Interactive report

Parvalbumin-positive projection neurons characterise the vocal premotor pathway in male, but not female, zebra finches

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Abstract

Parvalbumin (PV) and calbindin (CB) immunoreactivities were assessed in nucleus robustus archistriatalis (RA) of male and female zebra finches, together with retrograde labelling of RA neurons. The results of double and triple labelling experiments suggested that, in males, moderately and faintly PV-positive neurons were projection neurons, but that all intensely PV-positive cells were not. The latter, which are presumably interneurons, were also intensely CB-positive, and may correspond to the GABAergic inhibitory interneurons identified by others. In addition, the complete RA pathway and its terminal fields in the respiratory–vocal nuclei of the brainstem were strongly PV-positive. In female zebra finches, which do not sing, no evidence was found that PV-positive RA cells were projection neurons, yet the pattern of projections of RA neurons, as determined by anterograde transport of biotinylated dextran amine, was very similar to that of RA in males. Moreover, in females, RA neurons retrogradely labelled from injections of cholera toxin B-chain into the tracheosyringeal nucleus (XIIts) were abundant and included, in the lateral part of the nucleus, a population of cells that were as large as those in the male RA. Parvalbumin immunoreactivity was also present in RA and its projections in males of several other songbird species (northern cardinal, brown headed cowbird, canary) and in the female cardinal, which sings to some extent, but the labelling was not as intense as that in male zebra finches. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In oscine songbirds, such as zebra finches and canaries, there is a set of interconnected nuclei in the forebrain that is dedicated to the learning and production of song [2,20,21]. The output from this circuit is a spherical nucleus in the caudal telencephalon called robustus archistriatalis (RA). This nucleus is remarkable for a variety of reasons, not least of which is the fact that it has long descending projections that terminate directly on vocal motor neurons (tracheosyringeal nucleus or XIIIts) and on premotor respiratory nuclei in the medulla [20,23,31,32,36]. RA is thus intimately concerned with the control of vocal production via its influence on syringeal and respiratory muscles. The coordination of the activity of these functionally related but disparate muscle groups during singing may be effected via an inhibitory RA interneuron that has extensive dendritic Arborizations within the nucleus [27].

Another remarkable feature of RA in zebra finches and canaries is that it is sexually differentiated, being much larger in the adult male, who sings, than in the adult female, who sings much less or not at all [12,15,16,19]. The diameter of RA somata in the adult male zebra finch can be more than twice that of female RA somata [9], and male RA neurons also have larger dendritic fields [8]; yet RA neurons in the female are known to project upon XIIIts, as they do in the male, albeit providing a smaller terminal field [9]. In this last study, however, retrograde confirma-
tion of the origin of the projection to XItS in the female was based on a single case, which, until recently [12], was unique in the literature on this subject. Other projections of RA in females, such as those to the intercollicular region, have been described as somewhat different from those of RA in males [9], and no information is available on female RA projections to other targets identified in males, such as the respiratory premotor nuclei, which are presumably involved in calling, as well as in singing [9,32].

A third feature of RA, again in the male, is that many of its neurons are positive for the calcium binding protein parvalbumin [3], an immunoreactivity that has a developmental pattern reflecting that of the nucleus as a whole [4]. The presence of these PV-positive neurons, and of fewer calbindin-D$_{28K}$-positive neurons (CB-positive neurons [4]), in RA is curious, however, because it is not known whether they are projection neurons or have axons that remain within the confines of the nucleus, i.e. interneurons. Inhibitory interneurons in RA of adult male zebra finches have been suggested by immunostaining for GABA [10,24] and have been demonstrated by a combination of intracellular electrophysiology and staining. Spiro et al. [27] showed that neurons with fast action potentials and steep current–frequency relationships had small somata (~136 $\mu m^2$) with thin aspiny processes that extended throughout large parts of the nucleus. These neurons were found to be distinctly similar in morphology to RA neurons stained for glutamic acid decarboxylase (GAD), supporting the idea that they were inhibitory interneurons [27]. But since the PV-positive neurons thus far demonstrated in RA have been reported to have somewhat larger somata (12–17 $\mu m$ diameter in adult male zebra finch [3]), it is not clear whether they are likely to include the inhibitory interneurons identified by Spiro et al. [27] and Sakaguchi et al. [24]. On the other hand, if the PV-positive neurons originate long descending projections, then that would be unusual, since parvalbumin is not generally associated with such neurons (e.g. [11]).

In the present study, we sought to determine whether PV-positive and CB-positive neurons are present in RA of female as well as male songbirds, and whether or not these neurons are projection neurons. This involved labelling RA neurons by retrograde transport from XItS and double or triple labelling for parvalbumin and calbindin. We also assessed the presence of parvalbumin immunoreactivity within the whole vocal premotor pathway, and compared this with the RA pathway defined by anterograde tracing methods, in both males and females. The main focus of the study was on adult zebra finches, in which sexual differentiation of brain and vocal behavior is pronounced, but a small number of birds of other songbird species (cowbird, canary, northern cardinal) were included to assess the generality of the parvalbumin immunoreactivity of the RA pathway and to extend the findings to species in which the female sings, albeit less than the male.

2. Materials and methods

2.1. Subjects

Thirty-eight songbirds were used in this study: sixteen male and sixteen female zebra finches (Taeniopygia guttata), one male canary (Serinus canaria), two female and one male northern cardinals (Cardinalis cardinalis), one female and one male brown-headed cowbird (Melocephalus ater). The exact ages of the birds were unknown, but all were adult, species-specific song having been recorded or monitored in the male zebra finches, male and female northern cardinals, male canary and male cowbird. The cowbirds and cardinals were wild caught, the other species were obtained from commercial suppliers.

2.2. Anaesthesia

For all surgical procedures, the animals were anaesthetised either by intramuscular injections of a mixture of ketamine (50 mg/kg) and xylazine (20 mg/kg) or by inhaled isofluorane gas carried in oxygen. The procedures were carried out in accordance with the guidelines stipulated by the University of Auckland Animal Ethics Committee, and every effort was made to minimise the numbers of experimental animals used.

