Inhibition of Cell Proliferation in Black-Capped Chickadees Suggests a Role for Neurogenesis in Spatial Learning

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ABSTRACT: **Following development, the avian brain continues to produce neurons throughout adulthood, which functionally integrate throughout the telencephalon, including the hippocampus. In food-storing birds like the black-capped chickadee (*Poecile atricapillus*), new neurons incorporated into the hippocampus are hypothesized to play a role in spatial learning. Previous results on the relation between hippocampal neurogenesis and spatial learning, however, are correlational. In this study, we experimentally suppressed hippocampal neuronal recruitment and tested for subsequent effects on spatial learning in adult chickadees. After chickadees exhibited significant learning, we treated birds with daily injections of either saline or methylazoxymethanol (MAM), a toxin that suppresses cell proliferation in the brain and monitored subsequent spatial learning. MAM treatment significantly reduced cell proliferation around the lateral ventricles and neuronal recruitment in the hippocampus, measured using the cell birth marker bromodeoxyuridine. MAM-treated birds performed significantly worse than controls on the spatial learning task 12 days following the initiation of MAM treatment, a time when new neurons would begin functionally integrating into the hippocampus. This difference in learning, however, was limited to a single trial. MAM treatment did not affect any measure of body condition, suggesting learning impairments were not a product of non-specific adverse effects of MAM. This is the first evidence of a potential causal link between hippocampal neurogenesis and spatial learning in birds.** Develop Neurobiol 74: 1002–1010, 2014

Keywords: **adult neurogenesis; hippocampal neurogenesis; black-capped chickadee; spatial learning; methylazoxymethanol**

INTRODUCTION

Following development of the central nervous system, new neurons continue to be generated and integrated throughout the adult vertebrate brain. Patterns of proliferation and recruitment of adult-born neurons have been well described in several taxa. In mammals, new neurons are generated in the subgranular and subventricular zones and migrate to the dentate gyrus of the hippocampus (HP) and olfactory bulb, respectively (Kaslin et al., 2008). In the adult avian brain, new neurons are generated along the walls of the lateral ventricles and migrate radially throughout the brain, integrating not only in the HP and olfactory bulb but also in most of the remaining telencephalon (Kaslin et al., 2008; Vellema et al., 2010).

Additional Supporting Information may be found in the online version of this article.

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Although patterns of adult neurogenesis are well-documented, the function of most adult-born neurons in vertebrates remains unknown. For two brains areas in mammals, however, many current studies explore the involvement of new neurons in learning processes associated with the region in which they integrate: new neurons recruited to the olfactory bulb are involved in improving olfactory pattern segregation and forming olfactory memories (Lazarini and Lledo, 2011) and new neurons recruited to the mammalian HP are hypothesized to influence HP-mediated learning, including the formation of fear and spatial memories (Leuner et al., 2006; Deng et al., 2010).

Compared with mammalian neurogenesis, even less is known about the function new neurons serve in the adult avian brain. Studies on avian adult neurogenesis focus primarily on two sites of neuronal recruitment: HVC, a nucleus involved in the learning and production of birdsong (Tramontin and Brenowitz, 2000), and HP, associated with spatial cognition (Sherry and Vaccarino, 1989). In HVC, seasonal cues for breeding drive changes in neuronal recruitment and turnover to produce seasonal differences in neuron number throughout the year (Tramontin and Brenowitz, 2000). Pytte et al. (2012) reported a negative correlation between neuronal recruitment in HVC and song modification following deafening in adult male zebra finches and have proposed that new HVC neurons are involved in maintaining birdsong stereotypy. Similar to mammals, new neurons in the adult avian HP are hypothesized to be involved in spatial learning. A role for new HP neurons in spatial cognition is supported by positive correlations between spatial cognitive demand and levels of neuronal recruitment in the HP: LaDage et al. (2011) found that a migratory subspecies of white-crowned sparrows exhibited significantly higher levels of neuronal recruitment in the HP than a non-migratory subspecies, which the authors suggested was a function of navigational demand. Black-capped chickadees (*Poecile atricapillus*) deprived of opportunities to cache and retrieve seeds, a process that involves spatial learning (Sherry, 1984; Sherry and Vaccarino, 1989), exhibited significantly less HP neuronal recruitment compared with birds allowed caching and retrieval opportunities (LaDage et al., 2010).

