BRIEF REPORT

Differences in the Complexity of Song Tutoring Cause Differences in the Amount Learned and in Dendritic Spine Density in a Songbird Telencephalic Song Control Nucleus

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In our search for relations between vocal learning and neuron structure in the song control nuclei of songbird forebrains, we tested whether differential experience that leads to differences in adult song repertoire would affect dendritic spine density in HVc (also called high vocal center) and RA (robustus archistriatalis). We tape-tutored juvenile Eastern marsh wrens (*Cistothorus palustris*) with either 5 or 45 song types. As adults, the small repertoire group had learned mostly 5 or 6 song types, and the large repertoire group had learned 36 to 47. Wrens that learned the large song repertoires had a greater dendritic spine density for the most spiny neurons present in HVc (mean difference, 36%), but not in RA. Recent physiological evidence describes HVc as a premotor area coding syllables, motifs, and higher-order song patterns, and our data now clearly reveal that differences in the size of the song repertoire that is experienced lead to differences both in song learning and in the density of dendritic spines in HVc. In the forebrain song nuclei of these songbirds, as in some other vertebrate systems, differences in learning and performance are associated with differences in synaptic anatomy specifically in the region that organizes the learned pattern.   © 2000 Academic Press

The exact role that dendritic spines, a major class of synapses, play in synaptic function related to learning and memory is not completely understood (Harris & Kater, 1994; Shepherd, 1996), but several experimental learning paradigms in vertebrates demonstrate that spine number and memory formation are related. Increased spine density has been

found, for example, on rat cerebellar Purkinje cells with increased motor skill learning (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Anderson, Alcantara, & Greenough 1996), on rat basal dendrites of CA1 hippocampal pyramidal neurons with increased spatial learning (Moser, Trommald, & Anderson, 1994; Moser, Trommald, Egeland, & Andersen, 1997), and on neurons in the lobus parolfactorius of the chick following one-trial passive avoidance learning (Lowndes & Stewart, 1994). Because the dendritic spine is most commonly a postsynaptic site of glutamatergic input, these studies collectively suggest that an increase in the number of excitatory synapses underlies increased behavioral complexity.

Given these findings in other vertebrate systems, we set out to test whether we could affect dendritic spine density on song control neurons of songbird forebrains by providing differential experience that would lead to differences in the size of the song repertoire the birds learn and produce. Song in oscine birds is important to territorial defense and mate attraction. It is a learned, hormonally organized motor activity acquired early in development and an identified and relatively discrete cluster of interconnected brain areas (Fig. 1) controls its production. Song complexity varies by species, sex, and individual and can be readily recorded, quantified, and compared (for reviews of the neurobiology, see DeVoogd & Székely, 1998, and Volume 33, No. 5 of *Journal of Neurobiology*; for reviews of the behavior, see Kroodsma & Miller, 1996). For several reasons, we chose
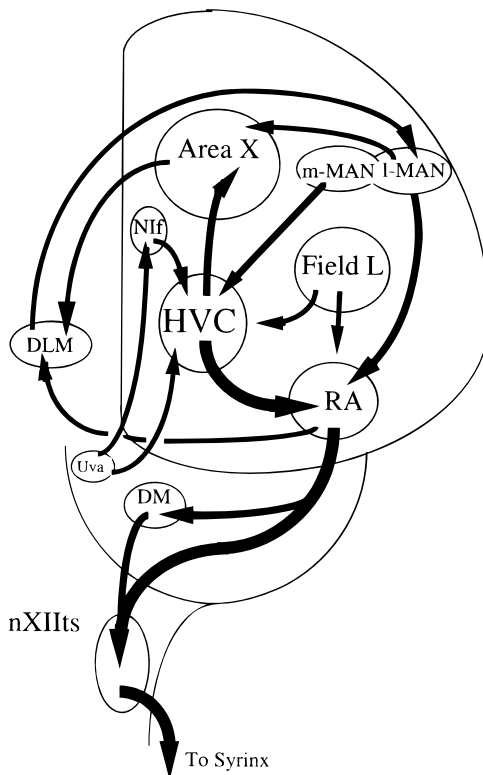

FIG. 1. Relations between major regions of the avian song system. Both HVC and RA are essential for song production, but differ in their function: HVc appears to organize the sequencing and timing of the components of song, whereas RA appears to direct the execution of individual song notes.

