 4e for a clear example). They were particularly numerous and long toward ventral, rostral, and medial, where they reached into adjacent neural structures such as A8, while sparing more lateral structures such as the LLD (Figure 4aa–ac,b).

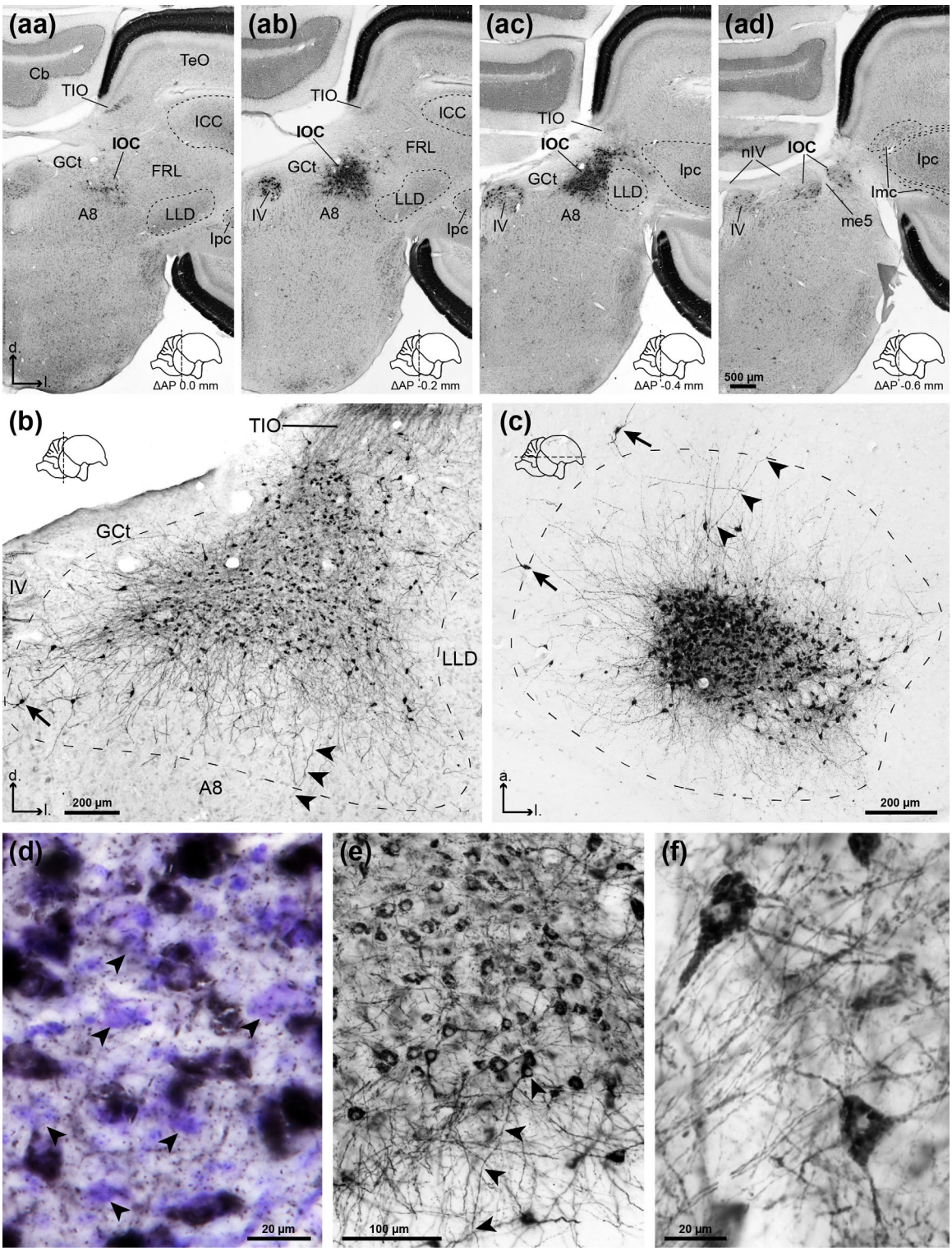

FIGURE 4.

3.2 | The tectal afferents to the IOC

Prompted by the unusual appearance of the IOC, we examined its afferents. In Neognathae, the strongest projection to the ION arises from neurons in L9-10 of the TeO (Cowan, 1970; Uchiyama et al., 1996; Woodson et al., 1991). Therefore, we injected PHAL (n = 2) and BDA (n = 2) into the TeO of the Chilean Tinamou in order to antero-gradely label the tectal afferents to the IOC, and RITC (red fluorescence) into the contralateral eye to retrogradely label the retinopetal IOC neurons. Like CTB, RITC reliably labeled the complete extent of retinal projections to the TeO (Figure 5a) as well as all retinopetal IOC neurons.

In all cases, the injection sites in the TeO were well-defined and lay predominantly centered in the intermediate tectal layers (L8–12). A representative case of an injection with BDA is shown in Figure 5. The injection site extended from L9 to L13 with no tracer spill or diffusion into other parts (Figure 5a,b). The nucleus isthmi pars parvocellularis (Ipc), which characteristically receives a topographic projection from L10 of the TeO, contained a confined band of labeled tectal fibers and retrogradelly labeled neurons. In all cases, BDA-labeled axons were present ventral to the inferior colliculus (IC; in birds also known as MLd, nucleus mesencephalicus lateralis pars dorsalis) and dorsal to the Ipc (Figure 5c), that is, in an equivalent position as the tecto-isthmal tract to the ION in Neognathae. While some of these fibers (see arrows in Figure 5c) bent ventrally toward the Ipc, others (see arrowheads in Figure 5c) could clearly be traced medially toward the IOC.

Tecto-IOC fibers were consistently found terminating in the IOC core region amidst the retrogradely labeled retinopetal neurons (Figure 5d–g). The terminals tended to branch (see arrows in Figure 5d,f) and diverge into delicate fields which sometimes spanned considerable areas (Figure 5f). Distal ramifications of the terminals contained many varicosities (Figure 5g), while fewer were also present on short side branches or *"en passant"* (see arrowheads in Figure 5d–f). Often, varicosities were found in immediate proximity to somata (see lower arrowheads in Figure 5e,g) or dendrites (see upper arrowheads in Figure 5g) of RITC-labeled cells, suggesting a monosynaptic projection from the TeO to the centrifugal IOC neurons. In other cases, varicosities were found without any immediate RITC-labeled target structure nearby (see e.g., upper arrowheads in Figure 5e). These may have either contacted more distal dendrites of centrifugal IOC neurons that were not well labeled by RITC (even compared with CTB; cf. Figure 4e,f), or interneurons (cf. Figure 4d). In the areas surrounding the IOC core, no tecto-IOC terminals were found in spite of the numerous radial IOC dendrites which traverse these regions (see above; Figure 4b,c).

These results show that the TeO in the Chilean Tinamou gives rise to a projection to the IOC core region, where widespread varicose ramifications form "divergent" terminals. As the injection sites were in all cases centered in the intermediate tectal layers, the cells-of-origin of the tecto-IOC projection were likely to be found there. Next, we did in vivo injections into the IOC in order to retrogradely label and identify these "tecto-IOC" neurons.

3.3 | The tecto-IOC neurons

CTB was injected into the isthmic region of 12 Chilean Tinamous, while contralateral intraocular RITC injections again served to reveal the IOC. In five cases, the injection site covered the IOC, while the remaining unsuccessful injections served as controls. Figure 6 shows sections from one representative case of a successful injection, amplified with TSA (see Methods). Although in this case the injection site was not completely centered in the IOC (Figure 6a), the existence of many CTB/RITC-double-labeled IO neurons (Figure 6b) demonstrated that a substantial portion of the IOC was covered by the CTB injection.