One limitation to studies on the function of new neurons in spatial learning is a reliance on correlations between levels of neurogenesis and behavior or life history. Without manipulating rates of neurogenesis, we cannot demonstrate a causal relationship between neuronal recruitment and spatial learning. In mammals, several techniques have been developed to manipulate levels of adult neurogenesis. Recent studies investigate learning in strains of mice expressing reduced levels of adult neurogenesis (e.g. Van der Borght et al., 2005). Targeted irradiation to the rodent HP completely depletes the pool of new neurons available for incorporation in the rodent HP (Tan et al., 2011). Adult neurogenesis can also be suppressed *in vivo* in adult mammals using systemic treatment with antimitotic agents, predominantly methylazoxymethanol (MAM). Two weeks of daily injections with MAM significantly reduced levels of HP neuronal recruitment in rats by up to 84% (Shors et al., 2001, 2002) with subsequent effects on HP-mediated learning.

In this study, we tested the effects of MAM treatment on HP neuronal recruitment and spatial learning in chickadees trained to search for sunflower seeds in a spatial array. Using a food-caching species in tests of spatial memory takes advantage of natural behavior exhibited in the wild to test hypotheses regarding spatial cognition (Healy and Hurly, 2004). Once all chickadees exhibited significant learning of a set of spatial locations, we required them to learn new sets of locations while we administered six daily injections of MAM or saline to each bird. Systemic administration of MAM over multiple days creates a "pulse" of reduced neuroproliferation, in which fewer neurons are available to integrate into the telencephalon (e.g. Shors et al., 2002). We predicted that if adult-born neurons are involved in spatial learning, MAM-treated birds should remember spatial locations less accurately than control birds starting between 9 and 15 days after the initiation of MAM injections, a time when adult-born neurons have reached their targets and begin establishing connections (Kirn et al., 1999). Conversely, we predicted that saline-treated birds should exhibit no learning impairments on the spatial task.

METHODS

Birds

Ten black-capped chickadees, four males and six females, were captured by Potter trap between October 2010 and January 2011 near the Western University campus in London, Ontario, Canada. Birds were housed individually on a 10:14 light:dark cycle and provided with food and water *ad libitum* except during brief periods of food deprivation as described below. Food was powdered sunflower seeds mixed with powdered Mazuri Small Bird Diet (PMI Nutrition International, Brentwood MO). All animals were handled and tested according to the guidelines of the Canadian Council on Animal Care (CCAC) and protocols approved by the University of Western Ontario Animal Use Subcommittee.

Studies using systemic MAM treatment to suppress neurogenesis have been criticized because MAM does not specifically target neurons but all cells with its antimitotic effects. Some researchers have argued that MAM-induced learning deficits may be associated with non-neuronal effects on cellular proliferation or general health of an animal (Dupret et al., 2005). To address these concerns, we monitored body weight over all training blocks and took several measures of body condition over the whole study using quantitative magnetic resonance scanning (QMR).

Testing Apparatus

Before testing, birds were randomly assigned to either the MAM or control group ($n = 5$ per group). Sex, determined at sacrifice (see below) was not known at the time of group assignment. Previous studies have found no sex difference in food-caching behavior, memory for cache sites, or relative size of the hippocampus in black-capped chickadees (Petersen and Sherry, 1996). Birds were tested in an indoor aviary measuring 2.74 m × 2.74 m with a one-way mirror in one wall to allow live behavioral scoring by an observer. The aviary contained four different tree branches supported vertically and arranged in an asymmetrical pattern. Each tree was numbered (Tree 1–4) and contained eight, evenly spaced holes (32 holes in total) drilled into the trunks and labeled with white, adhesive labels. Birds' home cages were attached to the wall of an adjoining holding room. A small door in each cage could be opened remotely to admit a bird to the testing aviary.

Training and Testing

Birds were food deprived at least 2 h before entering the aviary and training and testing procedures occurred between 11:00 am and 1:00 pm in 6-day blocks. To ensure birds had retrieved a reward from all 32 sites, we baited all holes with a sunflower seed fragment (sieved through 3 mm × 3 mm mesh) and plugged the holes with a piece of knotted red yarn. Each bird was released individually for 10 min in the testing aviary to find seeds. Because no bird recovered all 32 seed fragments in this time, birds were given an additional 10 min trial each following day in which only unvisited holes were baited and visited holes remained empty and unplugged. These additional trials continued daily until each bird retrieved a seed fragment from every hole.

