The retinorecipient pretectal projections to the oculomotor cerebellum in zebra finch ('Taeniopygia guttata') and Anna’s hummingbird ('Calypte anna')

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Sarina Azargoon

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

The retinorecipient pretectal projections to the oculomotor cerebellum in zebra finch (*Taeniopygia guttata*) and Anna’s hummingbird (*Calypte anna*)

Submitted by ___Sarina Azargo___ in partial fulfillment of the requirements for
the degree of ___Master of Science___
in ___Zoology___

**Examineving Committee:**

Douglas L. Altshuler, Professor, Zoology, UBC
__________________________________________________________
Supervisor

Douglas R. Wylie, Professor, Biological Sciences, University of Alberta
__________________________________________________________
Supervisory Committee Member

Benjamin J. Matthews, Assistant Professor, Zoology, UBC
__________________________________________________________
Additional Examiner

**Additional Supervisory Committee Members:**

Duncan Leitch, Assistant Professor, Zoology, UBC
__________________________________________________________
Supervisory Committee Member
Abstract

A major visual signal for control of posture and movement is optic flow - the global motion across the retina due to relative motion between an organism and its environment. In birds, the pretectum and accessory optic system of the midbrain consist of neurons that are sensitive to optic flow. The retinal recipient nuclei for these key pathways are the lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). Over a decade, research from pigeons (*Columba livia*) revealed that both retinal recipient optic flow nuclei send projections to the cerebellum. The vestibulocerebellum (folium IX) receives strong input from both LM and nBOR. The oculomotor cerebellum (folia VI-VIII) also received LM input.

Recently, the inputs to the vestibulocerebellum and the oculomotor cerebellum were measured in two additional species, Anna's hummingbirds (*Calypte anna*) and zebra finches (*Taeniopygia guttata*). The midbrain-cerebellar pathways differed among species in several unexpected ways. The hummingbird oculomotor cerebellum received half of it inputs from the LM and half from two other midbrain areas, the nucleus laminaris precommissuralis (LPC) and the nucleus principalis precommissuralis (PPC). In the zebra finch, the oculomotor cerebellum received 75% of its inputs from LPC and PPC. Thus, the recent work suggests important roles for LPC and PPC in oculomotor control. This result is surprising because in the pigeon, these two nuclei represent only 7.9% of the inputs to the oculomotor cerebellum. Relatively little is known about either LPC or PPC.
The goal of my thesis was to answer two questions about these circuits: 1) Do LPC and PPC neurons of zebra finches and hummingbirds that project to oculomotor cerebellum also receive retinal inputs? 2) Do retinal ganglion cells of hummingbirds and zebra finches project to other brain regions not currently described for birds? Both questions were addressed by injecting Cholera Toxin B with different fluorophores into the oculomotor cerebellum and the eye. These experiments revealed a novel one-synapse pathway from the eye to the cerebellum in LPC, but not in PPC. I also confirmed a second one-synapse pathway through the area pretectalis that had been proposed from earlier single injection studies.
Lay summary

A major visual signal for control of movement is optic flow—the global image motion resulting from self-motion. Optic flow processing begins with motion detectors in the midbrain. We focus on optic flow processing in birds because their visual circuits are extensive, well-described, and highly conserved with mammals. We know that the optic flow processing pathways are different amongst pigeons, hummingbirds, and zebra finches and here I help clarify the roles of two midbrain nuclei.
Preface

All the work presented in this thesis was conducted in the Altshuler laboratory at the University of British Columbia, Vancouver campus and in the Wylie laboratory at the University of Alberta in Edmonton, Alberta. The research in Chapter 2 of this thesis is original and unpublished. This project was inspired partly by a previous study, Pretectal projections to the oculomotor cerebellum in hummingbirds (*Calypte anna*), zebra finches (*Taeniopygia guttata*), and pigeon (*Columba livia*) by Gaede et al.

I worked with Dr. Andrea Gaede for all surgical procedures and histology. Data collection was further facilitated by methodological guidance from Dr. Cristian Gutierrez-Ibanez. All experimental procedures were approved by the UBC Animal Care Committee and conducted in accordance with guidelines set forth by the Canadian Council on Animal Care (A19-0113).

Throughout the entirety of the degree, I received guidance from my supervisors Dr. Douglas Altshuler and Dr. Douglas Wylie and my supervisory committee member, Dr. Duncan Leitch.
Table of contents

Abstract ........................................................................................................................................ iii
Lay summary .................................................................................................................................. v
Preface .......................................................................................................................................... vi
Table of contents ........................................................................................................................ vii
List of figures ..................................................................................................................................... ix
Abbreviations ............................................................................................................................... x
Acknowledgements ....................................................................................................................... xi

Chapter one: Introduction .............................................................................................................. 1
  1.1. Introduction .............................................................................................................................. 1
  1.2. Avian eye structure ................................................................................................................ 3
  1.3. The retinal ganglion cells of birds ....................................................................................... 5
  1.4. Optic flow processing ............................................................................................................. 6
  1.5. Anatomy of the optic tectum ............................................................................................... 7
  1.6. Anatomy of the AOS and the pretectum ............................................................................. 8
  1.7. Connectivity between the AOS and pretectum with the OCb ......................................... 11

Chapter two: Research .................................................................................................................. 14
  2.1. Introduction .............................................................................................................................. 14
  2.2. Methods .................................................................................................................................. 16
    2.2.1. Animals .......................................................................................................................... 16
    2.2.2. Surgeries and tracer injection ....................................................................................... 17
    2.2.3. Immunohistochemistry ............................................................................................... 20
2.2.4. Microscopy and image analysis ................................................................. 21

2.3. Results ........................................................................................................ 22

2.4. Discussion ................................................................................................... 27

Chapter three: Conclusion .............................................................................. 29

