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# Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees

(learning/neurogenesis/neuronal replacement)

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ABSTRACT **Neurogenesis and neuronal replacement in a population of wild free-ranging animals are described. Our subjects were adult black-capped chickadees (*Parus atricapillus*). Neuronal birth dates were determined by a single systemic injection of [$^3$H]thymidine, followed by release of the bird and its recapture 6 or more weeks later. Newly formed neurons appeared in the hippocampal complex during all times of year, but with a marked peak in the fall (October). New neurons were also added to the hippocampal complex of captive chickadees, but at levels only half as high as seen in the wild birds. Neurons born at different times of the year lived for a few months and then disappeared. We suggest that the neurons added are part of a process of neuronal replacement and that they are important for the acquisition of new spatial memories, a need that is particularly acute in the fall. Hormonal and experiential factors may determine the rate of neuronal replacement.**

Neurons are constantly added to the brain of adult birds (1–6) and other animals (7–13). In some cases this underlies a process of net growth (9–11), but in other cases addition makes up for cell loss, with no net gain in neuron numbers (14). We do not know what might be the significance of this latter form of neuronal replacement, or even if it occurs in wild free-ranging individuals. It has been suggested that neurons are permanently changed by the long-term memories they hold and that in small long-lived brains such "learned" cells must eventually be replaced to make room for new memories (15). The need for neuronal replacement may be particularly acute in birds because they are long lived and their airborne life-style places constraints on the size of their brains (15). If so, neuronal replacement in parts of the adult avian brain involved with the storage of information should peak just prior to times when much new information will be acquired.

We tested this prediction in the hippocampal complex (HC) of an adult free-ranging songbird that stores seeds in a seasonal manner. We focused on the HC because other authors have shown that this structure is relatively larger in storers than in nonstorers and have suggested that it plays an important role in spatial learning (16, 17). Black-capped chickadees (*Parus atricapillus*) are small (10–12 g) and very common in the forests of temperate North America. In late summer and early fall their diet shifts from insects to seeds (18). At that time, too, territorial boundaries cease to be defended and chickadees form flocks that persist throughout the fall and winter. Each flock has a home range of about 30 acres, approximately 3 times the size of a breeding territory (19, 20). This is a time of year, too, when the landscape changes drastically in its appearance, as trees change their color and then lose their leaves, followed later by the arrival of snow. As these social, residential, and dietary changes occur, and as the looks of the world are altered, chickadees start to hide a percentage of the seeds they find, one or very few items per storing site (21). In the wild, chickadees retrieve these seeds after a period of hours or days (21) but in captivity memory of cache sites lasts for several weeks (22). Recovery of stored seeds has also been reported in free-ranging willow tits (*Parus montanus*) after periods of up to 6 weeks, with a sharp decline thereafter (23). Evidence from this close relative suggests that such delayed recovery may occur also in wild black-capped chickadees. Retrieval of stored food requires that each bird have an updated map of its home range and that it remember where it stored its caches (24–27). Seed storing decreases as winter wears on (18).

If our hypothesis were correct, then a fall peak in spatial memory load would be preceded by a peak in the replacement of hippocampal neurons.

## MATERIALS AND METHODS

**Study Site.** The study was conducted at The Rockefeller University Field Research Center for Ecology and Ethology in Millbrook, New York. Millbrook is 80 miles to the north of New York City, in rural Hudson Valley. The Field Research Center comprises 1200 acres of natural habitats, including deciduous woodlands, meadows, and marshes.

