Auditory experience refines cortico-basal ganglia inputs to motor cortex via remapping of single axons during vocal learning in zebra finches

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Miller-Sims VC, Bottjer SW. Auditory experience refines cortico-basal ganglia inputs to motor cortex via remapping of single axons during vocal learning in zebra finches. J Neurophysiol 107: 1142–1156, 2012. First published December 7, 2011; doi:10.1152/jn.00614.2011.—Experience-dependent changes in neural connectivity underlie developmental learning and result in life-long changes in behavior. In songbirds axons from the cortical region LMAN\textsubscript{core} (core region of lateral magnocellular nucleus of anterior nidopallium) convey the output of a basal ganglia circuit necessary for song learning to vocal motor cortex [robust nucleus of the arcopallium (RA)]. This axonal projection undergoes remodeling during the sensitive period for learning to achieve topographic organization. To examine how auditory experience instructs the development of connectivity in this pathway, we compared the morphology of individual LMAN\textsubscript{core}→RA axon arbors in normal juvenile songbirds to those raised in white noise. The spatial extent of axon arbors decreased during the first week of vocal learning, even in the absence of normal auditory experience. During the second week of vocal learning axon arbors of normal birds showed a loss of branches and varicosities; in contrast, experience-deprived birds showed no reduction in branches or varicosities and maintained some arbor in the wrong topographic location. Thus both experience-independent and experience-dependent processes are necessary to establish topographic organization in juvenile birds, which may allow birds to modify their vocal output in a directed manner and match their vocalizations to a tutor song. Many LMAN\textsubscript{core} axons of juvenile birds, but not adults, extended branches into dorsal arcopallium (Ad), a region adjacent to RA that is part of a parallel basal ganglia pathway also necessary for vocal learning. This transient projection provides a point of integration between the two basal ganglia pathways, suggesting that these branches convey corollary discharge signals as birds are actively engaged in learning.

experience-dependent remodeling; axon remodeling; songbirds; topography

SENSORY EXPERIENCE during development refines topographic maps throughout the nervous system, and the resultant specificity of connections is essential for functional circuits to appropriately encode and transmit information. In zebra finches auditory experience refines the axonal connectivity of cortico-basal ganglia circuits during early vocal learning when birds are listening to and memorizing the song of a tutor (Iyengar and Bottjer 2002b; Iyengar et al. 1999). The cortical region lateral magnocellular nucleus of the anterior nidopallium (LMAN) provides the output of basal ganglia circuits necessary for vocal learning (Bottjer et al. 1984; Ölveczky et al. 2005; Scharff and Nottebohm 1991). LMAN consists of two subregions: LMAN\textsubscript{core} neurons project to vocal motor cortex [robust nucleus of the arcopallium (RA)], whereas LMAN\textsubscript{shell} neurons project to the adjoining region [dorsal arcopallium (Ad)]. Both core and shell regions of LMAN give rise to topographically organized basal ganglia loops that traverse the forebrain in parallel (Bottjer et al. 2000; Iyengar et al. 1999; Johnson et al. 1995) (Fig. 1A).

Although both core and shell circuits of LMAN undergo refinement during vocal learning, the axonal projection from LMAN\textsubscript{core} to RA undergoes dramatic remodeling on a scale that is rarely seen during neural development (Bottjer 2004; Iyengar et al. 1999). At the onset of vocal learning (20 days), neurons from small subregions of LMAN\textsubscript{core} send axons throughout RA, with little or no topographic organization. By 35 days, neurons from restricted subregions of LMAN\textsubscript{core} form terminal fields in specific subregions of RA, forming a topographic map that matches the adult pattern (Fig. 1B). In contrast to the LMAN\textsubscript{core}→RA projection, coarse topographic organization is already established by 20 days in the efferent pathway from LMAN\textsubscript{shell}→Ad as well as in the thalamic inputs to core and shell regions of LMAN (Fig. 1A) (Iyengar and Bottjer 2002a; Iyengar et al. 1999). Thus transformation from a highly diffuse pattern of connectivity to a topographically organized map is specific to the projection from LMAN\textsubscript{core} to vocal motor cortex (RA), and this pathway is known to control the production of juvenile song behavior in zebra finches (Aronov et al. 2008). Auditory experience is essential for the establishment of this topography: The LMAN\textsubscript{core}→RA projection of 35-day birds deprived of auditory experience is as disorganized as that of 20-day birds (Iyengar and Bottjer 2002b). This result is consistent with the well-known role of experience-dependent mechanisms in refining axonal connectivity (Kandler et al. 2009; Ruthazer and Cline 2004; Zhang and Poo 2001).

The wholesale lack of topographic organization seen in the LMAN\textsubscript{core}→RA projection during early development is atypical of most neural systems (O’Leary et al. 2007; Rubel and Cramer 2002), and the mechanisms whereby LMAN\textsubscript{core} axons are remodeled to achieve a precise map are unknown. Two processes could contribute to the topographic refinement of the projection from LMAN\textsubscript{core} to RA in juvenile birds. Individual axons could initially be exuberant and cover a large proportion of RA and then branches in incorrect positions within RA could be retracted, and/or individual axons could be initially mistargeted and then be repositioned to the correct location within RA with little net regression. To investigate this question, we reconstructed individual axons projecting from LMAN\textsubscript{core} to RA to determine how the morphology and position of axon arbors are shaped during development under normal conditions and in the absence of normal auditory experience.

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The core and shell pathways of LMAN play different roles during vocal learning in juvenile birds. The LMAN\text{shell} pathway does not directly influence motor output but is necessary for sequence learning and accurate imitation of a tutor song (Bottjer and Altenau 2010), whereas LMAN\text{core} drives motor activity in RA and hence vocal output (Aronov et al. 2008; Foster and Bottjer 1998; Mooney and Rao 1994). Thus LMAN\text{core} may serve primarily a motor function during learning, whereas LMAN\text{shell} may serve as a comparator circuit to evaluate vocal production (Bottjer 2004). We report here a novel projection: Half of all LMAN\text{core} axons in juvenile birds sent projections to both Ad and RA, providing a point of cross talk between LMAN\text{core} and LMAN\text{shell} pathways. This robust interconnection between these parallel basal ganglia loops carries information from the motor pathway for juvenile song production and provides a means of integrating information between the two pathways in juvenile birds actively engaged in learning.

**MATERIALS AND METHODS**

**Subjects.** Zebra finches were bred and raised by their parents in group aviaries at the University of Southern California. All procedures conformed to national regulatory guidelines and followed protocols approved by the Animal Care and Use Committee at the University of Southern California. Surgeries were performed on male zebra finches at 20 days (20 – 22; \(n = 4\)), 27 days (25 – 27; \(n = 5\)), or 35 days (34 – 36; normal \(n = 3\), white noise \(n = 4\)). Birds in the 20- and 27-day age groups remained with their parents in their group breeding aviary up until immediately before surgery and were then returned to the aviary after the procedure. Birds that received their surgery at 35 days were removed from the aviary with their parents and siblings at 15 – 17 days and placed in an individual cage (along with the nest box), which was housed in an acoustic isolation chamber through the end of the experiment. One group of 35-day birds could hear the vocalizations of their parents, siblings, and selves throughout the rearing period and thus had normal auditory experience, whereas a second group of 35-day birds (35-day white noise) was deprived of normal auditory experience by being raised in loud white noise. White noise was played through a speaker in the acoustic isolation box, and the noise level within the cage was 107 – 110 dB (SPL, A weighted) as measured every day of the experiment with a decibel meter at the top of the cage, resulting in a sound level of >100 dB throughout the cage. This procedure does not damage hair cells and has only a small temporary effect on hearing thresholds (Ryals et al. 1999; Zevin et al. 2004) but prevents birds from experiencing patterned auditory vocalizations. This level of noise prevents birds from being able to produce normal song as adults and disrupts the normal establishment of topographic organization of the LMAN\text{core}→RA pathway (Iyengar and Bottjer 2002b).