In the TeO of all successful IOC injections, retrogradely labeled neurons were found in L10, while other layers (except the deep layers L13-15; see Discussion) were devoid of labeled cells. Specifically, these presumptive tecto-IOC neurons lay in the more superficial portion of

FIGURE 4 Detailed anatomy of the Chilean Tinamou's isthmo-optic complex (IOC) labeled by intraocular injection of Cholera Toxin B subunit (CTB). (aa–ad) Antero-posterior series of coronal sections showing the neuroanatomical location and dimension of the IOC in the dorsal isthmus. It is found at the level of the trochlear nucleus (IV) and trochlear nerve (nIV), and is surrounded by the dorsal nucleus of the lateral lemniscus (LLD), formatio reticularis lateralis (FRL), central gray (GCt), and field A8. In the TeO, anterogradely labeled retinal projections are also CTB-positive (dark staining). Section planes with relative stereotaxic coordinates are indicated by the schemata at the bottom. (b) A coronal section through the IOC. The isthmo-optic tract (TIO) can be seen leaving the IOC dorsally. The IOC contains a core region densely packed with IO neurons and their dendrites. Some peripheral IO neurons are located relatively far away from the IOC core (arrow). Dendrites originating from the core region can be found extending away into surrounding neural structures, such as A8 (arrowheads). (c) A horizontal section through the IOC, showing the dense core region and few single IO neurons (arrows) lying at considerable distances from it. The radially extending core dendrites (arrowheads) are predominantly directed toward anterior and medial. (d) High power photomicrograph within the IOC core in a section counterstained with Nissl. Among the CTB-positive IO neurons (dark DAB-staining), many CTB-negative neurons (arrowheads) can be found, which might be interneurons. (e) Medium power photomicrograph of the IOC core region with its numerous multipolar IO neurons. Within the core, the dendritic neuropil has a very high density (see also f), which however rapidly declines at the border of the core region *(bottom)*. Some core neuron dendrites extend beyond the border (arrowheads), indicating that the IOC's radially extending dendrites (cf. b and c) may largely originate from within the core. (f) High power photomicrograph of two IOC neurons in the IOC core. Note their multipolar morphology and the dense dendritic neuropil that surrounds them. Orientation: *a*. anterior; *d*. dorsal; *l*. lateral. Abbreviations: *A8* field A8; *Cb*, cerebellum; *FRL*, formatio reticularis lateralis; *GCt*, central gray; *ICC*, central subnucleus of the inferior colliculus (in birds also known as nucleus mesencephalicus lateralis pars dorsalis, MLd); *Imc*, nucleus isthmi, pars magnocellularis; *Ipc*, n. isthmi, pars parvocellularis; *LLD*, dorsal n. of the lateral lemniscus; *me5*, mesencephalic trigeminal tract; *IV*, trochlear nucleus; *nIV*, trochlear nerve; *TeO*, optic tectum; *TIO*, isthmo-optic tract [Color figure can be viewed at wileyonlinelibrary.com]

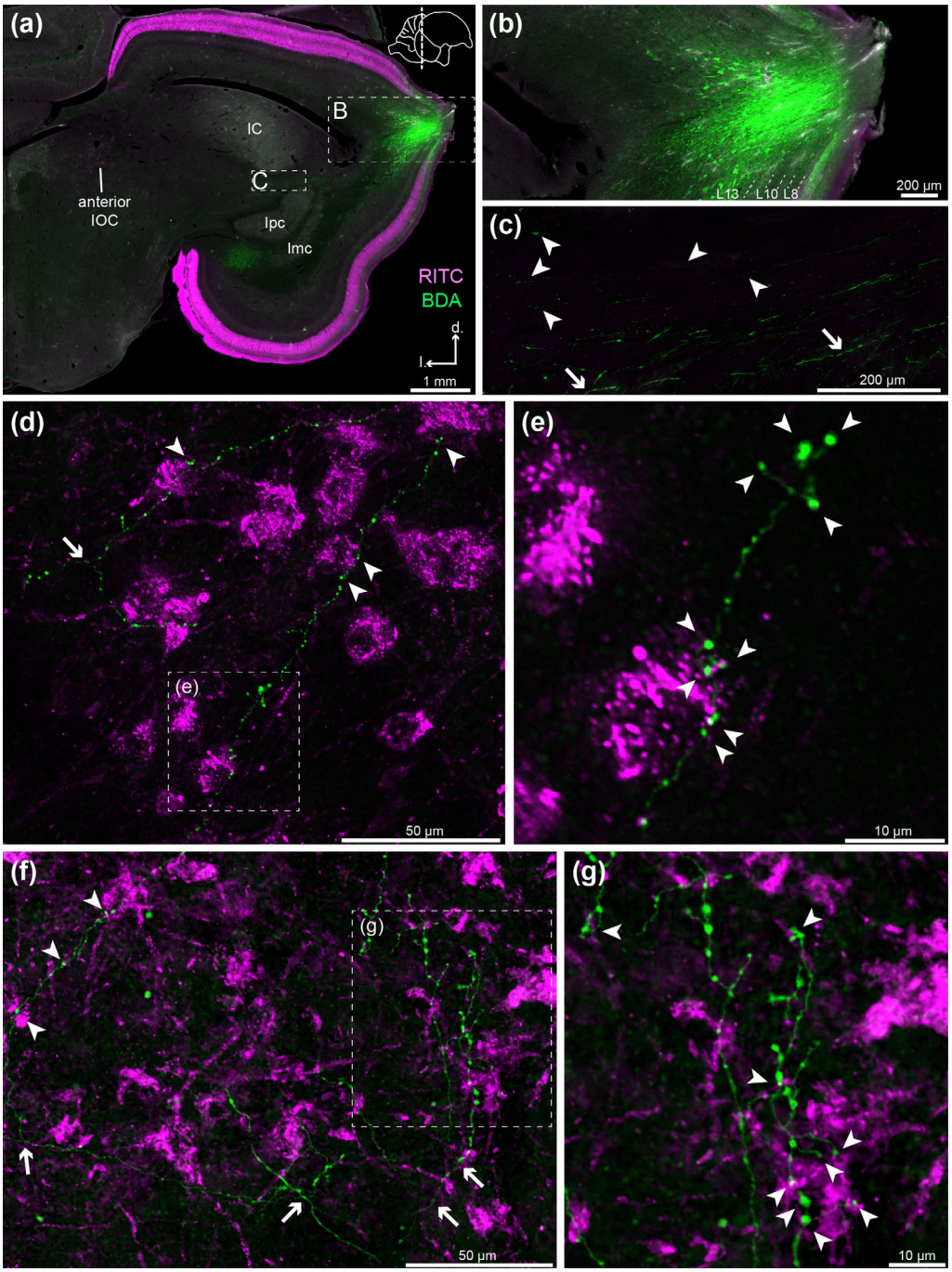

FIGURE 5 Tecto-IOC terminals after tracer injections into the TeO. Confocal microscopy images in transverse brain sections (section plane indicated by pictogram in upper right corner in a). *Magenta*: tracing after intraocular injection of Rhodamine B isothiocyanate (RITC). *Green*: Biotinylated dextran amine 3 kDa (BDA) injected into the TeO, revealed by Streptavidin-Alexa488. (a) Low-power photomicrograph of a coronal section containing the BDA-injection site in the TeO (green). The retinal projections to the TeO as well as the anterior portion of the IOC are labeled by RITC (magenta). (b) High-power photomicrograph of the tectal injection site, which was centered in the intermediate layers (L8–12). (c) BDA-labeled axons in the region of the tecto-isthmal tract between IC and Ipc (see inset in *a*). While some axons bend toward the isthmi nuclei Ipc and Imc (arrows), others run in direction of the IOC (see arrowheads). (d) BDA-labeled tecto-IOC terminals, which ramify (see arrow) among the IOC neurons, have a varicose appearance, and some varicosities are found in close proximity to IOC neurons (arrowheads). (e) Enlargement of inset in *d*. Varicosities can be found in immediate opposition to an IOC neuron soma (bottom arrowheads) as well as at some distance (top arrowheads). Short side-branches can be distinguished in both cases. (f) "Divergent" tecto-IOC terminal which splits into wide ramifications (arrows). (g) Enlargement of inset in *f*. Many varicosities of a terminal arborization lie in direct proximity to RITC-labeled IOC somata or dendrites (arrowheads), indicating synaptic contacts. Orientation: *d*. dorsal; *l*. lateral. Abbreviations: *IC*, inferior colliculus (in birds also known as nucleus mesencephalicus lateralis pars dorsalis, MLd); *Imc*, nucleus isthmi, pars magnocellularis; *Ipc*, n. isthmi, pars parvocellularis [Color figure can be viewed at wileyonlinelibrary.com]

