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Short communication

Neurons recruited in the nidopallium caudale, following changes in social environment, derive from the same original population

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ABSTRACT

Previously, we found that new neurons recruited into the nidopallium caudale in isolated birds were less labeled than those of communally housed birds, suggesting that different types of neurons may survive best under different conditions. Repetition of the experiment revealed no differences between groups, indicating that the new neurons were of the same generation. Hence, social environment does not appear to affect the type of newly recruited nidopallium caudale neurons.

Neurogenesis (birth of neurons) has been studied in many adult vertebrates, such as fish and reptiles [8,14], birds [18], and mammals, including humans [10,11]. In birds, neurogenesis occurs in the ventricular zone (VZ), from which the new neurons migrate to various brain regions. It takes between 10 and 40 days from birth until the new neurons are incorporated into existing circuits [3,12]. Since the total number of neurons in a specific area in the avian brain does not change, the assumption is that young neurons replace older ones [21]. It has been suggested that neurogenesis plays a role in the acquisition of long-term memory [16] and that the entire neuron, not only the synapse, is the basic unit of learning [17].

Previously [7], we treated male zebra finches with [^{3}H]-thymidine, which is a marker of DNA synthesis and therefore of cell birth. Following treatment, experimental birds were exposed to either a simple (isolation) or complex new social environment, and counts of new neurons were made at various intervals (40, 60 and 150 days) after the change in housing. One of the brain regions that we sampled was nidopallium caudale (NC), which is known to include auditory projections [24] and regions that are activated by playbacks of conspecific song [15]. One of our findings was that labeled neurons which were observed in this brain region 60 days after treatment, differed significantly between groups in the mean number of exposed silver grains per [^{3}H]-thymidine labeled neuronal nucleus (13.1 ± 3.3 grains in brains of birds exposed to isolation vs. 20.9 ± 3.8 grains in those exposed to a new complex social environment). This finding suggested that different kinds of neurons were surviving differently in these two social settings. More densely labeled neurons (with more exposed silver grains) occurred when birds were communally housed, while less labeled neurons (with less exposed silver grains) occurred when birds were kept in isolation.

The number of exposed silver grains is positively correlated to the amount of [^{3}H]-thymidine that is incorporated into the nuclei during S-phase, when DNA synthesis takes place. Accordingly, this previous finding of labeling differences can be explained by two suggested mechanisms: first, this could occur if the observed labeled neurons in each group had originated from different stem cell populations whose S-phase of DNA synthesis differed in duration. Under such a scenario, a longer S-phase would result in more labeling, because incorporation of [^{3}H]-thymidine into the dividing cells occurs over a longer period of time, and vice versa. Accordingly, if labeled neurons in brains of birds exposed to a complex social environment originate from a stem-cell population with longer S-phase, they will be relatively more densely labeled; whereas if labeled neurons in brains of birds exposed to isolation originated from a stem-cells population with shorter S-phase, they will be relatively less labeled. However, since to the best of our knowledge, there is currently no direct evidence that stem cells in the avian VZ differ in S-phase duration, we cannot provide support to this suggested mechanism from previous studies on birds. On the other hand, more generally, S-phase duration is known to differ between

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organisms and even between cells of the same organism [2]. Moreover, it has been shown that various environmental conditions can affect the replication check point (S-phase check point) [9,19,20], which in turn affects S-phase duration.

Another mechanism that could be suggested to explain this differential labeling between the groups relates to the number of divisions that a stem cell undergoes until the observed neuron is generated. If we assume that all stem cells had a similar duration of S-phase, then the initial uptake of [^{3}H]-thymidine was presumably comparable across groups, since it occurred before the onset of social differences between groups. Under such conditions, daughter neurons that originate from this mitotic event that occurred at the time of treatment are expected to reflect similar labeling. However, if successive rounds of stem cell divisions took place after the initial labeling, then a dilution of labeling occurred each time, yielding new generations of neurons with less labeling (i.e., fewer exposed silver grains per labeled neuron). Such pattern, of dilution of labeling which results from successive divisions, has already been shown before in the avian VZ [4,23]. Moreover, these two studies provide evidence that proliferation rate is non-uniform across the VZ. Taken together, the reduced labeling in the isolated birds could have ensued if, under this social setting, neuronal turnover is faster than in birds that are communally housed. If so, then the first generation of labeled neurons of the isolated birds died sooner than those of the communally housed birds and was replaced by a newer generation of neurons, which originated from a successive division of the same original stem cells, which now, in turn, had less [^{3}H]-thymidine. These newer neurons were those that we observed in the isolated birds and, as a consequence of the additional division/s of their stem cells, they had fewer exposed silver grains in their nuclei, in comparison with those in birds kept in a complex social setting.

