Decreased Neurogenesis Increases Spatial Reversal Errors in Chickadees (*Poecile atricapillus*)

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ABSTRACT: Adult hippocampal neurogenesis has been proposed to both aid memory formation and disrupt memory. We examined the role of adult hippocampal neurogenesis in spatial working and reference memory in black-capped chickadees (*Poecile atricapillus*), a passerine bird that relies on spatial memory for cache retrieval and foraging. We tested spatial working and spatial reference memory in birds that had received methylazoxymethanol acetate (MAM), a neurotoxin that decreases hippocampal neurogenesis. MAM treatment significantly reduced neurogenesis in the hippocampus quantified by doublecortin (DCX) labeling of newly divided and migrating neurons. MAM treatment had little effect on the working or reference memory but caused an increase in errors on the reference memory task following reversal. Working memory for recently visited spatial locations and reference memory for familiar spatial locations were thus unaffected by a reduction in neurogenesis. An increase in errors following reference memory reversal may indicate that adult hippocampal neurogenesis aids in pattern separation, the differentiation of similar memories at the time of encoding. © 2018 Wiley Periodicals, Inc. Develop Neurobiol 78: 1206–1217, 2018

Keywords: **neurogenesis; hippocampus; spatial memory; reversal; black-capped chickadee; methylazoxymethanol (MAM)**

INTRODUCTION

Adult neurogenesis, the production, migration, and differentiation of new neurons in the adult brain, may influence memory encoding, consolidation, and retrieval of memory. The exact function of adult neurogenesis, however, is not well understood. It has been proposed to aid in the formation of memory (Deng *et al*., 2010), and to promote forgetting (Frankland *et al*., 2014). In the avian brain, new neurons are generated along the walls of the lateral ventricles and migrate into the hippocampus, parts of the song control system, and other areas (Kaslin *et al*., 2008; Vellema *et al*., 2010).

The hippocampus plays a central role in learning, memory, spatial orientation, and behavioural inhibition (Clelland *et al*., 2009; Deng *et al*., 2010; Scarf *et al*., 2014). Spatial working memories are memories of the distinctive features of current spatial experience, while spatial reference memories are memories of the stable features of a spatial experience or a spatial environment (Olton *et al*., 1979).

Chickadees (*Poecile atricapillus*) store food and recover it by remembering the spatial locations of caches (Sherry, 1984; Healy and Hurly, 2004). Chickadees show a seasonal pattern in the recruitment of new neurons into the hippocampus (Barnea

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and Nottebohm, 1994), and an elevated level of hippocampal neurogenesis compared to non-storing species (Hoshooley and Sherry, 2007; LaDage *et al*., 2010). Chickadees experience a number of behavioural and ecological changes during the fall that are correlated with an increase in both hippocampal neurogenesis and food storing. Scatter-hoarding birds like chickadees form new memories about cache locations continually in the fall and winter, so neurogenesis may contribute to the creation of new memories. It is unclear, however, whether increased neurogenesis is a consequence of greater use of spatial memory or the result of endogenous factors that also promote food storing (Barnea and Pravosudov, 2011).

Marr (1971) proposed that little information about a single learned event is required to provoke its recall and presented a model of a neural structure for the storage of associations in memory. Marr proposed that the hippocampus acts as a competitive learning network that reduces the degree of overlap among activity patterns to facilitate storage with minimal interference from other activity patterns (Marr, 1971; Kesner *et al*., 1987). This reduction in overlap between similar memories is pattern separation: differentiating the cellular activation associated with two memories by reducing the average overlap of brain activation between them (Treves and Rolls, 1994). There is evidence that new neurons may be necessary for pattern separation in the dentate gyrus of adult mice (Clelland *et al*., 2009). When neurogenesis was inhibited, there were specific impairments in spatial discrimination on a radial arm maze and memory touch screen task. The ability to separate patterns or differentially encode small changes in similar or interfering inputs is particularly important for the accuracy of memory encoding (Clelland *et al*., 2009) and may require new neurons (Deng *et al*., 2010).

A further hypothesis proposes that young hippocampal neurons mediate pattern separation, while old ones facilitate pattern completion (Nakashiba *et al*., 2012). Transgenic mice born with the output of old granule cells inhibited showed enhanced pattern separation and reduced pattern completion in similar contexts which was abolished by the ablation of young granule cells (Nakashiba *et al*., 2012), suggesting that pattern separation required adult-born, or new, young granule cells, and that pattern completion required old granule cells to contribute to rapid recall. New neurons may be recruited into existing neural circuits and be involved in all stages of memory (Schneider and Gage, 2004), or they may be primarily necessary to avoid interference when new information is being learned (Wiskott *et al*., 2006; Deng *et al*., 2010).

