Involvement of the avian song system in reproductive behaviour

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The song system of songbirds consists of an interconnected set of forebrain nuclei that has traditionally been regarded as dedicated to the learning and production of song. Here, however, we suggest that the song system could also influence muscles used in reproductive behaviour, such as the cloacal sphincter muscle. We show that the same medullary nucleus, retroambigualis (RAm), that projects upon spinal motoneurons innervating expiratory muscles (which provide the pressure head for vocalization) and upon vocal motoneurons for respiratory–vocal coordination also projects upon cloacal motoneurons. Furthermore, RAm neurons projecting to sacral spinal levels were shown to receive direct projections from nucleus robustus arcopallialis (RA) of the forebrain song system. Thus, by indicating a possible disynaptic relationship between RA and motoneurons innervating the reproductive organ, in both males and females, these results potentially extend the role of the song system to include consummatory as well as appetitive aspects of reproductive behaviour.

1. Background

A major function of male song is to attract a female, and because singing is often correlated with reproduction, singing can be seen as a form of appetitive behaviour (courtship) that may be consummated by sexual union in which the male’s sperm is transferred to the female by the apposition of the birds’ cloacae (the avian reproductive organ). In some species of songbirds, including canaries, song is sung primarily by males, but even in species in which the female does not sing at all (e.g. zebra finch), similar pathways for vocal production are present in females as in males [1]. The motor pathway for song (figure 1) proceeds from HVC (proper name) to nucleus robustus arcopallialis (RA), which then projects directly to vocal motoneurons (XIIts) and to respiratory premotor neurons in nucleus retroambigualis (RAm) in the lower medulla. In turn, RAm projects upon spinal motoneurons that innervate expiratory muscles (which provide the pressure head for vocalization), and upon XIIts to effect vocal–respiratory coordination [2,3].

Female songbirds choose their sexual partner largely on the basis of their auditory assessment of various aspects of his song [4] and, given an appropriate hormonal state, they may respond by presenting the copulation solicitation display (CSD) [5], a suite of extensor postural responses resembling lordosis in mammals, which facilitates insemination. The neural mechanisms by which the CSD is evoked and manifest—and it can be evoked by the male’s song even in his physical absence [6]—are unclear, but in female canaries the elicitation and intensity of the CSD is under the control of highly specific, possibly innate, acoustic components of male song, called sexy syllables [7]. Moreover, although partial lesions of HVC cause female canaries to present the CSD indiscriminately, their CSDs remain most intensely evoked by canary sexy syllables [8]. This would seem to indicate that the pathway originating in HVC and descending via RA to RAm contributes in some way to the control of the CSD (figure 1). Although males do not normally present the CSD, cloacal positioning and associated movements are presumably as important as those in females for successful insemination and may, therefore, be controlled in part by the same pathways as those in females.
The cloaca is arguably the focus of the CSD for both the male and the female. Its external aperture (the vent) is enclosed by a sphincter muscle (mSC) [9], which in male Japanese quail has been shown electromyographically to be active during copulation and defaecation [10]. Furthermore, mSC motoneurons in quail receive a direct projection from RAm [11], but because quail are non-songbirds, they do not possess a forebrain song system. We ask here, therefore, whether the song system of songbirds is involved not only in appetitive aspects of reproductive behaviour (singing), but also in consummatory aspects (cloacal focus) of reproductive behaviour. Neuroanatomical evidence that it could be is presented below.

2. Material and methods

Subjects were 21 male and female canaries (Serinus canarius). Surgery was undertaken following induction of general anaesthesia by an intramuscular injection of ketamine (50 mg kg$^{-1}$) and xylazine (20 mg kg$^{-1}$). Injections of retrograde and anterograde tracers into brain nuclei (figure 2) were made with either air pressure or iontophoresis (2–4 μA, 7 s on and 7 s off for a total of 15–20 min), using tracer-filled glass micropipettes, guided to their targets stereotaxically [13] and electrophysiologically (e.g. [14]). For spinal injections, a laminectomy was performed over one or two sacral segments, respiratory movements were minimized by the use of vertebral clamps, and the pipette was inserted into the ventral horn. Injections in mSC were made under direct visual guidance.

Tracers used were unconjugated cholera toxin B-chain (CTB; Sigma-Aldrich, 1% in phosphate-buffered saline (PBS)), CTB Alexa 488 (Invitrogen, both 1% in PBS), and biotinylated dextran amine (BDA 3 K, Invitrogen, 10% in PBS). Although all tracers provided both anterograde and retrograde labelling, BDA and CTB Alexa 555 were used primarily for anterograde labelling of fibres and terminations, while CTB and CTB Alexa 488 were used primarily for retrograde labelling of cell bodies. Birds survived 2–4 days and were then euthanized prior to trans-cardiac perfusion with 50 ml normal saline.
followed by 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7–4. Brains and spinal cord segments were sectioned transversely on a freezing microtome at 35 μm and collected serially in PBS. Fluorescent sections were mounted on subbed slides, air dried and coverslipped with Citifluor (VWR). To reveal BDA, sections were incubated in streptavidin conjugated to horse-radish peroxidase (strep-HRP, 1 h at room temperature) and, after washing in PBS, treated with diaminobenzidine hydrochloride (DAB) and cobalt chloride to yield a black reaction product [15]. CTB was visualized by incubating sections in a goat anti-CTB polyclonal antibody (List Biological Laboratories, 1:33 000 dilution) overnight, then biotinylated rabbit anti-goat (Sigma-Aldrich, 1:300) 1 h, strep-HRP 1 h, and finally DAB with no cobalt, which yielded a brown reaction product [15].