**[$^3$H]Thymidine Administration.** Free-ranging chickadees were attracted to feeding stations, captured with mist nets, color banded, and released, starting during the winter of 1991 and continuing during the next 2 years. Treatment with [$^3$H]thymidine started during the following winter. All birds injected were adults and at least 1 year old; two had already been banded by another field worker 3 years earlier [wild black-capped chickadees become sexually mature at 1 year and can reach ages of up to 9–12 years (18); our assessment of adult age was based on prior color banding]. [$^3$H]Thymidine is incorporated into newly formed DNA during the S phase, which precedes cell division, and in this way acts as a cell birth marker (28). Nuclear labeling with [$^3$H]thymidine administered to adult birds occurs during the 60 min after injection; no significant labeling of dividing cells occurs thereafter (29). Each bird received a single dose of 50 $\mu$Ci of [$^3$H]thymidine (6.7 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) injected into the pectoral muscle. Radiation Control Permit no. 145-5, issued by The New York State Department of Environmental Conservation, allowed us to conduct this work. Different birds received this treatment at different times of year during the 2-year period of the study until eventually some birds were injected during most months of the year. Altogether, 74 adults of both sexes received [$^3$H]thymidine and were released immediately thereafter. Twenty-seven of these birds were recaptured 6 weeks later. The 6-week period of survival probably allowed enough time

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Abbreviations: HA, hyperstriatum accessorium; HC, hippocampal complex.

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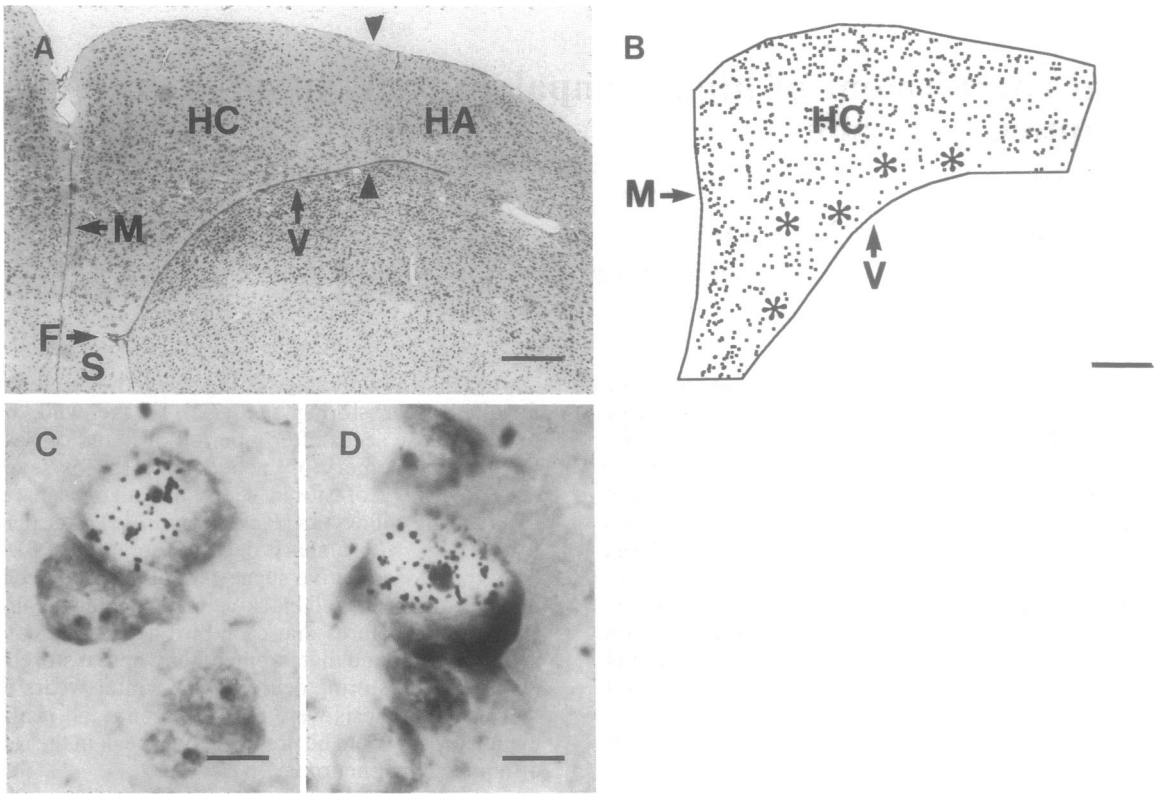


