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Growth and atrophy of neurons labeled at their birth in a song nucleus of the zebra finch

(brain/cell death/gender difference/estrogen)

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ABSTRACT **The robust nucleus of the archistriatum (RA) is one of the forebrain nuclei that control song production in birds. In the zebra finch (*Poephila guttata*), this nucleus contains more and larger neurons in the male than in the female. A single injection of tritiated thymidine into the egg on the 6th or 7th day of incubation resulted in labeling of many RA neurons with tritium. The size of tritium-labeled neurons and the tissue volume containing them did not differ between the sexes at 15 days after hatching. In the adult brain, tritium-labeled neurons and the tissue volume containing them were much larger in the male than in the female. Also, tritium-labeled RA neurons were large in females which received an implant of estrogen immediately after hatching. The gender differences in the neuron size and nuclear volume of the zebra finch RA are, therefore, due not to the replacement of old neurons by new ones during development but to the growth and atrophy of neurons born before hatching. Similarly, the masculinizing effects of estrogen on the female RA are due not to neuronal replacement but to the prevention of atrophy and promotion of growth in preexisting neurons.**

In many bird species, the male sings and the female does not. This sexual dimorphism in behavior finds its morphological correlates in the brain nuclei that control song (1). All song nuclei are larger in the male than in the female. The volume differences are due to differences in both cell size and number (2–5). In the zebra finch (*Poephila guttata*), the size and number of neurons in the robust nucleus of the archistriatum (RA), one of the forebrain song nuclei, are not different between the sexes until about 20 days after hatching. Gender differences in RA neuron size and number emerge rapidly between posthatching days 30 and 40 (3, 4). The simplest explanation for these observations is to assume that RA neurons grow in the male and undergo atrophy and death in the female (3). Another explanation is to assume neuronal replacement during development. New neurons continue to be born in the forebrain of the male zebra finch during the first month after hatching (6, 7). The original embryonic RA neurons may later be replaced by larger neurons in the male and smaller ones in the female. Female zebra finches that have received an implant of estrogen during the first 40 days of life develop larger neuron size and nuclear volume in their RA than normal females (2, 5, 8, 9). Estrogen implants either prevent neuronal atrophy and death or facilitate neuronal replacement in the RA. In both normal and estrogen-treated birds, the discrimination between neuronal replacement and growth or atrophy would be aided by the specific labeling of the neurons destined to occupy the embryonic RA. For this purpose, we labeled dividing neuroblasts with tritiated thymidine and observed their fate in both sexes from an early age to adulthood.

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MATERIALS AND METHODS

A colony of zebra finches in our animal quarters provided fertilized eggs of known dates of laying. Cells that were destined to become RA neurons were labeled with tritium derived from tritiated thymidine. We carried out tests to determine the single dose of tritiated thymidine necessary for obtaining controlled labeling of brain areas. [*methyl*-^{3}H]Thymidine with specific activity of 1 Ci/mmol (Research Products International; 1 Ci = 37 GBq) was dried and then resuspended in sterile saline to obtain an activity of 200 μCi/μl. Using a constant volume of 0.05 μl, we tested the following dosages per egg; 0.5, 2, 5, 10, and 20 μCi. Dosages smaller than 10 μCi produced little label in brain tissue. On the other hand, a dose of 20 μCi increased the mortality of the embryos. We, therefore, chose 0.05 μl and 10 μCi per egg as the most suitable set of conditions for the zebra finch. We determined the stage of embryogenesis most suitable for marking cells destined to become RA neurons by injecting tritiated thymidine into eggs staged 2, 4, 6, 8, 10, and 12 embryonic days. Embryonic day 0 was defined as the day when the first blood vessels appear on the yolk and day 13 is the day of hatching. Once we saw labeling of RA neurons, we tested the days preceding and following the day that gave the first positive results.

