Topography of Optic Flow Processing in Olivo-Cerebellar Pathways in Zebra Finches (*Taeniopygia guttata*)

Douglas R. Wylie[1], Andrea H. Gaede[1,2,3,*], Cristián Gutiérrez-Ibáñez[1,*], Pei-Hsuan Wu[3,*], Madison C. Pilon[1], Serena Azargoon[3] and Douglas L. Altshuler[3]

[1]Department of Biological Sciences, University of Alberta, Edmonton, AB, T6G 2E9, Canada

[2]Structure and Motion Laboratory, Department of Comparative Biomedical Sciences, Royal Veterinary College, London, United Kingdom

[3]Department of Zoology, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada

*The contribution of these authors was approximately equal.

To whom correspondence should be addressed: Douglas R. Wylie (dwylie@ualberta.ca)

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Abstract

In birds, the nucleus of the basal optic root (nBOR) and the nucleus lentiformis mesencephali (LM) are brainstem nuclei involved in the analysis of optic flow. One of the major projection sites of both nBOR and LM is the medial column of the inferior olive, which provides climbing fibres to the vestibulocerebellum. The organization of this pathway has been well documented in pigeons, but little is known in other birds. Recent works have highlighted that zebra finches show specializations with respect of optic flow processing, which may be reflected in the organization of optic flow pathways to the inferior olive. In this study, we use anterograde and retrograde tracers to characterize the organization of these pathways in the zebra finch. First, we found that the medial column in zebra finches consists of at least 8 subnuclei (i-viii) visible in Nissl-stained tissue. We then examined the projections of the LM and nBOR to the inferior olive using anterograde traces, followed by injections of retrograde tracers in the posterior cerebellum to determine the projections of the inferior olive. The projections from LM and nBOR to the inferior olive were bilateral, but much heavier to the ipsilateral olive, and showed a complementary pattern: LM projected to subnucleus i, whereas nBOR projected to ii and v. The retrograde experiments revealed that these subnuclei project to the vestibulocerebellum (folia IXcd and X), whereas the other medial column subnuclei project to IXab and the lateral margin of VII and VIII. The nBOR also projected ipsilaterally to the caudo-medial dorsal lamella of the inferior olive, which the retrograde experiment showed as projecting to the medial margin of VII and VIII. We compare these results with previous studies in other avian species.

List of Abbreviations

III	third cranial nerve
CbL	lateral cerebellar nucleus
CtG	central grey
D	nucleus of Darkschewitsch
DGCs	displaced ganglion cells
dl	dorsal lamella (of inferior olive)
gl	granule cell layer (of the cerebellum)
Glv	ventral leaflet of the lateral geniculate nucleus
GT	tectal grey
IC	interstitial nucleus of Cajal
ic	intercalated subnucleus (of the medial cerebellar nucleus)
im	intermediate subnucleus (of the medial cerebellar nucleus)
in	internal subnucleus (of the medial cerebellar nucleus)
Imc	nucleus isthmi, pars magnocellularis
IO	inferior olive
LM(m/l)	nucleus lentiformis mesencephali (medial/lateral subnuclei)
LPC	nucleus laminaris precommisuralis

67	mc/MC	medial column (of inferior olive)
68	ml	molecular layer (of the cerebellum)
69	nIII	oculomotor complex
70	nIV	trochlear nucleus
71	nBOR	nucleus of the basal optic root
72	PCl	Purkinje cell layer (of the cerebellum)
73	pcv	cerebellovestibular process
74	PT	nucleus pretectalis
75	RGCs	retinal ganglion cells
76	Rt	nucleus rotundas
77	Ru	nucleus ruber
78	SOp	stratum opticum
79	SP	nucleus subpretectalis
80	SpL	lateral spiriform nucleus
81	TeO	optic tectum
82	TrO	tractus opticus
83	vl	ventral lamella (of inferior olive)

VeL	lateral vestibular nucleus
VeM	medial vestibular nucleus
VeS	superior vestibular nucleus
VTA	ventral tegmental area
XII	twelfth cranial nerve
ZII	zebrin II (aldolase C)

1. Introduction

In all vertebrates, specialized visual pathways are involved in the analysis of optic flow, the motion that occurs across the entire retina during self-motion (Gibson, 1954). These visual pathways include retinal-recipient nuclei in the accessory optic system and pretectum (for reviews see Simpson, 1984; Gamlin, 2006; Giolli et al., 2006). In birds, these nuclei are the nucleus of the basal optic root (nBOR) of the accessory optic system (Brecha et al., 1980), and the pretectal nucleus lentiformis mesencephali (LM) (see Fig. 1) (Gamlin and Cohen, 1988a). The visual response properties of neurons in LM and nBOR are very similar; in both nuclei, most neurons have large receptive fields in the contralateral visual field and exhibit direction-selectivity in response to large-field visual motion (Morgan and Frost, 1981; Winterson and Brauth, 1985). The neuronal responses of these optic flow nuclei have been studied in several species of birds, including both pigeons and zebra finches (Burns and Wallman, 1981; Wylie and Frost, 1990; Wylie et al., 1994; Wylie and Crowder, 2000; Gaede et al., 2017, 2022; Smyth et al., 2022). While much of this work emphasizes the similarities (Crowder and Wylie, 2001; Ibbotson and Price, 2001; Gaede et al., 2022), species specific differences exist and these are likely related to the visual ecology of each species (Wylie et al., 1994; Iwaniuk and Wylie, 2007, 2020; Gaede et al., 2017; Smyth et al., 2022). With respect to visual response properties, in both pigeons and zebra finches, a heavy majority of LM neurons respond best to temporal-to-nasal motion (Winterson and Brauth, 1985; Wylie and Frost, 1996; Wylie and Crowder, 2000; Crowder et al., 2003; Gaede et al., 2017; Smyth et al., 2022) whereas in nBOR neurons are responsive to either upward, downward or nasal-to-temporal motion, with approximately equal proportions in both species (Wylie and Frost, 1990; Gaede et al., 2022). However, there are some species-specific differences. In particular, optic flow neurons in zebra finches tend to be tuned to stimulus velocity, whereas in pigeons and hummingbirds they show more tuning to temporal frequency

(Smyth et al., 2022). Differences also exist related to connectivity. In pigeons, projections from LM to the oculomotor cerebellum arise from the medial LM whereas in zebra finches (and hummingbirds) these arise from structures just medial to LM, the nucleus laminaris precommissuralis and nucleus principalis precommissuralis (Gaede et al., 2019).

One of the major projection sites of both nBOR and LM is the inferior olive (IO), which provides climbing fibre input to cerebellar Purkinje cells to produce complex spike activity (Eccles et al., 1966; Thach, 1968; Belcari et al., 1977; Simpson et al., 1996). This circuitry is outlined in Figure 1. In pigeons it has been shown that nBOR and LM project to the medial column (mc) of the IO (Fig. 1a_2,b_4) (Brecha et al., 1980; Gamlin and Cohen, 1988b), which in turn projects to folia IXcd and X (the vestibulocerebellum) (Fig. 1b_5) where complex spike activity is modulated by particular patterns of optic flow resulting from either self-rotation or self-translation (Graf et al., 1988; Wylie and Frost, 1991; Wylie et al., 1998a). In pigeons, the organization of inputs from LM and nBOR to the mcIO, as well as the organization of the projections from the IO to the cerebellum have been extensively documented (Gamlin and Cohen, 1988b; Wylie et al., 1997; Wylie, 2001; Pakan et al., 2010; Brecha et al., 1980). In contrast, nothing is known about these pathways in the zebra finch. Given the above-mentioned differences between pigeon and zebra finches in neural responses and connectivity of LM and nBOR, as well as documented differences in the organization of the IO and the cerebellum among birds (Vogt-Nilsen, 1954; Cunha et al., 2021), it is possible that species-specific differences also exist in the organization of visual pathways to the IO.

The mc of IO in zebra finches appears very complex compared to other avian species, consisting of numerous subnuclei, which we document in this manuscript. We also examined the projection of the LM and nBOR to the mc using anterograde techniques, and the projection from

the IO to the posterior cerebellum using retrograde techniques. We show that only some of the mc subnuclei receive projections from LM and nBOR, which in turn project to the vestibulocerebellum. Those subnuclei of the mc that receive input from LM and nBOR project mainly to folium IXcd[1], in addition to other folia in the posterior lobe.

