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Research Article

A putative RA-like region in the brain of the scale-backed antbird, *Willisornis poecilinotus* (Furnariides, Suboscines, Passeriformes, Thamnophilidae)

Jamily L.R. de Lima[1], Fabricio A. Soares[1], Ana C.S. Remedios[1], Gregory Thom[2], Morgan Wirthlin[3], Alexandre Aleixo[2], Maria Paula C. Schneider[1], Claudio V. Mello[3] and Patricia N. Schneider[1]

[1]*Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil.*
[2]*Museu Paraense Emilio Goeldi, Belém, PA, Brazil.*
[3]*Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA.*

Abstract

The memorization and production of song in songbirds share important parallels with the process of speech acquisition in humans. In songbirds, these processes are dependent on a group of specialized telencephalic nuclei known as the song system: HVC (used as a proper name), RA (robust nucleus of arcopallium), LMAN (lateral magnocellular nucleus of the nidopallium) and striatal Area X. A recent study suggested that the arcopallium of the *Sayornis phoebe*, a non vocal learner suboscine species, contains a nucleus with some properties similar to those of songbird RA, suggesting that the song system may have been present in the last common ancestor of these groups. Here we report morphological and gene expression evidence that a region with some properties similar to RA is present in another suboscine, the Amazonian endemic *Willisornis poecilinotus*. Specifically, a discrete domain with a distinct Nissl staining pattern and that expresses the RA marker RGS4 was found in the arcopallium where the oscine RA is localized. Our findings, combined with the previous report on the *S. phoebe*, suggest that an arcopallial region with some RA-like properties was present in the ancestor of both Suboscines infraorders Tyranni and Furnarii, and is possibly an ancestral feature of Passeriformes.

Keywords: vocal learning, RGS4, song nuclei, *in situ* hybridization.

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Introduction

Vocal learning is a rare trait present in three clades of birds: hummingbirds (Trochiliformes), parrots (Psitaciformes) and songbirds (Oscines, Passeriformes) (Zigmond *et al.*, 1973, Jarvis *et al.*, 2000, Wilbrecht and Nottebohm, 2003, Arriaga *et al.*, 2012). The memorization and production of song in avian species that learn their vocalizations share many important features with human speech (Doupe and Kuhl, 1999) and rely on a set of discrete telencephalic nuclei referred to as the song control system (Wilbrecht and Nottebohm, 2003, Jarvis, 2004, Arriaga *et al.*, 2012). Recent phylogenetic data have solidified the idea that parrots and songbirds are sister taxa, while hummingbirds are a separate group (Jarvis *et al.*, 2014) thus indicating that vocal learning, which is absent in the intervening clades, most likely evolved convergently among avian vocal learners (Jarvis, 2004).

Vocal control in avian vocal learners is dependent upon two main pathways. The posterior pathway is composed of HVC (used as a proper name) and RA (robustus nucleus of the arcopallium), which are localized in the caudal part of the telencephalon and are involved in the motor encoding of learned vocalizations. RA provides the major output of the song system; it influences vocal production through direct and indirect projections to brainstem motor neurons that innervate the syrinx, the vocal organ of birds. The anterior pathway resembles mammalian cortico-basal ganglia-thalamo cortical loops, and is more directly involved in vocal learning and plasticity. It includes two nuclei located rostrally in the telencephalon: Area X of the striatum (Area X) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Jarvis, 2004, Prather, 2013) (Figure 1A).

The Passeriformes are composed of two suborders: oscines (songbirds), with the largest number of species, and suboscines. The latter are further divided into two main infraorders: Tyranni and Furnarii. Representatives of the Tyranni are the tyrant-flycatchers, cotingas and phoebes, whereas antbirds are the most numerous species within the Furnarii infraorder. While traditionally viewed as vocal non-learners (Brenowitz *et al.*, 1991, Touchton *et al.*, 2014), there is also evidence that some suboscines, espe-

Send correspondence to Patricia N. Schneider. Instituto de Ciências Biológicas, Universidade Federal do Pará, 66055170 Belém, Pará, Brazil. E-mail: schneider@ufpa.br.