**Biotinylated dextran amine injections and immunohistochemistry.** On the day of surgery each zebra finch was anesthetized with 1.5% isofluorane and placed in a stereotaxic apparatus, and a small incision was made in the skin overlying the skull. Stereotaxic coordinates were used to approximate the location of LMAN\text{core}, and a small piece of isofluorane was placed in a stereotaxic apparatus, and a small piece of skin was removed from the avairy overlying the skull. The location of LMAN was then verified by measuring spontaneous activity with a glass pipette electrode (OD 20 μm) filled with 0.5 M NaCl. Injections were targeted to LMAN\text{core} by mapping the characteristic bursting activity of LMAN\text{core} neurons. To ionophorese the anterograde tracer biotinylated dextran amine (BDA; mol wt 10,000, 10% in sterile filtered PBS), pulses of positive current were applied to a silver wire (200 – 400 nA, 1 s on/1 s off) for 30 min through a glass pipette (OD 6 – 9 μm). All injections were targeted bilaterally, but in some cases an injection either missed LMAN\text{core} on one side or was so large that too many axons were labeled and hence single axons could not be
distinguished within RA. The total number of injections analyzed was 20 (n = 9 left, n = 11 right). After surgery birds were returned to their home aviary or cage for 2 days, after which they were deeply anesthetized with Equithesin (0.04 ml/10 g) and perfused through the heart with 0.7% saline followed by 4% paraformaldehyde plus 0.4% glutaraldehyde (pH 7.8). Brains were removed, postfixed for 24 h in 4% paraformaldehyde, and then cryoprotected in 25% sucrose for 24 h. Brains were then frozen in liquid nitrogen and sectioned in the coronal plane at a thickness of 50 μm on a cryostat; free-floating sections were collected in 0.02 M PBS.

Sections were first preincubated with DAB solution for 15 min and then exposed to DAB with 0.015% H2O2 for 3–5 min. Sections were mounted onto gelatin-coated slides, allowed to air-dry overnight, dehydrated in ethanol, defatted in xylene, and coverslipped with Permount (Fisher Scientific). After axon reconstructions, alternate tissue sections were counterstained for Nissl in order to visualize the borders of RA and LMANcore.

Axon arbor reconstructions. Figure 2, top, shows an example of an injection site; injections produced a cluster of labeled cells within a discrete area. Figure 3 shows the location of labeled cells compared with the Nissl-stained outline of LMANcore (injection site 18 is depicted in Fig. 2). Four injection sites included labeled cells outside LMANcore (4, 16, 17, and 18), and a total of 13 axons were traced from these four injection sites. All branches for 10 of 13 of these axons (including all of those from injections 4 and 17) projected exclusively to RA, indicating that these axons came from LMANcore and not from LMANshell. For injection sites 16 and 18 two of three axons and one of four axons, respectively, sent most of their branches to RA and a few to Ad, a pattern that was also seen in many axon arbors that emanated exclusively from LMANcore (see below). Labeled axon arbors from cell bodies located within LMANcore were reconstructed in RA in three dimensions under ×1,000 final magnification with a Leica DMR microscope with a motorized stage and Neurolucida software (Microbrightfield). Only well-labeled axons from injection sites that resulted in a small enough number of labeled axons to unambiguously trace each axon and all of its terminal branches were reconstructed. An example of labeled axon branches is shown in Fig. 2, bottom; axon arbors were well labeled to the ends of their terminal branches, and varicosities and endings were easily discerned. Markers were placed along the axon wherever there was a varicosity. To correct for shrinkage of the tissue during processing, the depth of every section of was measured at ×1,000 magnification for each axon arbor. The initial thickness (50 μm) was divided by the average measured thickness to calculate the shrinkage correction factor, and this correction was applied to each axon arbor in NeuroLucida. The shrinkage correction across all axon arbors was 6.84 ± 0.06 (mean ± SE).

Data analysis. Every axon arbor was traced in its entirety, including all branches both inside and outside RA. Branches outside RA were counted and characterized according to their location.

To assess whether age and auditory experience refine the morphology of individual LMANcore → RA axon arbors, we quantified only that portion of each axon arbor that fell within the boundaries of RA. For each axon arbor we calculated the number of endings (referred to as number of branches throughout this report), number of branch orders, total length of the axon arbor within RA, and number of varicosities. The mean length of branches for each axon arbor was calculated by dividing the total length (mm) of the axon arbor within RA by the total number of branches (endings) within RA. Varicosity density was calculated by dividing the number of varicosities by the total length of the axon arbor. The tangential extent of the terminal field of each axon arbor was determined by measuring the distance between the two endings that were furthest apart in the medial-lateral, dorsal-ventral, and anterior-posterior directions with Image Pro Plus software (Media Cybernetics). The volume occupied by each axon arbor was measured with a three-dimensional convex hull analysis using the Neurolucida program. The convex hull analysis generates a convex polygon drawn around the most distal axon arbor branch points and measures the volume within the entire axon arbor. As indicated above, only the volume of each axon inside the borders of RA was measured. For injection sites in 35-day normal and white noise birds that had more than one axon reconstructed we measured the combined convex hull volume of two axon arbors together for every possible pairwise combination of axons. This provided a measure of how close together axon arbors were within RA; pairs of axons that overlap with each other will occupy less space in combination than those that are far apart. The volume of RA was estimated by tracing the borders of RA on each section where it was present and multiplying the sum of the surface area of these contours by the section thickness. The percentage of RA occupied by a single axon arbor was calculated by dividing the convex hull volume of the axon by the volume of RA.
RESULTS

Iyengar et al. (1999) investigated the topographic organization of the axonal projection from LMANcore to RA by making the topographic arrangement seen in this study as well as in two previous studies from our lab (Fig. 1B) (Iyengar et al. 1999; Johnson et al. 1995). LMANcore was divided into five sections from medial to lateral as shown in Fig. 1B, and the injection sites were characterized according to their location in LMANcore. We concentrated on quantifying medial-lateral position within LMANcore because previous studies have shown that dorsal-ventral and rostral-caudal position do not substantially contribute to topography within RA. Axon arbors from injection sites in the medial or medial-intermediate parts of LMANcore were matched to the dorsal third of RA, those from the intermediate or lateral-intermediate parts of LMANcore were matched to the middle third of RA, and axon arbors from the lateral part of LMANcore were matched to the ventral third of RA. This alignment of topography was based on the published results of Iyengar and Bottjer (Iyengar et al. 1999; Iyengar and Bottjer 2002b) as shown in Fig. 1B and was also confirmed by this study (see RESULTS). The total length of the axon arbor, the number of endings, and the number of varicosities in each section were measured. Some injection sites spanned more than one region of LMANcore, axon arbors from these injection sites were matched to one of the two possible sections within RA based on which section had the greatest percentage in two of the three categories. This analysis provided a means to determine whether each axon arbor was targeted to the correct location within RA.

Nonparametric statistics were used to evaluate the data. The overall effect of age (20 days, 27 days, 35 days) was evaluated with Kruskal-Wallis tests. Individual comparisons between groups were evaluated with Mann-Whitney tests. All values in the text, tables, and figures are reported as means ± SE. The results of all statistical tests are shown in Table 1.

