VOLUME 67 NUMBER 6 MAY 2007

Developmental Neurobiology

FORMERLY JOURNAL OF NEUROBIOLOGY

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Neuronal Recruitment in Adult Zebra Finch Brain During a Reproductive Cycle

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Received 8 September 2006; accepted 19 December 2006

ABSTRACT: Previous studies suggest that adult neurogenesis and neuronal replacement are related to the acquisition of new information. The present study supports this hypothesis by showing that there is an increase in new neuron recruitment in brains of adult male and female zebra finches that coincides with the need to memorize vocalizations of nestlings before they fledge. We counted [^{3}H]-Thymidine labeled neurons 40 days after [^{3}H]-Thymidine injections. These counts were made in the parents' brains at the time eggs hatched, at the time juveniles fledged and still needed parental care, and at the time juveniles were already independent. We focused on nidopallium caudale (NC), a brain region which plays a role in sound processing. Recruitment of new NC neurons increased at the time the young fledged, followed by a significant decrease when the young reached independence. We suggest that this increase enables parents to recognize their own young when they are still dependent on parental feeding, yet easily lost among other fledglings in the colony. We saw no such increase in neuronal recruitment in the olfactory bulb, suggesting anatomical specificity for the effect seen in NC. We also found a preliminary, positive correlation between number of fledglings and number of new NC neurons in the parents' brain at fledging, suggesting that the number of neurons recruited is sensitive to the number of young fledged. © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 687–701, 2007

Keywords: **neuronal recruitment; avian brain; nidopallium caudale; memory; breeding cycle**

INTRODUCTION

Studies conducted during the last 20 years have suggested that for some tasks, the acquisition of new long-term memories is associated with the recruitment of new neurons, which in turn replace older ones of the same kind (Nottebohm, 1984, 2002a; Kirn et al., 1994; Kempermann, 2002). Neuronal replacement is seen, in this context, as a form of brain plasticity that enables organisms to adjust to environmental change (Gould et al., 1999; Sandeman and Sandeman, 2000). Three specific avian examples illustrate this relation. In the first one, recruitment of new neurons into a song system nucleus that encodes learned song (Hahnloser et al., 2002) peaks in adult individuals at the time they acquire new song (Kirn et al., 1994). In the second example, increases in new hippocampal neurons occur at a time when there is a sharp increase in the number of food items hidden and retrieved (Barnea and Nottebohm, 1994), a task thought to depend on hippocampal skills (Krebs et al., 1989; Patel et al., 1997; Biegler et al., 2001). In the third example, recruitment of new

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Contract grant sponsor: The Open University of Israel.
Contract grant sponsor: The National Institute for Psychobiology, Israel.
© 2007 Wiley Periodicals, Inc.
Published online 12 February 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/dneu.20379

neurons into the adult nidopallium caudale (NC) increases when a subject moves into an aviary where it encounters many conspecifics it had not met before (Lipkind et al., 2002; Barnea et al., 2006); the NC receives auditory inputs (Vates et al., 1996) and may be necessary for storing auditory memories. Taken together, these observations suggest that increases in new neuron recruitment are associated with expected or actual increases in memory load and that this is particularly so in parts of the brain that process and perhaps store this new information (Nottebohm, 1984, 2002b). The above evidence is circumstantial and correlational, but some recent studies in mammals, where neurogenesis has been manipulated, support a relation between neurogenesis and memory. For example, it has been shown that systemic treatment with a toxin that blocks cell proliferation and therefore recruitment of any new cells interferes with some types of hippocampal-dependent learning (Shors et al., 2001, 2002). Similarly, inhibition of neurogenesis in rats by irradiation interfered with hippocampal-dependent memory function (Winnocur et al., 2006). Nevertheless, the question whether there is a causal link between neuronal recruitment and learning and memory, and whether newly born cells participate in these processes is still controversial (for example, see review by Leuner et al., 2006).

Parent–offspring recognition is another example of a learning and memory task. Its distinct temporal and spatial definitions and the presumed involvement of auditory cues allow us to ask questions related to the underlying neuronal mechanisms. Parents work hard to benefit their offspring and must ensure that this effort benefits only their own offspring. In birds that defend breeding territories, nests are usually spaced at distances of tens or hundreds of meters and in them the risk of providing parental care to foreign young is relatively low. Any fledgling close to the nest is considered part of the family and enjoys parental care (Cullen, 1957; Hudson, 1979; Harris, 1983; Brown, 1998). However, in colonial species in which young leave the nest while parental care is still given, offspring from several nests may intermingle and the probability of misdirecting parental care increases (Burtt, 1977; Beecher et al., 1981; Davis and McCafferey, 1989). In such colonial species, parents must memorize the identity of their young before they fledge. This ability is found in various colonial birds such as swallows (*Hirundo rustica*: Medvin and Beecher, 1986; and *Tachycineta bicolor*: Leonard et al., 1997), terns (*Sterna bergii*: Davies and Carrick, 1962), cockatoos (*Cacatua roseicapilla*: Rowley, 1980), penguins (*Spheniscus demersus*: Seddon and Vanheezik, 1993), gulls (*Larus atricilla*: Beer, 1970), murrelets (*Ynthliboramphus antiquus*: Jones et al., 1987) and Piñon jays (*Gymnorhinus cyanocephalus:* McArthur, 1982).

Unlike in mammals, in which parent–offspring recognition relies on various combinations of visual (Ferreira et al., 2000), olfactory (Maletinska et al., 2002; Dobson and Jouventin, 2003; Phillips, 2003) and auditory (Illmann et al., 2002; Terrazas et al., 2003; Mathevon et al., 2004) cues, avian parents seem to recognize their offspring by their voices (Saino and Fasola, 1996; Jouventin and Aubin, 2002; Insley et al., 2003); no visual or olfactory cues are known to play a role in this recognition. Hence, parental care after fledging requires that parents learn and remember the distinctive vocalizations of their nestlings while the latter are still in the nest, and that they maintain or update these memories for as long as parental care is given (Soler et al., 1995; Lefevre et al., 1998; Insley et al., 2003). Species like the zebra finch (*Taeniopygia gutatta*), which produce more than one clutch per breeding season (Zann, 1996), have to go through this learning process more frequently than single clutch breeders. In zebra finches, nestlings develop by day 15 long tonal calls to which the parents reply selectively (Zann, 1984). After fledging, the long tonal calls of fledglings gradually develop, in both sexes, into distance calls which are also structurally unique to each individual. Zann (1996) cites an experimental study (McIntosh, 1983) which shows that parents discriminate the long tonal/distance calls of their own fledglings from those of others. Mother and father responded equally and responded to the calls of their offspring as young as 20 days of age.

In the present study, we tested the possibility that there is an increase in new neuron recruitment in NC of adult male and female zebra finches that coincides with the need to memorize the vocalizations of nestlings before they fledge. This brain region includes various auditory relays (Vates et al., 1996; Mello et al., 1998) that play a role in the processing and recognition of sounds, including those used in communication (Mello et al., 1995; Ribeiro et al., 1998) thus we suggest that it may also be involved in recognition of newly fledged offspring. We also looked at the olfactory bulb of some of our birds to compare our main results from NC with those from a brain region not thought to play a role in vocal communication.

