Neural projections from midbrain optic flow nuclei to the inferior olive and vestibulocerebellum of zebra finches (*Taeniopygia guttata*)

by

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B.Sc., National Cheng Kung University, 2017

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

December 2020

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

Neural projections from midbrain optic flow nuclei to the inferior olive and vestibulocerebellum of zebra finches (Taeniopygia guttata) submitted by Pei-Hsuan Wu in partial fulfillment of the requirements for the degree of Master of Science in Zoology

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Abstract

Global visual motion across the retina due to self-motion is called optic flow. Optic flow is an important idiothetic cue for locomotion control. In birds, the lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) of the midbrain process optic flow and transmit it to the cerebellum for integration with other sensory inputs.

The vestibulocerebellum (VbC), composed of folium IXcd and X, is important for visuomotor control and has been divided into several functional sagittal compartments, defined by multiple factors. Purkinje cells in the VbC differentially express a molecular marker, zebrin II, generating parasagittal zebrin immuno-positive bands alternating with zebrin immuno-negative bands. Both the LM and the nBOR project directly to the VbC as mossy fibers and are co-localized within immuno-positive bands. The LM and nBOR also project indirectly to the VbC via the medial column of the inferior olive (mcIO). The mcIO cells project as climbing fibers to the VbC. In pigeons, the LM projects to the caudal mcIO and the nBOR project to the rostral mcIO. These pathways have not been explored to the same detail in other avian species.

I dual-injected anterograde tracers of different fluorescence in the LM and the nBOR of zebra finches and traced the projections to the mcIO and VbC. Folium IXcd was also immunolabeled for zebrin II. I show that the zebra finch inferior olive has a more complex structure than previously reported in other birds. The nBOR axon terminals can be found in most of the mcIO subdivisions, whereas the LM terminals are mostly in the dorsal divisions. The zebrin expression and the mossy fiber distribution patterns are in general similar between zebra finches and pigeons. However, further analysis revealed that LM has more projections to per unit area of the immuno-positive bands, whereas more nBOR mossy fiber terminals were found in the area-corrected immuno-negative bands. The present study suggests that species differences in visuomotor pathways exist and zebra finches may serve as an important species to understand the evolution of the neuroanatomy that supports birds to perform different flight behaviors.
Lay Summary

Visuomotor control relies on inputs to the cerebellum from both the midbrain and the medulla oblongata. Among birds with different flight specializations, details of these connections seem to differ. Here, I describe visuomotor pathways in zebra finches, the first small bird species to be examined. As in other birds, the outputs of two midbrain visual nuclei are transmitted to different compartments of the vestibulocerebellum (VbC) and the inferior olive (IO) of the medulla. Moreover, IO signals are relayed back to the cerebellum. Notably, the zebra finch IO is more complex and stratified than in other bird species studied to date. Furthermore, I identified novel projections from two midbrain optic flow nuclei to the IO compartments. These results lead to two new questions: is the stratification of the IO an adaption for small birds? What types of visual information are carried by discrete projections from IO compartments to the cerebellum?
Preface

All of the work presented within this manuscript was conducted in the Altshuler laboratory at the University of British Columbia, under the guidance and supervision of Dr. D. L. Altshuler and Dr. D. R. Wylie. The text within this manuscript is original and unpublished material. The experimental research reported in Chapter 2 was conducted under UBC Animal Care Certificate number A19-0113 and was funded by the Canadian Institutes of Health Research (CIHR).

The research concepts were adapted from Pakan et al, 2010 and the experimental procedures were modified from Gaede et al, 2016 for the injection surgery, sample processing, and neural response analyses and from Wylie et al, 2017 for the immunohistochemistry. I was responsible for collecting and analyzing the data contained within Chapter 2. The image analysis was guided by Dr. D. R. Wylie and Dr. A. H. Gaede. The text within this document is my own original work, with editing provided by Dr. D. L. Altshuler, Dr. A. H. Gaede, and M. S. Armstrong. The information in the schematics of known visuomotor connections in figure 1 was conceptualized by me, and M. S. Armstrong helped to visualize the ideas.
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List of Abbreviations

AOS, accessory optic system
APH, area parahippocampalis
AVT, area ventralis of Tsai
CbL, lateral cerebellar nucleus
CbM, medial cerebellar nucleus
CtG, central gray
D, nucleus Darkshewitsch
DAO, dorsal accessory olive
DC, dorsal cap
DGCs, displaced ganglion cells
DIV A, nucleus dorsalis intermedius ventralis anterior
dl, dorsal lamella of inferior olive
DLL, anterior dorsolateral thalamus, lateral subdivision
DLM, anterior dorsolateral thalamus, medial subdivision
dMC, dorsal medial cell groups
DMCC, dorsomedial cell column of inferior olive
gl, granule layer of cerebellar cortex
GLv, ventral lateral geniculate nucleus
GT, tectal gray
GTc, caudal tectal gray
GTr, rostral tectal gray
Hp, hippocampus proper
IC, interstitial nucleus of Cajal
iMC, intermediate medial cell groups
IO, inferior olive
LM, nucleus lentiformis mesencephali
LMI, nucleus lentiformis mesencephali pars lateralis
LMm, nucleus lentiformis mesencephalipars medialis
LPC, nucleus laminaris precommissuralis
LTN, lateral terminal nucleus
MAO, medial accessory olive
mcIO, medial column of inferior olive
ml, molecular layer of cerebellar cortex
MTN, medial terminal nucleus
nBOR, nucleus of the basal optic root
nBORD, nucleus of the basal optic root, pars dorsalis
nBORl, nucleus of the basal optic root pars lateralis
nBORp, nucleus of the basal optic root, proper
NOT, nucleus of the optic tract
OCb, oculomotor cerebellum
OKN, optokinetic nystagmus
OMdl, oculomotor nucleus, dorsolateral subdivision
OMdm, oculomotor nucleus, dorsomedial subdivision
OMv, oculomotor nucleus, ventral subdivision
pcl, Purkinje layer of cerebellar cortex
pcv, processus cerebellovestibularis
PL, lateral pontine nucleus
PM, medial pontine nucleus
PO, principal olive
PPC, nucleus principalis precommisuralis
PT, nucleus pretectalis
Rt, nucleus rotundus
Ru, nucleus ruber (red nucleus)
SpL, lateral spiriform nucleus
TeO, optic tectum
VbC, vestibulocerebellum
vl, ventral lamella of inferior olive
VLO, ventrolateral outgrowth
vMC, ventral medial cell groups
Acknowledgements

I want to first thank the members of my supervisory committee: Dr. D. L. Altshuler, Dr. D. R. Wylie, and Dr. M. D. Gordon. My committee has continued to be patient and supportive. My research project changed significantly in the second year of my Master’s program. Several of the critical decisions I made were guided and supported by my committee members. Looking back now, I am glad that I decided to take on the challenge of doing this neuroanatomical study. From this project, I have gained a deeper understanding of the visuomotor systems in the bird species that I am, and will be, studying for the next few years. I would also like to express my sincere appreciation for Dr. A. H. Gaede and M. S. Armstrong. They helped me get through the hardest time and I have learned a lot from these two amazing ladies. There were several times that I was about to give up, but when I thought of the help they have provided, I bounced back and kept facing the challenges. Their support was necessary for me to finish this project. I would also like to thank V. Baliga, J. Theriault, E. Press, J. Wong, S. Senthivasan, L. Wood, F. Ciocca, and S. Azargoon for various kinds of help.

Big thanks to my family. They encouraged me to pursue my dream to study abroad and they are always supporting me no matter where I am. I would also like to thank my partner Kevin Ho. He has inspired me with several research ideas, even though we are studying very different topics. More importantly, during the past few months when I was focusing on finishing the project, he took care of my daily life and soothed me when I was in a bad mood. I am very lucky to have Kevin as my partner.

Lastly, I would like to thank for the CIHR. Their funding supported this research project.
1 Introduction

1.1 Two retinal recipient nuclei, LM and nBOR, are important for processing self-motion related visual cues

The ability to perceive and respond to a changing environment is crucial for executing complex behaviors, such as hunting, escaping, and courtship. These behaviours rely on multisensory integration. Aerial locomotion presents different challenges to terrestrial locomotion, and diverse bird species have evolved different strategies to overcome these challenges. One significant change is an enlarged midbrain, which contains several visual processing regions necessary for responding to rapidly changing visual stimuli, and birds with different life histories have evolved to strengthen different visual pathways [1]. Hummingbirds, which are a flying specialists that can hover and perform complex maneuvers, have a hypertrophied visual nucleus, lentiformis mesencephali (LM) [2, 3]. The LM and the nucleus of the basal optic root (nBOR) of the accessory optic system (AOS) are key midbrain regions for processing optic flow, which is defined as global visual motion across the retina due to subjective movement [4]. Optic flow, in conjunction with vestibular and proprioceptive information, provides important idiothetic cues and therefore is important for locomotor control.

Optic flow is processed by subpopulations of LM and nBOR cells, each featuring unique direction and spatiotemporal tuning. Each neuron has a preferred direction, defined as the direction of optic flow that elicits the highest firing frequency. Conversely, a stimulus presented in the opposite direction typically inhibits spontaneous firing, and thus is called the anti-preferred direction. The mammalian homologs to the LM and the nBOR have been clearly described. The avian nBOR is homologous to the medial and lateral terminal nuclei (MTN and LTN) in mammals, and the LM is homologous to the nucleus of the optic tract (NOT) [5]. In most tetrapods, the majority of LM neurons prefer forward motion (temporal-to-nasal) [5, 6]. In contrast, most nBOR cells prefer up, down, or backward (nasal-to-temporal) motion [5, 7]. In birds, much electrophysiological work has been done in pigeons, and LM and nBOR neurons are classified as fast or slow depending on their responses to sine-wave gratings moving in the preferred direction with different spatiotemporal frequencies [6, 8]. In pigeons, approximately two-thirds of LM cells are fast neurons that prefer high temporal frequencies (TFs) and low spatial frequencies (SFs) (velocity = TF/SF)
[7]. In contrast, most nBOR cells are classified as slow neurons (best excited by low TFs and high SFs) [7]. In the LM, almost all slow neurons prefer forward motion, whereas the direction preference for fast cells is more diverse [6, 9].

