

DEVELOPMENTAL NEUROSCIENCE

Site-specific regulation of adult neurogenesis by dietary fatty acid content, vitamin E and flight exercise in European starlings

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Keywords: adult neurogenesis, doublecortin, European starling, flight exercise, *Sturnus vulgaris*

Abstract

Exercise is known to have a strong effect on neuroproliferation in mammals ranging from rodents to humans. Recent studies have also shown that fatty acids and other dietary supplements can cause an upregulation of neurogenesis. It is not known, however, how exercise and diet interact in their effects on adult neurogenesis. We examined neuronal recruitment in multiple telencephalic sites in adult male European starlings (*Sturnus vulgaris*) exposed to a factorial combination of flight exercise, dietary fatty acids and antioxidants. Experimental birds were flown in a wind tunnel following a training regime that mimicked the bird's natural flight behaviour. In addition to flight exercise, we manipulated the composition of dietary fatty acids and the level of enrichment with vitamin E, an antioxidant reported to enhance neuronal recruitment. We found that all three factors – flight exercise, fatty acid composition and vitamin E enrichment – regulate neuronal recruitment in a site-specific manner. We also found a robust interaction between flight training and vitamin E enrichment at multiple sites of neuronal recruitment. Specifically, flight training was found to enhance neuronal recruitment across the telencephalon, but only in birds fed a diet with a low level of vitamin E. Conversely, dietary enrichment with vitamin E upregulated neuronal recruitment, but only in birds not flown in the wind tunnel. These findings indicate conserved modulation of adult neurogenesis by exercise and diet across vertebrate taxa and indicate possible therapeutic interventions in disorders characterized by reduced adult neurogenesis.

Introduction

The vertebrate brain exhibits continued neurogenesis throughout life (Kaslin *et al.*, 2008). The role of neurogenesis in organizing the developing nervous system is well known, and understanding the function and regulation of adult neurogenesis has important therapeutic potential. Unlike mammals, which integrate adult-born neurons strictly into the dentate gyrus of the hippocampus (HP) and olfactory bulb, birds continue to integrate new neurons throughout the telencephalon in adulthood (Kaslin *et al.*, 2008).

Modulation of adult neurogenesis in birds has been primarily studied in HVC (used as a proper name), a nucleus involved in the learning and production of birdsong (Zeigler & Marler, 2008), and in HP (Sherry & Hoshooey, 2010). Increased neuron recruitment in

HVC is associated with song modification (Pytte *et al.*, 2007, 2011) and is regulated by factors associated with seasonal reproduction (Tramontin & Brenowitz, 2000). Neuron recruitment in the avian HP also changes seasonally (but see Sherry & Hoshooey, 2010) and may mediate spatial learning in food-storing species (LaDage *et al.*, 2010). Although studies of adult neurogenesis in birds have focused on seasonality, adult neurogenesis in mammals is regulated by many other factors, including exercise and diet (van Praag, 2008; Park & Lee, 2011). We know nothing about how these other factors regulate neurogenesis in HVC, HP or other regions of the avian telencephalon that also exhibit adult neurogenesis (Vellema *et al.*, 2010).

We investigated whether three regulators of adult neurogenesis in mammals – exercise, dietary fatty acids and vitamin E – also regulate adult neurogenesis in a songbird (*Sturnus vulgaris*). Because migratory exercise occurs seasonally and diet changes *en route* during migration (insectivorous during the summer and omnivorous during the autumn and winter; Fischl & Caccamise, 1987), we hypothesized that diet and exercise might act as seasonal factors influencing neurogenesis in HVC and HP.

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Received 12 November 2013, accepted 16 November 2013

To increase exercise in a manner comparable to wheel-running in rodents (van Praag, 2008), we flew birds using a wind tunnel. Because exercise and migratory activity upregulate adult neurogenesis in the mammalian and avian HP, respectively (van Praag, 2008; LaDage *et al.*, 2011), we predicted that flight exercise would increase neuron recruitment throughout the telencephalon. Starlings are short-distance migrants and the wind tunnel flight simulated natural periods of sustained exercise that these birds would experience in nature.

We fed birds diets relatively enriched with either mono-unsaturated fatty acids (MUFAs) or poly-unsaturated fatty acids (PUFAs). Because dietary enrichment with specific PUFAs, such as docosahexaenoic acid, increases neurogenesis in the rat HP (Kawakita *et al.*, 2006), we predicted that birds fed a diet containing more PUFA would exhibit increased neuron recruitment throughout the telencephalon.

Finally, we enriched diets with one of two levels of vitamin E. We predicted that birds fed a diet with more vitamin E would exhibit reduced neuron migration and either unchanged or increased neuron recruitment, reflecting a reduction in both neuroproliferation and turnover in newly recruited neurons, respectively, as reported in the rat HP (Ferri *et al.*, 2003).

Materials and methods

Study species

We captured 39 adult male European starlings near London, Ontario, Canada, during the last 2 weeks of July 2009. Once captured, birds were held at the Advanced Facility for Avian Research at the University of Western Ontario in London, Ontario, Canada. Birds were maintained under constant ambient temperature of 20 °C in aviaries and initially kept under natural day length, adjusted once a week to match the natural photoperiod. From September 21 onwards, birds were maintained at 13 h 9 min light and 10 h 51 min dark. All experiments were carried out in accordance with the Guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures. Additionally, The Animal Use Subcommittee of the University of Western Ontario approved all treatment procedures.