METHODS

General

Two outdoor breeding colonies at The Meier Segals Garden for Zoological Research at Tel-Aviv University, Israel, provided zebra finches for the study. Experimental birds were kept in their breeding colony (2 × 2 × 2 m cages) until the age of 50–70 days, by which time they were already independent, could be easily sexed by their plumage and were

banded for individual identification using a randomly chosen unique color combination of 1–4 plastic rings (A.C. Hughes, Middlesex, England).

Experimental Design

Once banded, males and females were removed from their native colony and put in an outdoors standard cage (65 × 35 × 45 cm). A varied number of juveniles (depending on their availability in the breeding colony) were put together with a few adult birds (at least 150 days old) to reach an overall number of 12 individuals, 6 males and 6 females per cage. These cages were spaced not closer than 40 m from others, providing a self contained visual and auditory community. Birds in these cages followed the natural photoperiod (10.1–14.7 h of light per day) and were exposed to the range of mean daily temperatures (12–30°C). These conditions allow birds to breed throughout the year. Individuals in each of our experimental groups were obtained at various times of the year to ensure that our results were not affected by seasonal changes in temperature and photoperiod. The experimental birds were kept under these conditions until the age of 100–120 days, by which time they had all reached sexual maturity (typically at 90 days).

First Breeding Cycle. When experimental birds in a given standard cage reached the age of 100–120 days, all the 12 birds in that cage (experimental birds and unrelated adults) were transferred into a large outdoor aviary (1.5 × 1.5 × 2 m). The interior arrangement of all these aviaries was identical. We wanted the experimental birds to already be experienced parents when the experiment started and therefore provided them with nest boxes, nesting material (cotton wool and grass) and allowed them to breed for 60 days. At the age of 160–180 days, only experimental birds that succeeded in raising nestlings for at least 3 days post hatching, and could be therefore considered as "proven" breeders, continued to the next stage of the experiment as described below. The number of female and male experimental birds that became "proven" parents and could continue to the next stages was not necessarily equal because sometimes an experimental bird did not have a mate that had been brought up in the same manner and was of the same age. This is because, as described above, the number of potential experimental birds in a cage of 12 birds depended on their availability in the breeding colony at the time the cage was set up. Therefore, an experimental bird could breed either with another experimental bird of the opposite sex (in which case they would both continue to the next stages), or breed with an individual that was not part of the experimental cohort (in which case only the experimental bird would continue to the next stages). As a result, the number of females and males in each of the three experimental groups – Hatching, Fledging, Independence (see below) – was not equal.

Hormonal Rest. At the end of their first breeding cycle, the "proven" parents could have been at different reproductive stages, that is, with nestlings, fledglings, or completely post breeding. This variation probably affected their hormonal levels. In order to minimize this possible source of variation each of the "proven" breeders was housed singly in an aviary similar to the ones used for the previous stage, but without nest boxes, cotton wool and grass for the next 14 days.

Second Breeding Cycle. Following the "hormonal rest," an adult mate of the opposite sex was introduced into each cage of the "proven" breeder. The mate could have either been another "proven" experimental breeder or an unrelated adult, but in both cases this introduced bird was a new mate to the experimental bird. Therefore in all cases, experimental birds had different mates in each breeding cycle. As in the first breeding cycle, nest boxes, cotton wool, grass and artificial rain (by using a garden hose) were provided, to encourage breeding (Farner and Serventy, 1960). The conditions were similar to those provided during the first breeding cycle, only this time a single pair was housed in each aviary, which was in visual and auditory isolation from other zebra finches, which was effected by covering the lateral walls with burlap and placing it at least 50 m away from other aviaries.

Comparing New Neuron Counts in the Three Groups Tested

We wanted to compare new neuron recruitment in the brains of the "proven" breeders at three time points during this second breeding cycle: at hatching of their young; when their young fledged; and when their young reached independence and did not require further parental care. To do this, we had to calculate back, for each of the above timepoints, when to treat the "proven" breeders with the cell birth marker [^{3}H]-Thymidine (see following), in order to allow enough time for the new neurons born at the time of treatment to reach their final destination and differentiate before the brains were harvested. Our counts of [^{3}H]-labeled neurons focused on the Nidopallium caudale (NC; see following). In some of the brains we also looked at the olfactory bulb (OB), as an additional, reference region (see following for more details).

There are no data that tell us how long it takes a neuron born in adult zebra finches to reach destination in the NC and differentiate there. We know that in canaries it takes 1–2 weeks for a neuron born in adulthood to reach its destination in the High Vocal Center (HVC) and differentiate. Then, between weeks 2 and 3, there is an abrupt drop in the number of these neurons, so that only half of them remain at the end of the third week, and this number does not change further between then and the end of the fourth week (Kirn et al., 1999). However, another study that used zebra finches and focused on new neuron recruitment in telencephalon at the level of anterior commissure, showed that no cells with the phenotype of a differentiated neuron were found before day 20 after [^{3}H]-Thymidine administration and the counts of new neurons continued to increase until day 40 (Alvarez-Buylla and Nottebohm, 1988). Because of the uncertainty in our system (zebra finches and NC), regarding the time from treatment until the completion of migration and differentiation of the new neurons, it seemed reasonable to allow about 40 days from treatment until the

specific behavioral event that might affect the survival of these new neurons. By that time, we reasoned, the neurons born 40 days earlier would be ready for their tasks. We use the term "recruitment" to refer to these neurons, without presuming to know how many neurons had been born into that labeled cohort and how many had already been culled.

We divided the "proven" breeders into three groups, each tested at a different time during its second breeding cycle. We will refer to these groups as Hatching, Fledging, and Independence, indicating the offspring's stage at the time that we quantified, in the parents' brain, the presence of 40-day-old neurons. Each group was treated as follows:

Hatching. In this group we wanted to look at the recruitment of 40-day-old neurons in brains of parents soon after their young hatch. For this, we treated parents with [^{3}H]-thymidine during the first two days of the "hormonal rest" period, according to the following general calculation: 14 days hormonal rest, 6–7 days nest building, 5 days laying, 11–12 incubation days, plus 2–4 days after hatching of the young; this yields a total of about 38–42 days.

Fledging. In this group we wanted to look at the recruitment of 40-day-old neurons in brains of parents at the time their young fledged or soon thereafter, when their offspring still needed parental care. For this, we treated parents with [^{3}H]-thymidine during the first 2 days of incubation, according to the following general calculation: 11–12 incubation days, 25–26 days with nestlings, plus 2–4 days taking care of fledglings; this yields a total of about 38–42 days.

Independence. In this group we wanted to look at neuronal recruitment in brains of parents when their young reached independence and no longer required parental care. For this, we treated parents at the time their young left the nest, according to the following general calculation: during the first 14 days after fledging the young still needed parental care plus 25–26 days when the young are already independent; this yields a total of about 39–40 days. Nest boxes and nesting material were removed after the young left the nest to prevent the initiation of a new breeding cycle.

Calculations for timing of treatment with [^{3}H]-thymidine, in the three experimental groups, were based on our experience with zebra finches in our breeding colonies, which were also in agreement with data from others (reviewed in Zann, 1996). In this study we included only birds whose behavior conformed to our calculations. For example, if birds in the Hatching group did not build a nest within the 6–7 days after the end of the hormonal rest, they were excluded from the study. This was done to ensure that all birds in a group would be at the same breeding stage at the time they were killed. It limited, however, sample sizes in each experimental group.