Kempermann (2008) has proposed that adult neurogenesis provides a "neurogenic reserve" that allows the brain to maintain learning flexibility by recruiting new neurons from this reserve when there is new information to be learned. This hypothesis states that new neurons become incorporated into existing neural circuits only when there is a need for new learning. Kempermann's theory may explain mixed findings in research on the role of adult neurogenesis in memory because this theory states that learning deficits should be observed only when the reserve of "ready" neurons is depleted. Some subjects may fail to show behavioral changes in a task after neurogenesis manipulations because they have a reserve of new neurons ready to be incorporated as needed.

Barnea and Pravosudov (2011) suggest that birds are an ideal model for studying neurogenesis because they permit a combination of evolutionary, comparative, and neuroethological approaches. Many songbirds produce songs with seasonal variation that is associated with neuronal turnover in song control nuclei caused by neurogenesis. Food-caching birds, like chickadees, use memory-dependent behavior in learning the locations of scattered food caches. They have large hippocampi and experience neurogenesis linked to spatial learning. Blocking neurogenesis results in impaired spatial memory when cues to be remembered have little spatial separation, but not when cues have large spatial separation (Clelland *et al*., 2009), suggesting that new neurons may be needed for pattern separation. The naturally occurring memory-based behavior of chickadees and the ease of observing them in the wild and in the laboratory make them ideal for investigating the neural processes that underlie learning (Barnea and Pravosudov, 2011).

Neurogenesis can be manipulated experimentally using methylazoxymethanol acetate (MAM), a neurotoxin that disrupts DNA synthesis and suppresses cell proliferation in the brain without significantly changing the measures of body composition (Hall *et al*., 2014). In rats, MAM decreases neuroproliferation by 84% (Shors *et al*., 2002) and significantly alters hippocampal functions like spatial memory. In black-capped chickadees, MAM decreases neuroproliferation by 46% (Hall *et al*., 2014) and causes deficits in spatial reversal learning. The chickadee hippocampus is the site of high levels of neuroproliferation (Hoshooley and Sherry, 2007) and chickadees have been used successfully as models in neurogenesis research (Barnea and Pravosudov, 2011; Hall *et al*., 2014). A number of hypotheses for the function of adult neurogenesis specific to birds have been suggested. These hypotheses include: (1) adult neurogenesis is an epiphenomenon

remaining from development that serves no particular function; (2) new neurons are directly involved in learning; and (3) adult neurogenesis is necessary for the replacement of old neurons that have become damaged after intense use (Wilbrecht and Kirn, 2004).

We investigated the effect of decreased hippocampal neurogenesis on spatial working and reference memory in black-capped chickadees. Two groups of birds were tested in spatial working and reference memory tasks. The treatment group received MAM. The reference memory task required subjects to remember 6 out of 12 locations that consistently contained a food reward and was followed by reversal of the baited and unbaited sites. The working memory task required subjects to retrieve one food reward from 12 different locations and to keep track of which locations had already been searched within a trial. It was hypothesized that if neurogenesis aids memory, MAM-treated subjects would perform less well than controls. If neurogenesis disrupts memory, MAM-treated subjects should perform better than controls, due to decreased interference from new neurons.

METHODS

Birds

Twenty-four black-capped chickadees, 11 female and 13 male, were captured by Potter trap between September 2015 and November 2016 near the Western University campus in London, Ontario, Canada. The 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 restriction as described below. The light cycle was chosen to mimic the natural light cycle and to ensure food restriction was sufficient to motivate search for food during testing. Food provided in the birds' home cages 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 and the protocols were approved by the Western University Animal Care Committee.

