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Behavioural Brain Research 133 (2002) 31–43

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Research report

Social change affects the survival of new neurons in the forebrain of adult songbirds

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Received 25 June 2001; received in revised form 22 October 2001; accepted 22 October 2001

Abstract

Many new neurons are added to the adult avian brain. Most of them die 3–5 weeks after they are born (Nature (Lond.) 335 (1988) 353; J. Comp. Neurol 411 (1999) 487). Those that survive replace, numerically, older ones that have died (Neuron 25 (2000) 481). It has been suggested that the new neurons enhance the brain's ability to acquire new long-term memories (review in Sci. Am. 260 (1989) 74). If so, perhaps an increase in social complexity affects the survival of new neurons in a social species. To test this hypothesis, we treated adult zebra finches (*Taeniopygia guttata*) with [^{3}H]-thymidine immediately before introducing them into one of three different social environments that differed in complexity and killed them 40 days later. There was a significant difference between experimental groups in the number of [^{3}H]-labeled neurons in neostriatum caudale (NC), high vocal center (HVC) and Area X, three forebrain regions that are involved in vocal communication. In these regions, birds placed in a large heterosexual group had more new neurons than birds kept singly or as male–female pairs. Regulation of new neuron survival by extent of circuit use may be a general mechanism for ensuring that neuronal replacement is closely attuned to environmental change.

Keywords: Adult neurogenesis; New neuron survival; Neostriatum caudale (NC); High vocal center (HVC); Area X; Social change; Zebra finch (*Taeniopygia guttata*)

1. Introduction

It has been hypothesized that permanent changes in gene expression encode long-term memories, such that acquisition of these memories is like a final and irreversible step in cell differentiation. These changes, the hypothesis holds, would stand in the way of new learning [25]. If this hypothesis were correct, then perhaps the entire neuron, not the synapse, is the unit of learning [24]. If so, the number of neurons available for storing new long-term memories would be inversely related to the number of prior memories acquired. For animals such as songbirds, with small brains and long life spans, memory space could run out well before normal life expectancy. A solution to this dilemma would be to replace old, learned neurons, and the memories they held, by new ignorant neurons. This replacement should occur in those parts of the adult brain involved with the storage of specific information and it should peak at times of year when learned behaviors change or when information loads peak. This prediction has been met by observations of end of summer/early fall waves of neuronal recruitment in the high vocal center (HVC) of canaries, *Serinus canaria*

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0166-4328/02/$ - see front matter
PII: S0166-4328(01)00416-8

[19] and in the hippocampal complex (HC) of black-capped chickadees, *Parus atricapillus* [6,7], when the former learn new song and the latter engage in food catching.

However, temporal coincidence need not mean causality. For example, there is a seasonal recruitment of new neurons in the HVC of adult song sparrows, *Melospiza melodia*, though these birds do not change their song repertoire from 1 year to the next [33]. We wanted to look at new neuron recruitment under conditions of controlled environmental change.

We used zebra finches (*Taeniopygia guttata*), which, in the wild, are highly sociable birds that nest colonially and forage in flocks of hundreds [38]. The call and song of each male is learned and can be used to tell it apart from other individuals. Males and females form stable pair bonds and females recognize their mate by its song (review in [37]). It is possible that other sensory cues are also used for individual recognition, though this is not known. In nature, adult zebra finches often move from their native colony to other colonies and thus change the company they keep. The hypothesis we wished to test was that the complexity of the social environment into which an adult zebra finch is placed affects new neuron numbers in specific parts of the brain.

To test this hypothesis we injected adult zebra finches with a cell birth marker during the 6 days preceding social change. We knew, from earlier work, that [^{3}H]-thymidine injected systemically into an adult songbird has disappeared from its blood stream by the end of the second hour after injection [2]. Therefore, 2 h after the last injection the birds were placed in one of three social settings: (1) with 40 other adult male and female zebra finches (large group); (2) with one other adult of the opposite sex (pairs); or (3) singly. The aviaries into which birds in all three groups were placed were similar. In neostriatum caudal (NC), the number of new neurons added to the brain was larger in group (1) than in groups (2) and (3), and similar results were also obtained in high vocal center (HVC) and Area X, where group (1) was compared with group (2). These three brain areas have known functions associated with them: HVC and Area X are song system nuclei involved in vocal communication, and NC is involved with sound processing [5,15,22]. Since the new social settings were experienced after most of the new cells were born, we infer that the differences in new neuron numbers resulted from differences in survival of the new cells. Earlier studies, with other taxonomic groups, had shown that environmental complexity in general influences the number of new neurons present in the adult brain [16,17,29]. To the best of our knowledge, ours is the first report of differences in neuronal survival related specifically to differences in the complexity of the social setting into which an animal is placed.

2. Materials and methods

2.1. General

Two outdoor breeding colonies, at The Meier Segals Garden for Zoological Research at Tel-Aviv University, Israel, provided zebra finches for the study. When needed, we also used an additional outdoor breeding colony, from Kibbutz Eyal (about 30 km from Tel-Aviv). Experimental birds were kept in their breeding colony until the age of 45–60 days, when they became independent, and could also be easily sexed by their plumage. At that time, these juveniles were banded for individual identification, with a randomly chosen unique color combination of four plastic rings (A.C. Hughes, Middlesex, England), two on each leg. A previous study has shown that red, pink and black are sexually attractive for zebra finches [8], and therefore we used only these colors (red and pink rings for males; black, red and pink for females), to avoid behavioral differences which might occur as a result of different levels of sexual attractiveness. Once banded, juveniles from all three colonies were transferred into small indoor cages at the Zoological Garden (see below).

2.2. Experimental design

The birds we used in our experiments were removed from their native colony at 45–60 days of age. Each one of them was put in an indoors standard cage (65 × 35 × 45 cm), together with three other unrelated individuals, to avoid any stress that, in this very sociable species, might result from isolation. These three strangers consisted of another juvenile (same age, opposite gender), and two adults (a female and a male). Each such cage was visually isolated from the outside environment, and placed in a separate room, so that the experimental bird, which was kept in it, could not hear or see other zebra finches, except for the three individuals that were with it. Cages were exposed to natural illumination conditions (10.1–14.7 h of light per day).