[^{3}H]-Thymidine Treatment, Histology, and Autoradiography

New neurons were labeled by intramuscular injections of a radioactive form of thymidine ([^{3}H]-thymidine), which is a marker of DNA synthesis and therefore of cell birth (Sidman, 1970). Since the level of DNA synthesis that occurs during the S-phase of mitosis is approximately two orders of magnitude higher than that during DNA repair (Palmer et al., 2000), there is little risk of the latter contaminating the labeling for the former phenomenon. Each bird received three injections (6.7 Ci/mM; 50 μCi) into the pectoral muscle, at 12 h intervals: 6–7 am, 6–7 pm and 6–7 am of the following day. Forty days after last injection, birds were weighed, killed (overdose of anesthesia) and perfused with 20 mL of saline followed by 50 mL of 4% paraformaldehyde in 0.1 *M* sodium phosphate buffer, pH 7.4 (PB). As mentioned, this 40 days post-injection period allowed enough time for new neurons to migrate to their final destination and go through final anatomical differentiation (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1999). We refer to neurons at that stage of survival and differentiation as "recruited" neurons. After perfusion, brains were removed, weighed, placed in the same fixative for a minimum of 7 h, then embedded in polyethylene glycol, blocked transversely and cut at 6 μm intervals. Serial sections (every 10th; i.e. at intervals of 60 μm) were collected in PB and mounted on chromalum-coated 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).

Identification of Neurons Using Cresyl Violet and Immunohistochemistry, and Use of Neu-N Marker to Validate Cresyl Counts of New Neurons

Cells were identified as neurons by the size, shape, and staining properties of their nuclei: The nucleus had to be larger than 5 μm in diameter, round, with clear nucleoplasm, and one or two darkly stained nucleoli. All these features are readily apparent in tissue stained with cresyl violet, a Nissl stain. The reliability of cresyl violet-based identifications of new neurons in songbirds has been previously confirmed by electron microscopy in HVC (Goldman and Nottebohm, 1983; Burd and Nottebohm, 1985). In addition, HVC [^{3}H]-labeled cells with neuronal morphology have been identified as neurons also by intracellular neurophysiological recordings (Paton and Nottebohm, 1984), bolstering the validity of the Nissl stain identification. A neuron was considered to be [^{3}H]-labeled if, as a result of autoradiography, the number of exposed (black) silver grains over its nucleus 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 (Lipkind et al., 2002; Barnea et al., 2006).

We wanted to confirm the cresyl violet-based counts of [^{3}H]-labeled neurons using a neuron-specific stain. For this, four adult male birds that were not part of our experimental

groups were treated with [^{3}H]-thymidine similarly to the experimental birds and killed 40 days after the last injection. Their brains were fixed and cut. Alternate sections were stained with cresyl violet or with the neuron-specific marker anti-Neu-N (mouse monoclonal anti Neu-N, Chemicon International, Temecula, CA) that has been used successfully in mammals (Mullen et al., 1992; Wolf et al., 1996) and birds (Ling et al., 1997). In our material the Neu-N marker stained the nucleus and the cytoplasm of post-migratory neurons. This comparison was the same as in Lipkind et al. (2002) but used a new set of birds. The results of the comparison, applied both to NC and OB neurons, were very similar to those described in the earlier publication.

Mapping and Quantification

Nidopallium Caudale. Adult birds of various taxonomic groups use the vocalizations of juveniles to guide their parent–offspring recognition (Rowley, 1980; Beecher et al., 1981, 1986; Harris, 1983; Medvin and Beecher, 1986; Jones et al., 1987; Congdon, 1991; Barg and Mumme, 1994; Saino and Fasola, 1996; Leonard et al., 1997; Lefevre et al., 1998). We focused our interest on NC (Jarvis et al., 2005) because this brain region is known to include auditory projections (Vates et al., 1996) likely to play a role in the recognition of vocal signals (Leppelsack and Vogt, 1976; Mello et al., 1992, 1995; Mello and Clayton, 1994; Ribeiro et al., 1998).

NC boundaries are easy to recognize: The wall of the lateral ventricle and the surface of the brain define its medial, dorsal and lateral limits and the dorsal lamina of the archipallium (LAD) provides its ventral boundary. The rostro-caudal extent of our sampling in NC is shown in Figure 1. NC extends rostrally and caudally beyond the region we sampled; therefore, the volume of NC that we report refers only to the tissue bracketed between the arbitrary anterior and posterior limits that we set for our sampling, defined by distance from point zero in the rostrocaudal axis. Point zero, when looking at the whole brain from above, is over a blood sinus at the medial confluence of the two hemispheres and the rostral tip of the cerebellum, see Figure 1.

Ideally, one would want to sample as many sections as possible, but given the number of birds involved and time limitations, we settled for a spacing of 300 μm between tissue sections sampled (see following). We used a computerized brain mapping system (Neurolucida; Stereo Investigator, from MicroBrightField) to draw the boundaries of NC in each section sampled, to count neurons and to quantify other neuronal parameters, as described below. All mapping was done "blind" as to the experimental conditions. NC was sampled in all brains from the three experimental groups. Preliminary mapping showed no hemispheric differences in the number of labeled NC neurons per mm^3. Therefore, for purposes of characterizing neuronal recruitment in the three experimental groups we mapped sections only from the left hemisphere.

The following measurements were taken in all brains:

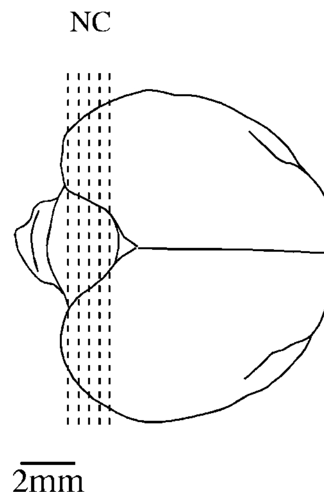

Figure 1 Top schematic view of the brain of an adult zebra finch; anterior is to the right, posterior to the left. Dashed lines indicate the position of the five frontal sections taken from Nidopallium Caudale (NC). Distance between sections is 300 μm. For anatomical details of the sections, see Figure 4 in Lipkind et al. (2002).

Estimate of NC Volume Sampled. This volume was defined by the position of the rostralmost and caudalmost sections used for counting [^{3}H]-labeled neurons. It was estimated for the NC of each bird by measuring the area of NC in each of the five sections used for counting [^{3}H]-labeled neurons, adding these areas and multiplying the sum by the sampling interval between sections (300 μm).

Number of [^{3}H]-Labeled Neurons per mm^3. In all brains, and in each of the five NC sections mapped in each brain, we used a 63X objective and the Neurolucida fractionator probe, to count the number of labeled neurons. In each section this count was made in at least 90 non-overlapping sampling squares (each of an area of 19,600 μm^2) that were randomly chosen by the software across the section. Labeled neurons were counted only if their nuclei completely appeared within the boundaries of the squares. We realize that this manner of counting led to a slight under estimate because we excluded neurons whose nuclei straddled the boundaries of the sampling squares, but since it was applied to birds in all groups we do not see it as a source of bias. In each of the five sections that were sampled, we also measured the area of NC and nuclear diameters of [^{3}H]-labeled neurons (see following). Based on the section thickness (6 μm) and nuclear diameters of labeled neurons, and by using the Abercrombie stereological correction equation (Guillery and Herrup, 1997), we could estimate the number of [^{3}H]-labeled neurons per unit volume in NC in each section mapped.