Diet groups

We randomly assigned starlings to one of four diet groups. Treatment groups were housed in separate aviaries. The four diets were isocaloric, had the same macronutrient composition (40% carbohydrate, 25% protein, 21% fat), and differed only in the relative amounts of plant oils that comprised the dietary fat and the amount of supplementary vitamin E. We used semi-synthetic agar-based diets to avoid the ambiguity in composition reported in diets prepared with raw foodstuffs (Murphy & King, 1982). All diets comprised water, agar (10906 USB Corp., Cleveland, OH, USA), glucose (D 16-10, Fisher Scientific, Houston, TX, USA), casein (12845 USB Corp.), cellulose (Celufil 13292, USB Corp.), salt mixture (Salt Mixture Briggs, 902834 MP Biomedicals, Cambridge, UK), amino acid mix (Langlois & McWilliams, 2010), vitamin mix (AIN Vitamin Mixture 76, 905454, MP Biomedicals, Inc.), mealworms (Exotic Nutrition Co., Newport News, VA, USA) and plant oils. We used a mixture of different plant oils (olive and sunflower oil) to produce diets that contained relatively more MUFAs (MUFA diet – 72% mono-, 13% polyunsaturated) or PUFAs (PUFA diet – 68% mono-, 17% polyunsaturated) than the other diet. Oleic acid (18 : 1) was the

predominant (> 97%) MUFA in both diets, whereas linoleic acid (18 : 2), an omega-6 fatty acid, and α -linolenic acid (18 : 3), an omega-3 fatty acid, were the predominant (> 98%) PUFAs in the two diets. The two diets differed in the relative amounts of omega-6 and omega-3 fatty acids – specifically, the ratio of omega-6 to omega-3 PUFAs was 22 for the MUFA diets and 38 for the PUFA diets (the omega-3 fatty acids were 0.5% of the total fatty acids in all diets, so the amount of omega-6 fatty acids produced these dietary differences in ratios). We supplemented these diets with either low or high levels of vitamin E (DL-alpha Tocopherol Acetate, 100559, MP Biomedicals, Inc.; 5 or 30 IU/kg diet, respectively) to produce four experimental diets – MUFA high vit E ($n = 10$), PUFA high vit E ($n = 9$), MUFA low vit E ($n = 10$) and PUFA low vit E ($n = 10$). The two levels of vitamin E enrichment used correspond to levels recommended for poultry diets with low or high PUFAs (5 and 30 IU, respectively; Scott *et al.*, 1982; Klasing, 1998). Aviaries had natural branches and birds had free access to water for drinking and bathing. *Ad libitum* food and water were supplied daily when the lights came on and aviaries were cleaned daily.

Untrained and flight-trained experimental groups

We trained birds with a flight schedule comparable to migration-like exercise. Half of the birds from each diet group were randomly assigned to a Control group ($n = 19$), housed in the large aviaries and never flown in the wind tunnel. Birds in the Flight group ($n = 20$) were flown in a wind tunnel designed for bird flight at the Advanced Facility for Avian Research at the University of Western Ontario (for details see Gerson & Guglielmo, 2011). Combined with the diet manipulations, exercise training produced a total of eight different treatment groups.

At the start of each flight-training block, a group of three birds was moved from their aviaries into a transport cage (1.2 × 0.7 × 1.8 m) that was then wheeled into the wind tunnel. We trained groups of three birds to fly voluntarily from the transport cage into the wind tunnel, at which point the access to the tunnel was closed. Birds flew within the closed flight section of the wind tunnel for prescribed lengths of time at an air speed of 12 m/s, 15 °C and 75% relative humidity (9.6 g H₂O/m³). Training was conducted for a given group of three birds over 14 consecutive days in which flight length was increased to a maximum of 180 min on day 11. On day 15, birds were flown for as long as they voluntarily flew (up to 4 h).

Each 14-day training period for groups of Flight birds was staggered by at least 3 days and all three-bird groups were flown over the total 58-day duration of the experiment.

Tissue collection

We killed one or two Flight birds in each group along with one or two randomly selected Control birds approximately every 3 days from 19 October to 16 December 2009. Flight birds were killed 2 days after their final flights (day 16 from start of flight training) to reduce the immediate effects of a long-duration flight. One to two days before this, we moved randomly selected Control birds from their aviaries to individual cages to control access to food and water on the day of tissue collection. Birds did not receive food on the day they were killed, but had access for 15 min to drinking water immediately after lights on. Between 5 and 7 h after lights on, birds were deeply anaesthetized with isoflurane and then killed by decapitation. Within 3 min, the brain was dissected from the skull and cut midsagittally to separate hemispheres. One randomly selected

hemisphere was immediately frozen and stored at -80°C for later fatty acid analysis. The remaining hemisphere was immediately submerged in 4% buffered paraformaldehyde for 8 days, cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) for 48 h, frozen on pulverized dry ice and stored at -80°C .

Brains were sectioned (40 μm) in the coronal plane using a cryostat and sections were collected in two alternating series. In each series, we collected every fourth section of the song control nuclei HVC, Area X and robust nucleus of the arcopallium (RA) and every eighth section of the telencephalon and HP. One series of tissue was stained to visualize NeuN immunoreactivity using a previously reported protocol (Newman *et al.*, 2010). The second tissue series was stained by immunohistochemistry to visualize expression of doublecortin (DCX) using previously reported protocols (Boseret *et al.*, 2007; Balthazart *et al.*, 2008; Hall & MacDougall-Shackleton, 2012).

Immunohistochemical protocols for visualizing DCX were adapted from previously reported techniques (Balthazart *et al.*, 2008). Briefly, tissue was removed from cryoprotectant and washed in PBS, incubated in 0.5% H_2O_2 in PBS for 30 min, blocked in 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in 0.3% Triton-X in PBS (PBS/T) for 60 min and then incubated with DCX primary goat antibody (diluted 1 : 250 in 0.3% PBS/T, Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalogue #sc-8066) overnight at 4°C . On the next day, tissue was washed in 0.1% PBS/T, incubated with biotinylated horse anti-goat secondary antibody (diluted 1 : 250 in 0.3% PBS/T; Vector Laboratories) for 1 h followed by ABC Elite avidin-biotin horseradish-peroxidase complex (Vector Laboratories) for 1 h. Tissue was then reacted with 0.04% diaminobenzidine solution (Sigma FastDAB) to visualize immunolabelled DCX. Tissue sections were then mounted on Superfrost Plus microscope slides (VWR, Radnor, PA, USA), serially dehydrated, cleared, and cover-slipped with Permount (Fisher). No immunostaining was found when the primary antibody was omitted.