Experimental birds were kept under these conditions until the age of 4–5 months, well after they normally reach sexual maturity (90 days). At that time each bird was treated with the cell birth marker [^{3}H]-thymidine (see below for details) and then placed in outside aviaries of standard size (see below) that differed in social environment. Overall, for each type of social environment we had a total of 10 experimental birds, five males and five females, to test for possible gender differences. The different types of social environments were as follows:

2.2.1. Isolates

Each one of the 10 experimental birds was placed singly in an aviary from where it could not see or hear other zebra finches. Isolation is probably not likely to occur in nature in this social species. Nevertheless, we included this oversimplified social environment as it enabled us to compare this situation, where no novel companies were provided at all, with the two following ones, where in both novel company was provided, but differed in how much.

2.2.2. Pairs

The 10 experimental birds were arranged into five pairs (a male and a female), one pair per aviary. Until paired, these birds had been strangers to each other. Each pair could not hear or see other zebra finches.

2.2.3. Large group

Each of the 10 experimental birds was introduced into an aviary which housed a pre-existing group of 40–45 adult male and female zebra finches, all of which were strangers to the experimental birds. There were three such large groups and experimental birds were added to them as they became available. No birds were removed from these large groups before the experimental birds themselves were taken out.

Birds in each of the three social settings were kept in large outdoor aviaries (1.5 × 1.5 × 2 m), which were visually blocked from the outside surroundings by covering the lateral walls with burlap, and placed 50 m or more from each other, so that birds in any given aviary could faintly hear those in other aviaries but were, otherwise, exposed predominantly to their own aviary's environment. The interior arrangement of all cages and number of objects inside (perches, food and water dishes) were identical.

2.3. [^{3}H]-thymidine treatment and histology

New neurons were labeled by intramuscular injection of a radioactive form of thymidine ([^{3}H]-thymidine), which is a marker of DNA synthesis and therefore of cell birth. This manner of labeling new neurons has already been used to quantify neurogenesis in developing and adult avian brain [5,7,15]. Experimental birds received injections of [^{3}H]-thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA; 50 μCi each) into their pectoral muscle, one injection per day on each of 6 consecutive days. Two hours after the last dose, the birds were introduced to their new environments, where they stayed for 40 days, until they were killed. This survival period allows enough time for neurons born at the time of injection to migrate to their final destination and go through final anatomical differentiation [3,18]. During this survival period there also was enough time for some of the new neurons to be culled [3,18].

Birds were weighed, then killed with an overdose of anesthesia 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. Brains, with a short segment of brain stem, were removed, placed in the same fixative and, after a minimum of 7 h in it, were embedded in polyethylene glycol, blocked transversely, cut at 6 μm intervals and processed for autoradiography as described in Ref. [7].

2.4. Identification of neurons using cresyl violet

Cells were identified as neurons by the size, shape and staining properties of their nuclei: large (more than 5 μm in diameter), round, with clear nucleoplasm and one or two darkly staining nucleoli. A neuron was considered to be [^{3}H]-labeled if the number of exposed (black) silver grains over its nucleus (as a result of autoradiography) was 20 times or higher than that of the background level, measured over the neuropil; this criterion usually required that a labeled neuron have a minimum of five grains over its nucleus (Fig. 1).

2.5. Identification of neurons using Immunohistochemistry

Sections from four other adult male birds that were not part of any of our experimental groups were used to confirm that the cells, on the basis as cresyl violet staining we identified as neurons were, indeed, neurons. For this we used a neuron-specific marker—anti-Neu-N (mouse monoclonal anti-Neu-N, Chemicon International, Temecula, CA), which was previously and successfully used as a neuron specific marker in mammals [23,36] and in birds [21]. In our material the Neu-N marker stained the nucleus and cytoplasm of post-migratory neurons. The four birds, which had been caged as a group, received the same [^{3}H]-thymidine treatment as the experimental birds, and were killed 40 days after the last injection. Their brains were fixed and cut, as were those for cresyl violet. Then sections were incubated in 0.01 M citrate buffer at 90 °C for 10 min, washed with 0.1 M phosphate buffer (PB) five times, for 10 min each, and then incubated in blocking buffer (PB containing 10% normal horse serum (NHS) and 0.3% Triton × 100), at room temperature (RT) for 30 min. Then, sections were incubated in primary antibody (anti-Neu-N, 1:200 in PB) with 0.3% Triton and 2% NHS, at 4 °C for 48 h, washed with PB with 0.1% Triton for 10 min, and followed by three additional washes with PB, 10 min each. After washing, sections were incubated in secondary antibody (biotinylated *anti*-mouse IgG, Vector Laboratories, Burlingame, CA; 1:200 in PB) with 0.3% Triton and 2% NHS, for 2 h at RT, and washed 3 times with PB for 5 min each. Sections were then exposed to avidin biotin peroxidase reaction (Elite-ABC kit, Vector Laboratories, Burlingame, CA; 1:100 in PB), at 90 min, then washed three times with PB for 5 min each, followed by

