Received: 19 April 2017 | Revised: 14 May 2017 | Accepted: 15 May 2017

DOI: 10.1002/cne.24247

RESEARCH ARTICLE

WILEY The Journal of Comparative Neurology

The ascending projections of the nuclei of the descending trigeminal tract (nTTD) in the zebra finch (*Taeniopygia guttata*)

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Funding information
NIH grant RO1NS029467 to Dr R.A. Suthers, Indiana University.

Abstract

In our traditional view of the avian somatosensory system, input from the beak and head reaches the telencephalon via a disynaptic pathway, involving projections from the principal sensory nucleus (PrV) directly to nucleus basorostralis (previously called nucleus basalis), whereas input from the rest of the body follows a trisynatic pathway similar to that in mammals, involving projections from the dorsal column nuclei to the thalamus, and thence to somatosensory wulst. However, the role of the nuclei of the descending trigeminal tract (nTTD) in this scenario is unclear, partly because their ascending projections have been examined in only one species, the mallard duck. Here we examine the ascending projections of the nTTD in the zebra finch, using in vivo injections of biotinylated dextran amine and verification of projections by means of retrograde transport of the beta subunit of cholera toxin. The results show a high degree of interconnectivity within the nTTD, and that these nuclei project to PrV. We also find a projection from nTTD to the contralateral thalamic nucleus uvaeformis, a multi-sensory nucleus connected to the song system. Furthermore, our finding of a projection from nTTD to the contralateral somatosensory thalamic nucleus dorsalis intermedius ventralis anterior (DIVA) is consistent with the well-known projection in mammals from nTTD to the ventrobasal thalamus, suggesting that the ascending trigeminal pathways in birds and mammals are more similar than previously thought.

KEYWORDS
Birds, DIVA, RRID: AB_10013220, somatosensory system, songbird, Uva

1 | INTRODUCTION

The avian somatosensory system can be subdivided according to the source of primary afferents into those from the body (including wings, legs, and claws)—which mainly project upon the dorsal horn of the spinal cord and dorsal column nuclei, and in some species to certain regions of the pons—and those from the beak and tongue, which mainly project upon the sensory trigeminal nuclei (Wild, 2015). The latter include the principal trigeminal nucleus (PrV), located dorsal and slightly rostral to the entrance of the sensory trigeminal nerve (Vs), and the nuclei of the descending trigeminal tract (nTTD), which extend throughout the brainstem from the caudoventral aspect of the entrance of Vs to the cervical dorsal horn.

Birds seem to depart largely from other amniotes in the organization of their ascending trigeminal pathways. In mammals and reptiles, both PrV and nTTD project to the dorsal thalamus, with nTTD additionally projecting to the superior colliculus (optic lobe in other vertebrates) (Molenaar & Fizaan-Oostveen, 1980; Pritz & Northcutt, 1980; Killackey & Erzurumlu, 1981; Bangma & Ten Donkelaar, 1982; Matsushita, Ikeda, & Okado, 1982; Wiberg, Westman, & Blomqvist, 1987; Desfilis, Font, & García-Verdugo, 1998; Veinante, Jacquin, & Deschênes, 2000). In turn, the avian PrV projects to the telencephalon via the quintofrontal tract, without any relay in the dorsal thalamus (Wallenberg, 1903; Carl Huber & Crosby, 1929; Wild, Arends, &

Abbreviations: AN, Nucleus angularis; Bas, Nucleus basorostralis; CuE, External cuneate nucleus; DH, Dorsal horn; DIVA, Nucleus dorsalis intermedius ventralis anterior; ICo, Intercollicular nucleus; LLi, Intermediate lateral lemniscal nucleus; MLd, Nucleus mesencephalicus lateralis pars dorsalis; NIf, Nucleus interfacialis; nTTD, Nuclei of the descending trigeminal tract; nTTDc, Nuclei of the descending trigeminal tract, pars caudalis; nTTDi, Nuclei of the descending trigeminal tract, pars interpolaris; nTTDo, Nuclei of the descending trigeminal tract, pars oralis; nXII, Motor hypoglossal nucleus; OI, Inferior olive; OS, Superior olive; PrV, Principal sensory trigeminal nucleus; Rt, Nucleus rotundus; TeO, Optic tectum; Uva, Nucleus uvaeformis; V, Trigeminal nerve; VIII, Octaval nerve.

Zeigler, 1985). Most studies of the avian trigeminal system have focused on this remarkable projection. The connections of the avian nTTD, however, have been thoroughly examined only in the mallard, using lesions, autoradiography, and HRP tracing techniques (Arends, Woelders-Blok, & Dubbeldam, 1984). According to this report, the avian nTTD also differ from the nTTD of mammals and reptiles in that they do not project to the optic tectum and in that their thalamic projections are scarce. This ascending projection was shown to originate from a minor proportion of neurons in the caudal region of the nTTD that send their axons to the caudal nucleus dorsolateralis posterior (DLPc) (Arends et al., 1984). A study of the sensory modalities of DLPc in the pigeon suggests that this projection would instead originate from a small population of cells scattered throughout the rostrocaudal extent of nTTD (Korzeniewska & Güntürkün, 1990). In the present work we provide a clearer picture of the connections of the avian nTTD using sensitive anterograde and retrograde neural tracers. Furthermore, we aimed to understand the organization of these projections in a songbird, given the potential importance of somatosensory feedback that the nTTD relay from the beak, upper vocal tract, tongue, and syrinx for the motor control of singing (Bottjer & Arnold, 1982; Bottjer & To, 2012, Faunes, Botelho, & Wild, 2017). We studied the efferent connections of the nTTD in the zebra finch (*Taeniopygia guttata*) using injections of biotinylated dextran amine (BDA 3 KDa) into different rostrocaudal levels of nTTD. We also verified these projections retrogradely with injections of the beta subunit of cholera toxin (CTB) into some of the putative midbrain and thalamic synaptic targets.

2 | METHODS

A total of 29 adult male and female zebra finches were used for this study. All procedures were approved by the Animal Ethics Committee of the University of Auckland.

2.1 | Tracer injections

The animals were anaesthetized with an intramuscular injection of an equal parts mixture of ketamine and xylaxine (50 and 20 mg/kg, respectively) and fixed in a custom-made stereotaxic frame with ear and beak bars. The beak was angled down with respect to the horizon by placing the confluence of the mid-sagittal and cerebellar sinuses ("Y" point) 0.3 mm caudal to inter-aural zero. Coordinates to reach the nTTD and some of their putative synaptic targets were obtained from an unpublished stereotaxic atlas of the zebra finch brain (M. Konishi) and guided by extracellular recordings.

After performing an incision in the scalp and a small craniotomy, tungsten microelectrodes (2–4 MΩ) were advanced to their targets in the brain using a Micro-Positioning Controller (MC-5B, National Aperture Inc., NH, USA). In order to avoid the bone that covers the dorsolateral aspect of the medulla, the electrodes were placed at lateral angles ranging between 13 and 15 degrees to reach the nTTD and PrV from a craniotomy located at the midline. To reach all other targets the electrodes were lowered vertically. Electrophysiological signals were amplified and filtered between 100 Hz and 10 KHz with a two-channel differential AC amplifier (AM systems, 1800, WA, USA), and monitored with a digital oscilloscope (TDS 2014, Tektronix, OR, USA) and a monitor speaker (MS2, Tucker Davis Technologies Inc., FL, USA). The tungsten microelectrodes were then replaced by tracer-filled glass micropipettes (World Precision Instruments 1B150F-4, FL, USA) having tip outer diameters of 15–20 microns.

The tracers were either 3 KDa BDA (Invitrogen, CA# D7135, Lot# 1412870, 10% in phosphate buffered saline, PBS) or CTB (Sigma, CA# C167-500UG, Lot#SLBK3733V, 1% in PBS). These tracers were delivered by iontophoresis (2–4 mA positive current, 7 s on-off cycle for a total of 15–20 min), and/or air pressure provided by a picospritzer (Parker, USA).

After the injections were placed, the pipettes were retracted and the surgical incision closed. The animals were left to recover from anesthesia and survive for 3–5 days to allow transport of the tracers before tissue processing.

2.2 | Tissue processing

The animals were anesthetized as described above and transcardially perfused with 0.9% saline and 4% paraformaldehyde (PFA). After >4 hr post fixation in 4% PFA, the brains were blocked in the coronal plane in the stereotaxic frame prior to removal from the skull. Then, they were placed in 30% sucrose buffer until they sank (usually 8 hr), embedded in 12% gelatin, postfixed again for 2 hr, placed in 30% sucrose until they sank again and cut in 35 micron-thick coronal sections on a freezing microtome. Sections were collected serially in PBS in three series (105 microns between sections).