Search for Rostro-Caudal Differences in New Neuron Recruitment. Previous studies in our lab (Lipkind et al., 2002; Barnea et al., 2006), which tested the effect of social environment on neuronal recruitment and survival, indicated that the caudal reaches of the NC volume sampled might be more sensitive to variables affecting new neuron

recruitment than the rostral ones. Since NC is a large brain area, these findings raised the possibility that NC might be composed, rostrocaudally, of successive domains that respond differently to environmental variables. Therefore we tested for this possibility by using the number of [^{3}H]-labeled neurons per mm^3, obtained from the five NC sections which we sampled along the rostro-caudal axis of NC.

Additional Measurements. From each brain we took the following additional measurements: estimate of neuronal density, mean nuclear diameter of all neurons, mean nuclear diameters of labeled neurons, and number of exposed silver grains per cell nucleus. However, for the same reason explained above, of NC being a large area, we wanted to test first whether any of these parameters show a rostral-caudal gradient. To do this we randomly sampled four brains in each experimental group, in which we measured these parameters in two sections: the 2nd and the 4th, representing the rostral and caudal halves of NC. As we shall see in the Results, a comparison of the data from these two sections did not find significant differences in any of the tested parameters. From this we concluded that these parameters do not show rostro-caudal gradient, and in the rest of the brains we measured them only in the 4th section. Accordingly, the data presented in the Results for these additional measurements are from the 4th section, and these data were also used for further calculations, such as the Abercombie correction. These additional measurements were obtained as follows:

Estimates of neuronal density. Neurons (labeled and unlabeled) were counted in eight squares each with an area 0.02 mm^2 (140 × 140 μm^2). The position of the squares was randomly chosen by the mapping software, using the Neurolucida fractionator probe. Neurons were counted only if their nuclei completely appeared within the boundaries of the squares. These counts yielded 155–224 neurons per brain. Again, this procedure of excluding neurons whose nuclei straddled the boundaries of the sampling squares resulted in a slight under estimate, but since it was applied to birds in all groups we do not see it as a source of bias. As explained above, these data, with the knowledge of the thickness of our tissue sections (6 μm), and of the nuclear diameter of all neurons in NC (see following), allowed us to estimate, using the Abercrombie stereological correction, the total number of NC neurons per unit volume in each section which was mapped.

Nuclear diameters of all neurons. In four of the eight samples taken in NC we measured nuclear diameters of total (labeled and unlabeled) neurons, yielding measurements of 73–130 neurons per brain. From these measurements we calculated, for each brain, the mean neuronal nuclear diameter in NC. This variable is important for comparing neuronal counts, because neurons with larger nuclei would tend to be over-represented in such counts. We used this information for the stereological correction to accurately estimate numbers of total neurons per mm^3.

Nuclear diameters of [^{3}H]-labeled neurons. Nuclear diameters of [^{3}H]-labeled neurons were similarly measured and yielded measurements of 7–17 labeled neurons per brain.

Number of exposed silver grains per cell nucleus. The number of silver grains per neuronal nucleus in NC was counted in each of the [^{3}H]-labeled neurons whose diameter was measured and whose [^{3}H]-labeling met our criterion for a labeled cell. From these counts we arrived, for each brain region, at a mean number of grains per [^{3}H]-labeled neuronal nucleus.

Olfactory Bulb. As explained above, we focused on NC because it is likely to play a role in auditory parent–offspring recognition. However, we wanted to look at another non-auditory brain region, which might also be involved in communication, to see whether neuronal recruitment differed between these two brain regions. For this, we chose the OB. However, because of their small size and their frontal and external position in the brain the OB was sometimes lost in dissection and so we obtained OBs for only some of the brains. The most caudal section of OB was at the point where the brain looked similar to that of the canary atlas at level A7.0 (Stokes et al., 1974). From this point we tried to collect and mount, rostrally, serial sections at 60 μm intervals, a process that yielded a maximum of 8–9 OB sections per brain. Because of the difficulties in preserving the OB, we could not obtain such complete sets of OB serial sections in all brains. We mapped (using the same system described earlier for NC) all available OB sections—an average of five sections per brain, equivalent to an average area of 1.2 mm^2.

All mapped sections were completely scanned for [^{3}H]-labeled neurons, which were counted and their nuclear diameters were measured. We used the largest OB section from each brain to estimate the neuronal density and nuclear diameters of all neurons. These measurements were obtained as described for the same parameters in the NC (see earlier). To estimate neuronal density we sampled four squares from the largest section, each with an area of 0.02 mm^2 (yielding 40–122 neurons per brain); nuclear diameters of all neurons were sampled in two of these four squares (yielding 33–67 per brain). We estimated, from all these data, using the stereological correction described above for NC, the total number of neurons and the number [^{3}H]-labeled neurons per OB unit volume in each of the brains analyzed.

An Additional, non-Breeding Group

As explained above, our experimental design included only breeders, tested at three stages during a breeding cycle (Hatching, Fledging, Independence). In addition, we wanted to compare the results from these groups with results obtained from non-breeding birds. For this we used birds from another, still unpublished study (Barnea et al., unpublished data), in which we kept birds in small colonies (total of 20 adult males and females) under similar conditions as those that our birds experienced during the first and second breeding cycles (for details see earlier). In that unpublished study, six males which hatched in one of the colonies grew up there with some other hatchlings, until

they reached adulthood. Then, still in their native colony, these six males were allowed to breed once, up to the time their young became independent. At the end of their first breeding cycle nests were removed, nest material was not provided and the birds could not breed. A month later, when they were 6 months old (same age as our birds), they were treated with [^{3}H]-thymidine (same protocol as the one used here), and killed 40 days later (same survival as the one in the present study). During that whole time, the birds continued to be housed in the same colony. The NC of these birds was mapped following the same protocol as here. Statistical comparisons between this group and the three groups in our present study are not in order because of a few reasons: the non-breeding individuals were not part of the original design of the experiment reported here; they were all males; and some of the treatment conditions were different. However, for the purpose of comparing our results with a non-breeding situation, we believe that the results from these birds can be used.

Statistical Analysis

The following data were subjected to statistical analysis: Body and brain mass, number of [^{3}H]-labeled neurons per mm^3, number of all neurons (labeled and unlabeled) per mm^3, mean nuclear diameter of all (labeled and unlabeled) neurons, mean nuclear diameter of labeled neurons only, and mean number of silver grains per neuronal nucleus of [^{3}H]-labeled neurons. Data that were expressed as number of cells per mm^3 or as silver grains per single neuron, were transformed, prior to the statistical analysis, by using the square root transformation. (These kinds 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 (Sokal and Rholf, 1995)). Throughout the statistical analysis, $p \leq 0.05$ was considered significant. For comparing numbers of labeled neurons per mm^3 obtained from several section levels, analysis of variance was performed using ANOVA (repeated measures). Because of the small group sizes we did not compare the results for males and females within each experimental group, but pooled the data from males and females. This seemed reasonable since our data did not show obvious sex differences and the question asked—does neuronal recruitment change by stage of the breeding cycle—did not make sex specific predictions because both parents feed the young and thus must identify them properly. Other data were analyzed by one-way ANOVA. Post hoc comparisons were carried out by the Bonferroni (HSD for unequal *N*) method. Correlation analysis for [^{3}H]-labeled NC neurons per mm^3 and the number of nestlings was made using the Pearson Correlation test.