Neuroanatomical measurements

To test for effects of exercise and diet on volumetric changes in the song nuclei HVC, Area X and RA, which occur seasonally along with changes in neurogenesis to HVC (Tramontin & Brenowitz, 2000), we measured volumes of these song control nuclei, along with volume of HP. All anatomical measurements we made on tissue reacted to visualize NeuN because song nuclei can be visually delineated by the larger and darker soma of cells and by cell density compared with surrounding neural substrate (Newman *et al.*, 2010). HP boundaries can also be distinguished in tissue reacted to visualize NeuN. For HVC, Area X, RA and HP, images were captured with a Leica DFC420 C camera mounted on a Leica DM5500 B microscope using a $5\times$ objective lens for HVC and RA, and a $1.5\times$ objective lens for Area X and HP. An experimenter blind to bird identity traced nuclei and region perimeters using Leica Application Suite software to yield cross-sectional areas. Volume was then calculated using the formula for a frustrum (truncated cone), accounting for sampling interval (160 μm) and consecutive frustra were summed to estimate total volume. If tissue processing prevented the delineation of a nucleus in a section, the experimenter estimated area using the average area of sections before and after the missing section. The same experimenter captured telencephalon images using a high-resolution (2400 d.p.i.) flatbed scanner with a transparency adapter and traced the perimeter of every eighth telencephalon image using NIH ImageJ to calculate cross-sectional areas to calculate telencephalic frustra volumes.

Neurogenesis quantification

We quantified adult-born neurons by immunostaining brain tissue for the production of DCX, an endogenous marker of immature neurons in the avian brain (Balthazart *et al.*, 2008). DCX expression is elevated in new neurons during migration and integration into new neural circuits and decreases upon neuron maturation. Furthermore, cells expressing DCX can be differentiated into migratory or recruited phenotypes based on elongate or spherical morphology, respectively (Balthazart *et al.*, 2008; Hall & MacDougall-Shackleton, 2012; but see Scott *et al.*, 2012 regarding migratory neuron morphology). We labelled neural tissue for DCX expression and quantified elongate and spherical doublecortin-immunoreactive (DCX-ir) cells to obtain local measures of neuron migration and recruitment, respectively. The major advantage of sampling neurogenesis using DCX over traditional measures (bromodeoxyuridine and tritiated thymidine) is that DCX is produced endogenously in neurons whereas these traditional markers must be injected into animals, dilute with subsequent cell divisions, are not expressed specifically in neurons and provide a snapshot of the fate of neurons generated only at the time of injection (Couillard-Despres *et al.*, 2005). Conversely, without knowing specifically how long DCX is expressed in new neurons (Balthazart *et al.*, 2008 suggest less than 30 days following proliferation), we are unable to test for shorter-term changes in neuronal migration and recruitment than those caused by the lengths of exercise and diet manipulations used here. We opted to use DCX because this marker is commonly used to identify the upregulation of neuronal recruitment in rodents following exercise manipulations of similar duration to ours (ex. Garrett *et al.*, 2012).

We quantified DCX reactivity in the striatum (Str), caudal and rostral nidopallium (cN and rN, respectively), HVC and HP near the lateral ventricles where new neurons are generated in the adult songbird brain (Vellema *et al.*, 2010). We selected these sampling areas based on previously reported sites of adult neurogenesis (Balthazart *et al.*, 2008) and we also selected rN and Str to sample migratory neurons close to the site of neuroproliferation (lateral ventricle), as they begin migrating tangentially throughout the telencephalon. Images of all regions of interest were taken using a $40\times$ objective lens on five adjacent tissue sections (intersection interval = 160 μm) in one randomly selected hemisphere (Fig. 1). No previous work has reported lateralization in DCX reactivity in HVC (Balthazart *et al.*, 2008). Each picture was compiled as a z-stack using Leica Application Suite from a series of images taken at a regular interval (0.63 μm) throughout the focal depth of the section using a Leica 420D camera with standardized optical settings. Compiling these photos created an image in which all cells and neurites were in focus.

Images were corrected for brightness and contrast and converted to 32-bit greyscale images. Two types of DCX-ir somas were counted (Fig. 2A and B). First, fusiform cells are identified by their long, bipolar cell body characteristic of migratory neurons en route to their point of integration. We also counted a second population of spherical, multipolar cells thought to represent recruited neurons that have begun integration into existing neural circuits (Balthazart *et al.*, 2008). In HP, only one DCX-ir cellular phenotype was found and this phenotype appeared similar to DCX-ir spherical cells (Fig. 2C).

Statistical analyses

All statistical analysis was performed using PASW (SPSS, Chicago, IL, USA) version 18. Birds were categorized by two levels of

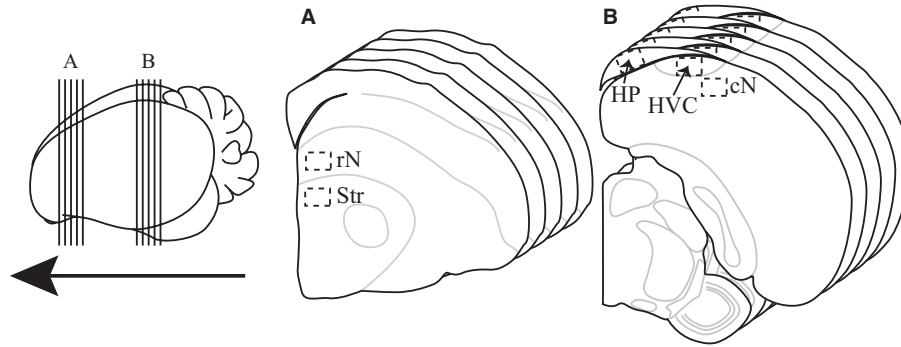


FIG. 1. Location of sampling squares (dashed outlines) used to sample doublecortin immunoreactivity across five coronal sections (intersection interval = 160 μm) in adult male European starling brains at two rostro-caudal locations (A and B). Locations of coronal sections (vertical lines) for sites A and B are depicted on a drawing of a starling brain from the sagittal view (far left; arrow points toward the beak). rN, rostral nidopallium; Str, striatum; HP, hippocampus; cN, caudal nidopallium; HVC, proper name (sometimes called high vocal centre).