Fig.1

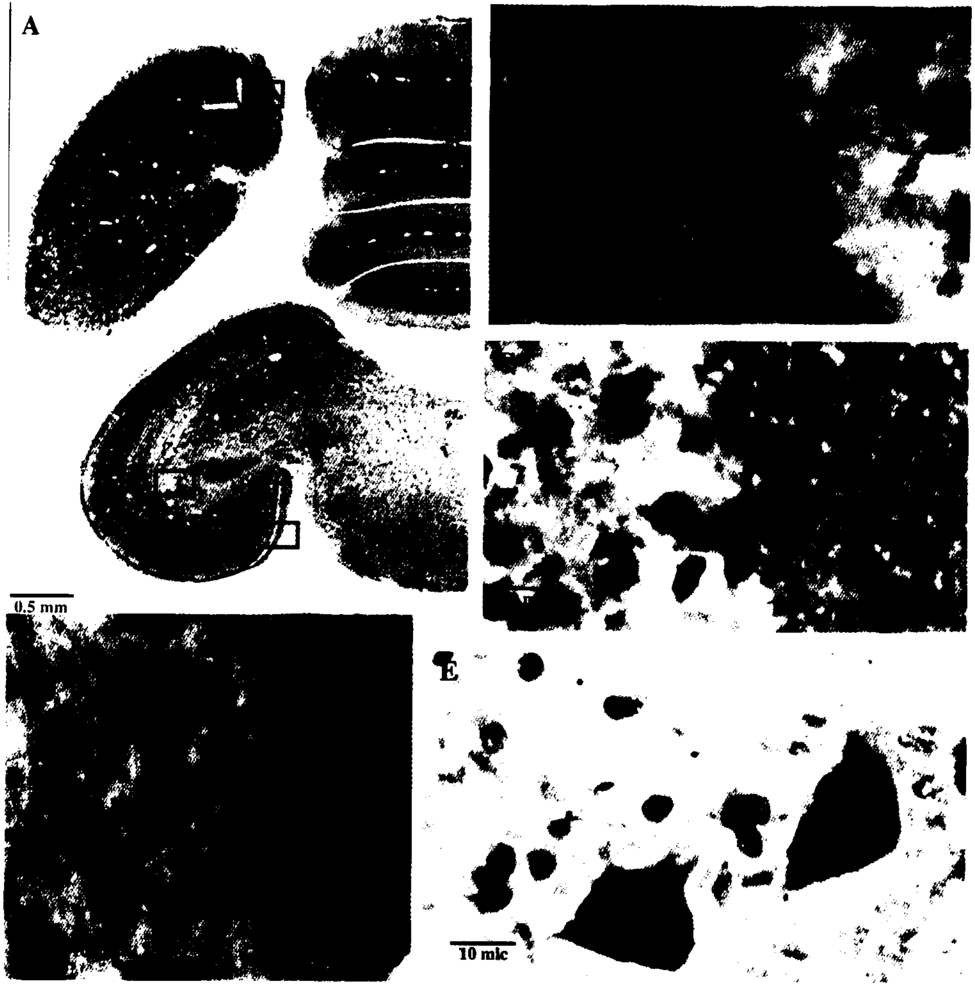

Fig. 2

Fig. 1. A [^{3}H]-thymidine labeled NC neuron, counterstained with cresyl violet (marked with an arrow).

Fig. 2. (A) Right half of a frontal section of a Zebra finch's brain, stained with Neu-N and counterstained with cresyl violet (Neu-N positive cells appear stained in brown, Neu-N negative cells appear stained in violet). The framed areas (which appear in × 1000 magnification) are as follows: (B) Part of the ventricular zone (VZ). The VZ (cell layer marked by an arrow) corresponds to the walls of the lateral ventricle and separates NC cells to the left (ventral), and hippocampal cells to the right (dorsal); (C) Part of tractus opticus (TrO) is seen on the right (containing only violet stained cells), and adjacent to it (on the left, with brown stained cells), a neuron-containing layer; (D) Part of neostriatum caudale (NC), containing neurons (brown stained cells) and glia (violet stained cells); and E) Part of nucleus isthmi pars magnocellularis (IM), containing neurons (large brown stained cells) and glia (smaller violet stained cells).

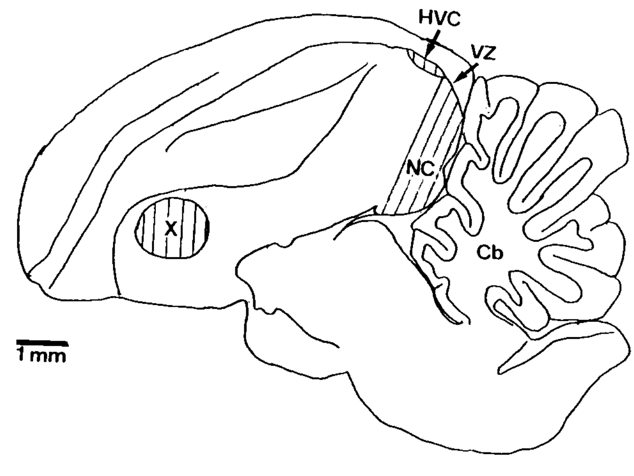

Fig. 3. A diagram of a sagittal section of a zebra finch's brain (left is rostral). Shaded areas indicate the anatomical regions which were sampled for neuronal quantification: neostriatum caudale (NC), high vocal center (HVC), and Area X. Ventricular zone (VZ) is indicated.

3,3′- diaminobenzidine tetrahydrochloride (DAB, Sigma, Israel) reaction (in PB containing 16% DAB and 0.01% H_2O_2), at RT, for 3–8 min. Finally, sections were washed three times with PB, for 5 min each and then processed for autoradiography [7].

We also performed a control, where we omitted the primary antibody. Control sections showed no staining. In addition, in order to confirm the neuronal specifity of the anti-Neu-N marker in our material, we prepared a few sections, which went through the same immunohistochemical procedure, and then were counterstained with either cresyl violet or methylene blue. This enabled us to see Neu-N negative, as well as Neu-N positive cells (Fig. 2A). The ventricular zone (VZ) and the outer layer of the optic tectum (tractus opticus, TrO), areas which are known to be free of neuronal somata, were examined and found to contain no Neu-N positive cells (Fig. 2 B & C, respectively). We also looked at other forebrain and optic tectum areas, which contain both neurons and non-neuronal cells: (NC), and nucleus isthmi pars magnocellularis (IM), and examined the cell morphology. In both areas, Neu-N negative cells had non-neuronal morphology, while cells with clear neuronal morphology were all Neu-N positive (Fig. 2D & E, respectively).

2.6. Mapping and quantification

We used a microcomputer system [4] to draw boundaries of the mapped areas, to count neurons, and to quantify other neuronal parameters, as described below. All mapping was done 'blind' as to the experimental conditions. Since, to the best of our knowledge, this study was the first to ask whether social variables affect new neuronal survival in adulthood, we screened, prior to mapping, the entire forebrains of a few birds from both genders and from each experimental group, to look for [^{3}H]-thymidine labeled neurons. This screening narrowed our interest to three candidate regions—NC, HVC and Area X—which are shown in Fig. 3. These regions were known to have a role in vocal communication [15,22,35] and met three criteria: they had defined boundaries, had [^{3}H]-labeled neurons and they interested us because of known functions associated with them. In all cases the number of new neurons is presented as the number of [^{3}H]-labeled neurons per mm^2 of area sampled; these areas are, of course, 6 μm thick. A description of the areas sampled is as follows:

