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Research report

Decreased proliferation in the adult rat hippocampus after exposure to the Morris water maze and its reversal by fluoxetine

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Abstract

Granular cell proliferation in the adult hippocampus decreases during aging and after chronic stress, while it can be increased by physical activity or treatment with the antidepressant fluoxetine. We investigated whether the physical and cognitive stimulation accompanied by stress in the commonly used Morris water maze affects the rate of proliferation and whether the induced changes can be influenced by antidepressant treatment with fluoxetine. Proliferating cells in the dentate gyrus were labeled by three injections of BrdU during the 24 h preceding sacrifice. Early differentiation to neuronal progeny was studied by immunohistochemical staining for doublecortin (DCX), a microtubule binding protein expressed in newborn neurons. Acquisition learning in the water maze for 15 days caused a significant decrease in granular cell proliferation in the granular cell layer of the hippocampus. The decrease in the number of BrdU- and DCX-positive cells was reversed to control levels by the use of fluoxetine during the water maze training. Fluoxetine treatment alone increased the number of BrdU-positive cells, but did not increase the number of DCX-positive cells. We conclude that the exposure of adult male rats to water maze acquisition trials is a stressful experience that significantly suppresses the production of new granular cells and that this stressful effect can be blocked by the concomitant administration of the antidepressant fluoxetine.
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Keywords: Antidepressant; BrdU; Doublecortin; Learning; Proliferation; Stress; Water maze; Neurogenesis

1. Introduction

New neurons are continuously born in the granular cell layer of the hippocampus throughout life in a number of mammalian species, including humans [1–6,8–13,15,17–19,22,23]. Newborn cells can be detected by labeling with [^{3}H] thymidine [3] or BrdU [1,2,4–6,8–13,15,17–19,22,23]. The neuronal phenotype can then be determined by using various neuronal markers such as doublecortin (DCX) [2,7,10].

Several conditions influence the rate of proliferation and the ability of the newborn cells to survive and functionally integrate. During aging and exposure to stress, granule cell proliferation is suppressed [9]; antidepressant treatment [5,12,13] and physical activity such as voluntary wheel running [23] promote proliferation. An enriched environment [15,23] contributes to the survival of newly generated granule cells, while some tasks of hippocampus-dependent learning contribute to survival [8], as well as to the proliferation [11] of BrdU-positive cells.

Stressful experiences increase corticosterone levels, which suppress proliferation [5,9]. Basal levels of corticosterone may influence proliferation throughout the entire lifespan. In the first two postnatal weeks, rats have low levels of corticosterone that are accompanied by a stress hyporesponsive period and a high rate of granular cell proliferation [9]. Basal levels of corticosterone increase with age, while the rate of proliferation declines [9]. The chronic application of several classes of antidepressants, including the selective serotonin re-uptake inhibitor fluoxetine, increases the rate of granular cell proliferation in rats [13]. Suggested molecular

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mechanisms for the antidepressants' effects on neurogenesis include the increased expression of the trophic factor BDNF [5,12,13]. Physical activity, such as voluntary wheel running for 12 days in a standard cage, increases the rate of proliferation [23]. This kind of physical activity also up-regulates the transcription of BDNF genes after only two nights [14] and has been shown to be additive to antidepressant treatment [16]. Hippocampus-dependent learning as short as 4 days of place acquisition in the Morris water maze (MWM), or the acquisition of 800 trials of a trace eye blink conditioned response, has increased the number of surviving BrdU-positive cells [8]. Animals exposed either for 8 weeks to an enriched environment [15] or for 30 days to voluntary wheel running [22], perform better in the hippocampus-dependent spatial learning in the MWM, presumably due to the contribution of newborn cells to hippocampal function.

We studied whether spatial learning in the MWM, which is a task that includes a certain level of both physical activity and stress, would affect the proliferation of granular cells in the hippocampus of adult rats. We examined whether the change in proliferative response to the MWM task is influenced by fluoxetine treatment. The rate of granular cell proliferation was evaluated by determining the number of BrdU-positive cells, and the number of newly born neurons was studied by staining cells with antibodies directed against doublecortin (DCX).

2. Materials and methods

2.1. Animals

The study was performed using 3-month-old male Wistar rats ($N = 20$; 320–340 g). The rats were housed under standard conditions, 2–3 per cage, with a 12 h light and dark cycle, and with access to water and food ad libitum. The experiments were approved by the ethical committee of the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic.