The LM and the nBOR are important for maintaining a stable retinal image with respect to the animal’s movement or the moving environment, a response called optokinetic nystagmus (OKN) [10-12]. Lesions to either of these two nuclei results in severe impairment of OKN responses [10-12]. In addition, several neuroanatomy studies demonstrate that both the LM and the nBOR project to the cerebellum directly as mossy fibers and indirectly through the olivocerebellar pathway [13]. The cerebellar regions that receive optic flow input integrate the visual signals with other sensory information (e.g., vestibular signals) and project to downstream cerebellar and vestibular nuclei to guide optokinetic responses [14, 15]. These neural connections suggest that the LM and nBOR play a critical role in driving motor responses to visual stimuli. Additionally, in pigeons, LM cells are tuned to different features of motion parallax, indicating that the LM could also play an important role in estimating the distance and depth of surrounding objects in relation to the moving bird [16].
1.2 Efferent and afferent projections of the LM and nBOR

The LM and the nBOR both receive direct visual inputs from the contralateral retina with some degree of retinal topography. The LM receives direct retinal input from retinal ganglion cells and displaced ganglion cells (DGCs), which are large ganglion cells found at the border of the inner nuclear layer and inner plexiform layer [17, 18]. However, the nBOR receives its primary retinal input from DGCs [18, 19]. Studies using retinal lesions and anterograde tracing with amino acid isotopes have proposed retinotopic maps for both nuclei [20, 21]. In the pigeon LM, the dorsal retina mapped ventrally and the ventral retina mapped dorsally [21]. Representations of the nasal and the temporal retina are mirrored between the two subdivisions of the LM, the lentiformis mesencephali, pars medialis (LMm) and the lentiformis mesencephali, pars lateralis (LMl). In the LMm, the nasal retina is mapped medially and the temporal retina is mapped laterally. Conversely, in the LMI, the nasal retina is mapped laterally and the temporal retina is mapped medially [21]. In the chicken nBOR, Ehrlich and Mark (1984) found that the ventrotemporal retina mapped mediodorsally, the nasal retina mapped medioventrally, and the dorsal retina mapped to the lateral part of the nBOR [20]. However, in pigeons, nBOR-projecting DGCs are broadly distributed in the retina and the retinotopy is rather coarse [18]. Further research is necessary to determine whether the nBOR has a clear retinotopy and if species differences exist.

In addition to retinal afferents, the LM also receives inputs from an important sensory cue processor, the optic tectum (TeO). The avian TeO is a complex laminated structure comprised of fifteen layers, whereas the mammalian homolog, the superior colliculus, has been divided into seven primary layers [22]. This large structure receives more than ninety percent of retinal projections in addition to other sensory inputs [1, 22]. While a direct connection from the TeO to the nBOR has yet to be shown, the nBOR has reciprocal projections with the LM and is considered to be the functional companion of the LM [23].

The two subdivisions of the LM, the LMm and the LMI, differ in their relative location in the pretectum [21] (figure 1A). The LMm sits medial to the LMI and appears rostrally earlier than LMI. Caudolateral to the LMI is the tectal gray (GT), which is immediately adjacent to the TeO [21]. All of these structures receive direct retinal inputs. Medial to the LMm is a non-retinorecipient nucleus, the laminaris precommissuralis (LPC), which is medially bordered by the nucleus principalis precommissuralis (PPC). The LM has several
important outputs and one of them is the bilateral projection to cerebellum folia VI to IX, in which the visual signal is integrated with other sensory information to guide motor behaviours. The majority of cerebellum-projecting LM neurons are found to terminate at VIc and IXcd and in the outer half of the granule cell layer [24]. In addition to the direct mossy fiber pathway to the cerebellum, the LM also projects ipsilaterally to the medial column of the inferior olive (mcIO), from which information is transmitted via climbing fibers to the Purkinje cell layer. Furthermore, the LM has ipsilateral connections with medial and lateral pontine nuclei (predominately PM), from which multiple sensory inputs are integrated and sent to the cerebellar granular layer. Other efferent projections of the LM include parts of the ipsilateral anterior dorsal thalamus and some ipsilateral structures along the midline of the mesencephalon [24-27]. One mesencephalic structure that receives LM input, the area ventralis of Tsai (AVT), sends optic flow signals to the hippocampus [28]. The hippocampus is an important region for path integration, using self-motion related idiothetic cues to calculate the global direction and distance moved in relation to a starting point [29, 30]. Projections from the LM to the thalamic nuclei are involved in distinguishing object-motion from self-motion, establishing three-dimensional space information, and multimodal sensory information integration [27]. A simplified schematic of the LM outputs is shown in figures 1B and 1C.

The LM is further classified by cell morphologies and their projections. Both the LMM and the LMI have large multipolar cells that project to folium IXcd of the cerebellum. LM neurons that project to the mcIO are medium-size fusiform cells located in the border between the LMM and the LMI. The nBOR-projecting LM cells are mostly small-size neurons in the LMI. The cell sizes are statistically different among the three types. In addition, a double-labeling experiment showed that IO-projecting cells do not send collaterals to folium IXcd or the nBOR [31].

Just caudal and medial to the LM is the rostral end of the nBOR. This structure has been divided into three parts, the nBOR proper (nBORp), the nBOR pars dorsalis (nBORd) and the nBOR pars lateralis (nBOR1) [32] (figure 1A). The nBORp is a dome-like structure that sits in the medial ventral wall of the midbrain. It comprises most of the nucleus and is made up of large multipolar, medium round and small spindle cells [32]. The nBORd is located at the dorsal and caudal margin of the nBORp and it consists of small spindle cells.
The nBORl is a thin lateral extend of the nBOR that sits right above the optic tract. The nBOR has reciprocal connections with the ipsilateral LMI [24, 32, 33]. Several efferent projections of nBOR are similar to the LM, including bilateral projections to cerebellum folium IX, mcIO, and AVT, as well as ipsilateral projections to the pontine nuclei (more in PM), and dorsolateral thalamus regions. There are other groups of mesencephalic structures (accessory oculomotor and perirubral areas) that receive bilateral nBOR inputs, although similar projections from LM were not discovered. These structures are involved in oculomotor and head movement control. Other efferents of nBOR include bilateral oculomotor complex and ipsilateral cerebellar nuclei, and the contralateral nBORd [32, 33]. A brief overview of the nBOR outputs is plotted in figures 1B and 1C.

The nBOR is also comprised of various morphologically distinct cell types, and different types of neurons project to different brain regions [34]. Similar to the LM, the cerebellum folium IXcd-projecting cells are a group of large multipolar cells found throughout the nBOR. Inputs to the mcIO arise from small neurons located in the dorsal region of the contralateral nBORp and the ipsilateral nBORd. However, unlike the LM, the mcIO-projecting nBOR cells have various cell shapes and are not limited to the fusiform type. Small cells (mostly fusiform cells) in both nBORp and nBORd project to the ipsilateral LMI.

By dual-injection of retrograde tracers of different colors into two different nBOR projection sites (cerebellum folium IXcd, IO, dorsal thalamus, or LM), none of the nBOR cells were double-labelled, indicating that, like the LM, individual nBOR cells do not project to multiple areas [34].

The cerebellum of birds has ten folia. Folia IXcd and X together make up the vestibulocerebellum (VbC) and folia VI-VIII comprise the oculomotor cerebellum (OCb). Folia I-V belong to the anterior lobe where no LM or nBOR terminals have been described. The avian folium IX is considered to be homologous to the mammalian uvula and folium X is homologous to the nodulus [35]. The lateral extent of folium IXcd and X are homologous to the mammalian flocculus and the two folia converge laterally to form a structure called the auricle, which is the lateral portion of the flocculus [36]. The LM projects to both the VbC and OCb, whereas nBOR projects mainly to the VbC [24, 32]. The axon terminals of neurons in both nuclei can be found in the ventral uvula (ventral lamella of folium IXcd) and the flocculus, but not in the nodulus [24, 32].
The inferior olive (IO) is the only source of the climbing fiber inputs to the cerebellum, and in birds, it can be divided into three main subdivisions [37]. The dorsal and ventral lamellae merge medially to form the medial column of the inferior olive (mcIO). The LM and the nBOR project predominately to the mcIO [24, 32].

Using retrograde tract tracing, Pakan and Wylie (2006) have provided a clear view of the two visual nuclei-cerebellar pathways for both VbC and OCb in pigeon [38]. In brief, the mossy fibers to IXcd are mainly from the LMI and the nBOR. Conversely, the LMm and tectopontine pathways are two major sources of mossy fiber inputs to the OCb. The climbing fiber input to the VbC comes from the mcIO, whereas the information to the OCb is from the dorsal and ventral lamellae of the inferior olive [38].
1.3 Mossy fiber and climbing fiber inputs to the vestibulocerebellar functional zones

Based on the distribution of climbing fiber inputs, physiological responses, efferent projections, and the molecular expression patterns, the VbC has been classified into several sagittally organized zones [39]. The Purkinje cells in the VbC respond to panoramic visual motion and are directionally tuned. Neurons modulated by different optic flow patterns are found across the entire VbC, and those with the same tuning are typically confined to the same sagittal region, thus forming discrete optic flow zones. Purkinje cell responses arise from the activity of inferior olive climbing fiber inputs. Each VbC optic flow zone receives olivo-inputs from discrete inferior olive regions that have similar optic flow tuning [40]. In addition to zones with similar response properties, molecular studies have shown that there are differences in the parasagittal organization of a number of biochemical markers. One of the most widely studied markers is zebrin II (36-kDa isoenzyme aldolase C). In pigeon cerebellum folium IXcd, this protein is differentially expressed by the Purkinje cells such that it forms several parasagittal stripes with alternating zebrin-positive and negative bands. The function of zebrin II is still not fully understood, but notably, each optic flow zone aligns with a pair of zebrin-positive and -negative bands [41, 42]. These organizational features together define the functional zones of the VbC (figure 1C).