After retrieving a fragment from all holes, birds remained in their home cages for a rest day and were weighed to obtain a pretreatment body weight measure. Day one of each block consisted of an Exploration Day (Fig. 1) in which 13 holes were randomly selected and baited with a seed fragment and all 32 holes were plugged with yarn. Each bird entered the aviary and was given as much time as needed to find seven of the 13 seeds before being returned to its home cage. The next 4 days (Retrieval days in Fig. 1) consisted of trials in which the seven holes previously visited on Exploration Day were baited and all 32 holes were plugged with yarn. For each trial, birds were given 5 min in the aviary to retrieve seed fragments. If all seeds were retrieved within five minutes, the trial was ended after the bird ingested the final fragment. Retrieval Days 1–3 included two trials separated by at least 50 min and Retrieval Day 4 included only one trial. The sixth day of each training block was a Rest day, during which the birds remained in their cages and body weights were measured. In the Exploration Day for the next block, 13 new holes were randomly selected and did not include any of the holes used in the previous training block. Because birds may begin this new training block by visiting previously baited holes from the previous block, we expected retrieval accuracy to start at or below chance levels in each training block. As birds begin to learn the locations of the baited holes for the new training block, we predicted that retrieval accuracy would improve over trials to significantly above chance by the end of each training block. We repeated training blocks four times for each bird before experimental manipulation. The order in which birds were run was randomized daily to prevent systematic differences in the time an individual bird had been food-deprived before testing. Kirn et al. (1999) demonstrated that new neurons become anatomically integrated in the adult avian brain anywhere between 9 and 15 days following their production. Because it is difficult to relate the recruitment and anatomical integration of new neurons to changes in behavior, we continued training birds before, during, and following MAM or saline treatment to ensure we would be

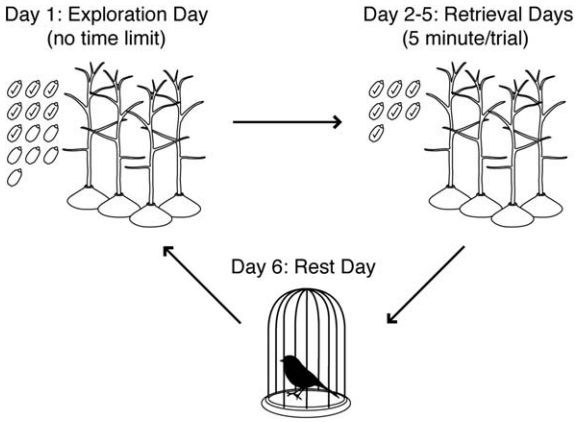

Figure 1 Days in each training block used to test spatial learning abilities in black-capped chickadees. On Day 1, Exploration day, birds are released into an aviary containing four trees, each with 8 holes drilled into the trunk (32 holes in total). Thirteen of 32 holes are baited with a sunflower seed fragment and all 32 holes are plugged with yarn. Birds search until they have successfully retrieved seven of the 13 hidden seed fragments. On Days 2–5, the seven sites where seed fragments were retrieved on Day 1 are re-baited and all 32 holes are plugged with yarn. Birds are released into the aviary for 5 min trials. Retrieval accuracy is scored as the number of sites where a seed fragment is retrieved in the first seven sites visited. On Day 6 of each training blocks, birds remain in their home cages and are weighed.

able to capture effects on behavior up to 15 days following the initiation of treatment.

Behavior Scoring

An observer blind to bird treatment condition observed all trials from behind a one-way mirror and recorded behavior. Retrieval accuracy was measured as the number of baited holes visited in the first seven sites from which the yarn was removed (seven of seven indicating perfect retrieval). The number of correct choices expected by chance was determined by simulating random choice among the 32 sites available. Seven choices were allowed in the simulation and seven sites were designated as correct. The simulation was repeated 100,000 times for each of 10 individuals and the mean number correct for this simulated group was compared with the observed mean performance of the birds. Correct choice of 2.8 or more baited sites exceeds the number expected by chance at $p < 0.01$. All birds reached this criterion by the end of four pretreatment blocks.

Suppression of Neurogenesis

Following four pretreatment blocks, lean mass, fat mass, total body water, and free body water of each bird was measured using QMR body-composition analysis (Gerson and Guglielmo, 2011). Starting on the Exploration Day, birds received a daily injection of either methylazoxymethanol-acetate (MAM group; 14 mg/kg; i.m.) dissolved in 0.1*M* phosphate buffered saline (PBS; pH = 7.4) or PBS vehicle (control group, i.m.) 1 h after lights on in the morning for every day in Block 5 (the treatment block). MAM is an antimitotic drug that reduces the number of adult-born neurons by causing DNA damage via the methylation of guanine residues (Matsumoto and Higa, 1966). MAM is commonly used to impair adult neurogenesis in rodent studies (Shors et al., 2001, 2002). In a pilot study, we treated chickadees with a dose of 7 mg/kg but found that this dose did not significantly reduce cell proliferation rates (Supporting Information Fig. S1). On the rest day of the treatment block, birds received an injection of bromodeoxyuridine (BrdU; 100 mg/kg; i.m.) 1 h after the injection of MAM or PBS. BrdU is a synthetic analog of the nucleotide base thymidine that incorporates into newly generated cells in the brain and is commonly used to quantify levels of adult neurogenesis in birds (Hoshooley and Sherry, 2007).