Table 1. Results of nonparametric statistical tests used to evaluate measures of axon arbor characteristics

<table>
<thead>
<tr>
<th></th>
<th>Kruskal-Wallis 20 d, 27 d, 35 d</th>
<th>Mann-Whitney 20 d vs. 27 d</th>
<th>Mann-Whitney 20 d vs. 35 d</th>
<th>Mann-Whitney 27 d vs. 35 d</th>
<th>Mann-Whitney 20 d vs. 35 d wn</th>
<th>Mann-Whitney 35 d vs. 35 d wn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length within RA</td>
<td>( P = 0.005 )</td>
<td>( P = 0.04 )</td>
<td>( P = 0.001 )</td>
<td>( P = 0.18 )</td>
<td>( P = 0.06 )</td>
<td>( P = 0.15 )</td>
</tr>
<tr>
<td>Volume</td>
<td>( H = 10.8 )</td>
<td>( U = 57 )</td>
<td>( U = 34 )</td>
<td>( U = 99 )</td>
<td>( U = 48 )</td>
<td>( U = 76 )</td>
</tr>
<tr>
<td>Percentage of RA occupied</td>
<td>( P = 0.14 )</td>
<td>( P = 0.04 )</td>
<td>( P = 0.16 )</td>
<td>( P = 0.90 )</td>
<td>( P = 0.07 )</td>
<td>( P = 0.83 )</td>
</tr>
<tr>
<td>Dorsal-ventral extent</td>
<td>( H = 3.9 )</td>
<td>( U = 57 )</td>
<td>( U = 77 )</td>
<td>( U = 133 )</td>
<td>( U = 49 )</td>
<td>( U = 106 )</td>
</tr>
<tr>
<td>Anterior-posterior extent</td>
<td>( P = 0.004 )</td>
<td>( P = 0.002 )</td>
<td>( P = 0.009 )</td>
<td>( P = 0.54 )</td>
<td>( P = 0.03 )</td>
<td>( P = 0.41 )</td>
</tr>
<tr>
<td>Medial-lateral extent</td>
<td>( P = 0.49 )</td>
<td>( P = 0.48 )</td>
<td>( P = 0.54 )</td>
<td>( P = 0.26 )</td>
<td>( P = 0.49 )</td>
<td>( P = 0.82 )</td>
</tr>
<tr>
<td>Number of branches</td>
<td>( H = 1.4 )</td>
<td>( U = 88 )</td>
<td>( U = 96 )</td>
<td>( U = 105 )</td>
<td>( U = 71 )</td>
<td>( U = 105 )</td>
</tr>
<tr>
<td>Number of branch orders</td>
<td>( P = 0.12 )</td>
<td>( P = 0.25 )</td>
<td>( P = 0.06 )</td>
<td>( P = 0.18 )</td>
<td>( P = 0.25 )</td>
<td>( P = 0.79 )</td>
</tr>
<tr>
<td>Number of varicosities</td>
<td>( H = 4.3 )</td>
<td>( U = 78 )</td>
<td>( U = 66 )</td>
<td>( U = 99 )</td>
<td>( U = 62 )</td>
<td>( U = 104 )</td>
</tr>
<tr>
<td>Varicosity density</td>
<td>( P = 0.002 )</td>
<td>( P = 0.13 )</td>
<td>( P = 0.001 )</td>
<td>( P = 0.02 )</td>
<td>( P = 0.24 )</td>
<td>( P = 0.02 )</td>
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<tr>
<td>Mean branch length</td>
<td>( H = 12.6 )</td>
<td>( U = 70 )</td>
<td>( U = 72 )</td>
<td>( U = 72 )</td>
<td>( U = 62 )</td>
<td>( U = 53 )</td>
</tr>
<tr>
<td>Topography</td>
<td>( P = 0.002 )</td>
<td>( P = 0.43 )</td>
<td>( P = 0.001 )</td>
<td>( P = 0.006 )</td>
<td>( P = 0.10 )</td>
<td>( P = 0.07 )</td>
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<tr>
<td>% Length</td>
<td>( H = 12.6 )</td>
<td>( U = 86 )</td>
<td>( U = 35 )</td>
<td>( U = 60 )</td>
<td>( U = 53 )</td>
<td>( U = 67 )</td>
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<tr>
<td>Topography</td>
<td>( P &lt; 0.001 )</td>
<td>( P = 0.19 )</td>
<td>( P &lt; 0.001 )</td>
<td>( P = 0.003 )</td>
<td>( P = 0.21 )</td>
<td>( P = 0.001 )</td>
</tr>
<tr>
<td>% Varicosities</td>
<td>( H = 17.9 )</td>
<td>( U = 74 )</td>
<td>( U = 16 )</td>
<td>( U = 54 )</td>
<td>( U = 60 )</td>
<td>( U = 28 )</td>
</tr>
<tr>
<td>Topography</td>
<td>( P = 0.002 )</td>
<td>( P = 0.73 )</td>
<td>( P &lt; 0.001 )</td>
<td>( P = 0.001 )</td>
<td>( P = 0.14 )</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>% Endings</td>
<td>( H = 0.5 )</td>
<td>( U = 96 )</td>
<td>( U = 21 )</td>
<td>( U = 36 )</td>
<td>( U = 56 )</td>
<td>( U = 55 )</td>
</tr>
</tbody>
</table>

RA, robust nucleus of arcopallium; d, days of age; wn, white noise.
restricted injections of an anterograde tracer into small sub-regions of LMANcore and analyzing the extent and location of label within RA. At the level enabled by such bulk injections, the results demonstrated that topographic organization between LMANcore and RA is established during the earliest stages of vocal learning, between 20 and 35 days. Here we asked how individual LMANcore axons are remodeled to achieve the topographic map in RA by labeling only a few neurons in LMANcore and reconstructing individual axon arbors. A total of 59 axon arbors were reconstructed from 20 LMAN injection sites in 16 birds: 13 axons from 6 injection sites in 20-day birds, 16 axons from 5 injection sites in 27-day birds, 17 axons from 5 injection sites in 35-day birds, and 13 axons from 4 injection sites in birds deprived of normal auditory experience by being raised in loud white noise (35-day white noise).

Qualitative overview of LMANcore axon arbors across all groups. LMANcore axons exit the dorsal border of LMAN and travel caudally within the mesopallial lamina (LaM). They exit the lamina at the level of HVC, traveling lateral to HVC and then coursing ventrally toward RA (Johnson et al. 1995). We began tracing each axon arbor as it descended toward RA just caudal to HVC. Figure 4 shows complete reconstructions of two axon arbors from each age group of normal birds (20, 27, and 35 days). Across all groups, 36 axons travelled ventromedially toward RA and entered the dorso-lateral border of RA and/or traveled through the medial portion of Ad and entered RA more ventrally (red axons in Fig. 4), whereas 23 axons traveled ventrally toward Ad in a position lateral to RA and then turned 90° and traveled through Ad to enter RA (black axons in Fig. 4). Just prior to reaching RA, 42 of the 59 axon arbors split into several main branches and sent multiple branches into RA. Interestingly, almost half of LMANcore axons across all groups sent terminal branches into Ad in a pattern that overlapped topographically with axonal inputs from LMANshell (see below).

Figure 5 shows axon arbor reconstructions within RA from three different LMANcore injection sites for each group. For clarity, two axon arbors (of 2–5 reconstructed) are shown from each injection site. LMANcore → RA axon arbors of 20-day birds were elaborate; These arbors had many branches and covered a large portion of RA. In contrast, axon arbors of 27-day birds were intermediate in complexity and extent. Axons emanating from the same injection site in 20-day birds tended to overlap in some areas but also sent branches to different areas of RA such that reconstructions of just a few axon arbors covered most of RA, whereas axon arbors of 35 day birds were located closer together within more restricted areas of RA. Axon arbors of 35-day birds raised in white noise had more branches, and in some cases axons from the same injection site sent branches to different locations within RA. Thus, in combination, axon arbors of birds reared in white noise tended to cover a relatively large portion of RA.