Eastern marsh wrens (*Cistothorus palustris*) for our subjects. Males sing a stable repertoire of song types each spring breeding season. In wild populations, individual males differ in the size of their repertoires, typically singing between 40 and 70 song types, with a mean repertoire size of about 54 song types (Canady, Kroodsma, & Nottebohm, 1984). In the laboratory, the song repertoire of adult males can be controlled by exposure to few or many song types during the early sensitive period (Brenowitz, Lent & Kroodsma, 1995).

Some of our methods are presented elsewhere, including a detailed description of the time of capture, housing conditions, tutoring regime, and song analysis of the 16 wrens used in the current study (Brenowitz et al., 1995). Briefly, 8- to 12-day-old nestlings were captured between 28 June and 8 July 1992 in marshes at the mouth of the Housatonic River in Connecticut and near Tivoli on the Hudson River in New York. At about 14 days, birds were assigned to two experimental groups destined to hear different tutoring tapes (assignment was random, except that siblings were placed in different groups). Tapes used for tutoring were composed from the recorded repertoire of a single male Eastern marsh wren. Eight male wrens were exposed to a small repertoire of 5 song types, and the other eight wrens were exposed to a larger repertoire of 45 song types, for 45 to 90 min per day, from 11 July through August. Repertoire size for the two groups of experimental birds was determined from stable, adult song in the spring of 1993.

After adult song repertoires had been tape-recorded, at approximately 10 months of age, all 16 birds were perfused with heparinized phosphate buffer (0.1 M, pH 7.4) followed by 4% paraformaldehyde fixative. The brains were postfixed for 12–24 h and stored in refrigerated phosphate buffer. Previous work has not detected any significant hemispheric differences in the sizes of telencephalic song nuclei or in spine density within them (DeVoogd & Nottebohm, 1981; Nixdorf, Davis, & DeVoogd, 1989; Brenowitz, Nalls, Kroodsma, & Horning, 1993). Therefore, the brains were hemisected so as to allow two sorts of analysis. One hemisphere, either right or left, was Nissl stained and used to examine gross morphology of the song system nuclei (results in Brenowitz et al., 1995), and the other hemisphere was Golgi stained for the present study (protocol based on Glaser & Van Der Loos, 1981). Hemispheres were immersed in Golgi–Cox fluid for approximately 5 weeks, dehydrated, embedded in celloidin, and sectioned at 80 μm. Sections were reacted with ammonia, counterstained with a mixture of methylene blue and thionin, and coverslipped.

We selected neurons in HVc (high vocal center) and RA (robustus archistriatalis) for analysis that were visually most spiny rather than attempting to select cells from specific classes. In HVc, these neurons most closely resembled neurons previously described in canaries as FD (furry dendrite) type, with spiny to very spiny dendrites and large, complex stellate arbors. In adult canaries, Nixdorf et al. (1989) contrasted this cell type with the TD (thick dendrite) type, mainly on the basis of differences in spine density. This distinction was not obvious in the wrens. The neurons in RA that were visually most spiny had morphology characteristic of Type IV neurons, previously described as having three to five primary dendrites with very thick, very spiny branches in the canary (DeVoogd & Nottebohm, 1981).

Both HVc and RA contain multiple classes of neurons, some of which are distinguished primarily by the density of spines on the dendrites. Although the class distinctions are clear across animals of the same sex and backgrounds (DeVoogd & Nottebohm, 1981; Nixdorf et al., 1989), it is not possible to rely on those distinctions when comparing

animals that differ in ways that may affect spine density. In the present study, by restricting our analysis to the visually most spiny neurons in RA and HVc, we could determine whether neuronal structure of the two wren groups differed. We could not resolve whether group differences were caused by spine addition or loss or whether differences occurred in all neuron classes.

We quantified dendritic spine density (spines/micrometer) on the Golgi-stained neurons at 100×. Because of dark staining and obscuring glial staining, spine density could not be measured in HVc for six birds and in RA for two birds. Spine density was quantified for the remaining birds using either our semiautomated data collection system (DeVoogd & Nottebohm, 1981; Nixdorf et al., 1989; $n = 5$ (large repertoire), 3 (small repertoire) for RA, $n = 5,5$ for HVc) or a Eutectics data collection system ($n = 3,3$ for RA). Spine counts were done blind to experimental condition. We counted spines in 10-μm sampling intervals from the soma to a distal tip of a dendrite, thereby obtaining a series of spine density values for a particular dendritic transmission pathway (termed a dendrite below). Several such dendrites were measured for HVc (mean for each animal: 7.4) and for RA (mean: 11.2). Spine density in these nuclei varies with many features, including neuronal class, transmission distance to the soma, dendritic orientation, and proximity to the margin of the nucleus (DeVoogd & Nottebohm, 1981, Nixdorf et al., 1989; Benton, Cardin, & DeVoogd, 1998). In order to minimize the effects of such influences, we took the peak spine density value from each dendrite that we had measured (usually from one of the samples between 30 and 60 μm from the soma) and averaged those values to derive a mean *peak* spine density value for RA and for HVc in each animal for subsequent analyses