Because BrdU labeling indicates levels of cell proliferation at the time of BrdU injection, BrdU labeling around the ventricular wall provided data on cell proliferation after 6 days of MAM (or saline) treatment even though several additional weeks of behavioral testing followed MAM treatment. Six days of MAM treatment introduced a temporal pulse of impaired cell proliferation expected to reduce the number of new neurons available for incorporation in the brain. We continued training and testing all birds for three additional blocks (23 days) following the first injection with MAM or saline to detect any impairment in spatial learning that might occur during reduced recruitment and incorporation of new neurons, as described for rodents (Snyder et al., 2005; Shors et al., 2012).

Tissue Collection and Processing

Two days following the last post-treatment block, birds were weighed and fat mass, lean mass, free body water, and total body water were recorded by QMR. Birds were then deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. Brains were dissected from the skull, submersed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in PBS for a minimum of 48 h, frozen on pulverized dry ice, and stored at −80°C until sectioning. Sex was determined at this time by examination of the gonads.

Brains were sectioned coronally (thickness = 40 μm). Once the subventricular zone (SVZ) was reached, as identified by whole-brain morphology, every fourth section in two alternating series was collected, until at least five sections containing SVZ and nine sections containing HP were collected from each brain. Brains were stored at 4°C until histology which occurred within 24 h of sectioning. One series of tissue, used for an anatomical guide to measure hippocampal area sampled, was stained to visualize NeuN immunoreactivity using previously reported protocol (Newman et al., 2010).

The second tissue series was stained to visualize BrdU labeling. Specifically, tissue was washed twice in PBS before being incubated in 2*N* HCl for 40 min, followed by a 10-min immersion in sodium borate in PBS. After two rinses in PBS, tissue was immersed in 0.5% hydrogen peroxide in PBS for 30 min at room temperature. Following three PBS rinses, tissue was incubated in 10% Goat Horse Serum (Vector Laboratories) in 0.3% Triton X-100 (Sigma) in 0.3% PBS (PBS/T) for 60 min at room temperature. Tissue was then moved directly from the serum into a solution of BrdU primary mouse antibody (1:500, Caltag, MD5000) in 0.3% PBS/T for 21 h at 4°C. On the next day, tissue was washed three times in 0.1% PBS/T and incubated with biotinylated goat anti-mouse secondary antibody (1:250, Vector Laboratories) in 0.3% PBS/T for 1 h at room temperature. After two rinses in 0.1% PBS/T, tissue was incubated in ABC Elite avidin-biotin horseradish-peroxidase complex (Vector Laboratories) for 1 h. Following two rinses in 0.1% PBS/T, tissue was reacted with 0.04% diaminobenzidene solution (Sigma) for 90 s to visualize antibody-avidin-biotin complexes and then rinsed four times with PBS. Sections were mounted on Superfrost glass slides (VWR) and left to dry overnight. Slides were dehydrated in a series of graded alcohol concentrations, cleared in xylene, and coverslipped.

BrdU Quantification

Tissue damage prevented quantification of BrdU-immunoreactive (BrdU-ir) cells in three birds along the

ventricular wall (one MAM-treated bird and two saline-treated birds) and four birds in HP (two MAM-treated birds and two saline-treated birds). We visually counted the number of BrdU-ir cells within 7 μm of the ventricular wall across five adjacent brain sections (intersection interval = 160 μm) that included the ventricular morphology associated with neuroproliferative zones [Alvarez-Buylla et al., 1990; Fig. 2(A)]. We also visually counted the number of BrdU-ir cells in HP across nine adjacent brain sections (intersection interval = 160 μm) to quantify neuronal recruitment in HP. We only sampled BrdU-ir cells that were darkly stained, with round or oval nuclei 5 μm or more in diameter (roughly the largest diameter found in all BrdU-ir cells observed). Although these criteria may include a small number of particularly large BrdU-ir glial cells and exclude particularly small BrdU-ir neurons, a similar sampling technique has been used extensively to sample neuronal recruitment in the chickadee HP (Hoshooley and Sherry, 2004, 2007; Hoshooley et al., 2005).