Bibliography ...................................................................................................... 32
List of figures

Figure 1. Pie charts illustrating, within the pretectum, the proportion of retrogradely labeled cells in the lateral lentiformis mesencephali (LMI), medial lentiformis mesencephali (LMm), nucleus laminaris precommisuralis (LPC), and nucleus principalis precommisuralis (PPC) from injections in the vestibulocerebellum (folium IXcd) and oculomotor cerebellum (folia VI/VII) ... 11

Figure 2. Tracer injection sites ................................................................. 18

Figure 3. Confirmation of injection sites in the oculomotor cerebellum and terminals in the optic tectum for zebra finches and hummingbirds ...................... 23

Figure 4. Retinal projections to the pretectum of zebra finches and hummingbirds resemble other birds ................................................................. 24

Figure 5. Further evidence for a previously undescribed one-synapse pathway from the retina to the oculomotor cerebellum through LPC ......................... 25

Figure 6. Further evidence for a one-synapse pathway from the retina to the oculomotor cerebellum through the area pretectalis ................................. 26
Abbreviations

AOS Accessory Optic System
CTB Cholera toxin subunit B
DGC Displaced ganglion cell
Glv ventral lateral geniculate nucleus
GTc caudal tectal gray
GTr rostral tectal gray
LMI lentiformis pars lateralis
LMm lentiformis mesencephali pars medialis
LPC nucleus laminaris precommisuralis
nBOR nucleus of the Basal Optic Root
nRt nucleus rotundus
OCb oculomotor cerebellum
PPC nucleus principalis precommisuralis
TeO optic tectum
VbC vestibulocerebellum
Acknowledgments

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Chapter one: Introduction

1.1. Introduction

Compared with mammals, birds have relatively large eyes. A bigger eye has more retinal ganglion cells and therefore higher spatial resolution. High visual acuity is thought to be essential for complex flight maneuvers such as avoiding collisions or capturing fast moving prey. In vertebrates, signals from the eye are transmitted to two general types of visual pathways: image-forming pathways and non-image forming pathways. The latter are used for fast reactive movements such as image stabilization, and recent evidence from avian visual neuroscience indicates that non-image-forming pathways may also be used for rapid movements in flight. The goal of my master’s thesis is to address gaps in our knowledge of how the output cells of the retina, the retinal ganglion cells are connected to the cerebellum, a key site for sensorimotor integration just upstream of pre-motor nuclei.

Extensive work with pigeons has led to a better understanding of the non-image forming pathways centered in the pretectum and accessory optic system (AOS). It was previously thought that birds had only two retinal recipient midbrain nuclei in this pathway: the pretectal Lentiformis Mesencephali (LM) and the nucleus of the basal optic root (nBOR) of the AOS.

A recent study sought to confirm this anatomical arrangement for zebra finches and hummingbirds because these bird species are also used for behavioral and electrophysiological studies related to flight control (Gaede, Gutierrez-Ibanez,
Armstrong, Altshuler, & Wylie, 2019). The study used retrograde injections to the oculomotor cerebellum and the vestibulocerebellum. These experiments led to the identification of two other midbrain inputs to the oculomotor cerebellum from two other avian midbrain visual areas, the nucleus laminaris precommisuralis (LPC) and the nucleus principalis precommisuralis (PPC) (Gaede et al., 2019).

According to the description by Gamlin and Cohen (1988), the LM consists of two subnuclei: LM pars lateralis (LML) and LM pars medialis (LMM). Medial to the LMM is a strip of small cells, the laminaris precommisuralis (LPC), which appears to be contiguous with the internal lamina of the ventral lateral geniculate nucleus (GLv) and it is composed of a lamina of very basophilic cells 14-16 pm in diameter. Medial to the LPC is the nucleus principalis precommisuralis (PPC), which resides lateral to the nucleus rotundus (Rt).

The anatomical connectivity of LPC and PPC has been largely ignored since 1988. It was, however, also known from tract tracing that the pigeon oculomotor cerebellum received weak input (7.9%) from LPC and PPC (Gaede et al., 2019). The strong connectivity from LPC and PPC to the oculomotor cerebellum discovered by Gaede et al. now suggests that these nuclei may have an important role in visuomotor control through the midbrain-oculomotor pathway.

To address this hypothesis, I made dual injections of two different fluorescent tracers in the eye and the cerebellum in both zebra finches and hummingbirds. One goal of these experiments was to determine if LPC and PPC are also retinal recipient. A second goal was to describe the range of retinal
projections more thoroughly from the retina of hummingbirds and zebra finches to other brain areas.

In this introductory chapter, I will review three research topics that form the intellectual foundation for my master’s research. I first describe what is known about the output cells of the retina in birds compared to mammals. I next review the anatomy of the AOS and the pretectum of the pigeon and to the extent known, other birds. Finally, I review what was previously known about connectivity of the AOS and pretectum connectivity to the cerebellum, especially to the oculomotor cerebellum.

1.2. Avian eye structure

There is no doubt that the ability of birds to perform fast movements and rapid maneuvers requires exceptional sensory abilities. The avian retina is a curved multi-layered structure that possesses one of the most sophisticated photoreceptor systems among vertebrates. It is capable of high visual acuity, color discrimination, and movement detection. It is also capable of a great range of movement detection and discrimination of slow moving objects (Meyer, 1977).

Characteristic features of the avian retina include five major layers and five major cell types. The five layers are the outer nuclear and plexiform layers, the inner nuclear and plexiform layers, and the ganglion cell layer. Five major classes of retinal neurons are also recognized: photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells.
Amongst these five cell types, there are three types of visual elements, rods, single cones, and double cones, which are characterized by their structural and chemical compartmentation. There are also other specialized structures such as diverse areas for acute vision (with one or two foveae), and an additional nutritive source, the pecten (Bischof, 1988) that are responsible for their exceptional physiological responses.