FIG. 2. Micrographs of elongate (A), spherical (B) and hippocampal spherical (C) neuron morphologies of cells expressing doublecortin in adult male European starling brains. Pictures are of brain tissue reacted to visualize doublecortin using diaminobenzidine taken at 40 \times objective magnification. Scale bar = 50 μm .

exercise (Control or Flight), two levels of dietary fatty acid composition (MUFA or PUFA) and two levels of vitamin E diet content (High or Low). GLM analyses compared main effects and interactions of these fatty acid, vitamin E and flight treatment factors for each dependent variable (see Supporting Information Tables S1 and S2). Hemispheres were analysed for telencephalon volume. HVC, RA, Area X and HP were analysed for total volume and volume as a percentage of telencephalon volume. HVC, Str, rN and cN were analysed for numbers of spherical and fusiform DCX-ir cells counted. DCX-ir cell counts were reanalysed in HVC using caudal nidopallium counts as a covariate because neuron migration to HVC and the surrounding caudal nidopallium is indiscriminate whereas recruitment and neuronal survival in HVC can be specific (Vellema *et al.*, 2010). DCX-ir cell counts in HP were analysed for only the spherical phenotype observed.

Results

A summary of all statistical results is given in Tables S1 and S2.

Neuroanatomy

Overall, there were minimal effects of our diet and exercise treatments on the size of song-control regions, HP or telencephalon. Significant effects on neuroanatomy tended to be limited to interactions with small effect sizes.

Telencephalon

We found a fatty acid \times vitamin E interaction effect on telencephalon volume ($F_{1,31} = 4.402$, $P = 0.044$). *Post-hoc* analysis (independent

sample *t*-tests) showed that, for birds fed a diet low in vitamin E, a diet enriched with PUFA significantly increased telencephalon volumes (mean \pm SE; $484.40 \pm 10.04 \text{ mm}^3$) compared with birds fed a MUFA diet ($446.20 \pm 11.93 \text{ mm}^3$; $t_{18} = 2.705$, $P = 0.015$).

Hippocampus

We found no significant main or interaction effect of exercise, fatty acid or vitamin E on HP volume. When HP volumes were reanalysed as a percentage of telencephalon, results were similar.

HVC

Control birds had slightly larger HVC volumes ($1.81 \pm 0.12 \text{ mm}^3$) than Flight birds ($1.51 \pm 0.11 \text{ mm}^3$; $F_{1,31} = 3.489$, $P = 0.071$) but this difference was not significant. When HVC volumes were reanalysed as a percentage of telencephalon volume, we found similar results.

Robust nucleus of the arcopallium

We found no significant main or interaction effects of exercise, fatty acid or vitamin E on RA volume. When RA volumes were reanalysed as a percentage of telencephalon volume, we found similar results.

Area X

We found a significant exercise \times fatty acid \times vitamin E effect on Area X volume ($F_{1,31} = 6.495$, $P = 0.016$). *Post-hoc* analysis revealed a significant effect of exercise in birds fed a MUFA diet and low vitamin E in which Flight birds exhibited a significantly larger Area X than Control birds ($t_8 = 2.33$, $P = 0.048$). When Area X volumes were reanalysed as a percentage of telencephalon volume, we found similar results.

Neuron migration and recruitment

In contrast to the few effects on neuroanatomy, our diet and exercise manipulations had substantial effects on DCX immunoreactivity in several brain regions, indicating a major effect on adult neurogenesis.

Hippocampus

Birds fed a MUFA diet had significantly more spherical DCX-ir cells in HP than birds fed a PUFA diet (Fig. 3; $F_{1,26} = 6.125$, $P = 0.020$). In addition to the main effect of fatty acids, we also

found a significant exercise \times vitamin E interaction effect on spherical DCX-ir cells in HP. *Post-hoc* analysis of this interaction revealed two significant effects. First, in birds fed a diet low in vitamin E, flight increased spherical DCX-ir cell counts in HP compared with Control birds (Fig. 3; $t_{16} = 2.59$, $P = 0.02$). Second, in Control birds, a diet with higher levels of vitamin E significantly increased DCX-ir spherical cells in HP compared with birds fed a low vitamin E diet (Fig. 3; $t_{16} = 2.29$, $P = 0.036$).

HVC

We found no significant main or interaction effects of exercise, diet or vitamin E on the number of DCX-ir fusiform or spherical cells in HVC. When DCX-ir cell counts were reanalysed using counts in cN as a covariate, similar results were found.

Striatum

We found no significant main or interaction effects of exercise, diet or vitamin E on the number of DCX-ir fusiform cells in Str. However, there were significant effects on the number of DCX-ir spherical cells. Flight training was associated with a slightly increased number of spherical DCX-ir cells in the Str (78.04 ± 4.03 cells) compared with Control birds (66.55 ± 4.03) but this effect was non-significant ($F_{1,29} = 4.071$, $P = 0.053$). More importantly, we found a significant exercise \times vitamin E interaction ($F_{1,30} = 5.144$, $P = 0.031$). *Post-hoc* analysis of this interaction revealed two

significant differences between groups. First, in Flight birds, a diet low in vitamin E significantly increased DCX-ir spherical cells in Str compared with birds fed a diet high in vitamin E (Fig. 3; $t_{17} = 2.68$, $P = 0.016$). Second, in low vitamin E birds, flight exercise significantly increased the number of DCX-ir spherical cells in Str compared with Control birds (Fig. 3; $t_{18} = 3.30$, $P = 0.004$). Thus, both exercise and vitamin E significantly affected the number of spherical DCX-ir cells, but interacted in their effects.

Rostral nidopallium

As for Str, we found no significant main or interaction effects of exercise, vitamin E or fatty acid on DCX-ir fusiform cells in rN (all $P > 0.05$), but we did find effects on DCX-ir spherical cells.