Although intriguing, we treated this finding of differential labeling between groups as preliminary, since it was based on only 12 brains (six per group). Furthermore, in each brain we sampled only one section of NC, and in it 10 randomly chosen ~0.02 mm^2 squares, which yielded a relatively small sample size of 6–18 labeled neurons per brain. In addition, this finding was found to be significant only between the 60-day survival groups; in the other groups (40 and 150 days) it was not significant, although in the former a similar tendency to less labeling in the isolated birds was observed. We therefore decided to repeat the experiment for its reproducibility, before exploring the possible mechanisms underlying it.

Male zebra finches were reared in three outdoor breeding colonies at the I. Meier Segals Garden for Zoological Research at Tel-Aviv University, Israel, and were banded at fledging with color plastic rings for individual identification. At the age of 45–60 days, when these males became independent and could be sexed by their plumage, each was removed from its native colony and housed outdoors in a standard cage (65 cm × 35 cm × 45 cm) with three other individuals: another juvenile male of a similar age, and two adults (a male and a female, over 150 days old). Birds in each cage could hear and see only their cage-mates. Experimental males remained in this setting until 120 days old, at which time each one received six daily intramuscular injections of [^{3}H]-thymidine (6.7 Ci/mM; 50 μCi). Each male was then placed in a large outdoor aviary (1.2 m × 1.2 m × 2 m), either individually or with a pre-existing group of 40–45 adults of both sexes, all of them strangers to the experimental bird. We shall refer to these two social settings as "isolation" and "complex", respectively. The aviary walls were covered with burlap and the distance between aviaries was approximately 50 m, so that birds in any given aviary could neither hear nor see those in the other aviaries. The interior arrangement (perches, food and water dishes) was the same for all aviaries. Experimental birds remained in the new social environment for 60 days. All cages and aviaries were exposed to natural illumination that changed seasonally (10.1–14.7 h of light per day). The range of mean daily temperature was 12–30 °C. Under these conditions our breeding stock is able to breed throughout the year. Consequently, and because experimental birds were obtained at all times of the year, seasonal changes in temperature and photoperiod are unlikely to have had an effect on the outcome of our study. The experiment was conducted in accordance with regulations and guidelines of the animal care and use committee of Tel-Aviv University.

The isolation group contained nine individuals and the complex group eight individuals. Sixty days following exposure to the new social setting, experimental birds were weighed, sacrificed with an overdose of anesthesia (0.06 ml of ketalar diluted ×10 followed by 0.06 ml of xylazine) and fixed with an intracardiac perfusion with saline (NaCl 0.9%) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3–7.4). Brains were removed, weighed, placed in the same fixative for 1 h, and transferred into PB in 4 °C. After 1–3 days, brains were dehydrated in alcohols, embedded in polyethylene glycol, blocked, and cut transversely at 6 μm intervals. Serial sections (every 20; i.e., intervals of 120 μm) were mounted on slides (Superfrost/PLUS), using a solution of 0.1% BSA (albumin bovine, minimum 98%) in PBS. Sections were incubated for 10 min in 0.01 M citrate buffer (pH 5.6–6) at 90–95 °C, then washed in PB for 5 min and incubated for 2 min in weak pepsin (pepsin stock; Sigma, 2.5% in PBS 1 M) diluted 1:19 in 0.1N HCl at room temperature (RT). After washing (PB, three times, 5 min each), sections were incubated in 3% H_2O_2 in PB for 20 min and then washed with PB twice, 5 min each. Blocking of unspecific binding sites was done by incubating for 1 h in blocking buffer (PB containing 10% normal horse serum and 0.3% Triton X-100). Sections were then incubated overnight at 4 °C, with the primary antibody that affixes specifically to neurons: anti-HuC/HuD mouse IgG2b, monoclonal 16A11 (Invitrogen; diluted 1:200). This antibody binds to HU protein, which is specific to neurons and is expressed at very early stages of neuronal differentiation, either concurrent or soon after terminal mitosis [5]. The next day, sections were washed and incubated for 2 h at RT with the secondary antibody biotinylated anti-mouse IgG (H_L) made in horse (Vector Laboratories; diluted 1:200). Sections were then washed and treated for 30 min with avidin–biotin–peroxidase complex (ABC R.T.U. Vectastain kit; Vector Laboratories) and incubated for 7 min with 16% DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma) in PB, which, in the presence of 0.01% H_2O_2, stains the neurons in the tissue brown. Sections were then washed with dH_2O, followed by delipidization in xylene/ethanol and coated with nuclear track emulsion NTB2 (Kodak). After 4 weeks of incubation at 4 °C the emulsion was developed and coverslipped with Aqua-Poly/Mount glue (Polysciences). The immunohistochemistry procedure described above yielded neurons that were stained brown and new neurons that were brown with silver grains.

