

An examination of avian hippocampal neurogenesis in relationship to photoperiod

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Received 23 March 2005; accepted 18 April 2005

More neurons are recruited into the hippocampus of adult black-capped chickadees in the fall than at other times of year. To determine whether declining photoperiod and photorefractoriness are cues stimulating this neuronal recruitment, we examined three groups of chickadees receiving bromodeoxyuridine injections 10 days before being killed: one group was photostimulated with long days (15L), a second group was held on 15L until photorefractory and another group was held on 15L until photorefractory, then

moved to short days (8L). Measures of neurogenesis revealed no significant differences in hippocampal neuronal recruitment. However, neuronal recruitment was increased in the hyperpallium apical of photostimulated birds. These results suggest that declining photoperiod *per se* is not a major factor regulating enhanced neuronal recruitment to the hippocampus in the fall. *NeuroReport* 16:987–991 © 2005 Lippincott Williams & Wilkins.

Key words: Adult neurogenesis; Avian; Hippocampus; Photoperiod; *Parus atricapillus*; *Poecile atricapilla*; *Poecile atricapillus*

INTRODUCTION

It is now well established that neurogenesis regularly occurs in the adult brain of many vertebrates [1,2]. In birds, new neurons are mitotically produced in the subventricular zone of the brain and then migrate along radial glial fibres to locations throughout the telencephalon [3–5]. Neurogenesis in the avian brain has been most extensively studied in the song-control system, especially in the song-control nucleus HVC [6]. More recently, neurogenesis has been examined in the hippocampus of a food-storing species, the black-capped chickadee (*Poecile atricapillus*; [7–10]).

Food-storing is an adaptive behaviour wherein chickadees, and some other species, store and retrieve hundreds to hundreds of thousands of individual food items over large areas throughout the fall and winter. Accurate retrieval of food requires the use of spatial memory and an intact hippocampus [11,12].

In the first study of neurogenesis in the chickadee hippocampus, Barnea and Nottebohm [7] found a seasonal pattern of neuronal recruitment; that is, of birds captured at various times of year, the highest level of recruitment was observed in the fall when chickadees are storing most. This increased recruitment primarily resulted from the increased survival of newly generated neurons. The finding of seasonally fluctuating neuronal recruitment supported Barnea and Nottebohm's hypothesis that adult-generated neurons in the avian hippocampus may be involved in processing spatial memories.

The proximate signals that cause neurons to be recruited into the hippocampus at a higher rate in the fall have yet to be determined. It is possible that the food-storing experience itself might trigger this process, possibly through heighte-

ned neuronal activity in the hippocampus. Data from juvenile chickadees support this hypothesis: more new neurons were recruited into the hippocampus when juvenile chickadees were permitted to engage in caching activity than when they were deprived of caching experience [9], indicating that food-storing experience is necessary for neuronal recruitment to the hippocampus during development. The same could be true for seasonal changes in adult recruitment of new neurons. Another factor that could initiate heightened neuronal recruitment in the fall is changing photoperiod. Decreasing day-length in the fall may be a cue that enhances hippocampal neuronal recruitment in anticipation of the heightened spatial memory processing demands associated with food-storing. Photoperiod is a primary cue used by many bird species to organize and regulate seasonal changes in the brain, physiology and behaviour [13]. Finally, the fall increase in neuronal recruitment to the hippocampus may be in response to an internal cue, such as the onset of photorefractoriness in late summer when birds spontaneously cease responding to the stimulatory effects of long days (LDs) and their reproductive system regresses [13]. The onset of this photorefractory state itself may be a cue to increase neuronal recruitment to the hippocampus in anticipation of autumn.

Our objective here was to test whether the onset of photorefractoriness or a declining photophase normally associated with autumn would increase recruitment of new neurons to the hippocampus in captive black-capped chickadees. Birds were switched from short days (SDs) to LDs for several weeks. One group of birds (LD photostimulated) was killed shortly after the switch to LDs. The other two groups were maintained on LDs until they

became photorefractory, at which point one group was switched to SD (SD photorefractory) while the final group remained on the same LD photoperiod (LD photorefractory). All birds received injections of the cell-birth marker bromodeoxyuridine (BrdU) 10 days before being killed. These three groups allowed us to determine whether the onset of photorefractoriness and/or declining day-length increased rates of neurogenesis.