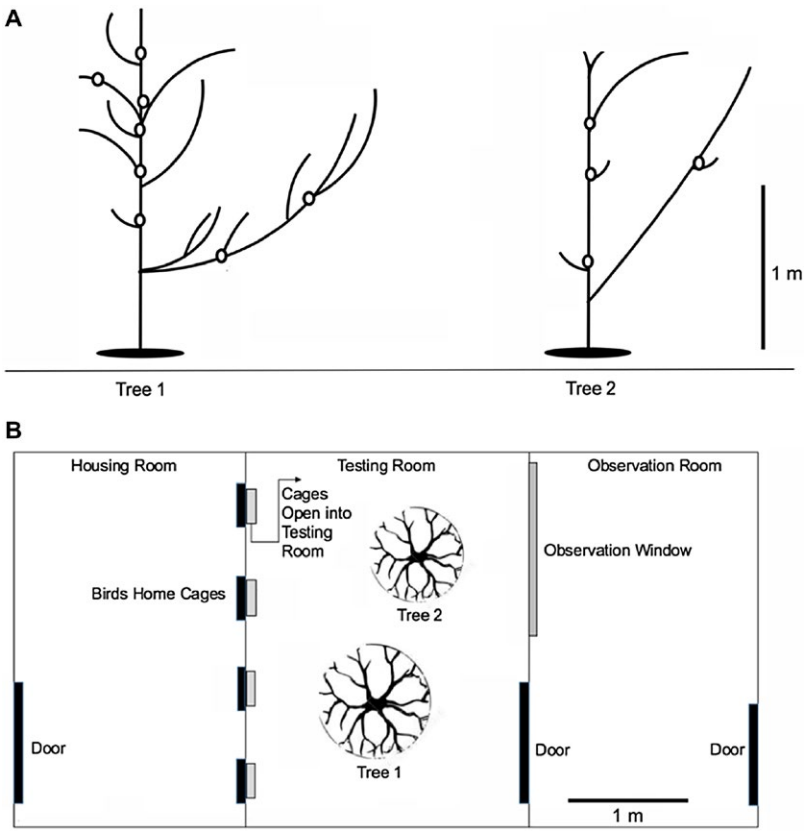

Figure 1 (A) Trees 1 and 2. Circles indicate food locations. (B) Plan view of housing, testing, and observation rooms. Reprinted from Guitar *et al.* (2017).

MAM-induced learning deficits may be associated with effects on nonneuronal cell proliferation or the general health of an animal (Dupret *et al*., 2005). To address this concern, we monitored fat mass, lean mass, free body water, and total body water of all birds over the study using quantitative magnetic resonance (QMR). The birds' body condition was measured the day preceding the first MAM or control saline treatment and following the last trial of testing.

Birds were randomly assigned to either the MAM or control group (n = 12 per group). The birds were tested in four cohorts (n = 6 per cohort) in a counterbalanced order: (working memory group (n = 6), reference memory group (n = 6), working memory group (n = 6), and reference memory group [n = 6]). The sex, determined at sacrifice, was not known at the time of group assignment. Previous studies have found no sex difference in the food-caching behavior, memory for cache sites, or relative size of the hippocampus in black-capped chickadees (Petersen and Sherry, 1996). The birds were tested in an indoor aviary measuring 2.7 × 2.7 m with a one-way mirror to allow live behavioral scoring by an observer blind to the birds' treatment condition (Figure 1). The aviary contained two tree branches supported vertically in stands. The branches contained drilled holes in which to conceal food which were labeled with white marker. Tree 1 was larger than Tree 2 and had 8 holes distributed over its 14 side branches. Tree 2 had 4 holes distributed over 6 side branches. The birds' home cages were attached to the wall of an adjoining holding room. An 0.2 × 0.2 m door in each cage could be opened remotely to admit a bird to the testing aviary.

Training and Testing

Birds were food deprived 2 h before entering the aviary and all training and testing occurred between 10:00 a.m. and 1:00 p.m. seven days a week until completion of the experiment. To ensure the birds experienced finding food at all 12 locations, all holes were baited with a sunflower seed fragment (sieved through 3-mm mesh) and plugged with a piece of knotted green yarn. Yarn was not used after habituation in training or testing to avoid visual cues to visited locations. Each bird was released individually for 10 min into the testing aviary to find the seeds. At the end of a trial, the lights were turned off and the birds returned independently to their home cages. The birds first experienced seven habituation trials in which food was found in all 12 locations covered by the knotted yarn. The criterion for habituation was retrieving 10 or more seeds within the first 12 searches for three days in a row. Twenty training trials were given for the birds to learn either the working or reference memory task, followed by a 20-day break before testing trials. Testing trials ranged from 32 to 39 days depending on the task.

Working Memory Task

The working memory task consisted of 32 trials in which all 12 locations were baited with a seed fragment. The birds were required to complete a within-trial working memory task and retrieve all 12 seed fragments. Revisits to holes previously visited within a trial were counted as working memory errors.

Reference Memory Task

In the reference memory task, six pseudo-randomly assigned locations were baited with a seed fragment for 35 trials for cohort two and 39 trials for cohort four. Locations were chosen such that the proportion of the baited locations on each tree was identical (four out of eight on Tree 1, and two out of four on Tree 2). The six holes varied among subjects, but each set was always matched between an MAM-treated and a control subject. To successfully complete a trial of the reference memory task, the birds were required to retrieve all six seed fragments. The birds searched until all six food rewards were found or until 10 min elapsed, at which point the trial ended. Revisits to holes previously visited, or visits to unbaited holes within a trial, were counted as memory errors. All birds experienced a single reversal during trials 33–39 in which the holes containing food were reversed from the bird's usual set of six to the opposite six holes.

After retrieving a fragment from all baited holes, the birds returned to their home cages for the remainder of the day. The order in which the birds were tested was randomized daily to prevent systematic differences in the length of time an individual bird had been food-deprived before testing. All injections of saline or MAM occurred on trials 6–11. 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 the birds before, during, and following MAM or saline treatment to ensure we would be able to capture the effects on behavior up to 21 days following the initiation of treatment.