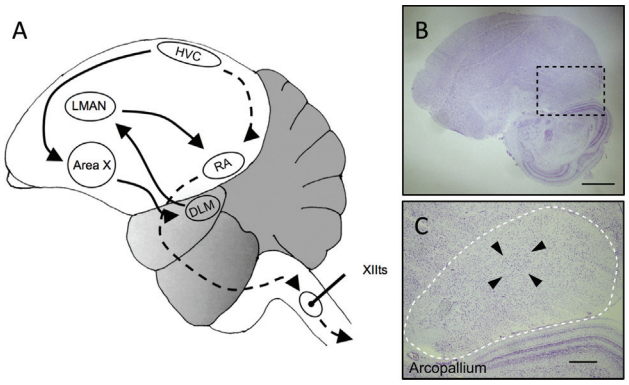

Figure 1 - The song control system of the Pale-breasted Thrush, *Turdus leucomelas*. (A) Schematic representation of the song control system in an oscine brain, showing the song production pathway (dashed arrows) and the song learning pathway (arrows) (Adapted from Nottebohm, 2005). (B) Nissl stained brain section of *T. leucomelas*, the arcopallium is located within the dashed line box. (C) The archopallium is demarcated by white dashed line, with arrowheads indicating the location of the RA nucleus. Scale bars are 20 μm (B) and 5 μm (C).

cially within the Cotingidae, may present at least some rudimentary forms of vocal learning (Irestedt *et al.*, 2002).

Early sudies utilizing cytoarchitectonics criteria in Nissl-stained sections suggested that suboscines lack the song system nuclei found in oscines (Brenowitz *et al.*, 1991). A recent study, however, provided evidence that the Eastern phoebe *(Sayornis phoebe)*, a tyrant flycatcher, has a rudimentary circuitry with a nucleus that shares some properties of the oscine RA, and that lesions to this region leads to vocal impairements similar to those observed with RA lesions (Liu *et al.*, 2013). Importantly, however, when their juveniles are raised in isolation, without exposure to tutor song, they develop normal conspecific adult vocalizations, indicating lack of vocal learning in this species (Brenowitz *et al.*, 1991). Similar results have been obtained with an antbird (*Hylophylax naevidoides*), providing support for a lack of vocal learning, among suboscines (Touchton *et al.*, 2014). Due to these conflicting findings, the extent to which vocal learning and associated brain structures are prevalent among suboscines remains unsettled.

In this study, we searched for the presence of song nuclei circuitry in yet another suboscines species, the antbird *Willisornis poecilinotus*, from an infraorder that has not yet been examined with regards to this trait. We found evidence of the presence of a rostro-ventral arcopallial area with some cytoarchitetonic features resembling those of song nucleus RA. Furthermore, we found evidence that RGS4, a marker of song nucleus RA in songbirds, is selectively expressed within this nucleus. These data, together with previous reports on *Sayornis phoebe*, suggests that some possible components of the circuitry associated with the emergence of vocal learning could be ancestral to Passeriformes.

Material and Methods

Sampling

A total of 6 male specimens of *Willisornis poecilinotus* and 2 male specimens of *Turdus leucomelas* were captured at the Refúgio de Vida Silvestre Metrópole da Amazônia, in Belém. Brains were dissected out and flash frozen in Tissue-tek embedding medium on dry ice and 100% ethanol. Cryosections (10μm) were made using a Leica CM1850 UV cryostat and placed onto colorfrost plus microscope slides, fixed in 3% paraformaldehyde, and stored at -80°C for further use. This study was approved by the Ethics Committe at the Instituto de Ciências Biológicas and performed in accordance to the special licence 20902-1 issued to MPDS by IBAMA/SISBIO.

Riboprobe synthesis

The cDNA clones for parvalbumin (PVALB) and for the regulator of G-protein signaling 4 (RGS4) were from the zebra finch brain cDNA ESTIMA collection, cloned in pbluescript II KS plasmids. As previously performed for the zebra finch, the specificity of these probes was verified by performing a blat alignment (using the UCSC genome browser) to the genome sequence of the golden-collared manakin (*Manacus vitellinus*), a subsocine species; the analysis showed alignment to the correct ortholog and lack of significant hits to other loci, supporting probe specificity in this avian suborder. Polymerase Chain Reaction (PCR) amplification was performed using forward and reverse primers (IDT) with a T3 overhang (Reverse: 5'-GTA AAA CGA CGG CCA GTG AG-3' and Forward: 5'-ATG ACC ATG ATT ACG CCA AG-3') to amplify the cDNA insert and T3 polymerase promoter using the vector as template. PCR was performed in 50 μL reaction volumes containing 39.2 μL of RNase free water, 1.5 μL of 10 mM $MgCL_2$, 5 μL of 10x buffer, 1 μL of each primer (0.5 μM) , 1 μL of dNTP mix (10 mM), 0.3 μL of Taq DNA Polymerase, and 1 μL of DNA template. The temperature profile consisted of preheating at 94 °C for 3 min, 32 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 10 min. Then, antisense riboprobes were synthesized using T3 RNA polymerase (Life Technologies) and DIG-labeling mix (Roche). The riboprobe reaction (Life Technologies) was performed in 20 μL reaction volumes containing 0.5 μL of RNAse inhibitor, 2 μL of DTT, 2 μL of DIG, 2 μL of 10x reaction buffer, 5 μL of template, 2 μL of T3 enzyme mix, and 6.5 μL of nuclease-free water.