To correct BrdU-ir cell counts in HP for the area of HP sampled, we scanned the adjacent sections in the NeuN tissue series using a high-resolution flatbed scanner and traced HP boundaries using ImageJ software in sections adjacent to those sampled in the BrdU series. Cell counting was done using a Leica DFC420 C camera mounted on a Leica DM5500 B microscope equipped with a 40× objective lens. Ventricular and HP BrdU-ir cell counts were each summed across left and right hemispheres and all sections to produce a single BrdU-ir cell count for each region in each brain. HP BrdU-ir cell counts were further divided by total HP area sampled to produce a BrdU-ir cells/mm^2 measure.

Statistical Analysis

All data were tested for normality using Shapiro-Wilkes tests (all $p > 0.05$). First, we compared BrdU-ir cell counts both along the ventricle and in HP using an independent-sample *t*-test with Treatment as a between-subjects factor (MAM vs. control).

To test for the effects of training and drug treatments on body weight, we compared bird body weight on the final day of the training blocks using a general linear model (GLM) including Block number as a within-subjects factor on five levels (Pretreatment and Blocks 1–4 including the Treatment Block as Block 1) and Treatment as a between-subjects factor on two levels (MAM vs. control).

To test for the effect of MAM on body condition, we compared lean mass, fat mass, total body water, and free body water before treatment to the end of the final post-treatment training block using a GLM including Pre- vs. Post-treatment as a within-subjects factor on two levels and Treatment as a between-subjects factor on two levels (MAM vs. control).

Finally, we compared mean retrieval accuracy on each day during the treatment and post-treatment blocks to a chance level (2.8 seeds retrieved, see above) in MAM- and saline-treated birds using one-sample *t*-tests. Whenever saline-treated birds retrieved fragments significantly above

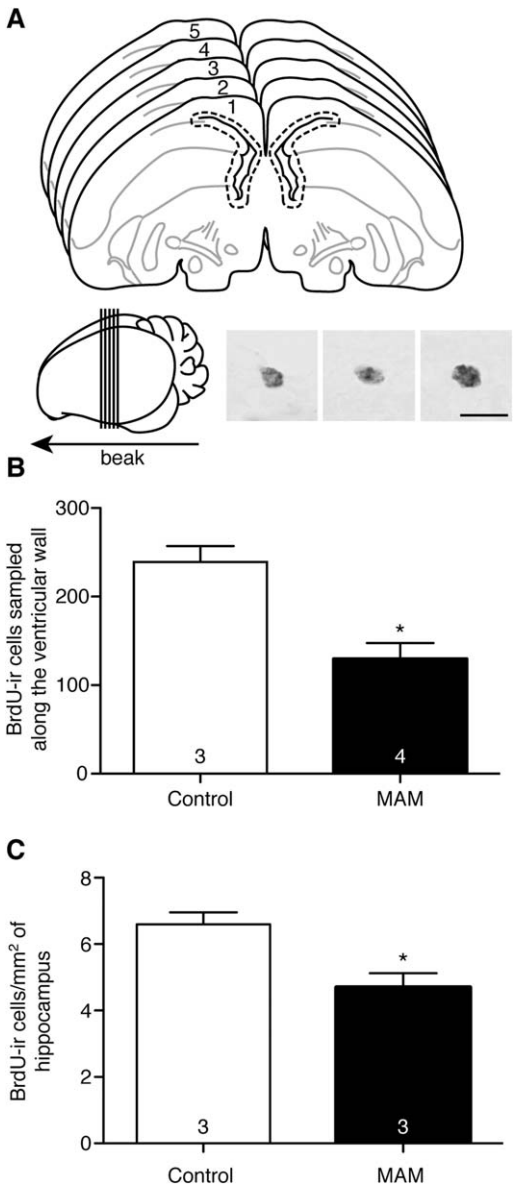

Figure 2 Quantification of neurogenesis around the lateral ventricles and neuronal recruitment in the hippocampus in adult black-capped chickadees by bromodeoxyuridine (BrdU) labeling. (A) Coronal sections of black-capped chickadee brains were used to quantify levels of adult neurogenesis around the ventricular walls. Lower left image is a sagittal view of the brain with vertical lines indicating the location of coronal sections used for quantification. Dotted line represents 7 μm boundary around the ventricular wall in which cells that incorporated BrdU were counted. Lower right image are three examples of BrdU-ir cells counted. Scale bar = 10 μm. (B) Number of BrdU-immunoreactive cells sampled around the ventricular wall in the brains of black-capped chickadees given daily injections of saline or MAM (14 mg/kg) for 6 days. * $p < 0.05$. (C) Number of BrdU-immunoreactive cells sampled in the hippocampus (corrected for the area sampled) of black-capped chickadees given daily injections of saline or MAM (14 mg/kg) for 6 days. * $p < 0.05$.

