Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 714–718, January 1996
Neurobiology

Recruitment and replacement of hippocampal neurons in young and adult chickadees: An addition to the theory of hippocampal learning

(neurogenesis/neuronal overproduction)

ANAT BARNEA* AND FERNANDO NOTTEBOHM

Field Research Center, The Rockefeller University, Tyrrel Road, Millbrook, NY 12545

Contributed by Fernando Nottebohm, October 2, 1995

ABSTRACT **We used [^{3}H]thymidine to document the birth of neurons and their recruitment into the hippocampal complex (HC) of juvenile (4.5 months old) and adult black-capped chickadees (*Parus atricapillus*) living in their natural surroundings. Birds received a single dose of [^{3}H]thymidine in August and were recaptured and killed 6 weeks later, in early October. All brains were stained with Cresyl violet, a Nissl stain. The boundaries of the HC were defined by reference to the ventricular wall, the brain surface, or differences in neuronal packing density. The HC of juveniles was as large as or larger than that of adults and packing density of HC neurons was 31% higher in juveniles than in adults. Almost all of the ^{3}H-labeled HC neurons were found in a 350-μm-wide layer of tissue adjacent to the lateral ventricle. Within this layer the fraction of ^{3}H-labeled neurons was 50% higher in juveniles than in adults. We conclude that the HC of juvenile chickadees recruits more neurons and has more neurons than that of adults. We speculate that juveniles encounter greater environmental novelty than adults and that the greater number of HC neurons found in juveniles allows them to learn more than adults. At a more general level, we suggest that (*i*) long-term learning alters HC neurons irreversibly; (*ii*) sustained hippocampal learning requires the periodic replacement of HC neurons; (*iii*) memories coded by hippocampal neurons are transferred elsewhere before the neurons are replaced.**

Black-capped chickadees (*Parus atricapillus*, order Passeriformes, family Paridae) are small common songbirds that live in the forests of temperate North America. Young ones hatch during May and June and fledge 16 days later. Fledglings follow their parents and are fed for another 2 weeks (1). Between independence and early August, 90% of juvenile chickadees of both sexes disperse over distances of 0.8–11.2 km (2, 3). A young chickadee spends the remainder of its life in the neighborhood where it settles during its first summer (1).

As the young birds follow their parents, disperse, and settle, they must memorize landmarks and learn the spatial relations that will allow them to return reliably to sources of food and shelter and avoid danger. The avian hippocampal complex (HC) is thought to help with this task (4, 5), but we know very little about the changes that HC undergoes from the time the young bird leaves the nest until the time it reaches sexual maturity. An earlier publication (6) showed that the hippocampus of adult free-ranging chickadees constantly adds new neurons and that these cells replace older ones. This process peaks in late summer–early fall. The present study compares the late-summer recruitment of new hippocampal neurons in juvenile and adult free-ranging chickadees.

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MATERIALS AND METHODS

Study Site. The present study was conducted at The Rockefeller University Field Research Center for Ecology and Ethology in Millbrook, NY, in rural Hudson Valley.

Animals. Six juveniles (approximate age 3 months, based on estimated hatching in second half of May; three males and three females) and five adults (three males and two females) were caught during the period of August 20–26, 1993, with mist-nets installed near feeding stations baited with sunflower seeds. Adults were recognized because they had been banded during the previous winter and so were at least 1-year-old. Juveniles were recognized by their darker skull (as seen through the skin, indicative of a thinner less-pneumatized skull wall), by the shape and coloration of the outer tail feathers (rectrices) (7) and by the very limited wear of all feathers.

[^{3}H]Thymidine Treatment. Each bird received a single intramuscular dose of 50 μCi of the cell birth marker [^{3}H]thymidine (6.7 Ci/mM; 1 Ci = 37 GBq; New England Nuclear) (8) and was then released. Nuclear labeling with [^{3}H]thymidine administered to adult birds occurs within 60 min after injection; no significant labeling of dividing cells occurs thereafter (9). Six weeks later the injected birds were recaptured very close to the original point of capture. Apparently, the juveniles had already settled into the neighborhood where they would live as adults. The birds were then killed. Based on previous studies in canaries, the 6 weeks of survival probably allowed enough time for the neurons born at injection time to migrate to their final destination and go through final anatomical differentiation (10, 11). Birds were treated under Radiation Control Permit 145-5, issued by The New York State Department of Environmental Conservation.