These qualitative differences are substantiated by quantitative measures made on the portion of each axon arbor located within the borders of RA (see MATERIALS AND METHODS), as described below.

Length and spatial extent of individual LMANcore→RA axon arbors decreased between 20 and 27 days and were not dependent on auditory experience. We determined the mean spatial extent of axon arbors within each group by measuring total length, tangential extent, and volume of each axon arbor within RA (Table 2). The tangential extent of each axon arbor was calculated in the dorsal-ventral, medial-lateral, and anterior-posterior axes by measuring the distance between the two endings that were furthest apart in each axis. The volume encompassed by each axon arbor within RA was measured by three-dimensional convex hull analysis (see MATERIALS AND METHODS). The percentage of RA encompassed by each axon arbor was calculated by dividing the volume of each axon by the total volume of RA. Because the volume of RA increases substantially between 20 days and adulthood (Bottjer et al. 1985; Herrmann and Arnold 1991; Herrmann and Bischof 1986; Johnson and Bottjer 1994; Konishi and Akutagawa 1985; Nordeen and Nordeen 1988), axonal projections with the same volume occupy a smaller percentage of RA at older ages than at younger ages (Iyengar and Bottjer 2002b; Leake et al. 2002; Rubel and Cramer 2002).

In normally reared birds the spatial extent of individual axons as measured by total length, volume, percentage of RA occupied, and dorsal-ventral extent decreased by almost half.
between 20 and 27 days and showed no further change between 27 and 35 days (Fig. 6, Table 2). All of these measures except for arbor volume showed statistically significant decreases as a function of age (Table 1). In each case individual comparisons showed a significant decrease between 20 and 27 days and no significant change between 27 and 35 days. Post hoc examination revealed that a few axon arbors in 35-day birds (3/17) had most of their branches close together but also extended one main branch with one or two endings in a different location (Fig. 5, black axon in 3rd column for 35-day birds). When these three arbors were removed from the analysis the decrease in volume of axon arbors with age was significant ($H = 6.43, P = 0.04$). Axon arbors of birds deprived of normal auditory experience by being raised in white noise decreased to the same extent as those of normal 35-day birds for each of these measures. Dorsal-ventral extent and percentage of RA occupied were decreased in arbors of 35-day birds reared in white noise relative to those of 20-day birds ($P = 0.03$), whereas total length and arbor volume tended to be lower ($P = 0.07$) (Table 1). None of these measures differed between normal and white noise.

Table 2. Analysis of length, tangential extent, and volume of individual axon arbors in RA

<table>
<thead>
<tr>
<th>Total No. of Arbors</th>
<th>Total Length, mm</th>
<th>Medial-Lateral Extent, $\mu$m</th>
<th>Dorsal-Ventral Extent, $\mu$m</th>
<th>Anterior-Posterior Extent, $\mu$m</th>
<th>Arbor Volume, $\mu$m$^3$</th>
<th>Volume of RA, $\mu$m$^3$</th>
<th>% of RA Occupied</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 d</td>
<td>13</td>
<td>$5.8 \pm 0.2$</td>
<td>$443 \pm 9$</td>
<td>$406 \pm 5$</td>
<td>$294 \pm 8$</td>
<td>$0.0210 \pm 0.0011$</td>
<td>$0.116 \pm 0.004$</td>
</tr>
<tr>
<td>27 d</td>
<td>16</td>
<td>$3.8 \pm 0.1$</td>
<td>$403 \pm 7$</td>
<td>$282 \pm 7$</td>
<td>$272 \pm 8$</td>
<td>$0.0119 \pm 0.0004$</td>
<td>$0.129 \pm 0.006$</td>
</tr>
<tr>
<td>35 d</td>
<td>17</td>
<td>$3.1 \pm 0.1$</td>
<td>$352 \pm 9$</td>
<td>$301 \pm 10$</td>
<td>$332 \pm 8$</td>
<td>$0.0155 \pm 0.0009$</td>
<td>$0.179 \pm 0.013$</td>
</tr>
<tr>
<td>35 d wn</td>
<td>13</td>
<td>$3.9 \pm 0.1$</td>
<td>$378 \pm 14$</td>
<td>$291 \pm 8$</td>
<td>$326 \pm 9$</td>
<td>$0.0136 \pm 0.0009$</td>
<td>$0.156 \pm 0.013$</td>
</tr>
</tbody>
</table>

Fig. 5. Reconstructions of LMANcore axon arbors branching within RA. Gray outlines show the Nissl-stained border of RA; coronal view, medial is to the left. At each age 2 axons from 3 different LMANcore injection sites are shown. Each color represents a single axon. Axon arbors frequently branched just before entering RA; for clarity, only the branches that enter RA are shown. Schematics on right show the location of each injection within LMANcore; numbers correspond to injection sites shown in Fig. 3. Scale bars, 100 $\mu$m.
Normally reared 35-day birds. White noise-reared 35-day birds orders, and higher number and density of varicosities than white noise had significantly more branches, higher branch between 27 and 35 days. In contrast, 35-day birds reared in between 20 and 27 days, followed by a significant decrease comparisons showed no difference in any of these measures.* 35-day white noise (wn) birds did not differ from those of normal 35-day birds in any of these measures. ‡

Fig. 6. Spatial extent of LMAN core axon arbors within RA decreased with age independent of normal auditory experience (all graphs show means + SE). Mean total length, volume, percentage of RA occupied, and dorsal-ventral extent of axon arbors decreased between 20 and 27 days. Measures of length and tangential extent are given in mm; volume is given in mm³. Axons of 35-day white noise (wn) birds did not differ from those of normal 35-day birds in any of these measures. *P < 0.05 compared with 20-day birds; ‡P < 0.005 compared with 20-day birds. See Table 1 for complete statistical comparisons.

There was no significant effect of age on either the medio-lateral or the anterior-posterior extent of axon arbors.

These results show that all groups, regardless of their exposure to normal auditory experience, showed a large net retraction in the spatial extent of individual axon arbors. Auditory experience did not play a significant role in the overall retraction of total length, volume, and dorsal-ventral tangential extent of axon arbors, as evidenced by the fact that 35-day birds raised in white noise showed decreases comparable to those observed in normal 35-day birds. We did not test 27-day old birds that had been deprived of normal auditory experience and thus cannot rule out the possibility that the spatial extent of axon arbors decreased more slowly in white noise birds compared with normal birds, but by 35 days the axon arbors of birds raised in white noise were as restricted as those of normal birds. It is notable that the decrease in axon arbor tangential extent was confined to the dorsal-ventral direction, since the mature topography between LMAN core and RA that is established by 35 days in normal birds is organized predominantly in the dorsal-ventral axis within RA (Fig. 1B; see below).