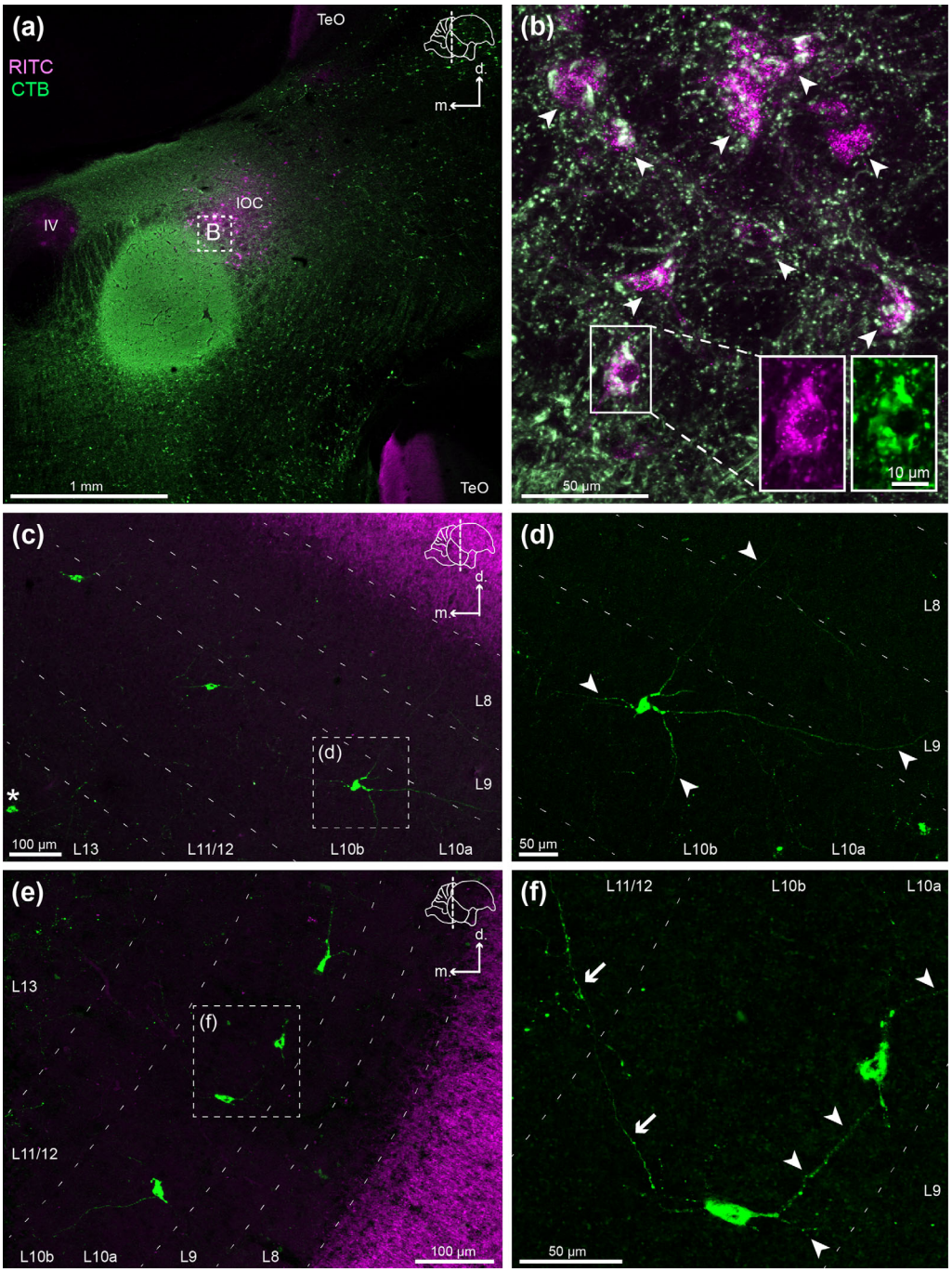

FIGURE 6 Tecto-IOC projection neurons in the TeO, retrogradely labeled by tracer injections into the IOC region. Confocal microscopy images of a representative case in which the injection partly covered the IOC. Transverse brain sections (section plane indicated by pictograms in upper right corners). *Magenta*: intraocularly injected Rhodamine B isothiocyanate (RITC). *Green*: Cholera Toxin B subunit (CTB) revealed by immunofluorescence (intensified by TSA; see Methods). (a) Injection site in the IOC region. (b) High-power photomicrograph of position indicated by inset in a. Although the center of the CTB injection only partly covered the IOC, many centrifugal visual neurons in the middle of the IOC were double-labeled with RITC and CTB (see arrowheads), indicating that the injection sufficiently spread into the IOC zones. (c–f) Presumptive tecto-IOC neurons in layer (L) 10a of the TeO retrogradely labeled by CTB, found in regularly spaced groups at various tectal positions. (c) Group of tecto-IOC neurons in the rostrodorsal TeO. An asterisk indicates a labeled neuron in L13, which probably belongs to a descending tectal projection that passes through the area of the injection. (d) High-power photomicrograph of one tecto-IOC neurons (see inset in *c*), showing its multipolar dendritic morphology (see arrowheads) (e) Group of tecto-IOC neurons in the caudoventral TeO, apparently with a narrower interneuronal spacing. (f) High-power photomicrograph of one of them (see inset in *e*). Some dendrites extend across the inter-neuronal space toward neighboring cells while others extend more radially through the TeO (see arrowheads). The axon which projects upon the IOC, splits off some collateral branches which terminate in L11/12 (see arrows) [Color figure can be viewed at wileyonlinelibrary.com]

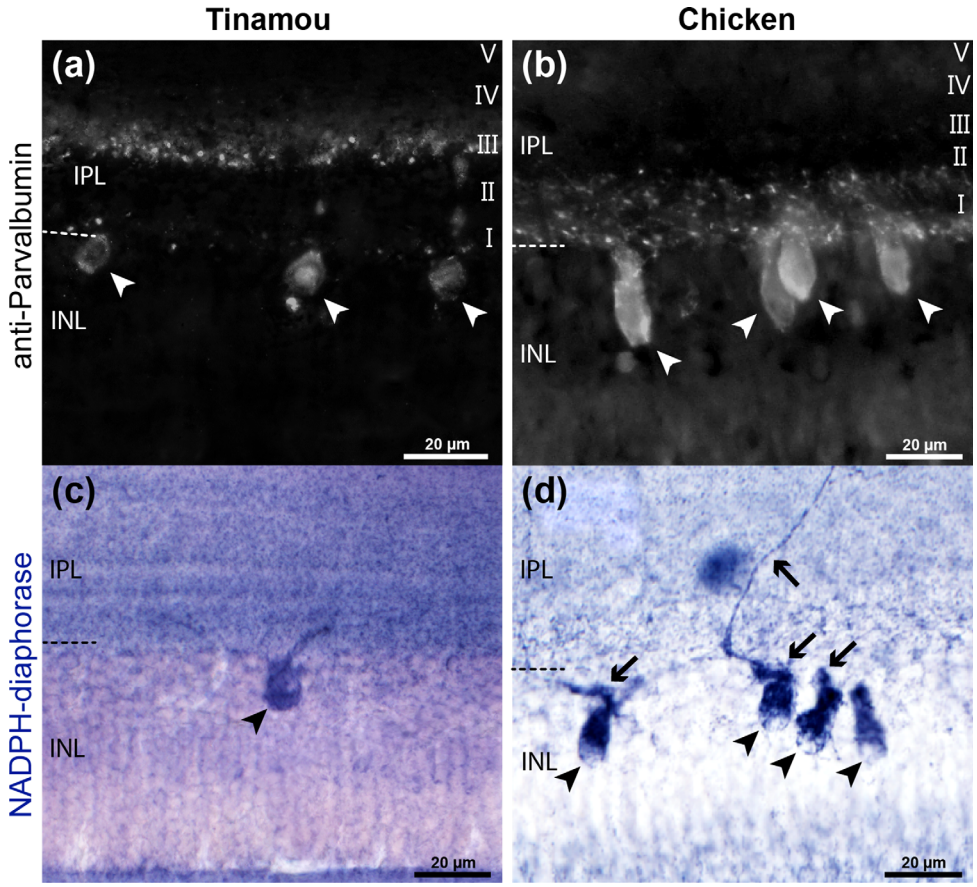

FIGURE 7 Histochemical comparison of centrifugal visual system elements in transverse sections of the retina of the Chilean Tinamou and the domestic chicken. (a and b) Anti-Parvalbumin immunofluorescence in the Tinamou (a) and the chicken (b). Labeled cell types are found in in the inner nuclear layer (INL) of both species (arrowheads). However, the Tinamou does not appear to possess cells which resemble the conspicuous elongated "association amacrine cells" (AACs) easily identified in the chicken (see arrowheads in b). (Note that the thickness of IPL sublamina I in b appears overrepresented due to the collapsed z-stack image.) (c and d) NADPH-diaphorase histochemistry in the Tinamou (c) and the chicken (d). In the chicken, this again labels the elongated AACs (see arrowhead in d) and also the "convergent" terminals (see arrows in d) from centrifugal ION fibers in the IPL, which synapse on AACs. In the Tinamou, although NADPH-diaphorase-positive cell types are present (see arrowhead in *c* for an example), none can be identified as AACs by any given criteria. Furthermore, no NADPH-diaphorase-positive "convergent" centrifugal visual terminals are found [Color figure can be viewed at wileyonlinelibrary.com]

L10, designated as L10a (Figure 6c,e). They were generally not very numerous, but in some parts of the TeO groups of them occurred together and formed a sparse, regularly spaced monolayer (Figure 6c,e). The interneuronal spacing varied from ~100–150 μm in the caudoventral TeO (Figure 6e) to ~300 μm in the rostrodorsal TeO (Figure 6c). The presumptive tecto-IOC neurons had oval-shaped somata and appeared to be multipolar (Figure 6d) possessing both obliquely horizontal and radially ascending/descending dendrites. Although some had an almost bipolar appearance (Figure 6c,f), they assumedly possessed additional dendrites not labeled by CTB. In cases where the dendrites were sufficiently labeled by CTB, they could be traced to ascend up to at least L8 and descend down to L10b (Figure 6d). Horizontal and obliquely horizontal dendrites extended across the intercell spacing toward the neighboring tecto-IOC neurons (see arrowheads in Figure 6f). In cases in which the axons were labeled, they were found to descend from the soma through the deep tectal layers (see arrows in Figure 6f), splitting off some thin collateral terminals with varicosities in L11-12 (see upper arrow in Figure 6f). Importantly, in the control cases where the injection site did not cover any part of the IOC but only nearby surrounding structures, no labeled neurons were found in L10 nor in other intermediate tectal layers. Only the deep layers (L13–15) also contained labeled neurons, which represented tectal pathways to (or passing through) areas surrounding the IOC (see Discussion).