Injections of thymidine in early embryos were made just next to a large blood vessel lying close to the embryo. After day 8, injections were made in any area devoid of blood vessels. Injections were made through a small hole cut on the eggshell, and the injection tip was inserted with a micromanipulator. The injection tip consisted of a tapered glass capillary with a tip diameter of 20 μm. We connected this capillary to the needle of a 1-μl Hamilton syringe after filling the syringe with mineral oil. When the glass tip was filled with 0.05 μl of tritiated thymidine, the interface between saline and oil became visible. We used this interface to see the flow of the injection vehicle into the egg. After injections, the hole was sealed with molten wax and the eggs were returned to their parents or given to foster parents. All injected eggs were identified by the injection date and age written on the eggshell and all chicks were individually marked.

Birds were deeply anesthetized with pentobarbital (Abbott) and perfused through the heart first with physiological saline and then with 10% Formalin. Brains were cut into 30-μm sections on a freezing microtome. Sections were then prepared for conventional autoradiography. The duration of exposure was 3 weeks at 4°C. Both the cross-sectional areas of tritium-labeled neurons and the tissue volumes containing them were measured by computer-aided planimetric methods. RA volumes were measured on sections prepared for autoradiography without counterstaining. We used a sample of five birds for each age, gender, and treatment group with the exception of the estrogen-treated adult group, which contained four birds. A sample of 50 tritium-labeled neurons

Abbreviation: RA, robust nucleus of the archistriatum.

was randomly chosen from each bird for the measurement of mean somal areas. These measurements were made on autoradiographic sections counterstained with cresyl violet. We used the known criteria such as a large pale nucleus, one or two distinct nucleoli, and dark cytoplasm to distinguish neurons from glial cells. Nine female chicks hatched from thymidine-injected eggs were given a subcutaneous implant of 50 μg of 17β-estradiol immediately after hatching. Five of these birds were used for autoradiography at posthatch day 15 and the rest were used in adulthood. The methods of making hormone pellets and subcutaneous implantation have been previously described (2).

RESULTS

A single injection of tritiated thymidine on the 6th or 7th day of incubation produced many tritium-labeled RA neurons in both sexes. The RA of such birds was clearly distinguishable without counterstaining at posthatching day 15 as a discrete volume of tissue occupied by tritium-labeled neurons (Fig. 1). None of injections made on days other than embryonic day 6 or 7 labeled a sufficient number of neurons to allow the identification of a song nucleus by tritium label alone. A comparison of labeled RA neurons between the sexes at day 15 showed no gender differences in either neuron size (Figs. 2 and 3*B*) or the tissue volume occupied by tritium-labeled neurons (Figs. 1 and 3*A*). A similar comparison in adulthood showed marked gender differences (Figs. 2 and 3). Tritium-labeled RA neurons and the volume they occupy were much larger in the male than in the female. Furthermore, adult RA volume and neuron size were larger in the male and smaller in the female than those of 15-day-old chicks of either gender. Both the size of tritium-labeled RA neurons and the tissue volume containing them were much larger in the estrogen-treated females than in untreated females. Although the differences in nuclear volume may reflect differences in neuron number, we did not count neuron number, because the density of labeled neurons in the RA was too variable to be useful for the estimation of neuron number.

DISCUSSION

We hypothesized earlier that gender differences in the volume and neuron size of the RA of the zebra finch arise because the neurons grow in the male and shrink and die in the female during the first month after hatching (3). Because