Behavioral Scoring

On the working memory task, retrieval accuracy was measured as the number of baited holes visited in the first 12 searches, where a score of 12 out of 12, that is, zero revisiting errors, indicates perfect performance. For the working memory task, revisits to a hole were scored as searches and the number of correct holes changed as searches proceeded. The number of correct choices expected by chance corresponds to an "occupancy problem" (Feller, 1967), as for the classic radial arm maze (Olton and Samuelson 1976). For 12 holes, all 12 of which are initially correct, and 12 searches, including revisits, the number of correct choices expected by chance equals 7.7 and the number of errors expected by chance equals 4.2.

On the reference memory task, retrieval accuracy was measured as the number of baited holes visited in the first six searches, where a score of six out of six, that is, zero visits to unbaited holes, indicates perfect performance. For the reference memory task, revisits were scored as searches and the number of correct choices expected by chance therefore corresponds to sampling with replacement from the binomial distribution. For 12 holes, 6 of which are correct, and 6 searches, including revisits, the number of correct searches expected by chance equals 3.0. The number of expected errors by chance is also 3.0. A search and revisit were defined as either eating the seed fragment found inside the hole, or inserting the beak into the hole, or looking directly into a hole at a distance of less than ~2 cm.

Suppression of Neurogenesis

Starting on trial six of testing in both the working and reference memory tasks, the birds received a daily injection following testing 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.) each day for six days. 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, 1996). MAM is commonly used to impair adult neurogenesis in rodents (Shors *et al*., 2001; 2002) and has been used effectively in chickadees (Hall *et al*., 2014). The lean mass, fat mass, total body water, and free body water of each bird were measured using QMR body-composition analysis (Gerson and Guglielmo, 2011) the day preceding the first treatment with MAM or saline and following the last trial of testing.

Doublecortin (DCX) immunohistochemical labeling was used to determine the number of new neurons in the hippocampus. DCX is expressed in proliferating progenitor cells and newly generated neuroblasts (Brown *et al*., 2003). DCX labeling around the ventricular wall provided data on cell proliferation after six days of MAM (or saline) treatment. Additional weeks of behavioral testing followed MAM treatment in order to capture the effect of neurogenesis. We continued testing the birds for 21–29 days following the last MAM or saline injection to detect any impairment in the spatial memory that might occur during reduced recruitment and incorporation of new neurons, as described for rodents (Snyder *et al*., 2005; Shors *et al*., 2012). DCX labeling was measured in five areas of the hippocampus: the subventricular zone (SVZ), the hippocampus excluding the subventricular zone (NONSVZ), the darkly staining V (V), the hippocampal cap (CAP), and the area parahippocampalis (APH) as described by Atoji and Wild (2006). Six days of MAM treatment introduced a temporal pulse of impaired cellular proliferation expected to reduce the number of new neurons available for incorporation in the brain.

Tissue Collection and Processing

The day following the last trial of testing, the birds were deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. Brains were removed, submersed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in PBS for a minimum of 40 h, frozen on pulverized dry ice, and stored at –80°C until sectioning. The sex was determined at this time by examining the gonads.

Brains were sectioned coronally (section thickness 40 μm). Once the SVZ was reached, as identified by whole-brain morphology, every 10th section in three alternating series was collected until no sections containing hippocampus remained. Brains were stored at 4°C until histological processing which occurred within 48 h of sectioning.

One tissue section series was labeled to visualize DCX. Specifically, the tissues were washed twice in PBS (pH 7.5) for 5 min with agitation before being incubated in 30% H_2O_2 for 15 min, followed by two more rinses in PBS. The tissues were then incubated in 10% Normal Horse Serum (Vector Laboratories) in 0.3% Triton X-100 (Sigma) for 1 h at room temperature with agitation. Tissues were then incubated with DCX primary antibody (C-18 sc-8066 Santa Cruz) 1:250 in 0.3% Triton X-100 (Sigma) overnight at 4°C.