In pigeons, Purkinje cells in the uvula and nodulus are modulated by panoramic visual cues resulting from translational movement along three axes; the vertical axis or the two horizontal axes oriented 45 degree to either side of the midline [43-45]. The flocculus, in contrast, responds best to rotational optic flow generated by rotational movement about the same axes as the translational zones in the uvula and nodulus [43, 45-47]. Four types of translational and two types of rotational motion have been identified and mapped in discrete parasagittal zones of the VbC (figure 1C). In the uvula and nodulus, there are four parasagittal zones. In the most medial zone, Purkinje cells respond best to contracting optic flow, which is a result of translational backward movement along the horizontal axis 45° to the midline. Lateral to this zone is an area that responds to two types of optic flow, expansion and ascent. The expansion cues arise from moving forward along the horizontal axis 45° to the midline. Ascent along the vertical axis generates downward visual motion. Lateral to this zone, Purkinje cells are modulated by upward optic flow, derived from descent along the vertical axis. The optic flow tuning in the most lateral zone in the uvula and nodulus is still
unknown. In the flocculus, there are four zones, two horizontal rotation (rH45) zones interdigitated by two vertical rotation (rVA) zones. Cells in the rH45 zones have the greatest responses to whole-field rotational patterns resulting from rotation about the horizontal axis oriented 45° to the midline. In the rVA zones, cells were most excited by yaw motion about the vertical axis. The same spatial reference frame is also shared by the vestibular semicircular canals and eye muscles [36], suggesting that the visual responses are well integrated with other proprioceptive signals and control the corresponding motor units.

Each cerebellar optic flow zone is subdivided by a pair of zebrin immuno-positive and immuno-negative bands, and in pigeons there are seven stripe pairs organized bilaterally in folium IXcd (from medial to lateral: P1 +ve/-ve to P7 +ve/-ve) [48] (figure 1C). The climbing fibers from discrete regions of the mcIO terminate in each optic flow zone and span the corresponding pair of zebrin bands [49, 50]. There is a clear topographical arrangement of projections from the mcIO to the translational and rotational zones in the VbC. Generally, the rotational zones receive dorsomedial mcIO inputs, whereas the climbing fibers projecting to the translational zones arise from the ventrolateral mcIO [51]. Within the lateral mcIO, cells in different rostrocaudal regions project to different translational zones. From caudal to rostral mcIO, the efferent targets shift gradually from contraction, to ascent and expansion, to descent zones. This climbing fiber organization corresponds to the medial-lateral parasagittal organization of the optic flow zones in the uvula and nodulus [52]. In the flocculus, the rostral mcIO projects to the two rH45 zones and the caudal mcIO projects to the two rVA zones [53-55]. Although there is a finer topography of the climbing fibers to the Purkinje cells in the zebrin immuno-positive and -negative bands [56, 57], Purkinje cells within the same zebrin stripe pair of an optic flow zone do not differ in their optic flow responses [41, 42].

In summary, the contraction zone aligns with the P1 positive and P1 negative medial zebrin stripe and acquires climbing fibers from the caudolateral mcIO; the expansion and ascent zone aligns with the P1 negative lateral and P2 positive medial bands and the climbing fibers are from the medial part of the lateral mcIO; and the descent zone matches P2 positive lateral and P2 negative bands and the climbing fiber inputs are from the rostral end of the lateral mcIO. In the flocculus, the two rVA zones correspond to the P4 +ve/-ve and P6 +ve/-ve stripes and receive caudal mcIO inputs; the rH45 zones correspond to P5 +ve/-ve and P7
+ve/-ve stripes and receive rostral mcIO inputs. A schematic of the VbC functional zones is presented in figure 1C. For simplicity, the topography of olivocerebellar connections is omitted. A comprehensive graphical abstract of these connections can be found in Pakan et al (2014) and Craciun et al (2018) [49, 56]. Also see review from Wylie, 2013 [39].

The anatomical and functional separation of the olivocerebellar pathways appears to be rooted in their optic flow input sources from the LM and the nBOR. The rostral mcIO receives more inputs from the nBOR bilaterally, whereas the caudal part has more afferents from the ipsilateral LM, in pigeons [58]. The caudal mcIO projects to the contraction and rVA zones where the types of optic flow that best stimulate the Purkinje cells include a forward motion component, either in the majority of, or on one side of, the panoramic visual field. In most species, the majority of LM neurons respond preferentially to forward motion. This may explain the large number of projections from the LM to the caudal mcIO. Similarly, the visual motion that best modulates the rostral mcIO, and the corresponding functional zones in the VbC, includes the preferred motions of the nBOR (i.e. up, down, or backward motion).

The LM and the nBOR also project directly to the cerebellum as mossy fibers. However, whether different functional zones receive different visual mossy fiber inputs has yet to be studied. Discrete injections of anterograde tract tracer into the LM and the nBOR have revealed more details of this pathway to the VbC [59]. The mossy fibers from both optic flow-detecting nuclei display similar distributions in the VbC in pigeons. The axon terminals (or mossy fiber rosettes) cluster in discrete regions of the VbC, aligning with zebrin-positive zones [59]. However, given that both the LM and the nBOR send projections to similar regions in the cerebellum, it is not fully understood what information is transmitted by these direct visual inputs.

Comparative research can provide another approach for understanding complex questions and is often used in neuroanatomical studies. Species differences exist in the projections from midbrain visual nuclei to the cerebellum in birds. For example, the oculomotor cerebellum receives a large proportion of pretectal inputs, and in pigeons, these primarily arise from the LMm [38, 60]. However, in hummingbirds and zebra finches, very few LMm projections are sent to folia VI-VIII. Instead, more than half of the pretectal inputs to the OCb arise from the LPC and PPC [60]. In the vestibulocerebellum, the proportion of
mossy fiber inputs that arise from the nBOR is also different between species: hummingbirds have the highest proportion of mossy fiber inputs from the nBOR (73.7%), then pigeons (51.6%), with the lowest proportion found in zebra finches (36.2%) [60]. Among the pretectal inputs to the VbC, all three species receive a large number of inputs from the LMI. In hummingbirds, almost 97% of the pretectal inputs arise from the LMI [60]. The differences observed in these neural connections may serve as the fundamental elements to support various behaviors relevant to each species. Importantly, this opens an interesting question: do birds with different flying abilities have different midbrain-cerebellar connections?

In this project, I focus on zebra finch visual nuclei-cerebellar connections with an emphasis on how the mossy and climbing fiber pathways differ from that of pigeons. The four research goals are: 1) to demonstrate whether the nBOR and the LM project to different areas in the inferior olive; 2) to identify the zebra finch zebrin stripe patterns; 3) to investigate whether the nBOR and the LM terminals are located in similar areas of the VbC when compared to previous pigeon studies; and 4) to determine how these axon terminals align with zebrin-immunoreactive zones.
Figure 1. Schematics of the lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) structures, output regions, and the visuomotor pathways. A: the LM and nBOR structures. The two subdivisions of the LM, the lentiformis mesencephali, pars medialis (LMm) and the lentiformis mesencephali, pars lateralis (LMI), are indicated in purple and dark blue, respectively. The laminaris precommissuralis (LPC) positioned medially to the LMm is light blue. Neighboring pretectal structures are shown in grey. All structures of the nBOR are magenta. Sections are 80 μm apart. See the list of abbreviations for the nomenclature of each nuclei.

B: LM and nBOR efferents. The LM projections are shown in blue; the nBOR projections are shown in magenta. Output targets are grouped by general function and the five pathways are presented in greyscale. See the list of abbreviations for the nomenclature of each nuclei.

C: visual mossy fiber and climbing fiber pathways to the oculomotor cerebellum (OCb, folium VI-VIII) and the vestibulocerebellum (VbC, folium IXcd and X). Top left: a sagittal view of the two visual pathways to the OCb and VbC. Mossy fibers are indicated with dotted lines: yellow to the OCb and green to the VbC. Both the LM and the nBOR project to the medial column of inferior olive (mcIO), from which climbing fibers project to the VbC, shown as a green solid line. The dorsal (dl) and ventral (vl) lamellae of the inferior olive, receiving sensory inputs from other regions (outside the focus of the present study), project to the OCb, shown as a yellow solid line. Bottom left: a coronal view shows the inputs and outputs of the left inferior olive, corresponding to cross section “a” in the sagittal view. Right: a coronal view of the functional zones in folium IXcd, corresponding to cross section “b” in the sagittal view. The VbC has seven zebrin stripe pairs. Each optic flow zone includes a pair of zebrin immuno-positive and –negative bands. The optic flow that best excites the Purkinje cells are plotted under each zone. The translational zones in the ventral uvula are shaded in light purple; the rotational zones in the flocculus are shaded in pink. The mossy fibers, plotted as a green dotted line, terminate in the granular layer. The climbing fibers from the mcIO, shown as a green solid line, innervate the Purkinje cells. See text in the introduction for more details.
2 Research Chapter

2.1 Introduction

Complex behaviors rely on the integration of self-motion-related sensory feedback. Whole-field visual motion resulting from self-movement, or optic flow, is one of the important idiothetic cues that animals use to adjust body orientation and locomotion [4]. In birds, two midbrain nuclei are critical for processing optic flow, the lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). Electrophysiological studies show that the LM and the nBOR are best excited by optic flow presented in the contralateral eye, and cells are tuned to specific directions and spatiotemporal frequencies [6-9]. The LM, for most bird species studied to date, has a bias for forward motion. Conversely, the nBOR prefers either upward, downward, or backward motions. The two optic flow-sensitive nuclei receive direct retinal inputs and project to several key sensorimotor nuclei (figure 1B) [19, 21, 24, 32]. Both the LM and the nBOR have bilateral projections to the vestibulocerebellum (VbC) via mossy fibers [24, 32] (figure 1C). In addition to this direct pathway, the two nuclei project to the medial column of the inferior olive (mclO), from which climbing fibers transmit optic flow information to the Purkinje layer of the VbC [24, 32] (figure 1C). The visual information is integrated with other sensory inputs in the VbC and the output from the VbC is important for maintaining a stable retinal image [61].

The VbC is organized into several parasagittal zones. The divisions are based on several factors, including the climbing fiber inputs, Purkinje cell responses to global visual motion, and biochemical marker expression patterns [39]. Purkinje cells in the VbC are modulated by different patterns of panoramic optic flow, and neurons with the same tuning are confined in the same sagittal region, creating several optic flow zones (figure 1C). In the medial VbC, which is comprised of the uvula (folium IXcd) and nodulus (folium X), Purkinje cells are modulated by optic flow resulting from translational movement, and three translational optic flow zones have been described in pigeons [43-45]. Conversely, Purkinje cells in the flocculus (the lateral extent of the folia IXcd and X) are maximally excited by rotational optic flow derived from either yaw or roll movement [43, 45, 47]. In folium IXcd, Purkinje cells differentially express a molecular marker, zebrin II, resulting in an alternating immuno-positive and immuno-negative zebrin stripe pattern. In pigeons, there are seven zebrin stripe pairs in the VbC [48]. A pair of zebrin immuno-positive and –negative bands
span each optic flow zone, and Purkinje cells within the same zebrin stripe pair did not differ in their optic flow responses [41, 42].