Total number of LMAN core→RA branches and varicosities decreased between 27 and 35 days and was dependent on normal auditory experience. Axon arbors of normal birds showed a large and significant decrease in number of branches, number of branch orders, and number and density of varicosities between 20 and 35 days (Fig. 7, Table 3). Individual comparisons showed no difference in any of these measures between 20 and 27 days, followed by a significant decrease between 27 and 35 days. In contrast, 35-day birds reared in white noise had significantly more branches, higher branch orders, and higher number and density of varicosities than normally reared 35-day birds. White noise-reared 35-day birds were not significantly different from normal 20-day birds in any of these measures (Table 1). In normal birds the reduction in number of branches was accompanied by a significant increase in mean branch length between 27 and 35 days, suggesting that shorter branches were preferentially lost and/or that remaining branches grew longer. This increase in branch length offset the loss in number of branches such that there was no net loss in total length between 27 and 35 days (Fig. 6). Axon arbors of 35-day white noise birds had significantly shorter branches than normal 35-day birds, as expected since 35-day white noise birds showed a retraction in total axon arbor length similar to that of 35-day birds but showed little or no loss of branches. Thus, in the absence of normal auditory experience, axon arbors maintained more, shorter branches, suggesting either that branches decreased in length but were not completely retracted or that new, shorter branches were added while longer branches were lost. In addition, normal auditory experience was necessary for the pronounced loss of varicosities that normally occurred between 27 and 35 days. Varicosities are probable sites of synapses (Jacoby and Marshall 2000; Morgenthaler et al. 2003; Shepherd and Harris 1998; Umeda et al. 2005), and previous studies have shown that the number and density of synapses made by LMAN core axons in RA decrease steeply between 25 and 53 days (Herrmann and Arnold 1991).

In summary, there was little reduction in the number of branches and varicosities on LMAN core→RA arbors between 20 and 27 days even though this corresponds to the time period when axon arbors showed the greatest decrease in their total length (Fig. 6). It is likely that branches and varicosities were dynamic between 20 and 27 days but branch addition and loss were balanced (O’Rourke et al. 1994; Ruthazer et al. 2003). Axon arbors then underwent a loss of branches (accompanied...
Table 3. Analysis of branches and varicosities of individual axon arbors

<table>
<thead>
<tr>
<th>Total No. of Arbors</th>
<th>No. of Branches</th>
<th>Branch Order</th>
<th>Branch Length, µm</th>
<th>No. of Varicosities</th>
<th>Varicosity Density, per mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 d</td>
<td>13</td>
<td>3.1 ± 1.1</td>
<td>11.7 ± 0.3</td>
<td>193 ± 4</td>
<td>144 ± 8</td>
</tr>
<tr>
<td>27 d</td>
<td>16</td>
<td>24.0 ± 0.9</td>
<td>10.6 ± 0.2</td>
<td>179 ± 5</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>35 d</td>
<td>17</td>
<td>13.5 ± 0.5</td>
<td>7.7 ± 0.1</td>
<td>246 ± 7</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>35 d wn</td>
<td>13</td>
<td>28.4 ± 1.9</td>
<td>9.6 ± 0.2</td>
<td>168 ± 6</td>
<td>101 ± 4</td>
</tr>
</tbody>
</table>

by an increased mean branch length) and a substantial loss of varicosities between 27 and 35 days. Experience was necessary for this reduction: Arbors of 35-day white noise birds had more (relatively shorter) branches and many more varicosities even though they had reduced total length, volume, and dorsal-ventral extent similar to normal 35-day birds. These results indicate that LMANcore→RA axon refinement occurs in two stages. The first stage consists of a net retraction in total length and spatial extent of the axon arbor, which results in a decreased extent in the dorsal-ventral direction and does not depend on experience. A second phase entails an experience-dependent remodeling of branches and substantial loss of varicosities. Since depriving birds of normal auditory experience disrupts the formation of topography that normally occurs between 20 and 35 days (Iyengar and Bottjer 2002b) it is likely that experience-dependent remodeling of branches and loss of varicosities is necessary for LMANcore→RA topography to achieve its final state.

**Topographic organization between LMANcore and RA.** The topographic map of LMANcore axons within RA is organized primarily from dorsal to ventral: Injections in medial LMANcore produce anterograde label in dorsal RA, and injections in lateral LMANcore label the ventral region of RA (Fig. 1B) (Iyengar and Bottjer 2002b; Iyengar et al. 1999; Johnson et al. 1995). The distribution of individual LMANcore→RA axon arbors agrees with this pattern of topographic organization. Figure 5 shows reconstructions of two different axon arbors for different injection sites from each group. Across all groups, axon arbors from more medial regions of LMAN (Fig. 5, left) tended to project to more dorsal regions of RA than axon arbors from more lateral injection sites (Fig. 5, right).

Despite the fact that topography is disrupted in 35-day birds reared in white noise (Iyengar and Bottjer 2002b), individual axon arbors of white noise birds were similar to those of normal 35-day birds in both total volume and percentage of RA occupied (Fig. 6). We therefore hypothesized that white noise rearing disrupts topography by stabilizing some axon branches to the wrong topographic locations such that axons from the same subregion of LMANcore project to different subregions within RA. To assess whether individual axon arbors in each group were targeted to the approximate correct topographic position within RA, we divided RA into thirds along the dorsal-ventral axis (Fig. 1B, Fig. 8). The location of each injection site within LMANcore was determined, and the percentage of each arbor that fell within the correct section of RA was measured based on previous observations of topography (see MATERIALS AND METHODS).

Most LMANcore axons were targeted to roughly the correct topographic location within RA. Even at 20 days, >50% of total axon length, varicosities, and endings were located within the correct section of RA; if axons were distributed randomly, only 33% would be expected to be targeted to the correct section (Fig. 8, Table 4). Topographic specificity improved during the following week such that by 27 days >70% of total axon length, varicosities, and endings were located within the correct section; there was no further change between 27 and 35 days. The overall increase in the percentage targeted to the correct section with age was not significant for endings but was significant for varicosities and approached significance for total length (Table 1). In both of the latter cases the increase in specificity of axonal targeting was significant between 20 and 27 days but not between 27 and 35 days. Although axons of 35-day white noise birds had slightly lower percentages in the correct section of RA than normal 35-day birds for all parameters, none of these differences approached significance. This pattern of results across the entire population of arbors is
consistent with the timing of changes described above: During an initial stage of refinement between 20 and 27 days axon arbors decreased their spatial extent, length, and volume to encompass less of RA (Fig. 6). Figure 8 shows that arbors became more restricted to a particular region of RA during this time, usually in the correct topographic location. These changes were independent of auditory experience; birds raised in white noise tended to be the same as normal birds in all of these measures.

However, a few axon arbors in both 20- and 27-day birds were targeted mostly outside of the correct topographic location within RA, as evidenced by the fact that they had <50% of their varicosities and endings in the correct section. Although mistargeted axons were found in 20-day (3/13 = 23%) and 27-day (2/16 = 12.5%) birds, no such axons were found in 35-day birds (0/17). Thus during the experience-dependent phase of remodeling between 27 and 35 days (Fig. 7) branches were repositioned and all axon arbors became targeted to the correct topographic location within RA in normal birds. Raising birds in loud white noise disrupted this process of refining arbors to the correct location: 3/13 axons (23%) in 35-day white noise birds had <50% of their varicosities and endings in the correct section of RA, similar to the pattern seen in 20-day birds.