FIG. 1. (*A*) Photomicrograph of a transverse section of the right dorsomedial forebrain, showing the boundaries used to define the HC. The dorsal, ventral, and medial boundaries of the HC, as seen in coronal sections, are the surface of the brain, the lateral ventricle (V) and the midline (M), respectively. Other abbreviations: F, fold; S, septum. The lateral boundary of the HC (arrowheads), where it meets the HA, is defined by an increase in cell density and a change in cell type from larger neurons to a mixture of both large and small neurons. (*B*) Schematic diagram of a transverse section through the central region of the HC, showing the location of unlabeled (small dots) and $^3$H-labeled (asterisks) neurons. (*C* and *D*) Two [$^3$H]thymidine-labeled hippocampal neurons. (Calibration mark for *A* and *B*, 250 μm; for *C* and *D*, 6 μm.)

for the neurons born at injection time to migrate to their final destination and go through final anatomical differentiation (1). Another 15 birds were recaptured after a longer interval. The remaining birds were not recaptured.

In addition to the wild birds, we had two captive groups of wild-caught adult chickadees. The captive birds were held in a large outside aviary, where they could store seeds, for at least 2 months before they received the single injection of [$^3$H]thymidine. One group ($n$ = 3) received [$^3$H]thymidine in May, the other one ($n$ = 4), in October of the same year.

**Histology and Mapping of the HC.** Birds were killed with an overdose of anesthetic [ketamine (Ketalar, Parke–Davis) and xylazine (Rompun, Haver)] and fixed by intracardiac perfusion with 20 ml of saline followed by 50 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB). The sex of each bird was established by looking at the gonads. Brains were removed, immersed in the same fixative for a month, embedded in polyethylene glycol (PEG), blocked transversely, and sectioned at 6-μm intervals. Serial sections (every 10th; intervals of 60 μm) were collected in PB and mounted on chromalum-coated glass slides. The sections were then delipidized in xylene/ethanol and coated with nuclear track emulsion NTB2 (Kodak). After 4 weeks of incubation at 4°C the emulsion was developed, and the sections were stained with 0.5% cresyl violet and coverslipped with Accu-Mount mounting medium (Baxter Scientific Products, McGraw Park, IL).

We used a microcomputer system (30) to draw the boundaries of the HC, following the criteria used by others (16, 17).† The rostral and caudal reaches of the HC were defined by the presence of a recognizable lateral boundary. This boundary was provided by the smaller and more densely packed cells of the hyperstriatum accessorium (HA) (ref. 16; and see Fig. 1*A*). Our definition of the HC by reference to the distinctness of its lateral boundary may underestimate the true size of this structure, a matter that can be resolved only by detailed 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 (31). We recognized three rostrocaudal subdivisions within the HC: a middle region showing a well-defined ventromedial "fold" (see Fig. 1*A*) and regions anterior and caudal to this middle region; all three regions had comparable rostrocaudal extents.

We used the volume of the HC and the packing density of its neurons to calculate the total number of HC neurons. We calculated the HC volume by multiplying the area of the HC seen in each section sampled by the distance between that section and the next one sampled (180 μm). We recorded the position of all $^3$H-labeled and unlabeled HC neurons (e.g., Fig. 1*B*) in sections spaced at 360-μm intervals (8–11 sections per bird). This procedure also yielded counts of cells in these two classes. A cell was defined as a neuron if it had clear nucleoplasm and one or two darkly staining nucleoli. A neuron was considered to be $^3$H-labeled if it had more than 20 times the background level of exposed silver grains over its nucleus (usually a minimum of 7 grains; see Fig. 1 *C* and *D*).