CTB was visualized by immunohistochemistry, as follows. All incubation steps were done at room temperature with gentle agitation and preceded by PBS washes (3× 10 min). First, the sections were incubated for 15 min in a solution of 0.3% H_2O_2 and 50% methanol in PBS to quench endogenous peroxidase activity. They were then incubated overnight in a primary antibody against CTB (anti-cholera B subunit made in goat, List Biological Laboratories, CA# 703, antibody RRID AB_10013220, Lot# 7032A6) diluted 1:33,000 in a blocking solution consisting of 0.4% triton and 2% normal donkey serum (Sigma, CA# D9663, Lot# SLBL4004V) in PBS. Next, sections were incubated for 1 hr in secondary antibody (biotinylated donkey anti-goat, Jackson Immuno, CA# 705–065-003, Lot# 67132) diluted 1:500 in blocking solution, and then for 1 hr in HRP-conjugated Neutravidin (Thermo Scientific, CA# 31001, Lot# QE317304) diluted 1:1,000 in 0.4% triton PBS. Finally, sections were incubated in a solution of 0.25 mg/mL diamonobenzidine hydrochloride (DAB), 0.2 mg/mL $CoCl_2$ in PBS (to produce a black reaction product), to which a few drops of 0.3% H_2O_2 were added to start the DAB-peroxidase reaction. The reaction was stopped after 1–3 min by transferring the sections to PBS. This procedure does not yield staining of control non-injected cases. BDA was visualized by incubating sections for 1 hr in HRP-conjugated neutravidin, followed by DAB.

The sections were mounted on gelatin-coated slides and left to dry. Then, they were cleared in an ascending series of ethanol followed by xylene; one series was left uncounterstained and at least one other

was counterstained with Cresyl Violet or Neutral Red. Sections were coverslipped with DPX mounting medium (Sigma).

2.3 | Data analysis and image processing

The sections were examined and photographed in a Nikon eclipse 80i microscope. Tracer labeled fibers and cells were mapped using Neurolucida software and a Nikon e800 microscope equipped with a computer-controlled stage (Micro Bright Fields). Photomicrographs were processed for brightness and contrast using Adobe Photoshop software and figures were composed using Adobe Illustrator.

3 | RESULTS

The cytoarchitecture of nTTD of the zebra finch is described in an accompanying article (Faunes & Wild, 2017). The nTTD can be subdivided, following the nomenclature used in mammals (Olszewski, 1950), into three subnuclei along the rostro-caudal axis: caudalis, interpolaris, and oralis. Caudalis is continuous with the trigeminal-recipient cervical dorsal horn and extends rostrally to the level of the obex. Interpolaris can be further subdivided into medial and lateral regions. The medial subdivision contains more densely packed cells, whereas in the lateral subdivision cells with larger coronal section areas are intermingled with the descending trigeminal tract (TTD) fibers. Roughly at the level of the vestibular VIIIth nerve entrance, oralis cells appear between the rostral tips of the two interpolaris regions. These cells can be distinguished as having larger coronal section areas and being more densely packed than lateral interpolaris cells. At the dorsolateral side of oralis there is a group of cells that receives lingual hypoglossal afferents (Wild, 1990). Oralis extends rostrally up to the caudoventral aspect of the entrance of Vs (Faunes & Wild, 2017).

3.1 | 3KDa BDA tracing

We first injected 3KDa BDA at different rostrocaudal levels of the nTTD and assessed anterograde transport of the tracer throughout the brain. Injection sites are depicted in Figure 1. In addition to these seven cases, seven injections that served as controls were located dorsal, medial, or ventral to nTTD. Control injections were located in the dorsal column nuclei (injection 2' in Figure 1b), external cuneate nucleus (injection 4' in Figure 1b,c), nucleus retroambigualis (injection 1' in Figure 1a,b) and the parvocellular reticular formation just medial to the nTTD at four different rostrocaudal levels (injections 3', which includes part of nucleus retroambigualis, 5', 6' and 7' in Figure 1b–e).

3.1.1 | Brainstem

Differently located control injections yielded different labeling patterns in the brainstem. Injections in the dorsal column and external cuneate nuclei produced labeled fiber terminals in the ipsi- and contra-lateral inferior olive and ascending fibers running through the contralateral medial lemniscus toward the intercollicular nucleus (ICo), as has been described for pigeons and a finch (Wild, 1989, 1997). The injection in retroambigualis labeled fiber terminals in the tracheosyringeal hypoglossal motor nucleus and other respiratory–vocal nuclei in the brainstem (as shown in Wild, 1993; Wild, Kubke, & Mooney, 2009). Injections in the parvocellular reticular formation produced labeling throughout the ipsi- and contra-lateral medullary parvocellular and magnocellular reticular formation. None of these control injections produced labeling in nTTD or PrV.

Injections at any rostro-caudal level of the nTTD labeled fibers and terminals throughout the rostro-caudal extent of nTTD and PrV, predominantly ipsilaterally (Figures 2a–e, a'–e', a"–e" and 3). Some retrogradely labeled cell bodies were found at different levels of the nTTD column, especially ipsilaterally and at levels caudal to the injection sites (red arrow in Figure 3a, Figure 4). Generally, the labeling produced throughout the sensory trigeminal column, including PrV, reflected the medio-lateral and dorso-ventral position of the injections in nTTD. For instance, injections limited to dorsomedial regions of nTTD caudalis, i.e., the mandibular nerve-recipient region of caudalis (Faunes & Wild, 2017), labeled medial parts of the trigeminal column and PrV (Figure 2a–e); whereas injections centered ventrolaterally in nTTD oralis labeled ventrolateral parts of the trigeminal column and PrV (Figure 2a"–e"). Similarly, an injection in the hypoglossal-recipient dorsolateral region of nTTD interpolaris and oralis (injection N° 5 in Figure 1e,f) produced labeling in the other hypoglossal-recipient regions of the trigeminal column (Wild, 1990; Faunes et al., 2017): a cytoarchitectonically distinct oval dorsolateral region of PrV, the lateral nTTD interpolaris and caudalis, and the medial cervical dorsal horn.

Even though the intra-trigeminal labeling could be partly due to tracer uptake by passing axons from nTTD and trigeminal ganglion cells, the fiber and somata labeling on the contralateral side suggests that at least some of it derives from intra-trigeminal connections. Projections to the contralateral nTTD seem to originate from fibers that cross the midline dorsal to the central canal in the cervical dorsal horn and at the level of the solitary nucleus (Figures 2a,b, a', a", b" and 3a,b). Taken together, these results suggest that sensory trigeminal neurons distributed in different parts of the trigeminal column, and which receive primary afferents in common, are interconnected.

In all cases, many fibers were seen leaving the caudalis and interpolaris regions of nTTD to distribute terminals to the surrounding parvocellular reticular formation (Figures 2b–d, b'–d', b"–d" and 3d). Some of these fibers course ventrally to terminate in the ipsilateral inferior olive, but most of them cross the midline ventrally to reach the contralateral inferior olive and the lateral reticular formation (Figure 2b,c, b', c', b", c"). Labeling at rostral levels of the brainstem was mostly contralateral. Labeled fibers join the contralateral medial lemniscus and course rostrally, reaching the area surrounding the contralateral superior olive and intermediate lateral lemniscal nucleus (Figure 2d–f, d'–f', d"–f"), where they originate numerous terminations. At the level of the isthmus, some of the labeled fibers turn dorsally toward the midbrain roof, and generate a distinct terminal field in the ICo surrounding the nucleus mesencephalicus lateralis pars dorsalis (MLd) (Figure 2f, f', f"), similar to what has been described for the projections of the dorsal column and external cuneate nuclei in pigeons (Wild, 1989, 1997). In none of the cases was any labeling found in the optic tectum or in MLd