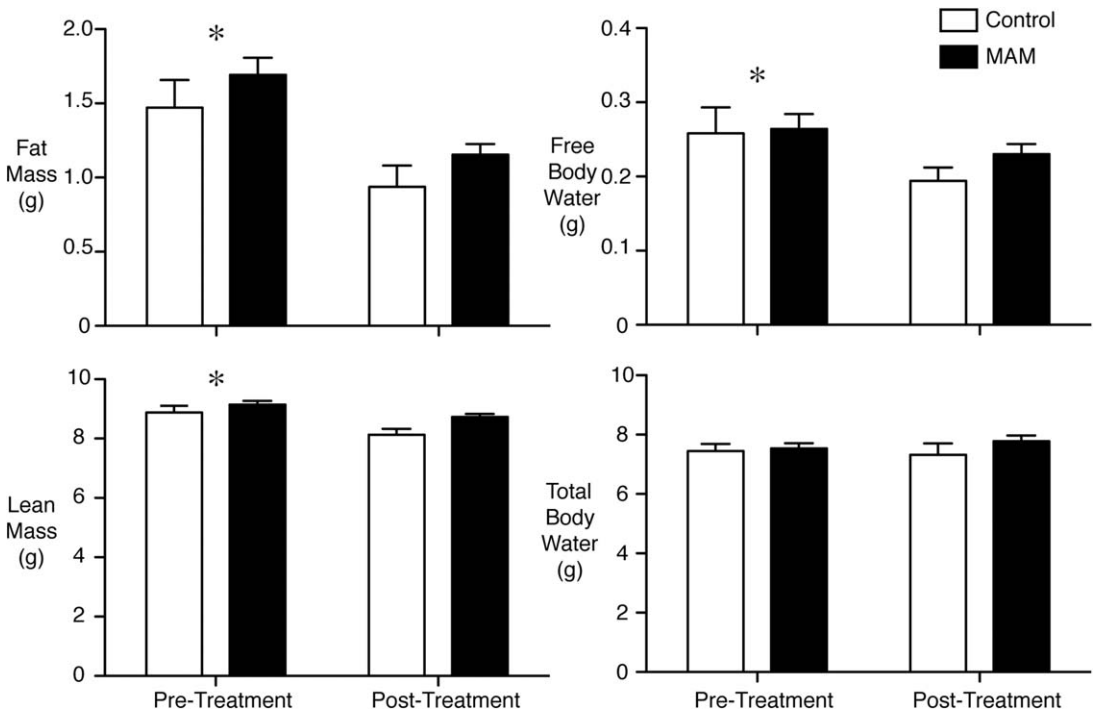

Figure 3 Body condition of black-capped chickadees treated with MAM or saline. Fat mass, lean mass, free water content, and total water content were measured before (Pre-treatment) and after (Post-treatment) by quantitative magnetic resonance scanning. Control $n = 5$, MAM $n = 5$. There were no significant differences between treatment groups. Asterisks indicate variables for which Pretreatment values, irrespective of treatment group, were significantly greater than Post-treatment values ($p < 0.05$).

chance levels, we ran an additional independent sample *t*-test for that trial to test whether control birds also retrieved significantly more seed fragments than MAM-treated birds.

RESULTS

Neurogenesis

Birds treated with MAM had significantly fewer BrdU-ir cells around the lateral ventricles [$t_5 = 4.160$, $p < 0.01$; Fig. 2(B)] and in HP [$t_4 = 3.401$, $p = 0.027$; Fig. 2(C)] than controls.

Body Condition

We found a significant effect of Block Number ($F_{1,8} = 7.527$, $p < 0.01$) on body weight but no significant interaction between Treatment × Block ($F_{1,8} = 1.626$, $p = 0.192$).

Lean mass ($F_{1,8} = 61.772$, $p < 0.01$), fat mass ($F_{1,8} = 60.841$, $p < 0.01$) and free body water ($F_{1,8} = 6.480$, $p = 0.034$) significantly decreased from pre- (lean = 9.01 ± 0.13; fat = 1.58 ± 0.11; free water = 0.26 ± 0.02) to post-treatment (lean = 8.46 ± 0.15; fat = 1.05 ± 0.08; free water = 0.21 ± 0.01; Fig. 3). This decrease, however, was observed in both MAM and control birds and no significant effect of Treatment was found on any measure of body condition (all $p > 0.05$).