2.3. Injection procedures

All injections in either RA or XItS were made using a combination of stereotaxis [28] and electrophysiological recording. The head skin was reflected from the midline, a burr hole made in the skull either over the caudal telencephalon or over the cerebellum, and the dura incised. A tungsten microelectrode (Frederick Haer; 3–5 M$\Omega$) was used to record multiunit activity (an indifferent electrode was inserted in a neck muscle) as it was passed either through the archistriatum and into RA, or through the cerebellum and into XItS. The dorsal border of RA was identified by a sudden increase in multiunit activity followed by a characteristic ‘bursty’ pattern of discharge through the depth of the nucleus [32]. RA in females was more difficult to locate because of its small size. XItS was identified by its characteristic respiratory rhythm in phase with expiration [33].

Following identification of the nuclei, a glass micropipette loaded with one of the tracers replaced the recording electrode and injections were made either iontophoretically (2–4 $\mu A$ positive current, 7 s on, 7 s off for a total time of 10–20 min) or using air pressure via a picospritzer (General Valve, Fairfield, NJ, USA).

The tracers used were: biotinylated dextran amine (BDA), dextran tetramethylrhodamine (Fluoro-ruby) and dextran fluorescein (Fluoro-emerald), (each 10 K M$_w$, lysine fixable; Molecular Probes), all 10% solutions in...
phosphate buffered saline (PBS); or Cholera toxin B-chain (CTB, List Biological Laboratories), 1% in PBS.

Unilateral injections of BDA were made in RA of five female and two male zebra finches, one female northern cardinal, and one male canary. Injections of one of the other tracers were made in XIlts of five female and nine male zebra finches. The XIlts injections were either unilateral or bilateral. If bilateral, different tracers were used to deposit different tracers on each side, e.g. CTB on one side and Fluoro-ruby on the other.

2.4. Lesions

Unilateral electrolytic lesions were made in RA in two male zebra finches and one male canary. Stainless steel 00 insect pins, insulated except at their tips, were used to first locate RA by recording multiunit activity, and then to produce a lesion by passing 0.25 mA anodal current for 30 s. In one of the zebra finches and in the canary, BDA was injected into RA on the opposite side of the brain. This was done to ensure that the unilateral lesioning did not in some way interfere with the projections of the contralateral RA and its associated PV-immunoreactivity.

2.5. Perfusion and sectioning

All birds were allowed to survive from 2 to 7 days depending on the tracer injected. Generally, in the afternoon, they were deeply anaesthetised with an overdose of ketamine and xylazine and perfused through the heart with saline, followed by fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4). The brains were removed from the skull and postfixed in the same fixative for 5–15 h. After cryoprotection in 30% sucrose in PBS for 15–48 h, the brains were sectioned at 35 μm in either the coronal or sagittal planes on a freezing microtome. The sections were collected serially in four series, one or more of which from each case was processed to reveal one or more of the tracers and, sometimes also parvalbumin and/or calbindin immunoreactivity. For some control studies, sections through RA were cut at 10 μm and collected in two serially adjacent series. One series was processed for parvalbumin (Section 2.6.3) and the other for calbindin immunoreactivity (Section 2.6.4).

2.6. Processing and immunochemistry

To improve penetrability of the immunochemical and histochemical agents, and to block non-specific peroxidase reactivity, most sections were pretreated with 50% aqueous methanol containing 1% H2O2 for 10–20 min. All immunochemical reactions were carried out on free-floating sections at room temperature in PBS containing 0.4% Triton X-100. All primary antibody incubations were overnight in duration, and all media contained normal horse serum (or rabbit serum for sections stained only for CTB). All other incubations were for 1–2 h duration. Between incubations, all sections were thoroughly washed in PBS. For all immunochemical procedures outlined below, the antibodies and ancillary agents used were carefully chosen and checked for cross reactivity between antibodies and/or epitopes.

2.6.1. BDA reactivity

Sections were incubated with streptavidin peroxidase medium (1:1000, Molecular Probes), followed by incubations in a 3,3′-diaminobenzidine (DAB) medium (0.025% DAB with 0.005% H2O2 and 0.002% CoCl2 in PBS). This resulted in a black opaque reaction product.

2.6.2. CTB immunoreactivity

Sections were incubated overnight in a goat anti-CTB medium (1:30 000, List Biological Laboratories), then with a biotinylated rabbit anti-goat antibody (1:200, Sigma). This was followed by incubations with streptavidin peroxidase and DAB (as above). In some cases, no CoCl2 was added to the DAB medium, which produced a brown reaction product.

2.6.3. Parvalbumin immunoreactivity

Sections were incubated overnight in a monoclonal anti-parvalbumin antibody medium (1:10 000 for opaque DAB staining, or 1:1000 for fluorescence microscopy. This antibody was raised against carp parvalbumin: Sigma, or SWANT 235, Basel, Switzerland), followed by a biotinylated horse anti-mouse antibody (1:200, Vector); then by incubations in either streptavidin peroxidase followed by DAB (as described above), or a variety of fluorescent streptavidin complexes (1:200, i.e. conjugated with either fluorescein, rhodamine, or Alexa 488, 546 or 568 depending on whether green or red fluorescence was required; all Molecular Probes products). These fluorescent labels were used primarily in Multiple labelling protocols.

2.6.4. Calbindin immunoreactivity

Two different anti-calbindin antibodies were used (both raised against chicken calbindin): For staining sections with opaque DAB reaction product, some sections were incubated with a monoclonal antibody (1:10 000, Sigma), then with a biotinylated horse anti-mouse antibody, followed by streptavidin peroxidase and DAB (as above for parvalbumin). Other sections destined for multiple labelling fluorescence microscopy were incubated with an antibody raised in rabbit (1:500, Emson, Cambridge, UK) [14,25], then with a biotinylated donkey anti-rabbit (1:200, Amersham) antibody, and the immunoreactivity was finally revealed using the variety of the streptavidin complexes described above, as well as streptavidin Alexa 350 (Molecular Probes).
2.7. Multiple labelling protocols

For the assessment of the presence of retrogradely labelled neurons and parvalbumin and/or calbindin immunoreactivity within RA, some of the procedures described above were combined using either black-brown opaque staining techniques, or by double or triple-coloured fluorescence techniques.