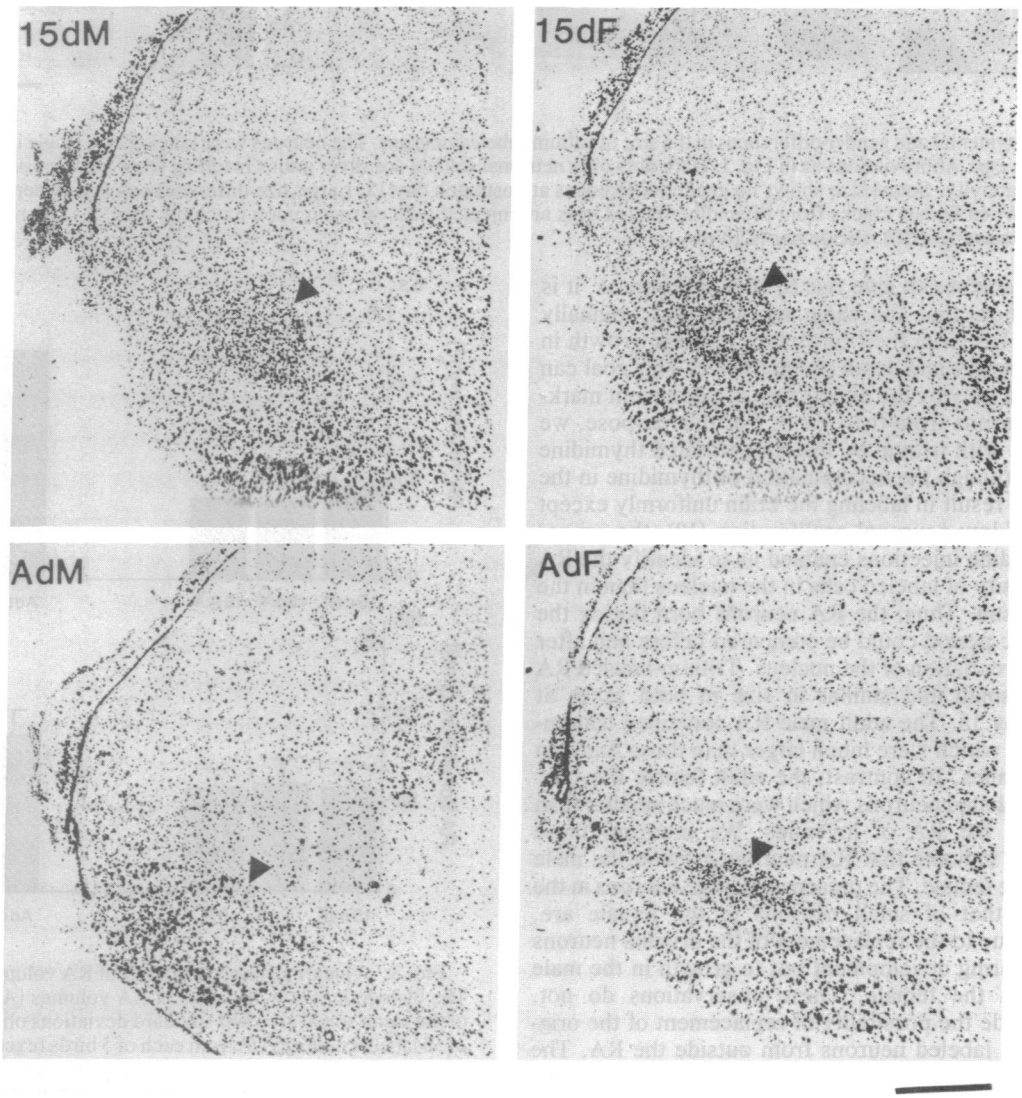

FIG. 1. Developmental and gender differences in RA volume occupied by tritium-labeled neurons. The neurons were labeled by a single injection of tritiated thymidine into the egg. The RA can be recognized by a higher density of tritium-labeled neurons as indicated by arrowheads. The size of the nucleus shows no gender difference at posthatch day 15. Marked size differences are found between the adult male and female. M, F, d, and Ad denote, respectively, male, female, day, and adult. (Scale bar is 0.5 mm.)

