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Persistent Increases In the Pool of Doublecortin Expressing Neurons in the Hippocampus Following Spatial Navigation Training

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Abstract

In addition to its role in neuronal migration during embryonic development, doublecortin (DCX) plays a role in hippocampal neurogenesis across the lifespan. Hippocampal neurons exhibit a high degree of synaptic plasticity while they are in the DCX phase. While previous studies have reported that behavioral training on hippocampus-dependent tasks can enhance neuron survival, little was known about the stage of development of those neurons and, particularly, whether a large pool of the surviving new neurons remains in the DCX phase for a prolonged period after training. Here we report that spatial navigation training increases the pool of neurons that are in the DCX phase four weeks after training ended. Thus, the stock of DCX-expressing neurons in the hippocampus is affected by whether a hippocampus-dependent task has been encountered during the preceding few weeks.

Keywords

Doublecortin; Memory; Neurogenesis; BrdU; Ki67

Introduction

Doublecortin (DCX) is a microtubule-associated protein that is expressed in neurons during differentiation and migration [9]. Mutations of the *DCX* gene can lead to devastating neuronal migration defects such as subcortical laminar heterotopia, or “double cortex” syndrome, and X-linked lissencephaly [7]. DCX is involved in the assembly and stabilization of non-centrosomal microtubules, is particularly enriched at the growth cones of the dendrites and axons of immature neurons, and is thought to have a role in the growth of neuronal processes and synaptogenesis [10, 18, 24].

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During adulthood, DCX is expressed in cells in the subventricular zone and the subgranular zone of the dentate gyrus, two areas where neurogenesis continues throughout the lifespan. In mice reared under standard laboratory housing conditions, DCX expressing neurons comprise about 17% of the neuronal population of the dentate gyrus of two-month-olds (young adults) but are rare in two-year-olds (senescent mice) [14]. The majority of DCX expressing cells in the adult dentate gyrus are immature neurons undergoing neurite elongation (>70%) while 20 percent are transiently amplifying progenitor cells [23]. Using bromodeoxyuridine (BrdU) as a mitotic marker, several studies have reported that training on behavioral tasks that require the hippocampus can enhance the survival of immature dentate gyrus neurons that were born either during the days leading up to behavioral training or while behavioral training was in progress [8, 11, 15].

Although the function of new neurons is not yet known, an intriguing possibility is that immature neurons encode new long-term memories. Computational modeling studies suggest that the availability of a pool of naïve neurons may enable the hippocampus to avoid catastrophic interference, a classic problem for connectionist neural networks in which exposure to a new stimulus pattern subsequently disrupts retrieval of older memories [17, 35]. However, neuron proliferation at the time of learning would not immediately contribute to new learning because the cell cycle, neurite extension, and integration of new cells into a network can take weeks. An economical strategy would be to maintain a stock of immature neurons that are available for encoding new experiences based on how often new hippocampus-dependent problems were encountered during the past several weeks [35]. Although previous research has established that behavioral training on hippocampus-dependent tasks can enhance neuron survival, it is not yet clear whether such training causes a long-lasting increase in the pool of neurons that are in the DCX phase. The present study provides data indicating that spatial navigation training increases the pool of neurons that are in the DCX phase for at least four weeks after training.

Materials and Methods

Subjects

Thirty-five male, Long Evans, hooded rats (3 months of age) were housed in groups of two (12 h light/dark cycle). All behavioral testing occurred during the light phase of the cycle. Rats had continuous access to food and water and were treated in accordance with the Institutional Animal Care and Use Committee regulations.

Spatial Learning

The Morris water task (MWT) was used for spatial navigation training [19]. Curtains that were suspended from a track attached to the ceiling enclosed the circular pool (1.83 m diameter). Four two-dimensional visual cues (0.8 m × 0.8 m) decorated with distinctive light and dark visual patterns were suspended from the ceiling and positioned at four cardinal positions relative to one another. The pool was filled with 28° C ($\pm 2^\circ$) water to which evaporated milk was added to make the water opaque. A Plexiglass cylinder, 10 cm in diameter, submerged 2 cm below the surface of the water, provided an escape refuge. A digital video camera mounted on the ceiling directly over the pool was interfaced to a

computerized behavioral tracking system (Ethovision; Noldus Information Technology, Wageningen, The Netherlands).