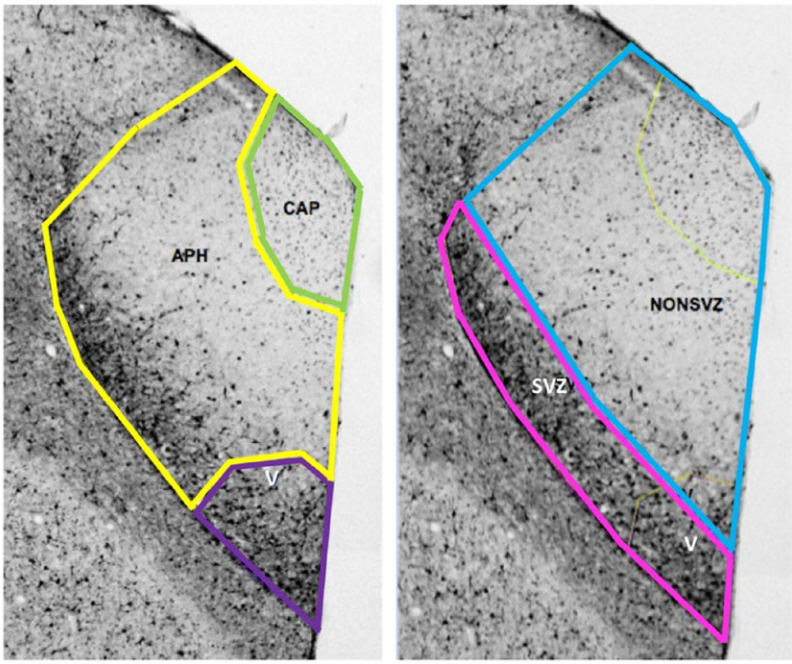

Figure 2 Five regions in which DCX-labeled cells were counted. On the left, V, APH, and CAP. On the right, SVZ and NONSVZ. Note that regions are not mutually exclusive. [Colour figure can be viewed at wileyonlinelibrary.com]

The following day, tissues were rinsed twice in 0.1% PBS/T for 5 min with agitation. The tissues were incubated with biotinylated secondary antibody Horse Anti-Goat IgG (BA-9500 Vector) 1:400 with 0.3% PBS/T for 1 h at room temperature with agitation. Next, the tissues were rinsed twice in 0.1% PBS/T for 5 min with agitation before incubating with ABC Elite avidin–biotin horseradish-peroxide complex (Vector) 1:200 with 0.3% PBS/T for 1 h at room temperature with agitation. The tissues were rinsed twice in 0.1% PBS/T for 5 min with agitation before it was reacted with 0.04% diaminobenzidene solution (Sigma) for 90 s to visualize antibody–avidin–biotin complexes before being rinsed 5 times with PBS. The sections were mounted on Superfrost glass slides (VWR) and left to dry for 48 h. The slides were dehydrated in a series of graded alcohol concentrations, cleared in xylene, and cover-slipped.

Doublecortin Quantification

We used the StereoInvestigator software (Version 10, Micro-brightfield, Colchester, VT) for all stereological measurements. We determined the boundaries of the hippocampal formation as described in Krebs *et al*. (1989), and Atoji and Wild (2006). The hippocampus was divided into SVZ and NONSVZ zones along with the V, CAP, and APH (see Figure 2). Five sections per bird were used for hippocampal measurements (400 µm apart) with a grid size of 280 µm for the NONSVZ, CAP, and APH regions, and a grid size of 180 µm for the SVZ and V regions. Appropriate grid sizes were determined in a pilot study. All sections were coded prior to the analyses so that all measurements were performed blind with respect to bird identity and experimental group.

The total number of hippocampal neurons were determined using the optical fractionator method (West *et al*., 1991), which combines the fractionator (multistage sampling scheme) with the optical dissector to allow for unbiased counting of neurons (Sousa *et al*., 1998). This method allows for the comparison of the number of neurons between the groups (West *et al*., 1991). Our analyses used a 60-µm counting frame and the 40× objective of a Nikon 90i Optiphot microscope connected to a PC running StereoInvestigator software. To evaluate the accuracy of sampling, we calculated the coefficients of error for neuron counts and the range of individual estimate which allows for the evaluation of the robustness of our sampling scheme (Schmitz and Hof, 2000; West, Slomianka and Gunderson ,1991). The variance of the estimates was low (less than 10%) for neuron counts in SVZ, NONSVZ, and APH. The variance of CAP and V

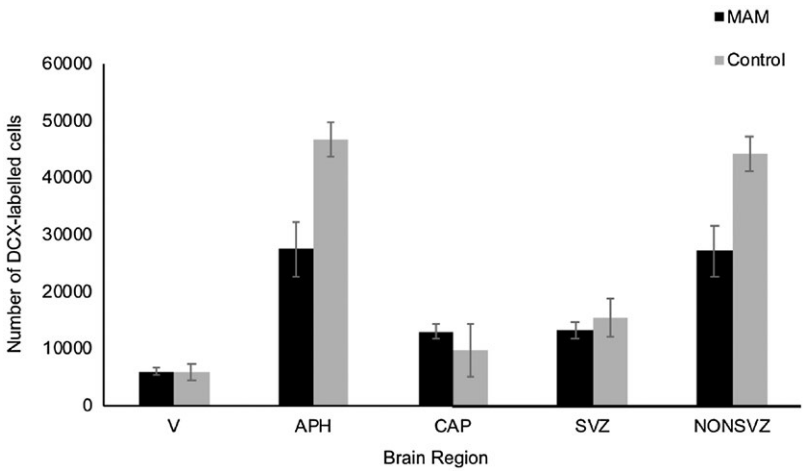

Figure 3 Cell counts for all five regions. In the NONSVZ and APH regions, control and MAM birds differed significantly in the number of DCX-labeled cells. Error bars equal ± 1 standard error of the mean.

regions was high, likely because of cell density differences within these regions.