Histology. Birds were killed immediately after recapture with an overdose of anesthesia and fixed by intracardiac perfusion with 20 ml of saline followed by 50 ml of 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). The sex of each bird was established by looking at the gonads. Brains were removed and treated for autoradiography as described (6).

Anatomy of the Hippocampal Complex. We recognized three subdivisions within HC. A middle region was defined by the presence of a ventromedial hippocampal "fold" (6). A line connecting the tip of this fold to the midline and intersecting the latter at right angles forms, with the fold, the ventral boundary of HC and separates HC from the septum. This boundary continues rostrally and caudally as a constriction of the tissue between the lateral ventricle and the midline. The HC rostral and caudal to the middle region will be referred to as the rostral and caudal HC, respectively.

Abbreviations: HA, hyperstriatum accessorium; HC, hippocampal complex; IZ, intermediate zone.

*Present address: Life Sciences, The Open University of Israel, Max Rowe Educational Center, 16 Klausner Street, P.O. Box 39328, Ramat-Aviv, Tel-Aviv 61392, Israel.

The rostral and caudal reaches of the HC were defined by the presence of a recognizable lateral boundary. This boundary occurred at the transition between the larger neurons of HC and the smaller and more densely packed neurons of the hyperstriatum accessorium (HA) (12). However, the exact position of the lateral boundary in rostral HC was difficult to establish in both age groups. Our definition of HC by reference to a distinct lateral boundary may underestimate the true size of HC, a matter that can only be resolved by future connectivity studies. At the rostral and caudal HC cut-off points that we used, the brain looked similar to that of a canary at atlas levels A4.0 and A0.2, respectively (13). We do not know whether HA continues as far caudal as do the small cells that define the lateral boundary of caudal HC.

Quantification. A previous study (6) did not find right–left and male–female differences in the number of labeled HC neurons and, therefore, all the data we present here come from right hemisphere analyses and pooled male and female material. Our criteria for recognizing a cell as an unlabeled or labeled neuron and our methods for mapping their position, measuring their nuclear diameter, estimating their packing density and calculating the volume of HC are as described in (6).

RESULTS

HC Boundaries and Dimensions. The lateral boundary of rostral HC coincided with a region of gradually changing cell sizes and packing densities, rather than with a sharp change, as described (12). Cells were smaller and more densely packed as one moved laterally. The gradual nature of this change in rostral HC results in an area of ambiguous affiliation—it could be HC, it could be HA, or it could be something else. The existing literature on avian hippocampus does not resolve this ambiguity (12, 14–16). For our analysis, we assigned to the ambiguous area, which we shall refer to as "intermediate zone" (IZ), boundaries of its own (shown in Fig. 1), and then estimated the overall volume of HC with and without IZ. The estimates for juveniles were 4.96 ± 0.41 mm^3 with IZ and 4.70 ± 0.44 mm^3 without IZ; for adults these values were 4.20 ± 0.60 mm^3 and 4.04 ± 0.57 mm^3, respectively. The difference between the juvenile and adult volume estimates that included IZ was significant ($U = 4$; $P = 0.045$), and that between the volume estimates that did not include IZ was marginal ($U = 5$; $P = 0.068$). The mean rostrocaudal extent of the HC was similar in juveniles and adults (3.1 ± 0.3 mm vs. 3.2 ± 0.3 mm, respectively).

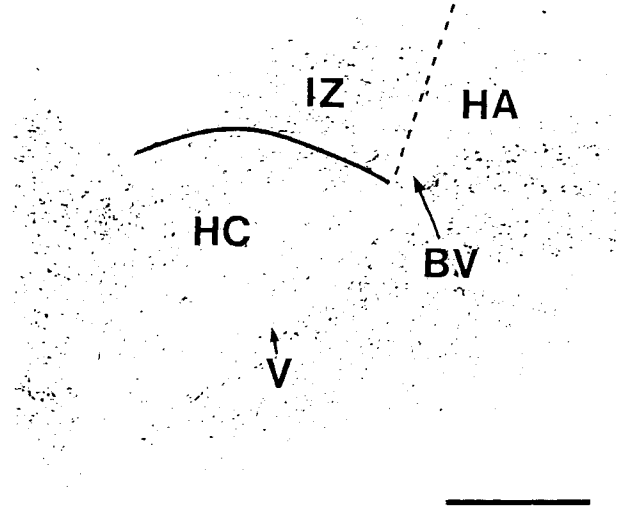

FIG. 1. Photomicrograph of a transverse section of the rostral hippocampal complex (HC) and adjacent areas of the telencephalon of a black-capped chickadee. The HC lies on the dorsomedial surface of the telencephalon above the lateral ventricle (V) and is a paired structure. Only the right side is shown, but part of the left side can be also seen. The lateral boundary in rostral HC is not obvious and seems to coincide with a region of gradually changing cell sizes and packing densities. The gradual nature of this change results in an IZ of ambiguous affiliation, confined between the dashed and solid lines. BV, blood vessel; F, fold. (Bar = 500 μm.)