The number of spherical DCX-ir cells counted in the rN (119.74 ± 3.86 cells) in birds fed the PUFA diet was slightly higher than birds fed a MUFA diet (109.25 ± 3.64 ; $F_{1,30} = 3.269$, $P = 0.081$) but this effect was non-significant. As for Str, we found a significant exercise \times vitamin E interaction effect on spherical DCX-ir cells in rN. *Post-hoc* analysis of this interaction revealed two significant differences between groups. First, in birds fed a low vitamin E diet, flight significantly increased the number of DCX-ir spherical cells in rN compared with Control birds (Fig. 3; $t_{18} = 3.72$, $P = 0.002$). Similarly, in Control birds, a diet high in vitamin E significantly increased the number of DCX-ir spherical cells in rN compared with birds fed a diet low in vitamin E (Fig. 3; $t_{17} = 2.59$, $P = 0.019$).

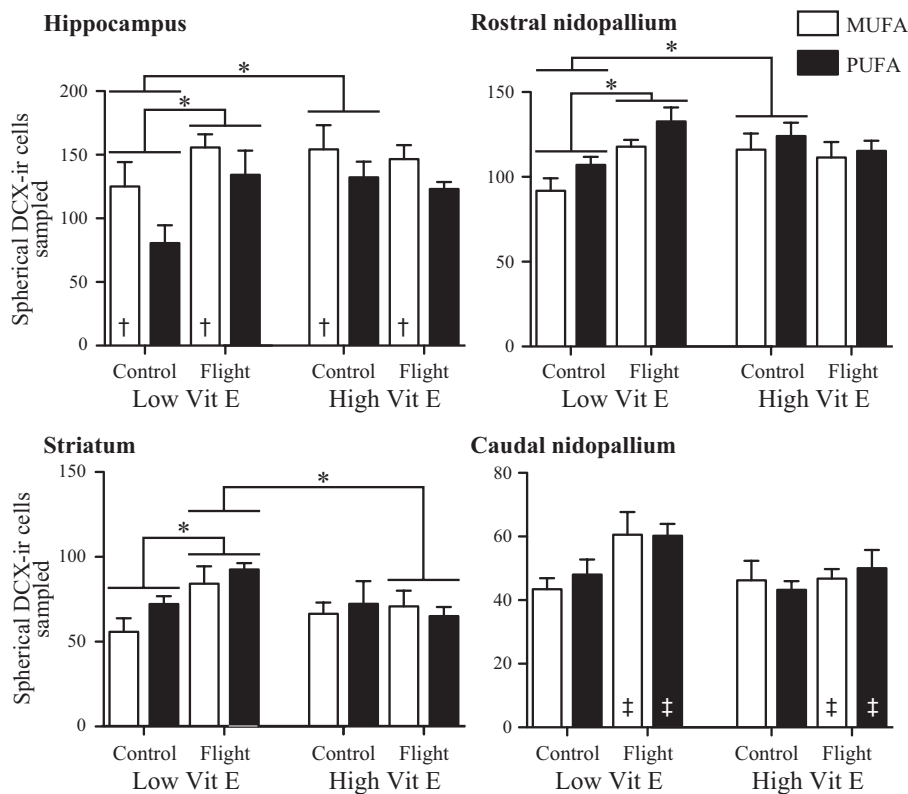


FIG. 3. Number of spherical neurons expressing doublecortin sampled in the hippocampus, striatum, and rostral and caudal nidopallium in adult male European starling brains. Starling treatment groups differed in the composition of fatty acids in the diet [relatively enriched monounsaturated fatty acids (MUFAs), white bars; relatively enriched polyunsaturated fatty acids (PUFAs), black bars], level of dietary enrichment with vitamin E, and exercise by regular flying in a wind tunnel. *Significant group differences ($P < 0.05$); †a significant increase ($P < 0.05$) in spherical doublecortin neurons in the hippocampus for a MUFA diet compared with PUFA diet; ‡a significant increase ($P < 0.05$) in spherical doublecortin neurons in the caudal nidopallium with flight exercise compared with control birds.

Caudal nidopallium

For DCX-ir spherical cells, we found a significant effect of exercise in which Flight birds had significantly more cells in cN than Control birds (Fig. 3; $F_{1,30} = 6.970$, $P = 0.013$).

For DCX-ir fusiform cells, we found a significant main effect of vitamin E treatment in which birds fed a diet high in vitamin E had more DCX-ir fusiform cells in cN than birds fed a diet low in vitamin E (Fig. 4; $F_{1,30} = 14.988$, $P = 0.001$). We also found a significant fatty acid \times vitamin E interaction for the number of fusiform DCX-ir cells in cN ($F_{1,30} = 6.448$, $P = 0.017$). *Post-hoc* analysis of this interaction revealed that in birds fed a PUFA diet, a diet high in vitamin E significantly increased the number of DCX-ir fusiform cells in cN compared with birds fed a diet low in vitamin E (Fig. 4; $t_{16} = 4.67$, $P < 0.001$) whereas this effect of vitamin E was not apparent for birds fed a MUFA diet ($t_{16} = 0.964$, $P = 0.348$).

Discussion

For the most part, neither diet manipulations nor flight exercise significantly affected neuroanatomical measures or neuron recruitment in the song-control system. The only exception was a three-way interaction of exercise, dietary fatty acid composition and vitamin E enrichment in the size of Area X. However, all three of our manipulations – exercise, dietary fatty acid composition and vitamin E dietary enrichment – influenced neuron migration and/or recruitment in a site-specific manner elsewhere in the telencephalon. Particularly notable was an interaction between vitamin E and exercise on neuronal recruitment in multiple sites of the telencephalon.

Influences on the song-control system

We found no significant effects of either dietary manipulation or exercise on song nuclei volumes or neuron recruitment to HVC, and therefore conclude that seasonal patterns in exercise and diet are not involved in stimulating neuroplastic changes in the song-control system.