MATERIALS AND METHODS

Animals, housing and photoperiodic manipulation: The birds in this experiment were also used in a parallel study of reproductive physiological responses to photoperiod (L.S. Phillmore, J.S. Hoshoooley, T.P. Hahn, S.A. MacDougall-Shackleton, in review). Eighteen adult black-capped chickadees were captured from the wild. Fourteen were captured near Kingston, Canada, and had been housed indoors for over 1 year on a natural photoperiodic schedule. Four birds were captured near London, Canada, and had been housed indoors for several months on a SD photoperiod of 8L:16D (L, light phase; D, dark phase). Immediately prior to this experiment, all birds were group-housed in one room, on SDs.

During the current study, birds were housed individually in cages within isolation chambers, two cages per chamber counterbalanced by sex, with *ad libitum* access to water, food and grit. Illumination within the chambers was approximately 77 lux during the light phase and 2 lux during the dark phase, as measured at cage level.

The first day that birds were placed in chambers and exposed to LDs was set as day 0 for reference to all other time points in the experiment. Because of a timer failure, all groups of birds were initially exposed to 24L:0D for 14 days; thereafter the constant LD photoperiod was 15L:9D. Throughout the experiment, birds underwent laparotomy and blood collection as part of a parallel study (L.S. Phillmore, J.S. Hoshoooley, T.P. Hahn, S.A. MacDougall-Shackleton, in review); these manipulations were equivalent across all three groups.

On day 26, six birds (LD-photostimulated group) received BrdU injections and were killed 10 days later. At the time of killing (day 36), these birds had developed gonads typical of birds in the breeding season. On day 71, when gonads and feather molt indicated that the remaining 12 birds were photorefractory, we transferred six of these birds (SD photorefractory group) to 8L:16D and the other six (LD-photorefractory group) remained on 15L:9D. These two groups received BrdU injections on day 75 and were killed 10 days later.

Bromodeoxyuridine injections and immunocytochemistry: All birds received a total of four injections in the pectoral muscle of 75 µg/g BrdU (Sigma-Aldrich, 0.015 g/mL) in 0.1 M phosphate-buffered saline (PBS; pH=7.4). Injections were all given on one day and were spaced 2 h apart. Birds were then killed 10 days later to allow new cells to migrate and begin expressing neuronal morphology. Thus, killing occurred on day 36 for the LD-photostimulated group and on day 85 for the two photorefractory groups. Birds were killed by an overdose of ketamine:xylazine 1:1, and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde (pH=8.5). Brains were removed from

the skull and left in 4% paraformaldehyde for 24 h, and then cryoprotected in 30% sucrose (in PBS) until saturated (about 24 h). The brains were then frozen on dry ice and stored at -80°C until further processing.

Brains were sectioned in the coronal plane at 30 µm thickness on a cryostat; three brains were processed at a time (one brain from each group). A set of every third section (90 µm intervals) throughout the brain was collected in PBS, mounted on gelatin-coated slides and Nissl-stained with thionin. These Nissl-stained sections were subsequently used to determine the volumes of three structures – the hippocampus, the hyperpallium apicale (formerly hyperstriatum accessorium) and the telencephalon. A second set of every 12th section (360 µm intervals) was collected in PBS for BrdU immunocytochemistry.

Free-floating sections to be stained for the presence of BrdU were first washed in PBS and then immersed in 2 N HCl for 40 min. Next, the sections were washed in a solution of 0.1 M sodium borate (with 0.5% HCl) for 10 min, washed in PBS, then immersed in 0.3% H₂O₂ for 30 min. They were then washed and immersed in 10% normal horse serum for 30 min and then immersed in anti-BrdU antibody overnight (BD Biosciences, Cat. No. 347580). Negative controls were included in each run by omitting the primary antibody for a few sections; these control sections lacked any nuclear or cytoplasmic staining.

Following immersion in the primary antibody, sections were washed in PBS and then incubated in a biotinylated secondary antibody for 1 h. Next, sections were washed then treated with an avidin-biotin horseradish peroxidase complex (Vector ABC kit) for 1 h. BrdU labelling was visualized using diaminobenzidine. Sections were mounted on gelatin-coated slides, dehydrated in a series of graded alcohol concentrations, cleared in solvent and then coverslipped using Permount (Fisher).

Morphology and cell counting: Hippocampal volume was calculated by measuring the region's area in every mounted section in the thionin-stained set (i.e. every 90 µm) in which the region appeared; area estimates were then combined using the formula for a truncated cone [14]. The boundaries used to define the area of the hippocampus were those reported by Sherry *et al.* [14]. The volume of the hyperpallium apicale was calculated in the same manner, but measurements were taken on every fourth mounted section (i.e. every 360 µm). The boundaries used to define the area of the hyperpallium apicale were those reported by Hoshoooley and Sherry [10]. Telencephalon volume was also calculated in the same manner, using measurements from every second mounted section (i.e. every 180 µm). All measurements were made using imaging software and digital images taken with a Spot Insight digital camera mounted on a Zeiss Axiophot microscope using the 5 × (hippocampus and hyperpallium apicale) or 1.25 × objective (telencephalon).