Retrieval Accuracy

Both control and MAM-treated birds retrieved significantly more sunflower seed fragments than expected by chance by retrieval Day 4 for the final pretreatment block (control Day 4 $t_4 = 4.50$, $p = 0.011$; MAM Day 4 $t_4 = 4.50$, $p = 0.011$; Fig. 4). Treatment with saline and MAM reduced retrieval accuracy to or significantly lower than chance during the Treatment block (Fig. 4). Both control and MAM-treated birds retrieved significantly more sunflower seed fragments than expected by chance by Days 3 and 4 of Post-Treatment Block 1 (Fig. 4). Over complete Post-treatment Blocks 2 and 3, both control and MAM-treated birds failed to retrieve a mean number of seed fragments that differed significantly from the number expected by chance on Day 4 (Fig. 4; $p > 0.05$). Within these two blocks, however, control

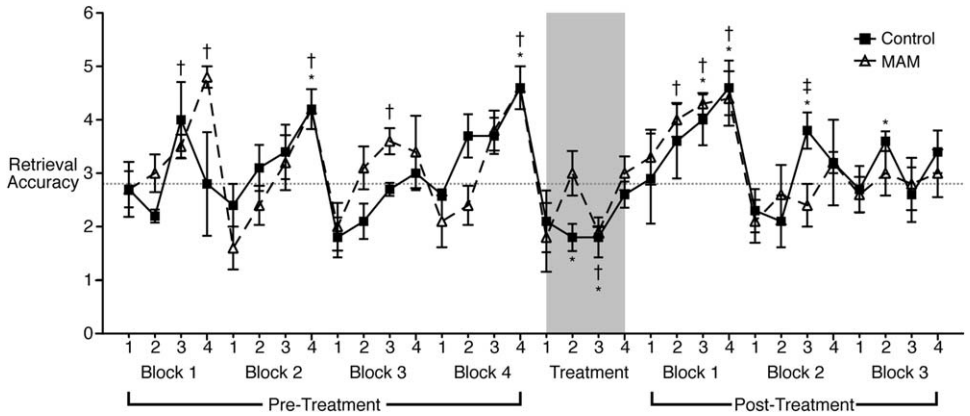

Figure 4 Accuracy of spatial memory before, during, and following six days of daily MAM or saline (Control) treatment. Treatment days are represented by the gray rectangle over the Treatment block. Control $n = 5$, MAM $n = 5$. Gray dotted line shows retrieval accuracy expected by chance (2.8). * Control birds significantly differ from chance ($p < 0.05$). † MAM birds differ from chance ($p < 0.05$). ‡ Control birds significantly differ from chance ($p < 0.05$) and retrieved significantly more fragments than MAM birds ($p < 0.05$). Error bars show ± SEM.

birds retrieved significantly more seed fragments than predicted by chance on Post-Treatment Block 2 Day 3 ($t_4 = 2.949$, $p = 0.042$) and Post-Treatment Block 3 Day 2 ($t_4 = 4.276$, $p = 0.013$) whereas MAM birds continued to retrieve seed fragments at the chance level on both of these days ($t_4 = 1.00$, $p = 0.374$ and $t_4 = 0.478$, $p = 0.658$, respectively; Fig. 4). Only on Treatment Block 2 Day 3, however, did control birds also retrieve significantly more seed fragments than MAM-treated birds ($t_4 = 2.670$, $p = 0.028$). On Post-Treatment Block 3 Day 2, treatment groups did not differ significantly in retrieval accuracy ($t_4 = 1.309$, $p = 0.227$).

DISCUSSION

Six daily injections with MAM significantly reduced neuroproliferation and HP neuronal recruitment in adult black-capped chickadee brains [Fig. 2(B,C)]. We also found some evidence of impaired ability in a spatial memory task in MAM-treated birds starting 12 days following the initiation of MAM treatment. This is the first study to provide evidence of a potential causal link between adult neurogenesis in the avian brain and spatial learning.

Suppressing Adult Neurogenesis

We found that six daily injections with MAM significantly reduced both neuroproliferation and HP neuronal recruitment in the brains of black-capped chickadees measured by BrdU-ir cell counts [Fig. 2(B,C)]. In rats, 6 or 14 daily injections with 7 mg/kg MAM significantly reduced the number of BrdU-ir cells in the dentate gyrus by up to 78% and 84%, respectively (Shors et al., 2001, 2002). Originally, we administered 15 daily injections of 7 mg/kg MAM to chickadees but found that this treatment did not significantly reduce the number of BrdU-ir cells sampled around the lateral ventricle (Supporting Information Fig. S1). Instead, we found that six daily injections of 14 mg/kg MAM significantly reduce cell proliferation by 46% in the adult chickadee brain. The reduced impact of MAM on levels of cell proliferation in the brains of chickadees compared with rats suggests that birds can clear MAM from circulation more quickly than rodents. Testing the impact of additional MAM doses and injection periods on neuroproliferation in the chickadee brain could help refine this method to achieve levels of neurogenesis suppression comparable to those reported in rodents (Shors et al., 2001).