3.4 | The centrifugal visual projection to the retina

In Neognathae, the main retinal target cells of the ION, the "association amacrine cells", are known to be positive for Parvalbumin (Fischer & Stell, 1999; Lindstrom et al., 2009; Sanna et al., 1992; Uchiyama & Stell, 2005) as well as NADPH-diaphorase, while the latter is additionally present in the prominent "convergent" centrifugal terminals (Fischer & Stell, 1999; Lindstrom et al., 2009; Morgan, Miethke, & Li, 1994; Nickla et al., 1994). In order to analyze whether these markers also reveal the retinal components of the palaeognathous centrifugal

visual system, we performed anti-Parvalbumin immunohistochemistry and NADPH-diaphorase histochemistry in transverse sections of the Chilean Tinamou retina. The chicken (*Gallus gallus*) served as a neognathous control.

In the Tinamou retina, various Parvalbumin-positive INL cell types were found, of which representatives are shown in Figure 7a. Their labeled processes stratified in IPL sublamina III, where they formed a clearly labeled band. None of the labeled cells in the Tinamou resembled the intensely Parvalbumin-positive AACs found in the ventral retina of the chicken (Figure 7b), which had the characteristic elongated morphology (Fischer & Stell, 1999; Lindstrom et al., 2009) and neurite stratification in IPL sublamina I (Fischer & Stell, 1999; Sanna et al., 1992). Analogous to these findings, NADPH-diaphorase histochemistry labeled various INL cell types in the Tinamou (see Figure 7c for an example), but none comparable to the elongated NADPH-diaphorase-positive AACs of the chicken (see arrowheads in Figure 7d). The latter were furthermore directly contacted by intensely NADPH-diaphorase-positive "convergent" centrifugal terminals (see arrows in Figure 7d), while in the Tinamou's retina no convergent NADPH-diaphorase-positive centrifugal fibers or terminals were observed.

Two different approaches were employed to label the centrifugal fibers in the Tinamou retina: First, we performed in vitro tracings with Dextran10K-Alexa546 from the optic nerve head of freshly dissected eye cups (see Methods), which were analyzed by confocal microscopy of horizontal (flatmount) retinal sections (Figure 8). The ganglion cell layer was strongly retrogradely labeled in many parts of the retina. In equatorial and ventral parts of the retina, the outer IPL (sublamina I) and border of the INL contained anterogradely labeled fibers with varicosities (Figure 8a). These presumptive centrifugal visual terminals were exclusively of the "divergent" type, forming a mesh of widespread horizontal ramifications (see filled arrowheads in Figure 8a). Their location at the IPL-INL border was corroborated by the presence of Parvalbumin-positive amacrine cells in the INL (see empty arrowheads in Figure 8b,c). The tracing also revealed retrogradely labeled displaced ganglion cells in the INL (see arrow in Figure 8a,c). Their dendrites also stratified in the IPL but had a smooth appearance which clearly distinguished them from the varicose centrifugal terminals (Figure 8a). The centrifugal terminals and displaced ganglion cell dendrites were mostly located in different strata. This can be seen in Figure 8d, which shows a reconstructed transverse section through the confocal z-stack volume, revealing two horizontal bands of Alexa-546 fluorescence signal. One band lay in sublamina II of the IPL and was primarily formed by displaced ganglion cell dendrites, which descended from the soma and

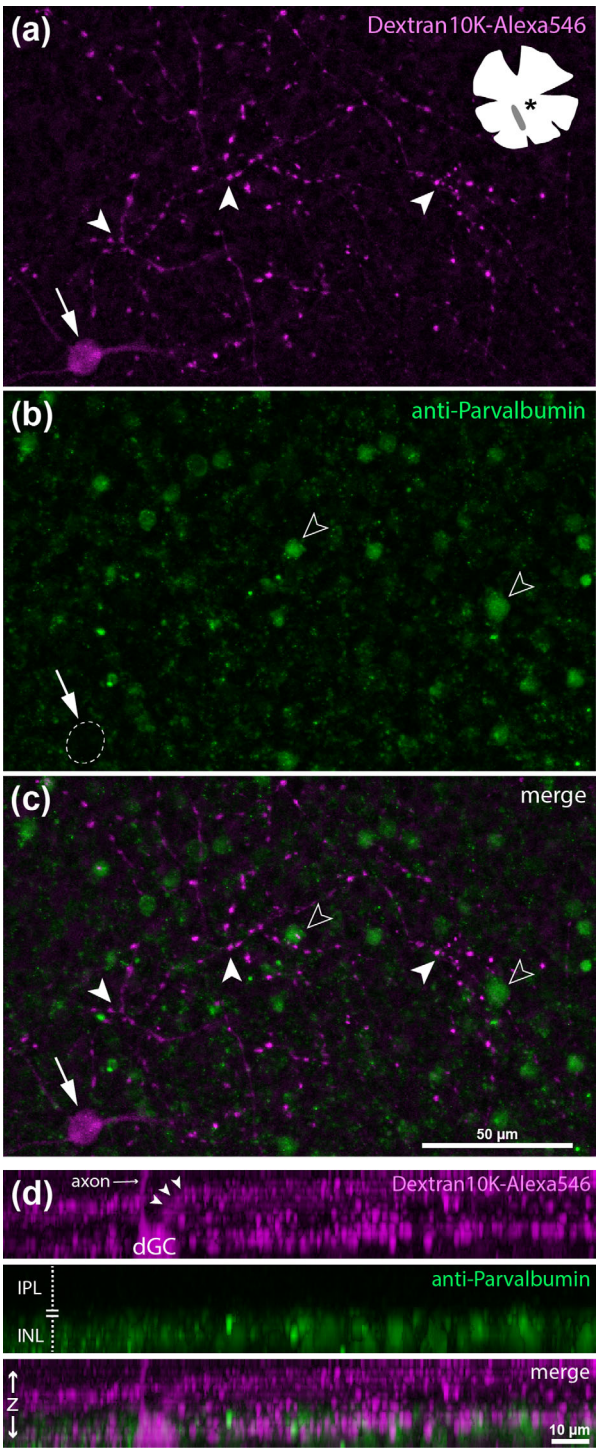

FIGURE 8 Centrifugal visual fibers and terminals in the Chilean Tinamou retina, labeled by in vitro tracing with Dextran10K-Alexa546. (a–c) Confocal photomicrograph in a horizontal retinal section at the level of the border between INL and IPL. Magenta corresponds to Dextran10K-Alexa546 (a), green to anti-Parvalbumin-immunofluorescence (b), and (c) shows the merge of both. Anterogradely labeled centrifugal terminals with many varicosities (magenta) can clearly be distinguished in the IPL. They lie in direct proximity to the INL marked by anti-Parvalbumin-labeled amacrine cells (green; see empty arrowheads). The terminals frequently ramify (see filled arrowheads) and form a big field. A retrogradely labeled displaced ganglion cell (dGC) is also present in the INL (note the smooth appearance of its dendrites). (d) Reconstruction of the *z* axis profile through the same confocal z-stack volume. The bulk of varicosity-rich centrifugal fibers (magenta) form a stratum in direct proximity to the INL. The INL can easily be identified by Parvalbumin-positive amacrine cells and a retrogradely labeled displaced ganglion cell whose axon ascends through the IPL. A second Dextran10K-Alexa546-labeled stratum is found where the dendrites of the displaced ganglion cell horizontally ramify [Color figure can be viewed at wileyonlinelibrary.com]