2.6.1. Caudal neostriatum

The wall of the lateral ventricle and the surface of the brain define the medial, dorsal and lateral walls of NC; the lamina archistriatalis dorsalis (LAD) provides the ventral boundary. NC was sampled in all brains from the three experimental groups. Preliminary mapping showed

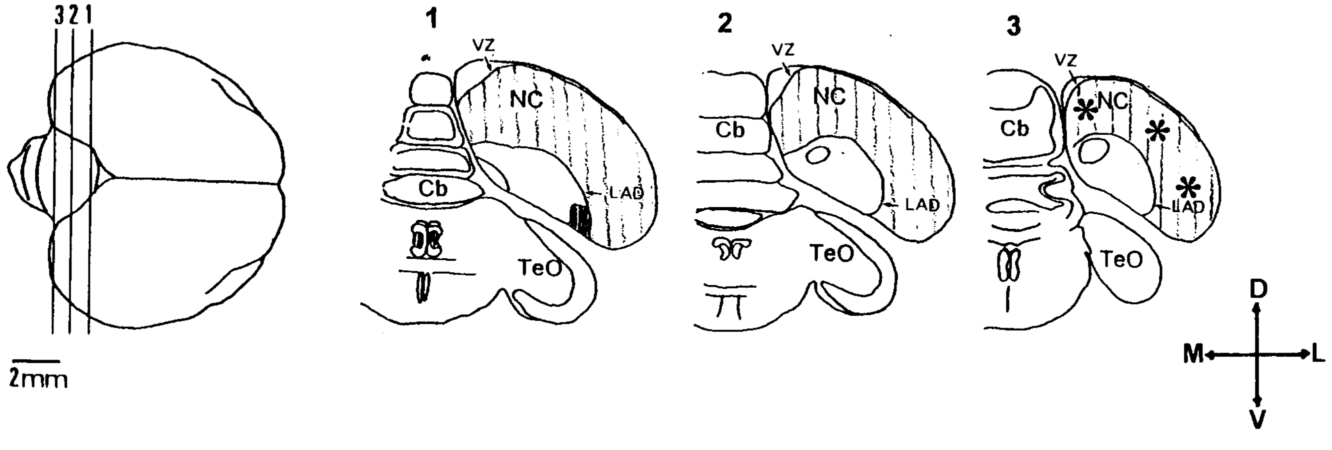

Fig. 4. Left: a top view of zebra finch's brain, (right is rostral). Vertical lines indicate the positions of the three mapped sections, which are also shown in frontal view on the right (up is dorsal). The distance between one section to the following is 600 μm. NC, neostriatum caudale (shaded); Cb, cerebellum; TeO, tectum opticum; VZ, ventricular zone; LAD, lamina archistriatalis dorsalis. Asterisks in section 3 represent sampling areas for some of the variables measured in NC (for details—see Section 2.6.1).

no hemispheric differences in the number of labeled neurons per mm^2. Therefore, in each bird we mapped three sections of the left hemisphere, spaced at 600 μm intervals (Fig. 4) and counted all [^{3}H]-labeled neurons in them (159–703 counts per brain, with the mean of 430 counts). The most rostral of these sections was at a point where the brain looked similar to that of the canary atlas at level P 0.6, i.e. caudal to the caudal end of HVC [32]. We also measured the area of each of the three sections, in order to calculate the number of labeled neurons per mm^2. In addition to counting the total number of [^{3}H]-labeled NC neurons in each of the three sections and estimating their number per mm^2, the following additional measurements were taken only from the most caudal NC section in all brains, as follows:

1. Total numbers of NC neurons (labeled and unlabeled) were counted in three 0.05 mm^2 squares: one near the medial boundary of NC, the second near the lateral boundary, and the third between the two previous ones (see Fig. 4, Section 3). The positions of these sampled squares were estimated by eye. These counts yielded 120–180 neurons per brain. The total number of counted neurons in each section was divided by the volume of these squares, to produce an estimate of neuronal density in NC (number of neurons per mm^3).
2. Nuclear diameters of NC neurons (labeled and unlabeled) were measured, as part of the same sample, in two arbitrary chosen microscope fields within each of the three squares described above (yield of 20–50 neurons per brain). From these measurements we could calculate, for each brain, the mean neuronal nuclear diameter in NC. This variable is important when estimating and comparing neuronal densities, because of the possibility that larger neurons might be over-represented, since they would often appear in two adjacent sections and counted twice. To avoid this possibility, there is sometimes a need to correct neuronal counts before estimating densities [9]. However, if nuclear diameters did not differ between experimental groups, the likelihood of splitting a neuron so that it appeared in more than one section would not differ between experimental groups. If so, there would be no need to correct neuronal counts and a simple comparison can be made. As we shall see in the section 3.1.2, that was the case in our experimental groups, and therefore we compared NC neuronal densities between groups without any need for corrections.
3. Nuclear diameters of [^{3}H]-labeled NC neurons were measured in three 0.2 mm^2 squares, located similarly as described above (and see Fig. 4, Section 3). From these measurements, which yielded 15–40 labeled neurons per brain, the mean nuclear diameter of [^{3}H]-labeled NC neurons were calculated for each brain.
4. The number of silver grains per neuronal nucleus in NC was counted in each of the [^{3}H]-labeled neurons whose diameter was measured. From these counts we arrived at a mean number of grains per each [^{3}H]-labeled NC neuronal nucleus per bird.

These last two variables were estimated to see whether we could make direct comparisons of numbers of labeled neurons per mm^2 between genders and between groups. If no significant differences existed, then we could assume that the probability of counting [^{3}H]-labeled neurons in this brain region was the same across genders and experimental groups, and therefore we could compare directly the number of labeled neurons per unit area without any correction for neuronal counts.

2.6.2. High vocal center

HVC was inspected only in males, since in females it is very small and sometimes hard to identify [26]. In males, HVC is clearly defined, bound dorsally by the ventricular wall that forms its roof and ventrally by abrupt changes in the cytoarchitechtonics of the adjacent neostriatal tissue. As we shall see below in the Section 3.2.1, two out of the three experimental groups—isolates and pairs—showed no differences in the number of labeled neurons per NC unit area. Therefore, in HVC we chose to focus just on one of these groups—pairs—and compared it with large group, because in both novel company was provided, but differed in how much.