Cell Packing Densities in Juveniles and Adults. The mean packing densities of neurons in the rostral, middle, and caudal regions of HC in juveniles and adults, respectively, were as follows: rostral HC (exclusive of IZ), 40,984 ± 4781 neurons per mm^3 and 31,292 ± 4075 neurons per mm^3; middle HC, 43,137 ± 3759 and 32,134 ± 4805; and caudal HC, 43,046 ± 8195 and 33,176 ± 4967. Differences between these three regions were not significant within each age group. However, the difference in packing density between same HC regions of juveniles and adults was significant (Mann–Whitney U test, in all cases $P < 0.05$). Since the estimated HC volume of juveniles was as large, or larger than that of adults, we infer, from the mean of the packing densities for the three regions (juveniles = 42,389 neurons per mm^3; adults = 32,200 neurons per mm^3) that the overall number of HC cells was at least 31% higher in juveniles than in adults.

Nuclear Diameters and Labeling. Nuclear diameters of unlabeled HC neurons were comparable in juveniles and adults (10.8 ± 0.6 μm vs. 10.5 ± 0.5 μm, respectively). Nuclear diameters of the ^{3}H-labeled HC neurons (Fig. 2) were significantly smaller in the juveniles than in the adults, but the net difference was modest (11.2 ± 0.6 μm vs. 12.1 ± 0.5 μm, respectively; $U = 3.5$ and $P = 0.04$). The mean number of exposed silver grains per labeled neuron was similar in both age groups (juveniles = 23 ± 2 vs. adults = 22 ± 2), suggesting that the S phase of the neuronal precursor cells also was similar. We infer that it is appropriate to compare directly the labeling indices in the two age groups.

Spatial Distribution and Labeling Index. The spatial distribution of ^{3}H-labeled HC neurons differed somewhat between both age groups. The mean distance from ^{3}H-labeled neurons to the nearest ventricular wall was 198 ± 22 μm in juveniles vs. 153 ± 21 μm in adults ($U = 1$ and $P = 0.01$). Despite this shift in mean distance to the ventricular wall, the vast majority of labeled neurons in both age groups (90% in juveniles and 95% in adults) was within a 350-μm band of tissue adjacent to the ventricular wall (see also ref. 6). The percentage of HC neurons that was labeled within this 350-μm band (all three HC regions combined) was 50% higher in juveniles (1.2 ± 0.4%) than in adults (0.8 ± 0.1%) ($U = 4$ and $P = 0.045$).

DISCUSSION

Understanding the role of the hippocampus in learning has been one of the great adventures of modern neuroscience (for reviews, see refs. 17–20). However, emerging theories, built around mammalian material, do not yet reflect the fact that hippocampal neurons continue to be added during development and in adulthood (21–26) and that many hippocampal neurons are constantly dying in adulthood (27). This process of birth and death of hippocampal neurons also occurs in adult chickadees (6). We will now evaluate the results of our comparison of adult and juvenile hippocampal neurogenesis and try to decipher what they mean.

What We Mean by "Recruitment." Many new HC neurons were being formed at 3 months of age, when our juvenile chickadees received the single injection of [^{3}H]thymidine. It seems unlikely that the act of netting the birds and giving them this intramuscular injection induced, by itself, an episode of