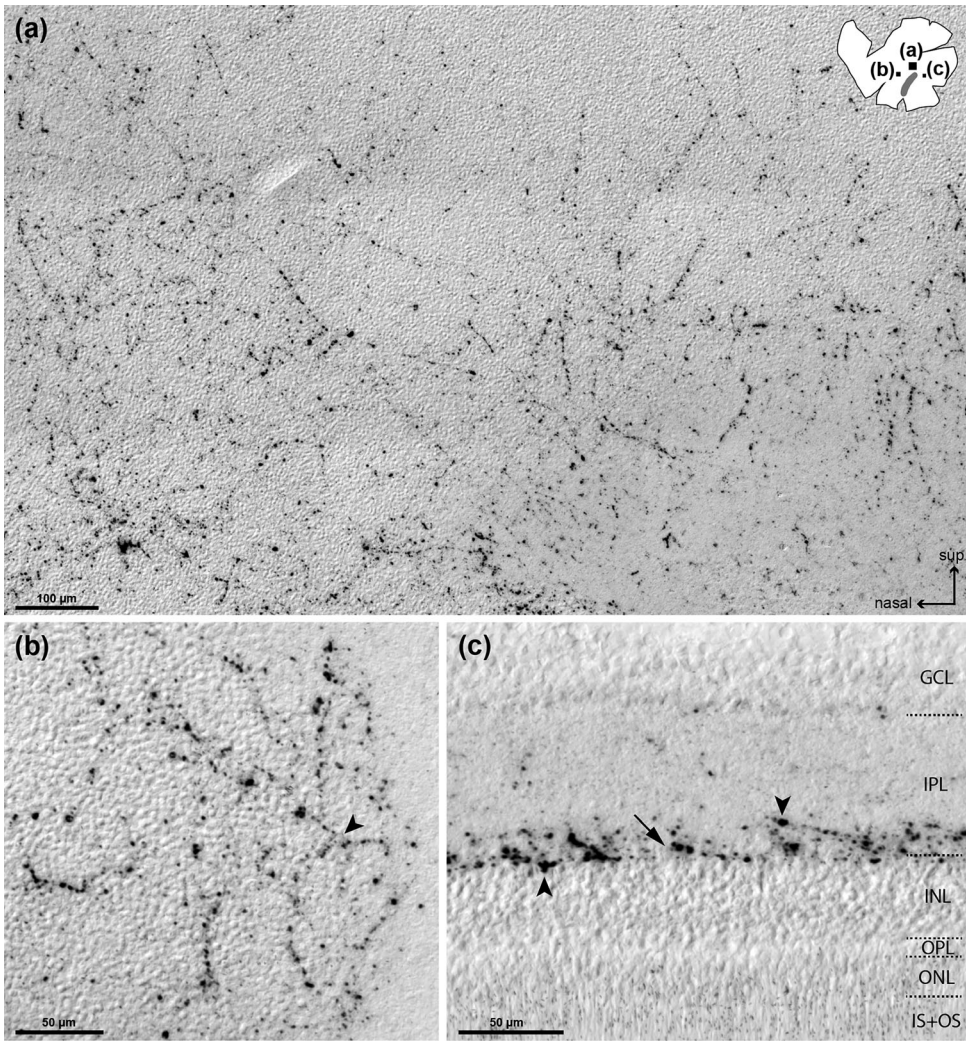

FIGURE 9 Centrifugal visual fibers and terminals in the Chilean Tinamou retina, labeled by in vivo tracing from the IOC. CTB-immunohistochemistry in the retina (TSA-enhanced; see Methods) after in vivo CTB-injections into the IOC (cf. Figure 5). The small pictogram in the upper right corner of A indicates the positions of the photomicrographs in the whole-mounted retina. (a and b) Horizontal sections of retinal whole-mounts. (a) Low-power differential interference contrast (DIC) photomicrograph at a central retinal position. A dense mesh of anterogradely labeled "divergent" centrifugal visual terminals is present in the retina at the border between IPL and INL (the cell-rich INL can be identified by the grainy appearance due to DIC). (b) Medium-power DIC photomicrograph at a nasal retinal position. An arriving centrifugal visual fiber terminal splits (see arrowhead) and ramifies into a varicosity-rich terminal. (c) High-power DIC photomicrograph of a transverse section through the retina. The centrifugal terminals (see arrowheads) mostly terminate around the border (see arrow) of INL and IPL, slightly protruding into the INL

stratified there (see arrowheads in Figure 8d; Wilson, Nacsa, Hart, Weller, & Vaney, 2011 Yang, Millar, & Morgan, 1989). Apart from those, only few varicosity-bearing fibers indicating centrifugal terminals were present here. The other more prominent band lay in IPL sublamina I at the border of (and protruding into) the INL, and (apart from a displaced ganglion cell soma) represented centrifugal terminals. In conclusion, the in vitro data suggest that most centrifugal terminals in the Tinamou lie in the IPL sublamina I adjacent to and protruding into the INL, while a minor portion might also terminate in IPL sublamina II.

Next, we processed the retinas of the in vivo IOC-injections for CTB-immunohistochemistry with TSA (see Methods), using again horizontal sections of retinal whole-mounts. Figure 9 shows differential interference contrast (DIC) photomicrographs from the contralateral retina of a representative case, which contained numerous centrifugal terminals of the divergent type. The majority of terminals were located in the equatorial and ventral retina. They formed a wide mesh of fibers (Figure 9a) and terminated in horizontal ramifications (Figure 9b). The granular surface of the INL can be distinguished to lie in the same focal plane as the centrifugal fibers and terminals. Transverse sections, produced in some parts of the retina due to tissue folds, confirmed that most centrifugal terminals lay at the border of IPL and INL (see arrow in Figure 9c), sometimes invading the INL (see lower arrowhead in Figure 9c), while a portion was present in sublamina I and presumably II of the IPL (see upper arrowhead in Figure 9c). Thus, our in vivo and in vitro data were in good agreement with each other. Furthermore, both

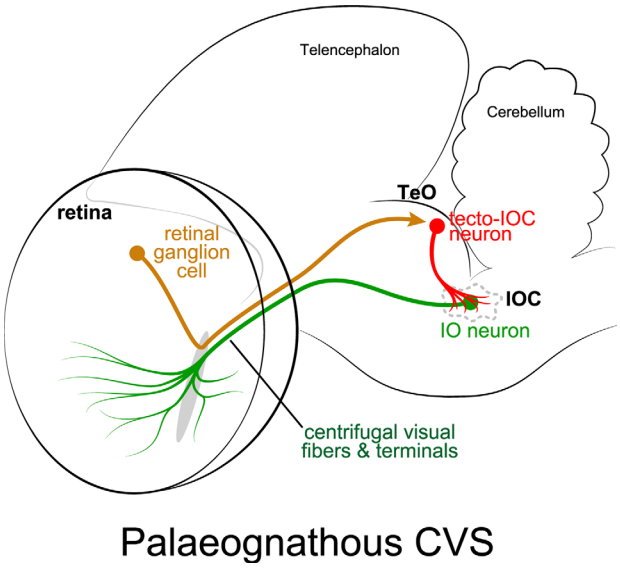

FIGURE 10 Schematic of the palaeognathous centrifugal visual system circuitry, updated by the new data. As shown by the present study, the isthmo-optic complex (IOC) of the Chilean Tinamou forms part of a comparable general circuitry as the isthmo-optic nucleus (ION) of Neognathae: It receives afferents from tecto-IOC neurons in the TeO and sends centrifugal visual efferents to the retina. In contrast to Neognathae with a well-developed ION, the tecto-IOC axons end as divergent terminals in the IOC, as do the retinopetal IOC fibers in the retina. The retinal targets of the IOC continue to be unknown, but the centrifugal projection seems to be asymmetrical as in Neognathae, since it is confined to the equatorial and ventral retina [Color figure can be viewed at wileyonlinelibrary.com]

indicated that the centrifugal fibers and terminals were topographically confined to the equatorial to ventral retina.

5 | DISCUSSION

The present study provides for the first time a detailed description of the centrifugal visual system of a palaeognathous bird. We have analyzed its circuitry in the Chilean Tinamou (*Nothoprocta perdicaria*) by in vivo and in vitro neural tracing experiments, focusing on the main components which have been studied in various neognathous species for over a century. Our findings indicate that while the Tinamou lacks a well-developed ION, its centrifugal visual neurons form a congregate IOC which receives divergent afferents from the optic tectum (TeO) and sends a prominent but exclusively divergent projection to the retina (Figure 10). Thus, similar to the Neognathous ION, the Palaeognathous IOC mediates a feedback circuit between the TeO and the retina. The elements of this centrifugal visual system circuitry, however, have notable structural and organizational differences. In particular, the multipolar morphology of the IOC neurons and their divergent terminals in the retina show parallels to the "ectopic centrifugal neurons" which lie outside the neognathous ION. These findings, which will now be discussed in detail, may have important implications for the understanding of evolution, development, and function of the centrifugal visual system, and lead the way for future comparative studies.