Nissl Staining

Cryosections were stained in an aqueous solution of 0.1% cresyl violet. After staining, the slides were dehydrated in absolute ethanol for 2 min; and cleared with

xylene for 2 min twice. The sections were then coverslipped using CytosealXYL (Richard-Allan Scientific) mounting medium.

In situ hybridization

In situ hybridization was performed according to a previously established protocol, with modifications (Lovell *et al.*, 2013, Carleton *et al.*, 2014). Brain sections were acetylated for 10 min in a solution of 0.33% acetic anhydride in water, rinsed twice with 2x SSPE buffer (0.02 M EDTA and 2.98 M NaCl in 0.2 M phosphate buffer) and dehydrated in 70%, 95% and 100% RNA-free ethanol. Each section was then hybridized with a solution (16 µL) containing 50% formamide, 2x SSPE, 1 µg/µl BSA, 1 µg/µL poly-A (New England Biolabs) in DEPC-treated water, and 1 µL of the DIG-labeled riboprobe. Slides were coverslipped, sealed by immersion in mineral oil, and incubated overnight at 65°C. On the following day, sections were rinsed and de-coverslipped in chloroform, washed briefly in 2x SSPE, and incubated serially for 1 h and 10 min at 65 °C in 2x SSPE containing 50% formamide, and twice in 0.1x SSPE for 30 min at 65°C. Sections were then rinsed briefly in TNT (TN: 100 mM Tris, pH 7.5; 150 mM NaCl/ TNT: TN + 0.3% Triton-X 100), demarcated using a pap pen (Life Technologies) and blocked for 30 min at RT in 200 µL of TNB (0.1 M Tris, 150 mM NaCl) blocking buffer (200 µL TNB/slide, 1% skim milk) and incubated overnight in TNB with an alkaline phosphatase conjugated anti-DIG antibody (Roche) (TNB + 0,3% skim milk + anti-DIG-AP; 1:600 dil.). Then, the slides were washed twice for 15 min in TMN (100 mM Tris, pH 9.5; 150 mM NaCl, 0.05 M MgCl), and incubated for 1–3 days in a detection solution containing the alcaline phosphatase substrates Nitro-Blue Tetrazolium Chloride (NBT)(Roche) and 5-Bromo-4-Chloro-3-Indolyl-phosphate p-Toluidine Salt (BCIP) (Roche) in a TMN solution with 1:1 chromogenic proportion. Slides were washed overnight in distilled water to remove salts, fixed in 4% paraformaldehyde, washed twice in distilled water, and coverslipped with Aquamount (Fischer Scientific). Slides were imaged using a Nikon SMZ1500 microscope and processed on NIS-Elements D 4.10.01 program (Nikon). For qualitative assessment of mRNA expression in brain tissue we compared the neuroanatomical expression of these two selected genes with data retrieved from the ZEBrA database (www.zebrafinchatlas.org).

Results

Cytoarchitectural analysis of the arcopallium of *W. poecilinotus* in Nissl-stained brain sections

As previosuly described in several songbird species, the set of song nuclei that controls vocal learning displays a characteristic cytoachitetonic organization and conserved locations within the forebrain. To establish whether similar nuclei are also present in the antbird *W. poecilinotus*, we first examined Nissl-stained parasagital brain sections of adult male Pale-breasted Thrush, *Turdus leucomelas* (Turdidae), a vocal-learning oscine species, thereby establishing a basis for comparison in a similar-sized oscine brain. The neuronal cells present in the song nuclei of this species are larger and noticeably distinct from the surrounding areas (Figure 1B,C). To characterize the forebrain of *W. poecilinotus*, we studied the general organization of the brain utilizing Nissl-stained parasagittal sections from adult males. After a thorough analysis, discrete nuclei with the large cells characteristic of HVC, LMAN and Area X in vocal learners were not observed in any of the Nissl-stained sections (Figure 2A,C,E). However, we found that the arcopallium contained discrete nuclei with large neuronal