Three different experimental groups were studied (Table 1). One group was trained on the Morris water maze with four trials (i.e., swims) per day over 8 d and one trial on Day 9 (Trained; n=11). Each trial lasted until the rat found the platform, or for 60 s. Rats remained on the platform for 5 s after each trail. Rats in a second group were each matched with a rat from the trained group and placed in the pool for the same amount of time (i.e., “yoked”) as a randomly chosen rat from the trained group, but no escape refuge was available (Swim Control; n= 17). On Day 38 rats returned to the pool for a 60 s probe trial with the platform removed. Finally, a group that was not exposed to the water maze task was also studied (Home Cage; n=7). Table 1 summarizes the regimens followed by each group.

Quantity and phenotype of newly born cells

Most rats in each group received bromodeoxyuridine (BrdU) injections (dissolved in 0.9% NaCl, 60 mg/ml, 60 mg/kg body weight; Sigma, St. Louis, MO). For those rats, starting on Day 13, intraperitoneal (ip) BrdU injections were given twice per week (Mondays and Thursdays) through Day 37 (see Table 1). In order to determine whether BrdU injections themselves produced changes in other cell markers, a subset of rats from each group did not receive BrdU injections [3, 5].

On Day 40 (2 days after probe testing), rats were deeply anesthetized with sodium pentobarbital (100 mg/kg), perfused with 5% sucrose in de-ionized (DI) water, followed by 4% PFA in DI water, using Perfusion One™ (Coretech Holdings, St. Louis, MO). Brains remained overnight in fixative, then were rinsed twice in 5% sucrose and placed in 30% sucrose for cryoprotection. Brains were sectioned at 50µm and every sixth section was collected for immunofluorescent labeling for BrdU, neuronal nuclei (NeuN), doublecortin (DCX), and Ki67.

For BrdU and NeuN staining, the tissue was washed, treated with 50% Formamide/2X SSC buffer for 2 hours at 65 °C, washed in 2X SSC, incubated with 2M HCl at 37 °C for 30 minutes, and washed over 2hrs. Tissue was then placed in primary antibody overnight: 0.3% triton X (Sigma, St. Louis, MO), 2% Normal Goat Serum (Jackson ImmunoResearch, West Grove, PA), 1:200 rat anti-BrdU (Accurate Chemical & Scientific Corp., Westbury, NY), and 1:1000 mouse anti-NeuN (Chemicon, Temecula, CA) in 0.1M PBS. Tissue was washed in 0.1M PBS and placed overnight in secondary antibody: 1:250 Goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR), and 1:500 biotinylated Goat anti-rat IgG (Chemicon, Temecula, CA) in 0.1M PBS. Tissue was washed and placed in streptavidin-Alexa 568 (1:500) in 0.1M PBS for 45 minutes. Tissue was washed then mounted onto subbed slides (1% gel, 0.2% chromalum) and cover slipped using glycerol mounting medium (1–3% n-propyl gallate in 1 part 0.1M phosphate buffer and 9 parts glycerol).

For DCX staining, tissue was washed and incubated in 0.3% H₂O₂ in 0.1M PBS for 25 min at room temperature. Tissue was washed then placed overnight in primary antibody solution: 1:1000 anti-DCX goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), 0.5% Triton-X, and 2% normal rabbit serum (Vectastain Elite ABC Kit Goat IgG - Vector

Laboratories, Burlingame, CA), in 0.1M PBS. Tissue was washed and placed in secondary antibody solution: 1:1000 Biotinylated anti-goat IgG (Vectastain Elite ABC Kit Goat IgG, Vector Laboratories), in 0.1M PBS for 1 hour at room temperature. Tissue was washed and incubated in ABC solution (Vectastain Elite ABC Kit Goat IgG - Vector Laboratories) for 45 minutes at room temperature. Tissue was washed and incubated in DAB with Nickel solution (Vector Laboratories) until color changed (2–10 min). Tissue was washed and mounted onto subbed slides (1% gel, 0.2% chromalum). Slides were dehydrated with an ascending series of ethanol, cleared in CitriSolv (Fisher Scientific), and coverslipped using Permount (Fisher Scientific) mounting medium.

For Ki67 staining, tissue was washed and incubated overnight in primary antibody solution: 1:500 Ki67 rabbit polyclonal (Novocastra, Newcastle upon Tyne, UK), 0.3% Triton-X, 1% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in 0.1M PBS. Tissue was washed and incubated for one hour at room temperature in secondary antibody solution: 1:1000 Anti-rabbit IgG biotinylated antibody (Vector Laboratories) in PBS. Tissue was washed and placed in 1:500 Streptavidin Alexa Fluor 568 conjugate (Molecular Probes) for 45 minutes at room temperature. Tissue was washed, mounted onto subbed slides (1% gel, 0.2% chromalum) and coverslipped using glycerol mounting medium (1–3% n-propyl gallate in 1 part 0.1M phosphate buffer and 9 parts glycerol).