CTB (black)-parvalbumin (brown) opaque staining was generally achieved by incubation of sections in a cocktail of the goat anti-CTB (1:30 000) and the mouse anti-parvalbumin (1:10 000) primary antibodies (as above). The CTB was stained black using the biotinylated rabbit anti-goat secondary antibody, streptavidin peroxidase and the DAB CoCl₂ (black) procedure. Parvalbumin immunoreactivity was revealed and rendered brown using the biotinylated horse anti-mouse secondary antibody, streptavidin peroxidase DAB (brown) procedure (as above).

CTB (red)-Parvalbumin (green) fluorescent staining was generally achieved by incubating sections in a cocktail of the anti-CTB (1:30 000) and anti-parvalbumin (1:1000) antibodies (described above). CTB was then stained red with a direct secondary, donkey key anti-goat Alexa 546 antibody (1:200, Molecular Probes). Parvalbumin immunoreactivity was next stained green, indirectly by incubations with the biotinylated horse anti-mouse secondary antibody (1:200), followed by streptavidin fluorescein (1:200).

Rhodamine dextran (red) and parvalbumin (green) fluorescent staining was achieved by incubating sections that contained retrogradely labelled red fluorescent RA neurons with the mouse anti-parvalbumin primary antibody (1:1000); then by incubations with the biotinylated horse anti-mouse secondary antibody (1:200), and finally streptavidin fluorescein (1:200).

Rhodamine dextran (red), parvalbumin (green) and calbindin (blue) fluorescent staining was achieved by incubating sections that contained retrogradely labelled red fluorescent RA neurons in a cocktail of the mouse anti-parvalbumin (1:1000) and rabbit anti-calbindin (1:500) antibodies. The parvalbumin was stained green using a direct secondary goat anti-mouse Alexa 488 antibody (1:200, Molecular Probes). Calbindin was subsequently stained for by incubating sections with a biotinylated donkey anti-rabbit secondary antibody (1:200), followed by incubation with streptavidin Alexa 350 (1:100 Molecular Probes).

Negative controls involved the omission of one or more of the primary antibodies, which always resulted in the absence of labelling. Internal control for multiple labelling of parvalbumin, calbindin and retrogradely labelled neurons was provided by the presence, in the same section, of different telencephalic and brainstem neurons that were singly fluorescent, i.e. that showed only red, green or blue fluorescence. If cross reactivity between the various procedures had been a problem, most cells would be expected to be fluorescent for more than one of the colours, which was not the case. However, in order to ensure that cross-reactivity was not a potential problem in the interpretation of the double labelling of cells with the mouse anti-parvalbumin and rabbit anti-calbindin antibodies, adjacent 10-µm frozen sections through the RA of a male zebra finch were incubated either with the anti-parvalbumin or the anti-calbindin antibody, at the same dilutions as given above for 35-µm thick sections. Parvalbumin was visualised (green) indirectly by using the appropriate biotinylated secondary antibody and streptavidin fluorescein, whereas calbindin was visualised (red) using the direct secondary goat anti rabbit Alexa 546 antibody.

These double and triple labelling procedures enabled the distributions and possible co-localisation of parvalbumin and calbindin and retrogradely labelled neurons in RA to be assessed with both bright field and fluorescence microscopy.

Sections to be examined for transported BDA were mounted on lightly subbed slides, air dried, dehydrated in alcohol, cleared in xylenes, and coverslipped with DePeX. One series was left uncounterstained, and one was counter-stained with cresyl violet. These sections were viewed in bright field and the projections were drawn using a macroprojector and camera lucida. Sections to be examined and photographed in the fluorescence microscope were mounted on slides and coverslipped either with Citifluor (Agar Aids) or Prolong (Molecular Probes) antifade agents. In some cases, the coverslips were subsequently removed and the section counterstained with cresyl violet.

Colour 35-mm photographic records were made of the same field of some of the multiply fluorescent sections, and of adjacent 10-µm sections in the cross-reactivity control case, in order to map the distributions of singly, doubly or triply labelled cells. Comparisons of the distributions of the different labels was achieved by scanning sets of three 35-mm slides (of red, green and blue fluorescence) of the same field, or of two 35-mm slides (of red and green fluorescence) of adjacent sections in the cross-reactivity control case, into PhotoShop (Adobe) with a Microtek ArtixScan 4000t slide scanner. These images were then carefully superimposed using various landmarks (e.g. blood vessels, see Fig. 4G–J), and combined to show two of the colours in the same image. Single and combined images were adjusted for evenness of illumination, brightness, contrast and colour balance. Cell sizes were measured using a 40× objective and drawing tube.

3. Results

3.1. RA in the zebra finch

In Nissl-stained sagittal sections, the adult female RA forms an ellipsoid or ovoid nucleus within the caudal archistriatum (Fig. 1A; see also [16]). In coronal sections it
Fig. 1. (A) Photomicrograph of Nissl stained parasagittal section through RA of female zebra finch; rostral to the left. (B) Photomicrograph of Nissl stained frontal section through RA of female zebra finch; medial to the left. (C) Photomicrograph of Nissl stained frontal section through RA of male zebra finch (outlined with arrow heads); medial to the left. (D) Neurons in RA of a female zebra finch retrogradely labelled with CTB from an injection centered on XIIIs, with spread to the medial part of nucleus retroambigualis; medial to the left (compare D with B). (E) Higher power view of the boxed area in the medial part of RA shown in (D). Note the cytoplasmic concentration of the CTB. (F) Higher power view of the boxed area in the lateral part of RA shown in (D). Compare the sizes of neurons in (E) and (F) and note the difference in packing density of labelled neurons. (G) Nissl counterstained version of (E). Note the many small, densely packed, basophilic cells between the retrogradely labelled cells (compare E and G). (H) Nissl counterstained version of (F). Note the smaller number and more dispersed nature of the small basophilic cells than in (G). LAD, lamina archstriatalis dorsalis; calibration bars = 100 μm; that shown in (H) applies also to (E, F and G).
Fig. 3. PV-positive cells and neuropil in RA of a male cowbird (A), a male cardinal (C) and a female cardinal (E). PV-positive terminal fields in XIIts of a male cowbird (B), in XIIts and nucleus retroambigualis (RAm) of a male cardinal (D) and in XIIts and RAm of a female cardinal (F). (A) and (B) are sagittal sections, rostral to the left; (C–F) are coronal sections, medial to the left. Calibration bars 5 100 μm for (A, B, C and E); 1 mm for (D) and (F).