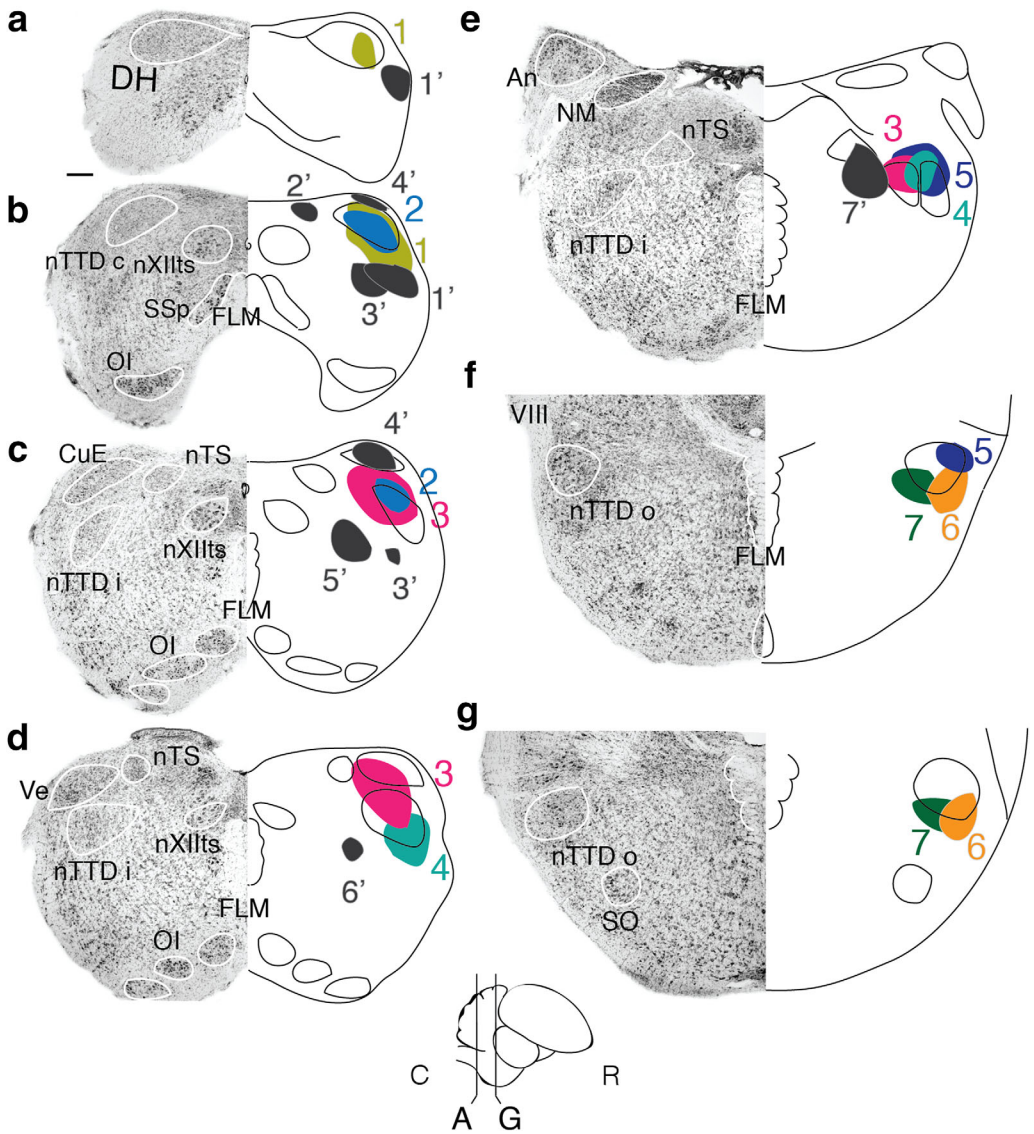

FIGURE 1 Schematic representation of the BDA injection sites. Coronal sections separated by 240 μm are depicted schematically from caudal to rostral and complemented by Nissl-stained material. Injection sites including the nTTD are depicted in colors (1–7), and control injection sites are depicted in black (1'–7'). Inset indicates in a lateral schematic view of the brain the location of the caudalmost (a, 2.94 mm caudal to inter-aural zero) and the rostralmost (g, 1.5 mm caudal to inter-aural zero) sections. Scale bar in a = 200 μm for all photomicrographs

itself. In all injections, a labeled contralaterally ascending fiber bundle that runs in the medial lemniscus at the bottom of the contralateral brainstem can be followed farther rostrally toward the thalamus.

3.1.2 | Thalamus

None of the control injections in the reticular formation produced labeling in the thalamus. As expected, control injections in the dorsal column and external cuneate nuclei produced labeling in the somatosensory thalamus i.e., the nucleus uvaeformis (Uva; Wild, 1994) and nucleus dorsalis intermedius ventralis anterior (DIVA). Uva is a multimodal sensory nucleus that provides sensory feedback to the song system in songbirds (Nottebohm, Paton, & Kelley, 1982; Wild, 1994; Akutagawa & Konishi, 2005), and is considered homologous to DLPc in other birds (Wild, 1994). It also receives an auditory projection from the lateral lemniscal nuclei, and a visual projection from the optic tectum (Wild, 1994; Wild, Krützfeldt, & Kubke, 2010). Uva projects to the interfacial nucleus (NIf) of the nidopallium and to HVC (Nottebohm et al., 1982; Wild, 1994). DIVA has been shown to receive a mainly contralateral projection from the dorsal column and external cuneate nuclei, and to project to the rostral hyperpallium apicale (HA) (Wild, 1987, 1989, 1997).

Our nTTD injections 4 and 5 (Figure 1d-f), in which the injection site included the lateral aspect of interpolaris, produced labeling of a distinct terminal field in the contralateral Uva (Figures 2g' and 5a,b), which seems to originate from fibers that leave the medial lemniscus at the level of the posterior commissure to course dorsally and then laterally (Figure 2g'). None of the other injections in the nTTD produced labeling in Uva, suggesting that in the zebra finch the nTTD projection

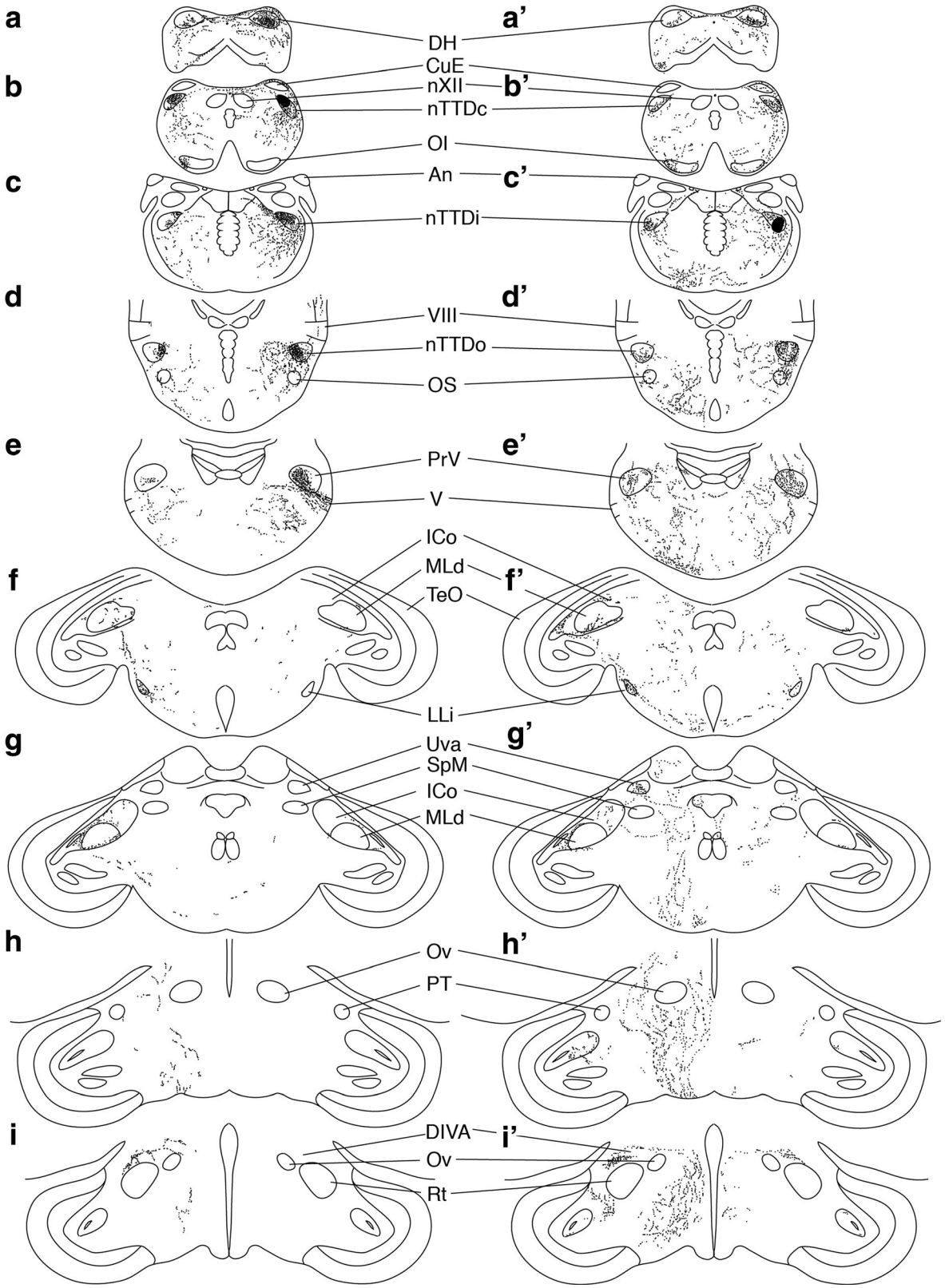

FIGURE 2 Schematic representation of the anterograde labeling obtained with BDA injections in caudalis (a-i, injection 2 in Figure 1), interpolaris (a'–i', injection 4 in Figure 1), and oralis (a"–i", injection 6 in Figure 1). Solid black areas represent the injection sites and short wavy lines and dots indicate the location of labeled fibers and terminals. Coronal sections separated by 480 μm are depicted from caudal (a, a', a") to rostral (i, i', i"). Inset indicates in a lateral schematic of the brain the location of the caudal-most (2.94 mm caudal to the inter-aural zero) and rostral-most (0.9 mm rostral to the inter-aural zero) sections