BrdU-, DCX-, and Ki67-positive cells were counted in brain sections that corresponded to plates 31, 35 and 44 of Paxinos [22]. BrdU-, Ki67-, and DCX-labeled cells in the granule cell layer and subgranular zone of the dentate gyrus were counted in both hemispheres from each section through either a 20 (DCX) or 40 (BrdU and Ki67) X objective. Two experimenters independently provided counts for each section and inter-rater reliabilities were consistently high (all r 's > 0.95). Composite means of the two experimenters' counts were used as dependent variables.

To quantify BrdU and NeuN co-expression, 30 BrdU-positive cells per animal were analyzed using an Olympus FluoView 1000 laser scanning confocal microscope. Cells were imaged through a 40 X (and 60 X whenever necessary) objective and Z-series of 0.5 μ m optical sections were merged to confirm co-expression. Ratios of cells that expressed BrdU alone and those that co-expressed BrdU and NeuN were determined.

Statistical analyses

All data analyses were based on ANOVA models and used Fisher's planned comparisons for post hoc tests (SPSS version 11.0.4 for Macintosh; Chicago, IL).

Results

Spatial learning

Rats in groups Trained and Swim Control received four daily swims in the water maze on days 1 through 8. For rats trained to locate the hidden platform, escape latencies significantly improved as a function of training day; $F_{(7,63)}=60.08$; $p < 0.001$; Fig 1a. Short escape latencies and direct swim paths were observed on Day 9 (single test trials), demonstrating excellent retention of the task learned on days 1 through 8. On Day 39, the

platform was removed from the pool for a 60 s probe test for rats in the Trained and Swim Control groups. An analysis of mean swim distance per trial on days 1 through 8 did not reveal any statistically significant difference between the groups (Fig 1b). Analysis of time spent in the target quadrant of the pool revealed a significant main effect of group; $F_{(1,26)}=29.55$; $p<0.001$; Fig 1c. Trained rats spent a significantly greater percentage of their time searching in the correct quadrant than did Swim Control rats.

Cell counts

DCX-, Ki67-, BrdU-, and BrdU/NeuN co-expressing cells in the subgranular and granule cell layers of the dentate gyrus were counted (Fig 2). In order to determine whether BrdU injections themselves affected DCX and Ki67 expression, one subset of rats from each experimental condition received BrdU injections ($n=21$) and the other subset did not ($n=14$). DCX and Ki67 expression were not significantly affected by BrdU injections; $F_{(1,32)}=1.1$; $p>0.3$. A significant main effect of group on DCX expression confirmed that training influenced the number of DCX-expressing neurons found in the dentate gyrus; $F_{(2,32)}=3.2$; $p<0.05$; Fig 3a. Trained rats had the most DCX-positive neurons and differed significantly from Home Cage ($p<0.04$) and Swim Control rats ($p<0.04$). Swim Control rats did not differ significantly from Home Cage rats in terms of the number of DCX-positive neurons observed. No significant group differences were noted in the number of Ki67-, BrdU-expressing, or BrdU- and NeuN-co-expressing cells observed (Fig 3 b and c).

Discussion

Rats that received eight daily sessions of spatial navigation training in the MWT provided clear evidence that they learned the location of the hidden escape platform and retained a memory of its location for 28 days. The ability to learn and retain the MWT depends on an intact dentate gyrus [6, 31, 33]. Within the dentate gyrus, more DCX expressing neurons were observed in rats that received spatial navigation training 30 days prior to the day they were sacrificed than in swim controls or home cage controls. The lack of an increase in DCX-expressing neurons in swim controls eliminates the possibility that swimming alone was responsible for the increase observed in the Trained group. Between-group comparisons in neuron proliferation made using BrdU and Ki67 as markers gave no indication of an effect of training on proliferation. BrdU is incorporated in DNA during the S-phase of the cell cycle. In rats, mitotic cells in the subgranular zone of the dentate gyrus are in the S phase for about 9.5 h, which comprises about 38% of the duration of the cell cycle [4]. BrdU is available for DNA incorporation for about 15 min after injection [16]. In the present study, BrdU injections began on the fifth post-training day and were repeated every other day until Day 37, two days before perfusions. If the spatial training procedure had increased the number of new neurons generated during the retention phase of this study, more BrdU labeled cells and BrdU/NeuN co-labeled cells should have been evident in tissue from trained rats than that taken from the control groups. Many BrdU and BrdU+NeuN co-labeled cells were observed in the dentate gyrus of rats in each group. However, no group differences in the number of BrdU labeled cells were found. Ki67 antibodies identify the cell cycle-associated protein, mKi67 [32]. Ki67 antibodies are sensitive to proliferating cells and identify cells in late G1, S, G2, and M phase of the cell cycle [26]. Ki67 positive cells were