Fig. 2. PV-immunoreactivity in RA (fluorescently stained using a biotinylated secondary antibody and streptavidin fluorescein) (A) of a male zebra finch — frontal section, medial to the left. Note that the nucleus is defined by the PV-positive neuropil and that the PV-positive cells vary in intensity from intense through moderate, to faint. (B) Ditto for female RA, but note that the nucleus is much more poorly defined (by arrowheads) and that the dorsolateral border is indistinct — compare with Fig. 1B. (C) Neurons in RA of a male zebra finch retrogradely labelled by an injection of rhodamine dextran into XIIts. The relatively sparse retrograde labelling in this case was chosen to highlight the double labelling seen in (E). (D) Neurons in RA of a female zebra finch retrogradely labelled by an injection of CTB centered on XIIts (CTB fluorescently stained using an Alexa 546 direct secondary antibody). (E) Superimposition of (A) and (C) to show double labelling of neurons in RA of male zebra finch — frontal section, medial to the left. Double labelled cells appear as yellowish and coincide only with moderately and faintly labelled PV-positive cells. The boxed area is shown at higher power in (G), where arrow heads point to three double labelled cells with moderate PV immunoreactivity, and arrows point to three intensely labelled PV-positive cells that are not double labelled. (F) Superimposition of (B) and (D). The boxed area is shown at higher power in (H), which shows that, apart from one suspicious cell at the arrowhead, retrogradely labelled cells are different from PV-positive cells. Calibration bars = 100 μm.
forms more of a slender arc, convex medially, often with a poorly defined dorsolateral border (Fig. 1B; see also [12,19]). The nucleus is typically half the width of the adult male RA (see Fig. 1C) and the individual cells are more tightly packed than in the male, especially in medial parts of the nucleus, where there is a preponderance of small cells (~5–10 μm mean somal diameter [16]). The smallest of these (~5 μm) tend to be basophilic (Fig. 1G), are never retrogradely labelled from XIIIts injections, and are probably glia ([9] and see below). Interspersed between the glia, and often approximated by them, are small neurons (~8–10 μm) that have a pale-staining cytoplasm. As one moves more laterally through the female RA, however, the cell size tends to become more variable, and there is an increasing number of larger cells that are as large as those in the male RA (12–20 μm, Fig. 1D and F).

RA may also be defined on the basis of retrograde labelling from target nuclei (Figs. 1D, 2C and D, and 4A and B). However, in the female, if different populations of RA neurons have different projection targets, as appears to be the case in the male [30], and has also been suggested in the female [12], then a total definition of RA based on retrograde labelling can only be achieved if all target nuclei are injected. In the present study, only XIIIts was a target nucleus for injection, although some of the larger injections also included parts of the adjacent nucleus retroambigualis (RAm), due to spread of tracer from the injection center. Retrograde labelling in RA of one of these cases (female zebra finch) is shown in Fig. 1D. In Nissl counterstained sections (Fig. 1G and H) it was clear that the retrogradely labelled cells were different from and generally larger than the small, basophilic RA cells described above, supporting the suggestion that these latter small cells are glia [9]. The neurons retrogradely labelled with CTB formed a mediolateral size and packing density gradient, with the smaller ones (8–10 μm in diameter) medially and more densely packed, and the larger ones (12–20 μm) laterally and more dispersed (Fig. 1D–H).

RA can also be defined on the basis of its densely labelled PV-positive neuropil [3,4]. In the male zebra finch, the clear border of this neuropil coincides with the border of the nucleus as defined in Nissl stained material (Fig. 2A). In the female zebra finch, the border of the PV-positive neuropil is much less clear (Fig. 2B), corresponding with the less well defined border of RA as defined in Nissl stained material (compare Fig. 1B). RA is also PV-positive in male brown-headed cowbirds and male northern cardinals (Fig. 3A and C). It is also relatively well defined by PV-positive neuropil in the female northern cardinal (Fig. 3E), which sings to some extent.

In both males and females, a substantial contribution to the PV-positive neuropil in RA is made by the processes of PV-positive cell bodies, which vary both in intensity and in size (Fig. 2A,B,G and H). Although their intensity varies continuously from very weak to very strong, the PV-positive cells may be conveniently categorized for the purposes of description as intensely labelled, moderately labelled and faintly labelled. These categories, however, do not also sort for cell size, for there was a range of sizes in each category. The intensely labelled cells measured in the order of 10–13 μm (smallest somal diameter), compared with the slightly larger, moderately labelled cells (12–15 μm). Weakly labelled PV-positive cells could not be measured with certainty due to the poor definition of their boundaries. PV-positive cell bodies of various intensities were also present in RA of cowbirds and northern cardinals (Fig. 3).

In the archistriatum outside RA in zebra finches, the PV-positive cell bodies also varied in intensity and size, and were less concentrated than in RA (Fig. 2A and B). In the male, the intensely labelled archistriatal cells were generally smaller than those in RA, whereas in the female the reverse tended to be true. Thus, PV-positive cells in the female RA were generally smaller than those in the male RA (compare Fig. 2G and H).