2. Methods

2.1 Animals: All experimental procedures were approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. Eighteen adult male zebra finches (*Taeniopygia guttata*, 13-16 g; Eastern Bird Supplies, Quebec, Canada) were used in this study. One bird (TG491) was perfused as described below, and the brain was extracted, sectioned and Nissl stained to provide a precise delineation of the IO (see below, Fig. 4). Eight of the birds were used for anterograde tracing experiments to examine the projections of nBOR and LM. Nine of the birds were used for retrograde tracing experiments to examine the projection from the inferior olive to the cerebellum.

2.2 Surgery: 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 and subcutaneous injections of 0.9% saline were given for hydration. Once anesthetized, the birds were placed in a stereotaxic frame designed for small bird neurosurgery (Herb Adams Engineering; Glendora, CA, USA). To align the head orientation to a zebra finch brain atlas (Nixdorf-Bergweiler and Bischof, 2007), ear bars were pinned against the otic process of the quadrate bone, which lies in the anterior part of the opening to the external

[1] Larsell (1967)used "folia" rather than lobules to describe the cerebellar cortex in birds.

acoustic meatus and the beak was secured to an adjustable beak bar. In this set-up, the head was pitched downward 45° to the horizontal plane.

2.3 Injections of Anterograde Tracer in LM and nBOR: A craniotomy was performed over the telencephalon to allow vertical penetrations with micropipettes into the LM and the nBOR on the right side of the brain. To ensure placement in LM and nBOR, extracellular activity of single units to moving large-field stimuli was recorded. We used glass micropipettes with tip diameters of ~20 µm filled with 2M NaCl, which were advanced through the brain using a microdrive (Frederick Haer & Co. Millville, NJ, USA). Extracellular signals were amplified, filtered and played over an audio monitor (A-M Systems). Upon isolation of a unit in LM or nBOR, the direction preference of the unit was qualitatively determined by moving a large (90x90°) handheld visual stimulus, consisting of black bars, wavy lines and dots on a white background, in the receptive field of the unit. With such stimuli, LM units can be easily identified (Pakan et al., 2010). Upon confirming the location of LM or nBOR with the recording, the pipette was retracted, emptied and refilled with a fluorescent dextran (10% in 10mM PBS), either Texas red (red; D3328; 3000 molecular weight; Invitrogen) or fluorescein (green; D3306; 3000 molecular weight; Invitrogen), through the tip by suction. The pipette was then repositioned at the recording location, and the dextran was injected iontophoretically (± 4.5 µA, 7 sec on, 7 sec off) for 15-40 min, followed by 5 min of rest. In some cases, after the first injection in LM or nBOR, a new micropipette was used to record from and inject in the other nucleus. At the end of the surgery, the craniotomy was filled with bone wax and the incision was sealed with cyanoacrylate (Vetbond, 3M).

2.4 Injections of Retrograde Tracer in the posterior cerebellum: To access the folia of the posterior cerebellum (VI, VII, VIII, IXab, IXcd and X), bone was removed from the dorsomedial

surface of the cerebellum, lateral to the midsagittal sinus. In some cases, the lateral margin of the VbC (IXcd and X) was accessed by removing the bone surrounding the semi-circular canals. The dura was removed, and glass micropipettes (20-30 mm tip diameter) were filled with cholera-toxin subunit B (CTB) conjugated with either AlexaFluor 488 (green, C22841, Thermo fisher) or AlexaFluor 594 (red, C34777, Thermo fisher). In three cases (TG452, TG459, TG466), the injections were made using a nanoinjector (Nanoject II, Drummond Scientific). The pipette was inserted into the targeted folium and multiple injections of 13.8 nl were made, one minute apart. In each of these three animals, 100-200nl in total were injected. In the other animals, the injections were made with iontophoresis (± 4 μA, 7 s ON, 7 s OFF, for 15 min), and were typically smaller in size. At the end of the injection period, the electrodes were left undisturbed for 5 min, and then withdrawn.

2.5 Brain Extraction and Sectioning: After four days in recovery, birds were deeply anesthetized with ketamine/xylazine (i.m.) and transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). The brains were then removed and stored in 4% PFA overnight at 4°C. For cryoprotection, brains were transferred into 30% sucrose in 0.01M PBS until they sank. Next, the brains were embedded in gelatin and again cryoprotected in 30% sucrose in PBS overnight. Brains were sectioned into three series at 40 μm in the coronal plane using a freezing stage microtome (American Optical Company, model 860; Buffalo, NY, USA). Except for those sections processed for zebrin II immunohistochemistry (see below), the tissue sections were mounted on gelatinized glass slides, dried, and stored at +4°C. For those anterograde cases where there was an injection in LM, a few drops of *SlowFade Gold antifade* reagent with DAPI (Invitrogen, Eugene, OR, USA) was applied to the slides with sections

through the pretectum. This was done because it assists in identifying the borders of LM subnuclei (Gutierrez-Ibanez et al. 2018).

2.6 Immunohistochemistry: In two of the anterograde cases (TG502, TG503), a few sections through cerebellar folium IXcd were immunostained for zebrin II (ZII; a.k.a., aldolase C) as described previously (Wylie et al., 2017) (see Table 1). This was done to determine if the mossy fibre terminals from LM and nBOR tend to align with the ZII +ve stripes as is the case in pigeons (Pakan et al., 2010). Free-floating sections were first washed with PBS three times, then blocked 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 2.5% normal donkey serum and the primary antibody to aldolase C (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. After washes in PBS (5 x 5 minutes), sections were transferred into Alexafluor-594 (red) or 488 (green) conjugated donkey anti-goat IgG (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. Finally, sections were washed five times, then mounted on gelatin-coated slides to be imaged.

In previous studies (e.g., Pakan et al., 2007), we used an anti-zebrin II antibody provided by Richard Hawkes that recognizes a single polypeptide band with an apparent molecular weight of 36 kDa in mouse and pigeon. Cloning studies showed it to be the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995; Pakan et al., 2007). The pattern of labeling seen in the zebra finch cerebellum with the anti-zebrin II used in the present study is essentially identical to that seen with the anti-zebrin antibodies used previously in several species of birds including pigeons *(C. livia)* (Pakan et al., 2007, 2010, 2011, 2014; Pakan and Wylie,

2008; Wylie et al., 2011, 2012, 2013, 2017; Graham and Wylie, 2012; Craciun et al., 2018; Long et al., 2018), chickens *(G.galllus domesticus)* (Marzban et al., 2010; Vibulyaseck et al., 2015), hummingbirds *(C. anna; S. rufus)* (Iwaniuk et al., 2009a), tinamous *(N. perdicaria)* (Corfield et al., 2015) and kiwi *(A. mantelli)* (Corfield et al., 2016).

2.7 Microscopy and Image Analysis: To acquire fluorescent images of the injection sites and terminal labelling, a couple of drops of PBS were applied to the slides and they were coverslipped. Sections were viewed with a compound light microscope (Leica DM6B, Concord, ON) equipped with TX2 (red), L5 (green) and DAPI (blue) fluorescent filters. Images were captured with either a DFC7000 T or K5 (both Leica, Concord, ON) camera using Leica Application Suite X imaging software. Adobe Photoshop was used to compensate for brightness and contrast.

After fluorescent images were acquired, the coverslips were removed and the slides were allowed to dry. Because we were interested in obtaining a precise delineation of the topographic projection from LM and nBOR to the subnuclei of the IO, and from the IO to the cerebellum, the sections through the IO were Nissl stained using thionin and coverslipped with Permount. Likewise, the sections through the injection sites were also stained for Nissl.

3. Results

3.1 Anterograde Experiments

3.1.1 Injections sites: Table 2 provides a list of the eight cases that received injections in the LM and/or nBOR. Note that three birds (TG502, TG503, TG513) received injections in both nBOR and LM. The first six cases in the list were used for detailed description of the terminal labelling

in the IO, and their injection sites are shown in Figure 2. Figure 2a_1-a_3 (and b_1-b_3) illustrate the procedure we used to localize the injection sites. A fluorescent image was obtained (Fig. 2a_2, b_2), the section was stained for Nissl (Fig. 2a_1, b_1), and a drawing was made of the overlay (Fig. 2a_3, b_3). Of the five LM injections, two were located rostrally (Fig. 2a,f), two were located caudally (Fig. 2d,e) and one was located at a mid-rostro-caudal level (Fig. 2c). These injections were 100-300μm in diameter, virtually confined to LM, and encroached upon both the medial and lateral subnuclei (LMm, LMl). The four nBOR injections were of similar size, confined to the nBOR, and localized to the anterior (Fig. 2g) or posterior (Fig. 2h-i) halves.