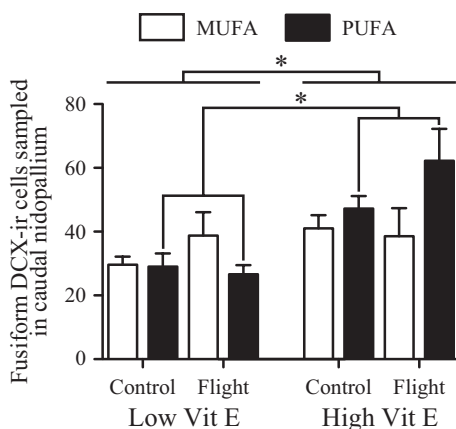


FIG. 4. Number of fusiform neurons expressing doublecortin sampled in the caudal nidopallium of adult male European starling brains. Starling treatment groups differed in the composition of fatty acids in the diet [relatively enriched monounsaturated fatty acids (MUFAs), white bars; relatively enriched polyunsaturated fatty acids (PUFAs), black bars], level of dietary enrichment with vitamin E, and exercise by regular flying in a wind tunnel. *Significant group differences ($P < 0.05$).

Exercise

Flight exercise significantly increased neuron recruitment in cN as measured by the number of spherical DCX-ir cells. Flight exercise was associated with increases in neuron recruitment in the rN and Str, but these differences were not statistically significant ($P = 0.081$ and $P = 0.053$, respectively).

Despite large increases in neuron recruitment and survival in the adult mammalian HP following exercise (van Praag, 2008), flight exercise enhanced neuron recruitment only in cN and had no main effect on neuron recruitment in the avian HP. This unexpected result is best explained by the significant regulation of exercise-induced neuron recruitment by dietary vitamin E – flight only significantly increased neuron recruitment in HP, St and rN when birds were also fed a diet low in vitamin E (see below).

The absence of a main effect of exercise on neuron recruitment in HP may be associated with the magnitude of our exercise manipulation. Both exercised and control birds were group-housed in large rooms that permitted free movement throughout the room and social enrichment, both of which modulate neuronal recruitment in the mammalian HP (van Praag *et al.*, 1999; Olson *et al.*, 2006). Fox *et al.* (2010), however, found no effect of group size, one factor indicative of social enrichment, on neurogenesis rates measured using DCX in HP of the black-capped chickadee (*Poecile atricapillus*).

Dietary fatty acids

Contrary to our prediction, a MUFA diet relatively low in PUFAs significantly increased neuron recruitment to the HP in male starlings. Mammals fed diets enriched with specific omega-3 fatty acids (docohehexanoic acid, a common fatty acid in the brain that can be produced from a C18 : 3 precursor) increased neurogenesis in both *in vivo* and *in vitro* preparations (Kawakita *et al.*, 2006). Although all diets in our study had similar amounts of omega-3 fatty acids, the MUFA diets that increased neuron recruitment to the HP had less omega-6 and, consequently, a lower ratio of omega-6/omega-3 fatty acids. Thus, our results are more consistent with those in lobsters, in which decreasing the ratio of omega-6/omega-3 fatty acids increased rates of adult neurogenesis (Beltz *et al.*, 2007). Beltz *et al.* (2007) hypothesized that specific fatty acids influence the recruitment and survival of adult-born neurons either by incorporation of fatty acids into the cell membranes or by the effect of fatty acids as signalling molecules in cytokine signalling pathways, stimulating neurotrophin expression in HP to promote new neuron recruitment and survival (Wu *et al.*, 2008). Analysis of fatty acid composition and expression of signaling molecules in both cytokine and neurotrophin pathways in brain regions exhibiting changes in neuron recruitment would delineate these potential mechanisms.

Vitamin E enrichment

Providing birds with a diet high in vitamin E significantly increased the number of migratory adult-born neurons in cN. This finding is in contrast to results in mammals showing that a higher level of dietary vitamin E enrichment reduced the number of newly-generated neurons in HP (Ciaroni *et al.*, 1999). Note that migratory cells sampled in this study might not have integrated into the same region in which they were sampled, but instead could be passing through the sampled region *en route* to another site of integration, which may explain the contrast between our results and the findings of Ciaroni *et al.* (1999) in rats.

Another possible explanation is the difference in vitamin E manipulation in our study compared with previous studies in mammals. Whereas we tested the effects of level of dietary enrichment of vitamin E on adult neurogenesis, previous studies compared animals fed diets completely lacking vitamin E with animals fed a diet supplemented with vitamin E, creating potential confounds from health disorders due to vitamin E deficiency (e.g. Ricciarelli *et al.*, 2007). Our low vitamin E treatment provided sufficient vitamin to preclude deficiency. We propose that our lower level of vitamin E may be comparable to a vitamin E-supplemented diet in previous studies. At lower levels, dietary vitamin E may reduce neuroproliferation and subsequent neuron migration to a point beyond which further enrichment appears to increase proliferation and/or recruitment, particularly to the cN of birds. Investigating the effect of multiple levels of dietary enrichment has also proven helpful in reconciling apparently conflicting results on the effect of vitamin E in the related field of exercise redox biology (Nikolaidis *et al.*, 2012).

Vitamin E × exercise interaction

Although our manipulations revealed effects of exercise, dietary fatty acid composition and vitamin E enrichment on neuron migration and/or recruitment in the avian telencephalon, most effects were site-specific. We consistently found a significant interaction between levels of dietary vitamin E enrichment and exercise affecting neuron recruitment in multiple brain regions. Specifically, flight exercise increased neuron recruitment in HP, Str and rN but only in birds that were fed a diet with a lower level of vitamin E enrichment. Conversely, a higher level of vitamin E enrichment decreased neuronal recruitment in Str in exercised birds. Lastly, a higher level of dietary vitamin E increased neuronal recruitment in HP and rN, but only in birds that were not flown in the wind tunnel. Because both vitamin E supplementation and exercise are known to increase the recruitment and survival of adult born neurons (Ferri *et al.*, 2003; van Praag, 2008), we suggest that both factors act through a shared signalling pathway.

Because the level of dietary enrichment with vitamin E has not been manipulated in this manner before in investigations of the effects of exercise neurogenesis, we would predict that standard rat chow diets may contain a level of vitamin E that is permissive for exercise to produce the increase in HP neuron recruitment, as is commonly reported (e.g. Kobilov *et al.*, 2011). Our data represent the first report of a non-additive interaction between exercise and a dietary supplement on adult neurogenesis. Examining the effects of vitamin E dietary enrichment on molecular signals known to be involved in exercise-induced neurogenesis, such as brain-derived neurotrophic factor, may delineate the interaction between exercise and diet.