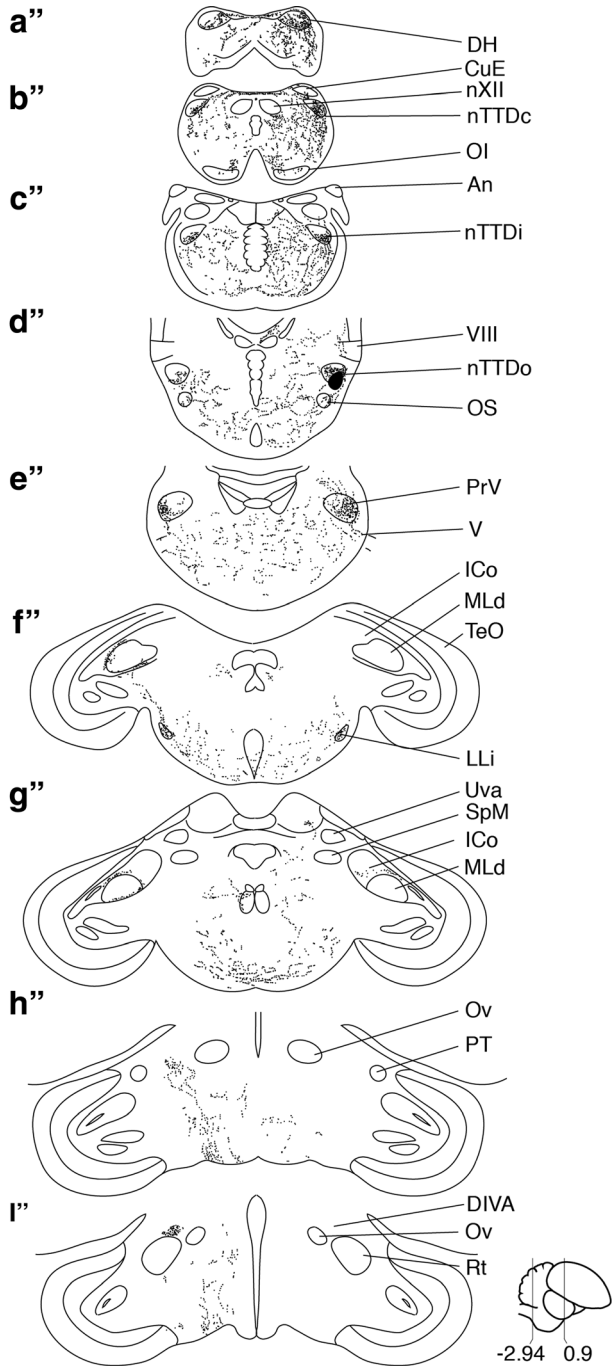

FIGURE 2 Continued

to Uva could originate from a specific cell population in the lateral interpolaris region.

In addition, all injections in nTTD produced labeling of a very distinct terminal field in the contralateral DIVA (Figures 2i, i', i" and 5c, d). In a few instances, a small amount of labeling was also found in the ipsilateral DIVA as well (e.g., Figure 2i'). Most of the fibers reaching DIVA come from the medial lemniscus, then ascend dorsally through the diencephalon, and approach DIVA from a medial position (e.g., Figure 2h,i, h', i', h",i").

3.1.3 | Cerebellum

In the present study, labeling patterns in the cerebellum following injections in nTTD were variable across cases, ranging from almost no labeling at all, to bilateral labeling restricted to lobule IX, to labeling throughout the entire cerebellum. However, to reach the nTTD our micropipettes traversed the cerebellum, and avoidance of tracer leakage from them was not always accomplished. In addition, the possibility cannot be ruled out that at least some of our cerebellar labeling could have resulted from tracer uptake by olivo-cerebellar passing fibers. An analysis of nTTD projections to the cerebellum should ideally include retrograde tracer injections in different cerebellar lobules, which was considered beyond the scope of this study (Arends et al., 1984; Arends & Zeigler, 1989).

3.2 | CTB tracing

Following our BDA experiments, we aimed to confirm the ascending projections from the nTTD to PrV, ICo, Uva, and DIVA. We injected CTB in these nuclei (at least two cases per target) and assessed the presence or absence of retrograde labeling in the nTTD.

3.2.1 | PrV

Two large CTB injections were made in PrV, and in both cases many labeled cells were found throughout the nTTD, mostly ipsilateral to the injection. Cells in the trigeminal-recipient dorsal horn were mostly located in the internal layers (Figure 6a), and throughout caudalis (Figure 6b), interpolaris (Figure 6c) and oralis (Figure 6d). In interpolaris, the medial region exhibits a higher cell density than the lateral region (Faunes & Wild, 2017) but many labeled cells were found in both regions. Cells were also labeled in the surrounding reticular formation (Figure 6). These results, combined with our BDA anterograde experiments, suggest that all subdivisions of the nTTD project upon PrV.

3.2.2 | ICo/MLd

Four CTB injections were made in ICo, two of them including MLd, and two in dorsomedial ICo, and none of them produced retrograde labeling in the nTTD. In the two cases where injections included MLd, many cells where labeled in the dorsal column nuclei, in nucleus retroambigualis, nucleus angularis, the superior olive, dorsal and ventral nuclei of the lateral lemniscus, and the contralateral MLd. Although a few cells were labeled at the borders of nTTD, the results suggest that most of the anterograde labeling found in ICo surrounding MLd in our BDA experiments was due to tracer uptake by DCN cells and possibly ICo-projecting passing fibers, and that a projection from nTTD to ICo, if present, is very small.

3.2.3 | Uva

One large CTB injection included Uva, and two smaller injections were almost restricted to this nucleus, e.g., the one depicted in Figure 7. In each of these three cases cell labeling was present in nTTD. A control injection located just dorsal to Uva, but not involving any of it, labeled no cells in nTTD. Most of the nTTD labeled cells in the Uva injection cases were located in the contralateral interpolaris, especially its lateral