TABLE 1
Mean Peak Spine Density in Telencephalic Song Nuclei of Eastern Marsh Wrens Tutored with Small (5 Song Types) or Large (45) Repertoires, Revealing a Significant Difference in Peak Spine Density of HVc but Not RA

HVc		RA	
Small repertoire group[a]	Large repertoire group	Small repertoire group	Large repertoire group
1.024 (8)	1.494 (9)	1.255 (10)	1.601 (11)
1.138 (6)	—	1.579 (15)	1.446 (10)
—[b]	1.507 (10)	1.515 (10)	1.658 (11)
1.059 (10)	1.255 (5)	—	1.516 (7)
—	1.109 (6)	—	1.273 (9)
0.827 (5)	1.336 (9)	1.100 (17)	1.650 (9)
—	—	1.864 (8)	1.517 (13)
0.857 (6)	—	1.559 (17)	1.697 (10)
Mean = 0.981 ± 134 (SD)	1.340 ± .150	1.479 ± .245	1.545 ± .139

Note. In parentheses are the number of dendritic pathways ("dendrites") measured in each nucleus (different dendrites usually came from different neurons). Spines were counted in 10-μm sampling intervals from the proximal portion of each dendrite to a distal tip, and the maximal density value from each dendrite was used for the analyses.

[a] Repertoire sizes for the listed birds were 5, 5, 5, 5, 5, 6, 6, and 12 for the small repertoire birds and 36, 39, 40, 40, 41, 41, 47, and 50 for the large repertoire birds.

[b] Data unavailable for some birds because either cells stained too darkly or glial staining obscured the view.

(see Table 1). Values from the large and small repertoire birds were compared with two-tailed, pooled t tests (Data Desk 6.0.1, Data Description Inc.). No hemispheric differences were found in these measures for either HVc or RA.

The results of our behavioral treatment were as expected: adult song repertoires in each experimental group were largely dictated by the tutoring regime (see Table 1). Birds tape-tutored with 5 song types learned to produce 5 to 12 acoustically normal song types, with a median of 5. Birds tape-tutored with 45 song types learned to produce repertoires ranging from 36 to 50 song types, with a median of 40.5. The songs of all birds appeared normal in structure in sonograms and to the ear, and the songs of all birds had good matches to the tutor songs in their repertoires.

In RA, we found that the difference in song repertoire size between the two wren groups was not related to peak spine density. Neurons did not differ in mean peak spine density between small and large repertoire birds ($t = 0.602$, $p = .558$; see Table 1, Fig. 2).

For HVc, however, song repertoire size and peak spine density were significantly related. Peak spine density for the wren HVc neurons was 36% greater for the large repertoire birds than for the small repertoire birds ($t = 3.746$, $p = .006$; see Table 1, Fig. 2). This effect was independent of the mean distance from the cell body at which the measures were taken.

Our results are the first to show that differential song exposure leads to differences in neuronal anatomy as well as differences in song learning. Birds that are exposed to and learn large repertoires have greater peak dendritic spine density in HVc than do birds that are exposed to and learn small repertoires. These results are consistent with data showing increased spine density with motor skill learning and spatial learning in rats (Black et al.,
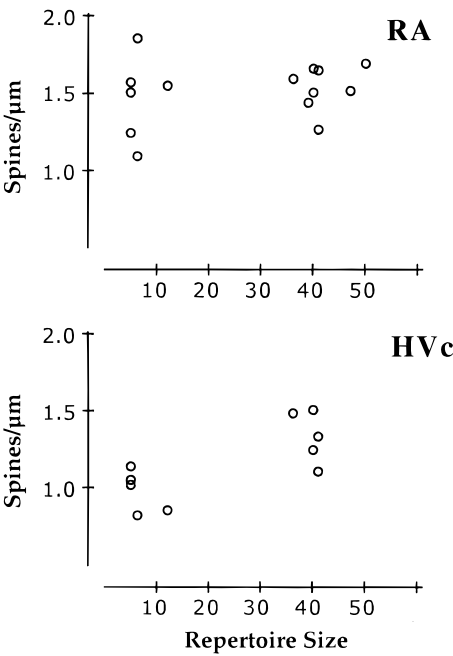

FIG. 2.   The relations between each animal's repertoire size and its mean peak spine density in RA and HVc. Differences in song tutoring and subsequent learning are associated with differences in spine density in HVc neurons but not in RA neurons.