3.1.2 *Projections of the LM and nBOR in Zebra Finches:* Entirely consistent with previous anatomical studies of the nBOR and LM in pigeons (Clarke, 1974, 1977; Brauth and Karten, 1977; Brecha and Karten, 1979; Gamlin and Cohen, 1988b; Wylie and Linkenhoker, 1996; Wylie et al., 1997, 1998b; Brecha et al., 1980), anterograde labelling was found in several nuclei in the brain. From injections in the LM, terminals were seen in the ipsilateral nBOR (Fig. 3b) and the dorsal thalamus (Fig. 3c), and bilateral labelling was seen in the IO (Fig. 3d). From the nBOR, terminals were also seen in the dorsal thalamus (not shown), the contralateral nBOR (Fig. 4f), and there was a very heavy projection to the ipsilateral LM that was directed to LMl (Fig. 4g). From nBOR injections, terminals were found bilaterally in all subdivisions of the oculomotor complex (nIII) (Fig. 4c), and the trochlear nucleus (nIV) (Fig. 4d). Terminals from the nBOR also targeted the accessory oculomotor nuclei bilaterally, with heavy labelling observed in the interstitial nucleus of Cajal (IC) and some labelling in adjacent structures including the central grey (CtG), nucleus of Darkschewitsch (D) and nucleus ruber (Ru) (Fig. 4e). As with the LM injections, terminal labelling was also found bilaterally in IO (Fig. 4b).

From the nBOR, a massive bundle of thick fibres travelled in the brachium conjunctivum to give rise to cerebellar mossy fibres (Fig. 5c, h). Collaterals from these fibres terminated in the medial and lateral cerebellar nuclei (Fig. 5h_1, h_2), as well as the cerebellovestibular process (pcv) (Fig. 5h_1, h_3). A few of these fibres also had collaterals terminating in the vestibular nuclei (not shown). Fibres from the LM also followed this course to the cerebellum but collaterals to the cerebellar nuclei, the pcv, and vestibular nuclei were not observed. The terminals of the mossy fibres from LM and nBOR are shown in Figure 5b-g, which end as rosettes (Fig. 5b) and tend to cluster into sagittal zones in the granular layer (gl) of folium IXcd (Fig. 5c, d, f, g). As has been shown in pigeons, the majority of these mossy fibres terminate adjacent to sagittal stripes of Purkinje cells that show a high level of zebrin II expression (Fig. 5f, g). Although the mossy fibres from nBOR mainly target IXcd, some were seen in folia VI-IXab. From the LM, again the target was mainly IXcd, but many were found in folia VI-VIII (Fig. 5e). No terminals were seen in X from either nBOR or LM.

In pigeons, different neurons in the LM and nBOR project to the IO and cerebellum. Large neurons of the nBOR, along with similarly sized neurons in LMm, LMl project as mossy fibres to the cerebellum; these are neurochemically distinct from those that project to the IO. The nBOR neurons that project to IO are small and confined to the dorsal subnucleus and the adjacent VTA. The LM neurons that project to the IO are located along the border between LMm and LMl (Gamlin and Cohen, 1988b; Wylie and Linkenhoker, 1996; Wylie, 2001; Pakan et al., 2006; Winship et al., 2006; Wylie et al., 2007, 2008; Iwaniuk et al., 2009b; Brecha et al., 1980).

A few descending fibres from LM and nBOR also target the pontine nuclei (not shown). Because all these observations are consistent with the aforementioned studies in pigeons, they

will not be discussed any further. The remainder of this section will focus on the bilateral projections to the IO (Fig. 3d, 4b), which targeted specific subnuclei.

3.1.3 The Subnuclei of the Inferior Olive in Zebra Finches: Figure 6a shows 20 serial coronal sections (40μm intervals) throughout the zebra finch IO from rostral (Fig. $6a_1$) to caudal (Fig. $6a_{20}$). The left and right sides are mirror images, with drawings of the subnuclei on the righthand side. The ventral lamella (vl), dorsal lamella (dl) and medial column are shown in white, light grey, and dark grey, respectively. In the zebra finch brain, the medial column (mc) spans the rostral 3/4ths of the IO (Fig. $6a_2$-a_{15}), and we identify at least eight subnuclei, i-viii, from caudal to rostral. The delineation into these subnuclei is motivated in part by the pattern of terminal labelling from nBOR and LM (see below). Caudally, the fibres of the twelfth cranial nerve (nXII; Fig. $6a_{12-16}$; dashed lines) can clearly be seen, and the mc is first present at around a_{15}. Three subnuclei oriented from dorsomedial (i) to ventrolateral (iii) are apparent and persist until a_{12}. At a_{11}, ii disappears and subnucleus iv emerges medial to i. Subsequently, at a_{10}, iii fades away, and v appears just ventral to i. We consider subnucleus v as a collection of subnuclei that can be difficult to delineate in some specimens. Nonetheless there appears to be dorsal, (V_a; Fig. $6a_{7-8}$; ventrolateral (V_c; Fig. $6a_{7-10}$) and ventromedial (V_b; Fig. $6a_8$) divisions. V_a and V_b appear to merge and continue rostrally as v_r (Fig. $6a_{4-6}$). V_b is clearly the smallest of these and is usually only seen in single coronal section. Ventromedially, subnucleus vi appears (Fig. $6a_{5-7}$), and lastly subnuclei vii and viii emerge laterally (Fig. $6a_{4-6}$) and continue towards the rostral end of the IO as subnuclei iv, v and vi all fade away.

For comparison, three sections are shown through the pigeon IO (from archival material) to illustrate the comparatively simple structure consisting of the dl, vl and mc that are not further divided into subnuclei (Fig. $6b_{1-3}$).

3.1.4 Projections to the Inferior Olive from LM and nBOR: In all cases, terminal labelling was observed bilaterally in the IO from injections in nBOR and LM (e.g., Fig. 3d, 4b). For both nBOR and LM, the projection was heavier to the ipsilateral, and for the LM the contralateral projection was very weak, sometimes appearing in only one or two sections through the IO. The three cases involving injections in both LM and nBOR (see Table 2) were particularly useful as it was clear that the nBOR and LM were targeting specific olivary subnuclei. This is shown in Figure 7 for case TG513. Fluorescent images of terminal labelling in seven sections through the IO (Fig. 7$b_{1\text{-}7}$; rostral to caudal) and the same sections stained for Nissl are shown (Fig. 7$a_{1\text{-}7}$). Fluorescent images of terminal labelling are shown from the other cases as well (Fig. 7$c_{1\text{-}7}$). En route to the IO, the fibres from LM course ventromedially to join those from the nBOR, and then travel caudally as described by (Brecha et al., 1980) for pigeons. They course through the ventral tegmentum just medial and dorsal to the lateral pontine nucleus, and caudal to the pontine nuclei they pass through the lemniscus spinalis between the ventral edge of the brainstem and the corpus trapezoideum. In the IO, the terminal fields of nBOR and LM are clearly complementary for the most part. Ipsilateral to the injections, terminals from the LM target subnucleus i of the mc throughout its rostrocaudal extent (Fig. 7$b_{2\text{-}7}$, $c_{2,4\text{-}6}$). Very little terminal labelling from LM was seen in other subnuclei in the ipsilateral IO: a few terminals were observed in lateral parts of v_a and v_c (Fig. 7$b_{2,3}$, c_2) and ii (Fig. 7b_6, $c_{5\text{-}6}$). From LM injections, the small amount of terminal labelling in the contralateral IO was observed in i, v_a and v_c (Fig. 7$c_{2,4}$; Fig. 3d). The projection from nBOR to the ipsilateral IO terminated heavily v_r (Fig. 7b_1, c_1) and v_b (Fig. 7b_3, $c_{2\text{-}3}$), as well as v_a and v_c. In v_a and v_c the labelling was heavier in the medial regions (Fig. 7$b_{2\text{-}3}$, $c_{2\text{-}3}$). Further caudally, labelling was heavy in ipsilateral ii (Fig. 7$b_{5\text{-}7}$, c_7) and, surprisingly, in a subnucleus of the dl, just lateral to the fibres of nXII (Fig. 7$b_{6,7}$, c_7). Otherwise on the ipsilateral side, labelling

was sparse, with a few terminals in iii (Fig. 7b6,7) and vi (Fig. 7b1, c1). The labelling in the contralateral IO was heavier from the nBOR compared to LM, but still much less than the labelling in the ipsilateral IO. V_b (Fig. 7b3, c2-3) and the medial aspects of v_a and v_c (Fig. 7b3,c3) in the contralateral IO contained a moderate amount of terminals from nBOR, and labelling was not insubstantial in i (Fig. 7b3-7, c1-3), v_r (Fig. 7b1, c1), and ii (Fig. 7b6,7). Very few terminals were observed in the contralateral iii (Fig. 7b6,7) and vi (Fig. 7b1, c1).