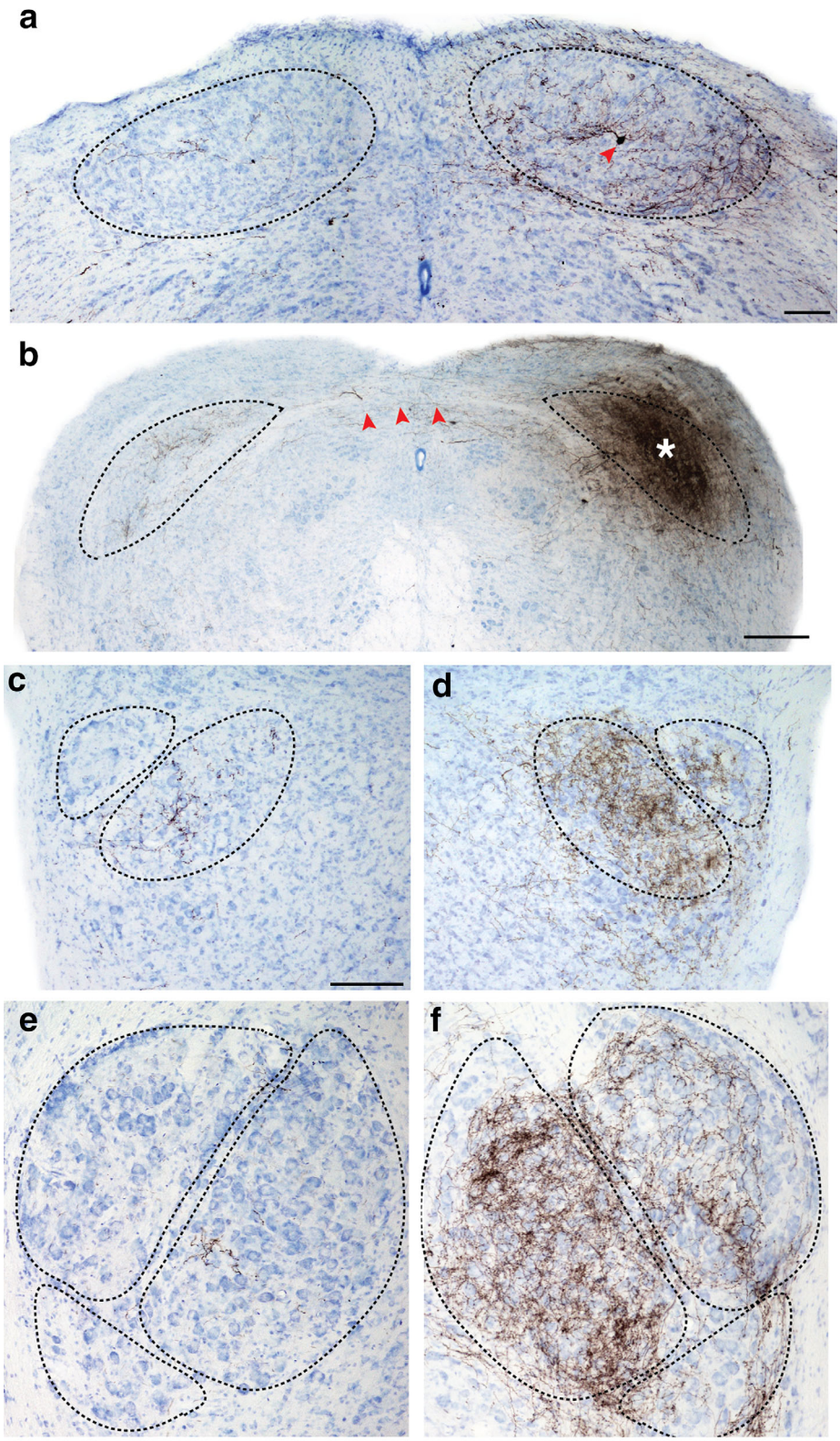

FIGURE 3 Bright field photomicrographs of coronal Nissl-counterstained sections showing BDA labeling in the cervical dorsal horn and trigeminal nuclei produced by an injection in the nTTD caudalis (injection 2 in Figure 1). (a) Terminal and retrograde (red arrowhead) labeling in the cervical dorsal horn. Left is the side contralateral to the injection. (b) Injection site (asterisk) and terminal labeling in the contralateral nTTD caudalis. Red arrowheads point to labeled fibers crossing contralaterally. (c, d) Terminal labeling in the contralateral (c) and ipsilateral (d) nTTD oralis, at the level where it exhibits a dorsolateral hypoglossal-recipient subdivision. (e, f) Terminal labeling in the contralateral (e) and ipsilateral (f) principal trigeminal nucleus. Medial is to the right in (c) and (e), and to the left in (d) and (f). Scale bar in a = 100 μm, in b = 500 μm, and in c = 100 μm for (c–f)

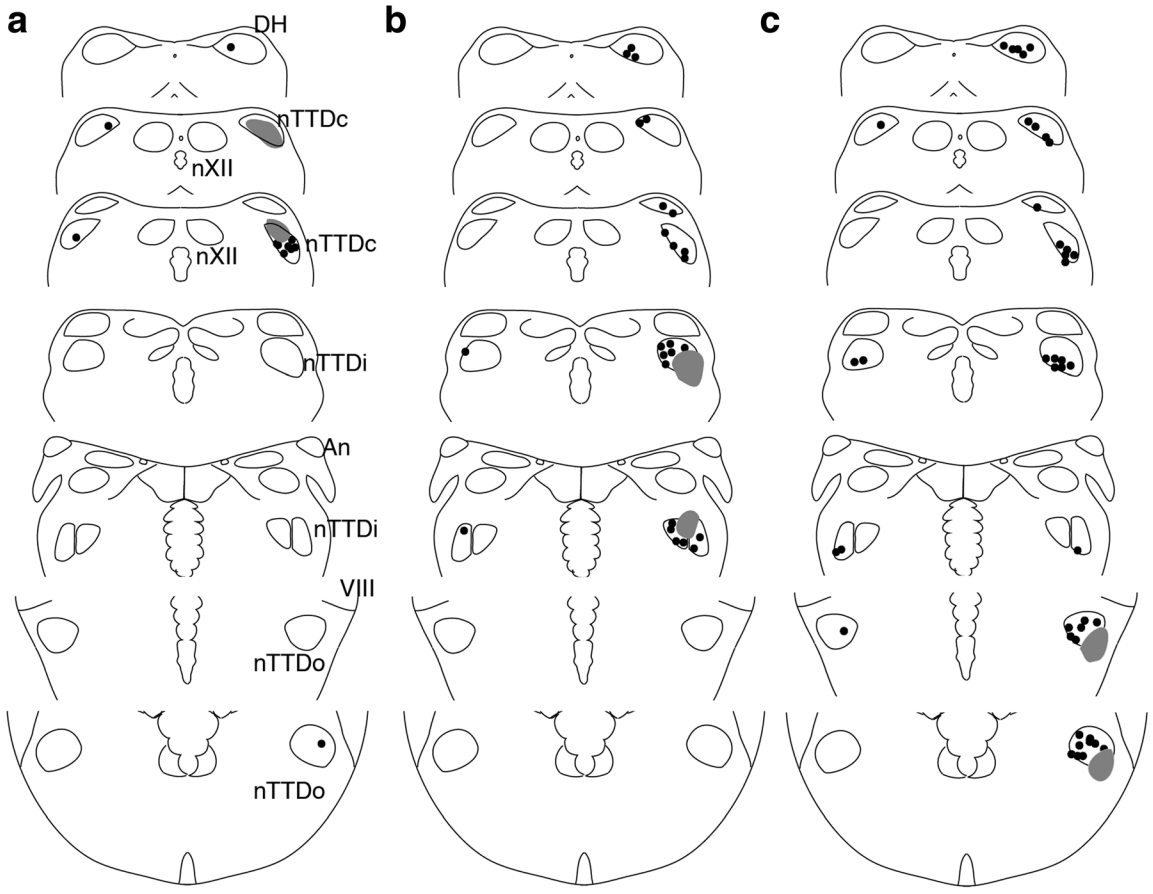

FIGURE 4 Schematic representation of the retrograde labeling obtained throughout nTTD with the same BDA injections depicted in Figure 2, in caudalis (a), interpolaris (b), and oralis (c). Coronal levels are the same as depicted in Figure 1. Grey areas represent injection sites, dots indicate labeled somata

region, and a few cells were also found in oralis. As expected from previous studies, our injections in Uva also yielded anterograde labeling in the ipsilateral NIf, and retrograde labeling in the ipsilateral optic tectum and contralateral dorsal column and external cuneate nuclei. Interestingly, a small population of cells in the ventral part of contralateral PrV was also labeled in these cases.

These results are in agreement with our anterograde BDA labeling experiments, and together they indicate that lateral interpolaris is the main source of the projection from nTTD to Uva.

3.2.4 | DIVA

Two large CTB injections that included DIVA and part of nucleus rotundus, one that included DIVA and part of nucleus ovoidalis, and one that was restricted exclusively to DIVA (depicted in Figure 8), produced labeled cells in oralis and interpolaris, mostly in the lateral region of interpolaris. A control injection just dorsal to DIVA but not including any of it did not label any cells in nTTD. DIVA injections also yielded anterograde labeling in the ipsilateral rostral Wulst, and retrogradely labeled cells in the contralateral dorsal column and external cuneate nuclei. Like Uva injections, these injections also produced labeling of a few cells in the ventral part of PrV.

Taken together, our results of BDA and CTB labeling reveal a previously unknown projection from nTTD to the contralateral somatosensory thalamus.