Previous works did not find hemispheric differences in [^{3}H]-thymidine labeling in HVC [1], and therefore we sampled the left hemisphere. The total number of [^{3}H]-thymidine labeled and of unlabeled neurons in HVC was counted in three sections, spaced at 180 μm intervals. The most rostral section was at the canary atlas level of AP 0.0 [32]. The number of [^{3}H]-thymidine labeled neurons per mm^2 was calculated for each mapped section as for NC.

As a result of the clearly defined boundaries of HVC, it was possible to obtain a fairly accurate estimate of HVC volume. This was done by measuring, in each of the sampled brains, the area of HVC in all sections that contained HVC (10–12 measurements per brain), and multiplying the sum of measured areas by the distance between the measured sections (60 μm). In addition, the average neuronal density in HVC was calculated by counting the total number of neurons in three HVC sections and dividing this number by the volume (in mm^3) of these three sections.

Nuclear diameters of all [^{3}H]-thymidine labeled neurons in HVC, in all three mapped sections, were measured (5–25 measurements per brain, with the mean of 18 measurements), and a mean nuclear diameter of [^{3}H]-thymidine labeled neurons in HVC was calculated. As for NC, and for reasons explained above, mean nuclear diameter of [^{3}H]-labeled HVC neurons was

Fig. 5. A double-labeled (with Neu-N and [^{3}H]-thymidine) NC Neuron (marked with an arrow).

calculated to see if we would be justified in making a direct comparison of numbers of labeled neurons per mm^2 between experimental groups.

Nuclear diameters of total neurons ([^{3}H]-thymidine labeled and non-labeled) were also measured, in two microscope fields that were positioned in arbitrarily chosen locations in HVC. These measurements were done only in one of the mapped sections (the middle one), and yielded 15–40 measured diameters per brain (mean of 28 measurements), from which a mean neuronal nuclear diameter in HVC was calculated. As already explained above for NC, this variable is important when estimating and comparing neuronal densities, to check whether there is a need to correct neuronal counts. As we shall see in the Section 3.2.2, there was no need for that, and therefore we compared HVC neuronal densities between groups without any corrections.

2.6.3. *Area X*

As for HVC, Area X information was collected from males kept as pairs or in large groups. Boundaries of Area X are recognized by changes in cytoarchitectonics, Area X's cells being, on average, smaller and more densely packed than those in the surrounding LPO tissue. Preliminary mapping showed no hemispheric differences in the number of labeled neurons per mm^2. Therefore, the total number of [^{3}H]-labeled neurons in Area X (44–150 counts per brain, mean of 99 counts) was counted in each brain, in three left sections, at 360 μm intervals, the most rostral section being at canary atlas level A 4.5 [28].

2.6.4. *Use of the Neu-N marker to validate the cresyl violet counts of new neurons*

We used two sections from each of the four brains treated to confirm neuronal identification of the new neurons: one at the level of Section 3 in Fig. 4, and the other one immediately adjacent. Both sections were incubated for autoradiography; one of the sections was counterstained with cresyl violet (Fig. 1), while the other one was reacted with Neu-N (Fig. 5). In the sections reacted with the Neu-N antibody, neurons were identified by Neu-N-labeling of the entire soma, while in the cresyl violet stained section they were identified by size, shape and staining properties of their nuclei, following the same criteria used in the rest of this study. We did this to test whether our identification of [^{3}H]-labeled neurons, based on cresyl violet nuclear staining properties, was reliable—i.e. was comparable to counts obtained when using [^{3}H]-labeling in conjunction with the Neu-N marker.

2.7. *Statistical analysis*

The following data were subjected to statistical analysis:

1. *NC*: Number of [^{3}H]-labeled neurons per mm^2; number of all neurons (labeled and unlabeled) per mm^3; mean nuclear diameters of total ([^{3}H]-thymidine labeled and non-labeled) neurons and of only [^{3}H]-labeled neurons; and mean number of silver grains per neuronal nucleus in [^{3}H]-labeled neurons.
2. *HVC*: Number of [^{3}H]-labeled neurons per mm^2; number of all neurons (labeled and unlabeled) per mm^3; volume of HVC; nuclear diameters of total ([^{3}H]-thymidine labeled and non-labeled) neurons and of only [^{3}H]-thymidine labeled neurons.
3. *Area X*: Number of [^{3}H]-labeled neurons per mm^2.
4. *Body weight*: Data that were expressed as number of cells per mm^2 or mm^3 were transformed, prior to the statistical analysis, by using the square root transformation. (This kind of data, i.e. number of discrete elements per unit, tend to have a Poisson distribution, and the suitable transformation for such a case is the square root transformation; [31]). Throughout the statistical analysis, $P \leq 0.05$ was considered significant. For data that were collected from several sections in a certain brain area (number of [^{3}H]-labeled neurons per mm^2 in NC, HVC and Area X), analysis of variance was preformed using ANOVA with group and gender (or only group, when data were obtained only from males) as between-subject factors, and section position as within-subject (repeated measures) factor. In all these cases no significant interaction was found between section position and gender or experimental group, and therefore data from all sections were pooled for further comparisons. Other data were analyzed by two-way ANOVA or t-tests (two-tailed). When results of the

overall analysis of variance were not significant for interaction between experimental group and gender, then data from males and females were pooled for post hoc comparisons, which were carried out by the Tukey (HSD for unequal N) method.

3. Results

3.1. Caudal neostriatum

3.1.1. Number of [^{3}H]-labeled NC neurons per mm^2

Sample sizes for males were: $N = 5$ in isolates, pairs and large group; and for females: $N = 5$ in isolates and pairs and $N = 4$ in large group. Overall analysis of variance revealed that gender differences and interaction between group and gender were not significant. There was a significant difference between experimental groups in the number of NC [^{3}H]-labeled neurons per mm^2 [$F_{(2,23)} = 14.02$, $P < 0.001$], with birds house with a large group having approximately 30% more NC [^{3}H]-labeled neurons per mm^2 than both isolates and pairs (Fig. 6). No significant differences were found between isolates and pairs.

3.1.2. Nuclear diameters of NC neurons and neuronal densities

Sample sizes for both genders were $N = 5$ for isolates and pairs, $N = 4$ for birds housed with a large group. No significant gender or group differences and no significant interaction between group and gender were found in mean nuclear diameters of total ([^{3}H]-labeled and non-labeled) NC neurons, which ranged between 7.0 and 8.4 μm. Similarly, no such differences were found in mean diameters of [^{3}H]-labeled NC neuronal nuclei, which ranged between 7.0 and 10.6 μm; and in mean neuronal densities, which ranged between 123 000 and 260 166 neurons per mm^3.