Based on the pattern of terminal labelling we observed across all cases, Figure 8 offers a summary of the projections of LM and nBOR to the IO using the drawings of the subnuclei from Figure 6. The terminal fields of LM and nBOR are shown, respectively, with green and magenta, respectively, and a more saturated colour represents a stronger projection. The right side is ipsilateral to the injection sites. The projection of LM is mainly directed at subnucleus i through its rostro-caudal extent (Fig. 8a5-15). Terminals from LM are sparse otherwise, but some were apparent in the lateral aspects of v_a and v_c (Fig. 8a7-10). The projection to the contralateral IO from LM was very weak, but the few fibres observed terminated in the lateral aspect of i, v_a and v_c (Fig. 8a7-11). The projection from nBOR to the ipsilateral IO was very heavy in all parts of v: v_r (Fig. 8a4-6), vb (Fig. 8a8), and the medial parts of v_a and v_c (Fig. 8a7-10). Further caudally, ii was heavily labelled as was a subnucleus in the dl, just lateral to nXII (Fig. 8a12-15). The projection from nBOR to the contralateral IO was heaviest to v_b and the medial aspect of v_a (Fig. 8a7-8) but also included v_r, v_c, i and ii (Fig. 8a4-15). Except for the odd apparent en passant swelling, subnuclei iii, iv, vi vii and viii were devoid of labelling from nBOR and LM.

3.2 Retrograde Experiments

3.2.1 Injections sites: Retrograde tracers were injected in the cerebellum of nine zebra finches. Although we only describe in detail the retrograde labelling in the IO, as most of the injections encroached upon the granule cell layer, retrogradely labelled neurons were observed in several nuclei that are known to provide mossy fibre input to the avian cerebellum. Consistent with previous studies we observed retrograde labelling in LM, nBOR, the medial spiriform nucleus, the vestibular and cerebellar nuclei and the pontine nuclei (Brodal et al., 1950; Clarke, 1974, 1977; Karten and Finger, 1976; Gamlin and Cohen, 1988b; Wild, 1992; Pakan and Wylie, 2006; Pakan et al., 2006, 2008; Wylie et al., 2007, 2008; Iwaniuk et al., 2009b; Gaede et al., 2019; Gutiérrez-Ibáñez et al., 2022; Brecha et al., 1980).

All of the injection sites are shown in Figure 9, and the distribution of retrograde labelling in the IO subnuclei is indicated in Table 3 and shown in Figures 10, 11 and 12. While six of the animals received a single injection (Fig. 9a-c,g-i), three received injections of both red and green CTB in different folia (Fig. 9d-f). These experiments allow for comparison of the retrograde IO labelling from the injections in IXcd and X, which we expected to occur in the subnuclei receiving input from nBOR and LM, with injections in VII, VIII and IXab, which we expected to label other subnuclei. Photomicrographs of retrograde labelling in the IO from each of the cases is shown in Figures 10 and 11, and drawings of serial sections through the IO are shown for five of the cases in Figure 12.

3.2.2 Retrograde labelling in the IO from injections in the Vestibulocerebellum (IXcd and X): There were four cases in which the injection of CTB was confined to folia IXcd and/or X. In case ZF16-08, the injection was centred on the midline of the ventral lamella of IXcd and about 500 μm in width (Fig. 9a). Olivary labeling was observed bilaterally but confined to mc subnucleus ii in four serial sections in the caudal mc (Fig. 12a). The labelling was heavy and the

neurons densely packed (Figs. 10a). In case ZF16-16, the injection was also at the midline but in the anterior end of folium X (Fig. 9b). It was smaller, about 400μm in width and appeared not to cross the midline as olivary labelling was only observed in the right (contralateral) IO in three serial sections. As with case ZF16-08, mc subnucleus ii was heavily labelled with densely packed cells (Fig. 10d$_1$). Smaller clusters of labelled cells were seen in mc subnuclei i, and v$_b$ (Fig. 10d$_2$). In case ZF17-12, the red injection was in the flocculus, the lateral protuberance of the vestibulocerebellum (Larsell, 1967). It was small, about 350 microns in width and confined to IXcd (Fig. 9d). Retrograde labelling was heavy throughout subnuclei i and v (Figs. 10c, 12b). With respect to v, the heaviest labelling was found in v$_a$, v$_b$ and v$_r$, but sparse labelling was seen in v$_c$ (Fig. 10c$_2$, 12b$_{1-5}$). The injection in case ZF16-12 was also in the flocculus and included the lateral poles of both IXcd and X (Fig. 9c). The retrograde labelling was confined to the rostral half of mc subnucleus i (Fig. 10b) and only observed in two serial sections.

3.2.3 Retrograde labelling in the IO from injections in folium IXab: The injection of red CTB in case ZF16-10 was the only one restricted to IXab. About 500μm in width, it was centred in the white matter, just left of the midline (Fig. 9f). Olivary labelling was very heavy throughout the rostro-caudal extent of mc subnuclei iii and iv (Figs. 11a, 12d). A few scattered cells were also observed in v$_c$ (Fig. 12d$_{2-4}$), v$_b$ (Fig. 12d$_3$), vii and dl (Fig. 12d$_1$). The only other case involving an injection in IXab was case ZF16-09. The red injection was anterior in the white matter subserving both IXab and IXcd (Fig. 9e$_1$). Consistent with the other cases involving IXcd, retrograde labelling was observed in mc subnuclei i (moderate in caudal regions), ii (heavy), and v (Figs. 10e$_{1,2}$, 12c). In v, the labelling was heavy in v$_c$ (Figs. 10e$_2$, 12c$_{2-4}$) but absent otherwise except for a few cells in v$_a$ and v$_r$ (Figs. 10e$_3$, 12c$_{1-3}$). Presumably from the involvement of the injection in IXab, labelling was also heavy in iv (Figs. 10e$_2$, 12c$_{2-4}$), moderate in iii (Figs. 10e$_2$,

12c2-6) and very heavy in vi (Figs. 10e3, 12c1-2). A cluster of cells in the medial part of vl was also seen in several serial sections (Figs. 10e2, 12c2-5).

3.2.4 Retrograde labelling in the IO from injections in folia VII and VIII: There were six cases that received injections in folia VII or VIII (Table III). The green injection in case ZF16-10 was small (<300μm in width) and abutted the midline of VIII (Fig. 9f). This injection did not appear to cross the midline as retrograde labelling was only observed in the right (contralateral) IO. Labelling was largely restricted to the dl, although there were a few scattered cells in iii (Figs. 10a, 12d). The heaviest concentration of labelling was caudo-medially in the dl (Figs., 11a2, 12d6). Rostrally, these green labelled cells in dl were just lateral to, and somewhat intermingled with the cluster of red labelled cells in iii from the injection in IXab (Fig. 11a1, 12d1-4). A very similar pattern of labelling was observed from the green injection in ZF16-09. This injection was also small (about 350μm wide) and abutted the midline, but in folium VII (Fig. 9e2,3). Retrograde labelling was restricted to the medial half of dl, with the emphasis to mid-caudal regions (Fig. 12c4-5)). The green injection in case ZF17-12 was larger (>800μm wide) and extended more laterally (Fig. 9d). Most of the retrograde labelling was in dl (Fig. 10c2) but extended more laterally and rostrally compared to cases ZF16-09 and ZF16-10, and the density of labelling was highest more rostrally (Fig. 12b1-3). A few cells were also seen in the medial vl (Fig. 12b1), and iii (Fig. 12b4-6). The injection in case TG452 was also in VIII, but larger (≈1mm wide) and extended more laterally (Fig. 9g). Heavy labelling was observed in both the dl and the medial vl (Fig. 11c). For case TG466, the injection was quite large, spanned folium VIII, and was ellipsoid in shape. The rostro-lateral extent of the injection is shown in Figure 9i, but in more caudal sections the injection shifted medially and crossed the midline. In the left side of the IO, retrograde labelling was restricted to the caudo-medial dl, with a few scattered cells in iii (Fig.