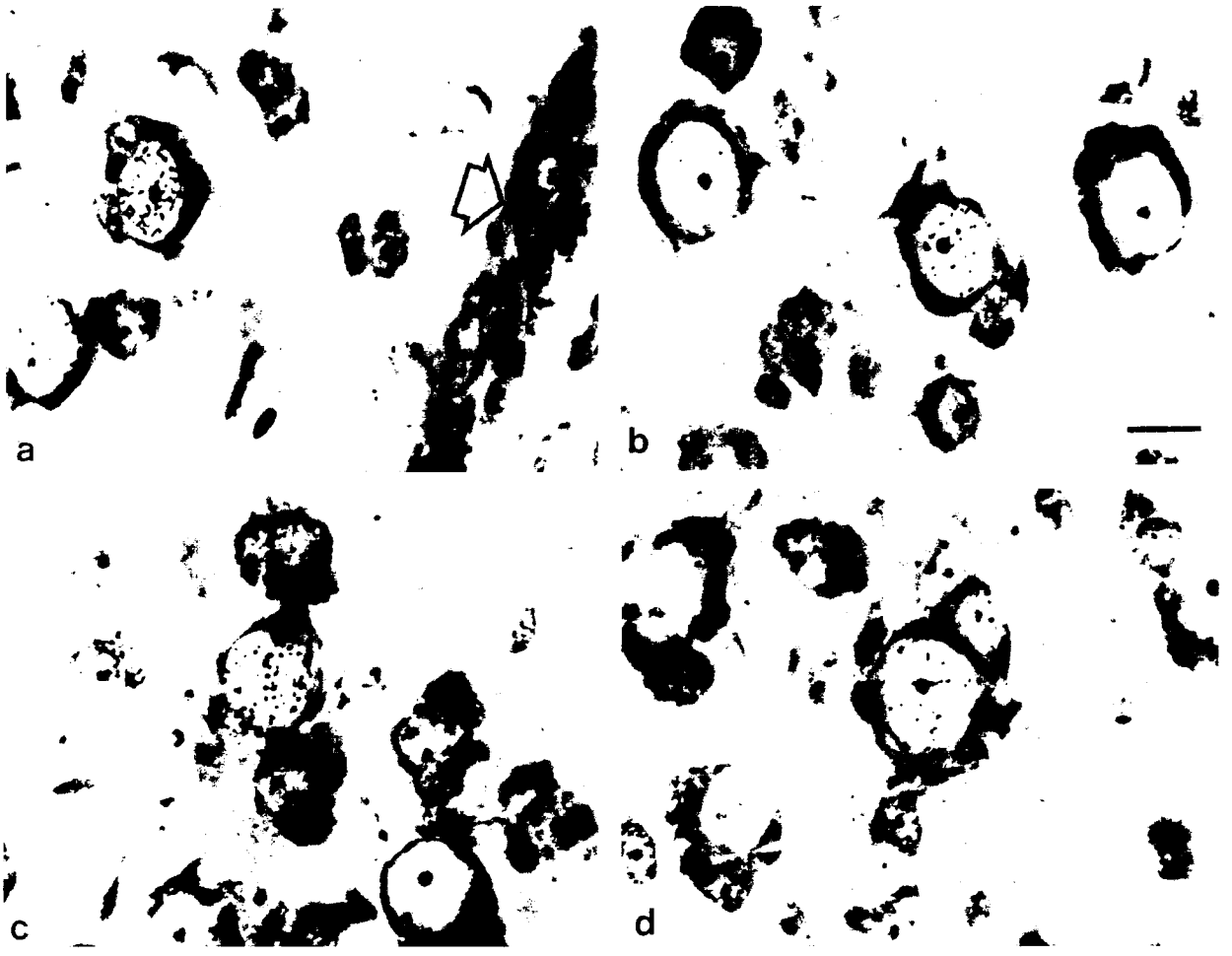

FIG. 2. Photomicrograph of labeled HC neurons in juvenile (*a* and *b*) and adult (*c* and *d*) black-capped chickadees. Small black dots over the clear neuronal nuclei correspond to ^{3}H labeling. Large single (*a*, *b*, and *d*) or double (*c*) dark dots correspond to nucleoli. The labeled neurons are members of a class of relatively large HC neurons. Open arrow in *a* points to ventricular zone cells that form the ventral boundary of HC. The dorsal and ventral walls of the lateral ventricle are tightly apposed, so that one sees no patent ventricular lumen. (Bar = 10 μm.)

neurogenesis. More likely, new neurons were added every day during the time of year when the birds were treated with the cell birth marker. However, we do not know how many neurons were born at the time of injection and if this number differed with the age of the birds. Birth rates could have been similar in juveniles and adults, but if so, then fewer of the new neurons lived for as long as 6 weeks in the adults. Thus, "recruitment" refers to the number of neurons that had been added and were still present at 6 weeks. Intriguingly, the daily rate of neuronal recruitment may have been significantly higher than the 1.2% we saw in juveniles and the 0.8% we saw in adults because the S phase of the dividing cells was, in all likelihood, shorter than 24 h.