Statistical Analysis

We compared DCX cell counts along the ventricle in SVZ, NONSVZ, V, APH, and CAP using independent samples *t*-tests with treatment (MAM vs. control) as the between-subjects factor.

To test for the effects of MAM on body condition, we compared lean mass, fat mass, total body water, and free body water before and after the treatment with paired sample *t*-tests.

To test for the effects of MAM on reference and working memory performance, we conducted two repeated measures ANOVAs with trial as a within-subject factor and group as a between-subject factor. A separate repeated measured ANOVA was conducted for the reversal phase of the reference memory task. A Greenhouse–Geisser correction factor was used.

RESULTS

Neurogenesis

Six daily injections with MAM significantly reduced hippocampal neuroproliferation in the brain of chickadees measured by DCX-labeled cell counts in the NONSVZ, $t(22) = 3.149$, $p < 0.01$, and the APH, $t(22) = 3.426$, $p < 0.01$ (see Figure 3). Hall *et al*. (2014) found chickadees experienced a significant reduction in neuroproliferation by MAM of 46% using the same dosage of 14 mg/kg. We found a 38% reduction in neurogenesis in the hippocampus NONSVZ, and a 42% reduction in the area parahippocampalis APH. No differences were found in SVZ, V, or CAP suggesting that Atoji and Wild's (2006) argument for distinguishing between these areas is supported by differences in neuronal recruitment.

Body Condition

QMR body condition measures did not differ significantly before and after MAM or saline administration.

Working Memory Accuracy

There was no significant difference in the number of errors made between MAM and control subjects, $F(1, 10) = 1.12$, $p > 0.05$, $\eta_p^2 = 0.10$, and both groups performed significantly better than chance: MAM, $t(31) = 36.28$, $p < 0.001$; control, $t(31) = 35.88$, $p < 0.001$. There was no effect of trial, $F(7.01, 70.11) = 1.88$, $p > .05$, $\eta_p^2 = 0.16$, and no significant interaction between the group and the trial $F(7.01, 70.11) = 1.37$, $p > .05$, $\eta_p^2 = 0.12$ (see Figure 4).

Reference Memory Accuracy

Trials 1_32

There was no significant difference in accuracy between MAM and control subjects, $F(1, 10) = 2.05$, $p > 0.05$, $\eta_p^2 = 0.17$, and both groups performed significantly better than chance: MAM, $t(31) = 29.20$, $p < 0.001$; control, $t(31) = 21.24$, $p < 0.001$. There was a

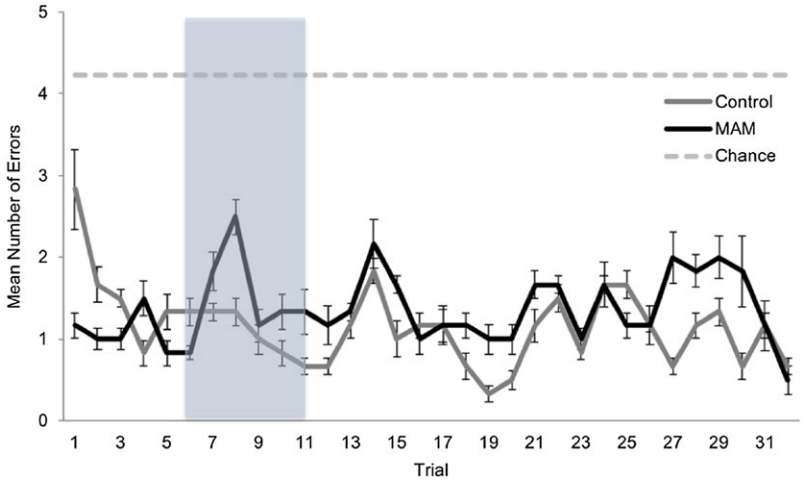

Figure 4 Mean number of working memory errors of MAM-treated subjects and control birds. MAM treatment occurred between trials 6 and 11 indicated by the vertical bar. A reduction in the number of new neurons in the hippocampus would be expected from approximately trial 21 onwards. Error bars equal ± 1 standard error of the mean.

significant effect of trial, $F(6.68, 66.78) = 3.85$, $p < .05$, $\eta_p^2 = 0.28$, such that fewer errors were made as trials progressed. There was no interaction between the trial and the group $F(6.68, 66.78) = 1.10$, $p > 0.05$, $\eta_p^2 = 0.10$ (see Figure 5).