12e5-6). On the right side, there was heavy labelling in the vl and dl (Figs. 11d, 12e), as well as a distinct cluster in the rostral part of viii (Fig. 12e1). A cluster was also observed in iv (Fig. 12e4,5), in addition to a few scattered cells in iii and vii (Fig. 12e2,6). This diverse pattern of labelling was also observed in case TG459. The injection spanned the entire medio-lateral extent of the left side of folium VIII (Fig. 9h). The injection did cross the midline, as several retrogradely labelled cells were observed in the medial extreme of the caudal dl, and two cells were seen in iii. On the right side, labelling was heavy throughout vl and dl (Fig. 11b). In addition, there was a cluster of labelled cells in iv (Fig. 11b5-6), a moderate amount of labelling in vii and viii (Fig. 11b1-2), and a few scattered cells in iii.

4. Discussion

Optic flow is initially analyzed by two retinal recipient nuclei in the avian brain: nBOR and LM (Brodal et al., 1950; Clarke, 1974, 1977; Karten and Finger, 1976; Brecha et al., 1980; Gamlin and Cohen, 1988b; Wild, 1992; Pakan and Wylie, 2006; Pakan et al., 2006, 2008; Wylie et al., 2007, 2008; Iwaniuk et al., 2009b; Gaede et al., 2019; Gutiérrez-Ibáñez et al., 2022), which are, respectively, homologous to the terminal nuclei and nucleus of the optic tract in mammals (Simpson, 1984; Gamlin, 2006; Giolli et al., 2006). nBOR and LM neurons have large receptive fields and show direction selectivity in response to large-field motion (Burns and Wallman, 1981; Morgan and Frost, 1981; Winterson and Brauth, 1985; Wylie and Frost, 1990, 1996; Wylie and Crowder, 2000; Crowder et al., 2003; Gaede et al., 2017, 2022; Smyth et al., 2022). Such response properties are unique, insofar as visual neurons in other retinal recipient areas have

448 smaller receptive fields with large inhibitory surrounds and prefer "object" motion as opposed to
449 optic flow (Frost et al., 1990, 1994; Frost, 2010).

450 *4.1 The projections of LM and nBOR are similar in zebra finches and pigeons:* In this report, we
451 first examined the projections of the LM and nBOR in zebra finches, using anterograde
452 techniques. En masse, the projections are much the same as reported in pigeons (see Figs. 3-5).
453 The LM projects ipsilaterally to nBOR, the dorsal thalamus and the pontine nuclei, and
454 bilaterally to IO, and the cerebellum (folia VI-VIII, IXcd) as mossy fires. The nBOR also
455 projects ipsilaterally to LM, the dorsal thalamus and the pontine nuclei, contralaterally to the
456 nBOR, and bilaterally to the posterior cerebellum (folia VI-IXcd) as mossy fibres, IO, the
457 trochlear nucleus, all subdivisions of the oculomotor complex (nIII), the interstitial nucleus of
458 Cajal (IC) and some adjacent structures including the central grey (CtG), nucleus of
459 Darkschewitsch (D) and nucleus ruber (Ru). With the exception of the mossy fibre projection to
460 the cerebellum, these bilateral projections are all much heavier to the ipsilateral side. En route to
461 the cerebellum, the mossy fibres from nBOR, but not those from LM, have collaterals
462 innervating the cerebellar nuclei, the vestibular nuclei and the cerebellovestibular process (pcv).
463 Several papers have detailed these projections in pigeons (Clarke, 1974; Brauth and Karten,
464 1977; Clarke, 1977; Brecha and Karten, 1979; Brecha et al., 1980; Gamlin and Cohen, 1988b;
465 Wild, 1989; Wylie and Linkenhoker, 1996; Wylie et al., 1997, 1998b). Together, the present
466 study of zebra finches and these previous pigeon studies suggest that the optic flow pathways are
467 highly conserved. Focussed studies may reveal species differences in some of these projections.
468 For example, Gaede et al. (2019) examined the mossy fibre projection from the LM to the
469 cerebellum in zebra finches and pigeons using retrograde techniques and found that there were
470 similarities as well as differences. In both species the projection to IXcd was mainly from the

lateral subnucleus of LM (LMl). The projection to the oculomotor cerebellum (VI-VIII), however, was mainly from the medial LM (LMm) in pigeons, but arose from the adjacent nucleus nucleus laminaris precommisuralis (LPC) and nucleus principalis precommisuralis (PPC) in zebra finches. Whereas the LMm and LMl are retinal-recipient, the LPC and PPC are not (Gamlin and Cohen, 1988a).

4.2. A Comparison of the Projections of LM and nBOR to the Inferior Olive in Zebra Finches and Pigeons: Our main finding pertains to the projection of LM and nBOR to the IO. Previous studies using anterograde techniques in pigeons (Brecha et al., 1980; Gamlin and Cohen, 1988b; Wylie et al., 1997; Pakan et al., 2010) showed that both LM and nBOR project to the medial column (mc) of the IO, which resides medial to the cranial nerve XII (see Fig. 6b). Wylie (2001), using retrograde techniques, and Pakan et al. (2010b), using anterograde techniques, showed that there was a differential projection from LM and nBOR: the projection from the LM is heavier to the caudal mc, whereas that from the nBOR is heavier to the rostral mc. The projection from LM is ipsilateral and from a distinct population of spindle-shaped medium-sized cells along the border between LMm and LMl (Gamlin and Cohen, 1988b; Wylie, 2001; Pakan et al., 2006). The projection from nBOR is bilateral and arises from neurons in the dorsal and caudal margins of nBOR (Brecha et al., 1980; Wylie, 2001; Wylie et al., 2007). Insofar as the mc of zebra finch IO contains several subnuclei we consider it more complex than that of the pigeon (see Fig. 6). In the zebra finches we found that not all subnuclei of the mc received projections from the LM and nBOR. Moreover, there was a clear differential projection to the subnuclei of the medial column. Whereas the LM mainly targeted subnucleus i, the nBOR targeted subnuclei ii and v (see Figs. 7, 8). As in pigeons, the projection from nBOR was bilateral, but heavier to the ipsilateral side. The projection from LM was also bilateral, which has not been reported in

pigeons, although the projection to the contralateral side was weak. The other subnuclei of the mc, iii, iv, vi, vii and viii, did not receive inputs from LM and nBOR suggesting they are not involved in processing optic flow information. We also found that the nBOR in zebra finches projects to a discrete area in the caudal dl (see Figs. 7b[6,7], 7c[7], 8a[12-15]). This projection has not been reported in pigeons.

4.3 Olivo-Cerebellar Circuits in Zebra Finches, Pigeons and Chickens: We performed retrograde experiments to determine the projections of the mc subnuclei to the posterior cerebellum in zebra finches. In Figure 13a, we summarize our findings and compare them with data from the pigeon (Fig. 13b) and chicken (Fig. 13c). The pigeon scheme (Fig. 13b) was initiated by Arends and Voogd (1989), modified with respect to the vestibulocerebellum (folia IXcd and X) by Pakan and Wylie (Pakan and Wylie, 2006) and refined by Gutierrez-Ibanez et al. (2022). The chicken scheme (Fig. 13c) is gleaned from Vibulyaseck et al. (2015). For pigeons, both Arends and Voogd (1989) and Pakan and Wylie (2006) had the pattern depicted in folia VII-VIII extended into IXab. However, Pakan and Wylie (2006) and Gutierrez et al. (2022) did not have any cases involving IXab, and for Arends and Voogd (1989) it is not reported if any injections were in IXab. For this reason, we have left IXab blank.