Double cones and rods are responsible for luminescence vision of birds, mediating the vision in bright and dim light, respectively and color vision is mediated by single cones, based on expression of SWS1, SWS2, Rh2 and LWS cone opsins (Hart & Hunt, 2007). In daylight conditions, birds use color vision to discriminate large objects, and luminance vision to detect details and motion.

The bipolar cells are interneurons in the retina, and they effectively transfer information from rods and cones to ganglion cells. The bipolar cells are located in the inner nuclear layer and there are two types of bipolar cells in birds, outer (or large) and inner (or small) bipolar cells (Sefton, 1975).

The horizontal cells of the outer plexiform layer mediate the lateral spread of visual activation in the retina, and there are two distinguished types of horizontal cells in birds, brush-shaped and stellate (Sefton, 1975).

Function of the amacrine cells is similar to horizontal cells in transferring information laterally across the retina. Amacrine cells differ in their morphology and neurotransmitter type. Amacrine cells may also be responsible for shaping the complex receptive fields seen in some retinal ganglion cells and this complexity
usually correlates with the amount of amacrine cell input. The predominance of complex responses and directional selectivity in avian retinal ganglion cells compared to those in mammals may be attributable to amacrine circuitry.

1.3. The retinal ganglion cells of birds

Retinal ganglion cells (RGCs) process and convey information from the retina to visual centers in the brain. These output neurons comprise subpopulations with distinct structure and function (Wassle & Boycott, 1991). Ganglion cells are a morphologically heterogeneous population and may be distinguished by variations in somatic size, configuration, and lamina of dendrite distribution in the inner plexiform layer, as well as by axon caliber (Ramón y Cajal, 1892). Most ganglion cells are located in the ganglion cell layer proper, immediately above (distal to) the layer of optic nerve axons and there are some large ganglion cells along the inner margin of the inner nuclear layer which have been referred as the displaced ganglion cells, and are found in all vertebrate classes, particularly conspicuous in avian retinas (Witkovsky & Stell, 1973).

There are 2 million or more retinal ganglion cells in chick, pigeon, and quail compared to 1 million RGCs in rhesus monkey and humans (Thompson, Palacios, & Varela, 1992). In general, nocturnal animals and deep-sea fish tend to have lower quantities of ganglion cells and several adaptations have been discussed with respect to the bird's ability to detect movement. (Dacey, 1999; Wassle & Boycott, 1991). RGCs have been shown to possess highly specialized responses to particular movements and some of them are good motion detectors and may prefer a specific
direction of stimulus movement, whereas others are sensitive to the orientation of the stimulus but not its direction. There are five classes of retinal ganglion cells in the pigeon, each of which differs in its response to a particular movement, e.g., vertical edge, horizontal edge, general edge, directional moving edge, convex edge (Maturana & Frenk, 1963).

Retinal ganglion cells give rise to efferent nerve pathways from the retina that terminate upon a number of distinct central nervous system structures including the optic tectum, dorsal and ventral thalamus, pretectum, and the accessory optic nuclei (Ramón y Cajal, 1892).

1.4. Optic flow processing

The visual pathway and nuclei are very well developed in birds (Harvey J. Karten, 1969; Meyer, 2000). The avian visual system, like the mammalian visual system, consists of a very large number of anatomical structures arranged in parallel pathways, which overall show a remarkable resemblance to the organization found in tetrapods.

There are two major visual pathways from the retina to the telencephalon of birds: the thalamofugal (or lemnothalamic) and the tectofugal (or collothalamic) pathways. The thalamofugal pathway runs from the retina to the principal optic nucleus of the thalamus (OPT) to the visual Wulst (Shimizu & Bowers, 1999) and the tectofugal pathway proceeds from the retina to the optic tectum, and then to the thalamic nucleus rotundus (n.Rt) of the thalamus, and finally to the ectostriatum in dorsal ventricular ridge (DVR) (Benowitz & Karten, 1976; Butler & Hodos, 2005).
There is also another neural pathway from the retina where the retinal-recipient nuclei in the Accessory Optic System (AOS) and pretectum project to many areas in the brain (Brecha, Karten, & Hunt, 1980; Gamlin & Cohen, 1988b, 1988a) and it is responsible for analyzing the optic flow, the pattern of visual motion at the moving eye (Gibson, 1954). In birds, the key nuclei in this pathway are called the nucleus of the basal optic root (nBOR) of the accessory optic system (Brecha et al., 1980) and the nucleus lentiformis mesencephali (LM) of the pretectum (Gamlin & Cohen, 1988b, 1988a). LM and nBOR then project to the oculomotor cerebellum (folia VI-VIII) and folia IXcd of the vestibulocerebellum (Clarke, 1977; Pakan et al., 2006). These three pathways closely parallel respectively the colliculo-pulvinar-extrastriate pathway, the retino-geniculocortical pathway and the accessory optic pathway of mammals (H. J. Karten, Fite, & Brecha, 1977; Pettigrew & Konishi, 1976).

The tectofugal system of the pigeon seems remarkably well-adapted to respond to various aspects of object motion while ignoring most classes of self-induced visual motion. This is in contrast with the visual processing that was found in the pigeons’ accessory optic system, which conversely seems well suited to extract important features of self-induced visual motion (Frost, Wylie, & Wang, 1990).

1.5. Anatomy of the optic tectum

The avian optic tectum, the first station of the tectofugal pathway, receives topographically (Hamdi & Whitteridge, 1954; Schmidt, Engelage, & Bischof,
ordered input exclusively from the contralateral eye. Arrangement of layers in the optic tectum in the birds shows a similar arrangement to that in the reptile (Carl Huber & Crosby, 1929; Jungherr, 1945).