2.2. Morris water maze (WM) acquisition training and drug administration

After initial handling, the rats were randomly assigned to one of four groups and then underwent 15 days of one of four different treatments. The first (C – control) group received a daily i.p. injection of 1.5 ml saline for 15 days. The second group (WM – water maze) received a daily i.p. injection of saline and underwent four trials per day of acquisition training in the WM for 15 days. The third group (F – fluoxetine) received a daily i.p. injection of fluoxetine (Chemos CZ, Prague, Czech Republic) for 15 days, 5 mg/kg diluted in saline. The fourth group (FWM – fluoxetine treated and water maze exposed) received an i.p. injection of fluoxetine 1 h prior to learning and underwent the same acquisition training in the water maze as did the second group.

The acquisition training in the MWM took place during four consecutive trials per day. The Morris water maze apparatus was a circular tank 196 cm in diameter filled with cold water ($22 \pm 2\,^{\circ}$C). A submerged invisible platform 10 cm in diameter was kept in one quadrant for 10 days and then moved to another quadrant on days 11 and 13. This modification of the acquisition learning, together with a change in light conditions on day 11, was introduced in order to keep the animals learning and motivated to solve the task under new conditions. The trials were always initiated from different positions in the water tank. After finding the submerged platform, the rats were allowed to rest on the platform for 10 s and then the next trial started. The cut-off time was 60 s.

On day 15 all animals in all groups received an i.p. injection of 18 mg/ml BrdU (5-bromo-2-deoxyuridine) (Sigma Aldrich, Prague, Czech Republic) solution in distilled water (75 mg/kg of body weight). The BrdU injection was repeated after 8 and 16 h (a total of three injections in 24 h). The animals were sacrificed 24 h after the first BrdU injection by barbiturate overdose (200 mg/kg) and transcardialy perfused by phosphate buffered saline pH 7.4 (PBS) followed by freshly made 4% paraformaldehyde in PBS.

2.3. Tissue processing and immunohistochemistry

The brains were removed from the skull and stored in 4% paraformaldehyde for at least 3 days, then transferred to solutions with ascending concentrations of sucrose to a maximum of 30%. After saturation, 40 μm thick coronal sections were cut on a freezing sliding microtome (Microm, Walldorf, Germany). Sections for BrdU immunohistochemistry were processed the following day; additional sections were stored in a cryoprotectant containing 30% ethylene glycol, 30% glycerine and 40% PBS. The antibodies used were a mouse monoclonal directed against 5-bromo-2-deoxyuridine and diluted 1:100 (Roche Applied Science, Basel, Switzerland), goat anti-doublecortin C-18 diluted 1:500 (Santa Cruz Labs, Santa Cruz, USA), and Cy3-conjugated rabbit anti-rat GFAP diluted 1:200 (Sigma Aldrich, Prague, Czech Republic). For peroxidase immunohistochemistry to detect BrdU, peroxidase-conjugated rabbit anti-mouse IgG (whole molecule) diluted 1:100 (Sigma Aldrich, Prague, Czech Republic) and peroxidase-conjugated goat anti-mouse IgG (whole molecule) diluted 1:50 (Sigma Aldrich, Prague, Czech Republic) were used as secondary antibodies with diaminobenzidine (DAB substrate kit, Vector Laboratories, Burlingame, USA) as the substrate. For DCX immunostaining, the C-18 primary antibody was detected using a Vectastain Elite ABC kit (goat IgG) (Vector Laboratories, Burlingame, USA). For indirect immunofluorescence, a series of secondary antibodies was used. Alexa Fluor 594-conjugated goat anti-mouse IgG diluted 1:500 (Molecular Probes, Eugene, USA) or Alexa Fluor 488-conjugated goat anti-mouse IgG diluted 1:200 (Molecular Probes, Eugene, USA) were used for the detection of BrdU, while Alexa Fluor 488-conjugated donkey anti-goat IgG diluted 1:200 (Molecular Probes, Eugene, USA) was used for the detection of DCX. For BrdU immunostaining, the following procedure was used: The sections were incubated in 3 N HCl for 20 min, washed in distilled H_2O, then neutralized in 0.1 M borate buffer for 10 min. Endogenous peroxidase was then quenched in 0.3% H_2O_2 in methanol for 30 min. Subsequently, the sections were incubated in 5% fetal bovine serum in PBS for 20 min, then overnight with anti-BrdU at $4\,^{\circ}$C. After rinsing in PBS, the slices were incubated for 30 min at room temperature in peroxidase-conjugated goat anti-mouse IgG diluted in PBS with 5% fetal bovine serum, followed by rinses and a 30 min incubation in peroxidase-conjugated rabbit anti-mouse IgG. The peroxidase reaction was detected by a solution of DAB in PBS and 0.06% H_2O_2.