5.1 | The istmo-optic complex

An ION is clearly present in most neognathous birds (Gutiérrez-Ibáñez et al., 2012, 2014). It characteristically consists of small monopolar neurons ~15 μm in diameter, which in Neognathae with well-developed ION form a lamina around a central neuropil containing their dendrites (Clarke & Caranzano, 1985; Cowan, 1970; Güntürkün, 1987; Li & Wang, 1999). The morphologically different ectopic centrifugal neurons, which lie outside the proper ION, are more heterogeneous in size and generally multipolar (Clarke & Cowan, 1975; Cowan & Clarke, 1976; Hayes & Webster, 1981; Li & Wang, 1999; Médina, Repérant, Miceli, Bertrand, & Bennis, 1998; O'Leary & Cowan, 1982; Weidner, Desroches, Repérant, Kirpitchnikova, & Miceli, 1989; Weidner, Repérant, Desroches, Miceli, & Vesselkin, 1987; Wolf-Oberhollenzer, 1987). The ION presents a high variety of morphologies with different levels of complexity, but in most species (with some noteworthy exceptions such as Procellariiformes and Pelicaniformes) it is at least clearly distinguishable as a nucleus (Gutiérrez-Ibáñez et al., 2012). Therefore, the reported complete absence of an ION in Palaeognathae, represented by the Southern brown kiwi *Apteryx australis* (Craigie, 1930), the ostrich *Struthio camelus* (Verhaart, 1971), and more recently the Chilean Tinamou (Gutiérrez-Ibáñez et al., 2012), has been striking. However, our previous (Krabichler et al., 2015) and present data have shown that intraocular tracer injections in the Chilean Tinamou retrogradely label numerous centrifugal visual neurons in the dorsal isthmus, ~4100 contralaterally and 300 ipsilaterally projecting cells on each side (Krabichler et al., 2015). While their location suggests that they correspond to the neognathous ION, they do not form a distinct nucleus but a diffuse cellular group, which we have termed IOC. The finding that Palaeognathae do in fact possess a substantial number of IO neurons, although differently organized than an ION, is interesting. Foremost, the relatively large and multipolar IOC neurons resemble neognathous ectopic centrifugal neurons rather than ION neurons. Since also crocodiles, the closest living relatives of birds (Figure 2), possess exclusively "ectopic-like" centrifugal neurons (Ferguson, Mulvanny, & Brauth, 1978; Médina, Repérant, Ward, & Miceli, 2004), we previously hypothesized that the Tinamou's IOC neurons may correspond to the "ectopic-like" centrifugal neurons of the sauropsidian ancestors, while the "true" ION first evolved in Neognathae after their divergence from the Palaeognathae (Krabichler et al., 2015).

On the other hand, the IOC of the Chilean Tinamou has a much more congregate organization than the IO neurons of crocodiles, which lie dispersed over a widespread area around the isthmus (Médina et al., 2004). In fact, the dense IOC "core" region which contains the majority of the centrifugal neurons, is somewhat reminiscent of the neognathous ION, while relatively few neurons lie scattered over a wider area like ectopic centrifugal neurons (Figure 4b,c). Thus, the question of how IOC, ION and ectopic centrifugal neurons relate to one another may not have a simple answer. In Neognathae, developmental evidence suggests that ION neurons and ectopic centrifugal neurons arise from the same embryonic precursors: They are generated over the same time period (Clarke, 1982), are subjected to the same degree of naturally occurring cell death during embryogenesis (O'Leary & Cowan,

1982), and initially have the same morphology, which subsequently changes within the developing ION by dendritic reorientation and cytolamination (Clarke & Kraftsik, 1996). The differentiation into the segregated populations of ION and ectopic centrifugal neurons might therefore be epigenetically induced by different morphogenetic influences resulting from their relations and milieu interactions, rather than because they represent inherently separated cell lineages (O'Leary & Cowan, 1982). Accordingly, the Tinamou's IOC could constitute an "undifferentiated" organizational layout in which no dendritic reorientation and cytolamination occur, rather than represent either an "ectopic centrifugal neurons" or an "ION lineage." Clearly, this issue cannot be resolved based solely on morphology, but needs to consider more meaningful comparative criteria. In the present study we have focused on the hodology of the palaeognathous centrifugal visual system to determine whether its IOC has comparable connections as the neognathous ION.

5.2 | Tectal afferents to the IOC

The ION of Neognathae receives its major afferents from tecto-ION neurons in the TeO. In the chicken, quail, and pigeon, these neurons form a narrowly spaced monolayer in L9–10 of the TeO (Crossland & Hughes, 1978; Miceli et al., 1993, 1997; Uchiyama et al., 1996; Uchiyama & Watanabe, 1985; Woodson et al., 1991;) and their axons are thought to make one-on-one contacts on single ION cells (Li, Hu, & Wang, 1999; Uchiyama et al., 1996; Woodson et al., 1991). In addition, the ION/ectopic centrifugal neurons of neognathous birds possess extratectal afferents (Médina et al., 1998; Miceli et al., 1997, Miceli, Repérant, Bertrand, & Rio, 1999, Miceli, Repérant, Rio, Hains, & Medina, 2002; Repérant et al., 1989), which according to results from transneuronal tracing with RITC (Miceli et al., 1997) and more recently transsynaptic viral vectors (Mundell et al., 2015), originate in several brainstem regions such as the mesencephalic reticular formation, pontine reticular formation, and ventral tegmental area (formerly ventral area of Tsai; Reiner et al., 2004). However, it is still unknown if the extratectal afferents project to ION cells or ectopic centrifugal neurons or both.

Our tracer injections into the TeO of the Chilean Tinamou showed a clear tectal projection to the IOC (Figure 5). Moreover, the tecto-IOC fibers appeared to take the same path as the tecto-ION fibers in Neognathae, which converge into the tecto-isthmal tract ventral to the third (i.e., tectal) ventricle (Cowan, 1970; Uchiyama et al., 1996; Uchiyama & Watanabe, 1985; Woodson et al., 1991). However, unlike the topographically confined ("convergent") tecto-ION terminals described in Neognathae (Uchiyama et al., 1996; Wylie, Gutiérrez-Ibáñez, Pakan, & Iwaniuk, 2009), tecto-IOC axons were thin and possessed "divergent" varicose terminals, which ramified over wider areas within the IOC core (Figure 5e,g). Due to this widespread configuration, the terminals from each tecto-IOC neuron may contact various IOC neurons. Interestingly, no tectal terminals were observed in the areas surrounding the IOC core, even though they contained many radially extending IOC dendrites (Figure 4b,c). This might indicate that extratectal rather than tectal afferents project onto these dendrites. However, the lack of tectal fibers in these regions might also be a result of the small sizes of the tectal injections. In the future, this issue could be resolved with the help of retrogradely transsynaptic viral vectors.

We identified the tectal neurons giving rise to the tecto-IOC projection by tracer injections into the IOC. Neurons were retrogradely labeled in both intermediate (L10a) and deep (L13–15) layers. The L13–15 neurons most likely represented projections to tegmental structures such as the field A8 (formerly anterior portion of locus coeruleus; Reiner et al., 2004), or pathways which traverse peri-IOC regions such as certain descending projections (Reiner & Karten, 1982) or the tecto-tegmento-tectal pathway (Stacho, Letzner, Theiss, Manns, & Güntürkün, 2016). In fact, labeled L13–15 neurons have also been reported in chicken and pigeons following injections into tegmental areas ventrolateral to the ION, while injections confined to the ION never labeled such cells (Woodson et al., 1991).

By all evidence, the cells labeled in L10a (Figure 6) represented the tecto-IOC neurons. (i) They were located in a similar layer as their neognathous counterparts and likewise appeared to form a regularly spaced monolayer. (ii) They were consistently found in all successful IOC injections. Control cases with injections outside the IOC failed to label any neurons in intermediate tectal layers (i.e., L8–12), analogous to what has been reported in Neognathae (Reiner & Karten, 1982). (iii) Axons of the L10a neurons were sometimes observed with collateral terminals in deep tectal layers L11–12 (Figure 6f), which is similar to findings in the quail (Uchiyama, 1989; Uchiyama et al., 1996; Uchiyama & Watanabe, 1985). (iv) The tectal injections which labeled terminals in the IOC were centered in intermediate tectal layers.

The tecto-IOC neurons of the Chilean Tinamou show some differences from their counterparts in Neognathae. First, they appear to have a sparser distribution. The tecto-ION neurons of the chicken (Crossland & Hughes, 1978; Woodson et al., 1991), the quail (Uchiyama et al., 1996; Uchiyama & Watanabe, 1985), and the pigeon (Miceli et al., 1993; Woodson et al., 1991), lie in a narrowly spaced monolayer around the TeO. In accordance with their assumed one-on-one projection to ION cells, their total number has been estimated to 7000–10,000 in the quail (Uchiyama et al., 1996), 9,500 in the chicken (Clarke, Rogers, & Cowan, 1976), and 12,000 in the pigeon (Woodson et al., 1991). Our results suggest that the Chilean Tinamou tecto-IOC neurons represent a smaller population with broader inter-cell spacing (100–300 μm; Figure 6) than the tecto-ION neurons of Neognathae such as the quail (50–100 μm; Uchiyama et al., 1996) and the pigeon (30–175 μm; Woodson et al., 1991). Though our data do not permit us to provide a confident estimation of the number of tecto-IOC neurons, they could (by interpolating the numbers and intercell spacing in Neognathae) amount to a few thousand. It should be noted that even though this may suggest similar numbers of tecto-IOC and IOC neurons in the Tinamou (see above), a precisely tuned 1:1 ratio as in Neognathae is unlikely, because the tecto-IOC projection is divergent rather than 1-on-1 as the tecto-ION projection.

Another difference regards morphology. The tecto-ION cells of Neognathae such as quail (Uchiyama et al., 1996), pigeon (Woodson et al., 1991), and chicken (Heyers, Luksch, & Redies, 2004) are multipolar, with a conspicuous "willow-like" appearance, which results from

thick dendrites bending downward to deeper layers and arranging themselves into radial columns (Uchiyama & Watanabe, 1985). While the Tinamou's tecto-IOC neurons are also multipolar, their dendrites are not arranged in columns but extend horizontally and obliquely over wider tectal areas (Figure 6). Interestingly, these dendrites often spread toward neighboring tecto-IOC neurons, which might permit their population to functionally cover the entire TeO with their receptive fields.