abundant in our samples, but no group differences in Ki67 labeling were detected. Together, the BrdU and Ki67 labeling results suggest that spatial training did not increase cell proliferation during the post-training phase of the study. Therefore, the increase in DCX-expressing neurons observed in this study suggests that spatial training increased the survival of cells born before, during, or during the first four days after the spatial training regimen, and that many of these new neurons remained in an immature developmental state until Day 40.

One concern with the use of the yoked swim control is that because they cannot find the platform rats in the yoked control group may become stressed over repeated trials and stress hormones might inhibit the pool of neurons of interest in this study. Stress hormones can indeed suppress hippocampal neurogenesis [12]. We would be concerned about the possibility that forced swimming without a platform in the pool may have suppressed neurogenesis in that group if fewer DCX-, BrdU-, or Ki67-positive cells had been observed in the yoked controls than in the home cage controls. However, no differences between yoked controls and home cage controls were detected suggesting that brief swims without the platform were not sufficiently stressful to suppress neurogenesis in the present situation.

Several previous studies have reported that training on hippocampus-dependent learning and memory tasks enhances new neuron survival [8, 11, 15]. Training on a trace eyeblink conditioning task, which depends on the hippocampus, enhances the survival of neurons that formed prior to training for at least 60 days [15]. In a study that used a training procedure that closely resembled the one used in the present study, spatial training did not affect survival of BrdU positive cells that formed during first four days of an eight day training regimen [8]. During the last four days of training, however, more BrdU positive cells were detected in a spatially trained group than in controls and more BrdU positive cells that formed during the final four training days survived for 28 days [8].

In contrast with the above-mentioned studies that reported positive effects of behavioral training on hippocampal neuron survival, one recent study reported that neuron proliferation and DCX labeling was reduced by watermaze training [21] and a second study failed to observe enhanced neuron survival after watermaze training [29]. The present study differed from the one reported by Namestkova et al. (2005) in several key respects. Namestkova et al. (2005) used colder water than we did ($22^{\circ} \pm 2^{\circ} \text{C}$ in their study versus $28^{\circ} \pm 2^{\circ} \text{C}$ in our study), executed all of the trials consecutively without opportunity for subjects to return to home cage (i.e., they used 10 sec ITIs with the rats going straight from the escape platform back into the cold water whereas our rats were placed in a warm, dry holding chamber for 5 min between each trial), and shifted the location of the escape platform during final two days of testing. Furthermore, Namestkova et al. (2005) sacrificed their rats on the final day of swim training whereas we waited one month before sacrificing the rats. Thus, the procedure that Namestkova et al. (2005) used may have been more stressful to the rats than the procedure that we used and, furthermore, by sacrificing their rats on the final day of watermaze training they were more likely to detect a stress effect than we were by waiting one month before sacrificing the rats. With respect to the effects of watermaze training on neuron survival, although the present study was not designed explicitly to resolve the discrepancies between the results of other laboratories [1, 28, 29], our study clearly provides

additional evidence favoring the view that spatial navigation training promotes the long-term survival of neurons while they are in the DCX phase.

The time it takes for new neurons to progress from the cell cycle to maturation ranges from about three days to several weeks [23]. Although it was already clear from previous studies that behavioral training on hippocampus-dependent tasks could enhance neuron survival, little was known about the stage of development of those neurons and, particularly, whether the surviving new neurons were in the DCX phase. The present study suggests that many neurons remain in the DCX phase for at least four weeks after spatial navigation training. A key question that remains is whether neurons serve a unique function in the hippocampus while they are in the DCX phase.

Immature neurons are known to possess certain physiological properties that distinguish them from mature neurons. For example, long-term potentiation (LTP), an activity-dependent enhancement of synaptic plasticity that has been proposed as a mechanism of information storage in the nervous system [20], can be induced at a lower threshold and decays more slowly in immature hippocampal neurons than in mature neurons [25, 30]. Evidence that immature neurons may encode new stimuli was reported in a study that quantified the expression of the immediate-early genes *c-fos* and *Arc* (activity-regulated cytoskeletal-associated protein) in groups of mice that received BrdU injections 1, 2, 4, 6, or 8 weeks prior to training on a hippocampus-dependent task; the Morris water maze task [13]. Both *c-fos* and *Arc* expression were detected in a greater percentage of neurons that were 4–8 weeks old than in younger neurons or in neurons that were not BrdU positive (i.e., neurons of all ages). Thus, there does appear to be a developmental time course for neuronal sensitivity to spatial stimulation that begins sometime around the fourth week after mitosis.