2.4. Images and cell counting

Coronal brain sections were cut through the entire hippocampal formation containing the granular cell layer. An Axioskop 2 plus microscope (Carl Zeiss, Germany), equipped with a 63× Plan Neofluar objective, was used to count BrdU-positive cells visualized by DAB as well as DCX-positive cells labeled with the fluorophore Alexa 488. Every sixth section was used for counting BrdU-positive cells. Cells were counted separately in the ventral and dorsal blades of the granular cell layer and in the area of the hilus. Cells that were located more than two cells away from the sub-granular zone were considered to be in the hilus. The quantification of DCX-positive cells was done in three sections from each animal. Only cells that showed the typical appearance of an unstained nucleus surrounded by staining in the cell matrix and membrane were counted. Fluorescent images shown on our micrographs were acquired on a confocal microscope (TCS-NT, Leica).

2.5. Statistical analyses

All analyses were performed using Sigmaplot 2000 (SPSS Inc., Chicago, IL). The number of BrdU-positive cells in the granular cell layer was counted for each hippocampus in every sixth slice, while the number of DCX-positive cells was counted for each hippocampus in three sections from every animal; comparisons between groups were made using Student's *t*-test.

3. Results

The rate of proliferation following the four different treatments was quantified as the number of BrdU-positive cells, i.e. the number of cells that integrated BrdU into their DNA during the last 24 h of life, in three hippocampal regions: the inner and outer blade and the hilus of the dentate gyrus. BrdU-positive cells were present in all the examined groups of animals. BrdU-positive cells were detected mostly in the sub-granular zone (SGZ) of the granular cell layer (GCL), often appearing with irregularly shaped nuclei in typical clusters (Fig. 1A, F–L). In the area of the hilus, they were mostly present as pairs or small clusters, often visible only as single ovoid nuclei (Fig. 1A). There were no significant differences in the distribution of BrdU-positive cells between the two blades of the GCL in any of the groups. Some of the cells in the clusters were in close apposition to astrocytic processes, which often formed loops or baskets around the newborn cells (Fig. 1F, G–I, J–L).

Estimates of the effect of the treatments on actual neurogenesis were made using antibodies directed against DCX (Fig. 1B–E). The antibodies to DCX typically stained the periphery of the soma and the proximal processes; the nucleus of the cell was not stained (Fig. 1D). DCX-positive cells and their processes were seen in the SGZ of the GCL, but none were found in the hilus (Fig. 1B and C). Cells often appeared apposed to each other or even alone, distributed without any particular pattern, although they were always present at the junction of the two blades of the GCL (Fig. 1B). The data are presented as raw data and represent the mean numbers of BrdU- or DCX-positive cells per hippocampus in a single slice.

Fig. 2 summarizes the effects of the treatments, i.e. Morris water maze acquisition training (WM), fluoxetine treatment (F), and fluoxetine treatment during water maze acquisition (FWM), on the number of BrdU- or DCX-positive cells per hippocampus in one slice (mean ± S.E.M.). The number of BrdU-positive cells per hippocampus in control animals ($N = 5$) was 17.4 ± 1.38 ($n = 60$) in the GCL and 6.97 ± 0.68 ($n = 60$) in the hilus (Fig. 2A and B). The number of DCX-positive cells was 69.48 ± 4.22 ($n = 30$) (Fig. 2C).

Acquisition training in the water maze significantly reduced granular cell proliferation ($N = 5$). The mean number of BrdU-labeled cells in the GCL was decreased by 27% (12.73 ± 1.22, $n = 60$, $p > 0.01$), while in the area of the hilus the decrease was only 15% (5.92 ± 0.59, $n = 60$, n.s.) (Fig. 2A and B). The number of DCX-positive cells was 23% lower than in controls (53.83 ± 3.30, $n = 30$, $p > 0.05$) (Fig. 2C).