4 | DISCUSSION

In contrast to the well-known quintofrontal projection that carries fibers from PrV directly to nucleus basorostralis (Bas, previously called nucleus basalis; Wallenberg, 1903; Berkhoudt, Dubbeldam, & Zeilstra, 1981; Wild et al., 1985), the ascending projections of the avian nTTD have been largely neglected, with the exception of the work by Arends et al. (1984) in the mallard. In the present study, the projections of nTTD were examined with sensitive anterograde and retrograde tracers in the zebra finch. We show that the nTTD subnuclei are highly interconnected and project upon PrV, predominantly ipsilaterally, and that there are two projections to the contralateral dorsal thalamus. One originates mainly from cells in lateral interpolaris and terminates in Uva, while the other originates from cells in both interpolaris and oralis and terminates in DIVA. Whether the cells originating the projections from the nTTD to their different targets are segregated within these nuclei and whether the terminal fields originating from different nTTD subnuclei are segregated in PrV and DIVA, are matters that will require

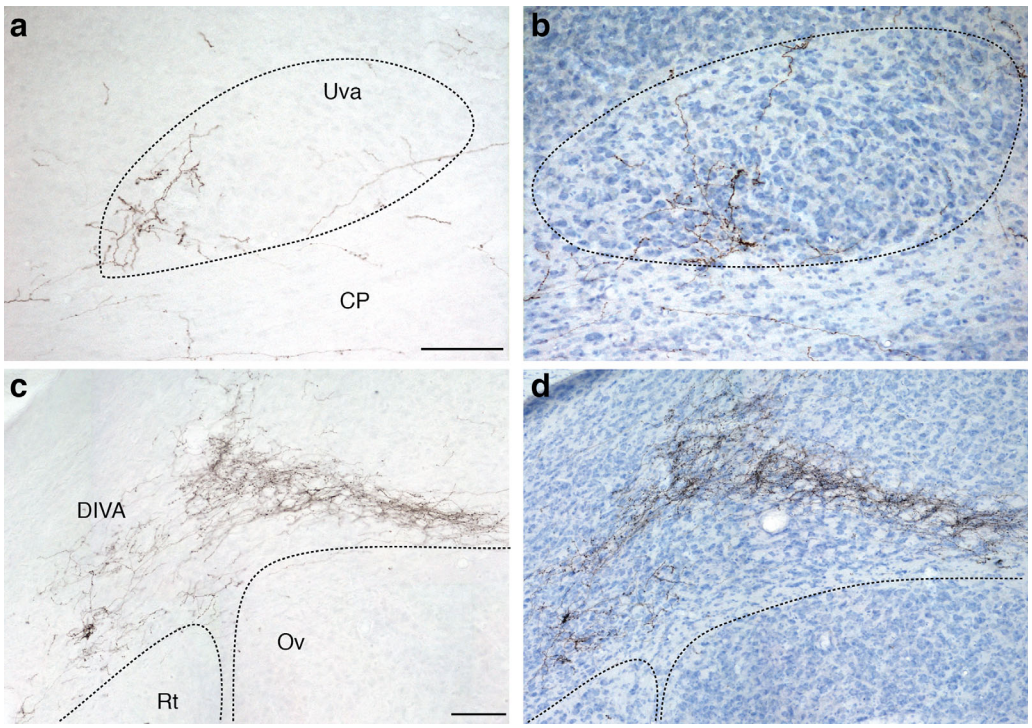

FIGURE 5 Bright field photomicrographs showing BDA labeled terminal fields in non-counterstained (a, c) and Nissl-counterstained (b, d) coronal sections of the contralateral Uva (a, b), and DIVA (c, d) after an injection in the nTTD (injection 4 in Figure 1). Medial is to right. Scale bars = 100 μm, scale bar in (a) for (a-b) and in (c) for (c-d).

further investigation. Trigeminal projections to the cerebellum were not addressed in this work. A scheme of the ascending projections of the sensory trigeminal complex of the zebra finch is presented in Figure 9.

4.1 | Comparison to previous findings

Our results partially confirm previous reports of trigeminal projections in the mallard and pigeon. Intra-trigeminal projections were described in the mallard by Arends et al. (1984), who showed that projections arising from interpolaris reach oralis and the ventral part of PrV bilaterally. Our results contrast with those of Arends et al. (1984) in showing that these bilateral projections originate mostly from cells in the caudalis and interpolaris nTTD and that they target cells located throughout the rostro-caudal extent of the nTTD and the PrV.

In agreement with Arends et al. (1984), we could not confirm retrogradely a putative projection from nTTD to the ICo/MLd, which was suggested by the anterograde tracing experiments. It thus appears that the real origin of this projection is the dorsal column nuclei (Wild, 1995). Also in agreement with Arends et al. (1984), we did not find any evidence of a projection from the nTTD to the optic tectum, which contrasts with findings of projections from nTTD to the contralateral midbrain roof (the optic tectum and/or the torus semicircularis) in representatives of most other main vertebrate groups, including cyclostomes (De Arriba & Pombal, 2007), actinopterygians (Northcutt, 1982; Yamamoto, Kato, Okada, & Somiya, 2010), anurans (Zittlau, Claas, & Münz, 1988), mammals (Aitkin, Kenyon, & Philpott, 1981; Killackey & Erzurumlu, 1981; Wiberg et al., 1987), and lepidosauromorphs (Molenaar & Fizaan-Oostveen, 1980; Dacey & Ulinski, 1986). Information about the presence or absence of this pathway in crocodilians could tell us whether it was lost early in archosaurians or closer to the origin of the avian lineage.

Regarding the projections to the contralateral dorsal thalamus, our results differ from those of Arends et al. (1984) and Korzeniewska and

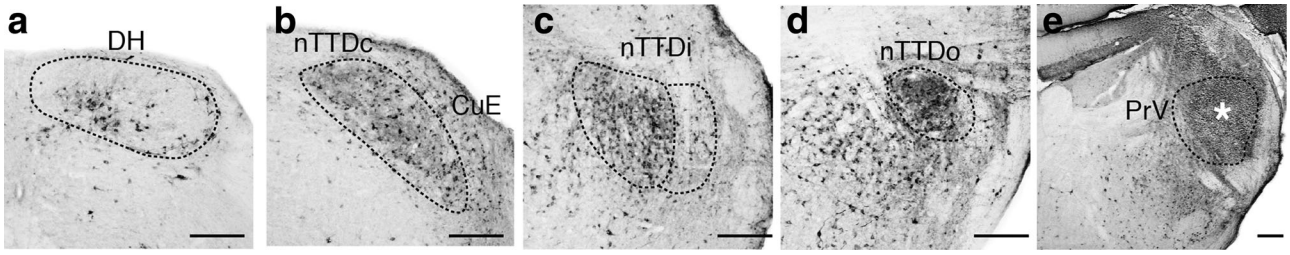

FIGURE 6 Example of retrograde labeling obtained with a CTB injection in PrV. Bright field photomicrographs showing CTB labeling in (a) cervical dorsal horn, (b) caudalis, (c) interpolaris, (d) oralis, (e) injection site in PrV, white asterisk indicates the center of the injection site. Scale bars = 200 μm

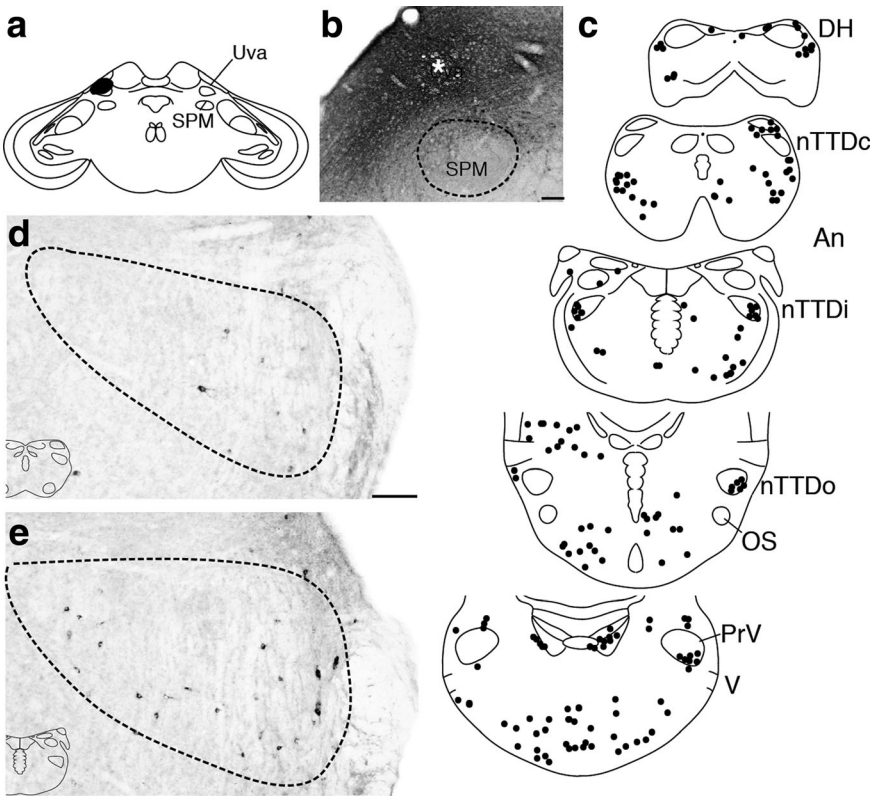

FIGURE 7 Retrograde labeling in nTTD obtained after a CTB injection in Uva. (a) Schematic representation of the location of the injection site in the coronal plane. (b) Brightfield photomicrograph showing the injection site. White asterisk indicates the center of the injection. (c) Schematic representation (from caudal to rostral) of the location of retrogradely labeled cells (black dots) in the trigeminal nuclei. (d, e) Bright field photomicrographs showing CTB labeling of somata at the level of interpolaris. Insets represent the rostrocaudal level of the coronal sections. Scale bars = 100 μm. Scale bar in (d) for (d, e)