This pattern suggests that normal auditory experience is necessary to refine the position of a subset of arbors that are mostly mistargeted. To assess this idea we compared the percentage of varicosities, length, and endings in each section of RA for the mistargeted arbors of 20-day birds versus those of 35-day white noise birds (Fig. 9). All six of the mistargeted axons emanated from the intermediate-lateral portion of LM_{AN_{core}} and therefore should have projected to the middle section of RA (gray shading, Fig. 9). Figure 9, top, shows three axons (red) from 20-day birds that had the majority of their arbors in the wrong section of RA (Table 5). As described above, most axons of 20-day birds covered a large area of RA

<table>
<thead>
<tr>
<th>Total No. of Arbors</th>
<th>% Length</th>
<th>% Varicosities</th>
<th>% Endings</th>
<th>No. &lt; 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 d 13</td>
<td>53.9 ± 1.3</td>
<td>56.4 ± 1.0</td>
<td>62.3 ± 1.3</td>
<td>3</td>
</tr>
<tr>
<td>27 d 16</td>
<td>71.2 ± 1.0</td>
<td>71.6 ± 1.1</td>
<td>70.6 ± 1.4</td>
<td>2</td>
</tr>
<tr>
<td>35 d 17</td>
<td>67.4 ± 1.2</td>
<td>70.0 ± 1.0</td>
<td>73.2 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td>35 d wn 13</td>
<td>64.4 ± 1.9</td>
<td>65.7 ± 2.2</td>
<td>63.1 ± 2.0</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4. Percentage targeted to correct section of RA

Fig. 9. Examples of axons with <50% of their varicosities in the correct section of RA in 20-day and white noise-reared 35-day birds. In each example the gray-shaded region highlights the middle section of RA to which axon arbors were matched based on the location of the injection site in LM_{AN_{core}}. Some axons project to the wrong location in normal 20-day birds and in 35-day birds deprived of normal auditory experience. Top: the red axon in injection site 6 split into 5 branches just distal to the view shown here. Bottom: injection site 18 spanned both the lateral and lateral-intermediate sections of LM_{AN_{core}}, so axon arbors could have been targeted to either the intermediate or the ventral section of RA. However, most of the orange axon was targeted to the dorsal section of RA, whereas the maroon axon arbors traversed RA dorso-ventrally and was less spatially refined, with varicosities spread throughout all 3 sections of RA. See Table 5 for quantitative data. Numbers correspond to injection sites shown in Fig. 3. One axon was reconstructed from injection sites 1, 3, and 19; 4 axons were reconstructed from injection sites 6 and 18 (only 3 are shown from injection site 6 for clarity). Scale bars, 100 μm.
but nevertheless had >50% of their arbors in the correct section of RA (e.g., black and green axons from injection site 6). Of the three mistargeted axons in 35-day white noise birds, one axon had >90% of its varicosities, length, and endings in the incorrect (ventral) section of RA (injection site 19). The two other mistargeted axon arbors came from a single injection site (18) that spanned lateral-intermediate sections of LMANcore and thus should have been targeted to either the intermediate or ventral section of RA. However, the orange axon arborized primarily within the dorsal section of RA and the maroon axon spread across all three sections of RA (Fig. 9, bottom). Two additional axon arbors from injection site 18 (green and black) were correctly targeted. The similarity of these results between normal 20-day birds and 35-day white noise birds shows that in the absence of normal auditory experience ectopic axon arbors either are not repositioned or are repositioned incorrectly such that arbors emanating from the same injection site in LMANcore are spread out and project to different locations within RA.

As an additional test of whether axon arbors from the same LMANcore injection site projected to the same location within RA or were more spread out, we measured the total volume encompassed by two axon arbors together for every possible pairwise combination of axon arbors in normal and white noise 35-day birds. Despite the fact that the average volume of individual axon arbors was the same in normal and white noise 35-day birds (Fig. 6), the average volume encompassed by two axon arbors (Fig. 8, bottom right) was more than twice as large in white noise birds (0.1050 ± 0.0068 mm³) compared with normal birds (0.0448 ± 0.0009 mm³). This difference was not significant (U = 133, P = 0.12), primarily because the variance in the total volume of two axons was much higher in white noise than in normal 35-day birds. We therefore examined the volume of every pairwise combination of axons separately for each injection site. In normal 35-day birds the mean volume of two axons ranged from 0.0115 ± 0.0008 mm³ to 0.0587 ± 0.0048 mm³ (n = 4 sites). Of four injection sites in white noise birds, one (Fig. 5, 2nd column) had axon arbors that overlapped (in roughly the correct location) and the mean volume of two axon arbors combined was low (0.0158 ± 0.0006 mm³); however, these two arbors had substantially more branches (28.3 ± 4.7) and varicosities (77 ± 11) than normal 35-day birds (Table 3). In the second injection site (Fig. 5, 3rd column) two of four axons were mistargeted and the mean volume of two axons was 0.1957 ± 0.0157 mm³, which is more than three times the highest value seen in normal birds (U = 1, P < 0.001). All of the axons from the third injection site (Fig. 5, 1st column) projected to the correct section of RA, but the mean volume of a pair of axons was 0.1021 ± 0.0112 mm³, almost double the highest value for normal 35-day birds (U = 4, P = 0.01). The fourth injection site labeled only one axon, and this axon projected to the completely wrong topographic location within RA (Fig. 9, 1st column, bottom). Thus three of four injection sites in birds raised in white noise had axon arbors that projected to the wrong location within RA and/or axon arbors from the same injection site were spread out, showing that in the absence of normal auditory experience axon arbors from the same location within LMANcore were not located in close proximity to each other within RA.

This overall pattern of results shows that LMANcore→RA topography is disrupted when ~23% of axons arborize mostly within the wrong location within RA, as predicted by the results for normal 20-day birds. Figure 10 shows the location within RA of six axons from three different injection sites for both normal and white noise 35-day birds. The arbors in the white noise birds came from the three injection sites with axons that either did not overlap or were targeted to the incorrect location and size of injection sites within LMANcore. The color of the axon arbors in the 2 bottom images correspond to the injection site of the same color. Middle: location within RA of axon arbors from each injection site in normal 20-day birds. Bottom: the axon arbors of 35-day white noise birds were less topographically organized. Axon arbors with asterisks have <50% of their varicosities in the correct section of RA. Numbers correspond to injection sites shown in Fig. 3.
location within RA; corresponding injection sites were chosen from normal birds. Normal 35-day birds demonstrated a typical pattern of topographic organization: Axons from the most medial injection site (red in Fig. 10; n = 2) projected to more dorsal parts of RA, axons from mid-LMANcore projected throughout the center of RA (green; n = 3), and an axon from lateral LMANcore projected to the ventral portion of RA (blue; n = 1). Three of the six axons in white noise birds projected to the wrong location within RA: An axon emanating from mid-LMANcore (green; n = 1) projected to the most ventral portion of RA instead of the middle region, and two axons from the lateral injection site (blue; n = 2) covered the full extent of the dorso-ventral axis in RA. Although six axons are shown in both normal and white noise birds, topography is grossly disrupted in birds reared in white noise because of the presence of mistargeted arbors, even though not all arbors are mistargeted.

Axon arbors of birds reared in white noise differed from those of normally reared birds in three respects: Axons maintained more branches and varicosities (Fig. 7), axons from the same location in LMANcore were less close together within RA (Fig. 8), and some axon arbors projected to the completely wrong topographic location within RA (Figs. 9 and 10). Axons from each of the four white noise-reared birds in this experiment exhibited at least one of these characteristics. Thus in the absence of normal auditory experience even though individual axons became spatially constricted (Fig. 6), topography was disrupted through the maintenance of a larger number of branches, some of which were located within inappropriate regions of RA.