1990; Anderson et al., 1996; Moser et al., 1994, 1997) and with passive-avoidance learning in chicks (Lowndes & Stewart, 1994).

Our results also help us better understand how song repertoire size and different aspects of HVc anatomy are related. Learned repertoire size and HVc volume are positively correlated in domestic canaries (Nottebohm et al., 1981), zebra finches (Ward, Nordeen, & Nordeen, 1998), and wild marsh wrens (Canady et al., 1984). For the same marsh wrens used in this study, wrens that learned small or large song repertoires did not differ in HVc volumes, but, within the large repertoire group, the association between repertoire size and HVc volume approached significance (volume of RA also did not differ between groups but was significantly related to repertoire size within the large repertoire group; Brenowitz et al., 1995). These data suggest that significant brain–behavior associations in the correlation studies occurred because brain influenced behavior rather than the other way around. In other words, the correlations were not effects of learning but rather resulted from individual differences in the size of HVc, and a large HVc permitted birds to learn a large repertoire. Thus, genetic or developmentally determined differences in cell number may act to constrain the size of the repertoire that can be acquired (Brenowitz & Kroodsma, 1996; Ward et al., 1998). Our current data further suggest that differences in songbird repertoire size that result from differential opportunity for learning are associated with differences in the number of spine synapses. In future studies on song learning, we hope that differences in morphometry of individual cells are considered along with cell number and nucleus volume. Future studies would also benefit from more precise, quantitative morphometric analyses of discrete cell classes (Benton et al., 1998). We would expect that measurements on neuronal classes defined using criteria that are independent of spine density, such as connectivity or neurotransmitter, would find differences in total spine number that are the basis for our differences in peak spine density.

Our data on spine densities in HVc and RA also contribute to emerging ideas on the functions of these two nuclei. In zebra finches (*Taeniopygia guttata*), for example, RA appears to control discrete syringeal muscles so as to produce different notes, but HVc codes syllables, motifs, and higher-order temporal patterning of song (Vu, Mazurek, & Kuo, 1994; Yu & Margoliash, 1996; Margoliash, 1997). RA connectivity might therefore be expected to vary with the number of distinct notes that are produced. Marsh wren song types seem acoustically and spectrographically similar; they typically begin with a nasal buzz, followed by a few brief introductory notes and a trill of repeated syllables consisting largely of a series of brief tonal notes (Kroodsma, 1989). A repertoire of 5 to 6 song types might well exhaust most of the spectrographic range of muscular control. Another 35 to 40 song types might then not require much more connectivity within RA; perhaps any enhanced anatomy needed either would be beyond the resolution of the counting methods we used or would be represented in structural aspects of RA that we did not measure. In contrast, differences in peak dendritic spine density in HVc may represent an increased network space for coding additional song variants.

Our laboratory efforts are intended to help us understand the neurobiology of song learning in the wild and how natural variation in opportunity to learn songs influences neuronal connectivity. Among our laboratory wrens, both groups received the same amount of exposure to song and both learned their song models accurately; furthermore, wrens from both the small and the large groups sang vigorously (Brenowitz et al., 1995). The only difference in song that we observed between wrens in the two groups was repertoire

size: we had artificially limited the wrens tutored with only 5 song types to an abnormally small repertoire. In nature, birds presumably learn as many songs as they are capable of learning, and we would predict a relationship between repertoire size and spine density there, too. Because repertoire size among wild birds varies far less than in our two laboratory groups, however, demonstrating the relationship in nature may prove difficult.

The results of our laboratory study, however, are unequivocal: differences in the complexity of juvenile experience with song cause differences in spine density in these songbird forebrains. Eastern marsh wrens assigned to learn a large song repertoire developed greater spine density in HVc than did wrens assigned to learn a small repertoire. The localized induction of synaptic plasticity that we found in HVc is consistent with recent physiological evidence describing HVc as a premotor area coding syllables, motifs, and higher-order song patterns. The differences in experience in these wrens thus influence song learning and performance and synaptic anatomy specifically in the region that organizes the learned pattern.

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