Within the optic tectum, several cell types are distributed in a distinct manner in 15 distinguishable layers (Ramón y Cajal, 1892). The axons of retinal ganglion cells show distinct termination patterns in tectal layers 2, 3, 4, 5b, and 7 (Hayes & Webster, 1975; Repérant & Angaut, 1977) in the pigeon and the zebra finch. The stratum griseum centrale (SGC, layer 13) is the major output source of the tectal projection to the thalamic nucleus rotundus (RT). From there visual information is transferred to the telencephalic ectostriatum (Gamlin & Cohen, 1986).

1.6. Anatomy of the AOS and the pretectum

Specialized visual pathways are involved in the analysis of optic flow, the motion that occurs across the entire retina during self-motion (Gibson, 1954). In all vertebrates, retinal-recipient nuclei of the accessory optic system and pretectum form visual pathways that process global visual motion (Giolli, Blanks, & Lui, 2006; Simpson, 1984). The key nuclei involved in these specialized pathways of birds include the nucleus of the basal optic root (nBOR) of the accessory optic system (Brecha et al., 1980) and the nucleus lentiformis mesencephali (LM) of the pretectum (Gamlin & Cohen, 1988b, 1988a).

These pathways are involved in generating the optokinetic response to facilitate retinal image stabilization (Waespe & Henn, 1987). The visual response
properties of neurons in LM and nBOR are very similar; in both nuclei, most neurons have large receptive fields in the contralateral visual field and exhibit direction-selectivity in response to large field visual motion (Morgan & Frost, 1981; Winterson & Brauth, 1985).

It was first reported that only retinal ganglion cells (RGCs) in the ganglion cell layer, but not displaced ganglion cells (DGCs), project to LM (Bodnarenko, Rojas, & McKenna, 1988; Fite, Brecha, Karten, & Hunt, 1981). It was suggested that, at least in pigeons, zebra finches and hummingbirds (Gutierrez-Ibanez et al., 2018), DGCs may project to LM. DGCs are a specialized subset of retinal cells, which are found at the margin of the inner nuclear layer (INL) and inner plexiform layer (IPL) rather than the ganglion cell layer (Fite et al., 1981; H. J. Karten et al., 1977; Reiner, Brecha, & Karten, 1979). In pigeons, DGCs also project to nBOR, which then projects directly to the accessory optic nuclei and the accessory optic nuclei of the pigeon. nBOR sends a strong mossy fibre projections to the vestibulocerebellum (Benowitz & Karten, 1976; Clarke, 1977; H. J. Karten et al., 1977). Thus, the displaced ganglion cells are the source of a major bisynaptic pathway originating in the retina and projecting upon the cerebellum via the nucleus of the basal optic root (nBOR) (Brecha et al., 1980; H. J. Karten et al., 1977).

The LM, but not other visual nuclei, is hypertrophied in hummingbirds relative to other birds (Iwaniuk & Wylie, 2007). This enlargement may represent a neural specialization related to hovering flight. Hummingbirds are very sensitive
to small changes in their visual environment while hovering and will drift to compensate for optic flow in all directions (Goller & Altshuler, 2014). In nearly all tetrapods studied to date, the typical pattern observed is that LM neurons prefer temporonasal (back-to-front) motion across the retina, and nBOR neurons prefer naso-temporal (front-to-back), upward or downward motion (Fite, 1985; Hoffmann & Schoppmann, 1981; Ibbotson, Mark, & Maddess, 1994; McKenna & Wallman, 1985; Mustari & Fuchs, 1990; Winterson & Brauth, 1985; D. R. W. Wylie & Crowder, 2000). However, in hummingbirds, a different pattern of response properties in the LM emerged (Gaede, Goller, Lam, Wylie, & Altshuler, 2017). The majority of LM neurons do not prefer temporo-nasal motion; instead, there is a more uniform distribution of preferred directions, with cells preferring upward, downward, and naso-temporal motion as frequently as temporo-nasal motion (Gaede et al., 2017). Consistent with other tetrapods, there is a strong population-level preference for temporo-nasal motion among LM neurons of zebra finches and pigeons. Furthermore, hummingbird and zebra finch LM neurons prefer higher velocities of visual motion than pigeon LM neurons (Gaede et al., 2017). This suggests a role for the LM in responding to high-speed visual motion during hovering and collision avoidance in hummingbirds.

The connections of LM and nBOR are extensive and include structures involved in axial motor control, oculomotor control, and nuclei in other visual pathways (Brecha et al., 1980; Clarke, 1977; Gamlin & Cohen, 1988a)
1.7. Connectivity between the AOS and pretectum with the OCb

Flight demands acute multisensory integration and sophisticated motor control. The cerebellum is much larger in birds compared to non-avian reptiles (Husband & Shimizu, 2001) and it is traditionally implicated in motor control (Ito, 1984) and is clearly a site of multisensory integrations (Bower, 1997; Paulin, 1993), therefore it is important for flight (Kornhuber, 1974).

In vertebrates, retinal-recipient nuclei of the accessory optic system and pretectum form visual pathways that process global visual motion. These pathways are responsible for generating the optokinetic reflex to maintain retinal image stabilization (Waespe & Henn, 1987). Projections from LM, nBOR, and other...
visual nuclei converge in the oculomotor cerebellum (folia VI-VIII) and folium IXcd of the vestibulocerebellum where sensorimotor control is coordinated (Clarke, 1977; Pakan & Wylie, 2006). Additionally, Feenders et al. (2008) found increased activity in folia VI and IXcd during flight by using the expression of immediate-early genes as an indicator of neural activity (Feenders et al., 2008).