Reversal Trials 33–35

There was no significant difference in accuracy between MAM and control subjects, $F(1,10) = 0.31$, $p > 0.05$, $\eta_p^2 = 0.003$, and neither group's performance differed from chance: MAM, $t(2) = 1.14$, $p > 0.05$; control, $t(2) = 0.09$, $p > 0.05$. There was a significant effect of trial $F(1.80, 18.00) = 9.10$, $p < 0.05$, $\eta_p^2 = 0.48$, such that trial 33 (M = 4.50, SE = 0.22) differed significantly from trial 34 (M = 3.67, SE = 0.36) and trial 35 (M = 2.75, SE = 0.26) but 34 and 35 did not differ from each other (Figure 6). A significant interaction between the trial and the group was also found $F(1.80, 18.00) = 4.31$, $p < 0.05$, $\eta_p^2 = 0.30$. For the control group, trial 35 (M = 2.33, SE = 0.37) differed from trials 33 (M = 4.17, SE = 0.31) and 34 (M = 4.33, SE = 0.51). In the MAM-treated group, trial 33 (M = 4.83, SE = 0.31) differed significantly from trials 34 (M = 3.00, SE = 0.51) and trial 35 (M = 3.17, SE = 0.37).

Reversal Trials 33–39

The second cohort (n = 6) was tested during reversal for three additional trials to determine whether MAM-treated subjects were reversing differently than controls. There was a significant difference in accuracy between MAM and control subjects on trial 36 of reference memory reversal, $F(1,4) = 7.2$, $p = 0.05$, such that control subjects were more accurate than MAM-treated subjects. There was no significant difference in the overall accuracy in trials 33–39 between MAM and control subjects, $F(2.05, 9.76) = 2.05$, $p > 0.05$, $\eta_p^2 = 0.34$, and neither group's performance differed from chance: MAM, $t(6) = 1.96$, $p > 0.05$; control, $t(6) = 0.07$, $p > 0.05$, and there was no interaction between the trial and the group $F(2.05, 9.76) = 1.65$, $p > 0.05$, $\eta_p^2 = 0.29$.

DISCUSSION

Suppressing Adult Neurogenesis

We found that six daily injections with MAM significantly reduced neuronal recruitment in the hippocampus NONSVZ zone and APH with low coefficients of error (see Table 1). These areas are partially overlapping in our regional definitions (Figure 2) but correspond to areas regarded as avian hippocampus (Krebs *et al*., 1989; Atoji and Wild, 2006). We were not surprised to find no differences in neuroproliferation in the V and SVZ because perfusions occurred three weeks after the last MAM treatment day, and neuroproliferation in these regions is likely to have returned to normal levels in that three-week period. We would

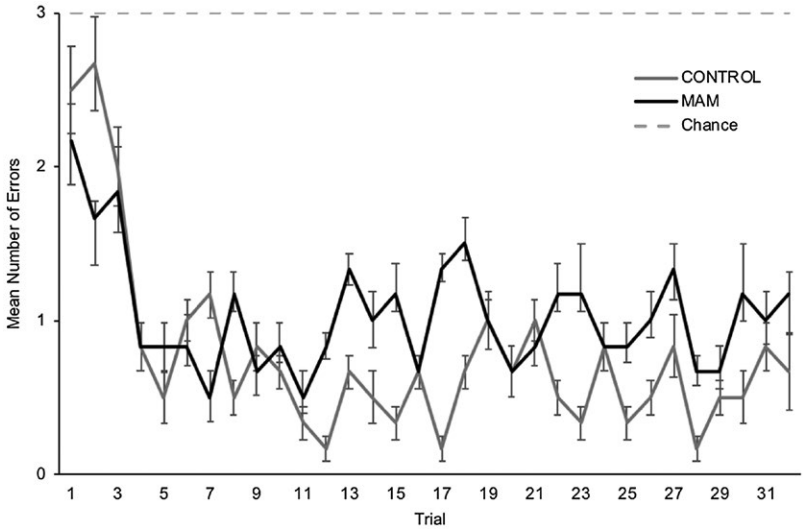

Figure 5 The mean number of reference memory errors of MAM-treated and control birds. MAM treatment occurred between trials 6 and 11 indicated by the vertical bar. A reduction in the number of new neurons in the hippocampus would be expected from approximately trial 21 onwards. Reversal occurred following trial 32. Error bars equal ± 1 standard error of the mean.