In most avian species, the mcIO is further divided into three anatomically distinct subgroups (dorsal, medial, and ventral mcIO). However, in pigeons, these subdivisions are not clear. Nevertheless, different regions of the pigeon mcIO are differentially innervated by the LM and the nBOR, and the climbing fibers from these regions project to different VbC functional zones. In pigeons, more LM axon terminals have been found in the caudal mcIO, while more nBOR inputs are found in the rostral mcIO [58]. In the rostral mcIO, the climbing fibers project to the rH45, descent, ascent, and expansion optic flow zones in the VbC (figure 1C). In these zones, the optic flow that best excited the Purkinje cells contained either upward, downward, or backward motions [52]. In the caudal mcIO, the climbing fibers project to the rVA and contraction optic flow zones (figure 1C). In these zones, the optic flow that activates the Purkinje cells includes forward motion either in the entire or partial visual field [53-55]. As mentioned above, in most avian species, the LM is biased toward forward motion, whereas the nBOR prefers either up, down, or backward motion. The topographical connections presented in pigeons suggest that different visual information is conveyed in different olivocerebellar pathways. The majority of studies in these visuomotor pathways are carried out in pigeons, thus, it is unknown how the visual signals are transmitted in most other avian species where the mcIO is more differentiated.

In birds, the direct visual mossy fiber pathway has only been investigated in pigeons. Mossy fiber inputs from the LM and the nBOR cluster in discrete regions of the VbC, aligning with zebrin immuno-positive bands [59]. It is still unknown how visual information is processed in different VbC compartments with different zebrin immunoreactivity. However, it seems that immuno-negative bands receive visual inputs mostly via the climbing fibers, whereas immuno-positive bands receive not only the climbing fibers but also visual mossy fibers. Whether this is a general trend for most avian species is yet to be confirmed.

A recent comparative study performed in our lab shows that species differences in visuomotor pathways exist, and smaller bird species seem to have distinct pathways for transmitting optic flow signals to the cerebellum [60]. Zebra finches, compared to pigeons that usually walk and forage on the ground, use forward flight as their primary mode of locomotion and foraging. During forward flight, zebra finches often perform flap-bounding,
which is a flight style consisting of flapping phases alternating with flexed-wing bounds [62]. This flight style is also common for many small bird species. Forward flight may contain special visual stimuli. The behavioral demands may be supported by neuroanatomy. In addition, zebra finches have long been used to study vocal learning, and a number of powerful molecular tools have been adapted from typical model organisms to this bird species [63, 64]. Conversely, the visual circuits have not gained much attention. As a forward flight specialist and with the possibility of using molecular tools established in other model organisms, zebra finches are a great model to study the avian visuomotor pathways.

In this study, I use dual-injections of anterograde tracers and zebrin immunostaining to investigate the visuomotor pathways in zebra finches. I injected different color fluorescent dextran tracers in the LM and the nBOR and traced the neural projections to the inferior olive and the cerebellum folium IXcd. The LM and the nBOR terminals were overlaid to see if they project to different areas of the inferior olive and whether they have different projection patterns in folium IXcd of the cerebellum. The cerebellum was also immunolabeled for zebrin II to determine whether the mossy fibers from the LM and the nBOR, like pigeons, align with zebrin immuno-positive bands.
2.2 Methods

2.2.1 Animals

Three male adult zebra finches (*Taeniopygia guttata*, 13-15g) were used for neuronal tract tracing. The bird IDs were TG497, TG502 and TG503. Birds were obtained from Eastern Bird Supplies, Quebec, Canada. Prior to the surgery, they were housed with other male zebra finches in a temperature, humidity, and dark-light cycle-controlled room. Bird seed, clean water, and cuttlebones were available ad libitum. Leafy greens, peas and corn were provided periodically to supplement their diet. All experimental procedures were approved by the University of British Columbia Animal Care Committee (A19-0113).

2.2.2 Surgery and tracer injection procedures

Birds were anesthetized by intramuscular injection of a ketamine and xylazine mixture (65 mg/kg ketamine and 8 mg/kg xylazine) in the pectoral muscle. Supplemental doses were given as necessary. Once anesthetized, feathers on the head, and around the ears, were trimmed, and the birds were placed in a stereotaxic frame specifically designed for small bird neurosurgery (Herb Adams Engineering; Glendora, CA, USA). To hold the head firmly and to align the head orientation to a zebra finch brain atlas (Konishi, unpublished), ear bars were pinned against the otic process of the quadrate bone, which lies in the anterior part of the opening to the external acoustic meatus. A beak bar was placed in the beak and the upper beak was secured to the bar. A subcutaneous injection of 150 μl 0.9% saline was given for hydration. A small amount of Marcaine (1 to 2 μl) was injected at the surgery site before beginning the incision. To zero coordinates for use with the brain atlas, a craniotomy was performed above the y-sinus. The y-sinus is the convergence of three sinuses—the mid-sagittal and cerebellar sinuses—and is the reference point for the electrode. Prior to placing the bird in the frame, a glass micropipette (20 μm tip; A-M Systems, Inc., catalog# 626000) was zeroed against the right ear bar to set inter-aural zero in the anterior-posterior (A-P) axis. The pipette was then moved 0.3 mm caudal to inter-aural zero for centering the y-sinus. The medial-lateral (M-L) axis and head angle were adjusted so that the tip of the pipette sat at the center of the y-sinus. The final head angle was pitched 45° down from the horizontal plane. A second craniotomy was performed over the right telencephalon to expose the surface of the brain and allow access to the LM and the nBOR.
To ensure the injections were made in the correct sites, extracellular multi-unit responses to large-field moving patterns were recorded in the LM and the nBOR. The glass microelectrode was filled with 2M NaCl and a silver wire was placed inside the electrode (ID = 20 μm). Extracellular activity was amplified (10k gain; 100 Hz high pass and 3k Hz low pass filter; A-M Systems, Inc., Model 3000 differential amplifier; Sequim, WA, USA), sampled at 50 kHz (Cambridge Electronic Design., Micro 3; Cambridge, UK) and recorded using Spike 2 software (Cambridge Electronic Design). To identify visually responsive cells, the bird’s left eye was taped open and a drawing of black dots and lines was waved in front of the eye. Once a responsive site was found, a computer-generated large-field random dot pattern was presented to the bird [65]. The monitor (144 Hz, 83° × 53°, ASUS) was positioned 30 cm from the bird and tangent to the viewing eye. The visual stimulus was composed of a plane of randomly positioned black dots (2.1° diameter) on a white background. The plane moved at 12.6°/sec in eight directions 45° apart. Each sweep consisted of 4 seconds of motion, followed by a 4 second pause, in each of the eight directions. The change of visual stimuli and the cell responses were recorded for at least 5 minutes.

After the recording, the electrode was raised and the 2M NaCl was removed. The same electrode was refilled with fluorescent dextran, either Texas red (red; D3328; 3000 molecular weight; Invitrogen) or fluorescein (green; D3306; 3000 molecular weight; Invitrogen), through the tip by suction. A clean silver wire was placed in the electrode and connected to an iontophoresis machine (Stoelting Co; Wood Dale, IL, USA). The pipette was subsequently lowered down again to the same depth where the recording was made. The dextran was injected iontophoretically for 15-25 min, followed by 5 min of rest. The LM and the nBOR coordinates, injected tracers, and detailed injection parameters for each bird are listed in Table 1. After completing the first injection, a new electrode was used to record and inject at the other nucleus. When changing pipettes, the new pipette was re-zeroed at the y-sinus using the previous inter-aural zero. At the end of the surgery, the craniotomy was filled with bone wax and the skin was sutured with cyanoacrylate (Vetbond, 3M). The birds were recovered for 4 days to allow for transport time.
Table 1. Summary of the LM and the nBOR coordinates, tracer injected, and the iontophoresis injection parameters for the three samples used in this study. The AP is the anterior-posterior and ML is the medial-lateral coordinates, which are relative to the inter-aural zero and the y-sinus zero, respectively.

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<td>TG502</td>
<td>TG503</td>
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<tr>
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<tr>
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<td>Red dextran</td>
<td>Green dextran</td>
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<tr>
<td>Injection parameters</td>
<td>+7 μA; 15 min; 7 sec on/off</td>
<td>-4.5 μA; 20 min; 7 sec on/off</td>
<td>+4.5 μA; 25 min; 7 sec on/off</td>
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<tr>
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<td>Coordinates</td>
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<td>AP: 0.72; ML: 0.95</td>
<td>AP: 0.72; ML: 0.75</td>
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<tr>
<td>Injected tracer</td>
<td>Red dextran</td>
<td>Green dextran</td>
<td>Red dextran</td>
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<td>Injection parameters</td>
<td>+4.5 μA; 15 min; 7 sec on/off</td>
<td>+4.5 μA; 25 min; 7 sec on/off</td>
<td>+4.5 μA; 20 min; 7 sec on/off</td>
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2.2.3 Histology and Immunohistochemistry

After four days in recovery, birds were deeply anesthetized by injecting 150 to 170 μl ketamine and xylene mixture (65 mg/kg ketamine and 8 mg/kg xylazine) in the pectoral muscle. Subsequently, birds were transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in 0.01M phosphate-buffered saline (PBS, pH 7.4). The brains were removed from the head and immersed in 4% PFA overnight at 4 ℃. To cryoprotect the tissue, the brains were then transferred into 30% sucrose in 0.01M PBS for another 24 hours at 4 ℃. After the brains sank to the bottom of the 30% sucrose solution, they were imbedded in gelatin blocks. The gel blocks were immersed in 4% PFA for 2 hours and then transferred into 30% sucrose overnight at 4 ℃ for cryoprotection. The gel-blocked brains were cut at 40 μm steps in the coronal plane with a freezing stage microtome. The sections were stored in individual wells containing 0.01M PBS. Sections were collected in three series for each brain and each series contained sections from the rostral end of the midbrain to the caudal end of the cerebellum. For TG502 and TG503, all sections of the first series were mounted on gelatin-coated slides and imaged for dextran labeled neural projections. Sections that contained vestibulocerebellum in the second and the third series were used for immunohistochemistry study. For TG497, all series were mounted on gelatin-coated slides.
and imaged for fluorescent labels. After the imaging, the slide containing the inferior olive structures from one series was Nissl stained.