Güntürkün (1990) in the location of the cells of origin of the projection to DLPc/Uva. Both groups found this projection to originate from a small population of cells, either in caudalis (Arends et al., 1984) or throughout the rostro-caudal extent of nTTD (Korzeniewska & Güntürkün, 1990). In contrast, our results suggest that this projection originates from a group of cells located in interpolaris. The projection from nTTD to DIVA that we show in the present work was not described by Arends et al. (1984) in the mallard, and neither did we confirm a projection from DIVA to Uva that might be similar to the projection from DIVA to DLPc described by Korzeniewska and Güntürkün (1990) in the pigeon.

Beyond possible species differences, the use of different tracers or tracing methods likely contributes significantly to differing results. Arends et al. (1984) used lesions, tritiated leucine, and horseradish peroxidase (HRP), while Korzeniewska and Güntürkün (1990) used HRP, wheat germ agglutinin-HRP, Fast blue, and rhodamine isothiocyanate. Furthermore, while studying the projections of the dorsal column and external cuneate nuclei, Wild (1989) made injections of wheat germ agglutinin-HRP in DLPc and DIVA of the pigeon and did not find significant retrograde labeling in the nTTD. In the present study, 3kDa BDA was used primarily as an anterograde tracer, while CTB was used as a retrograde tracer. Both tracers transport in anterograde and retrograde directions (Fritzsch, 1993; Luppi, Sakai, Salvert, Fort, & Jouvet, 1987; Reiner et al., 2000; Angelucci, Clascá, & Sur, 1996); however, our own observations indicate that 3 kDa BDA is more efficient as an anterograde tracer and CTB as a retrograde tracer. Furthermore, the retrograde transport of 3 kDa BDA was instrumental in determining the interconnections between nTTD subnuclei, and the anterograde transport of CTB provided good confirmation of the location of the injections in PrV, Uva, and DIVA.

4.2 | Implications of a trigeminal input to Uva in songbirds

Songbirds have a sophisticated motor control system for song production, in which feedback loops at multiple levels are thought to play important roles in the circuit's function (Ashmore, Renk, & Schmidt, 2008; Schmidt & Wild, 2014; Alonso, Trevisan, Amador, Goller, & Mindlin, 2015). During singing, movements and adoption of different positions of the beak, upper vocal tract, and tongue act to filter the sound produced at the syrinx (Hoese, Podos, Boetticher, & Nowicki, 2000; Riede, Suthers, Fletcher, & Blevins, 2006; Ohms, Beckers, Ten Cate, & Suthers, 2012; Riede, Schilling, & Goller, 2013; Suthers, Rothgerber, & Jensen, 2016). In addition, the syrinx itself has sensory receptors that are innervated by fibers that project to the sensory trigeminal nuclei (Bottjer & Arnold, 1982, Faunes et al., 2017), although the

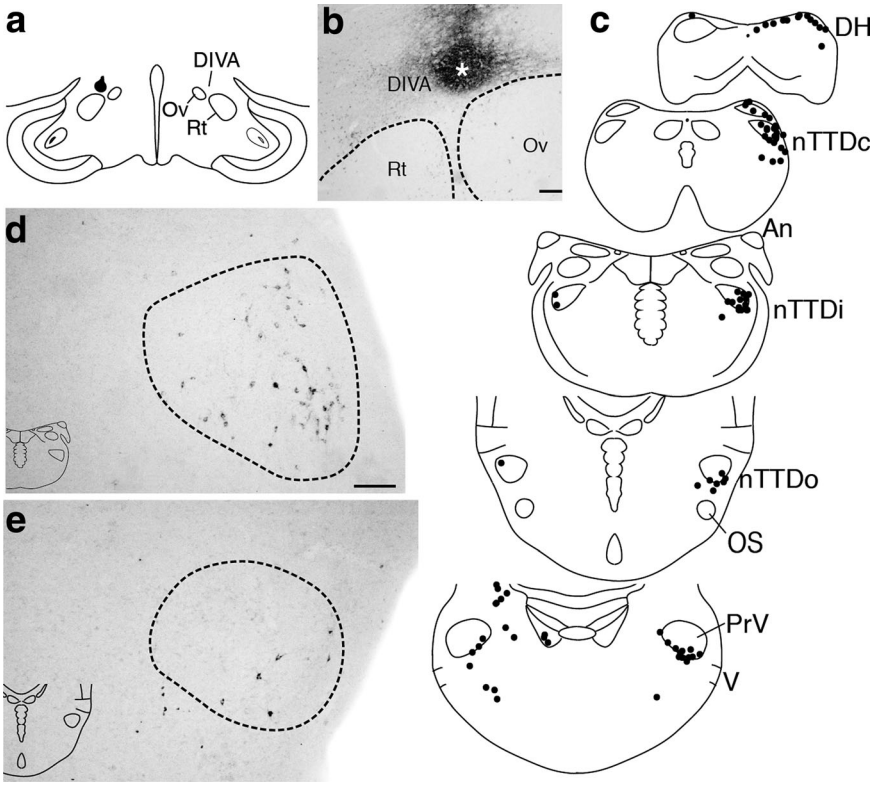

FIGURE 8 Retrograde labelling in the nTTD obtained after a CTB injection in DIVA. (a) Schematic representation of the location of the injection site in the coronal plane. (b) Brightfield photomicrograph showing the injection site. White asterisk indicates the center of the injection site. (c) Schematic representation (from caudal to rostral) of the location of retrogradely labeled cells (black dots) in the trigeminal nuclei. (d, e) Bright field photomicrographs showing CTB labeling of somata at the levels of interpolaris (d) and oralis (e). Insets represent the rostrocaudal level of the coronal sections. Scale bars = 100 μm. Scale bar in (d) for (d, e)

nature of these receptors is not known specifically. Nevertheless, it seems likely that trigeminal sensory feedback could be involved in the control of singing (Bottjer & To, 2012). Uva provides a multimodal sensory input to the song system (Nottebohm et al., 1982; Wild, 1994; Akutagawa & Konishi, 2005) and our finding of a projection from interpolaris to Uva suggests that sensory feedback from the beak, upper vocal tract, tongue, and syrinx could reach the song system through this portal. The apparent absence of a specific interpolaris-DLPc projection in ducks and pigeons (Arends et al., 1984; Korzeniewska & Güntürkün, 1990) might indicate that the interpolaris-Uva projection in zebra finches is a songbird specialization.

4.3 | Comparison with mammals

Most of our knowledge of the organization of central trigeminal pathways concerns the mystacial pad whiskers of rodents. These whiskers, which are endowed with multifarious low-threshold mechanoreceptors, are moved in a rhythmic way when the animal is actively exploring the tactile environment, a behavior known as whisking (reviewed in Deschênes & Urbain, 2009; Bosman et al., 2011). The central trigeminal pathways involved in whisking are highly developed, and the somatosensory brainstem, thalamus, and cortex contain cytoarchitectonically distinct units called barreloids, barrelletes, and barrels, respectively; each bearing cells with receptive fields dominated by a single whisker (Woolsey & Van der Loos, 1970; Ma, 1991; reviewed in Deschênes & Urbain, 2009; Bosman et al., 2011).

4.3.1 | Intra-trigeminal connections

As in birds, the mammalian trigeminal nerve enters the brainstem and branches into an ascending tract that ends in the PrV and a descending tract that ends in a column of nuclei equivalent to the avian nTTD, the spinal trigeminal nuclei (Ramón y Cajal, 1909; Astrom, 1953), which are divided into the oralis, interpolaris, and caudalis subnuclei (Olszewski, 1950; Astrom, 1953; Torvik, 1956). These subnuclei are highly interconnected, and projections from caudalis and interpolaris upon oralis and the PrV are especially abundant (Panneton & Burton, 1982; Ikeda, Tanami, & Matsushita, 1984; Nasution & Shigenaga, 1987; Jacquin, Chiaia, Haring, & Rhoades, 1990; Voisin, Domejean-Orliaguet, Chalus, Dallel, & Woda, 2002). For example, the PrV of rats receives a glutamatergic projection from caudalis and a GABA/glicinergic projection from interpolaris (Furuta et al., 2008). These intra-trigeminal connections are thought to be involved in shaping the receptive fields of trigeminal projection neurons, thus gating the ascending flow of somatosensory activity produced by different stimuli arising from different behavioral states (Jacquin et al., 1990; Chiang, Hu, Hu, Dostrovsky, & Sessle, 2002; Timofeeva, Lavallée, Arsenault, & Deschênes, 2004; Furuta et al., 2008; Deschênes & Urbain, 2009).