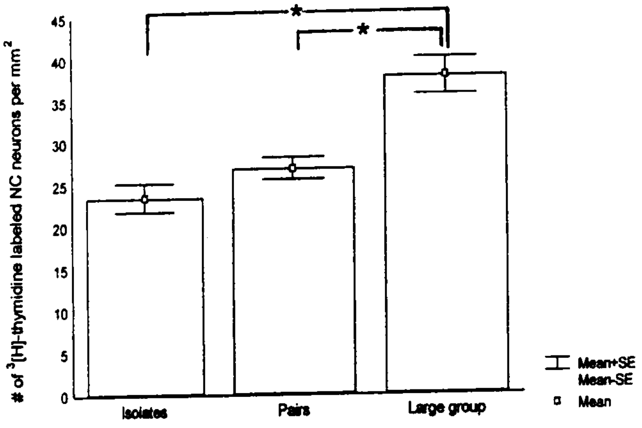

Fig. 6. Number of NC [^{3}H]-labeled neurons per mm^2 (mean ± SE) in the three experimental groups. $^*P < 0.05$.

3.1.3. Mean number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus

Sample sizes are the same as for mean nuclear diameters of [^{3}H]-labeled NC neurons see Section 3.1.2. We tested for differences in number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus between brains which came from different dipping batches, in order to see whether small differences in the autoradiographical procedure affected the number of grains per nucleus. However, no significant differences were found. A significant gender difference was found in mean number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus [$F_{(1,22)} = 5.82$, $P = 0.025$], with males having more grains per labeled nucleus than females (19.5 ± 4.96 grains vs. 15.6 ± 4.06 respectively). Since the interaction between gender and experimental group was not significant, we pooled the data from females and males and found a significant difference between experimental groups [$F_{(2,22)} = 4.74$, $P = 0.019$], with birds housed with a large group and in pairs having more exposed silver grains per [^{3}H]-labeled NC neuronal nucleus than isolates (19.7 ± 4.9 and 18.96 ± 4.1 vs. 14.36 ± 4.0, respectively).

3.2. High vocal center

3.2.1. Number of [^{3}H]-labeled HVC neurons per mm^2

There was a significant difference between experimental groups ($N = 5$ in each group; $F_{(1,8)} = 10.588$, $P \leq 0.001$]. The males housed with a large group had two-and-a half as many [^{3}H]-labeled HVC neurons per mm^2 as males in pairs (Fig. 7A).

3.2.2. HVC volumes, nuclear diameters and neuronal densities

There was no significant difference in HVC volume and in average neuronal densities in HVC between males in the large group and pairs. Similarly, no significant differences were found in either mean nuclear diameter of [^{3}H]-thymidine labeled neurons or in mean nuclear diameters of total (labeled and unlabeled) neurons. Therefore, total neuronal counts were similar in the two groups.

3.3. Area X

There was a significant difference between experimental groups ($N = 5$ in each group; $F_{(1,7)} = 9.75$, $P = 0.017$]. Males housed with a large group had approximately twice as many [^{3}H]-labeled neurons per mm^2 as males kept in pairs (Fig. 7B).

3.4. Neu-N labeling

Since only four brains were sampled for the immunohistochemical labeling, we did not analyze the data

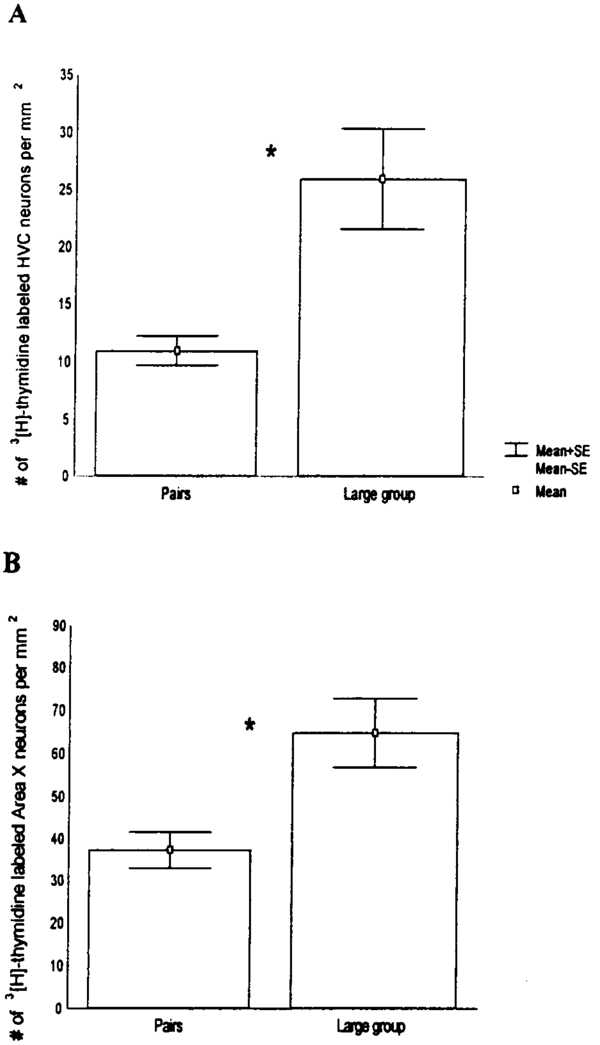

Fig. 7. Number of [^{3}H]-labeled neurons per mm^2 (mean ± SE) in (A) HVC and (B) Area X, in males of two experimental groups: pairs and large group. *$P < 0.05$.

Table 1
Numbers of [^{3}H]-labeled NC neurons per mm^2 in Neu-N stained sections (where neurons were identified by Neu-N-labeling), and in adjacent cresyl violet stained sections (where neurons were identified by the size, shape and staining properties of their nuclei), in the four sampled brains

Section	Brain			
	1	2	3	4
Neu-N	33.3	19.5	18.8	24.3
Control	34.0	17.6	15.9	26.1

statistically, and the results are presented in Table 1. For each of the four brains, the numbers of [^{3}H]-labeled NC neurons per mm^2 which were counted in two adjacent sections reacted with cresyl violet and anti-Neu-N antibody were very similar in all four brains.