To estimate the number of new neurons recruited into the hippocampus and the adjacent hyperpallium apicale, all sections labelled for BrdU and containing the area of interest were examined. BrdU immunoreactive (BrdU-ir) neurons in the areas of interest were counted exhaustively. That is, all new neurons appearing on a section in the given structure of interest were counted and this was repeated for all sections in the series. BrdU-ir nuclei were identified according to morphological criteria [15]: darkly stained, round or oval

nuclei approximately 10 μm in diameter were counted. Smaller BrdU-ir nuclei were observed in the stained material but were not counted, because they likely were glial nuclei. This exhaustive approach to cell counting likely provides just as accurate, if not more, estimations as using a stereological methodology in which estimates are obtained from much smaller, randomly sampled regions. For example, in the songbird song-control system, similar estimates of neuron number were obtained using either stereological methods or systematic nonstereological methods of the type used in the present study [16]. To estimate the density of BrdU-ir, we divided the average number of labelled neurons per section of a given brain region by the mean sectional volume of the region to yield an estimate of number of BrdU-ir neurons/ mm^3 . To estimate the total number of new neurons in the hippocampus and hyperpallium apicale, we multiplied the density of BrdU-ir in each structure by the volume calculated for that structure. Because of low levels

of background staining in the BrdU-ir tissue, it was not possible to discern the boundaries of the hyperpallium apicale in the tissue from some of the brains, and it was therefore not possible to obtain measurements of neuronal recruitment into this structure in all brains. All volume measurements and cell counts were made by an observer blind to treatment group.

Statistical analyses: Separate one-way ANOVAs were used to examine the density of BrdU-ir in the hippocampus and hyperpallium apicale, the total number of BrdU-ir neurons in the hippocampus and hyperpallium apicale, the volume of the hippocampus and hippocampus volume as a proportion of telencephalon volume. Male and female data were pooled, as several previous studies have found no effect of sex on neurogenesis or hippocampal volume in black-capped chickadees [7,8,17,18].