3.2. Are the RA projection neurons also PV-positive?

Double labelling for both retrogradely transported tracer and parvalbumin was carried out in a series of male and

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**Fig. 4.** (A) Parasagittal section through RA of a male zebra finch in which CTB was injected in XIIIts — retrogradely labelled neurons appear as black and are confined to the ventral two thirds of the nucleus [30]. The section was also immunolabelled to reveal parvalbumin-like reactivity; PV-positive cells appear as various intensities of brown throughout the nucleus. Many RA neurons were presumably double labelled for PV and CTB, but this cannot be resolved in this preparation. (B) RA of a male zebra finch (frontal section, medial to the left), which received a large injection of rhodamine dextran into XIIIts, with spread outside the nucleus to include parts of nucleus retroambigualis. (C–E) Different views of the same frontal section of RA of a male zebra finch (medial to the left). (C) PV immunoreactivity (stained green using a biotinylated secondary antibody and streptavidin fluorescein); (D) CB immunoreactivity (stained red with a direct secondary, Alexa 546 antibody); (E) the superimposition of (C) and (D) — double labelled cells appear as a pale yellow. (F) Higher power view of the boxed area in (E). Again, the double labelled cells appear as pale yellow or yellow-green, but pure green PV-positive cells can be seen, as can a few pure red CB-positive cells. (G) Cells in RA of a male zebra finch retrogradely labelled from an injection of rhodamine dextran in XIIIts. (H) The same section and field in (G) stained green for PV immunoreactivity (using an Alexa 488 direct secondary antibody). Note the same cell at the arrowhead in both (G) and (H) is double labelled for moderate PV immunoreactivity and rhodamine dextran, i.e. is a PV-positive projection neuron. Note also the three intensely labelled PV-positive cells at the arrows, which are not double labelled and hence are probably not projection neurons. These same three cells appear in (I), which is the same section and field as in (G) and (H), but is immunolabelled for anti-CB, (stained blue using a biotinylated secondary antibody and streptavidin Alexa 350 and viewed with UV excitation). (I) shows (G) and (I) superimposed to show the same three cells as in (H) and (I), which are not retrogradely labelled; i.e. CB-positive RA cells are not projection neurons. Asterisks located next to blood vessels are used to align the different images. Calibration bars = 100 μm.
female zebra finches. Fig. 4A shows RA in a male zebra finch that received an injection of CTB in XIIIts; the section was subsequently stained for anti-PV. Retrogradely labelled neurons appear black and are largely confined to the ventral two thirds of the nucleus, as expected on the basis of previous studies of RA topography with respect to its target nuclei [30]. The PV-positive cell bodies appear as various shades of brown and are distributed throughout all parts of the nucleus. While there is a strong suggestion that many projection neurons are PV-positive in this material, the data remain equivocal because of the inability to resolve black and brown in the same cell. In fluorescent material, double labelling of RA cells with retrogradely labelled tracer and anti-PV was clearly present in males, but only with respect to the moderately and some faintly PV-positive cell types (Fig. 2A,C,E and G). Intensely labelled PV-positive cell bodies were never retrogradely labelled (Figs. 2G and 4G and H), and no retrogradely labelled cell body was ever intensely positive for parvalbumin. In the female RA, retrogradely labelled RA cells were numerous following XIIIts injections of CTB (Figs. 1D, 2D) but none of them was definitely PV-positive (Fig. 2B,D,F and H).

3.3. Further identification of the intensely labelled PV-positive RA neurons

Double labelling for parvalbumin and calbindin was carried out both in sections from birds not receiving injections of retrograde tracer, and from birds that received injections of rhodamine dextran in XIIIts. In the male zebra finch, every intensely labelled PV-positive cell in RA was also intensely positive for anti-CB (Fig. 4C–F), and none of such doubly labelled cells were retrogradely labelled (Fig. 4G–J). As in other cases described above, some moderately or faintly labelled PV-positive cells were also retrogradely labelled (Fig. 4G and H), suggesting that these were projection neurons. But we observed no RA cells that were doubly labelled for anti-CB and retrograde tracer (Fig. 4J), i.e. CB-positive RA cells do not appear to be projection neurons. Since moderately or faintly labelled PV-positive and CB-positive RA cells formed different populations, which could be seen in the same section that also contained cells intensely labelled with both anti-PV and anti-CB (Fig. 4F), an internal control for cross reactivity was provided. In addition, cross reactivity as an interpretation of double labelling with anti-PV and anti-CB antibodies in the cocktail procedure (see Multiple labelling protocols) was ruled out on the basis of the results of labelling of 10-μm sections through RA with either anti-PV or anti-CB alone. Fig. 5 shows clearly, that when two such adjacent sections are accurately superimposed, those RA cells that were cut in two or three during sectioning, and were intensely labelled for anti-PV, were also intensely labelled for anti-CB. Fig. 5 also confirms that retrograde labelling that resulted from a XIIIts injection of rhodamine dextran, was confined to RA cells that were moderately or faintly labelled for anti-PV. These cells were always calbindin negative.

In the female zebra finch, double labelling for anti-PV and anti-CB showed that some intensely labelled PV-positive cells were also intensely positive for anti-CB (not shown). However, there were many fewer of these doubly labelled cells, owing to the relative sparseness of CB-positive neurons in the female RA. Triple labelling was not performed in the female.

Outside RA, in the neostriatum, there were also many cells double labelled for anti-PV and anti-CB, but there were also many PV-positive cells that were not CB-positive, providing additional internal controls for cross-reactivity in the same section that contained RA.