In this study, the immunohistochemistry followed the protocol of Wylie et al (2017) [57]. Sections were first gently washed in wells with PBS three times, for 5 min each time, followed by blocking with 10% normal donkey serum (Jackson Immunoresearch Laboratories) in 0.4% PBS-Triton for 1 hour at room temperature. Subsequently, individual sections were incubated in 1 ml solution containing 2.5% normal donkey serum and the primary anti-zebrin antibody (1:1,000; goat-polyclonal; sc-12065, Santa Cruz Biotechnologies, Santa Cruz, CA; RRID: AB_2242641) in 0.4% PBS-Triton at 4 ℃ for 5 days. Sections were then gently washed in wells with PBS five times, for 5 min each time, and then incubated in a secondary antibody, Alexafluor-594 (red) or 488 (green) conjugated donkey anti-goat antibody (diluted 1:100 in 0.4% PBS-Triton and 2.5% normal donkey serum; Jackson Immunoresearch Laboratories: red RRID: AB_2340432; green RRID:AB_2340428) for 4 hours at room temperature. For both TG502 and TG503, the second series was immunoreacted with the red secondary and the third series used the green secondary. Finally, the sections were washed five times, for 5 min each time, and mounted on gelatin-coated slides to be imaged.

The Nissl staining was done on two slides of TG497 to outline the structure of the inferior olive subnuclei. The slides were delipidized in chloroform for 40 min, followed by five 1-min rehydration steps in ethanol with serial dilution rates from 100%, 95%, 70%, 50%, to 30%. The slides were subsequently immersed in thionin buffer for 1 min, followed by 3 min in thionin stain and back into thionin buffer for 15 sec. After the staining steps, the slides were dehydrated again in ethanol with the opposite serial dilution rate, from 30%, 50%, 70% (with a few drops of acetic acid), 95%, to 100%. Finally, the slides were immersed in Hemo-De (Hemo-De, Inc; Keller, TX, US) to remove extra ethanol, then cover-slipped with Permount.
2.2.4 Spike sorting and direction tuning analysis

The extracellular recordings were analyzed with the spike sorting program in Spike2 (Cambridge Electronic Design). To capture spikes (wave-marks) from raw traces (waveform), I first manually selected a triggered threshold to exclude the noise, then I zoomed in on one spike to set the template window to encompass the full spike. Spikes that crossed the threshold were isolated and compared to the existing templates. If a spike could not be sorted into any template, a new template was created. If over 60% of a spike matched to a template, the spike was sorted into that template unit. Finally, using the trigger overdraw function in Spike 2, templates with similar shapes were grouped together, resulting in spike activity data for a single cell. The grouping of templates was also confirmed by cluster analysis of principal component data.

The spike sorted results were further analyzed for direction tuning with Matlab (R2019b; MathWorks; Natick, MA). The scripts used were from Gaede et al (2017) [65]. The Matlab programs generated peristimulus time histograms (PSTHs; bin size = 200 ms) for each 4 s stimulus. The results were plotted as polar plots with R (version 3.6.1), showing the average firing rates (spikes/sec) as a function of the eight stimulus directions as well as the mean baseline activity.

2.2.5 Microscopy and image analysis

The injection sites, the fluorescent labeled axon terminals, and the zebrin immunolabeling results were viewed with Zeiss A.1 microscope equipped with the appropriate fluorescence filters (rhodamine and GFP). The photos were taken with a Zeiss AxioCam MRc camera and Zeiss ZEN 2012 software. Nissl-stained sections were imaged under brightfield with the same microscope and camera.

To show the spatial relationship of the LM and the nBOR axon terminals in the inferior olive and cerebellum, the two fluorescent channels were merged in Fiji [66]. The LM and the nBOR projection patterns in folium IXcd were also shown separately. The image stitching, figure annotations, brightness, and contrast adjustment were done manually in Adobe Photoshop.

Because the immunolabeling fluorescent signal is stronger than the two tracer fluorophores, to be able to align mossy fiber terminals with zebrin stripes, I immunolabeled
alternate series with red and green secondary antibodies. The fluorescent images of the same section were first stitched and adjusted for the contrast and brightness with Adobe Photoshop. Then the divisions and numbering of zebrin bands were identified in accordance with Pakan et al (2007) [48]. To show the mossy fiber distribution in different zebrin bands, a zebrin photo was taken first, then the fluorescent filter was changed to the color of the rosettes without changing the image area. The two fluorescent photos were merged with Fiji and the merged photos were stitched and annotated with Photoshop.

To outline the inferior olive structure, seven 120 μm-spaced sections from series 1 and one section from series 3, which is 40 μm rostral to the last section in series 1, were Nissl-stained and imaged (bird ID, TG497). The brightfield inferior olive photos from the same section were stitched into a single image with Photoshop. The inferior olive structure was delineated using the Nissl-stained sections in Adobe Illustrator. According to previous inferior olive studies in other bird species [37, 67, 68], the avian inferior olive has three main divisions, the ventral and dorsal lamellae (vl, dl), and the medial column (mcIO). In many bird species, the mcIO can be further divided into dorsal, medial, and ventral subgroups. In zebra finches, the inferior olive structure is more complex than the previously reported divisions. Thus, the nomenclatures are tentatively given as numbers and letters. Based on the available anatomical landmarks (e.g., the course of the hypoglossal nerve (cranial nerve XII), midline of the brain, rostral and caudal end of the IO, and the overall shape of the medulla), the IO was first divided into four main groups, denoted as number 1 to 4 for dl, mcIO, vl, and an additional group in the rostral IO, respectively. Within some of these main groups, cells are clustered into several subgroups. The subdivisions were labeled with letters. However, not all subdivisions were obvious in all sections and the shape and location of each subgroup change significantly over the 120 μm interval between neighboring sections in TG497. Therefore, another bird (TG486) that was not directly involved in this study, and did not have critical injections in the brain, was used to create a full series of Nissl-stained sections at 40 μm intervals for the entire IO. These sections were used to help determine the subgroup identities in TG497. The subgroups were assigned based on their shape changes across sections, the relative location to the anatomical landmarks and to each other, and the cell morphologies or Nissl staining properties.

To map the LM and the nBOR axon terminals in the inferior olive, the merged
fluorescent images from TG497 were superimposed on the IO structure outlines of the corresponding section. The areas containing LM terminals were demarcated by yellow ellipses and the nBOR terminals were demarcated by blue ellipses using Adobe Illustrator. Terminal labeling in TG502 has not been Nissl-stained because these samples will be re-imaged with a high-resolution microscope for publication. The IO outlines from similar sections in TG497 were used as an estimation of the IO borders for the LM and the nBOR projections in TG502.

The mossy fiber terminals (rosettes) in each zebrin stripe were counted using the Fiji cell counter plugin. Rosettes in all immuno-positive or -negative bands from all sections were summed and normalized to the total rosettes counted across all sections. The area of the granular layer for each zebrin band was measured with Fiji. The area of bands with the same immunoreactivity were summed and normalized to the total area across all sections. The average rosette number in positive bands was normalized to the average area of the positive bands. This calculation was repeated for the negative bands.
2.3 Results

2.3.1 Dual injections in the LM and the nBOR

I injected 3k molecular weight dextran of two different colors in the LM and the nBOR of three male zebra finches. Images of the injection sites for each case are presented in figure 2 and all of the injection sites were confirmed with extracellular recordings. For TG502 and TG503, the computer-generated visual stimuli were also used to create direction tuning curves for the LM and nBOR cells.

In most bird species, LM neurons are biased toward forward motion [6]. In this study, all recorded LM neurons were excited by forward motion. The representative raw trace and direction tuning curve from TG502 are shown in figures 2B and 2C, respectively. In the direction tuning curve, it is clear that the cell was tuned to 0 degrees (forward) and was inhibited by global visual motion in the opposite (“null”) direction.

Conversely, nBOR cells are almost equally likely to be tuned to either up, down, or backward motion [7]. In TG502, the nBOR cells recorded were tuned to upward motion, whereas in TG503, the nBOR cells were modulated by downward motion. The representative raw trace and direction tuning curve from TG502 are shown in figures 2E and 2F, respectively. In the nBOR, neurons of pigeons are topographically arranged by direction preference such that most up cells are located in the dorsal nBOR whereas down cells are found in a region more ventral to the up cells [69]. This topographic mapping of direction tuning in the nBOR was also seen in this study with zebra finches. As shown in figures 2D and 2H, the injection in TG502 was centered in the dorsal part of the nBOR, whereas in TG503, the brightest tracer injection spot was located in the ventral site. The spread of the tracer covered all of the nBOR. Note in TG503, there was a tract of tracer along the micropipette path up to the mesencephalic reticular formation. This is not a concern because this area does not project to the cerebellum or the inferior olive. The nBOR injection in TG497 was mainly in the medial part of the nucleus (figure 2J).
Figure 2. Dual injections in the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). A, G, and I: microscopic photos of injection sites in the LM. In A, red dextran was injected in TG502. In G and I, green dextran was used for TG503 and TG497. D, H, and J: images of injection sites in the nBOR. In D, green dextran was injected in TG502. In H and J, red dextran was used for TG503 and TG497. All scale bars equal 100 μm. B, E: raw traces of cell responses to wide-field motion of a dot field for the LM and the nBOR, respectively. Data are from TG502 and from one sweep. Each sweep consisted of 4 sec of motion, followed by a 4 sec pause. The arrows above the raw traces show the direction of motion. C, F: direction tuning curves of single LM and nBOR neurons. The mean firing rate was plotted as a function of the motion direction. 0°, 90°, 180°, and 270° represent forward, downward, backward, and upward motions, respectively. Light grey circles indicate the spontaneous firing rate. Dark grey dots are the firing rate at each sweep, and the orange dots are the mean firing rate across sweeps.
2.3.2 Unique LM and nBOR projections in different areas of the mcIO

To map the LM and the nBOR projections in the inferior olive, the structures of each subnucleus of the IO were first delineated from two Nissl stained sections of TG497. The avian IO can be divided into three main parts, the ventral and dorsal lamella (vl, dl), and the medial column (mcIO) [37, 68]. In most birds, the mcIO can be subdivided into dorsal, medial, and ventral subgroups [67, 68]. However, the subdivisions of the mcIO are not as clear in pigeons as they are for many other avian species [37]. In this study, I found that the zebra finch inferior olive structure has more than 3 subgroups within the mcIO. A series of Nissl-stained sections and schematics of the IO structure outlines are presented from caudal to rostral in figures 3A and 3B. The intervals between sections are 120 μm for sections 1 to 7 and 40 μm between sections 7 and 8. Because this highly differentiated inferior olive structure has not been reported in other birds, the nomenclatures are tentatively given as numbers one to four for dl, mcIO, and vl, and an additional main group, respectively. The additional main group (group 4) can be found in the rostral sections, located at the ventral wall of the medulla and close to the midline (figure 3A, B). Within some of the main divisions, the cells are clustered into several distinct groups, generating five mcIO and three vl subgroups, indicated with letters. The subgroups were assigned based on their relationship with each other and the relative location and shape change across sections. Some anatomical landmarks (e.g., hypoglossal nerve) were also used to separate different groups. To verify these divisions, another zebra finch (TG486) that was not directly involved in this study was used to create a full series of inferior olive Nissl images with a finer step size (40 μm). The rostral-caudal range of zebra finch inferior olive is around 840 μm, which is similar to the sparrow (870 μm), flycatcher (840 μm), and swallow (870 μm) [68]. The mcIO begins at the rostral end of the IO and spans 500 to 600 μm.