RESULTS

Nidopallium Caudale

Sample sizes in each group were as follows: $N = 7$ in Hatching group (4 females and 3 males); $N = 7$ in Fledging group (4 females and 3 males); and $N = 6$ in Independence group (2 females and 4 males). Overall, our results come from 20 individual Zebra finches.

NC Volume. There was no significant difference ($p = 0.45$) in the sampled NC volume between experimental groups: Hatching 15.74 ± 2.87 mm^3; Fledging 16.52 ± 4.78 mm^3; Independence 18.40 ± 2.92 mm^3.

Nuclear Diameters of all NC Neurons and of Just [^{3}H]-Labeled Neurons. As explained under Methods, we first looked for possible rostro-caudal gradients in nuclear diameters of NC neurons, by comparing the 2nd and 4th sections in a sample of 12 brains (four brains per group). No significant differences were found between these sections in either mean nuclear diameters of all NC neurons (9.5 ± 0.9 μm vs. 9.4 ± 0.9 μm; $N = 12$; $p = 0.67$), or between mean nuclear diameters of just [^{3}H]-labeled neurons (11.4 ± 1.1 μm vs. 12.2 ± 0.9 μm; $N = 12$; $p = 0.09$). Therefore we compared in all 20 brains neuronal diameters between experimental groups only in the 4th section of NC, as representative for the whole region mapped. No significant differences were found between experimental groups in nuclear diameter of total NC neurons (mean 9.4 ± 0.8 μm; range: 5.2–15.6 μm; $N = 20$; $p = 0.99$) or of [^{3}H]-labeled NC neurons (mean 12.2 ± 1.1 μm; range: 7.7–16.2 μm; $N = 20$; $p = 0.43$). Therefore, comparisons of total neuron number and [^{3}H]-labeled neuron number per unit area of NC could have been made directly between the experimental groups without the need to modify our counts using stereological corrections. However we did the stereological corrections as we transformed our counts of number of neurons per unit area to number of neurons per unit volume.

Estimates of Neuronal Density. Here too, we first looked for possible rostro-caudal gradients in this parameter, by comparing NC neuronal densities in the 2nd and 4th sections in a sample of 12 brains (four brains per group). No significant differences were found between these sections in estimates of neuronal number per unit volume (mean of 63,455 ± 9987 neurons/mm^3 vs. 73,298 ± 10,029 neurons/mm^3; $N = 12$; $p = 0.07$). Therefore in all brains we compared estimates of neuronal density between experimental groups only in the 4th section of NC, as a representative for the whole region mapped. No significant differences were found between experimental groups in estimates of neuronal density ($p = 0.36$), and mean estimates were as follows: Hatching group: 80,719

± 21,910 neurons/mm^3 (N = 7); Fledging group: 91,120 ± 20,482 neurons/mm^3 (N = 7); Independence group: 76,155 ± 12,943 neurons/mm^3 (N = 6). Total mean neuronal density was 82,990 ± 19,214 neurons/mm^3 (n = 20).

Mean Number of Exposed Silver Grains per [^{3}H]-Labeled NC Neuronal Nucleus. No significant differences were found in number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus between brains that came from different dipping batches (p = 0.11). This indicates that possible differences in autoradiographical procedure, which might have existed between batches, did not affect the number of grains per nucleus.

As in previous parameters, no significant differences were found between the 2nd and 4th NC sections, in a sample of 12 brains (four brains per group), in mean number of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus (14.1 ± 3.1 gains vs. 15.1 ± 3.1; N = 12; p = 0.36). Therefore in all brains we compared number of grains per [^{3}H]-labeled NC neuronal nucleus between experimental groups only in the 4th section of NC, as a representative for the whole region mapped. No significant differences were found between experimental groups in numbers of exposed silver grains per [^{3}H]-labeled NC neuronal nucleus (p = 0.45), and mean numbers were as follows: Hatching group: 16.4 ± 3.0 gains per labeled neuron (N = 7); Fledging group: 14.7 ± 3.2 (N = 7); Independence group: 17.0 ± 4.1 (N = 6). Total mean number of exposed grains per labeled neuron was 15.9 ± 3.4, (N = 20).

Number of NC [^{3}H]-Labeled Neurons per mm^3 as a Function of Breeding Stage. We wanted to know whether different stages during a breeding cycle affect the recruitment of new NC neurons per mm^3 in the parent brain. However, since we sampled five sections along the rostro-caudal axis in each brain, we first had to establish whether we could pool the results from all sections. A statistical analysis, which used data from all brains, did not find any significant rostro-caudal differences in number of [^{3}H]-labeled neurons per mm^3 between sections (p = 0.46), and no significant interaction between section position and experimental group (p = 0.8). Therefore, for each experimental group, data were pooled from all five sections.

Analysis of the number of [^{3}H]-labeled neurons per mm^3 revealed significant differences between experimental groups ($F_{(2, 19)} = 7.262$, $p = 0.005$), with birds in the Fledging group having significantly more labeled neurons per unit volume than birds in the Independence group (p = 0.04). No significant differences were found between Hatching and Fledging (p = 0.37) or between Hatching and Independence groups (p = 0.12), (Fig. 2). Because of the relatively large standard deviations of neuronal densities which we got in the tested groups, we also calculated the percentages of NC [^{3}H]-labeled neurons as a function of breeding stage (Fig. 3). Statistical analysis revealed similar results: significant differences between experimental groups ($F_{(2, 19)} = 4.508$, $p = 0.027$), with birds in the Fledging group having significantly higher percentages of labeled neurons than birds in the Independence group (p = 0.03), and no significant differences between Hatching and Fledging (p = 1) or between Hatching and Independence groups (p = 0.1).

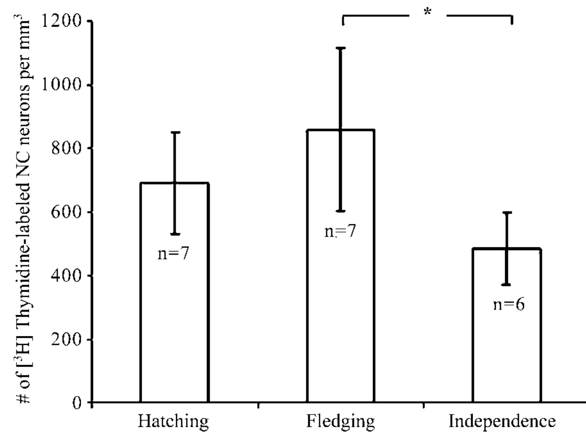

Figure 2 Number of NC [^{3}H]-labeled neurons/mm^3 in the three experimental groups. Data are presented as means (± S.D.); * indicates $p \leq 0.05$. n = sample size.