The NC stretches rostrocaudally for a distance of 1200 μm. From this area, five sections were scanned for our samples of [^{3}H]-thymidine neurons. The middle section incorporated the robust nucleus of arcopallium at its largest diameter; in the canary atlas [22] this would correspond to level P1.2. Two more sections were then obtained, rostrally and caudally, respectively, at distances of 360 and 600 μm from the middle section. We used a computerized brain-mapping system (Neurolucida; Stereo Investigator; MicroBrightField) to draw boundaries of the NC in each section sampled, enter the position of labeled neurons, and count the exposed silver grains in them. All mapping was done "blind" as to the experimental conditions. Our previous studies [1,6,7,13] had found no hemispheric differences and therefore we mapped sections only from the left hemisphere. In all brains, and in each of the five NC sections mapped in each brain, we used a 63× objective and the Neurolucida fractionator probe, to sample labeled neurons. In

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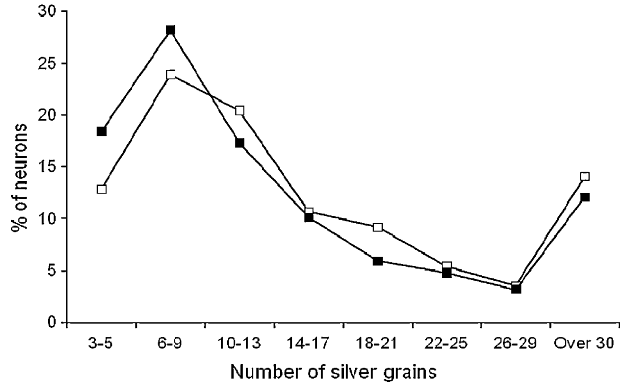

Fig. 1. Distribution of the number of exposed silver grains in [^{3}H]-labeled NC neurons in brains of birds held in isolation (closed squares) and in a complex social setting (open squares).

order to obtain at least 20 labeled neurons per section, we sampled at least 21 non-overlapping sampling squares (each of an area of 19,600 μm^2) that were randomly chosen by software across the section. Using this method we scanned 4–100% of the total section area, depending on the density of the labeled neurons in each section. Labeled neurons were sampled if their nuclei completely appeared within the boundaries of the squares or if they touched two specific lines (upper or right line). This protocol yielded 1–129 labeled neurons per section (mean of 20.6 ± 14.7; N = 84 sections) in which we counted the number of exposed silver grains per cell nucleus. Total sample sizes per brain ranged between 40 and 262 labeled neurons (mean of 102 ± 45.6; N = 17 brains). From these counts we arrived, for each brain, at a mean number of exposed silver grains per [^{3}H]-thymidine labeled NC neuronal nucleus.

Prior to the statistical analysis data were transformed by square root. We tested for group differences in the number of grains per neuron using ANOVA with repeated measures, in which the variables were social setting (isolation or complex) and sections were the repeated measure.

No significant differences were found in number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus between brains of birds kept in different social settings ($F_{(1,15)} = 1.428$; $P = 0.25$). The mean number of silver grains per labeled neuron in the isolation group was 13.1 ± 2.3, while in the complex group it was 14.6 ± 2.8. In addition, no differences were found between groups in the distribution of exposed silver grains in labeled neurons (Fig. 1).

The similar number of exposed silver grains found in NC neurons in both experimental groups (isolation and complex social setting) contrasted our previous finding [7], which had indicated a significant difference between these groups. This discrepancy could have been due to the small sample sizes in our earlier work, which might have led to an incorrect conclusion. This explanation is supported by the fact that in that previous study the significant difference was found only between the 60-day survival groups and not for the other two groups (40 and 150 days). Furthermore, our other studies [1,6,13], which similarly tested the effect of various social settings on new neuronal recruitment in the NC, also did not find differences in the number of exposed silver grains per labeled neuron between experimental groups. The same holds true in regard to the distribution of exposed silver grains in NC neurons. The only case in which we observed a differential distribution was between the 60-day survival groups [7]. In all other cases cited above, as well as in the present study, no such pattern was found. Taken together, the similar number and distribution of exposed silver grains in labeled neurons that were recruited into the NC as a response to a change in the social environment, found in the present study as well as in our previous ones, indicates that these new neurons result from a single mitotic event and, therefore, are a part of the same generation. Hence, in contrast to our earlier study, we conclude that changes in the social environment do not affect the type of new neurons recruited in the NC of the adult zebra finch brain.

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