To trace the neural projections from the LM and the nBOR to different subgroups of the inferior olive, the fluorescent labeled axon terminals were superimposed onto the IO structure outlines. The results are shown in the schematics in figure 3B (blue and yellow shaded areas represent the nBOR and LM axon terminals, respectively). The nBOR axon terminals can be seen in both sides of the IO and in almost all subgroups of the mcIO, except for 2a. The additional main group, group 4, also receives nBOR inputs. Conversely, the LM terminals were predominately in the side of the IO ipsilateral to the injection site and were mostly in
the dorsal divisions, 2c and 2d. There were a few LM projections found in the contralateral side at the lateral edge of 2d. Two representative images from TG502 are presented in figures 3C and 3D. As shown in the schematic, the green dextran labeled nBOR terminals were significant in both sides of the IO, whereas the red dextran labeled LM projections were mainly in the ipsilateral side. Figures 3C and 3D match the fifth and the sixth sections in the schematic, respectively. Note in figures 3C and 3D, the outlines of the inferior olive were derived from the close sections in TG497 because the TG502 sections were not Nissl-stained. The microscope images show an important projection pattern: the density of the nBOR axon terminals was not equal between contralateral and ipsilateral sides in different rostro-caudal sections. In the rostral section, there were more nBOR terminals on the ipsilateral side (figure 3D). By contrast, in the caudal section, more nBOR projections were seen on the contralateral side (figure 3C). In addition, in the caudal section, it is clear that on the ipsilateral side, the LM terminals were denser and dorsal to the nBOR terminals. A previous study in pigeons found that the projections from the LM and the nBOR were predominantly in the caudal and rostral mcIO, respectively [59]. This was not seen in this study as the nBOR terminals spanned almost the whole mcIO, although this observation is derived from a single case (TG497).
**Figure 3.** Zebra finch inferior olive subnuclei and the terminal labeling pattern in the IO from injections in the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). A: series of Nissl-stained sections from TG497, spaced by 120 μm for section 1 to 7 and 40 μm between 7 and 8. The eight sections of the IO are placed in caudal to rostral order. B: corresponding schematics of the inferior olive structure outlines and the superimposed LM and nBOR projections. Blue shaded area represents the nBOR terminals; yellow shaded area represents the LM terminals. The gray dotted lines show the course of the hypoglossal nerve (XII). The four main IO structures are labelled with numbers (1 for dl; 2 for mcIO; 3 for vl; 4 for the additional main group). Lower case letters label the subgroups within the main structure. Scale bar equals 200 μm. C, D: microscopic photos from TG502. Red tracers label the LM terminals and green tracers label the nBOR terminals. The interval between the two sections is 120 μm. Scale bars equal 100 μm.
2.3.3 LM and nBOR terminals in the granular layer of the cerebellum folium IXcd

In all cases, the injections in the LM and the nBOR resulted in terminal labeling in the folium IXcd. Representative images of the mossy fiber projections from TG503 are presented in figure 4. The axons from the two midbrain visual nuclei coursed through the brachium conjunctivum cerebellopetal, entered the cerebellum via the white matter, and then terminated in the granular layer closely adjacent to the Purkinje layer (figure 4C-4E). Several parasagittal clusters of rosettes can be found bilaterally for both the LM (figure 4A) and the nBOR (figure 4B) injections. Although in this case, the LM terminals may seem sparser than that of the nBOR, this could be due to the injection site, injection size, or the labeling properties of different dextran tracers. As seen in TG502 where the red dextran was injected in the LM, many rosettes can be clearly observed (figure 6). When the two fluorescent channels were merged, clusters of rosettes from the LM and the nBOR almost overlapped in the same parasagittal areas (figure 4C-4E). The overlapping of mossy fiber inputs from the LM and the nBOR were also found in pigeons [59].
Figure 4. Representative photos of the mossy fiber labeling in the cerebellum folium IXcd. A, B: microscopic photos of mossy fiber terminals (rosettes) in folium IXcd from injections in the LM (green) and the nBOR (red). Data are from TG503. C, D, and E: enlarged images of boxes in A and B. The LM and nBOR terminal labeling were merged. ml, molecular layer; pcl, Purkinje cell layer; gl, granular cell layer. Scale bars in A and B are 100 μm. Scale bars in C to E are 50 μm.
2.3.4 Zebrin expression pattern in the cerebellum folium IXcd of zebra finches

Zebrin II is a highly conserved protein in vertebrates and the parasagittal zebrin stripe patterns have been described for many species [48, 67, 70]. This study is the first to report zebrin expression patterns of zebra finches by including a series of coronal sections of cerebellum folium IXcd from two zebra finches with immunostaining for zebrin II. One series was immunostained with red secondary, while the other series, which was 40 μm rostral to the former series, was immunostained with green secondary. Four sections with red and three sections with green zebrin staining from TG502 were presented in a caudal to rostral order (figure 5). The interval between the red and green sections was 160 μm. Similar to pigeons [48], the zebrin was differentially expressed by Purkinje cells and was expressed in the somata and the dendrites, but not in the nuclei. The borders between zebrin immunopositive and immuno-negative bands were clear in the ventral lamella and in most of the stripe pairs. In addition, like pigeons, the medial stripe pairs (1 +/- and 2 +/-) were wider than the rest of the stripe pairs. A noticeable difference of the zebrin expression pattern between zebra finches and pigeons is that the narrow immunopositive stripe found in some caudal sections in pigeons was located in the 1- region, whereas this thin band was observed in 2+ in zebra finches. This thin stripe created two notches in the 2+ band and was only presented in the caudal sections (figures 5A and 5B). In more rostral sections, a single gap in the middle of 2+ can be seen, especially in the ventral lamella.
Figure 5. Zebrin II stripe patterns in the zebra finch cerebellum folium IXcd. Coronal sections with folium IXcd from TG502 were immunostained for Zebrin II. From A to G: microscopic photos of the folium IXcd, placed in caudal to rostral order. The interval between red and green sections is 160 μm. The numbering of bands are shown in the center of each band. A, C, E, and G: sections stained with red secondary. B, D, and F: sections stained with green secondary. The white dotted lines indicate the borders between immuno-positive and -negative bands. The triangles point at gaps within 2+ zebrin-positive band. Scale bars equal 100 μm.

2.3.5 LM and nBOR mossy fiber rosettes in different immunoreactive zebrin bands

Mossy fibre images were superimposed onto the zebrin immunostaining results to examine the distribution of rosettes in different zebrin bands. Three sections with LM terminals and three sections with nBOR terminals from TG502 were chosen for representation (figure 6). Sections were placed in caudal to rostral order with 240 μm interval between sections. The rosettes can be found in almost every band. To quantify the results, the total amount of rosettes in immuno-positive or immuno-negative bands was normalized to the total rosettes counted across sections. From the nBOR injection, there were more rosettes in the positive bands (63.46%) than in the negative bands (36.54%). From the LM injection, there were also more rosettes in the positive bands (77.43%) than in the negative bands (22.57%). The data were from TG502 alone. Similar results were also observed in pigeons. In the pigeon study, it is shown that bilateral nBOR projections are more in the positive bands (79.9%) than in the negative bands (20.1%) and the LM also projects more to the positive (91.1%) than the negative bands (8.9%) [59]. The bias toward positive bands is more significant in the LM than in the nBOR for both pigeons and zebra finches.

There are two potential pitfalls of directly using these numbers to compare the distribution of mossy fiber terminals in different immunoreactive bands. First, the areas of some zebrin bands are larger than others. As shown in figures 5 and 6, it is clear that most of the positive bands are larger than the negative bands within the same stripe pair. Intuitively, the larger area may receive more mossy fiber terminals. In addition, depending on the rostrocaudal extent where the section is located, some bands may just begin to appear laterally. For example, in the most rostral section included in the analysis, band 5+ just
started to appear on the sides, and no further lateral bands were on this section. Thus, there are no data points for the negative band of the fifth stripe pair (figure 5G). To compensate for the area effects and to examine the mossy fiber distributions with less bias, the average rosette number in immuno-positive and immuno-negative bands were normalized to the average area of the immuno-positive and immuno-negative bands, respectively. When the area is considered, the results show a diverged distribution of mossy fiber terminals from the LM and the nBOR. For the nBOR, more area-corrected rosettes were found in negative bands (1.15) than in positive bands (0.93). Conversely, the LM had more axon terminals in the positive (1.14) than in the negative bands (0.71) per unit area of zebrin bands. No statistical analysis was performed because these data came from a single case.
Figure 6. Representative patterns of mossy fiber terminals in cerebellum folium IXcd and zebrin expression. A to C: three coronal sections with LM terminals (red) and green zebrin staining. D to F: three coronal sections with nBOR terminals (green) and red zebrin staining. Data are from TG502. All sections are placed from caudal to rostral order and the interval between sections is 240 μm. The white dotted lines indicate the borders between immuno-positive and -negative bands. Scale bars equal to 100 μm.
2.4 Discussion

Zebra finches are a great candidate for studying visuomotor signal integration. The avian visuomotor pathways have been studied mainly in pigeons, some in chickens, and a few in zebra finches and hummingbirds. Among these bird species, hummingbirds hover when feeding from flowers; chickens, although mostly feed on the ground, can perform some transient flight. Pigeons can fly a long distance but they often walk while foraging. Zebra finches use forward flight as the main locomotion and foraging method. During forward flight, zebra finches often perform flap-bounding, which is a flight style consisting of flapping phases alternating with flexed-wing bounding phases [62]. This flight style is also common for many small bird species. Furthermore, zebra finches have long been used for studying vocal learning pathways and a number of powerful molecular tools have been adapted from typical model organisms to this species [63, 64]. Despite the characteristic flight behavior, and the potential use of the molecular tools established in rats and mice, zebra finches have seldom been investigated for their visuomotor processing pathways. To elucidate the connections between visual nuclei and the cerebellum, I used anterograde tract tracing to map the projections from the LM and the nBOR to the inferior olive and cerebellum. In addition, I used immunohistochemistry to examine the zebrin stripe patterns in the cerebellum folium IXcd in zebra finches. The results were compared to previous pigeon and chicken studies, from which we have a better understanding of visuomotor pathways. Here I provide evidence for several unique inferior olive structures and projection patterns that appear to only exist in zebra finches. Because these structures have not been documented in other avian species, I will discuss them in comparison with some mammalian olivocerebellar connections to shed light on their potential role in visual signal integration in zebra finches.