LMANcore axons extend branches in Ad only in juvenile birds. Previous work from our lab suggested that only LMANshell and the dorsal region of the caudolateral nidopallium (dNCL) send axonal projections to Ad (Fig. 1A) (Bottjer et al. 2000; Iyengar et al. 1999; Johnson et al. 1995). However, analysis of single LMANcore axons in juvenile birds revealed a novel projection: Across all groups 27 of 59 axon arbors of LMANcore neurons sent branches into Ad as well as RA. Three axons also sent a single branch to ventral arcopallium (Av), a region that receives a strong projection from LMANshell and a sparse projection from LMANcore (e.g., red axon of 20-day bird in Fig. 4) (Johnson et al. 1995). The LMANcore branches projecting to Ad were topographically organized. Figure 11 shows axon arbors projecting to RA and Ad from three injection sites in 35-day birds; axons coming from medial LMANcore extended branches within medial Ad, whereas axons from mid-LMANcore tended to project throughout the middle of Ad and axons from lateral sites in LMANcore extended branches to lateral portions of Ad. This pattern corresponds to the topographic map formed by the LMANshell→Ad projection in both juvenile and adult birds (Iyengar et al. 1999; Johnson et al. 1995). Axon arbors from 20-day, 27-day, and 35-day white noise birds showed this same general pattern (see Ad axons in 20-day bird, Fig. 4). The black axon arbor of the 27-day bird in Fig. 4 is an exception and appears to project more laterally than would be expected. Three axon arbors projected to Ad in 35-day white noise birds, and all three were in approximately the correct topographic location. The proportion of axon arbors with branches projecting to Ad and the mean number of axon branches and varicosities within Ad were the same at all ages (Table 6). Thus the number of branches and varicosities on LMANcore→Ad arbors does not change between 20 and 35 days even as LMANcore→RA arbors are being pruned. Thus single projection neurons in LMANcore have different mechanisms for establishing regional morphology of their axon arbors depending on their postsynaptic target. Although birds reared in white noise had fewer axons with branches projecting to Ad and fewer branches and varicosities in Ad, none of these differences was significant.

In contrast to juvenile birds, LMANcore axon arbors of adult birds did not send branches into Ad. Qualitative examination of 11 axon arbors from 4 adult birds showed that their entire terminal fields were restricted to RA; none sent terminal

### Table 6. LMANcore→RA axons send branches to Ad only in juvenile birds

<table>
<thead>
<tr>
<th>Total No. of Arbors</th>
<th>Arbors with Branches in Ad</th>
<th>Mean No. of Branches in Ad</th>
<th>Mean No. of Varicosities in Ad</th>
<th>Arbors with Branches in Av</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 d</td>
<td>13</td>
<td>5</td>
<td>7.2 ± 1.2</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>27 d</td>
<td>16</td>
<td>10</td>
<td>7.2 ± 0.3</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>35 d</td>
<td>17</td>
<td>8</td>
<td>8.0 ± 0.4</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>35 d wn</td>
<td>13</td>
<td>4</td>
<td>3.0 ± 0.2</td>
<td>26 ± 4</td>
</tr>
</tbody>
</table>

LMANcore, core region of lateral magnocellular nucleus of anterior nidopallium; Ad, dorsal arcopallium; Av, ventral arcopallium.

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branches to Ad. (Individual axon arbors could not be distinguished in these adults because the injection sites were too large.) Thus the axonal projection from LMANcore changes qualitatively as well as quantitatively during development: LMANcore axons send information into the LMANshell pathway only in juvenile birds that are actively engaged in vocal learning. Furthermore, the matched topography of core and shell axons within Ad implicates Ad as an important site of integration between these two pathways and suggests that the shell pathway may function in one or more aspects of sensorimotor integration in juvenile birds as they are learning to imitate a tutor song. The LMANcore→RA projection is necessary to drive vocal production in juveniles but not adults (Aronov et al. 2008). Hence core neurons may carry a corollary discharge signal to Ad that is conveyed to LMANshell circuitry and compared with auditory feedback and the tutor template (Bell et al. 1983; Poulet and Hedwig 2007). Although LMANcore axons did not send branches into Ad in adult birds, some sent sparse branches into Av, as reported previously (Johnson et al. 1995). Thus only the projection from core neurons to Ad, but not to Av, is developmentally regulated.

DISCUSSION

We examined the morphology of individual LMANcore→RA axon arbors as a function of age and auditory experience to determine how topographic connectivity in this pathway is established during early stages of vocal learning. Axon arbors were elaborate at the onset of vocal learning; they had many branches and covered a large portion of RA. In addition, axons of cell bodies in the same location of LMANcore tended to project to different regions of RA. As early stages of vocal development progressed, LMANcore→RA axon arbors were modified to refine topographic organization in two main phases: Individual axon arbors first retracted to become more spatially refined within the dorsal-ventral axis, and then mistargeted axon arbors were repositioned to achieve the correct topographic location within RA. Only the second of these two processes was dependent on auditory experience.

Mechanisms for establishing topographic maps during development. Interestingly, despite the fact that axon arbors in 20-day birds were larger and encompassed a relatively large proportion of RA, the majority were nevertheless targeted to approximately the correct location (Figs. 5 and 8). Topographic specificity within RA increased in 27-day birds, not only because of a net retraction in total length, volume, and dorsal-ventral extent of arbors but also because of the growth of RA volume overall. This net regression represents an unusual means of achieving topographic precision, since almost all studies of map formation have found that the absolute size of axonal projections grows during development (Antonini and Stryker 1993; Borrell and Callaway 2002; Leake et al. 2002; Sretavan and Shatz 1986). In contrast, decreases in the relative size of axonal projections due to growth of the postsynaptic target represent a common mechanism for achieving topographic precision (Rubel and Cramer 2002). We found that both absolute regression and relative spatial refinement due to target growth contributed to an increase in map precision within RA during the first week of vocal learning (20–27 days) and that neither of these mechanisms depends on normal auditory experience.

Tracer injections that include small subregions of LMANcore produce anterograde label that ramifies throughout most of RA in 20-day birds but is much more restricted in 35-day and adult birds (see Fig. 4 in Iyengar et al. 1999). Although we found that core axons are targeted to roughly the correct location within RA at the onset of vocal learning (20 days), the enlarged volume and extent of arbors at this age, plus the presence of almost 25% largely mistargeted arbors, contribute to a substantial decrease in overall topographic organization compared with older ages. Likewise, 35-day birds reared in white noise show a lack of overall topographic organization comparable to that of normal 20-day birds (Iyengar and Bottjer 2002b). This lack of topographic organization in 35-day white noise birds is not due to expanded size of single arbors but to having arbors with a larger number of relatively short branches (Fig. 7) and to maintaining roughly 25% of arbors with a substantial number of ectopic branches (Figs. 9 and 10). In addition, birds reared in white noise fail to eliminate varicosities, indicating that synaptic pruning is restricted in the absence of normal auditory experience. Thus a wholesale lack of topographic organization occurs in 35-day birds reared in white noise despite normal spatial retraction between 20 and 27 days (Fig. 6), suggesting that remodeling of specific branches contributes to retargeting axon arbors to the correct locations within RA.

Because there is no loss of LMANcore→RA projection neurons during development or in deafened birds, it is unlikely that mistargeted axon arbors are entirely eliminated (Burek et al. 1991; Nordeen et al. 1992). This pattern indicates that the experience-dependent phase of axonal refinement seen here involves more precise mechanisms of establishing neural connections than in the initial experience-independent phase, such as elimination of mistargeted axon branches and synaptic pruning (Borrell and Callaway 2002). Projections to and from LMANcore are mediated largely by NMDA receptors; virtually all the current at LMANcore→RA synapses is carried via NMDA receptors (Bottjer 2005; Mooney and Konishi 1991; Stark and Perkel 1999). NMDA receptors could serve as a filter for gating correlated activity or bursting patterns from LMANcore (Kao et al. 2008), providing a mechanism for the degradation of synapses that fail to drive the postsynaptic target. Blocking NMDA receptors in the visual system during early development results in an increase in synaptic density on axon arbors; similarly, we found that depriving birds of normal auditory experience results in an increase in the number of branches and varicosities (Colonnesi and Constantine-Paton 2006; Johnson et al. 1999; Ruthazer et al. 2003). In the absence of activity or NMDA receptors, most retinal axons are targeted to their correct topographic location, but some arbors or branches are incorrectly localized, disrupting topography (Cline and Constantine-Paton 1989; Simon et al. 1992). Our results also show that birds raised in white noise have disrupted topography in RA because some branches and in some cases the entire axon arbor are maintained in incorrect topographic locations. These results are consistent with the idea that patterned auditory input is necessary to drive correlated activity in order to selectively eliminate mistargeted branches to refine topography. Another mechanism that can lead to selective degradation is activity-dependent presynaptic release of brain-derived neurotrophic factor (BDNF) binding to the p75 receptor on inactive branches (Cao et al. 2007; Singh et al. 2008). LMANcore axons anterogradely transport and
release BDNF, and the p75 receptor is expressed in RA, suggesting that pruning of inactive branches by BDNF could play a role in LMANcore→RA axon arbor refinement (Johnson et al. 1997).