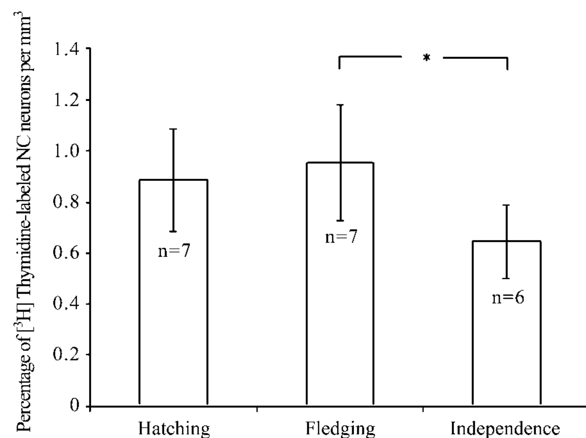

Figure 3 Percentage of NC [^{3}H]-labeled neurons in the three experimental groups. Data are presented as means (±S.D.); * indicates $p \leq 0.05$. n = sample size.

Number of NC [^{3}H]-Labeled Neurons per mm^3 as a Function of Number of Nestlings. We found a positive and significant correlation between the number of nestlings and recruitment of new NC neurons per mm^3 in the brain of parents killed soon after the young fledged (Fledging group), as presented in Figure 4 ($r = 0.778$; $p = 0.018$, $N = 8$): the more nestlings a parent had to raise, the more new neurons were recruited into the NC in its brain. Tests for this correlation in the Fledging group used the data from the seven parents included in the Fledging group in Figure 2. The number of juveniles fledged by parents in this group ranged from 1 to 3 nestlings per parent. We added to the data of these seven parents data from another parent (not included in the Hatching group in Fig. 2), whose single nestling died 3 days after hatching, and therefore this parent was considered as a parent that bred but did not have to memorize the identity of any offspring at fledging time. Accordingly, the data point for this parent was entered at the zero nestling number. Because of the small sample size we could not test for sex differences. Yet, the data in Figure 4 suggest that no sex differences occurred.

We also calculated the percentages of NC [^{3}H]-labeled neurons as a function of number of nestlings (Fig. 5). Statistical analysis revealed similar results, that is, a positive correlation between number of nestlings and recruitment of new NC neurons in the brains of parents in the Fledging group ($r = 0.686$; $p = 0.048$, $N = 8$).

Olfactory Bulb

For reasons explained under Methods, OB was mapped only in some brains, from the Fledging and Independence groups. In each group we could map

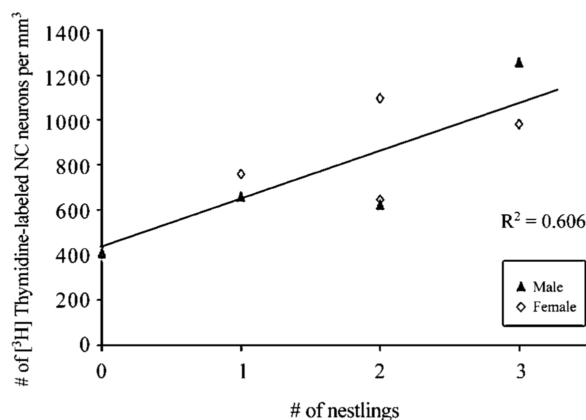

Figure 4 Mean number of [^{3}H]-labeled neurons/mm^3 in NC of parents, as a function of the number of nestlings they have raised. (The two individuals which raised three nestlings formed a pair).

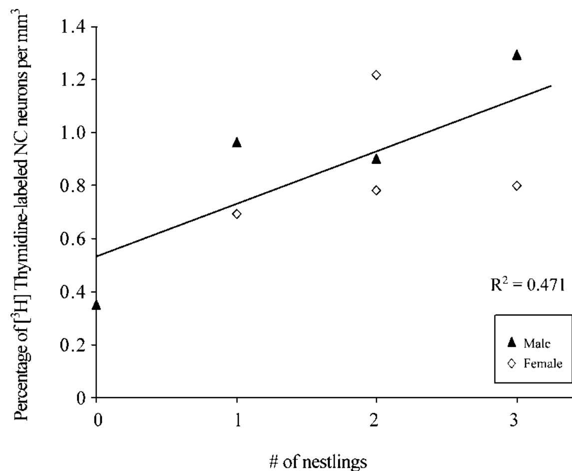

Figure 5 Mean percentage of [^{3}H]-labeled neurons in NC of parents, as a function of the number of nestlings they have raised. (The two individuals which raised three nestlings formed a pair).

three brains (1 female and 2 males). For similar reasons as in NC, data from males and females were pooled. As already explained above, although the OB data were derived from only a fraction of the brains studied, we present them here because they enable us to compare results from two parts of the forebrain serving two different sensory modalities potentially involved in communication. It must be remembered that zebra finches spend a good deal of time in close contact and grooming each other's feathers and members of a pair often sleep perched next to each other and in close contact, so that the exchange of olfactory signals would not seem out of the question.

Nuclear Diameters of all OB Neurons and of Just [^{3}H]-Labeled Neurons. No significant differences were found between the two experimental groups (Fledging and Independence) in nuclear diameter of total OB neurons (means of 7.3 $\pm$ 0.4 μm and 6.7 $\pm$ 0.3 μm, respectively; $N = 3$ for each group; $p = 0.07$), or in nuclear diameter of [^{3}H]-labeled OB neurons (means of 8.9 $\pm$ 0.7 μm and 8.5 $\pm$ 0.8 μm; $N = 3$ for each group; $p = 0.51$).

Estimates of Neuronal Density. No significant differences were found between the two experimental groups (Fledging and Independence) in estimates of neuronal density (mean of 75,278 $\pm$ 4,493 neurons/mm^3 and 78,610 $\pm$ 6,877 neurons/mm^3; $N = 3$ for each group; $p = 0.97$).

Number of OB [^{3}H]-Labeled Neurons per mm^3 as a Function of Breeding Stage. Analysis of the mean

number of [^{3}H]-labeled neurons per mm^3 revealed no significant difference between two experimental groups Fledging and Independence (means of 574.0 ± 119.0 labeled neurons per mm^3 and 666.8 ± 190.6 labeled neurons per mm^3, respectively; $N = 3$ for each group; $p = 0.52$). We also calculated percentages of OB [^{3}H]-labeled neurons per mm^3 in these two groups, and statistical analysis revealed similar non-significant results, with means of 0.10% ± 0.01% and 0.12% ± 0.07%; $N = 3$ for each group; $p = 0.6$).

Neu-N Labeling

The numbers of [^{3}H]-labeled NC neurons per mm^2 that were counted in two adjacent sections (one reacted with cresyl violet and the other with anti-Neu-N antibody) of four birds were as follow: 283 vs. 309; 515 vs. 509; 268 vs. 298; 281 vs. 295. These results are very similar in all cases and were in line with the results of similar comparisons reported previously (Lipkind et al., 2002; Barnea et al., 2006). We take this as validation of our use, for quantification purposes, of sections stained with cresyl violet in which neuronal identity was established by the size, shape, and staining pattern of nuclei—relatively large, with clear nucleoplasm and a single or double, darkly staining nucleolus. Counts of [^{3}H]-labeled OB neurons also were very close when comparing cresyl and Neu-N stained material; this is important for there are no prior published accounts of [^{3}H]-labeling, and thus inferred new neuron recruitment, for the OB of songbirds.