The avian inferior olive has been divided into three main groups, the dorsal (dl) and ventral (vl) lamellae, and the medial column of the inferior olive (mcIO). The mcIO is the main inferior olive structure to receive optic flow information from the LM and the nBOR. The mcIO in many bird species is further differentiated into three subdivisions, dorsal, medial, and ventral mcIO [68]. Even in chickens that only perform transient flight, the mcIO has three subdivisions referred to as ventral, intermediate, and dorsal medial cell groups (vMC, iMC, and dMC) [67]. Interestingly, pigeons are the only exception found to date that
do not have clear subdivisions in the mcIO [37, 68]. In the present study, I found that the zebra finch inferior olive is more stratified than any avian species previously studied. Aside from the three main groups (dl, vl, and mcIO), there is an additional group positioned ventromedially in the rostral inferior olive. Within the mcIO, cells are clustered into several subgroups and a total of five distinct subgroups could be identified. This complex structure requires further neuroanatomical and electrophysiological investigation to determine whether cells in different subgroups are functionally different. Nevertheless, the present work shows that the subnuclei receive differential projections from optic flow-sensitive LM and nBOR cells. The nBOR terminals could be found in almost all subdivisions of the mcIO (except 2a), whereas the LM terminals were primarily in the dorsal divisions. Given the known directional preferences of LM and nBOR neurons, this could suggest that cells in the dorsal divisions of mcIO respond to forward optic flow, while the other divisions are more excited by up, down, or backward motion. Whether the directional tuning in different subgroups of mcIO is clearly separated has yet to be verified. Very few projections from the nBOR and the LM were found in the dl and vl, which is similar to previous pigeon studies [24, 32].

The mammalian inferior olive is more complex than birds. In mammals, the inferior olive has three major subnuclei, principal olive (PO), dorsal accessory olive (DAO), and medial accessory olive (MAO), and four small subnuclei: subnucleus β, dorsomedial cell column (DMCC), dorsal cap (DC), and ventrolateral outgrowth (VLO) (see the 3D structures of each divisions in Sugihara and Shinoda, 2004) [70]. The caudal MAO is further divided as a, b, and c subnuclei. The DC, DMCC, β nucleus and the b subnucleus in the caudal MAO are four IO subdivisions known to be important for eye movement control (see review from Barmack, 2006) [71]. The homology of the IO between birds and mammals is not fully understood. Based on morphological characteristics alone, the avian vl is homologous to the mammalian PO, the dl to the DAO, and the mcIO to the MAO [68]. However, based on the olivocerebellar connections, Arends and Voogd (1989) proposed that the mammalian PO, MAO and DAO are equivalent to the avian mcIO, dl and vl, respectively [37].

The majority of work examining connections between visual nuclei and the inferior olive in birds has been carried out in pigeons. In this species, the LM and the nBOR project to the caudal and rostral mcIO, respectively [58]. While studies in other avian species are sparse, similar connections have been documented in several mammalian species. Previous
mammalian studies have shown that the nucleus of the optic tract (NOT, the mammalian homolog of LM) projects directly to the caudal DC [72-74]. The nBOR homolog in mammals, the medial and lateral terminal nuclei (MTN and LTN), indirectly project to the rostral DC and VLO [75-77]. The DC is homologous to the mcIO in birds, according to Arends and Voogd (1989). The rostral-caudal division of different visual inputs to the inferior olive is conserved between mammals and pigeons. However, this topography was not clearly seen in the present study. In zebra finches, the nBOR projections were found in the entire rostrocaudal extent of the mcIO, and the LM terminals were found in rostral sections that also contained nBOR terminals. Instead of the rostral-caudal topography, the two visual inputs to the mcIO are clearly separated in the dorsal-ventral axis in all cases. This unique projection pattern of the two visual nuclei further emphasizes that zebra finches, and perhaps bird species that perform more forward flight, have different functional divisions in the inferior olive, compared to animals that forage on the ground like mammals and birds that often walk like pigeons. However, additional birds are required to further elucidate the extent of these projections and extending this study to additional species with different flight strategies could provide a fuller picture.

While the olivocerebellar connection has yet to be described in zebra finches, the climbing fiber input from different inferior olive subnuclei to different parts of the cerebellum has been demonstrated in pigeons and chickens [56, 57, 67]. The neurons in the dorsal and ventral lamellae of the IO project to the oculomotor cerebellum (OCb) in both pigeons and chickens [38, 67]. The OCb, comprised of folia VI to VIII, is thought to be important for obstacle avoidance and moving through cluttered environments, because it receives both global optic flow and local motion information from key visual nuclei [13]. The optic flow inputs predominantly arise from the LM, and to a lesser extent the nBOR, whereas the local motion signals arise from the optic tectum (TeO). In addition, the pontine nuclei also terminate in the OCb as mossy fibers. The pontine nuclei receive inputs from multiple sensory regions, including the ipsilateral TeO, LM, and the cerebral cortex. Previous electrophysiological recordings in pigeon have shown that pontine nuclei respond to directional motion from the contralateral visual field [25]. As in mammals, the avian pontine nuclei relay information from the cerebral cortex to the cerebellum. However, in birds, the medial spiriform nucleus is thought to play a stronger role in this connection [78]. While the
majority of the mcIO in pigeons does not project to the OCb, in chickens, the dMC, vMC, and a small part of the iMC are found to be additional sources of climbing fibers to the OCb, aside from the dl and vl [67].

Interestingly, Gaede et al (2019) found that the OCb in zebra finches has very few nBOR mossy fiber inputs (only about 4%) and almost 96% of the inputs arise from the pretectum [60]. Furthermore, within the pretectum, the LPC and PPC, instead of the LM, are the main inputs to the OCb [60]. If the mcIO-OCb connection found in chickens also exists in zebra finches, perhaps the LM and the nBOR inputs to the OCb are via this indirect olivocerebellar connection through some subdivisions of the mcIO. Further neuroanatomical investigation is necessary to uncover these potential connections.

The vestibulocerebellum (VbC, folia IXcd and X) receives optic flow information either directly via mossy fibers from the LM and the nBOR or via indirect inputs from olivocerebellar projections (figure 1C). The Purkinje cells in these folia respond to panoramic optic flow, resulting from translational or rotational self-movement. Cells with the same optic flow tuning are located at the same sagittal compartment, creating several optic flow zones in the VbC (figure 1C). The VbC integrates self-movement related sensory information, and is considered to be the key cerebellar region for adjusting body orientation and flight trajectory of the birds in open environments [13]. In pigeons, the olivocerebellar inputs to the VbC arise from the mcIO and these connections are topographically arranged. The rostral mcIO, which receives more nBOR inputs, projects to descent, ascent, expansion, and rH45 optic flow zones [56-58]. In these optic flow zones, the optic flow that activates the Purkinje cells contain either upward, downward, or backward visual motions, corresponding to the preferred direction of the nBOR. The caudal mcIO, which receives more LM inputs, projects to contraction and rVA zones [56-58]. In these zones, the optic flow includes a forward motion (the LM preferred direction) either in the entire or partial visual field. In chickens, this connection is mainly from the iMC, though in this study they did not investigate the topographical relationship between iMC and VbC [67].

Similar topographical olivocerebellar connections with different visual input sources exist in mammals. The DC and VLO are the main inferior olive nuclei that project to the flocculus, the homolog of the lateral extent of folium IXcd and X in birds. The caudal DC responds to horizontal optokinetic stimuli and projects to zones in the flocculus related to
horizontal eye movement, whereas the rostral DC and the VLO are modulated by upward motion and project to floccular zones that are related to vertical eye movement [36]. As previously described, the caudal DC receives more NOT (the LM homolog) inputs whereas the rostral DC and VLO receive indirect inputs from the MTN and LTN (the nBOR homolog). Whether these topographical arrangements observed in pigeons and mammals exist in other bird species requires further research. Future research on the projections of each mcIO subdivision to the VbC in zebra finches may provide more understanding of avian visuomotor pathways.

In many vertebrates studied to date, the zebrin immuno-positive and immuno-negative bands within a stripe pair are innervated by distinct regions of the inferior olive. Although the mcIO in pigeons is not well differentiated, the climbing fiber inputs to the zebrin immuno-positive and immuno-negative bands were found to originate from separate but adjacent regions of the mcIO [56, 57]. Similarly, in rats, the zebrin positive and negative bands receive climbing fiber inputs from different but adjacent IO subnuclei [70, 79].

The visual mossy fiber relationship to zebrin stripes has seldom been studied. In addition, not all mammals have this direct pathway [75, 80]. Here I compare my findings with a similar study carried out in pigeons. The results observed in the present study are similar to their findings [59]. The mossy fiber terminals (rosettes) from the LM and the nBOR overlap within the same parasagittal areas, predominately in the zebrin immuno-positive bands. However, when the number of rosettes is normalized to the area of each band, the LM and nBOR mossy fiber pathways seem to be diverged in different zebrin immunoreactive bands. More nBOR rosettes were found in the area-corrected negative bands, while LM inputs are likely to be more in the positive bands. Since the area effects were not dissected in the pigeon study, it is not known if per unit area of a zebrin band received more inputs from one visual nucleus versus the other. However, Pakan and colleagues stated that the mossy fiber terminals from the nBOR were more horizontally dispersed than that of the LM [59]. It is possible that the diverged mossy fiber pathways found in the zebra finches also exist in pigeons.