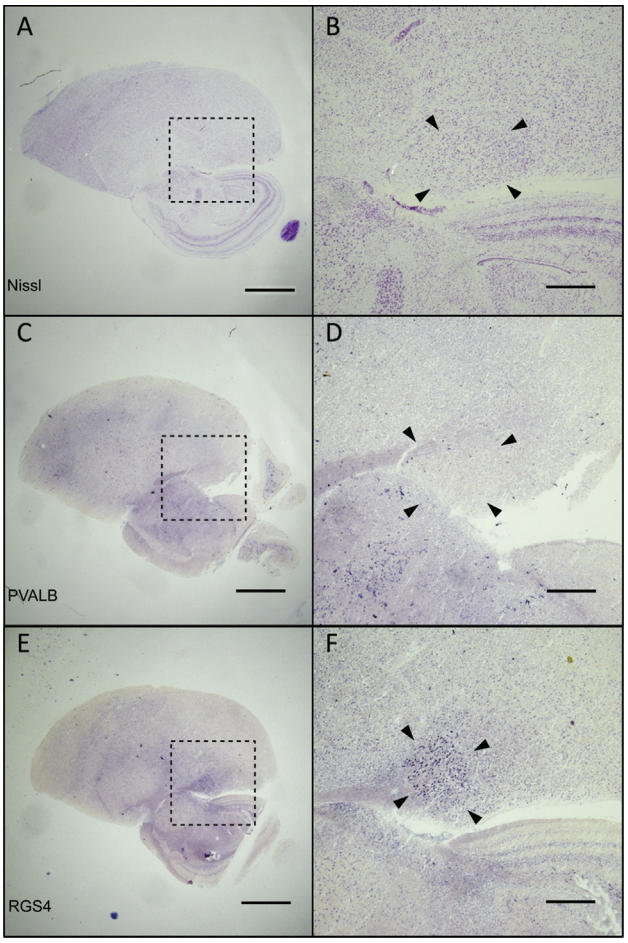

Figure 2 - An RA-like region in the brain of the antbird *Wllisornis poecilinotus*. (A) Nissl stained section of the *W. poecilinotus* brain, with the arcopallium region denoted by a dashed line box. (B) RA like nucleus characterized by the presence of large neuronal cells that are distinct from the surrounding cells. (C) Expression pattern of the song nuclei marker PVALB in a brain section of *W. poecilinotus* and (D) within the arcopallium. (E) Expression pattern of the song nuclei marker RGS4 in a brain section of *W. poecilinotus* and (F) within the arcopallium. Arrowheads indicate the location of the RA-like nucleus. Scale bars are 20 µm (A, C, E) and 5 µm (B, D, F).

cells resembling those of song nuclei in oscines, in particular a prominent one within the rostro-ventral region (Figure 2A,B and Figure S1); this prompted us to further examine a putative RA-like nucleus utilizing molecular criteria.

Expression pattern of oscine song nuclei markers in *W. poecilinotus*

To further investigate the presence of song nuclei in *W. poecilinotus*, the expression pattern of PVALB and RGS4 were analyzed. These markers were chosen as they represent conserved markers of song nuclei, including RA, in songbirds (Lovell *et al.*, 2008) and ZEBrA database (www.zebrafinchatlas.org) and in other avian vocal learners (Hara *et al.*, 2012). Similar to our Nissl staining results, neither PVALB nor RGS4 expression was detected in putative HVC, LMAN and Area X nuclei (Figure 2C-E). Although PVALB expression was not detected in the arcopallium or in any putative song control nucleus (Figure 2C and D), it was observed in the cerebellum (Figure S2), indicating at least partial probe cross-hybridization. Nevertheless, we found enriched RGS4 mRNA in the arcopallium (Figure 2E,F and Figure S3), within a rostro-ventral region that overlapped with the location of the distinct cytoarchitectonic region detected by Nissl staining.

Discussion

Our data provide evidence for the presence of a distinct region within the arcopallium of *W. poecilinotus,* a suboscine representative, with some cytoarchitectonic and molecular similarities with the RA song nucleus of songbirds and its analogous nuclei in hummingbirds and parrots. The cytoarchitetonic analysis of Nissl-stained brain sections revealed distinct large cells within a discrete domain in the arcopallium, as characteristic of RA and other song nuclei in songbirds. To further examine the presence of areas with song-nuclei properties in a suboscine species, we analized the expression of two song nuclei markers. RGS4 is a regulator of G protein signaling that controls the expression of NMDA-receptors in the forebrain on song learners (Saugstad *et al.*, 1998). This gene has androgen-related action and its expression is highly enriched in the RA nucleus of songbirds (Saugstad *et al.*, 1998, Wild *et al.*, 2005, Lovell *et al.*, 2008). Our results showed enriched mRNA expression of this song nuclei marker within the arcopallium of *W. poecilinotus*, suggesting the presence of a potential RA-like region in a suboscine representative. However, no differential expression was observed in HVC, Area X or in the LMAN putative areas in this species.