Arends and Voogd (1989) described VII and VIII as containing 3 sagittal zones: a medial A zone that receives input from dl (medium and light blues in Fig. 13) , a middle C zone that receives input from vl (dark blue), and a lateral E zone that receives input from the ventral mc (yellow). Arends and Voogd (1989) divided the A zone into A1 (light blue) and A2 (medium blue) zones that receive input from the caudo-medial and rostro-lateral dl, respectively. These projections were confirmed in subsequent studies (Lau et al., 1998; Pakan and Wylie, 2006; Gutiérrez-Ibáñez et al., 2022). Based on data from the present study, a highly similar

organization exists in the zebra finches (Fig. 13a). An A1 zone receiving input from the caudo-medial dl is supported by the cases with injections in VII and VIII abutting the midline (cases ZF16-09 green, Fig 12c; ZF16-10 green, Fig. 12d) as well as those cases where the injection crossed the midline and labelling was restricted to the caudo-medial dl in the left IO (TG466, Fig. 12e; TG459). Injections that involved progressively more lateral regions of VII and VIII injections provide evidence for an A2 zone receiving input from the rostro-lateral dl (ZF17-12, Fig. 12b) and a C zone receiving input from the vl (TG452, TG459, TG466; see also Table 3). The two cases with the injections spreading most laterally (TG459, TG466) showed labelling in mc subnuclei iv, vii and viii, regions of the mc that do not receive input from LM and nBOR. In effect, these subnuclei resemble the ventral area of the mc in pigeons that projects to the E zone. In chickens, a very similar pattern was revealed by Vibulyaseck et al. (2015). The drawing of the A zone is similar, but more nuanced: as a gradient from medial to lateral receiving olivary input from the dl with a gradient from caudo-medial to rostro-lateral (Fig. 13c). The lateral E zone receives input from two of the three nuclei of the chicken mc; the ventral and intermediate (vMC; iMC) (Fig. 13c). Vibulyaseck et al. (2015) have further shown that these cerebellar zones align with the aldolase C (a.k.a., zebrin II) stripes.

From our injections in IXab in zebra finches, although we only had two injections to consider, we conclude that the majority of the olivary input arises from the areas of the mc that do not receive input from LM and nBOR: subnuclei iii, iv and vi (Fig. 12c,d; Fig 13a). Unlike the injections in VII and VIII, there was little labelling in vl and dl. Although there are no comparable data from pigeons, in chickens Vibulyaseck et al. (2015) show that much of the input to IXab arises from the two subnuclei of the mc, vMC and iMC (Fig. 13c). It remains to be seen if these areas of the chicken mc receive input from LM and nBOR, but we expect not.

Vibulyaseck et al. (2015) show the A zone (blue) extending into IXab, but by and large IXab could be considered an expanded E zone.

From injections in the vestibulocerebellum (IXcd and X) in zebra finches, retrograde labelling was only observed in areas of the mc subnuclei that receive input from LM and nBOR: subnuclei i, ii and v (red in Fig. 13a; Fig. 12a,b; Table 3). The projection from the mc to IXcd and X has been extensively studied in pigeons. In Figure 13b we show this projection as two sagittal stripes in the vestibulocerebellum, with the medial half of mc projecting to the lateral vestibulocerebellum (dark red) and the lateral half of mc projecting to the medial vestibulocerebellum (light red) after Lau et al. (1998). In fact, this projection is much more intricate, with at least 12 adjacent areas in the mc projecting to particular sagittal zones in the vestibulocerebellum, each aligned with the zebrin II stripes in IXcd (Wylie et al., 1999, 2017; Crowder et al., 2000; Winship and Wylie, 2003; Pakan et al., 2005, 2007, 2014; Pakan and Wylie, 2008; Craciun et al., 2018). Moreover, the climbing fibres from the mc to each of the sagittal zones carry information associated with a particular pattern of optic flow resulting from self-rotation and self-translation (Wylie and Frost, 1991, 1993, 1999; Wylie et al., 1993, 1998a; Winship and Wylie, 2001; Pakan et al., 2011; Graham and Wylie, 2012; Wylie, 2013). It is very likely that projection from the mc to the VbC in zebra finches shows a finer organization as well. For example, in case ZF16-08, where the injection was right on the midline, retrograde labelling was only observed in the caudal region of subnucleus ii (Fig. 12a), whereas in ZF16-12, where the injection was more lateral, retrograde labelling was only observed in subnucleus i (Table 3). We speculate that each of subnuclei i, ii and v, or parts thereof, project to particular sagittal bands within the VbC, similar to what is observed in pigeons. In chickens, Vibulyaseck et al.

(2015) reported that the olivary input to IXcd and X arises from the dorsal subnucleus of mc (dMC, red in Fig. 13c). We predict that dMC in chickens receives input from LM and nBOR.

The present data emphasizes that, in zebra finches, optic flow information from LM and nBOR proceeds to the vestibulocerebellum as climbing fibres from a subset of subnuclei within the mc (Fig. 13a). This mirrors the connectivity in pigeons, however, the mc is not obviously divided into separable subnuclei. There is one important difference between zebra finches and pigeons. In zebra finches, the nBOR projects to an area of dl. This projection is directed to the caudo-medial region of the ipsilateral dl (Fig. 7b[6,7],c[7]; Fig. 8a[12-15]). This implies that optic flow signals from nBOR are reaching the A1 zone in the posterior cerebellum as climbing fibres. No such projection has been reported in pigeons and recording studies have not noted climbing fibre mediated optic flow responses outside of the vestibulocerebellum. However, there is a bilateral mossy fibre projection from LM to zone A1 (Gutiérrez-Ibáñez et al., 2022).

4.5 Conclusion

Our results show that while the general organization of optic flow pathways to the inferior olive (IO) of birds is conserved, some species-specific differences exist. Namely, we found that the medial column (mc) of the IO of the zebra finch is organized in several distinct subnuclei, which receive differential optic flow inputs and have differential projections to the cerebellum (Figs. 10-13). This adds to the growing evidence that in birds there are species-specific specializations in the neural pathways that are involved in the analysis of optic flow (Gaede et al., 2017, 2019, 2022; Smyth et al., 2022) and further suggests that the organization of these neural pathways may be related to the visual ecology and optokinetic demands of each species.

584 Zebra finches are small birds that show higher wingbeat frequency and maneuverability than
585 larger birds like pigeons and chickens (Tobalske et al., 2005; Donovan et al., 2013). Ebbesson
586 (1984) has previously suggested that in the brain, a sign of system specialization is the degree of
587 parcellation of the structures. Thus, the presence of subnuclei in the inferior olive of the zebra
588 finch, particularly of the medial column, may reflect a higher level of specialization in optic flow
589 analysis. Using the same logic, we predict that medial column of hummingbirds, which are
590 among the fastest and most maneuverable birds (Dakin et al., 2018), will also present a high
591 degree of parcellation.

Conflict of Interest Statement

The authors have no conflict of interest.

Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: DRW, DLA, AHG, CG-I. Performed experiments and processed tissue: AHG, P-HW, CG-I, SA. Microscopy and Image Acquisition: MP, CG-I, P-HW. Data Analysis: DRW, CG-I, AHG, P-HW. Drafting of the article: DRW, AHG, P-HW, CG-I. Construction of figures: DRW, CG-I. Critical revision of the article for important intellectual content: DLA. Obtained funding: DLA, DRW. Student supervision: DLA, DRW, CG-I, AHG. Much of the anterograde data formed the MSc thesis for P-HW(Wu, 2020).

DATA AVAILABILITY STATEMENT

The data and images that support the findings of this study are available from the corresponding authors upon request.

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848

Figure captions

Figure 1. Optic flow olivo-cerebellar pathways in the avian brain. **a_1** shows a para-sagittal section through the zebra finch brain indicating the major structures of interest for the present study: the medial column (mc) of the inferior olive (IO), the nucleus of the basal optic root (nBOR) and the pretectal nucleus lentiformis mesencephali (LM). The dashed vertical lines in a_1 indicate that coronal sections through each of these structures are show in **a_2**, **a_3** and **a_4**. In **a_2** the entire IO is indicated on the right side of the brain (shaded grey) while on the left side the mc is shown (shaded black). In **b**, a schematic of the circuit to the cerebellum is shown. From the retina (**b_1**), both retinal ganglion cells (RGCs) and displaced ganglion cells (DGCs) project to the LM (**b_2**). The DGCs are found along the border of the inner plexiform and inner nuclear layers (**b_1**) (Karten et al., 1977). The nBOR (**b_3**) receives projections primarily from DGCs (Karten et al., 1977; Reiner et al., 1979; Fite et al., 1981; Gutierrez-Ibanez et al., 2018). Neurons in both nBOR and LM project to the mc of the IO (**b_4**) (Brecha et al., 1980; Gamlin and Cohen, 1988b; Wylie et al., 1997). Neurons in the mc project to the contralateral vestibulo-cerebellum (folia IXcd and X) as climbing fibres (**b_5**) (Arends and Voogd, 1989; Lau et al., 1998; Crowder et al., 2000, p 200; Winship and Wylie, 2003; Pakan et al., 2005). For other abbreviations, see list. Scale bar =1mm, applies to **a_{1-4}**.