Fluoxetine treatment significantly increased proliferation in the granular cell layer. When compared to controls, the mean number of BrdU-positive cells in the hippocampus of fluoxetine-treated rats ($N = 5$) was increased by 26% in the GCL (21.9 ± 1.86, $n = 60$, $p > 0.05$) and by 15% in the hilus (8.03 ± 0.64, $n = 60$, n.s.) (Fig. 2A and B). The number of DCX-positive cells was, however, 14% lower than in controls (59.43 ± 4.27, $n = 30$, n.s.) (Fig. 2C).

Fluoxetine treatment during acquisition learning in the water maze maintained the proliferation in the GCL at control levels, but in the hilus the number of BrdU-positive cells was lower than in controls ($N = 5$). The mean number of cells per hippocampus was similar to control animals in the granular cell layer (19.45 ± 1.73, $n = 60$, n.s.), but was significantly decreased by 33% (4.7 ± 0.51, $n = 60$, $p > 0.01$) in the area of the hilus (Fig. 2A and B). The number of DCX-positive cells was similar to control levels (69.23 ± 4.28, $n = 30$, n.s.) (Fig. 2C).

Fig. 3 demonstrates the learning curves of the two groups of animals ($N = 5$) that underwent the Morris water maze learning task (WM and FWM). In both groups, four animals showed an improvement in acquisition learning during the first 10 days and all animals showed improvement by day 15. The differences between the average latencies of the five animals receiving an i.p. injection of either fluoxetine (5 mg/kg) (FWM) or saline (WM) 1 h prior to the WM task were not significant. Modification of the task on days 11 and 13 presented a new challenge to the animals as a result of the changed location of the platform and additional cues such as the lightening conditions in the room. The new learning conditions initially caused a prolongation of the latency. Subsequently, learning of the new spatial situation was induced, as indicated by the descending learning curves.

We conclude that acquisition training in the MWM significantly reduced the proliferation of granule cells in the GCL. Fluoxetine treatment increased the number of proliferating cells in the GCL. The application of fluoxetine during the acquisition of spatial learning in the MWM main-