One possible interpretation is that the arcopallium of *W. poecilinotus* contains a nucleus analogous to oscine RA that is possibly part of a vocal control circuitry and has some involvement in vocal control. While connectivity and functional data in this species are still lacking, such an interpretation would be consistent with that suggested by recent data obtained in the Eastern phoebe, a tyrant flycatcher suboscine. That study used gene expression analysis to demonstrate singing related activation of a discrete arcopallial domain, and lesions to demonstrate that some vocal changes occur after the loss of their so-called RA-like region (Liu *et al.*, 2013). Taken together, these studies suggest that an arcopallial region with some similarities to song nucleus RA and thus possibly representing a rudimentary form of RA is present in suboscines species from both tyranni and furnarii infraorders (Figure 3). Such a nucleus could have been present in the common ancestor to oscines and suboscines and thus possibly be ancestral to Passeriformes.

In vocal learning birds, RA and its analogs in parrots and hummingbirds are the main output of the circuitry involved in learned vocalizations, and are thought to play central roles in controlling learned songs (Patton *et al.*, 1981, Nottebohm *et al.*, 1982). Namely, they receive projections from HVC and LMAN and project to motorneurons that innervate the syrinx, the main avian vocal organ (Vates and Nottebohm, 1995). The neuronal activation patterns in RA during singing correlate with specific features of song (Yu and Margoliash, 1996, Hahnloser *et al.*, 2002) indicating that it plays an important role in the neuronal encoding of learned song patterns. The role that an RA-like region might play in suboscines is not understood, especially in species where there is no compelling evidence of vocal learning. Also, we did not observe the same expression pattern using the song nuclei marker PVALB, indicating that, the RA-like region, represents the rudimentary form of RA. By analogy to songbirds, one would expect such a region to project to vocal and respiratory motorneurons in the brainstem, and to be functioanally active during vocal behaviors. While such evidence is still lack-

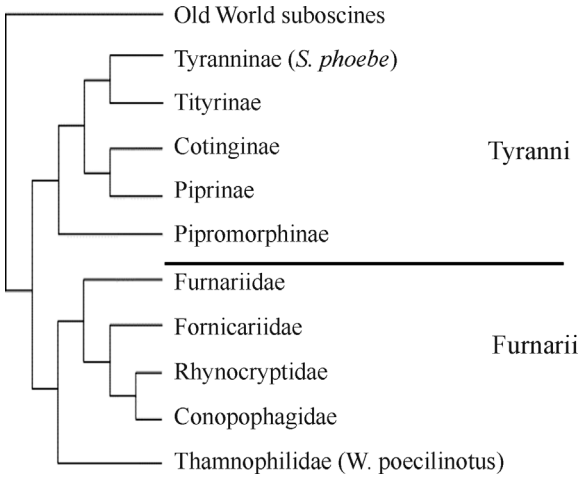

Figure 3 - Phylogenetic tree of the suborder suboscines. New world suboscines are divided into the infraorders Tyranni and Furnarii. Putative RA-like nuclei have been found in *S. phoebe* (Liu *et al.*, 2013) and in *Willisornis poecilinotus* (this study).

ing, the area identified here is now an interesting candidate target for such studies.

Importantly, however, there are alternative interpretations to our findings. For example, the domain of enriched RGS4 expression is located rostro-ventrally, underneath the occipitomesencephalic (OM) tract, thus not entirely consistent with the expected location of an RA-like nucleus based on songbirds. Rather, this domain is very reminiscent of a rostroventral arcopallial domain of high RGS4 expression that can also be seen in the zebra finch (ZEBrA atlas; www.zebrafinchatlas.org) and could subserve other, as yet undetermined function(s), such as the auditory processing circuit, part of the conserved auditory pathways, as it sends descending projections to the auditory thalamus and midbrain (Wild *et al.*, 2005). Furthermore, some of the induced gene expression and lesion effects observed in the Eastern phoebe study could be interpreted by considering that the so-called RA-like nucleus may be part of a conserved auditory processing circuit (Wild *et al.*, 2005), analogous to the region immediately outside of RA (also called the RA cup) in a songbird (Mello *et al.*, 1998). That region shows singing-induced auditory activation (Mello and Clayton, 1994), but possible effects of lesions have not yet been examined in a songbird.

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Supplementary Material

The following online material is available for this article:

Figure S1 - Nissl stained section of the *W. poecilinotus* brain.

Figure S2 - Expression pattern of PVALB in a brain section of *W. poecilinotus.*

Figure S3 - RA-like region in the brain of the antbird *W. poecilinotus*

This material is available as part of the online article from http://www.scielo.br/gmb.

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