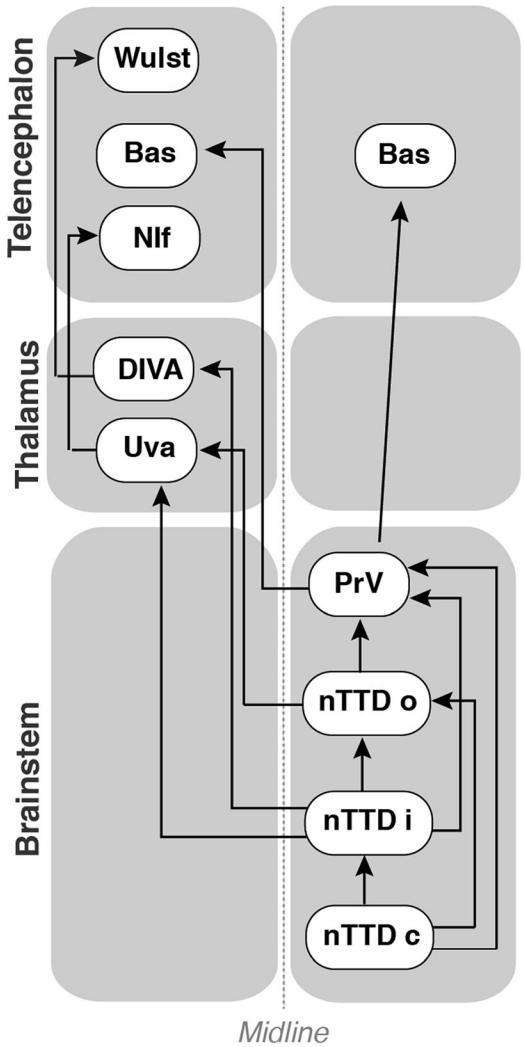

FIGURE 9 Schematic depiction of the ascending projections of the sensory trigeminal complex in the zebra finch.

In the zebra finch we found a similar pattern of intra-trigeminal connections, where each nTTD subnucleus projects upon the nTTD subnuclei rostral to it and to PrV. As in mammals, these are probably involved in shaping the receptive fields of trigeminal projection neurons (Silver & Witkovsky, 1973).

4.3.2 | Thalamic projections

Even though the somatosensory systems of mammals and birds share a generally similar organization, their trigeminal systems seem to differ greatly, due to the peculiar organization of the ascending trigeminal projections in birds. The present work, however, indicates that these trigeminal systems are less divergent than previously thought.

In mammals, the organization of the ascending trigeminal pathways can be seen as a specialization of the general somatosensory system, in that it involves thalamic and cortical structures adjacent to those involved in somatosensory reception from the body (Dawson & Killackey, 1987; Diamond, Armstrong-James, & Ebner, 1992). In rodents, whisker inputs to the thalamorecipient somatosensory cortices arrive over at least four parallel pathways that originate in the principal and spinal interpolaris trigeminal nuclei. Two lemniscal pathways originate from cells in the PrV and reach the barrel cortex in S1 through the barreloids in the thalamic ventral posterior medial nucleus in the thalamus, one that is composed of cells with single-whisker receptive fields and one composed of cells with multi-whisker receptive fields. A third extralemniscal pathway originates from multi-whisker responding interpolaris cells and reaches S2 and the inter-barrel areas in S1 through a region of the ventral posterior medial nucleus where no barreloids are discernible. The fourth, paralemniscal pathway, originates from multi-whisker responding cells in interpolaris and reaches S1, S2 and the motor cortex through the posterior thalamic nucleus, which is adjacent to the ventral posterior medial nucleus (reviewed in Deschênes & Urbain, 2009). Much less information about ascending trigeminal pathways is available for reptiles, although there are reports of similar trigeminal projections that reach the dorsal cortex through a relay in the dorsal thalamus in lizards (Desfilis et al., 1998; Desfilis, Font, Belekhova, & Kenigfest, 2002).

The homology between the thalamocortical somatosensory pathways found in mammals and reptiles and the avian lemniscal pathway that reaches HA via DIVA is generally accepted (Wild, 1997; Medina & Reiner, 2000). However, in contrast to the somatosensory system of mammals, the avian lemniscal somatosensory pathway has seemed thus far to lack an evident trigeminal component. There are some reports of somatosensory activity from the beak and/or head in the dorsal thalamus (including Uva and DIVA, Korzeniewska, 1987; Korzeniewska & Güntürkün, 1990). However, the projection from nTTD to the contralateral DIVA described in the present study is the first anatomical evidence of a trigeminal component in this lemniscal pathway.

The most distinctive trait of the avian somatosensory system is the direct projection from PrV to nucleus Bas in the rostral telencephalon, which is absent in other vertebrates (even though there are some indications that this projection might also be present in reptiles, e.g., Ten Donkelaar & Boer-Van Huizen, 1981). Although the predominant inputs to Bas are trigeminal, other kinds of input have been amply described, including whole body somatosensory, auditory, and vestibular inputs (review in Wild, 2015). Interestingly, the body somatosensory inputs originate, in the barn owl, in an external pontine nucleus (Wild, Kubke, & Carr, 2001), or, in the budgerigar, in a subprincipal nucleus lying immediately ventral to PrV (Wild, Reinke, & Farabaugh, 1997). In the present study CTB injections in DIVA and Uva produced small groups of labeled cells in the ventral part of PrV, but whether this constitutes another peculiarity of the quintofrontal system (i.e., one having a population of cells that projects to both Bas and the dorsal thalamus), or whether these cells are simply displaced oralis cells, similar to those described in the pigeon by Wild and Zeigler (1996), is not clear. Further studies on afferent input to Bas in other avian species and a determination of the presence or absence of a homologous nucleus in crocodylians are needed to understand whether the quintofrontal system evolved first as a trigeminal specialization and acquired whole body somatosensory and auditory functions later, or it became secondarily dominated by trigeminal input.

Our finding of DIVA projecting cells in the trigeminal nuclei suggests that, in birds, a trigeminal lemniscal pathway has been conserved at the same time as a novel ascending trigeminal pathway has evolved that targets the telencephalon directly, and it renders unnecessary the postulation that Bas might be a rostral displacement of an apparently absent trigeminal component of the somatosensory dorsal thalamus (Cohen & Karten, 1974). Nonetheless, it is unclear whether the somatosensory pathways that traverse Uva and the quintofrontal system bear homologies to any of the multiple mammalian trigeminal pathways. The broad trigeminal intratelencephalic and descending projections involving Bas could constitute circuits homologous to mammalian cortical circuits (Wild et al., 1985; Atoji & Wild, 2012), even if the underlying individual cell populations might not be strictly homologized in a 1:1 fashion (Striedter, 1999; Faunes, Francisco Botelho, Ahumada Galleguillos, & Mpodozis, 2015). Studies of the detailed connections of these pathways, like those that have been advanced for the visual and auditory avian pallia (Krützfeldt & Wild, 2004, 2005; Wang, Brzozowska-Prechtl, & Karten, 2010; Ahumada-Galleguillos, Fernández, Marin, Letelier, & Mpodozis, 2015) are needed to establish fair comparisons to the much better known mammalian somatosensory system.

ACKNOWLEDGMENTS

We thank the Centre for Brain Research of the University of Auckland. We also thank Sara Fernández for her kind help in some steps of this work.

CONFLICT OF INTEREST

The authors declare that there is no actual or potential conflict of interest in relation to this article.

AUTHOR CONTRIBUTIONS

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: JMW. Acquisition of data: MF, JMW. Analysis and interpretation of data: MF, JMW. Drafting of the manuscript: MF. Critical revision of the manuscript for important intellectual content: JMW. Obtained funding: JMW. Study supervision: JMW.

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How to cite this article: Faunes M, Wild JM. The ascending projections of the nuclei of the descending trigeminal tract (nTTD) in the zebra finch (*Taeniopygia guttata*). *J Comp Neurol*. 2017;00:000–000. https://doi.org/10.1002/cne.24247