The Evidence for Replacement. Neuronal replacement is a numerical concept that applies to populations of cells—new cells replace older ones. It does not mean, necessarily, that a new cell slips into the position vacated by an older one. We know that the number of HC neurons remains constant in adult chickadees. Therefore, in them the recruitment of new neurons must be accompanied by replacement. However, we do not know if the new neurons added in juveniles replaced older ones or were still part of a process of net growth. It is clear, though, that by 4.5 months there had been an overproduction of hippocampal neurons.

Why Overproduce Hippocampal Neurons? Overproduction of neurons during early development has been described in various parts of the central nervous system (28–31). This overproduction may occur to ensure that enough cells with the "right" connections are in place in the fully formed brain and spinal cord, before the young animal starts to lead an independent life (31). However, our juvenile chickadees had reached adult body size and at the time they were killed had been leading a life that differed little, if at all, from that of adults.

Work on the marsh tit (*Parus palustris*), a close relative of the black-capped chickadee, showed that the number of HC neurons is comparable in 2-week-old nestlings and in adults (32). We show that in black-capped chickadees, there are more HC neurons in 4.5-month-old juveniles than in adults. Thus, data from these two species suggest that an excess of HC neurons is generated after the bird leaves the nest when, in all likelihood, it is already using its hippocampal functions.

It has been proposed that the role of some neurons—e.g., those of the mammalian subplate—is restricted to a well-defined developmental stage (33); once that stage is over, those neurons disappear (31). However, the late overproduction seen in HC brings another possibility to mind. Juveniles learning about a novel environment for the first time may require more neurons than adults, for whom this environment is familiar. The eventual reduction in the number of HC neurons present at 4.5 months need not compromise adult hippocampal efficiency. In addition, memories acquired by juveniles may persist if these memories are transferred to another part of the brain, as suggested for primates (34).

Why Add New Neurons? Neuronal recruitment was higher in our juveniles than in our adults. Earlier work showed that

neuronal recruitment in adult chickadees is higher in late summer–early fall than during the rest of the year and that the rate of recruitment is higher in free-ranging than in captive chickadees. The question, then, is what do juveniles, a free-ranging life style and late summer–early fall have in common that promotes a heightened recruitment of new neurons in chickadee hippocampus? We suggest that all three variables listed above increase the opportunities and need for learning (6). Perhaps birds that have committed many of their HC neurons to storing long-term memories and have not yet transferred these memories to elsewhere in the brain, have "run out of space." They must add new HC neurons before they can add new memories (35).

The suggestion that the recruitment of new neurons favors learning is based on three assumptions that may apply to some but not necessarily to all neural systems. Assumption 1 is that the whole neuron, not the synapse, is the unit of learning. Assumption 2 is that acquisition of a long-term memory is accompanied by a permanent and irreversible change in gene expression, so that learning can be likened to a last step in cell differentiation. Assumption 3 is that a neuron that has been altered in this manner cannot do it again. If these assumptions hold true, then it is easy to see how an animal that acquires more and more long-term memories is left with fewer and fewer neurons that can be committed to new learning. This view of learning is, of course, very different from that postulated by Eccles (36), who thought that the modifiability of synapses and the enormous array of possible synaptic relations between neurons guaranteed virtually unlimited learning. However, our view is compatible with that of Goelet *et al.* (37), who showed that the acquisition of long-term memories involves growth of dendrites, formation of new synapses, and changes in genomic expression.

It is possible that the irreversible cellular differentiation postulated for the long-term acquisition of hippocampal memories falls in a class by itself. Neurons, such as those of the larval tobacco moth (*Manduca sexta*), that undergo a profound respecification during metamorphosis are at the other end of a continuum of reversibility of neuronal commitments (38).

Why Replace Neurons? Widespread neuronal replacement may have evolved to its highest expression in birds and may be related to the need to maximize the learning potential of the brain while keeping its weight down (39). Birds live longer than mammals of comparable weight. Black-capped chickadees can live up to 12 years in the wild (1), six times longer than a mouse of comparable weight. A hippocampus that accumulated memories over several years might have to be much larger than it is and it might have to grow with age. Instead, the HC of adult chickadees is no bigger than that of juveniles and in fact includes fewer neurons. In contrast, the granular cell layer of the rodent hippocampus becomes thicker with age and includes more neurons (23, 24). Thus, though both birds and mammals constantly add new hippocampal neurons, birds may rely on neuronal replacement more heavily than mammals.