Studies that have used various techniques to block adult neurogenesis suggest that hippocampus-dependent memories require a population of immature neurons. The antimitotic agent methylazoxymethanol acetate (MAM), which inhibits neuronal precursor proliferation, impairs rats' performances on trace eye blink conditioning and trace cued fear conditioning, two Pavlovian conditioning tasks that depend on the hippocampus, while sparing non-hippocampal "delay" eye blink conditioning [27]. MAM also impairs object recognition memory, depending on the interval that separates the sample and recognition phases of the task [2]. MAM-treated subjects perform as well as controls when delays of 15-m or 1-h between the sample and recognition components of the object recognition memory task were used but not when 24- and 48-hour retention intervals are used. Similarly, on delayed visual cue non-match-to-sample task, blocking hippocampal neurogenesis via exposure to X-irradiation disrupts performance when retention intervals of two and four minutes are used but not when retention intervals of 1 minute or less are used [34]. Therefore, it appears that object and spatial discrimination tasks can be learned even without immature neurons, but forgetting is rapid.

Further evidence that immature neurons are required for long-term retention of spatial information was provided by a study that exposed rats to whole brain irradiation four weeks prior to training on the Morris water maze. Blocking neurogenesis did not interfere with rats' abilities to initially learn to locate the hidden platform refuge or to retain the spatial

information for up to one week after acquisition training. However, irradiated rats demonstrated poor retention of spatial information compared to normal controls that were tested two or four weeks after acquisition training [29]. Thus, long-term retention, but not acquisition or short-term retention, of spatial information requires a population of immature neurons.

In summary, the present study found that many of the neurons remain in the DCX phase, a developmental stage during which they express a high degree of plasticity, for up to one month after training on a spatial learning task. One approach to the question of whether the population of DCX positive cells in the hippocampus is controlled by the recent memory load placed on the hippocampus would be to assess whether systematically varying the number of hippocampus-dependent tasks different groups are trained to solve would produce corresponding changes in the number of DCX positive cells observed. Although such an experiment would be complicated by potential confounding factors such as between-experimental condition differences in physical activity, the discovery of a strong positive relationship between learning history and the population of neurons in the DCX phase would reinforce the view that neurons in the DCX phase are used to encode novel stimuli for long-term storage.

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Fig 1.

Escape latencies of rats trained to search for a hidden escape platform. The platform was in a fixed location on days 1–9 (A). Mean swim distance of swim controls and spatially trained rats (B). Results from a probe trial with the escape platform removed (C). Data are expressed as mean % of the total trial (60 s) that rats in the trained and swim control groups spent in the correct pool quadrant. Above each bar are swim paths of a representative (i.e., median performer) from each group. Bars represent standard error of the mean.

Fig 2.
Photomicrographs of representative examples of DCX-positive cells observed via light microscopy at 40 X (A), Ki67-positive cells (red) observed with epifluorescence at 40 X (B), and BrdU/NeuN co-labeled cells (yellow) and BrdU-positive cells (red) imaged with confocal microscopy at 60 X (C). Scale bars are 50 μ m.

Fig 3.
Cell counts as a function of group. DCX- (A), Ki67- (B), and BrdU- (C) positive cells per rat in Swim Control (SC), Home Cage (HC) and Trained groups. Percentage of BrdU-positive cells that co-expressed NeuN (D). Bars represent standard error of the mean.

Table 1

Schedule of swim sessions (*s*), single retention trial (*r*), BrdU injections (*b*), and probe test (*p*).

Group	Day																																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38					
Home Cage													<i>b</i>		<i>b</i>				<i>b</i>								<i>b</i>												<i>b</i>		<i>b</i>		
Swim Control	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>r</i>			<i>b</i>			<i>b</i>				<i>b</i>				<i>b</i>			<i>b</i>													<i>b</i>		<i>b</i>	<i>p</i>	
Trained	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>r</i>			<i>b</i>			<i>b</i>				<i>b</i>				<i>b</i>			<i>b</i>														<i>b</i>		<i>b</i>	<i>p</i>