Body and Brain Mass

No significant differences were found in either body mass ($p = 0.68$; measured before birds were killed) or brain mass ($p = 0.43$; measured after perfusion) between experimental groups. Mean body mass was 13.2 ± 1.3 g; $N = 20$, and mean brain mass was 0.43 ± 0.03 g; $N = 20$.

An Additional, non-Breeding Group

As explained in Methods, we used an additional group of non-breeding males (from another, still unpublished study from our laboratory), for a comparison with the three groups of breeders in our study. The mean NC neuronal recruitment in this additional group was found to be 345 ± 141 new neurons per mm^3. This value is the lowest in comparison with our groups; it is lower than the one observed in the Hatching group (689 ± 160 new neurons per mm^3) and even lower than that observed in the Independence group (486 ± 111 new neurons per mm^3). Similar tendency occurs when calculating percentages of NC [^{3}H]-labeled neurons, and making the same comparison: the non-breeding birds had a mean percentage of 0.37% ± 0.17% new neurons, less than the Hatching group (0.89 ± 0.02%) and even less than the Independence group (0.65 ± 0.15%). For reasons explained above we did not make statistical analysis between the non-breeding group and the three breeding groups in our present study. However, for the purpose of comparing our results with a non-breeding situation, we believe that the results from this additional group can be used, to show that neuronal recruitment in a non-breeding stage decreases to the lowest level observed.

DISCUSSION

Neuronal Recruitment in NC of Parents' Brains Increases at the Time Their Young Fledge

The results we present here show that the recruitment of new neurons into the nidopallium caudale (NC) of breeding zebra finches increases at about the time their young fledge; our data do not allow for greater temporal resolution than this. These results are consistent with our initial hypothesis which was based on the following previous observations and rationales: (1) NC plays an important role in the processing of sounds (Vates et al., 1996); (2) the calls of fledglings convey their individual identity (McIntosh, 1983; Stoddard and Beecher, 1983); (3) the fitness of parents gains when they selectively feed their offspring (Hamilton, 1964); (4) in systems where neuronal recruitment occurs in adulthood, this recruitment peaks when new memories replace older ones (Nottebohm, 2002b).

We looked at neuronal recruitment in the NC of zebra finch parents at three stages during the breeding cycle: soon after hatching, after young fledged from the nest but still needed parental care, and when young were independent and no longer needed parental care. We found that the recruitment of new NC neurons was highest at about the time when the young fledged and were still in need of parental care. We suggest that this increase in neuronal recruitment facilitates the parent–offspring recognition that occurs in this species (McIntosh, 1983), at a time when the young are most vulnerable, still dependent on parental feeding, yet, because of increased mobility, easily lost among the throng of other fledglings from the same colony. The increase in neuronal recruitment seen soon after the young fledged was followed by a significant decrease when the young

reached independence and parental care was no longer given. This finding is also in line with the observations that the response of parents to vocalizations of their own fledglings decreases when the young reach 32–35 days of age (about 10 days post fledging and a few days before independence), and ceases completely soon afterwards (McIntosh, 1983). Since the number of [^{3}H]-thymidine labeled neurons was not significantly different at hatching and at fledging, the scaffolding for the vocal recognition of offspring may already be under construction by the time the young hatch and tracking by voice may start before they fledge. Our results and interpretation have a precedent. A wave of new neurons is added to the olfactory bulb of female mice shortly before pups are born, perhaps enabling, in this manner, their quick olfactory recognition and subsequent maternal care (Shingo et al., 2003). The same study has also shown that this recruitment of new neurons is mediated by prolactin. Similarly, we are currently conducting a follow up study, focusing on hormonal changes in zebra finch parents during a reproductive cycle, and their possible effect on neuronal recruitment and survival.

Neuronal recruitment in the NC of non-breeding males was the lowest observed, in comparison with the three breeding groups. The magnitude and direction of these differences are so marked as to suggest that neuronal recruitment into the NC of parents' brains during a breeding cycle builds up from a lowest level in non-breeding birds to a gradual rise when their young hatch, stays high or reaches a peak when they fledge, drops significantly when the young reach independence and drops further if a period of non-breeding follows the breeding cycle.

Other interpretations for the differences in neuronal recruitment during a breeding cycle cannot be completely ruled out at this stage and need not be mutually exclusive. For example, the addition of new neurons to the NC of zebra finch parents might be related to the need to recognize vocalizations of adult conspecifics, with whom the parents may interact in a natural setting; or it could represent a heightened sensitivity to the calls of their mates, with whom they must coordinate their reproductive cycle. However, these interpretations seem less compelling in light of the evidence that zebra finches use the calls of their offspring for recognition (McIntosh, 1983), a need that is restricted to a part of the breeding cycle. Since our breeding pairs were kept acoustically and visually isolated from other adult finches, no interactions with other adult conspecifics occurred. Moreover, it has been shown that members of a pair always recognize the calls of their partners (Silcox, 1979; McIntosh, 1983). A possible and interesting way to further test our hypothesis and interpretation regarding the role of the new NC neurons would be to look at the recruitment of NC neurons during the reproductive cycle of a non-colonial species, in which the incentive to discriminate between one's own offspring and those of others is less acute. It is possible, of course, that parents remember the food begging call of each of their offspring even before they fledge, to help them distribute food more evenly, and this need may not differ between colonial and non-colonial breeders.

Our data do not fully address the issue of whether the changes which we observed in NC neuronal recruitment were specific to this region, or occurred in all parts of the forebrain where neuronal recruitment was in evidence. In an attempt to address this question, we compared the NC results with observations in the olfactory bulb (OB), another brain region that might play a role in communication, albeit not auditory. Although OB data were derived from a fraction of the brains studied, and represent only two groups, they show a different pattern of neuronal recruitment than the one in NC, with no marked (nor significant) difference between the Fledging and Independence groups. In addition, levels of neuronal recruitment were much lower in the OB than in NC (0.10% and 0.12% vs. 0.95% and 0.65%, respectively). These observations, even if partial, are in line with previous instances of region-specific changes in neuronal recruitment associated with seasonal or social variables (Barnea and Nottebohm, 1994; Barnea et al., 2006).

Body and brain mass did not differ significantly between experimental groups and from this and other observations we infer that all birds remained in good health. In addition, since NC is a large brain area, we first looked, in some of the brains, for systematic rostro-caudal differences in some of the parameters which we measured: diameters of all neurons or of just [^{3}H]-labeled neurons, total (labeled and unlabeled) neuronal packing densities, and number of exposed silver grains per cell nucleus. We did not find such differences. Therefore we assumed that measurements of these parameters, taken in all brains from the caudalmost NC section, were representatives of the whole NC area sampled. When doing so we found no group differences in diameters of all neurons, or of just [^{3}H]-labeled neurons, and in total neuronal packing densities. Therefore, as explained in the Results section, we could have calculated new neuronal recruitment directly from our raw counts of number of neurons per unit area. Nevertheless, we converted these data into the customary estimate of numbers per unit volume, using appropriate stereo-

logical corrections. This should make it easier to relate the extent of neuronal recruitment that we observed to future studies of the same phenomenon in this and other species.