The projection to and from both the LM and nBOR have been studied extensively in pigeons (Douglas R Wylie, Gutiérrez-Ibáñez, Gaede, Altshuler, & Iwaniuk, 2018; Douglas Richard Wylie, 2013). In pigeons, zebra finches and hummingbirds, the projections to the vestibulocerebellum largely arise from the lateral LM, with fewer inputs from the medial LM, LPC and PPC. In the pigeon, most LM projections to the oculomotor cerebellum originate from the medial LM, with fewer inputs from the lateral LM and a small portion of projections come from LPC and PPC, however in the zebra finch, the majority of the projections to the oculomotor cerebellum comes from PPC and then LPC rather than LM. In the hummingbird, the majority of the projections to the oculomotor come from the LMI and then PPC and LPC and LMm send the least number of projections to the oculomotor cerebellum (figure 1) (Gaede et al., 2019). Little is known regarding the functional role of the LPC and PPC, though Gamlin and Cohen (1988a) have shown that a few LM projections terminate in the pigeon PPC. Visual motion processing demands are likely to differ in birds with diverse flight behaviors, and this may be reflected in the functional neuroanatomy of each species.
Additionally, in pigeons the nBOR projects preferentially to folium IXcd of the vestibulocerebellum (Pakan & Wylie, 2006)(Pakan et al., 2006). These pathways of optic flow to the cerebellum of birds have been proposed to serve different functions in visuo-motor control, particularly during flight (Wylie et al., 2018).
Chapter two: Research

2.1. Introduction

Avian flight requires a rapid transformation of visual information into motor output for tasks such as avoiding collisions, landing, capturing prey, and avoiding predators. Our understanding of the visual pathways that are important for flight control derives from decades of research, mostly with pigeons (*Columba livia*) (Brecha et al., 1980; Gioanni, Palacios, Sansonetti, & Varela, 1991; Pakan et al., 2006). There are several brain nuclei that receive retinal input and thus represent the early stage of a visual pathway (Clarke, 1977; Gamlin & Cohen, 1988b; Krabichler, Vega-Zuniga, Morales, Luksch, & Marín, 2015; Mpodozis, Letelier, Concha, & Maturana, 1995; Pakan & Wylie, 2006; Douglas R Wylie, Kolominsky, Graham, Lisney, & Gutierrez-Ibanez, 2014). The distinct visual pathways are homologous among vertebrates and convey visual signals used for accomplishing different tasks (Butler & Hodos, 2005; Knudsen, 2020; McKenna & Wallman, 1985, 2010).

The major visual signal for control of posture and movement is optic flow -the global motion across the retina due to relative motion between an organism and its environments (Gibson, 1954; S. & Gibson, 1951). Previous work with pigeons has demonstrated that optic flow is processed in the pretectum and accessory optic system of the midbrain (Gamlin, 2006; Giolli, Blanks, & Lui, 2006; Simpson, 1984; Simpson, Giolli, & Blanks, 1988; Brecha et al., 1980; Frost, Wylie, & Wang, 1990; Wylie, Gutiérrez-Ibáñez, Gaede, Altshuler, & Iwaniuk, 2018). The
retinal recipient nuclei for these key pathways are the Lentiformis Mesencephali (LM) and the nucleus of the basal optic root (nBOR) (Douglas R Wylie et al., 2018, 2014). Both of these retinal recipient optic flow nuclei send projections to two cerebellar regions, the vestibulocerebellum (folium IX) and the oculomotor cerebellum (folia VI-VIII) (Brecha et al., 1980; Gaede et al., 2019; Pakan et al., 2006).

Recently, the inputs to the vestibulocerebellum and the oculomotor cerebellum were measured in two additional species, Anna's hummingbirds (Calypte anna) and zebra finches (Taeniopygia guttata). This tract tracing study revealed several unexpected results in what was previously thought to be a highly conserved network. First, for both species, the majority of pretectal inputs to the oculomotor cerebellum came from the nucleus laminaris precommisuralis (LPC) and the nucleus principalis precommisuralis (PPC), a total of 51.2% of the inputs in Anna’s hummingbird and 73.9% in the zebra finches. In contrast, it was shown that in pigeons, 92.1% of total inputs to the oculomotor cerebellum are coming from the LM. The LPC and PPC together represent only 7.9% of the inputs to the pigeon oculomotor cerebellum. This was shown by quantifying the proportion of retrogradely labeled cells in the lateral subdivision of the LM, the medial subdivision of the LM, the LPC, and PPC from injections in the vestibulocerebellum and oculomotor cerebellum (Gaede et al., 2019). Given that the LPC and PPC are now understood to be major sources of visual information to the oculomotor cerebellum, at least in zebra finches and
hummingbirds, it would be highly informative to learn about their anatomy and physiology. The first question addressed in this thesis is whether LPC and/or PPC are retina recipient in zebra finches and hummingbirds. I addressed this question using a dual tract tracing study. Anterograde injections to the retina in three zebra finches and in one hummingbird were accompanied by retrograde injections to the oculomotor cerebellum (folia VI). The new discovery of LPC and PPC projections in hummingbirds and zebra finches also illustrates how little is known about the visual neuroanatomy of bird species other than pigeons, and to some extent chickens (Ehrlich & Mark, 1984a, 1984b; Luksch, Karten, Kleinfeld, & Wessel, 2001; Verhaal & Luksch, 2013) Thus, a second aim of my thesis was to determine if there are any previously unknown retinal projections in hummingbirds and zebra finches.