Impacts on Retrieval Accuracy

Before treatment with saline or MAM, all birds were retrieving seed fragments significantly above chance by the final day of all training blocks (Fig. 4). During daily injections with saline or MAM, all birds retrieved seed fragments at or significantly below chance (Fig. 4). This reduction in performance is likely due to stress associated with daily handling and injections during the treatment block because all birds exhibit significant spatial learning in the first post-treatment training block (Fig. 4). Furthermore,

retrieval accuracies significantly below chance during the treatment block are attributed to birds retrieving 2 or fewer fragments during training trials. The return to significant learning in post-treatment Block 1 suggests that MAM treatment has no effect on spatial ability from 6 to 9 days after the initiation of MAM administration. We predicted that it would take at least 9 days before any effect of MAM on spatial learning would be manifest, to account for the time required for the MAM-reduced pool of adult-born neurons to migrate away from the ventricle and begin integrating into the brain (following the time periods tested in Kirn et al., 1999). A similar delay between MAM treatment and effects on HP-mediated learning processes has been reported in rats (Shors et al., 2002).

Following post-treatment training Block 1, we found evidence that MAM treatment affected spatial abilities in chickadees. Specifically, MAM-treated birds failed to retrieve seed fragments above chance for the remainder of training. Control birds, in contrast, retrieved significantly more fragments than chance on Day 3 of post-treatment training Block 2 and Day 2 of post-treatment training Block 3; however, only on the former of these two days did control birds also retrieve significantly more fragments than MAM-treated birds (Fig. 4). These results suggest that MAM-suppressed neurogenesis may impair spatial ability in adult black-capped chickadees at a point when affected neurons are integrating into the telencephalon. Some previous studies using MAM treatment or targeted irradiation to reduce adult neurogenesis in rodents failed to report subsequent effects on spatial navigation in the Morris water maze (Shors et al., 2001, 2002; Snyder et al., 2005). Leuner et al. (2006) however, have argued that the Morris water maze may be too insensitive to detect changes in spatial ability following neurogenesis suppression. This criticism is supported by reported impairments in spatial ability in a place recognition task and the Barnes maze in rats following irradiation-reduced adult neurogenesis (Rola et al., 2004). Food-caching bird species have long been tested in indoor aviaries using food storage and retrieval tasks to test spatial abilities (Barnea and Pravosudov, 2011) and our findings suggest this task is likely sufficient to begin detecting changes in spatial learning following suppression of HP neuronal recruitment.

Although control birds showed significant spatial learning on some days during post-treatment training Blocks 2 and 3, both control and MAM-treated birds ceased to show learning curves in these training block of the kind seen in pretreatment and the first post-treatment training blocks (Fig. 4). A potential explanation for this discrepancy is pro-active interference (Shettleworth, 2009). Specifically, having learned which sites were rewarded and unrewarded in earlier training blocks may have impeded learning in later blocks in which a combination of previously-rewarded and -unrewarded sites could contain seed fragments. Because control birds retrieved fragments above chance twice during this period, our results suggest that control birds were better able to cope with this potential interference than MAM-treated chickadees. Fewer training blocks or more potential baited spatial locations might reduce potential pro-active interference in later blocks. Furthermore, testing the effects of MAM-reduced neurogenesis on performance in multiple spatial cognition paradigms has worked to identify the role of new neurons in spatial learning in mammals (Leuner et al., 2006) and would likely complement and help clarify our findings on retrieval accuracy here.

Body Condition

Although we found that body weight fluctuated between training blocks and lean mass, fat mass, and free body water decreased following treatment and post-training (Fig. 3), none of these changes in body condition were related to MAM treatment, and can instead be ascribed to the effects of daily injections and fragment retrieval training. Our results on body condition suggest that impaired fragment retrieval accuracy in post-treatment training blocks can be explained specifically by MAM's effects on neuroproliferation and not negative effects on general health of the birds.

Conclusion

In this study, we provide the first evidence of the causal relationship between adult neurogenesis and spatial learning in adult black-capped chickadees. Chickadees treated with MAM exhibited suppressed cell proliferation around the lateral ventricles and reduced neuronal recruitment in HP and subsequent impairments in learning on a spatial task. Spatial impairments were not observed until 10 days following the initiation of treatment, a time when new neurons have begun incorporating into functional neural circuits. Direct manipulation of adult neurogenesis makes it possible to further explore the function of adult-born neurons in the adult avian telencephalon.

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