In the present study, I showed the projections from the LM and the nBOR to the medulla oblongata and the cerebellum in zebra finches. While several visuomotor pathways are conserved between mammals and pigeons, it is unknown whether similar connections
exist in other avian species that display different flight behaviors and have species-specific visual cue processing demands. In zebra finches, the highly differentiated mcIO and the unique distribution of the LM and nBOR axon terminals in mcIO subnuclei suggest: 1) they are more likely to have fine divisions of visual cue processing, 2) different types of visual information are directed to different inferior olive subgroups, and 3) it is possible that the olivocerebellar connections are different from the proposed models in pigeons and mammals. Zebra finches use forward flight as their primary mode of locomotion and the unique flap-bounding flight style is shared by many small bird species. Forward flight in small bird species may require them to evolve different visuomotor pathways. Future study using zebra finches may provide clues for understanding the evolution of the visuomotor systems in different bird species with different flight specialities.
3 Concluding Chapter

The sensorimotor system underlying various locomotion styles has long been of interest. Understanding the mechanisms of animal movement has potential for biomedical and engineering applications, but also has the potential to reveal fundamental principles of animal form and function. Bird flight is a remarkable behavior, and requires multiple adaptations in anatomy, physiology, and neural architecture. One of them is the visual system. The optic tectum of birds is massively enlarged relative to other vertebrates and nearly half of the avian brain, by volume, is dedicated to vision [22]. Presumably, these adaptations are what allow birds to respond to rapidly changing visual stimuli when navigating a three-dimensional space. In addition, birds of different flight specialities (e.g., hovering hummingbirds, nocturnal foraging owls) have further customised their visual processors in response to the specific visual stimuli they encounter in their routine behaviour [1]. In the present study, I examined the connections from the two optic flow nuclei in the midbrain to the cerebellum and medulla oblongata in zebra finches. This species has been an important animal model for vocal learning. My thesis presents data that suggests these birds are also excellent model organisms for studying visuomotor integration.

Self-movement generates global visual motion across the retina and this visual cue is known as optic flow [4]. The lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) are the two nuclei in the avian midbrain that process this information. Like all optic flow neurons, the LM and the nBOR neurons are tuned to specific direction and spatiotemporal frequencies of global visual motion [5]. The LM is biased toward forward motion in most species. Conversely, the nBOR neurons may prefer up, down or backward motion. The outputs of the LM and the nBOR terminate at several sensorimotor integration regions, including two regions of the cerebellum and the inferior olive of the medulla oblongata [24, 32]. There are two routes for the optic flow information to reach the cerebellum, the direct mossy fibers from the LM and the nBOR and the climbing fiber pathway via the inferior olive. I injected anterograde tracers of different fluorophores in the LM and the nBOR and tracked the projections of these nuclei to the inferior olive and different sagittal compartments of the cerebellum.

The inferior olive has three main divisions (dl, vl, and mcIO) in most bird species and the mcIO can be further divided into three subgroups (dorsal, medial, and ventral mcIO) [68].
The LM and the nBOR project mainly to the mcIO [24, 32]. Within the pigeon mcIO, the rostral part receives more nBOR inputs bilaterally whereas the caudal region is innervated more by the ipsilateral LM [58]. Similar connections between optic flow nuclei and the inferior olive have also been reported in mammals [72-77]. In the present study, I found that the mcIO of zebra finches is more differentiated than in any bird species reported so far [67, 68]. More than three well delineated subgroups were found in the zebra Finch mcIO and there is one additional main group in the rostral IO. The projections from the nBOR can be found in most of the mcIO subdivisions and in the additional group, whereas the LM axon terminals are mainly in the dorsal subdivisions. Similar to pigeons, the nBOR projections are bilateral and the LM projects mainly to the ipsilateral side. However, the rostrocaudal topography of the LM and the nBOR projections are not as clear in the zebra finches as in pigeons and mammals. Instead, it is clearer that the LM and nBOR axon terminals are separated in the dorsal-ventral axis. The complicated inferior olive structures, along with the unique visual nuclei projection patterns, suggest that the olivocerebellar connections in zebra finches may differ from the known topography shared by pigeons and mammals [36].

The visual mossy fiber pathways of birds have been studied mostly using pigeons. The LM projects to both the oculomotor cerebellum (OCb, folia VI to III) and the vestibulocerebellum (VbC, folia IXcd and X). The nBOR projects mainly to the VbC. I focus on the two visual nuclei projections to the VbC. In the VbC, there are several sagittal compartments, each with unique visual responses to panoramic self-motion related visual motion and each receives inputs from distinct regions of the inferior olive [56, 57]. In addition, the Purkinje cells in folium IXcd differentially express a biochemical marker, zebrin II, such that it forms parasagittally organized stripes with alternating zebrin-positive and -negative bands [48]. In this study, I immunolabeled the zebrin II in zebra Finch VbC and superimposed the mossy fibers of the LM and the nBOR. Generally, the zebrin stripe patterns in zebra finches were similar to that of pigeons [48]. The mossy fiber terminal patterns from the two visual nuclei were also similar to previous observations in pigeons [59]. The projections from the LM and the nBOR clustered in similar sagittal regions and most of the rosettes from both visual nuclei aligned with the zebrin immuno-positive bands. However, in this study, I further normalized the rosette numbers to the area of the zebrin bands. When the area effects were considered, per unit area of the immuno-negative bands received more
nBOR inputs, whereas the immuno-positive bands have more LM mossy fibers. This may suggest that different sagittal compartments in the VbC differ not only in the sources of the climbing fibers but also in their visual mossy fiber inputs.

The findings in the present study add further evidence to the hypothesis that birds with different flight specializations have evolved differences in their sensorimotor pathways to process species-specific visuomotor challenges. The special inferior olive structures and visual nuclei projection patterns found in the zebra finches open opportunities to revisit the visuomotor connections previously described in pigeons. Further research on these visual pathways in zebra finches, together with the known clues from pigeons and chickens, may accelerate our understanding of the evolution of visuomotor integration systems in different bird species. However, it should be noted that this project is just a pilot study and more work is necessary to draw any formal conclusions.

Limitations and future directions

In the present study, most of the findings are supported with sufficient sample size, but several observations should be confirmed with additional samples. In pigeons, the LM and the nBOR inputs to the mcIO are clearly separated in the rostral and caudal axis [58], but I found these visual nuclei projections to the inferior olive did not have a clear rostrocaudal topography in zebra finches. Instead, in all cases used in this study, the dorsal-ventral separation of the LM and nBOR inputs is more obvious. Because the rostrocaudal extent of the LM and nBOR inputs were determined from one bird (TG497), whether the lack of rostrocaudal topography is general for zebra finches or is just for these specific injection sites is yet to be verified. However, given that there are more subdivisions in the mcIO of zebra finches, it is possible that the visual nuclei-mcIO topography is different between zebra finches and pigeons. Despite this caveat, the unique neuroanatomical features found in the zebra finches open a new door for comparative study of the avian visuomotor pathways.

The majority of research on avian visuomotor pathways has been performed with pigeons. Although there are some studies using chickens, these studies tend to focus on the olivocerebellar connections with less attention paid to upstream optic flow inputs [67]. Research on other bird species is even sparser. It has been reported that the LM in hummingbirds has unique directional and spatiotemporal tuning to optic flow [2, 65].
However, relatively little is known about their visuomotor connections. A recent comparative study from our laboratory has provided evidence that pigeons, zebra finches, and hummingbirds differ by the proportion of LM and nBOR inputs to different regions of the cerebellum [60]. Among these birds, hummingbirds hover when feeding from flowers; pigeons often walk and forage on the ground; and chickens are only capable of transient flight. Zebra finches use forward flight as their primary mode of locomotion and foraging. Collectively, the previous work and the current study suggest that forward flight specialization may be associated with specific modifications to visuomotor pathways in birds.

To test my hypothesis, the first and the most important experiment is to conduct an electrophysiological and an anterograde tract tracing study in the IO subdivisions. In this study, the IO subgroups were tentatively given as numbers and letters because these structures are newly found in birds and it is unknown whether neurons in different subgroups have different cell responses and outputs to the cerebellum. Therefore, the recording of cell responses to visual motion will reveal if these subgroups are physiologically different. Given that in chickens, the OCb is also innervated by subdivisions of the mcIO [67], it is worth testing if any subdivisions of the mcIO found in zebra finches also have projections to the OCb. An anterograde labeling experiment will reveal whether each IO subgroup projects to different regions of the cerebellum.

In pigeons, the rostral and caudal mcIO receive different amounts of LM and nBOR inputs, and project to different optic flow zones in the VbC [56, 57]. In addition, neurons in distinct regions of the mcIO have similar optic flow responses to the Purkinje cells that they innervate in the VbC optic flow zones [40]. In zebra finches, the stratified inferior olive and the dorsal-ventral separated LM and nBOR inputs in the mcIO suggest that various visual cues involved in forward flight may be directed into different subgroups of the IO. Each IO subdivision subsequently transmits the various sensory information to different sagittal compartments of the cerebellum. Because the Purkinje cell activities arise from the climbing fiber inputs, if different subnuclei of the IO have different visual inputs and cell responses and project to different regions of the cerebellum, it is possible that zebra finches have different optic flow zone organizations in the cerebellum. To examine this, an electrophysiological study could be done in the cerebellum. This could reveal whether the
optic flow zones found in pigeons exist in zebra finches and are organized in similar ways.

For another anatomical study, it would be informative to inject a retrograde tracer in different functional zones of the cerebellum. The retrograde tract tracing can give more detail of the topographical arrangements of the olivocerebellar connections. In addition, it can test if different functional zones receive mossy fibers from subgroups of LM and nBOR cells. The anterograde tracing in the present zebra finch and previous pigeon study show that the projections from these visual nuclei were largely overlapping in the VbC. However, the anterograde tracers injected in the LM and the nBOR usually cover a large area of these nuclei; it is still not known if a finer topography exists or if cells projecting to different zebrin bands are morphologically different.

Finally, the hypothesis that the subdivisions of the IO are associated with forward flight specialization can be assessed using existing Nissl stained brains from diverse species. Such collections have already been used to establish hypertrophies in different brain areas (e.g., hippocampus, LM) [3, 81, 82]. The present study points to the IO as another potential site of interest for anatomical, physiological, and comparative study.
References

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