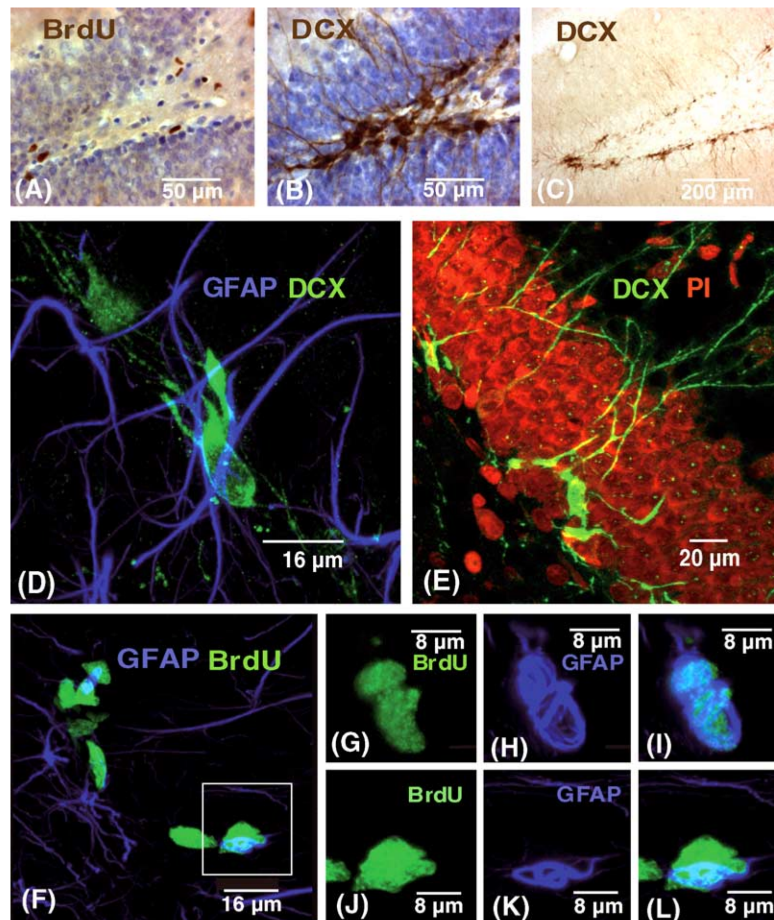

Fig. 1. Photomicrographs of a rat hippocampus stained with anti-BrdU and anti-DCX. The nuclei in the GCL are counterstained with Harris hematoxylin. (A) Example of BrdU-positive cells (brown) at the junction of the granular cell layer. The proliferating cells are present in the sub-granular zone and in the hilus. (B) Typical appearance of DCX-positive cells – newborn neurons – (brown) with their processes extending through the GCL. Most of the DCX-positive cells can be found at the junction of the internal and external blades of the GCL. (C) An illustrative photomicrograph of clusters of DCX-positive cells (brown) lining the sub-granular cell layer. The cells are extending long processes through the GCL. Confocal microscopy images of coronal sections through the rat hippocampus; (D) typical appearance of DCX-positive cells (green) in the sub-granular zone extending their processes parallel to the GCL, doublestained with anti-GFAP (blue). (E) DCX-positive cells (green) extending their processes through the SGZ; the nuclei of granular cells are counterstained with propidium iodid (PI, red). (F) A cluster of BrdU-positive cells (green) in the granular cell layer surrounded by astrocytic processes (blue). (G–L) Images of BrdU-positive cells surrounded by astrocytic processes. GFAP staining (H – blue) and BrdU staining (G – green) are shown separately. (J–L) A higher magnification image of a BrdU-positive cell (J – green) from figure F, showing its close apposition to a GFAP-positive process (K – blue).

tained the rate of proliferation in the GCL at control levels but led to a decreased number of proliferating cells in the hilus.

4. Discussion

Our study shows that spatial learning acquisition trials in the Morris water maze and fluoxetine treatment affect the rate of cell proliferation in the neurogenic region – the granular cell layer – and in the hilus of the dentate gyrus in the hippocampus. Complex stimulation and learning in the water maze for 15 days resulted in a down-regulation of granular cell proliferation. This is a very surprising finding since learning has generally been considered as a factor that positively influences the generation of new neurons in adulthood. It has been clearly demonstrated that hippocampus-dependent learning of temporal or spatial events enhances the survival of newborn cells. Proliferation, on the other hand, remained at control levels after training with the trace paired eyeblink protocol [8] and after 12 days of MWM acquisition training [23]. In an experiment done by Lemaire et al., 5 days of spatial task learning in the Morris water maze did increase the rate of proliferation [11]. This finding seems to be contradictory to our results; however, there are important differences between the studies. The length of exposure to the MWM task was only 5 days in the Lemaire et al. study, and the BrdU injections were given just before the training session for 5 days, thus also labeling cells that had been proliferating before exposure to the MWM. Five days of exposure led to an increase in the rate of proliferation, but in our study, 15 days of MWM training caused a decrease in the proliferation rate

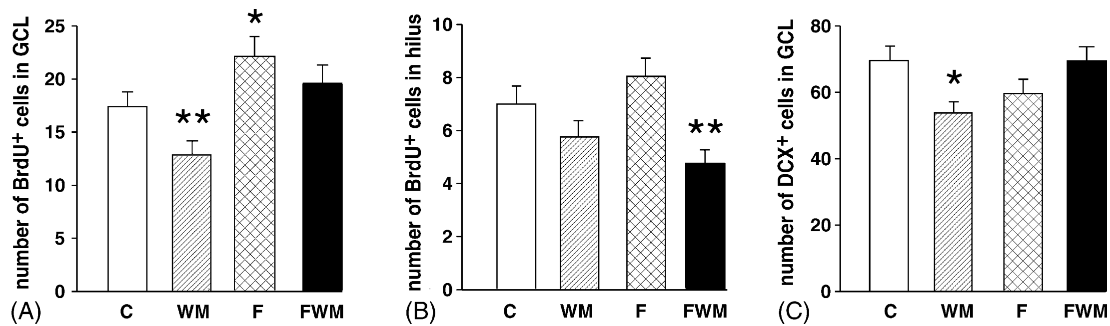

Fig. 2. The numbers of 5-bromo-2-deoxyuridine (BrdU)- or doublecortin (DCX)-positive cells in the dentate gyrus in rats (C – controls, WM – Morris water maze trained, F – fluoxetine treated, FWM – WM trained and fluoxetine treated). The values are expressed as mean ± S.E.M. per single hippocampus in a slice. (A) The number of BrdU-positive cells in the granular cell layer was decreased by 27% ($p > 0.01$) after water maze stimulation (WM) and increased by 26% ($p > 0.05$) after fluoxetine treatment (F), when compared to control animals (C). The administration of fluoxetine blocked the negative effect of water maze training on proliferation (FWM). (B) Fluoxetine increased non-significantly proliferation in the area of the hilus by 15% (F) when compared to control animals (C); MWM stimulation caused a non-significant decrease of 15% (WM) and in combination with fluoxetine treatment caused a significant decrease of 33% ($p > 0.01$) (FWM). (C) The number of DCX-positive cells – newborn neurons – in the sub-granular zone was decreased after stimulation in the water maze by 23% ($p > 0.05$) (WM) and after fluoxetine treatment non-significantly by 14% (F), when compared to controls (C). Fluoxetine treatment during the acquisition training in the water maze did not cause an increase in the number of DCX-positive cells (FWM).