Conserved, but site-specific regulation of adult neurogenesis

We report that three regulators of adult neuroplasticity in the mammalian brain, dietary vitamin E, dietary fatty acid composition and exercise, influence neuron migration and recruitment in the avian telencephalon. Beltz & Sandeman (2003) also note similarities in the regulation of adult neurogenesis between mammals and decapod crustaceans, including enhanced neuron recruitment in the adult brain by environmental enrichment and diet. Here, we demonstrate that neurogenesis in the adult avian brain is modulated by similar factors. Examining the cellular signalling pathways and genetic signals controlling neurogenesis in different taxa could reveal to what extent these regulatory processes are conserved in vertebrates.

Clarifying how the regulation of adult neurogenesis is conserved across species may produce new models for research on diseases of the nervous system, such as depression, characterized by a reduction in neuron recruitment (Jacobs, 2002).

Although our manipulations did not influence plasticity in the song-control system, the absence of differences in neuron recruitment to HVC shows that the effects of exercise and diet on neuron recruitment in the telencephalon were site-specific. The differences in which factors regulate adult neuronal recruitment in different brain regions may provide clues to the function of new neurons recruited to different regions of the brain. In support of a link between the site-specific regulation and function of neuronal recruitment, the act of singing, which may be considered a form of exercise, upregulates neuronal recruitment to HVC (Li *et al.*, 2000; Sartor and Ball, 2005). This finding suggests that the functional involvement of a brain region in a behaviour may underpin site-specific changes in neuronal recruitment. This may also explain why our flight manipulation had no influence on neuronal recruitment to HVC, which is involved in the production of birdsong and not flight.

Site-specificity has also been reported in rodents, where exercise enhanced HP neuron recruitment without influencing recruitment to the olfactory bulb (Brown *et al.*, 2003). In mammals, new neurons only integrate into HP and the olfactory bulb. Birds, fish and reptiles may provide better models for examining site-specific adult neurogenesis because they continue to incorporate new neurons throughout much of the adult brain (Kaslin *et al.*, 2008).

Conclusions

The avian brain exhibits continued neuron migration and recruitment throughout the telencephalon in adulthood. Because most studies on neuroplasticity in the avian brain are restricted to the song-control system or HP, we know nothing regarding the functions of new neurons elsewhere in the avian telencephalon or how their migration and recruitment are regulated. We tested the effects of three regulators of adult neurogenesis, exercise, dietary fatty acids and dietary vitamin E, on adult neuron migration and recruitment in male European starlings and found that all three factors significantly influenced neuron migration and/or recruitment in a site-specific manner. We also found a consistent interaction between exercise and vitamin E on the upregulation of adult neurogenesis, suggesting that the influence of these factors on the brain are linked and context-specific.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. Main and interaction effects of exercise, dietary fatty acid ratio and vitamin E on neuroanatomy in adult, male European starlings with significance values. In each cell, the top value is the *F* value and the lower number is the *P* value for each main or interaction effect.

Table S2. Main and interaction effects of exercise, dietary fatty acid ratio and vitamin E on the number of fusiform and spherical doublecortin (DCX) neurons sampled in the brains of adult, male European starlings with significance values. In each cell, the top value is the *F* value and the lower number is the *P* value for each main or interaction effect.

Table S3. Treatment assignment and brain measures for each European starling used in this study.

Acknowledgements

This work was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (Grant no. 217831) and the National Science Foundation of USA (Grant no. IOS-0748349). The authors declare no competing financial interests.

Abbreviations

cN, caudal nidopallium; DCX, doublecortin; HP, hippocampus; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; RA, robust nucleus of the arcopallium; rN, rostral nidopallium; Str, striatum.