All evidence combined suggests that the palaeognathous Chilean Tinamou possesses a TeO-IOC projection originating from a population of tectal neurons in L10a. Their morphological dissimilarities from the neognathous tecto-ION neurons could be regarded as evidence that they do not correspond to those but perhaps to a different lineage of neurons projecting to the ectopic centrifugal neurons. However, there is only limited evidence for a "tecto-ectopic" projection in Neognathae (Clarke, 1985; O'Leary & Cowan, 1982; Uchiyama et al., 1996; Woodson et al., 1991), and the identity of the putative "tecto-ectopic" neurons is unknown. By contrast, the tecto-IOC and tecto-ION neurons could indeed be homologous, and their main differing characters, for example the wide-spread dendrites of the tecto-IOC neurons, could be morphogenetically induced by factors such as their wider intercell spacing. In any case, the connectivity between the TeO and the centrifugal visual system seems to be an ancestral characteristic of archosauria (Figure 2), since there is evidence that the IO neurons of crocodiles receive a tectal projection. Tracer injections into the TeO of *Caiman crocodilus* have been reported to label fibers and some terminals in its IO area (Ferguson et al., 1978). However, the tectal fibers have not been conclusively shown to contact the IO neurons, and the putative tecto-IO neurons have so far not been identified. The tecto-ION neurons of pigeons can be conveniently labeled by retrograde transneuronal transport of intraocularly injected RITC (Miceli et al., 1997, 1993), while this has unfortunately failed both in crocodiles (Médina et al., 2004) as well as in preliminary experiments performed by us in the Chilean Tinamou. Possibly, very dense connections are necessary for detectable transneuronal RITC transport to occur. In the future, retrograde trans-synaptic viral vector tracing may facilitate comparative studies of the afferents to centrifugal visual neurons in birds and reptiles (Mundell et al., 2015).

5.3 | Centrifugal visual fibers to the retina

In Neognathae such as the chicken (Lindstrom et al., 2009), Japanese quail (Uchiyama & Ito, 1993), and pigeon (Hayes & Holden, 1983; Woodson et al., 1995), centrifugal fibers from both ION cells and ectopic centrifugal neurons project to the retina via the isthmo-optic tract (Catsicas, Catsicas, & Clarke, 1987b; Cowan & Powell, 1963; Crossland & Hughes, 1978; Galifret et al., 1971; Hayes & Webster, 1981; Wallenberg, 1898) and terminate within the exterior segment of the IPL or at the interior edge of the INL (Chmielewski et al., 1988; Dogiel, 1895). ION axons form striking "convergent" terminals (Fischer & Stell, 1999; Lindstrom et al., 2009; Morgan et al., 1994; Nickla et al., 1994) on "association amacrine cells" (Ramón y Cajal, 1893, 1911; Mariani, 1982; Uchiyama & Stell, 2005), which are readily identifiable by their striking morphology and strong staining for Parvalbumin and NADPH-diaphorase (Fischer & Stell, 1999; Lindstrom et al., 2009, 2010; Uchiyama & Stell, 2005; Wilson et al., 2011; present results, Figure 7b). The axons of ectopic centrifugal neurons form "divergent" terminals, which widely ramify in the IPL over a greater area of retinal space (Chmielewski et al., 1988; Dogiel, 1895; Fritzsch et al., 1990; Hayes & Holden, 1983; Lindstrom et al., 2009; Maturana & Frenk, 1965; Uchiyama & Ito, 1993; Woodson et al., 1995;). They have been suggested to contact displaced ganglion cells (Dogiel, 1895; Hayes & Holden, 1983; Maturana & Frenk, 1965; Nickla et al., 1994) and "flat" amacrine cells (Dogiel, 1895; Maturana & Frenk, 1965).

In the Chilean Tinamou retina, we exclusively found "divergent" centrifugal visual fibers by in vitro as well as in vivo tracing experiments. The absence of the "convergent" type was corroborated by the lack of any strongly-labeled NADPH-diaphorase-positive structures in the INL/IPL that would resemble the pericellular terminal nests in the chicken (Figure 7d). The divergent terminals formed a dense varicosity-rich mesh in equatorial and ventral parts of the retina (Figures 8 and 9a), similar to the divergent terminals of ectopic centrifugal neurons in the pigeon (Woodson et al., 1995; compare their figure 15 with our Figure 9a). Within the retina, the terminals were located in the outermost IPL and partially entered the innermost INL (Figures 8d and 9c), which was in the in vitro tracings (Figure 8) identified by Parvalbumin-positive amacrine cells (Fischer & Stell, 1999; Hamano et al, 1990; Sanna et al., 1992) and retrogradely labeled displaced ganglion cells (Karten, Fite, & Brecha, 1977; Wilson et al., 2011). Interestingly, in Neognathae only the convergent ION terminals have been described to enter the INL (Dowling & Cowan, 1966; Lindstrom et al., 2009; Ramón y Cajal, 1911), while the divergent ones remain in the IPL (Chmielewski et al., 1988; Dogiel, 1895; Fritzsch et al., 1990).

The findings that the IOC retinal terminals in the Chilean Tinamou have a divergent morphology and do not form prominent NADPH-diaphorase-positive terminals on Parvalbumin-positive AACs, could be seen as supporting the notion that the IOC neurons correspond to ectopic centrifugal neurons of Neognathae. Unfortunately, our data have not revealed the actual targets of the IOC fibers in the retina. Because the IPL sublamina where the displaced ganglion cells dendrites ramify contained only few (if any) centrifugal terminals (Figure 8d), displaced ganglion cells are unlikely to be the major targets.

An intriguing question is whether the Tinamou possesses cells which correspond to the neognathous AACs but receive divergent IOC terminals instead of convergent ones. As one possibility, such palaeognathous AACs could be among the Tinamou's NADPH-diaphorase- and Parvalbumin-positive INL neurons, but morphologically different from chicken AACs. However, very few Parvalbumin-positive processes are found in sublamina I of the Tinamou's IPL (Figure 7a), although this is in chicken the main distribution zone of the AAC processes and therefore strongly labeled (Figure 7b; Fischer & Stell, 1999; Hamano et al., 1990; Sanna et al., 1992). Another possibility could be that palaeognathous AACs exist, but either ancestrally or due to secondary loss do not express both Parvalbumin and NADPH diaphorase (or at least not sufficiently for detection by histochemical methods). Finally, AACs might be an evolutionary novelty only found in Neognathae.

This also leads to the question how the neognathous AACs, the convergent centrifugal fibers, and the intimate connection between the two originally evolved. It should be noted that divergent centrifugal visual fibers terminating at the border of IPL and INL are the prevalent configuration in almost all vertebrates (Repérant et al., 2007, p. 171), including humans (Repérant & Gallego, 1976). Convergent fibers, on the other hand, have only been reported in neognathous birds with a well-developed ION. Interestingly, their connection with the AACs is not entirely exclusive: In the pigeon, the convergent ION fibers form loose pericellular nests with many collaterals (Dogiel, 1895; Dowling & Cowan, 1966; Hayes & Holden, 1983; Maturana & Frenk, 1965; Woodson et al., 1995), and even the dense pericellular nests in the chicken split off side branches, which form synaptic boutons on other unknown targets (Lindstrom et al., 2009).

Thus it is conceivable that the convergent terminals first emerged in Neognathae by reinforcing a specific connection (i.e., with the AACs) out of a pool of originally existing "divergent" connections, along with the differentiation of the centrifugal visual system into an ECN and an ION pathway. Consequently, rather than being a completely *de novo* evolutionary innovation of Neognathae, cells homologous to the AACs could exist in Palaeognathae such as the Chilean Tinamou, and perhaps even in crocodiles. Furthermore it is possible that the high level of plasticity, which is indicated by the variability of the ION among Neognathae (Gutiérrez-Ibáñez et al., 2012), is retained at all levels of the avian centrifugal visual system. Thus, in Neognathae with poorly developed or absent ION such as storks (Showers & Lyons, 1968) and sea birds (Procellariiformes and Pelicaniformes; Gutiérrez-Ibáñez et al., 2012), a reversal to an undifferentiated centrifugal visual system configuration similar as in the Chilean Tinamou might again be found, including the terminals and their targets in the retina. In the future, broad comparative studies of the retinal centrifugal visual system components in Neognathous and Palaeognathous birds as well as crocodiles could give new insights into evolutionary and developmental principles of the avian centrifugal visual system.