3.5. Body weight

No significant differences were found in body weight (measured before the birds were killed), between genders or between experimental groups. Mean body weight was 12.9 ± 1.2 g ($N = 29$).

3.6. Behavioral observations

The behavior of the experimental birds during the 40 days spent in the different social environments was monitored by direct observations. We inventoried the incidence of various behavioral patterns performed by the birds. A companion paper (in preparation) will describe the methods and results of these observations in detail. For the purpose of the present report we will only note that a given social setting did not cause the same, or a similar effect on all, or most, of the monitored behavioral patterns. In other words, the effect of social environment on the different behavioral patterns, varied: some activities, such as feeding and drinking, were comparable in all groups. Others, such as flying between perches, were more frequent in birds kept as isolates or as pairs than in birds kept in a large group (Fig. 8A). And still other behaviors, such as preening, were more common in the large group than in birds kept as isolates (Fig. 8B). Unfortunately, we did not quantify amount of singing by birds kept under the three conditions and this, in retrospect is a pity since it is known that amount of singing affects the survival of new HVC neurons [20]—but of course this variable would only apply to males.

4. Discussion

We manipulated a single variable—social complexity—by introducing adult birds of comparable age to standard outdoor aviaries where they were kept as isolates, in pairs, or in a large group. Immediately prior to the social change, the birds had received for 6 days daily injections of the cell birth marker [^{3}H]-thymidine. Body weight did not differ between genders or between groups at the time all experimental birds were killed 40 days later; from this we infer that all birds remained in good general health. Similarly, no differences were found in mean diameter of total neurons (labeled and unlabeled) in NC or HVC, and therefore, as explained in Section 2.6.1, we could calculate neuronal densities without any corrections for neuronal count. Neuronal densities in these two brain areas did not differ between genders or between experimental groups, and from this we infer that number of labeled neurons per mm^2 can be compared directly between different experimental groups. Our results indicate that differences in social settings affected the number of new neurons in the

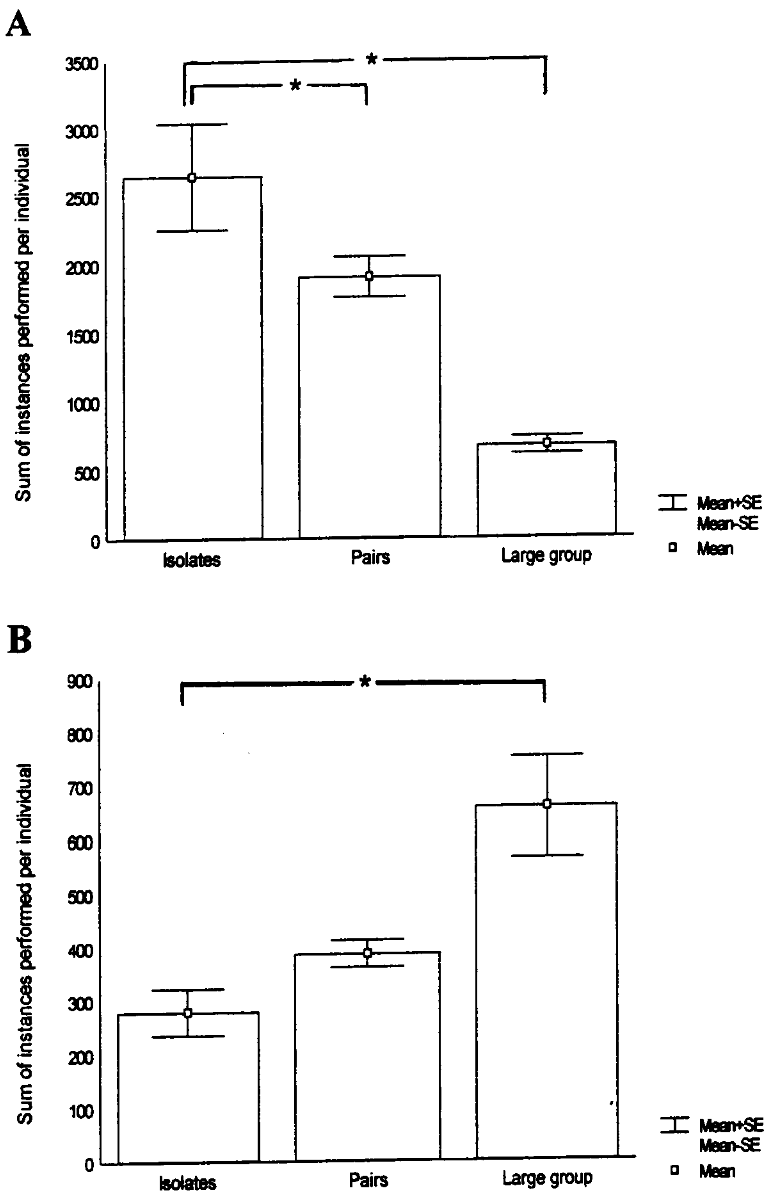

Fig. 8. Mean (± SE) of the sums of instances of: (A) flying between perches; and (B) preening, that were observed for each individual during 12 observations (15 min each), that were conducted during the 40 days that the experimental birds spent in the novel social environment. $*P \leq 0.05$ (Kruskal Wallis test).

three tested brain regions—NC, HVC and Area X—which are all part of brain pathways used in vocal communication.

4.1. Reliability of neuronal identification

There was a very good correspondence between our counts of [^{3}H]-labeled NC neurons that used cresyl violet and those that used the Neu-N antibody (Table 1). We infer that our counts of [^{3}H]-labeled neurons that used the cresyl violet stain were minimally contaminated by non-neuronal cells. Since the criterion for neuronal identification using the cresyl stain was that candidate cells have round, clear nuclei with nuclear diameters no smaller than 5 μm, we believe that we probably excluded from our cresyl violet counts all migrating neuroblasts, whose nuclei are elongated, dark and small [5]. However, if there were any adult microneurons with nuclear diameters smaller than 5 μm, they also would have been excluded. However, no size structures were imposed on the Neu-N data and so if new microneurons were present, they were a very small contingent. Neu-N did not stain migrating neuroblasts.