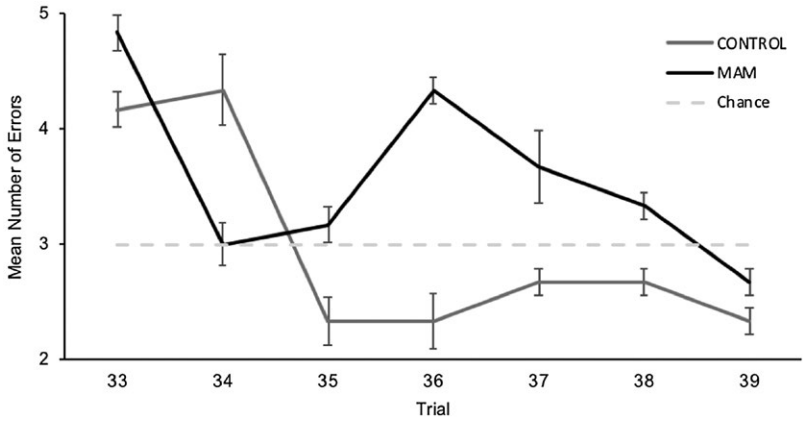

Figure 6 Reference memory errors in trials 33–39. Reversal began in trial 33. All 12 subjects in each group completed trials 33–35. Six subjects from each group completed trials 36–39. Error bars equal ± 1 standard error of the mean.

Table 1 Schmitz–Hof Coefficients of Error for Counts of DCX-Labeled Cells

Area	Mean CE	Range
Subventricular Zone (SVZ)	0.08	0.05–0.09
Non-Subventricular Zone (NONSVZ)	0.07	0.07–0.09
Area parahippocampalis (APH)	0.07	0.05–0.09
Darkly Staining V (V)	0.12	0.09–0.17
Hippocampal Cap (CAP)	0.15	0.11–0.22

Note: Six daily injections with MAM significantly reduced neuronal recruitment in the hippocampus NONSVZ zone and APH with low coefficients of error.

expect the effects of our manipulation to be detectable in the hippocampus away from the neuroproliferative zone. No differences were found in the CAP region likely because of the large coefficient of error for sampling cell counts. DCX-labeled cells in the CAP were both sparse and clustered.

Working and Reference Memory Accuracy

We predicted that if neurogenesis aids memory, MAM-treated subjects would perform worse than controls, whereas if neurogenesis disrupts memory, MAM-treated subjects would perform better than controls. There are multiple reasons for there being no difference in working or reference memory performance between MAM-treated subjects and controls. One possible explanation is that a reduction in neurogenesis greater than the 38-42% we found in NONSVZ and APH, respectively, is necessary for hippocampal memory processes to be disrupted. Another is that decreased neurogenesis does not influence performance of well-learned spatial working and reference memory tasks. According to Frankland *et al*. (2014), a decrease in neurogenesis should cause less forgetting and increased pattern completion especially in a retrograde fashion. There is evidence from rodent studies, however, that reduced neurogenesis can both fail to affect performance on the Morris water maze (Shors *et al*., 2001, 2002 ; Snyder *et al*., 2005) and, at other times, impair spatial ability in place recognition tests (Rola *et al*., 2004). Manipulations of neurogenesis can have multiple contradictory effects on spatial memory.

We did find an effect of reduced neurogenesis during reversal on the reference memory task. As can be seen in Figure 6, MAM-treated birds made more errors during reversal and required several trials before their number of errors decreased to the performance level of control birds, although MAM-treated and control birds differed significantly only on trial 36. Acquisition of new information that conflicts with previously stored information may be impeded if neurogenesis is reduced after original learning, as we observed in reference memory reversal. Reducing hippocampal neurogenesis can impair anterograde memory formation in rats (Deng *et al*., 2009) and prevents new hippocampal memory formation in the trace eyeblink condition paradigm (Shors *et al*., 2001). These results fit with the prediction of Wiskott *et al*. (2006), and Frankland *et al*. (2014) that new neurons are necessary to avoid interference when new information is added to memory. Increasing the length of the reversal and using multiple reversals would be useful to examine the effects of reduced neurogenesis on the acquisition of conflicting information in more detail.

Some theories provide alternate explanations for the given results. It is possible that no changes in performance were observed during working or reference memory learning because committed neurons in an existing memory circuit have a survival advantage when neurogenesis levels change (Leuner *et al*., 2004). If neuronal changes occur alongside established circuits, they may coexist with, rather than alter, established synaptic connections (Frankland *et al*., 2014). The effect of reduced neurogenesis may also be dependent on factors such as age at the time of neurogenesis reduction (Martinez-Canabal *et al*., 2012), the number of neurons targeted (Ko *et al*., 2009), the maturational stage of the targeted neurons at the time of learning (Gu *et al*., 2012), and the type of behavioural task used to assess learning and memory (Shors *et al*., 2002).

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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