3. Results
To identify cloacal motoneurons, three males and two females received injections of CTB in mSC. Retrogradely labelled motoneurons were located at the ventrolateral periphery of the ventral horn throughout several sacral spinal segments caudal to the closure of the rhomboid sinus (figure 2a), as in Japanese quail [10,11]. Axons of these motoneurons were observed in the ventral root and their dendrites extended both medially and dorsally. To identify putative premotor neurons in the brainstem, two males and two females received air pressure injections of CTB in the ventral horn of one or two sacral spinal cord segments shown to house cloacal motoneurons. As in Japanese quail [11], the injections in canaries retrogradely labelled neurons in the caudal part of RAm (figure 2d), and in several other groups of neurons throughout the brainstem. Of these, only RAm receives a descending projection from the forebrain song system [16]. Four males and four females received injections of CTB 555 in RAm to label descending projections to the spinal cord. Each of these birds also received an injection of CTB 488 in mSC. The results of these combined injections confirmed the putative premotor nature of RAm with respect to motoneurons innervating mSC, in both males and females (figure 2b), and confocal scanning laser microscopy confirmed the presence of presumptive axo-dendritic appositions (figure 2c). These findings are directly comparable to those found in

\[ \text{(a) a longitudinal section of sacral spinal cord showing cloacal (mSC) motoneurons (green) retrogradely labelled by an injection of CTB 488 into the muscle. (b) RAm axons (red) in relation to mSC motoneurons (blue-green) in the ventral horn (vh) of the sacral spinal cord (female; transverse section). (c) Confocal projection of some contents of (b). (d) RAm neurons retrogradely labelled by an injection of CTB into the ventral horn of an upper sacral spinal cord segment (male). (e) RAm neurons retrogradely labelled from a similar injection of CTB (brown) embedded within the terminal field of BDA-labelled RA axons (black; female). An RA terminal field in RAm in a male canary, at approximately the same rostrocaudal level as the section shown in (d), is shown in fig. 3a of Wild et al. [12].} \]

\[ \text{(f) Neurons in female RA (f) and DM (g) retrogradely labelled from an injection of CTB 555 centred on RAm (MLd in (g) = avian auditory midbrain). Scale bars, 100 μm. At right of each group of photographs are shown coronal semi-sections housing the nuclei shown in figure 1. The sites of the injections are indicated by tracer-filled pipettes, and retrograde and anterograde directions of intra-axonal travel are indicated by arrows.} \]
male Japanese quail [11]. RAm injections also retrogradely labelled RA and several groups of neurons in the brainstem, including the dorsomedial nucleus of the intercollicular complex (DM), as previously described [15] (figure 2f,g). Finally, two males and two females each received an injection of BDA in RA combined with an injection of CTB into the sacral spinal cord to determine the relation of RAm neurons retrogradely labelled by spinal cord injections to the descending projections of RA—the output of the forebrain song system (figure 2c).

4. Discussion

In general, these investigations suggest that the song system may not only control vocal–respiratory activity characteristic of singing, but may also influence muscles involved in consummatory aspects of reproductive behaviour. In fact, the representation of non-vocal–respiratory components of the CSD in the song system’s motor pathway could suggest that vocal learning evolved within a motor circuit associated with reproductive behaviour. Specifically, the results show that RAm in canaries, like RAm in quail [11] and like the homologous nucleus retroambiguus (NRA) in mammals [17,18], is functionally diverse, housing not only respiratory and vocal premotor neurons [16,19], but also premotor neurons activating muscles involved in mating behaviour. However, unlike RAm in non-songbirds or NRA in mammals, RAm neurons in canaries that project upon motoneurons involved in reproductive behaviour receive a putative direct projection from the output of the telencephalic song system, RA. Probably, as in all other birds [15], RAm in canaries also receives a projection from DM, which could mediate hypothalamic sexual drive, although further work is required to substantiate this [11]. In sum, we describe a neural pathway that putatively connects RA with cloacal motoneurons diusynaptically, thereby providing the link between the song and reproductive systems necessary to mediate a continuum of appetitive and consummatory behaviours.

How might this link function? It is proposed that, similar to NRA circuits in mammals [20], neuronal circuits within RAm are physiologically reconfigured from their usual respiratory–vocal configuration to one that is organized for the production of reproductive behaviour. In mammals, it is the input from periadventitial grey (PAG) to NRA [21] that is proposed to stimulate the NRA reconfiguration for lordosis [20], and in birds the input from DM—which is proposed as equivalent to parts of PAG [15]—might do likewise for the RAm reconfiguration mediating the CSD. But in female canaries, given an appropriate hormonal state, the reconfiguration could also be triggered by HVC neurons that are specifically tuned to the sexy syllables of male song. Perhaps these same neurons are also involved in the production of female-specific trills, which are significantly correlated with CSDs [22].

In future studies, it will be important to establish the functional connectivity of the described pathways, to determine whether RAm projects upon other CSD motoneurons, and to what extent the pathways differ between males and females, possibly influenced by hormonal factors [23].

Ethics. All procedures were approved by the Animal Ethics Committee of the University of Auckland, Protocol no. 001177, and the research was conducted under project nos 3626763 and 3704273.

Data accessibility. The data (images) upon which the conclusions are based are included in the main text of the paper.

Authors’ contribution. J.M.W conceptualized the study, performed the experiments, and wrote the paper. J.F.B. made substantial contributions to the analysis and interpretation of the results, provided critical revision of important intellectual content of the paper, drew figure 1 and organized figure 2. Both the authors gave final approval of the version of the paper to be published, and agree to be accountable for all aspects of the work.

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