2.2. Methods

2.2.1. Animals

Three adult male zebra finches (Taeniopygia guttata, 17-21g) and one adult male Anna’s hummingbird (Calypte anna, 4.66g) were used in this study. The zebra finch IDs were TG459, TG466 and TG483 and the hummingbird ID was CALAN261. Zebra finches were acquired from a commercial supplier (Eastern Birds, Quebec, Canada), and were group housed in 92cm x 46cm x 46cm cages with other male zebra finches. Food, water, and cuttlebone was provided ad libitum. Peas, corns, and greens such as lettuce and spinach were provided twice weekly. The hummingbird was captured on the campus of the University of British
Columbia and housed in a 61cm x 61cm x 91cm cage with ad libitum fresh artificial nectar (NektarPlus) and sugar water. The zebra finches and hummingbird were housed in separate rooms, but both had controlled temperature, humidity, and dark-light (12:12) cycle. All experimental procedures were approved by the University of British Columbia Animal Care Committee in accordance with the guidelines set out by the Canadian Council on Animal Care (A19-0113).

2.2.2. **Surgeries and tracer injection**

Surgeries were performed using a custom-built stereotaxic frame designed for small bird neurosurgery with integrated gas delivery (David Kopf instruments, CA, USA). First, the beak was fixed on the beak bar inside a detachable anesthesia mask that was specifically designed for each bird species. The mask was connected to a source of an inhalant anesthetic which was a mixture of isoflurane and oxygen. The induction isoflurane dose was 1.5% for zebra finches and 1.2% for the hummingbird.

Once the birds were under anesthesia, they were moved with the bar to the stereotax and their head was secured using ear bars. The maintenance dosage of isoflurane was 1.2% for Zebra finches and 0.3% for the hummingbird. During the surgery, the isoflurane dosage was increased ~0.3%-0.4% higher than the maintenance dose. The gas mixture was delivered at a constant flow (300 mL/min) using the customized masks. The small space between the mask and the bird was covered to prevent isoflurane leakage.
Once it was established that birds were in the surgical plane, the feathers, skin, bone, and dura mater overlying the cerebellum particularly folia VI and VII (oculomotor cerebellum) were removed. These sites were identified by anatomical markers. 150 μl of subcutaneous saline was injected under the skin of the neck to keep the tissues hydrated.

![Diagram](image)

**Figure 2. Tracer injection sites.** Tracer injections to the oculomotor cerebellum and the eye were used to establish visuomotor connectivity. Injections of neuronal tracer cholera toxin B (CTB)-Alexa Fluor 488 in the oculomotor cerebellum (folia VII) and cholera toxin B (CTB)-Alexa Fluor 594 in retina using nano-injector and Hamilton syringe, respectively.

Two injections were made, first to the oculomotor cerebellum and second to the eye. The injection to the left side of oculomotor cerebellum was made using a Nano injector with a glass micropipette (tip diameter 20–30 μm), The tip of the micropipette was moved down to the granule cell layer of the target folium of the
cerebellum and cholera toxin B (CTB)-AlexaFluor 488 [green] was delivered. The intent was to retrogradely label neurons that project as mossy fibers to the granular layer. In two of the three zebra finches, 200 nl of CTB 488 (7 injections of 13.8 μl with five-minute gaps) was injected in the folia VII of the cerebellum in lateral and medial positions (a total of 400 nl) in two depths of cell layers. In the other zebra finch and in the hummingbird, 100 nl of CTB 488 was injected in the folia VI of the cerebellum in medial position and in two depths. Following the injections to the oculomotor cerebellum, the craniotomy was filled using bone wax, and the skin was closed by cyanoacrylate (Vetbond, 3 M).

The eye injections were also made on the left side, ipsilateral to the oculomotor cerebellum injection. A small incision was made dorsal to the eye socket using a scalpel. The needle of a Hamilton syringe was gently passed through the sclera and 5 μl of 0.83% cholera toxin B (CTB)-AlexaFluor 594 in PBS with 2% dimethylsulfoxide [DMSO] was injected into the eye's vitreous humour. The diffusion of the tracer was confirmed with an ophthalmoscope and then the skin was closed with cyanoacrylate (Vetbond, 3 M).

Following the injection surgery, birds were kept in isolation cages for 3-4 days to allow sufficient time for CTB tracer transport. The final animal procedure was transcardial perfusion to fix the tissues. The birds were brought into a deep anesthetic plane using an intramuscular injection of ketamine and xylazine (65 mg/kg ketamine and 8 mg/kg xylazine). They were perfused transcardially with
saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.4).

2.2.3. Immunohistochemistry

After the perfusion, the brains were taken out of the skull and were fixed in 4% PFA overnight at 4°C in the fridge and then cryoprotected in 30% sucrose in 0.01 M phosphate buffered saline (PBS, pH 7.4) at 4°C. Brains sank to the bottom after 24 hours at 4°C. The brains were next gel blocked and then the blocks were fixed again in 4% PFA for 2 hours and then for the purpose of cryoprotection, the brains were transferred to 30% sucrose in 0.01 M phosphate buffered saline (PBS, pH 7.4) overnight at 4°C.

Using a freezing stage microtome (Feather blade holder No. 160E), the brains were sectioned in the coronal plane with 40 μm thickness. All the brain sections were immersed in individual wells containing 0.01M phosphate buffered saline (PBS, pH 7.4). The sections were divided into two series. Series 1 was immediately mounted on gelatin-coated superfrost slides for fluorescent imaging followed by Nissl staining.