Interestingly, the thalamic inputs to LMANshell [from ventromedial portion of medial dorsolateral nucleus of thalamus (DLMVM); Fig. 1] also show large net regression during vocal development, although coarse topography is already established in the DLMVM→LMANshell pathway by 20 days of age (Iyengar and Bottjer 2002a). Thus large-scale regression may contribute to refinement of the coarse topographic map in LMANshell but is not necessary for its initial establishment. Despite the large-scale pruning seen in both the DLMVM→LMANshell and LMANcore→RA pathways, the contribution of such regression to the establishment of topographic maps (in RA vs. LMANcore) is dissimilar. In the case of the thalamocortical projection from DLMVM to LMANshell, the coarse topographic map is maintained throughout vocal development as axon arbors regress and the postsynaptic target first grows and then regresses. The lack of coarse topography in the connection of LMAN at the onset of vocal learning is apparently unique to the LMANcore→RA projection, and may relate to the motor function of RA (see below).

Functional aspects of topography in the projection from LMANcore to RA. What is the function of LMANcore→RA topography? The topography we describe for LMANcore axons within RA corresponds to the topography between RA and its vocal motor and respiratory targets. The dorsal portion of RA is innervated by axons from medial LMANcore and projects to midbrain and hindbrain regions that control respiration (Wild 1993, 2004). The ventral two-thirds of RA projects to the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which controls the vocal organ (syrinx). Intermediate RA is innervated by intermediate LMANcore and projects to the rostral portion of nXIIIs, which in turn controls the ventral muscles of the syrinx; ventral RA is innervated by lateral LMANcore and projects to the caudal portion of nXIIIs, which controls the dorsal muscles of the syrinx (Vicario 1991). The ventral syringeal muscles control the fundamental frequency of vocalizations, and the dorsal syringeal muscles control the timing of sound production for each side of the syrinx independently to influence the timing and amplitude of phonation (Goller and Suthers 1996; Suthers and Zollinger 2004). LMANcore→RA topography is likely to be essential for controlling these three aspects of vocal behavior in juveniles and enabling LMANcore to influence the acoustic features of adult vocalizations (Aronov et al. 2008; Kao et al. 2005; Sober et al. 2008).

Several important milestones in vocal learning occur during the time that LMANcore axons are refined to establish topography within RA. Birds have formed an auditory memory of their tutor song by ∼35 days (Böhner 1990) and are just beginning to produce incipient song vocalizations. In addition, a large number of axons from HVC are growing into RA (Akutagawa and Konishi 1994; Foster and Bottjer 1998; Mooney and Rao 1994). This timing suggests that the achievement of topography coincides with onset of the sensorimotor integration phase of vocal learning. HVC drives RA to produce vocal output in older juveniles and adults, whereas LMANcore drives vocalizations in juvenile birds (Aronov et al. 2008). In both juveniles and adults LMANcore is necessary for the production of variable vocalizations (Bottjer et al. 1984; Ölveczky et al. 2005; Scharff and Nottebohm 1991). Mature LMANcore topography within RA may be necessary for young juvenile birds to initiate subsong (babbling) and explore vocal-motor space, as well as for subsequent vocal development. For example, the topographic organization of LMANcore→RA axons may provide a scaffold to allow HVC axons to find their postsynaptic partners within RA. As more HVC axons grow into RA and vocalizations become more mature, then LMANcore→RA topography could allow vocal output to be changed in a directed manner to more closely match the learned tutor template (Kao et al. 2005; Tchernichovski et al. 2001; Thivierge and Marcus 2007).

Iyengar and Bottjer (2002b) demonstrated that the LMANcore→RA projection of birds raised in white noise becomes topographically organized by adulthood (rather than as a coarse map), even though topography is completely prevented during the time when it would normally form (i.e., by 35 days). Thus it seems likely that individual axon arbors of birds reared in white noise would become better targeted in adults, but it is impossible to test whether fine-scale synaptic specificity would be disrupted. It should be noted that the prevention of topography at the normal time in vocal development is likely to have permanent deleterious effects, even if topography is established at a later time. For example, as noted above, in normal birds topographic organization in the LMANcore→RA projection is already formed prior to the time when the majority of HVC axons are growing into RA (∼35 days) but is almost completely lacking in 35-day birds reared in white noise.

Zebra finches exposed to tutor song only between 20 and 35 days ultimately produce good copies of that song (Böhner 1990). Birds begin to produce their first song-related vocalizations at ∼35 days of age, and because they are still capable of learning from a tutor for some time after 35 days, there is overlap between auditory memorization of the tutor song and sensorimotor integration under normal circumstances. However, in our study exposure to white noise was largely restricted to the auditory phase of vocal learning, suggesting that auditory experience is largely responsible for instructing the development of LMANcore→RA topography. Of course, we cannot rule out that exposure to white noise also alters early aspects of vocal production, which could also exert an influence on the formation of topography in this pathway.

A novel projection: integration between core and shell pathways of LMAN. An important new discovery of this study is that half of the axons projecting from LMANcore in juvenile birds, but not adults, send axon branches to Ad, which had previously been thought to receive input exclusively from LMANshell and dNCL (Bottjer et al. 2000). Thus the LMANcore→Ad projection serves as a point for cross talk between the two parallel basal-ganglia loops formed by core and shell projections only in juvenile birds that are actively engaged in vocal learning. If LMANcore is a motor circuit and LMANshell acts as a comparator circuit (Bottjer and Altenau 2010), then LMANcore neurons could be sending efference copy to Ad of signals being conveyed to RA, allowing for iterative comparison between LMANcore-driven motor output and its auditory consequences. In 45-day birds lesions of Ad have no immediate effect on vocal behavior, supporting the idea that the shell pathway receives and processes corollary discharge but has no direct role in vocal motor output (Bottjer and Altenau 2010).
By the time birds reached adulthood, no LMANcore axons extended branches in Ad. Thus the existence of this pathway is restricted to the time when juvenile birds are learning and suggests that a forward model of vocal motor commands may form an important part of the learning process (Bass et al. 1994; Bell 1989; Sommer and Wurtz 2002).

Three axon arbors in juvenile birds also sent a branch to Av (ventral arcopallium), consistent with the demonstration of Johnson et al. (1995) that both LMANcore and LMANshell send projections to Av in adult birds. Av sends a sparse projection back to ipsilateral LMANshell and a stronger projection to LMANshell on the contralateral side. Thus Av may provide another point of cross talk between the core and shell pathways in both juvenile and adult birds, as well as a place of coordination of the LMANshell pathway across the two hemispheres of the brain.

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AUTHOR CONTRIBUTIONS

Author contributions: V.C.M.-S. and S.W.B. conception and design of research; V.C.M.-S. performed experiments; V.C.M.-S. analyzed data; V.C.M.-S. and S.W.B. interpreted results of experiments; V.C.M.-S. prepared figures; V.C.M.-S. drafted manuscript; V.C.M.-S. and S.W.B. edited and revised manuscript; V.C.M.-S. and S.W.B. approved final version of manuscript.

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