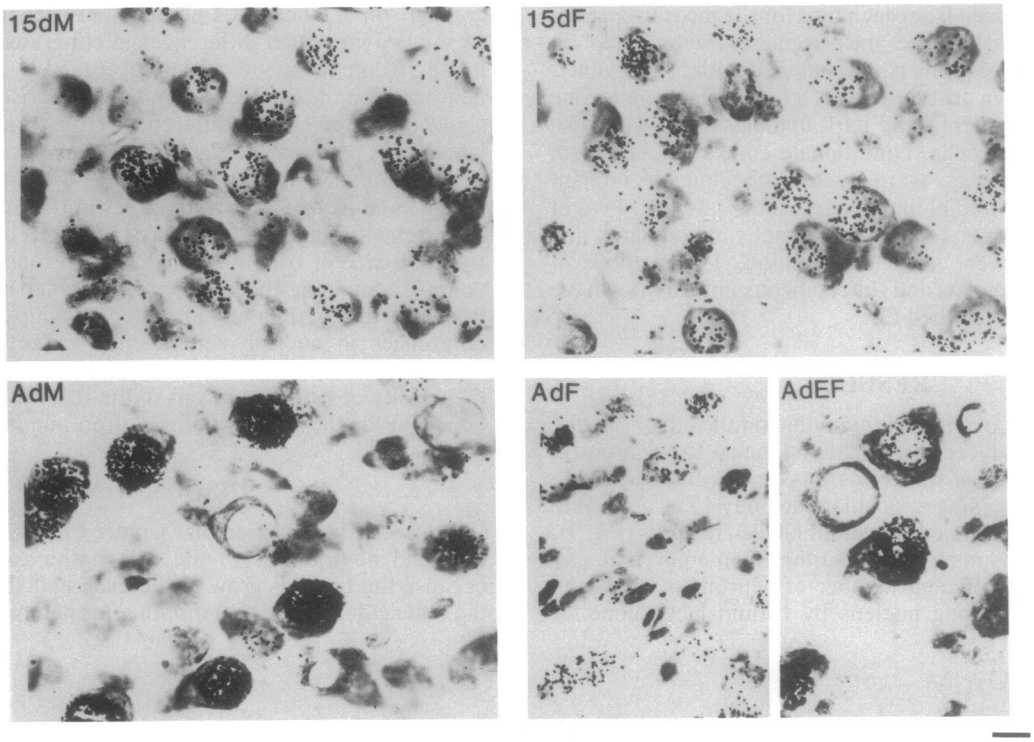

FIG. 2. Developmental and gender differences in the size of tritium-labeled neurons. The neurons were labeled by a single injection of tritiated thymidine into the egg. Abbreviations as in Fig. 1. Dark dots over neuronal somata are silver grains resulting from the exposure of photographic emulsion to radioactivity. Somal size shows no gender differences at posthatch day 15. Large size differences emerge when the birds are kept until adulthood. When female chicks from thymidine-treated eggs are injected with estrogen upon hatching, their RA neurons are large when examined in adulthood (AdEF). (Scale bar is 10 μm.)

neuronal replacement can give rise to such differences, it is necessary to show that the same neurons that originally migrated to the RA from their birthplace undergo growth in the male and atrophy and death in the female. This goal can be accomplished only by the attachment of permanent markers on those neurons from their birth. For this purpose, we labeled neurons with tritium by injecting tritiated thymidine into the egg. Although the accumulation of thymidine in the egg is known to result in labeling the brain uniformly except for the areas of low neuronal proliferation (10), the proper timing of thymidine injections enabled us to identify the RA by a higher density of labeled cells in the nucleus than in the surrounding tissue. Thus, the RA neurons born during the same embryonic period could be compared before and after the sexual differentiation of the nucleus. Tritium-labeled RA neurons were small and similar in size in both sexes at posthatching day 15. The adult male RA contained tritium-labeled neurons which were much larger than those found in the RA of juveniles. In contrast, the adult female RA contained tritium-labeled neurons which were much smaller than those found in the RA of juveniles. We interpret these observations to indicate that RA neurons grow in the male and shrink in the female. The presence of large neurons in the male RA and that of small neurons in the female are, therefore, not due to the replacement of the original neurons by new ones during development but to growth in the male and atrophy in the female. These observations do not, however, exclude the possibility of replacement of the original neurons by labeled neurons from outside the RA. The above possibility can be ruled out only by exclusive labeling of embryonic RA neurons.