It is worth noting that mean estimates of total (labeled and unlabeled) neuronal packing densities, in the NC in the three experimental groups, showed relatively large variability, with standard deviations of about 20%. There are precedents to such variability in our previous studies (Lipkind et al., 2002; Barnea et al., 2006). This variability is probably not due to processing the brains in separate batches (which could lead to varying degrees of shrinkage of the sections), because no correlation was found between higher packing densities and smaller nuclear diameters, neither in the present study, nor in the two previous ones cited above. Another possibility is that this high variability in total neuronal packing densities might have to do with sex differences, because when we pooled the values for all females and compared them with those of all males, the numbers were higher in females and this result was close to significance ($F_{(2, 17)} = 3.498, p = 0.08$). If true, then having used both sexes in the same study yielded an unexpected and interesting outcome, which might be related to brain function. However, sample sizes of each sex in our study were too small for a reliable sex comparison, and therefore all we can say at this stage is that this issue deserves further and more systematic investigation, which we intend to do.

Number of exposed silver grains per cell nucleus also showed no group differences. Assuming that [^{3}H]-thymidine had comparable access to the dividing cells in all three groups and that the duration of the S-phase was similar in all three groups, differences between groups in number of [^{3}H]-labeled cells can be taken at face value. We realize that the variable we followed—number of new neurons present 40 days after their birth—does not distinguish between the contributions of number of neurons born, number of neuroblasts that migrate successfully, number of new neurons that differentiate and connect into existing circuits and the proportion of the latter cells that then survived. Other studies have shown that the survival of neurons in caudal nidopallium is particularly sensitive to the complexity of social context (Lipkind et al., 2002; Barnea et al., 2006) and so it is possible that had our birds been in groups closer in size to those found in natural colonies, neuronal recruitment would have been higher. That effect could have been disproportionately larger for parents with offspring at the fledging stage because the presence of more offspring from other pairs would have complicated offspring recognition.

The 40-day survival that we chose for the present study and the exact timing of the injections with respect to stage in the breeding cycle were based on earlier work in canaries, not zebra finches, and in other forebrain regions (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1999; see Methods for more details). These studies indicated that it can take this long for newly born neurons to migrate and differentiate in adult brain. Stronger or weaker differences between groups might have been obtained if the timing of injections had been shifted by a few days or if the birds had been killed at 30 or 60 day survivals, instead of at 40. Hence, there is still much room for refining what is the best time for quantifying the full impact of the breeding stage variable and the duration of this effect.

Correlation Between Number of Fledglings and NC Neuronal Recruitment in the Parents' Brains?

In addition to the main finding, that neuronal recruitment in the brain of a zebra finch adult is affected by that individual's stage in its breeding cycle, other preliminary observations suggest that this relation might be further refined by a positive correlation between number of juveniles fledged and number of new NC neurons in the parents' brains, present at fledging or a few days later (Fig. 4). Body and brain mass (which serve as indications for general good health) of the birds which are included in Figure 4 were similar to the overall means of all the birds in our study. Moreover, no correlations were found between body or brain mass and number of fledglings or number of new NC neurons in the parents' brains. From this, we infer that all birds in this group were in good general health and that it is unlikely that poor condition lead to having few young and/or few new neurons. We suggest, instead, that the excess number of neurons recruited into the parental NC at the time of fledging is sensitive to the number of young fledged. This correlation had not been predicted and should be treated with great care since it is based on a small number of breeding adults. Future effort will be directed at building up the sample of parents and extending the range of number of fledglings per clutch; if the correlation holds up, it will give us the first simple metric relating stimulus complexity and number of new neurons added. In retrospect, a relation between the number of new neurons and the number of fledglings would fit well with the idea that the size of the memory load influences the number of new neurons recruited. It should be noted that stage in the breeding

cycle is a predictable event, while number of young that fledge is not. If the relation between number of fledglings and number of new neurons holds up, then it means that neuronal recruitment can respond not only to predictable environmental changes but also to unpredictable ones, which occur as the breeding cycle progresses. It could be a nice example of the impact of a relatively simple and natural variable on new neuron recruitment, based on the experience of parents looking after their young.

One of the birds we used to establish the correlation between number of fledglings and number of new neurons lost its young 3 days after hatching, and therefore in Figures 4 and 5 was entered at the zero nestling number. This bird is interesting because it links two experimental groups: On the one hand, it belongs to the Fledging group, because it was treated and killed at the same time as the other birds in this group, though it cared for its young for only a few days. It yielded the lowest number of new neurons of all birds in the Fledging group. By the same criterion of no longer having any dependent young (zero fledglings), it also could have been included in the Independence group and its score was similar to that of other birds in that group. Thus the outcome fits our original expectation: having fledglings to look after favors a higher recruitment of new neurons; absence of young, because they died or became independent, decreases neuronal recruitment. However, it is worth noting again that at this stage, the correlation which we found between number of fledglings and NC neuronal recruitment should be regarded as suggestive and not final proof for such a relation. We are now expanding our observations on this matter.

Overview

The common denominator between the present and earlier studies is that when we look at a part of the brain that receives new neurons and then look at conditions that augment the memory load for that part of the brain, the number of new neurons recruited increases markedly as the memory load increases. This is true for nucleus HVC and song learning (Kirn et al., 1994), it is true for the hippocampus and food cashing and retrieval (Barnea et al., 1994), and it is true for caudal neostriatum and social complexity (Lipkind et al., 2002, Barnea et al., 2006 and present study). It would be a mistake, though, to assume that under all of these conditions information load is the only variable that matters. We know, for example, from work done in other bird species, that hearing the food-begging calls of nestlings prolongs the parental stage; interruption of this auditory feedback is soon followed by the onset of a new breeding cycle suggesting, that the parental hormonal profile is maintained by the food begging calls (Nottebohm and Nottebohm, 1971). Information (processed as neuronal activity) could act on circuits—and on neuronal recruitment—as units of data that must be remembered (therefore our use of the term ‘‘information load’’), or by virtue of altering systemic physiological processes. Our studies do not discriminate between these two.

The most intriguing aspect of adult neurogenesis is, perhaps, that it is part of a spontaneous turnover of cells. Therefore, the very conditions that favor the survival of some cells, must induce the death of others or these two sets of conditions must occur in linear succession. At present, we know more about the conditions that favor recruitment of new neurons, than about the way in which the same conditions lead to the demise of existing cells. Perhaps as attention focuses on new variables and disregards older ones, the cells that dealt with the prior condition become underused and vulnerable. This would be one way for a limited space memory system to apportion resources in a manner that tracks environmental change.

Though a relation between learning and adult neuronal recruitment was first described and studied in adult birds (Barnea and Nottebohm, 1994; Kirn et al., 1994), the phenomenon is not restricted to them. A review of the mammalian literature on this topic can be found in Leuner et al. (2006). However, whereas work with mammals has tended to favor training conditions originally introduced by comparative psychologists, resulting in learned skills that occur in an artificial context, the work on birds has tended to focus on naturally occurring behaviors that the birds express spontaneously. Despite this procedural difference, it is encouraging that studies in both groups of animals seem to draw similar conclusions - peaks in information load that must be responded by behavioral adjustments, favor the recruitment of new neurons.

We thank the staff of the Meyer Segals Garden for Zoological Research at Tel-Aviv University for their help. Thanks are also due to two anonymous referees for useful comments.

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