3.4. RA pathway is PV-positive in male but not female zebra finches

In males, the whole of the RA pathway, including the tracts and terminal fields, were intensely PV-positive (Fig. 6A,C,E–G). The medial part of the occipitomesencephalic tract (OM), which carries the RA axons out of the hemisphere, was positive (Fig. 7B), as was the rest of the tract as it courses through the diencephalon and brainstem (Fig. 6E). PV-positive terminal fields were present in all the nuclei known to receive projections from RA in male zebra finches and canaries [32,36, Fig. 8, present study], viz. the dorsomedial nucleus of the intercollicular complex (DM; Fig. 7C), the nucleus infra-olivaris superior (IOS), the ventrolateral nucleus of the rostral medulla (RVL), nucleus parabigualis (Pam), nucleus retroambigualis (RAm; Fig. 6C), and XIIIts and SH (Figs. 6F and 7D). The densest immunolabelling was in the supraphyoglossal area (SH) where there were peri-somatic concentrations around XIIIts and RAm cell bodies (Fig. 6G). In contrast, in females these tracts and terminal fields were largely absent (Fig. 6B and D). Occasionally, faint labelling of the OM tract was present in the lower medulla, but no terminal fields were present in either RAm or XIIIts which occur at these levels (Fig. 6D). Note, parenthetically, the absolute size difference between the male and female medullary

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Fig. 5. (B) and (C) PV (stained green using a biotinylated secondary antibody and streptavidin fluorescein) and CB immunoreactivity (stained red using an Alexa 546 direct secondary antibody), respectively, in adjacent 10-μm thick frontal sections through the ventral region of RA. (A) Superimposition of (B) and (C) to show some cells that were stained with both anti-PV and anti-CB antibodies, separately applied to each section (e.g. up arrows). Arrowheads directed leftwards point to cells that were intensely positive only for PV, while arrowheads directed rightwards point to cells that were only CB positive. The boxed area in B shows one cell that was retrogradely labelled by an injection of rhodamine dextran into XIIIts — note the particulate nature of the label, in contrast to the generally solid labelling of PV and CB positive cells. Calibration bar= 50 μm.
Fig. 6. PV immunoreactivity defines the RA pathway in male but not female zebra finches. (A) PV-positive cells and neuropil in male RA and intermediate archistriatum — frontal section, medial to the left. (B) Ditto for female RA. (C) PV immunoreactivity in XIIIts and RAm of male zebra finch. (D) Absence of PV labelling in XIIIts and RAm of female zebra finch. The medullary size difference between C and D is real — see text. (E) Parasagittal section (rostral to the left) through the brainstem of a male zebra finch. Note the PV-labelled occipitomesencephalic tract (OM) tract and terminal field in RAm. (F) PV immunoreactivity in XIIIts and the suprahypoglossal area (SH) of a male zebra finch (sagittal section; rostral to the left). (G) Higher power view of the boxed area in (F) to show the dense PV immunoreactivity in SH and perisomatic densities around XIIIts motoneurons. Calibration bars = 1 mm for (A–E); 100 μm for (F) and (G).
Fig. 7. Lesions of RA in male zebra finches depletes PV immunoreactivity in the RA pathway. (A) Large lesion of the left medial archistriatum, including RA. Note the PV-positive cells in the intermediate archistriatum. (B) Frontal section through the diencephalon showing PV immunoreactivity in the right medial OM tract (which carries RA axons out of the hemisphere [32]) and in the right OM subjacent to nucleus ovoidalis (Ov). Note the absence of labelling of these structures on the left. (C) Frontal section through the mesencephalon showing PV labelling of DM and OM on the right, but not on the left. (D) Frontal section through the caudal medulla showing PV labelling of Xllts and Ram on the right, but not on the left. DLM: 3v and 4v, 3rd and 4th ventricles; III, nucleus nervi oculomotorii; SSp, nucleus supraspinalis; DM, dorsomedial nucleus of the intercollicular complex. Calibration bars = 1 mm.

sections at comparable rostrocaudal levels (compare 6C and D). The reliability of this difference is presently unknown, but in two females treated with the anti-PV antibody and sectioned coronally, a markedly smaller medullary cross section than in the male was evident.

The PV-negativity of the RA projections in the female zebra finch is consistent with the general lack of RA cells doubly labelled for retrograde tracer and parvalbumin (see above).

3.5. PV-immunoreactivity of the RA pathway in other species

A similar pattern of results to those in male zebra finches was present in the male canary, cardinal and cowbird, and in the female cardinal, although the immunostaining was much weaker than in the male zebra finch, at the same or even more concentrated antibody dilutions (compare Figs. 3 and 6). Staining of the RA pathway in the female cowbird was absent, although PV-positive structures were present in other parts of the brain, e.g. cerebellum.

3.6. RA seems to be the major origin of the PV-positive projections to Xllts

When RA was electrolytically lesioned in the male zebra finch and canary, the PV-positive tracts and terminal fields that were present on the unlesioned, contralateral side were almost completely PV-negative on the ipsilateral side (Fig. 7), suggesting that RA was at least the major source of the PV staining of OM and its terminal fields. This conclusion is supported by the finding of very few PV-positive cell bodies in any of the other sources of projections to Xllts. A few faintly labelled PV-positive cell bodies were found in DM and RVL.