4.2. An effect on recruitment or survival

[^{3}H]-thymidine was administered before social change. We know, from earlier observations [2] that systemically injected [^{3}H]-labeled thymidine disappears from the blood stream rather quickly, so that there is little if any left by 2 h after injection, which was the reason why we chose this delay between the last [^{3}H]-thymidine injection and social change. Therefore, group differences in number of [^{3}H]-labeled neurons must have resulted from differences in recruitment or survival. By 'recruitment' we mean the incorporation of new neurons into a particular region. New neurons are born in the vz zone [3,10]. The destination of their subsequent migration could respond to local signals that draw them, e.g. into HVC, NC or Area X. Our data do not tell us whether group differences in new neuron numbers resulted from differences in the recruitment of new neurons into a particular region or differences in their survival after they got there, but earlier observations suggest that survival is the likeliest explanation. For example, Kirn et al. [18] have shown that 2 weeks after [^{3}H]-thymidine injection new HVC neurons have adopted a post-migratory phenotype; 7 days later 50% of these new cells have disappeared. Experience-based winnowing of post-migratory cells continues even after then [20]. It seems likely that in the present study, too, counts of new neurons were affected by the effects of experience on the survival of new cells.

4.3. The dynamics of neuron numbers: hints from silver grain counts

As explained under Section 2.6.1, counts of labeled neurons might be influenced by their nuclear diameters. We found no evidence that mean nuclear diameter of labeled neurons differed between groups in the two parts of the brain (NC and HVC) where this was checked. On the strength of this observation, group comparisons were made directly, with no size corrections.

Counts of labeled neurons can also be influenced by the number of exposed silver grains over their nuclei. In NC, where this was checked, the count was lower in the isolates than in the birds housed in a group or as pairs. The uptake of [^{3}H]-thymidine was presumably comparable across groups, since it occurred before the onset of social differences between groups. A dilution of label in the isolate birds could have resulted if some of the neurons initially labeled had short survivals and were replaced by a new set of neurons born from the same original stem cells. If so, the mean survival of new NC neurons may have been lower in the isolates than in the paired or group-housed birds. This scenario is in line with the observation that the death of replaceable neurons results in a higher number of new neurons of the same kind [30], though it is not known if this higher number results from an effect of the vacancies on production, recruitment or survival.

The situation described above for isolates did not arise for birds housed as pairs. The number of exposed silver grains per labeled neuron in them was no different from that in group housed individuals. Therefore, differences in numbers of new neurons between those two social settings resulted most likely from differences in recruitment or survival. However, if it is true that long-term (e.g. 40 days) survival of new neurons is possible only if the new cell finds a vacancy created by the death of another cell, then one could infer that the greater number of new neurons present at 40 days in the large group birds resulted from the fact that, after the experimental animals were transferred to a large group there was an initial mortality of pre-existing cells that was greater in group housed than in pair housed birds. The important conceptual point, here, is that the primary effect might be cell death, rather than cell survival.

4.4. Interpreting the effect of social setting

It is hard to imagine a variable that is more complex than 'social setting'. Yet this does not make it less real. How did social setting influence neuronal survival? Had survival depended, as suggested for mice [17], just on overall level of activity, then perhaps it should have been higher in the birds kept as isolates or in pairs, because they flew more between perches than birds kept as a group. However, there were other behaviors, such as social preening, that were more frequent in the group-housed birds than in the others.

The incidence of singing and calling probably differed between our experimental groups. Directed singing is part of courtship and requires the presence of a partner. It is safe to assume that directed singing did not occur in isolates. However, male zebra finches also produce undirected song. We know that the firing of Area X neurons increases when male zebra finches produce undirected song [14], but we do not know if undirected song was more frequent among males housed singly, in pairs or in a colony. We did not quantify how much males sung and the reason for this was practical. Data gathering was done by sight and it was difficult, when observing the group-housed birds, to quantify singing amidst the din of colony sounds. We know, from other work, that neuronal survival in one brain region (HVC) is greater in singing than in non-singing birds [20], an effect that, in HVC, seems to be mediated by BDNF (brain derived neurotrophic factor) [20,28].

Though amount of singing is a likely variable for differential survival of HVC and Area X neurons, the increased number of new NC neurons in male and female group-housed birds probably resulted from the richer auditory environment—female zebra finches do not sing. We do not know if social complexity may have affected new neuron survival in still other ways—for example, by differences in stress level or by the load of social information the brain had to handle. The lack of difference in NC between isolates and birds in pairs suggests that we are not dealing with an effect of stress that might result from an overly simplified social environment, because that effect, for a social species such as the zebra finch, should have been maximal in isolates. This lack of difference in NC between the two groups also suggests that the amount of stimulation provided by a single companion is not enough, and that it takes more than this to see an effect. Therefore, it could be that pair formation, bonding, and the constant exchange of social signals between members of the pair does not, by itself, result in a noticeable change in new neuron survival, and that the system has to be pushed further into the realm of social complexity to see an effect.

4.5. *A relation between new neurons and replacement?*

Previous studies have shown that increases in the number of new neurons in a particular part of the avian brain are preceded by an increased culling of existing neurons [19,30]. The present study did not yield data on neuronal death. However, in the specific instance of HVC, neuronal densities and volumes were comparable in males housed as pairs and in those housed in a large colony, yet numbers of new neurons in the latter group were twice those in the former. We suspect that, as in previous reports, increases in new neuron numbers were preceded or accompanied by a matching demise of older ones.

4.6. *Overview*

The present study shows that social setting can be added to the list of variables that regulate the number of new neurons in the adult vertebrate brain. A tentative precedent for this already has been described in crayfish, an invertebrate [29]. Other variables thought to affect the numbers of new neurons are environmental complexity [7,17,27], exercise [20,34], stress [11,13] and learning [12]. It is too early to speculate whether the underlying mechanism—e.g. pathway use and related neurotrophins—is the same in all these cases.

Acknowledgements

This work was supported in Israel by The Open University of Israel, The National Institute for Psychobiology, and The Adam Super-Center for Brain Research. In the USA, support came from PHS grant MH18343. We would like to thank C. Scharff, M. Grossman and L. Willbrecht for their help in histology and immunohistochemistry, I. Gelernter for the statistical analysis, and the stuff of The Meier Segals Garden for Zoological Research for their technical help.

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