The midbrain regions (18-22 sections) from series 2 were used for immunohistochemical labeling for calretinin (CR). These sections were washed five times in 0.01 M phosphate buffered saline (PBS, pH 7.4), each time for 5 minutes on a shaker at room temperature. The next step was blocking with 10% normal donkey serum (Jackson Immunoresearch Laboratories) and 0.4% Triton X-
100 in PBS for 1 hour at room temperature. The sections were next transferred to 1 ml solutions of 2.5% normal donkey serum, 0.4% Triton X-100 and a rabbit polyclonal antibody for CR (1:2000; Swant Inc., Switzerland; immunogen: recombinant human calretinin; rabbit polyclonal, Cat-#7,697, RRID: AB_2721226) and incubated for 48 hours. Sections were again washed five times in 0.01 M phosphate buffered saline (PBS, pH 7.4), each time for 5 minutes on the shaker at the room temperature and then incubated in 1 ml solutions containing 2.5% normal donkey serum, 0.4% Triton X-100 and AMCA (blue) - conjugated donkey anti-rabbit IgG (H + L) (1:200, Jackson Immunoresearch Laboratories; Cat# 711-155-152, RRID: AB_2340602) for 2 hours at room temperature. Finally, the sections were rinsed in PBS, 5 times and each time for 5 minutes and mounted on gelatinized superfrost slides. The remaining sections from series 2 were also mounted on slides as described for series 1.

2.2.4. Microscopy and image analysis

Images were acquired on the Leica DM6 B upright microscope with the Leica sCMOS Microscope Camera K5 and LAS-X software (Leica Microsystems, Wetzlar, Germany). Helicon Focus 7 software was used to process the multifocus pictures. Brightness, contrast adjustment, labelling retinal terminals, and labeling fluorescent cells, were done manually with Adobe Photoshop and Adobe Illustrator.
2.3. Results

We began by asking two questions: first, do the LPC and PPC neurons of zebra finches and hummingbirds that project to oculomotor cerebellum also receive retinal inputs?

To address this question, dual injections of fluorescent tracers, CTB 488 in the eye and CTB 594 in the oculomotor cerebellum were made (figure 2). Panels a

Figure 3. Confirmation of injection sites in the oculomotor cerebellum and terminals in the optic tectum for zebra finches and hummingbirds a) Injection in the folia VII of the zebra finch cerebellum. b) Same as a but for hummingbird. c) The retinal terminals of the optic tectum in zebra finch. d) Same as c but for hummingbird. TeO, optic tectum. Scale bars = 1 mm.
and b in figure 3 depict the injection cites in the oculomotor cerebellum of zebra finches and hummingbird. These images confirm on target injections of green CTB 488 in folia VII of the oculomotor cerebellum in both birds.

Retinal efferents form the superficial layers of the optic tectum (tectal layers 2, 3, 4, 5b, and 7) (Engelage & Bischof, 1993; Hayes & Webster, 1975; Repérant & Angaut, 1977). Figure 3 panels c and d illustrate red labeled stratum opticum, which indicates that the injections in the eyes reached the retinal ganglion cells and their terminals in the zebra finch, the entire layer has even red labelling. Thus, this eye injection reached the whole retina. In contrast, in the hummingbird stratum opticum, the red fluorescence is partial. This result suggests that the injection to the hummingbird eye reached most, but not all the retina. Thus, the answer question 2 (presence of undescribed retinal recipient brain regions) is not very clear in the hummingbirds compared to the zebra finch.
Figure 4. Retinal projections to the pretectum of zebra finches and hummingbirds resemble other birds  
a) Low magnification image showing terminal labeling in zebra finch.  
b) Low magnification image showing terminal labeling in the LM of hummingbird.  
c) Zebra finch pretectum.  
d) Hummingbird post pretectum.  
e) High magnification version of cells in LMI/ LPC/LMm for zebra finch.  
f) High magnification image of cells in hummingbird LMI/ LPC/LMm.  
Glv, ventral lateral geniculate nucleus;  
GT, tectal gray;  
LMI, lentiformis pars lateralis;  
LMm, lentiformis mesencephali pars medialis;  
LPC, nucleus laminaris precommissuralis;  
nRt, nucleus rotundus;  
PPC, nucleus principalis precommissuralis;  
TeO, optic tectum.  
Scale bars = 0.1 mm.
Figure 4 depicts projections from the retina to both subunits of LM (LMl and LMm), GLv, and partially to LPC in zebra finches and hummingbirds. The projection sites are generally similar to what has been observed in other avian taxa. We found evidence for a previously undescribed one-synapse pathway from the retina to the oculomotor cerebellum through LPC but not PPC.

As shown in figure 5, there are cells that are projecting to folia VII to the oculomotor cerebellum in LM, LPC and PPC as shown by Gaede et. al. 2019. There are cells in LPC that are projecting to the oculomotor cerebellum and at the same time overlap with retinal inputs, providing further evidence for a one synapse
pathway from the eye through the oculomotor cerebellum via LPC. Little was known about the LPC in birds, but my results suggest that the neurons may have an important role in processing the optic flow prior to sending this visual signal to the cerebellum.

![Figure 6. Further evidence for a one-synapse pathway from the retina to the oculomotor cerebellum through the area pretectalis.](image)

To answer the second question, which was whether the retinal ganglion cells of hummingbirds and zebra finches project to other brain regions that are not currently described for birds, I looked at other brain areas. The most significant results were in the area pretectalis. According to Gamlin et al. 1988, there are retinal projections to the area pretectalis in pigeons and there are also cells in the area that were projecting to the oculomotor cerebellum. However, this earlier result

Figure 6. Further evidence for a one-synapse pathway from the retina to the oculomotor cerebellum through the area pretectalis. a) Low magnification image of area pretectalis cells in zebra finch. b) Same as in (a) but at higher magnification. c) Same as (a) but for hummingbirds. d) Same as (b) but for hummingbirds. SpM, medial spiriform nucleus; TeO, optic tectum. Scale bars = 0.1 mm.
was deduced from separate injections and without confirmation of cell contacts. The dual injections now confirm that these two input and output cells are located in the same area and have at least a modest degree of contact. Thus, I can provide evidence for another one-synapse pathway from the retina to the oculomotor cerebellum through the area pretectalis in both zebra finches and hummingbirds (figure 6).