Nuclear diameters were measured by using the displacement of a computer cursor viewed through a camera lucida. We measured the nuclear diameter of all $^3$H-labeled HC neurons in all brains, and that of 100 or more unlabeled HC neurons in 10 brains (5 randomly chosen from October and 5

†Our study made no attempt to discriminate between the hippocampus and the more caudal parahippocampal areas, and so, following others (16, 17), we refer to these two as the HC.

from February–March). In each of these 10 brains we sampled HC sections at 720-μm intervals, from rostral to caudal, yielding an average of five to six sections. In each section, the distance between the midline and the lateral HC boundary was divided so that three evenly spaced points along that distance were determined. At each such point we drew a line that connected the ventricle and the dorsal HC boundary. Each neuron that touched the line was measured. Nuclear diameter information was used to correct our counts so that larger cells were not over-represented (32). Counts corrected in this manner were used to estimate total cell numbers.

To ascertain whether neuronal recruitment into the HC was higher than in adjacent tissue, we scanned for labeled neurons in the HA lateral to the HC–HA boundary, up to a distance of 3 mm from the midline, on the same side on which we had done the HC sampling. The amounts of HA and HC tissue sampled were comparable. In addition, we looked for labeled neurons in two sections through the center of the left and right ectostriatum of the October and February–March samples. The ectostriatum was chosen because it receives inputs from the thalamus and is the primary visual relay of the telencephalon (33), and therefore it would be involved in the acquisition of spatial memories.

## RESULTS

**Free-Ranging Birds.** *Six-week survival.* There were no right–left and male–female differences in the number of labeled HC neurons found during the various times of year, and therefore all the data we present here come from right-side analyses and pooled male and female material. In addition, there were no seasonal or rostrocaudal trends in the mean nuclear diameter of $^3$H-labeled and unlabeled HC neurons. However, the mean nuclear diameter of all $^3$H-labeled neurons was slightly larger (11.5 μm; Fig. 1 *C* and *D*) than that of unlabeled ones (10.9 μm; paired *t* test, $t_{(9)} = 2.05$; $P = 0.07$). There was no seasonal trend in the mean number of silver grains (21.8 ± 6.7) exposed over the nucleus of labeled HC neurons.

The distribution of labeled HC cells was not random. Instead, 95% of these cells was found in a band of tissue extending from the ventricular wall to a distance of 350 μm (Figs. 1*B* and 2). In addition, the percentage of HC neurons that was labeled was significantly higher in rostral than in middle HC (paired *t* test, $t_{(22)} = 2.64$; $P = 0.015$), and both of these regions showed significantly more labeled neurons than did caudal HC ($t_{(22)} = 3.49$; $P = 0.002$; and $t_{(22)} = 5.38$; $P < 0.0001$, respectively).

There was a marked seasonality in the occurrence of labeled HC neurons (Fig. 3). Birds that received [$^3$H]thymidine in October had a higher percentage (mean = 0.55%) of labeled HC neurons than birds that received this marker during the rest of the year ($U = 7.5$; $P < 0.0001$). We had enough individuals to run a statistical comparison with the October birds at only two other times of year. The percentage of labeled neurons was significantly higher in October than in August (0.37%; $U = 6$; $P = 0.02$) and than in February–March (0.15%; $U = 0$; $P = 0.002$). We also tested the significance of the seasonal effect by fitting a polynomial curve to the monthly distribution of individual values arranged so that the October values were at the end of the curve. This curve (Fig. 3) had an *r* of 0.821, suggesting that the month during which a sample was obtained accounted for 67% of the variability in neuronal labeling. By comparison, the mean percentages of $^3$H-labeled neurons in the HA in October and February–March were, respectively, 0.09 ± 0.07 and 0.05 ± 0.02; these values did not differ significantly between these two times of the year ($U = 15$; $P = 0.317$). There were no labeled neurons in the ectostriatum.