What Variables Regulate Neuronal Replacement? *Does stress play a role?* Corticosterone is necessary for the survival of the granule cells in the rat hippocampus (27, 40). High corticosterone levels—a response to stress—dampen the rate of production of these same cells (41–43). We do not know if corticosterone regulates hippocampal cell death and neurogenesis in birds but data on hand do not show a simple relation between the basal corticosterone levels of adult black-capped chickadees sampled at various times of year (H. Schwabl, A.B., and F.N., unpublished observations) and neuronal recruitment levels during those times (6). Moreover, basal corticosterone levels are very similar in adult free-ranging and aviary-held black-capped chickadees (H. Schwabl, A.B., and F.N., unpublished observations), yet the neuronal recruitment rates are twice as high in the former as in the latter (6). From this, corticosterone levels do not emerge as a likely common denominator that accounts for the observed differences in neuronal recruitment rates. Unfortunately, we do not know whether corticosterone levels differ between juveniles and adults.

The role of experience. Experience may regulate the production and survival of HC neurons. Healy *et al.* (32) report that the HC of young marsh tits deprived of the opportunity to cache and retrieve cached food (a form of spatial learning) has fewer neurons and fewer apoptotic cells than that of experienced birds of the same age. In the same vein, we have found that the recruitment of new HC neurons is lower in captive than in free-ranging adult chickadees (6). The aviary environment of the captive birds is, presumably, a good deal simpler than that of the wild birds and probably offers many fewer learning opportunities. If environmental novelty and the acquisition of memories regulates the recruitment of neurons and their survival, then perhaps a juvenile first settling in the area where it will live has much more to learn about it than an adult. The heightened recruitment of hippocampal neurons in juveniles may reflect this more intense learning. Elsewhere we have argued that the late summer–early fall peak in the recruitment of new HC neurons in adult black-capped chickadees can also be related to expanded learning opportunities. The argument for a role of experience in neuronal recruitment stands whether the effects of experience are direct or mediated via hormonal changes.

Do HC Neurons Learn? Avian hippocampal neurons may acquire and store memories, even if the storage function is not permanent, as has been suggested for primates (e.g., ref. 34). Support for the idea of temporary storage comes from clinical observations that show that hippocampal lesions are usually accompanied by the loss of memories acquired during a limited time before the lesion (20, 44). The naturally occurring replacement of hippocampal neurons provides a rationale for why memories should be transferred out of the hippocampus. Young and adult birds and mammals may program their loss of hippocampal neurons so that it occurs after the memories held by these hippocampal neurons have been transferred to other parts of the brain.

A Special Population of HC Neurons. Neuronal recruitment was particularly marked in the 350-μm-wide band of HC tissue adjacent to the lateral ventricle (6). If, as we suggest, the recruitment of new neurons is related to learning, then the neurons in this band of tissue must be privy to novel information and must have molecular programs for long-term learning. These neurons may be functionally homologous to the granule cells of the mammalian dentate gyrus. These latter cells are the only ones that continue to be produced and apparently replaced in adult mammalian hippocampus (21–23, 27, 45).

Overview. We suggest that the excess of hippocampal neurons produced in juvenile chickadees is related to the need to learn much novel environmental information. If, as in mammals, the information learned is eventually transferred from HC to another, more permanent brain storage site, then the number of HC neurons found in juveniles can be cut back to that found in adults without loosing the memories acquired. Similarly, the replacement of HC neurons may reintroduce learning potential without compromising memories transferred elsewhere. Anatomically, our observations draw attention to a paraventricular layer of the avian HC—homologous to the granule cell layer of mammalian hippocampus?—that receives the great majority of the HC neurons produced by juveniles and adults.

The hippocampus has provided major insights on the neurobiology of learning, including the importance of long-term potentiation (46) and the role of *N*-methyl-D-aspartate receptors (47). We now suggest that the neuron, not the synapse, is the unit of learning and that this hypothesis offers a logic for the replacement of otherwise healthy hippocampal neurons. Theories of hippocampal learning that overlook the fact that

the hippocampus constantly adds and looses neurons are seriously incomplete.

We are particularly grateful to various colleagues that helped with several aspects of this study: Arturo Alvarez-Buylla for the computer-assisted collection and analysis of data, Enrique Mira-Domenech and Hubert Schwabl for assistance with field work, and Philippe Rousselot for helping with photography. In addition Drs. David Vicario, Philippe Rousselot, and Marta Nottebohm gave us useful comments on the text. This work was supported by Public Health Service Grant 18343 and by generous help from Mr. Herbert Singer, Mr. Howard Phipps, and the Mary Flagler Cary Charitable Trust.

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