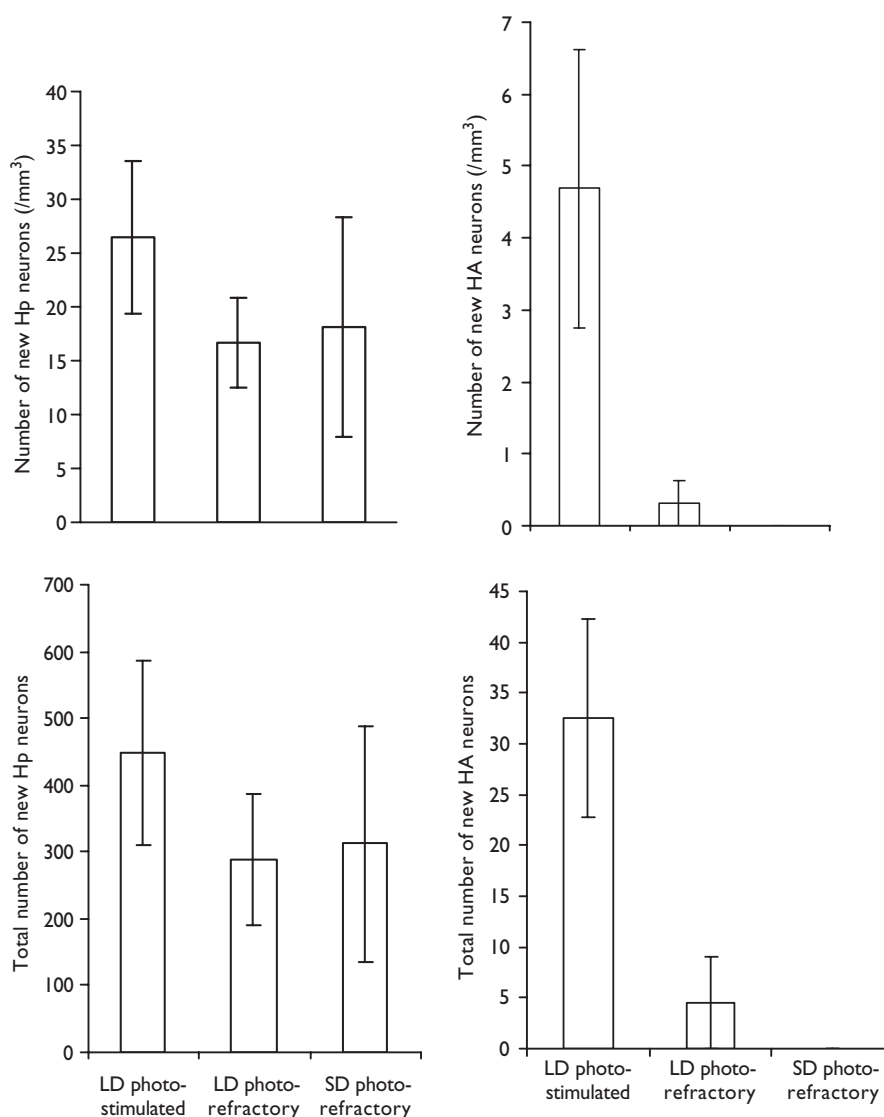


Fig. 1. Left panel: Mean (\pm SEM) density of new hippocampal (Hp) neurons (top), and total number of new hippocampal neurons (bottom) across photoperiod groups. Sample sizes were long day (LD) photostimulated, $n=6$; LD photorefractory, $n=5$ and short day (SD) photorefractory, $n=6$. Right panel: Mean (\pm SEM) density of new hyperpallium apicale (HA) neurons across the different photoperiodic conditions (top), and the total number of new hyperpallium apicale neurons (bottom). Sample sizes were: LD photostimulated, $n=4$; LD photorefractory, $n=4$ and SD photorefractory, $n=2$.

RESULTS

We found no effects of our treatment groups on neural recruitment to the hippocampus. In the hippocampus, no significant differences among treatment groups were observed in either the density of BrdU-ir [$F(2,16)=0.450$, $p=0.646$] or the total number of new neurons [$F(2,16)=0.982$, $p=0.399$; Fig. 1]. We also examined the hyperpallium apicale, a region adjacent to the hippocampus, to determine whether a shortened photoperiod or photoperiodic condition would affect the recruitment of new neurons into this structure. Although we did not find a statistically significant difference among the groups in the density of BrdU-ir [$F(2,9)=3.668$, $p=0.081$], we did observe a trend towards a greater density of newly recruited neurons in photostimulated birds (Fig. 1). A significant difference between the treatment groups was observed in the total number of new neurons in the hyperpallium apicale [$F(2,9)=5.335$, $p<0.05$]. Post-hoc analyses (LSD) revealed that there were more new neurons in the hyperpallium apicale of LD-photostimulated birds than in either of the two groups of photorefractory birds, which were not significantly different from each other.

Hippocampal volume did not differ significantly across the photoperiodic conditions examined [$F(2,17)=0.005$, $p=0.995$] nor did we find any significant differences when hippocampal volume was assessed as a proportion of the total telencephalon volume [$F(2,17)=0.133$, $p=0.877$; Fig. 2].

DISCUSSION

This study was conducted to determine whether photoperiod or photoperiodic condition is involved in the regulation of seasonally fluctuating neurogenesis in the hippocampus of black-capped chickadees. A decrease in day length did not significantly alter the level of recruitment of new neurons into the hippocampus. We also examined the hyperpallium apicale to determine whether photoperiod or photoperiodic condition would affect levels of neuronal recruitment into this structure. In the hyperpallium apicale, we observed a greater amount of total neuron recruitment in LD-photostimulated birds than in either of the two photorefractory groups, which did not differ significantly from each other. Finally, our photoperiodic manipulation was not found to have a significant effect on the volume of the hippocampus.

Although there is a known seasonal pattern of neurogenesis in the song-control system [19], more research is required to determine how photoperiod influences the recruitment of new neurons in the adult brain. Most work conducted to date has concentrated on the hormonal control of neurogenesis rather than the effects of photoperiod and photoperiodic condition. In the song-control region HVC, photoperiod is involved in the regulation of apoptosis, a process that is now increasingly studied in conjunction with neurogenesis. Switching male canaries from an LD photoperiod (14.5L:9.5D) to a SD photoperiod (8L:16D), induced a significant increase in apoptosis in HVC in just 2 weeks [20]. Targeted cell death in HVC leads to an increase in the recruitment of new neurons into that structure, a process termed neuronal replacement [21]. It may be possible that in chickadees, the decreasing day-length in the fall may act as a cue inducing an increase in hippocampal apoptosis. Subsequently, this potential increase in hippocampal cell death could act as a signal leading to enhanced recruitment