2.4. Discussion

The goal of my thesis was to answer two questions about these circuits: 1) Do LPC and PPC neurons of zebra finches and hummingbirds that project to oculomotor cerebellum also receive retinal inputs? 2) Do retinal ganglion cells of hummingbirds and zebra finches project to other brain regions that are not currently described for birds? Both questions were addressed by injecting Cholera Toxin B with different fluorophores into the oculomotor cerebellum and the eye (figure 2). The injections were accurate as seen in figure 3 and 4. These injections revealed a possible one synapse connection between the eye and the oculomotor cerebellum through LPC but not PPC (figure 5). There was also evidence for a second one synapse pathway found through the area pretectalis (figure 6).

I know that the injections in the retina was complete in the zebra finch as we can see that in the zebra finch optic tectum, the superficial layers (tectal layers 2, 3, 4, 5b, and 7) are completely labeled with red fluorescent tracer (CTB 594), however the optic tectum of the hummingbird is partially labeled by the red CTB
which is indicative of the partial injection in the eye (Engelage & Bischof, 1993; Hayes & Webster, 1975; Repérant & Angaut, 1977) and further study is required to confirm the data for this species.

Little was known regarding the functional role of the LPC and PPC, only Gamlin and Cohen (1988a) have shown that a few LM projections terminate in the pigeon PPC. Here I describe a previously unknown one-synapse pathway between the eye and the oculomotor cerebellum through the LPC. Considering that the oculomotor cerebellum is part of an extensive visuo-motor network that incorporates visual information from several direct and indirect sources, LPC being one of the direct sources of inputs, I suggest an important role for this nucleus in visuomotor processing in zebra finches and hummingbirds. However, this strong connection is only seen in the LPC and not PPC so further study is needed to address the role of PPC in processing the optic flow. Such studies can contain tracer injections in the PPC and tracing the terminals and then injecting tracers in the recipient areas to determine the connections between PPC and other brain areas (Gaede et al., 2019; Pakan et al., 2006).

In conclusion, visual motion processing demands are likely to differ in birds with different flight behaviors, and this may be reflected in the functional neuroanatomy of each species. Further investigation examining the responses of LPC and PPC neurons to visual motion (FU, XIAO, GAO, & WANG, 1998) may elucidate the roles of these nuclei in visuomotor processing in birds with different flight strategies.
Chapter three: Conclusion

A major visual signal for control of posture and movement is optic flow - the global motion across the retina due to relative motion between an organism and its environment. We know optic flow sensitive neurons exist in the brains of many vertebrates (Carl Huber & Crosby, 1929; Gaede et al., 2017; Hoffmann & Schopmann, 1981; Ibbotson et al., 1994; Simpson, 1984) however, the question of how exactly optic flow is used to guide behaviour remains poorly understood. Moreover, the question remains whether the pathways of optic flow processing are conserved across species.

Larsell (1967) suggests that strong fliers tend to have a large posterior cerebellar lobe, particularly in folia VI and VII, and these folia are significantly smaller in flightless birds (Iwaniuk & Wylie, 2007). These folia are also a part of a much more extensive visuo-motor network that incorporates visual information from several direct and indirect sources (Gamlin & Cohen, 1986, 1988a). These findings suggest that any new data in relation to these folia could be of an importance for understanding the visuo-motor integration.

In a recent study on the connections to the oculomotor cerebellum of the zebra finch, hummingbird, and pigeons by Gaede et al. 2019, it was shown that despite the similarities, these three bird species are different in terms of the connections from the midbrain to the oculomotor cerebellum. In the zebra finch, the oculomotor cerebellum received 75% of its inputs from LPC and PPC, in the
hummingbird 51.2% and in the pigeon, only 7.9% of the inputs to the oculomotor cerebellum originated from these two nuclei and the rest of the projections came from LM in all three species. In conclusion researchers have shown that LM, nBOR, PPC and LPC project as mossy fibers to the oculomotor cerebellum (Brecha et al., 1980; Clarke, 1977; Gaede et al., 2019). However, despite the fact that the connections of LM and nBOR to other brain areas are well understood (Brecha et al., 1980; Gutierrez-Ibanez et al., 2018) the function and connectivity of LPC and PPC to other brain areas and the eye remains unknown. Thus this thesis explored the possible (Gaede et al., 2019) new connections between the eye and the oculomotor cerebellum by performing two simultaneous tracer injections with different fluorescent colors, one in the retina, targeting the retinal ganglion cells and another in the oculomotor cerebellum targeting the mossy fibers and the results suggest that the visual pathway of optic flow processing is more complex than previously thought.

My research contributes to our understanding of new anatomical connections between the eye and the oculomotor cerebellum in zebra finches and hummingbirds through LPC and the area pretectalis. Both these connections are thought to be one-synaptic as we observe overlap between the retinal ganglion cell terminals and the projections to the folia VII of the oculomotor cerebellum.

We now know that these two areas, the LPC and the area pretectalis, are involved in the optic flow processing, however the connections of these areas especially LPC to other brain areas need to be studied more thoroughly. I propose
performing surgeries and injecting antero- and retro-grade tracers in the LPC and looking at its cells and their terminals closely. Additionally, we know that PPC is sending projections to the oculomotor cerebellum in zebra finches and hummingbirds but there are many questions regarding these projections, such as: where are these projections coming from? Are they projecting to other brain areas? In other to answer these questions, further study is needed, and neural tracing can be performed like explained for LPC.

We explored the anatomical connectivity of LPC and PPC to the eye and the oculomotor cerebellum, but their function remains unknown. LPC might be responsive to global visual motion with unique tuning properties and it can be researched using electrophysiological methods (Gaede et al., 2017; Winterson & Brauth, 1985).

In conclusion, this brain areas, LPC and PPC need to be studied more thoroughly to get a better understanding of their roles in flight and/or other complicated tasks.
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