5.4 | Implications for functional hypotheses of the centrifugal visual system

The striking nature of the avian centrifugal visual system has led to a long-standing debate about its functional and behavioral significance (Repérant et al., 1989) and many hypotheses have been put forward (reviewed in Wilson & Lindstrom, 2011). Previous studies have almost exclusively focused on Neognathae with well-developed, specialized ION. Their centrifugal visual system is generally thought to constitute an excitatory feedback loop (Li et al., 1999; Pearlman & Hughes, 1976) with a well-defined topography as demonstrated both anatomically (Crossland & Hughes, 1978; McGill et al., 1966a, 1966b; Uchiyama et al., 1996; Wylie et al., 2009) and physiologically (Holden & Powell, 1972; Li, Xiao, Fu, & Wang, 1998; Miles, 1972; Uchiyama, Nakamura, & Imazono, 1998). Functionally, this presumably results in a considerable spatial resolution due to the high number of ION neurons, "one-on-one" projecting tecto-ION neurons, and "one-on-one" convergent ION fibers to retinal AACs. However, the ION size and complexity among Neognathae are highly variable. In fact, small or indistinguishable ION are found in many species that are neither basal nor necessarily closely related (Feyerabend, Malz, & Meyer, 1994; Gutiérrez-Ibáñez et al., 2012; Shortess & Klose, 1975; Weidner et al., 1987).

The Chilean Tinamou represents an excellent model for studying a centrifugal visual system with an undeveloped ION and only divergent fibers. While the participation of the TeO in the circuit and the congregate IOC organization suggest that the system is in some way topographically organized, the divergent connections between its components should result in a comparatively low spatial resolution. This makes it unlikely that the Chilean Tinamou's centrifugal visual system is involved in any spatially accurate mechanisms of visual attention. The well-differentiated ION has for instance been suggested to represent an adaptation in ground-feeding birds, for functions such as retinal spot-lighting or attention scanning during pecking (Galifret et al., 1971; Holden, 1990; Marín et al., 1990; Uchiyama, 1989; Uchiyama et al., 1998; Uchiyama & Barlow, 1994). Although the Chilean Tinamou is an exclusively ground-living and ground-feeding bird (Cabot, 1992), it is improbable that its IOC is involved in such tasks. However, because the IOC projection targets only the equatorial to ventral retina similar to both the convergent and divergent fibers in Neognathae (Lindstrom et al., 2009; Woodson et al., 1995), it might in some way serve for switching attention between coarse retinal regions such as from ventral to dorsal (Clarke, Gyger, & Catsicas, 1996; Gutiérrez-Ibáñez et al., 2012); or for some rough form of covert spatial attention by "illuminating" retinal regions without eye-head movements (Ohno & Uchiyama, 2009). Wilson & Lindstrom (2011) suggested on grounds of the asymmetrical centrifugal projection that well-developed ION could serve for detecting aerial predators by switching attention from shadows on the ground toward the sky. However, this hypothesis is contradicted by the existence of many aerially predated Neognathae with small ION (Gutiérrez-Ibáñez et al., 2012). Likewise, the Chilean Tinamou has not developed a more specialized ION despite being heavily preyed on by diurnal raptors (Figueroa & Corales, 1999; Jiménez & Jaksić, 1989).

In the end, such functional comparisons between the Chilean Tinamou and Neognathae must consider the different organization of the ION-based and IOC-based centrifugal visual system, which probably imply different functions as well. Due to the structural similarities between the IOC and the neognathous "ectopic centrifugal neurons", the IOC might even possess a functional role similar to those rather than the ION. While the ectopic centrifugal neurons have unfortunately been barely investigated, recent studies with lesion experiments have suggested that the chicken centrifugal visual system (especially the ectopic centrifugal neurons) might play a role in regulating eye development (Dillingham et al., 2013, 2017). In order to test this hypothesis, similar studies could be performed in the Tinamou.

Apart from that, the Tinamou could serve as a model for studying the functional role of extratectal afferents to the centrifugal visual system, which may for example contact the numerous radially extending IOC dendrites. The structures into which these dendrites extend could give a first hint toward their possible roles. For example, many

of them are found in the central gray, which has recently been proposed as being implicated in tonic immobility to predator attacks in pigeons (Melleu, Lino-de-Oliveira & Marino-Neto, 2017). Interestingly, a striking behavior of the Tinamou is that it freezes when in danger (and only as a last resort jumps up and flies forcefully away; Cabot, 1992). Thus, IOC dendrites might receive projections from central gray neurons or their affiliated circuits, modulating the centrifugal visual system during tonic immobility, possibly affecting visual attention. While this is of course very speculative, it may be worthwhile to study the radially extending IOC dendrites and the extratectal IOC afferents in detail.

Our present study stresses the importance of comparative neurobiology. Comparison can reveal the context in which neuronal circuits have evolved, and this is ultimately also important for understanding their functions. With respect to the centrifugal visual system, we suggest to widen the range of avian (and reptilian) model organisms, in order to study its plasticity and how it may correlate with phylogeny as well as different life-styles and behaviors. Palaeognathous birds hereby represent an important link due to their long evolutionary divergence from Neognathae, and in this respect the Chilean Tinamou is becoming increasingly established as a valuable model for comparative neurobiology (Corfield et al., 2015; Krabichler et al., 2015).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: QK, TVZ, GM, HL. Acquisition of data: All in vivo and in vitro experiments were conducted at the Universidad de Chile. Most of them were done by QK during several research visits, while a few complementary experiments were done by MF, DC, TVZ and GM. Histology and histochemistry were performed by QK at the Universidad de Chile and at the Technische Universität München in Germany. Analysis and interpretation of data: QK, TVZ, CG, HL, GM. Drafting of the manuscript: QK. Critical revision of the manuscript for important intellectual content: TVZ, CG, HL, GM. Obtained funding: GM, HL. Study supervision: HL and GM.

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Appendix C – Further contributions of the author

C1. Co-author contributions to peer-reviewed articles

- Hoffmann, Susanne, Tomas Vega-Zuniga, Wolfgang Greiter, Quirin Krabichler, Alexandra Bley, Mariana Matthes, Christiane Zimmer, Uwe Firzlaff, and Harald Luksch. 2016. "Congruent Representation of Visual and Acoustic Space in the Superior Colliculus of the Echolocating Bat *Phyllostomus discolor*." *European Journal of Neuroscience* 44 (9): 2685–97. doi:10.1111/ejn.13394.

  Role of the author: Visual field measurments, histological processing of retinal tissue, stereological analysis of retinal ganglion cell topography, interpretation of data, participation in drafting of corresponding parts of the manuscript, revisions and critical comments on the full draft of the manuscript.

- Vargas, Alexander O., Quirin Krabichler, and Carlos Guerrero-Bosagna. 2016. "An Epigenetic Perspective on the Midwife Toad Experiments of Paul Kammerer (1880–1926)." *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 328 (1–2): 179–92. doi:10.1002/jez.b.22708.

  Role of the author: Investigation of Paul Kammerer's original scientific literature in German, analysis and reinterpretation of Kammerer's reported data in light of the new model proposed by Alexander Vargas, translation of relevant sections into English (including the translation of a major part

of one paper which was published as a supplementary file to the main article), revisions and critical comments during drafting of the manuscript.

- Niederleitner, Bertram, Cristian Gutierrez-Ibanez, Quirin Krabichler, Stefan Weigel, and Harald Luksch. 2017. "A Novel Relay Nucleus between the Inferior Colliculus and the Optic Tectum in the Chicken (*Gallus Gallus*)." *Journal of Comparative Neurology* 525 (3): 513–34. doi:10.1002/cne.24082.

  Role of the author: Interpretation of data, preparation of figures 1 and 11, revisions and critical comments during drafting of the manuscript.

C2. Poster presentations at scientific conferences

- Krabichler, Quirin, Harald Luksch, Tomas Vega-Zuniga, Gonzalo J. Marín, Cristian Morales, and Jorge Mpodozis. Retinal topography and central visual projections of a palaeognath bird, the Chilean Tinamou (*Nothoprocta perdicaria*). Poster session presented at: 10th Göttingen Meeting of the German Neuroscience Society; 2013 Mar 13-16; Göttingen, Germany.
- Krabichler, Quirin, Denisse Carrasco, Tomas Vega-Zuniga, Cristian Morales, Harald Luksch, & Gonzalo J. Marín. Conspicuous Features in the Visual System of a Basal Bird, the Chilean Tinamou (*Nothoprocta perdicaria*). Poster session presented at: X Annual meeting of the Chilean Society of Neuroscience; 2014 Oct 1-4; Valdivia, Chile.
- Krabichler, Quirin, Tomas Vega-Zuniga, Denisse Carrasco, Cristián Gutiérrez-Ibáñez, Gonzalo J. Marín, & Harald Luksch. The centrifugal visual system of a palaeognathous bird, the Chilean Tinamou (*Nothoprocta perdicaria*). Program No. 429.10. 2015 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2015. Online.

- Krabichler, Quirin, Cristián Gutiérrez-Ibáñez, Natalie Wossidlo, Denisse Carrasco, Tomas Vega-Zuniga, & Harald Luksch. Comparative study of the distribution of centrifugal visual fibers and their targets in avian retinae. 8th European Conference on Comparative Neurobiology (ECCN). April 7-9, 2016. Munich, Germany.

C3. Contributions to conference abstracts

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