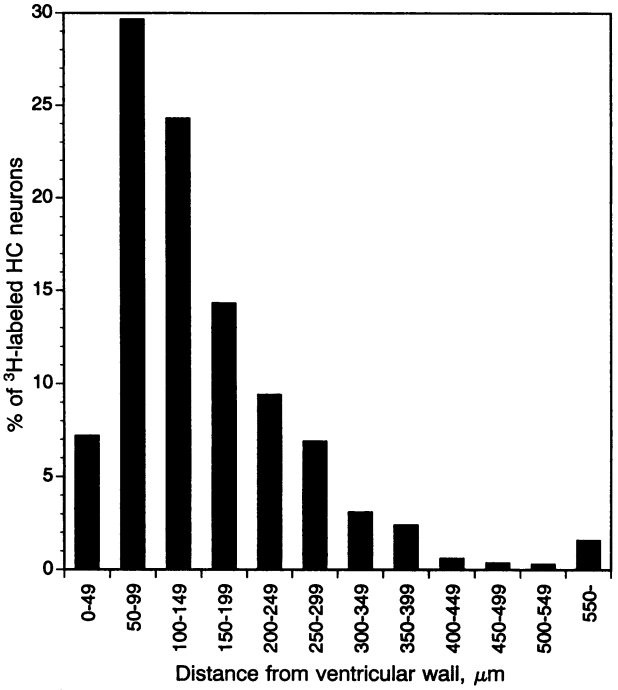


FIG. 2. Population distribution, normalized for each individual and averaged for the group of 27 birds, of distance between [$^3$H]thymidine-labeled HC neurons and the nearest ventricular wall; $n = 655$ neurons; mean distance from ventricular wall ± SE = 165 ± 32 μm.

There were no significant differences between the total number of HC neurons in the October birds (mean ± SE: 143,823 ± 25,418) and February–March birds (170,684 ± 46,418; $U = 13$; $P = 0.21$).

*Longer survivals.* The anatomical distribution of HC $^3$H-labeled neurons was very similar at 6 weeks and at longer survival times. The mean (±SE) distance from the nearest ventricular wall was 165 ± 32 μm at 6 weeks ($n = 27$ birds) and 161 ± 57 μm ($n = 13$ birds) for longer survival times. However, approximately half of the new neurons present 6

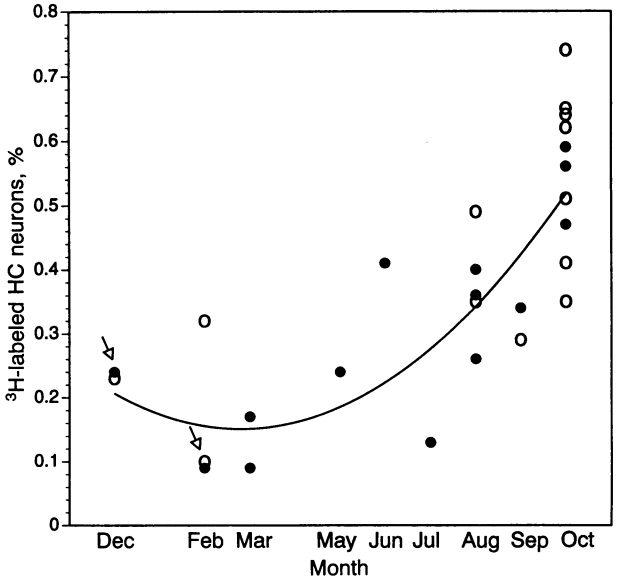


FIG. 3. Percentage of $^3$H-labeled HC neurons present in adult birds recaptured 6 weeks after [$^3$H]thymidine injection. ○, Females; ●, males. The two arrows point at individuals that were of unknown age when color banded 3 years before injection. The line is a polynomial regression with $r^2 = 0.67$.