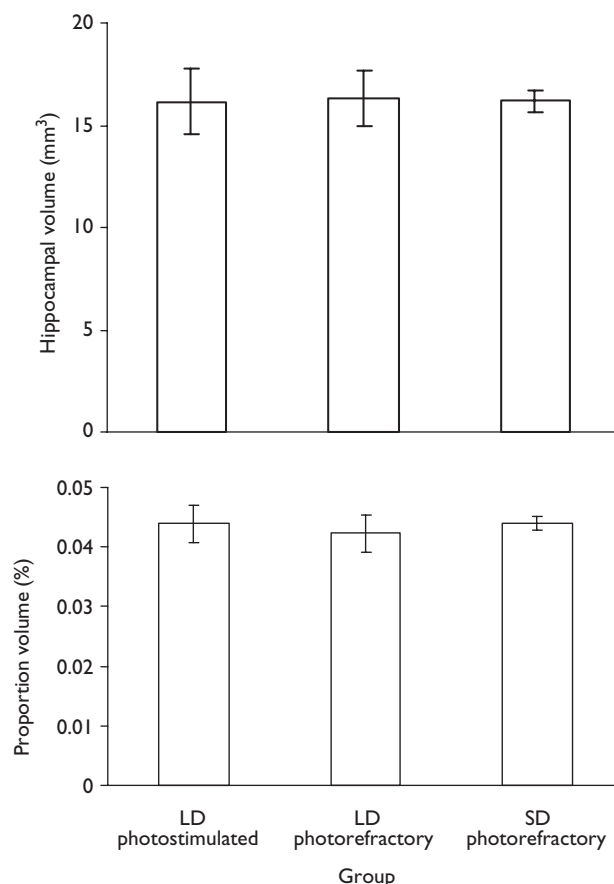


Fig. 2. Mean (\pm SEM) hippocampal volume (top) and hippocampal volume as a proportion of the total telencephalon volume (bottom) in the three groups of birds: long day (LD) photostimulated, $n=6$; LD photorefractory, $n=6$ and short day (SD) photorefractory, $n=6$.

of new neurons. Such a process could lead to the previously observed seasonal pattern of hippocampal neuronal recruitment [7].

We found a significantly greater amount of total neurogenesis in the hyperpallium apicale of LD-photostimulated birds than in either group of photorefractory birds (Fig. 1). In Barnea and Nottebohm's study of seasonal chickadee neurogenesis [7], no seasonal fluctuations in neuronal recruitment in this structure were found; however, in their study, birds were not killed until 6 or more weeks after cell-birth marker injection (in our study birds were killed 10 days after administration of the cell-birth marker). It is possible that seasonal differences may be found in the death rate of new neurons in this structure that are absent by 6 weeks. Alternatively, some of the neurons that originate from the hyperpallium apicale might not be destined to become incorporated into that structure but instead might be migrating en route to another target structure, such as HVC, which lies directly beneath the hyperpallium apicale on the opposite side of the ventricle. New neurons are known to be recruited into HVC at a higher rate in the fall [19], so it might be possible that our LD-photostimulated birds have more new neurons in the hyperpallium apicale because fewer are migrating away from the structure to other downstream targets such as HVC. Whether these new neurons then become incorporated into the hyperpallium

apicale remains unanswered, although the results of the previous study by Barnea and Nottebohm [7] suggest that many of the new neurons we observed in the hyperpallium apicale in the spring would not persist in this structure for as long as 6 weeks. Determining whether our hypothesis has merit would require further study, varying the time course of BrdU administration and photoperiod manipulations.

The lack of increase in neural recruitment to the hippocampus following a switch to short days is consistent with other studies in which photoperiodic manipulations that induced changes in food-storing behaviour failed to induce accompanying changes in the volume of the hippocampus [22,23]. It has been reported that chickadees in the wild show an increase in the size of the hippocampus in the fall [18]. It is possible that manipulations in the laboratory do not produce changes in hippocampal volume because of the effects of captivity. However, the volumes of song-control regions in captive chickadees do change in response to photoperiod manipulations [23], and other studies have failed to observe volumetric changes in the hippocampus in the field [7,10]. Resolving the discrepancy between field and lab studies of seasonal changes in hippocampal volume requires further study, but the current data suggest that photoperiod may not be an important factor regulating the growth of this structure.

CONCLUSION

The results of the current study suggest that photoperiod does not play a large role in enhancing the recruitment of new neurons into the hippocampus of the adult black-capped chickadee in the fall. The factors that lead to enhanced recruitment of adult-generated neurons into the chickadee hippocampus in the fall remain to be determined.

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Acknowledgements: Funding was provided by the Natural Sciences and Engineering Research Council (NSERC), Canada. We thank David Sherry for advice, Anne Marie Polistuk for assisting with microscopy work and Laurie Bloomfield and Bridgette Szekeres for providing us with chickadees. All research was carried out with relevant environmental and animal care permits.