Figure 2. Photomicrographs and illustrations of coronal sections through the injection sites in the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). The injection sites for all cases used in examining the topographical projection to inferior olive are shown, and the case number is indicated in the upper right corner (see Table 1; 9 injections in 6 cases). The injection site in LM for case TG502 is shown in **a_1-a_3**, and the injection in nBOR for case TG513 is shown in **b_1-b_3**. Nissl-stained sections (**a_1, b_1**) were used to identify the borders of

nuclei and the corresponding fluorescent image of the injection site in the same section (**a_2**, **b_2**) was then superimposed on the outlines (**a_3**, **b_3**). Drawings of the remaining LM injection sites are shown in **c-f**, and drawings of the other nBOR injections are shown in **g-i**. In the drawings, the injection site is indicated by central dark region surrounded by the penumbra in a lighter shade (magenta or green). The red injections have been pseudo-coloured as magenta. The medial and lateral subnuclei of LM are indicated (LMm, LMl). The injection is centered in the LMm for TG513 (**c)** and TG499 (**d**). In TG496 (**e**) and TG503(**f**), the injections are centered in LMl. The arrows in **b_1** and **b_2** indicate two micropipette tracks. The right track was associated with the injection. For other abbreviations, see list. All scale bars = 500 μm.

Figure 3. Terminal labelling from injections in the nucleus lentiformis mesencephali (LM). A sagittal drawing of the brain is shown in **a** with vertical lines indicating the rostro-caudal locations of the coronal sections shown in **b-d.** Photomicrographs of terminal labelling in the ipsilateral nucleus of the basal optic root (BOR) (**b**), the dorsal thalamus (**c**) and the inferior olive (IO) (**d**)are shown. In **b**, axons from the LM terminate mainly in the dorsal parts of nBOR, but also in the adjacent ventral tegmental area (VTA). For **c**, both a Nissl-stained image and the fluorescent terminal labelling are shown to emphasize that these terminals target the dorsal thalamus but not the nucleus rotundas (nRt). In this and subsequent figures, the case numbers from which each of the sections are taken are shown in the upper right of each panel, andthe red terminal labelling has been pseudo-coloured as magenta. m = medial, l = lateral. Scale bars = 100 μm in **b, d**; 400 μm in **c**.

Figure 4. Photomicrograpohs of terminal labelling from injections in the nucleus of the basal optic root (nBOR). A sagittal drawing of the brain is shown in **a** with vertical lines indicating the rostro-caudal locations of the coronal sections shown in **b-g. b** shows terminal labelling

bilaterally in the inferior olive (IO). **c** shows terminal labelling in the oculomotor nucleus (nIII) (**c**). Both a Nissl-stained section (**c_1**) and a flurorescent image (**c_2**) are shown to emphasize that terminals were seen in all divisions of nIII; ventromedial (vm), dorsomedial (dm) and dorsolateral (dl). **d** shows terminal labelling in the trochlear nucleus (nIV). **e** shows terminal labelling in the accessory oculomotor region, which mainly targeted the interstitial nucleus of Cajal (IC). **f** shows terminals in the contralateral nBOR and adjacent ventral tegmental area (VTA). **g** shows terminal labeling in the ipsilateral nucleus lentiformis mesencephali (LM). Both a section stained for Nissl (**g_1**) and a fluorescent image (**g_2**) are shown to highlight that the terminals are almost exclusive to the lateral subnucleus of LM (LMl) rather than the medial subnucleus (LMm). m = medial, l = lateral. For other abbreviations, see list. All scale bars = 100 μm.

Figure 5. Mossy fibre labelling in the cerebellum from injections in the nucleus of the basal optic root (nBOR) (**c, g, h**) and lentiformis mesencephali (LM) (**b, d- f**). **a** shows a sagittal section of the brain indicating the nomenclature of the folia of the cerebellum with Roman numerals. The mossy fibres terminate as beautiful strings of mossy fibre rosettes in the granule cell layer (gl) of the cerebellar cortex (**b**). From nBOR, the vast majority of these terminals were in folium IXcd. From LM, in addition to those in IXcd, many terminals were also found in folia V-VIII (e.g., **e**). In **f** and **g**, the sections have been immunoprocessed for zebrin II (ZII), which is expressed in some Purkinje cells. In **f**, a green secondary was used to contrast with the red (magenta) terminals from LM. In **g**, a red secondary (pseudo-coloured magenta) was used to contrast with the green terminals from nBOR. Shown in **h_1,** the mossy fibres from nBOR, but not those from LM, have collaterals innervating the cerebellar and vestibular nuclei. Terminals can be seen in the superior, medial and lateral vestibular nuclei (VeS, VeM, VeL), but much heavier

labelling was seen in the medial and lateral cerebellar nuclei (CbM, CbL). The two rectangular insets in **h_1** indicate the locations of **h_2** and **h_3**, which show higher magnification of the terminals in the intercalated (ic) subnucleus of CbM and the cerebellovestibular process (pcv), respectively. All sections shown are in the coronal plane. ml = molecular layer, PCl = Purkinje cell layer, m = medial, l = lateral. For additional abbreviations, see list. Scale bars =100 μm in **c-g**, **h_2, h_3**; 50 μm in **b; 250** μm in **h_1**.

Figure 6. The inferior olive (IO) in zebra finches (a) and pigeons (b). **$a_{1\text{-}20}$** show coronal serial Nissl-stained sections through the rostral (**a_1**) caudal (**a_{20}**) extent of the zebra finch IO, at 40 μm intervals. The left side is a mirror image of the right side, with shading to indicate the medial column (mc) in dark grey, the ventral lamella (vl) in light grey, and the dorsal lamella (dl) in an intermediate grey. The dashed lines indicate the fibres of the twelfth cranial nerve (XII), which are most visible in the more caudal sections (**a_{12}-a_{16}**) and separate the mc from the dl and vl. The mc has been divided into several subnuclei (i-viii, incl. $v_{a\text{-}c}$). **$b_{1\text{-}3}$** show three Nissl-stained coronal sections of the pigeon IO, at rostral, mid, and caudal levels. Note that the division of the IO into several subnuclei is less apparent in pigeons compared to zebra finches. See text for additional details. Scale bars = 500 μm.

Figure 7. Photomicrographs of the terminal labelling in the inferior olive in zebra finches from injections of anterograde tracers in the nucleus of the basal optic root (nBOR) and lentiformis mesencephali (LM). **a** and **b** show data from case TG513, where green and red anterograde tracers were injected in the LM and nBOR, respectively. (The red has been pseudo-coloured magenta) Seven serial sections from rostral (top) to caudal (bottom) are shown. **b_1-b_7** show photomicrographs of the fluorescent terminal labelling and **a_1-a_7** show the same sections subsequently stained for Nissl. The white lines in **b** outlining the subnuclei of the IO (i-viii) were

obtained from drawings of the corresponding Nissl-stained sections. In **a**, the distance (in μm) of each section from the caudal-most section (**a_7**/**b_7**) is shown (e.g., section **a_1**/**b_1** is 320 μm rostral to **a_7**/**b_7**). **c_1**-**c_7** shows data from the five other cases, where the sections are from approximately the same rostro-caudal levels as those in the corresponding panels in **a**/**b** (e.g., **c_1** is from approximately the same rostro-caudal level as **a_1**/**b_1**). The colour of the tracer and nuclei injected are indicated at the top right of each panel (e.g., **c_1** is from case TG497 where red (magenta) tracer was injected in nBOR). The right sides of the photomicrographs are ipsilateral to the injection sites. See text for detailed description. dl = dorsal lamella. Scale bar = 500 μm, applies to all.

Figure 8. A summary of the projections from the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) to the inferior olive (IO). Drawings of the IO from rostral (**a_1**) to caudal (**a_{15}**) are shown based on the Nissl-stained sections from the case shown in Figure 4. The projections from the LM and nBOR are shown in green and magenta, respectively, and more saturated colour represents a stronger projection. The right side is ipsilateral to the injection sites. As in Figures 4 and 5, lower case roman numerals identify the subnuclei of the medial column (mc). Grey shading indicates regions where terminal labelling was absent: dark, medium and light greys indicate the mc, dorsal lamella (dl) and ventral lamella (vl), respectively. See text for a detailed description. Scale bar = 500 μm.