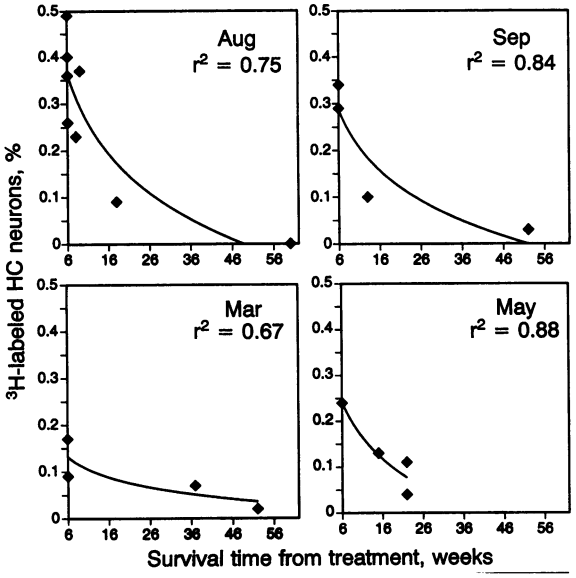


FIG. 4. Survival curves of $^3$H-labeled HC neurons from birds that were treated with [$^3$H]thymidine in August, September, March, or May and that survived the indicated numbers of weeks before they were killed. Only curves with a minimum of three survival times are shown.

weeks after labeling had disappeared by 16 weeks, and very few were still present 1 year after [$^3$H]thymidine injection (Fig. 4). The rate of disappearance was faster in rostral HC than in middle or caudal HC (Fig. 5).

**Captive Birds.** The mean percentage of labeled HC neurons in the captive birds was twice as high in October (0.25%) as in May (0.13%) ($U = 0.5$; $P = 0.0518$). In both captive groups the percentage of labeled HC neurons was half as high as in free birds injected at the same time. We tested for the significance of this difference only in the October samples ($U = 0$; $P = 0.005$), and for these samples the difference in the percentage of labeled neurons was particularly acute in rostral HC (Fig. 6).

## DISCUSSION

The results presented here indicate that relatively large neurons in a ventral layer of the HC are constantly born in the adult chickadee brain, and that a peak in the recruitment of these cells occurs in the fall. Since both mean nuclear diameters of $^3$H-labeled and unlabeled HC cells and the intensity of $^3$H-labeling over the individual neuronal nuclei showed no seasonal trends, we can assume that the percentages of labeled HC neurons that we observed at different times were not influenced by changes in either neuronal size or uptake of [$^3$H]thymidine by neuronal precursors. The

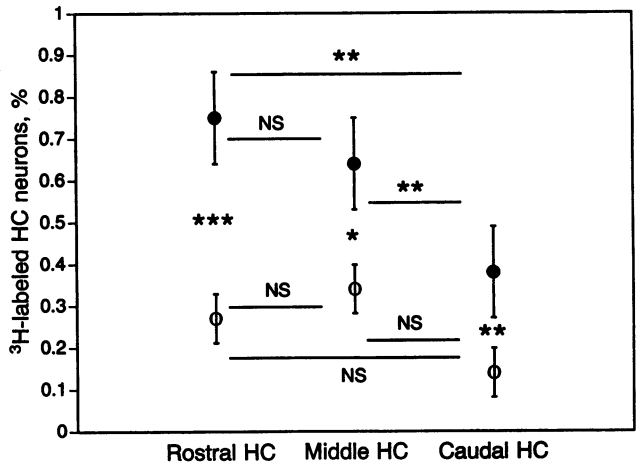


FIG. 6. Mean (±SE) percentage of $^3$H-labeled HC neurons in the three rostrocaudal subdivisions of the HC of black-capped chickadees injected with [$^3$H]thymidine in October. ●, Free-ranging birds ($n = 10$). ○, Captive birds ($n = 4$). Differences between groups were not significant (NS) or reached significance levels of $P < 0.01$ (*), $P < 0.001$ (**), or $P < 0.0001$ (***).

percentages of labeled HC neurons that appear in the text and in Figs. 3–6 represent the fraction of all the HC neurons that was labeled. However, since 95% of the labeled neurons were in a 350-$\mu$m-wide band adjacent to the ventricle, within that band the fraction of labeled cells was approximately 3 times higher.