Tritium-labeled RA neurons in estrogen-treated females were much larger than those of juveniles and normal females. This finding lends support for the interpretation that the masculinizing effects of estrogen on RA neurons are not due

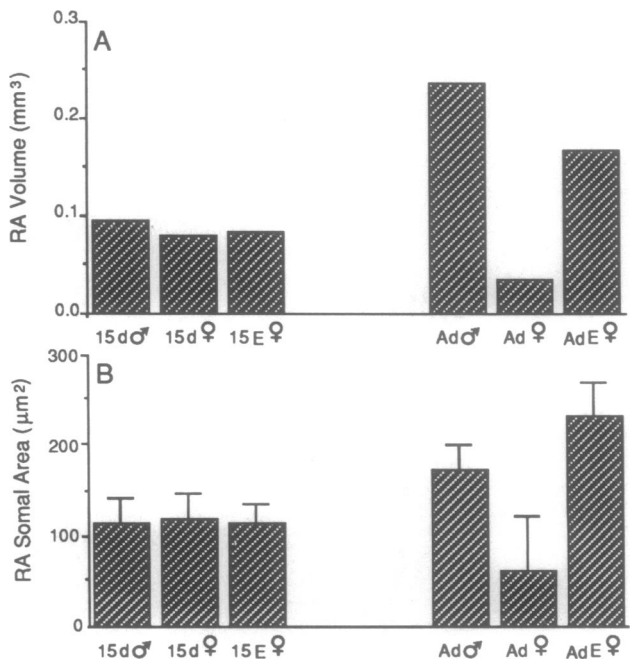

FIG. 3. Quantitative comparisons of RA volume and neuron size. The histograms show the median RA volumes (*A*) and the averages of the mean somal area and standard deviations obtained for a sample of 50 labeled neurons (*B*) from each of 5 birds (except $n = 4$ for adults treated with estrogen as juveniles) belonging to each age and gender class. Neither RA volume occupied by tritium-labeled neurons nor the size of the neurons shows gender difference at posthatch day 15. The RA of estrogen-treated females (E♀) is similar to that of normal males or females at day 15. In the male, both RA volume and neuron size are larger in adulthood than at day 15, while the converse is true of the female.

to neuronal replacement but to the prevention of atrophy and death and the induction of growth in the neurons born during embryogenesis (5, 9).

Our results do not allow us to assess the amount of neuronal death and birth after hatching. The death and birth of neurons occur in the forebrain song nuclei of the zebra finch during the first month after hatching. In both canaries and zebra finches, the neurons born after hatching originate in the ependymal layer of the lateral ventricle and migrate to all parts of the forebrain (6, 7, 11, 12). The RA of the male zebra finch, however, receives few or no new neurons after posthatching day 6 (6, 7). In our earlier report (3), we inferred neuronal death in the female RA from a decrease in the volume of the RA. Because both the number and size of neurons contribute to differences in the volume of a nucleus, it is necessary to discriminate between the two variables. The most direct method is to count dying neurons. The timing of changes in the number of dying neurons appears to be correlated with that of size reduction in the female RA (13). A larger proportion of pyknotic neurons occurs in the female RA than in the male. Furthermore, in the female RA, more neurons appear to die between days 20 and 30 than before or after this period. This finding is consistent with the observation that the decline in the volume of the female RA is steeper during this period (3). All these findings, taken together, indicate that the posthatch recruitment of neurons contributes little to the development of gender differences in the RA of the zebra finch, while the death of the original neurons in addition to atrophy is responsible for the decrease in the volume of the female RA.

The growth, atrophy, and death of neurons have been implicated for the development of gender differences in other forebrain song nuclei, including the caudal portion of the ventral nucleus of the hyperstriatum (HVc) and the magnocellular nucleus of the anterior neostriatum of the zebra finch, although marked neurons have not been observed in these nuclei (3–5). Other factors that contribute to the development of gender differences are the continued recruitment of new neurons to the male HVc and the area X during the first month after hatching (6, 7) and the delayed arrival of HVc axons in the male RA (3).

Epigenetic processes, particularly those mediated by gonadal steroids, control the gender-specific fate of homologous cells born to both sexes in other body organs and tissues. The present study suggests that this mode of sexual differentiation takes place in the RA of the zebra finch.

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