Figure 9. Injections of retrograde tracers in the cerebellum. Drawing of coronal sections through the injection sites for nine cases are shown (**a_2, b-d, $e_{1,3}$, f-i**) and photomicrographs are shown for two of the injections (**a_1, e_2**). Six cases involved the injection of a single tracer, either red (**a-c**) or green (**g-i**) cholera toxin subunit B (CTB), whereas in three cases both coloured tracers were injected in different folia (**d-f**). The red injections have been pseudo-coloured magenta. In the

964 drawings the molecular, granular and white matter layers are shaded as grey, black and white,
965 respectively. The injections are drawn as duotone, with core a saturated magentaor green, and the
966 penumbra as a less saturated colour. All scale bars = 1.1mm.

967 **Figure 10.** Photomicrographs of coronal sections through the inferior olive showing retrogradely
968 labelled cells from injections in the cerebellum. For those shown in **a**, **b** and **c**, the fluorescent
969 image as well as a photomicrograph of the subsequently Nissl-stained section are shown to
970 illustrate how the borders of the olivary subnuclei were determined for the fluorescent images
971 (white dashed lines). The retrogradely labelled cells from injections of red CTB have been
972 pseudo-coloured magenta. In the upper right of the panels, the case number is indicated, as well
973 as the colour of cholera toxin subunit B employed, and the location of the injection (e.g., **c** is
974 from case ZF17-12 where red (magenta) and green CTB were injected in folia IXcd and VII,
975 respectively). For b-e, the left side is medial. i, ii, iii, iv, v_a, v_b, v_c, v_r, vi, vii, viii are the subnuclei
976 of the medial column. dl = dorsal lamella, vl = ventral lamella. All scale bars = 250μm except $\mathbf{d_1}$
977 = 100μm. See text for detailed description.

978 Figure 11. Photomicrographs of coronal sections through the inferior olive showing retrogradely
979 labelled cells from injections in the cerebellum. In the upper right of the panels, the case number
980 is indicated, as well as the colour of cholera toxin subunit B employed, and the location of the
981 injection (e.g., **a** is from case ZF16-10 where red and green CTB were injected in folia IXab and
982 VIII, respectively). The retrogradely labelled cells from injections of red CTB have been pseudo-
983 coloured magenta. **b** shows a series of 4 sections from case TG459 from rostral ($\mathbf{b_{1,2}}$) to caudal
984 ($\mathbf{b_{7,8}}$). Both the Nissl sections (left) and fluorescent images (right) are shown. For all panels the
985 left side is medial. i, ii, iii, iv, v_a, v_b, v_c, v_r, vi, vii, viii are the subnuclei of the medial column. dl
986 = dorsal lamella, vl = ventral lamella. All scale bars = 250μm.

Figure 12. Drawings of serial sections from five cases are shown illustrating the distribution of labelling in the inferior olive (IO). Sections are arranged rostral (top) to caudal (bottom). The sections do not span the same rostro-caudal extent among cases, and the sections are not necessarily at equal intervals within each case. Sections were chosen to illustrate the extent of labeling for each case. On the top section for each case, the case number as well as the colour (red or green) and folia for each injection is indicated. Each dot (red or green) shows the location of a retrogradely labelled cell. A few dots in **d** are yellow, to indicate double-labelled neurons. In cases ZF16-08 (**a**) and TG466 (**e**) as the injection crosses the midline there was bilateral labelling, whereas in the others labelling was restricted to the right IO. The dashed vertical line indicates the midline. i, ii, iii, iv, v_a, v_b, v_c, v_r, vi, vii, viii are the subnuclei of the medial column. dl = dorsal lamella, vl = ventral lamella. Scale bars = 250μm (applies to all). See text for detailed description.

Figure 13. A comparative description of the olivo-cerebellar projection among three species of birds: zebra finches (**a**), pigeons (**b**), and chickens (**c**). The scheme for the zebra finches is based on the present study. That for the pigeon is based on Arends and Voogd (Arends and Voogd, 1989), Pakan and Wylie (2006) and Gutierrez-Ibanez et al. (2022), and the chicken scheme is gleaned from Vibulyaseck et al. (2015). For each species, four coronal sections through the inferior olive are shown, rostral (top) to caudal, with colour coding indicating the projection to the posterior cerebellum (folia VII-X). For the pigeon and zebra finches, the projection is shown to an idealized coronal section through the cerebellum. For the chick, the projection is shown as projected onto the surface of an unfolded cerebellar cortex. i, ii, iii, iv, v_a, v_b, v_c, v_r, vi, vii, viii are the subnuclei of the medial column (mc). The medial column in chicken contains three

subdivisions: dorsal, intermediate and ventral (dMC, iMC, vMC). dl = dorsal lamella, vl = ventral lamella. See text for detailed description.

1011 Table 1. Summary of antibodies used.

1012

	Antibody	Host/ isotype	Immunogen	Supplier	Catalog#/RRID	Conc.
Primary	Aldolase C (N-14)	Goat/ polyclonal	Epitope mapping of N-terminus of human Aldolase C	Santa Cruz	Cat#: sc-12065 RRID: AB_2242641	1:1000
Secondary	AlexaFluor-594 donkey antigoat IgG	Donkey	Goat IgG	Jackson ImmunoResearch Labs	Cat#: 705-585-003 RRID: AB_2340432	1:100
Secondary	AlexaFluor-488 donkey antigoat IgG	Donkey	Goat IgG	Jackson ImmunoResearch Laboratories	Cat#: 705-545-003 RRID: AB_2340428	1:100

1013

Table 2. Injections of anterograde tracers into the pretectal nucleus lentiformis mesenscephali (LM) and nucleus of the basal optic root (nBOR) for the seven cases used to determine projections to the inferior olive. Photomicrographs and/or illustrations of the injections appear in Fig. 1 as indicated. All injections were on the right side of the brain.

Case	LM injection	nBOR injection
TG496	Texas Red (Fig. 1i)	
TG497		Texas Red (Fig. 1e)
TG499	Fluorescein (Fig. 1h)	
TG502	Texas Red (Fig. 1a)	Fluorescein (Fig. 1b)
TG503	Fluorescein (Fig. 1c)	Texas Red (Fig. 1d)
TG513	Fluorescein (Fig. 1g)	Texas Red (Fig. 1f)
TG540	Fluorescein	
TG541	Fluorescein	

Table 3. Retrograde labelling in the Inferior Olive from injections in various folia (VII to X) in the zebra finch cerebellum The folium (or folia) injected for each case is indicated as well as the presence of retrogradely labelled inferior olivary cells in the subnuclei of the medial column (i-viii), as well as the dorsal lamella (dl) and ventral lamella. The presence of labelling in the caudo-medial portion of the dl that receives projections from the nucleus of the basal optic root (nBOR) is also indicated (dl-nBOR). A qualitative scale is used to indicate the relative amount of labelling in each olivary area: heavy (+++); moderate (++) and sparse (+).

					Retrograde Labelling in Olivary Subnuclei (heavy +++; moderate, ++; sparse +, none = -)									
case	**Folium**	i	ii	iii	iv	v_a	v_b	v_c	v_r	vi	vii	viii	dl	vl
ZF16-08	IXcd	-	+++	-	-	-	-	-	-	-	-	-	-	-
ZF16-12	IXcd X	+++	-	-	-	-	-	-	-	-	-	-	-	-
ZF17-12 (red)	IXcd	+++	-	-	-	+++	+++	+	+++	-	-	-	-	-
ZF16-16	X	++	+++	-	-	-	++	-	-	-	-	-	-	-
ZF16-09 (red)	IXcd IXab	++	+++	++	+++	+	-	+++	+	+++	-	-	-	++
ZF16-10 (red)	IXab	-	-	+++	+++	-	+	+	-	-	+	-	+	-
ZF16-09 (green)	VII	-	-	-	-	-	-	-	-	-	-	-	+++	-
ZF16-10 (green)	VIII	-	-	+	-	-	-	-	-	-	-	-	+++	-
ZF17-12 (green)	VII	-	-	+	-	-	-	-	-	-	-	-	+++	+
TG452	VII	-	-	-	-	-	-	-	-	-	-	-	+++	+++
TG459	VIII	-	-	+	++	-	-	-	-	-	++	++	+++	+++
TG466	VII	-	-	+	++	-	-	-	-	-	+	++	+++	+++