3.7. RA projections in female zebra finches

We reinvestigated the projections of RA in female zebra finches using BDA (compare [9]). Although the size and density of these projections was less than in the male (compare [32]), the pattern of projections was very similar (Figs. 8 and 9). As in the male, there was a discrete terminal field in DM, with no additional labelling of ICo (Figs. 8B and 9A). Other discrete terminal fields were
Fig. 8. (A–I) Rostrocaudal series of schematic camera lucida drawings of RA projections (depicted as short wavy lines) in a female zebra finch, as defined by an injection of BDA in the right RA (shown as black in (C), with a small amount of spread ventrally shown as hatched). Terminations are found in dorsal DLM (A), DM (B), IOS (D), RVL (E), PAm (F), RAm (G–I) and XIIIts (E–I). Note the contralateral fibers in (E–I). Ai, Archistriatum intermedium; Cu, nucleus cuneatus; DLM, nucleus dorsolateralis anterior thalami, pars medialis; FLM, fasciculus longitudinalis medialis; ICc, central nucleus of the inferior colliculus; IOS, nucleus infra-olivaris superior; LAD, lamina medullaris dorsalis; MV, nucleus motorius nervi trigemini; NIIL, nervus oculomotorius; III, nucleus nervi oculomotorii; OI, nucleus olivaris inferior; OM, tractus occipitomesencephalicus; OS, nucleus olivaris superior; PAm, nucleus parambigualis; RA, nucleus robustus archistriatalis; RAm, nucleus retroambigualis; Ru, nucleus ruber; RVL, ventrolateral nucleus of the rostral medulla; SpM, nucleus spiriformis medialis; TPC, nucleus tegmenti pedunculopontinus, pars compacta; TTD, nucleus et tractus descendens nervi trigemini; Uva, nucleus uvaeformis; VeM, nucleus vestibularis medialis; VeL, nucleus vestibularis lateralis; VI, nucleus abducentis; Vc, nucleus descendens nervi trigemini, pars caudalis; X, nucleus nervi vagi; XIIIts, nucleus tracheosyringealis.
Fig. 9. Photomicrographs depicting the terminations of RA projection neurons in a female zebra finch, as defined by an injection of BDA in RA. (A) Terminations in DM; (B) in XIIIts rostral to the obex — note the few contralateral fibers (at arrow); (C) Darkfield shot showing terminations in XIIIts and SH caudal to the obex; (D) Fibers and terminations in the contralateral RAm; (E) Fibers and terminations in XIIIts, SH and RAm — section lightly counterstained with cresyl violet; (F) higher power view of the boxed area shown enclosing part of XIIIts in (E). Note the perisomatic terminal densities around some XIIIts motoneurons; (G) Higher power view of the boxed area shown enclosing part of RAm in (E). Note again the perisomatic terminal densities around some RAm cell bodies. V, tectal ventricle. Calibration bars = 100 µm.
present in IOS, RVL, PAm, RAm, XIIIts and the suprahypoglossal area (SH), where the terminations were densest (Fig. 9C). Moreover, the terminations in XIIIts and the respiratory premotor nuclei (e.g. RAm) included many that appeared to be axosomatic (Fig. 9F and G). There were also a few terminations in the dorsolateral part of DLM, as in the male (Fig. 8A; see also [32]). Curiously, in the best case, there were more contralateral projections in the medulla than in any male thus far observed (Fig. 9B and D; compare [36]). A very similar pattern of ipsilateral RA projections was present in the female cardinal, but the RA injection in this single case was only partially on target, thereby revealing only a portion of the descending tract.

4. Discussion

In songbird species in which the female sings little or not at all, sexual differentiation of the brain during early development results in a female RA that is much reduced in size compared with that in the male [1,9,12,15,16]. However, in the female zebra finch the number of neurons reported to be present in the RA of adult birds is apparently quite variable, as witnessed by the figures of 6950 (range 6000–7600) given by Gurney [9], 2900 (range 2400–3700) given by Konishi and Akutagawa [16], and ~8000 given by Johnson and Sellix [12]. In the respective studies, these figures compare with the proportionally less variable counts of 16 400, 13 600 and ~12 000 for numbers of neurons in the adult male RA. In the present study, this variability in the numbers of neurons in the adult female RA was supported by the findings of apparently different overall sizes of RA in different birds, although neuron density was not measured. Only about 22% of RA neurons in the adult female zebra finch are projection neurons having XIIIts as their target, compared with more than twice as many in the male [12]. The maximum somal diameter of neurons in the adult female RA has been reported to be about 8 μm [9,16], but a variation in cell density or size across the nucleus has not previously been noted. In the present study, both Nissl staining and retrograde labelling of RA neurons showed that cells in more lateral parts of the female nucleus, as seen in frontal sections, were less densely packed than in more medial parts of the nucleus and generally approximated the sizes of male RA neurons (12–20 μm). This observation appears to be supported by the findings of Johnson and Sellix [12; see their Fig. 5C], but its significance is unclear.

Nevertheless, it is clear from the present results that in the adult female, RA has a substantial projection to XIIIts and all the other respiratory–vocal nuclei that have been identified in the male [32]. From rostral to caudal, there was sparse labelling of the dorsolateral part of DLM, which in the male is the origin of a feedback loop to a forebrain circuit involving the nucleus 1MAN [29], and there was specific labelling of DM in the midbrain. This latter is new evidence, since Gurney [9] described diffuse projections that encompassed both DM and the surrounding intercollicular nucleus (ICo) in the female. This result was probably due to spread of his tritiated amino acids outside RA to archistriatal regions that are now known, in the male, to project to regions of ICo that surround DM [17,35]. In the rest of the brainstem there were substantial projections to IOS, to RVL and to both PAm and RAm, as well as to XIIIts and the suprahypoglossal area (SH). As in the male, the great majority of these projections were ipsilateral, and there was strong evidence suggesting that the terminations on XIIIts and RAm cells were frequently axosomatic. There was also good evidence for sparse contralateral labelling of XIIIts and RAm, even more so than in males (Fig. 8D; compare [36]).

The question is, what function do these RA projections perform in the non-singing female zebra finch? As Simpson and Vicario [26] asked with respect to the female HVC and RA, are the projections simply a remnant of a developmental program that is only fully expressed in males, or do they have some other, as yet unexplained raison d’etre? Perhaps they are involved in calling, which presumably requires the activation of abdominal respiratory muscles, at the least, and, if the call is acoustically modulated, then syringeal activation would be required in addition. However, Simpson and Vicario [26] showed that the ‘long’ or ‘distance’ call of the female zebra finch, which is characteristically longer than that of the long call of the male [38,39], has a lower fundamental frequency than that of the male and lacks certain learned features such as a fast frequency modulation, was largely unaffected either by cutting the tracheosyringeal nerves bilaterally, or by lesioning RA or HVC. Simpson and Vicario [26] concluded, therefore, that their “data suggest that the HVC–RA–nXIIIts–syringeal pathway is essential for production of the learned vocalizations made by male zebra finches but is not needed for production of the unlearned female call” (p. 1555).