References

- Balthazart, J., Boseret, G., Konkle, A.T.M., Hurley, L.L. & Ball, G.F. (2008) Doublecortin as a marker of adult neuroplasticity in the canary song control nucleus HVC. *Eur. J. Neurosci.*, **27**, 801–817.
- Beltz, B.S. & Sandeman, D.C. (2003) Regulation of life-long neurogenesis in the decapod crustacean brain. *Arthropod Struct. Dev.*, **32**, 39–60.
- Beltz, B.S., Tlustý, M.F., Benton, J.L. & Sandeman, D.C. (2007) Omega-3 fatty acids upregulate adult neurogenesis. *Neurosci. Lett.*, **415**, 154–158.
- Boseret, G., Ball, G.F. & Balthazart, J. (2007) The microtubule-associated protein doublecortin is broadly expressed in the telencephalon of adult canaries. *J. Chem. Neuroanat.*, **33**, 140–154.
- Brown, J., Cooper-Kuhn, C.M., Kempermann, G., van Praag, H., Winkler, J., Gage, F.H. & Kuhn, H.G. (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur. J. Neurosci.*, **17**, 2042–2046.
- Ciaroni, S., Cuppini, R., Cecchini, T., Ferri, P., Ambrogini, P., Cuppini, C. & Del Grande, P. (1999) Neurogenesis in the adult rat dentate gyrus is enhanced by vitamin E deficiency. *J. Comp. Neurol.*, **411**, 495–502.
- Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H.G. & Aigner, L. (2005) Doublecortin expression levels in adult brain reflect neurogenesis. *Eur. J. Neurosci.*, **21**, 1–14.
- Ferri, P., Cecchini, T., Ciaroni, S., Ambrogini, P., Cuppini, R., Santi, S., Benedetti, S., Pagliarini, S., Del Grande, P. & Papa, S. (2003) Vitamin E affects cell death in adult rat dentate gyrus. *J. Neurocytol.*, **32**, 1155–1164.
- Fischl, J. & Caccamise, D.F. (1987) Relationships of diet and roosting behavior in the European starling. *Am. Midl. Nat.*, **117**, 395–404.
- Fox, R.A., Roth, T.C., LaDage, L.D. & Pravosudov, V.V. (2010) No effect of social group composition or size on hippocampal formation morphology and neurogenesis in mountain chickadees (*Poecile gambeli*). *Dev. Neurobiol.*, **70**, 538–547.
- Garrett, L., Lie, D.C., Hrabé de Angelis, M., Wurst, W. & Höflter, S.M. (2012) Voluntary wheel running in mice increases the rate of neurogenesis without affecting anxiety-related behaviour in single tests. *BMC Neurosci.*, **13**, 61.
- Gerson, A.R. & Guglielmo, C.G. (2011) Flight at low ambient humidity increases protein catabolism in migratory birds. *Science*, **333**, 1434–1436.
- Hall, Z.J. & MacDougall-Shackleton, S.A. (2012) Influence of testosterone metabolites on song-control system neuroplasticity during photostimulation in adult European starlings (*Sturnus vulgaris*). *PLoS ONE*, **7**, e40060.
- Jacobs, B.L. (2002) Adult brain neurogenesis and depression. *Brain Behav. Immun.*, **16**, 602–609.
- Kaslin, J., Ganz, J. & Brand, M. (2008) Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philos. T. Roy. Soc. B.*, **363**, 101–122.
- Kawakita, E., Hashimoto, M. & Shido, O. (2006) Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. *Neuroscience*, **139**, 991–997.
- Klasing, K.C. (1998) Nutritional modulation of resistance to infectious diseases. *Poultry Sci.*, **77**, 1119–1125.
- Kobilo, T., Liu, Q.-R., Gandhi, K., Mughal, M., Shaham, Y. & van Praag, H. (2011) Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learn. Memory*, **18**, 605–609.
- LaDage, L.D., Roth, T.C., Fox, R.A. & Pravosudov, V.V. (2010) Ecologically relevant spatial memory use modulates hippocampal neurogenesis. *Proc. Biol. Sci.*, **277**, 1071–1079.
- LaDage, L.D., Roth, T.C. & Pravosudov, V.V. (2011) Hippocampal neurogenesis is associated with migratory behaviour in adult but not juvenile sparrows (*Zonotrichia leucophrys* ssp.). *Proc. Biol. Sci.*, **278**, 138–143.
- Langlois, L. & McWilliams, S.R. (2010) Protein requirements of an omnivorous and granivorous songbird decrease during migration. *Auk*, **127**, 850–862.
- Li, X.C., Jarvis, E.D., Alvarez-Borda, B., Lim, D.A. & Nottebohm, F. (2000) A relationship between behavior, neurotrophin expression and new neuron survival. *Proc. Natl. Acad. Sci. USA*, **97**, 8584–8589.
- Murphy, M.E. & King, J.R. (1982) Semi-synthetic diets as a tool for nutritional ecology. *Auk*, **99**, 165–167.
- Newman, A.E.M., MacDougall-Shackleton, S.A., An, Y.-S., Kriengwatanana, B. & Soma, K.K. (2010) Corticosterone and dehydroepiandrosterone have opposing effects on adult neuroplasticity in the avian song control system. *J. Comp. Neurol.*, **518**, 3662–3678.
- Nikolaidis, M.G., Kyparos, A., Spanou, C., Paschalis, V., Theodorou, A.A. & Vrabas, I.S. (2012) Redox biology of exercise: an integrative and comparative consideration of some overlooked issues. *J. Exp. Biol.*, **215**, 1615–1625.
- Olson, A.K., Eadie, B.D., Ernst, C. & Christie, B.R. (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus*, **16**, 250–260.
- Park, H.R. & Lee, J. (2011) Neurogenic contributions made by dietary regulation to hippocampal neurogenesis. *Ann. NY Acad. Sci.*, **1229**, 23–28.
- van Praag, H. (2008) Neurogenesis and exercise: past and future directions. *Neuromol. Med.*, **10**, 128–140.
- van Praag, H., Christie, B.R., Sejnowski, T.J. & Gage, F.H. (1999) Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc. Natl. Acad. Sci. USA*, **96**, 13427–13431.
- Pytte, C.L., Gerson, M., Miller, J. & Kim, J.R. (2007) Increasing stereotypy in adult zebra finch song correlates with a declining rate of adult neurogenesis. *Dev. Neurobiol.*, **67**, 1699–1720.
- Pytte, C.L., Yu, Y.-L., Wildstein, S., George, S. & Kim, J.R. (2011) Adult neuron addition to the zebra finch song motor pathway correlates with the rate and extent of recovery from botox-induced paralysis of the vocal muscles. *J. Neurosci.*, **31**, 16958–16968.
- Ricciarelli, R., Argellati, F., Pronzato, M.A. & Domenicotti, C. (2007) Vitamin E and neurodegenerative diseases. *Mol. Aspects Med.*, **28**, 591–606.
- Sartor, J.J. & Ball, G.F. (2005) Social suppression of song is associated with a reduction in the volume of a song-control nucleus in European starlings (*Sturnus vulgaris*). *Behav. Neurosci.*, **119**, 233–244.
- Scott, M.L., Nesheim, M.C. & Young, R.J. (1982) *Nutrition of the Chicken*. M.L. Scott & Associates, Ithaca, NY.
- Scott, B.B., Gardner, T., Ji, N., Fee, M.S. & Lois, C. (2012) Wandering neuronal migration in the postnatal vertebrate forebrain. *J. Neurosci.*, **32**, 1436–1446.
- Sherry, D.F. & Hoshoooley, J.S. (2010) Seasonal hippocampal plasticity in food-storing birds. *Philos. T. Roy. Soc. B.*, **365**, 933–943.
- Tramontin, A.D. & Brenowitz, E.A. (2000) Seasonal plasticity in the adult brain. *Trends Neurosci.*, **23**, 251–258.
- Vellema, M., van der Linden, A. & Gahr, M. (2010) Area-specific migration and recruitment of new neurons in the adult songbird brain. *J. Comp. Neurol.*, **518**, 1442–1459.
- Wu, C.-W., Chang, Y.-T., Yu, L., Chen, H., Jen, C.J., Wu, S.-Y., Lo, C.-P. & Kuo, Y.-M. (2008) Exercise enhances the proliferation of neural stem cells and neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice. *J. Appl. Physiol.*, **105**, 1585–1594.
- Zeigler, H.P. & Marler, P. (2008) *Neuroscience of Birdsong*. Cambridge University Press, Cambridge.