There were relatively few labeled neurons in the HA and their numbers were comparable in early fall and late winter. In addition, there were no labeled neurons in the ectostriatum. Taken together, these observations suggest that there was anatomical specificity for the relatively high and seasonally focused recruitment of new neurons into the HC. Since the peak in neuronal recruitment occurred at a time when changes in life-style and use of space can be inferred to generate an acute need for new spatial memories, our data support the hypothesis we set out to test.

The mean distance from labeled HC neurons to the nearest ventricular wall was comparable at 6 weeks and at longer survivals. We infer that the new cells had already reached their final destination at 6 weeks. Our data also show that the new neurons lived for a few months and then disappeared. Presumably they were replaced by other new neurons, as the total numbers of HC neurons remained comparable during different times of the year.

Hippocampal damage has been linked to memory impairment in humans, monkeys, and rodents. This impairment affects recent memories, but not older ones (34). It will be fascinating to find out if the neurons that are added to and deleted from the hippocampus of adult chickadees are in-

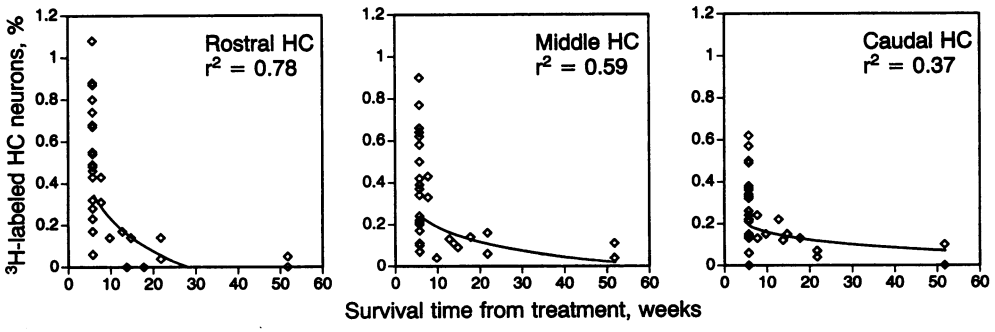


FIG. 5. Survival curves of $^3$H-labeled neurons in the rostral, middle, and caudal HC of birds that were treated with [$^3$H]thymidine at different times of the year and survived the indicated numbers of weeks before they were killed.

volved in learning, and if so whether they are the final repositories of memories or if, as described in mammals, they are just temporary guardians of such memories.

Neuronal addition and neuronal turnover were more marked in the rostral HC than in the rest of the HC. This suggests that memory acquisition and turnover may occur at different rates and perhaps with different degrees of detail in different parts of the HC. In addition, neuronal recruitment occurred at a higher rate in the free-ranging than in the captive birds. Perhaps memory acquisition and the ratio of "learned" to "unlearned" cells regulate, to some extent, the recruitment of new cells and the demise of older ones. If so, this effect seems to be particularly marked in the rostral HC. The rostral HC emerges as a particularly propitious part of the brain in which to study how experience affects neuronal replacement and how this relates to memory formation. New insights on hippocampal and neuronal function may come from studies of long-lived animals in which hippocampal plasticity is pushed to the limit and where it can be related to the everyday needs of a natural existence.

We suggest that the research reported here is paradigmatic. It offers a frame, a method, and a rationale for comparing the replacement of hippocampal neurons in storers and nonstorers, migrants and residents, and birds living in evergreen and deciduous forests, so as to uncover the pressures, rules, and mechanisms that govern the constant rejuvenation of this part of the adult brain.

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