Whatever its function in the female, the RA pathway is also different from that of the male with respect to its almost total lack of parvalbumin immunoreactivity, which is correlated with the lack of cell bodies in RA that were double labelled for anti-PV and retrograde label. Thus, while there are many cell bodies in the female RA that stain positively for anti-PV, none of them was positively identified as doubly labelled by injections of retrograde tracer in XIIIts. In contrast, in the male RA, all retrogradely labelled XIIIts-projecting neurons were also stained faintly or moderately for anti-PV, but not all PV-positive cell bodies were also retrogradely labelled. There are two reasons relevant to the last part of this statement, one technical and the other substantive. In the case depicted in Fig. 2, relatively few RA neurons were retrogradely labelled from the XIIIts injection, so the presence of many
PV-positive RA cell bodies that were not also retrogradely labelled in the same sections is not surprising. However, in other cases such as those depicted in Figs. 4A and 4B, where many more RA neurons were retrogradely labelled, there were still many PV-positive cells that were not double labelled. Some of these were faintly or moderately labelled for PV, reflecting the subctal nature of the retrograde labelling, even in these cases; but others were intensely labelled, and these intensely labelled PV-positive cells were never retrogradely labelled in any of the cases. Thus, in the male, but not in the female zebra finch, the XIIIs projecting RA neurons are characterised by their faint-to-moderate PV immunoreactivity, and this reactivity also characterises the whole pathway and terminal fields. However, such is the density of the terminations of RA axons in RAm, XIIIs and particularly SH, that the PV immunostaining in these structures appears intense, rather than faint or moderate. The RA lesion data strongly suggest that the PV staining of these fields derives from RA and not from other sources of afferent nuclei.

The intensely labelled PV and CB cell bodies in the male RA do not project to XIIIs, and since they are not confined to a particular RA region, such as the dorsal `cap' that projects to nuclei other than XIIIs (e.g. DM and the respiratory premotor nuclei [12,31,32,36], it seems likely that they are not projection neurons at all. Their soma size is similar to that of the GABAergic RA neurons identified by others [10,24,27], which could suggest that the intensely PV- and CB-positive RA cells also contain GABA. As suggested by Spiro et al. [24], such inhibitory interneurons would be strategically placed to link and coordinate the activity of otherwise unconnected groups of RA projection neurons that might innervate functionally different motor (syringeal) and premotor (respiratory) neurons in the medulla. GABA-containing cells have also been identified in RA of the female zebra finch [24], and these, too, could be coincident with the intensely PV- and CB-positive cells identified in the female RA in the present study, but whether such cells also have an inhibitory function in the non-singing female is not known.

Given the postulated general role of calcium binding proteins in intracellular calcium buffering [7], the potential co-localization of high levels of parvalbumin and calbindin in these GABAergic interneurons of the male RA would be consistent with their intrinsic physiology of fast action potentials that fire at very high frequencies in response to depolarizing currents, as in other systems [13,18,27]. More specifically, Caillard et al. [5] have suggested that in such high frequency GABAergic interneurons (such as cerebellar stellate cells synapsing on Purkinje cells), synaptic transmission during bursts could be maintained by parvalbumin quickly reducing in the presynaptic terminal residual calcium associated with the preceding action potential, and by preventing cumulative facilitation so that synaptic strength is maintained at resting levels. In the GABAergic interneurons of the songbird RA, high levels of parvalbumin and calbindin would be conducive to their postulated role of mediating the tonic to phasic transitions in firing patterns during singing, when HVc afferent activity is transformed into the phasic bursts that characterise RA activity [27,37].

There are few instances in the CNS where both parvalbumin and calbindin co-localise in the same neurons, notable exceptions being cerebellar Purkinje cells of mammals and birds ([4, unpublished observations 7]), which are also GABAergic, and rat dorsal root ganglia [6]. In the present study in the adult male zebra finch, all intensely labelled PV-positive cells were doubly labelled for anti-CB, whereas the moderately labelled PV-positive and CB-positive cells formed separate populations. In a developmental study of PV and CB immunoreactivity in the zebra finch, Braun et al. [4] showed that PV- and CB-positive cells in RA had different developmental histories and differed markedly in numbers, the CB-positive cells being relatively sparse and generally surrounded by CB-negative neuropil. We also found that CB-positive RA cells and neuropil to be much less dense than the PV-positive RA cells and neuropil. However, the source of the dense PV-positive RA neuropil might not be totally intrinsic to the nucleus; some of it could result from the projections of HVc and/or lMAN cells, some of which are also PV-positive [3,4, Wild, unpublished observations].

As mentioned in the Introduction, parvalbumin is not usually associated with long projection neurons, the vast majority of PV-positive neurons in the CNS being interneurons [7]. However, Preuss and Kaas [22] identified a population of cells in layer V of M1 (Betz cells) and parts of somatosensory cortex in primates (Macaca and Galago). It is of related interest that RA is located in a part of the avian archistriatum that has been likened, on the basis of its long descending projections to a variety of brainstem sensory and motor nuclei, to layers V–VI of somatosensory motor cortex of mammals ([20,40]; see also [32,34,35]). The presence of PV-positive neurons in RA and surrounding intermediate archistriatum is thus consistent with the findings of Preuss and Kaas [22] of PV-positive neurons in layer V of somatic cortex in primates.

The PV immunostaining of the RA pathway in the male canary, cardinal and cowbird was never as strong as it was in the zebra finch, it was weakest in the female cardinal, and was absent in the female cowbird. The reasons for the weaker staining in males of other species are not clear. All were adult, established singers, and the perfusion and immunohistochemical protocols were similar to those used in the zebra finches; in fact, lower final dilutions of antibody were also used to try and overcome this difference, but to no avail. However, the fact some staining was detectable in the female cardinal, which is known to sing to some extent, and not in the female zebra finch or cowbird, which do not sing, could suggest that parvalbumin immunoreactivity of RA projection neurons might...
be correlated with the ability to sing and not with gender. But this suggestion must remain speculative until further tests are carried out on a range of other species in which the female sings.

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References