in animals of similar age. The MWM challenge represents not only spatial learning and physical activity, but it also has a stressful component. The motivation for finding the location of the hidden platform is to escape from a life-threatening situation in a novel, unpleasant environment of cold water. The different results of the two studies suggest that the benefit from proliferation enhancing factors during the test, such as physical activity, may be diminished by prolonged exposure to a stressful experience.

Our results can be compared to a study in which mice were exposed to either 12 days of water maze acquisition or only to swimming [23]. In that study, in part investigating proliferation, the number of labeled cells was not different between the test groups and controls when BrdU was administered every day during all 12 training days. The down-regulating effect of stress was probably masked by a normal rate of neurogenesis prior to the decline in the proliferative rate during the water maze training. Supporting arguments for our results can be also found in the work of Shors et al. [19], who suggested that the acquisition of spatial memory in the water maze may be a task that requires only a small percentage of new neurons; trace eye blink and fear conditioning were impaired by blocking neurogenesis with cytostatics [18,19], but spatial learning performance in the water maze was unaffected by such blockade [19].

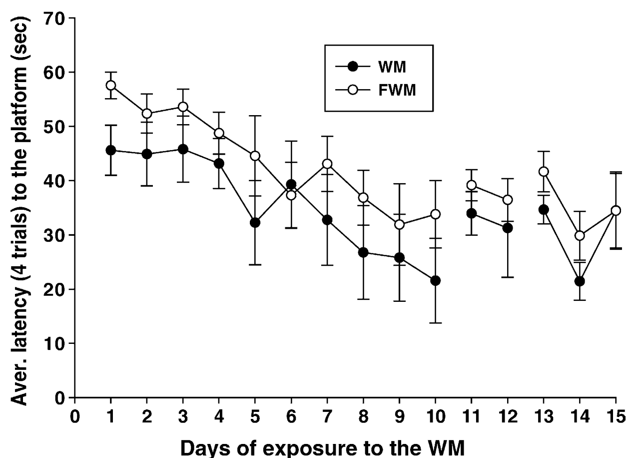

Fig. 3. A graph presenting the learning curves of the two groups of animals exposed to the Morris water maze learning task for four trials a day; shown are the average latencies ± S.E.M. The animals received an i.p. injection of either fluoxetine solution (5 mg/kg; FWM) or of saline (WM) 1 h prior to the task. The spatial conditions remained standard for 10 days, then on days 11 and 13 the position of the platform and the light conditions in the experimental room were changed in order to keep the animals learning (the breaks in the learning curves indicate the changes in the conditions). There is great variation among animals in both experimental groups, and the differences between the groups are not significant.

The administration of fluoxetine to animals that were not exposed to the WM increased the rate of proliferation as expected in the examined regions (Fig. 2A and B). Proliferation was very high in the hilus of the DG. The work of Cameron et al. in 1993 suggested that the neurogenic region in the hilus, observed in the early postnatal period, may persist to adulthood [3]. One to 24 h after labeling newborn cells with [^{3}H] thymidine, the majority of positive cells was observed in the area of the hilus. Three weeks after labeling a majority of positive cells was observed in the granular cell layer, suggesting that the cells migrate to the granular cell layer [3]. This suggestion cannot, however, be fully supported by our observations that there were no DCX-positive cells present in the area of the hilus. Animals treated with fluoxetine for 15 days did not show a greater number of DCX-positive newborn neurons (Fig. 2C). The higher proliferation rate caused by the chronic application of fluoxetine was not reflected in the number of the DCX-positive cells. These results suggest that fluoxetine treatment increases proliferation but not the differentiation and survival of newborn neurons.

Evidence for a stress-related decrease in proliferation after water maze learning is provided by the reversal of the effect

of stress by the application of fluoxetine. The animals in the WM trained group that received fluoxetine showed similar numbers of BrdU-positive cells as did the control rats. The significant decrease in proliferation seen in the hilus remains unexplained.

Immunohistochemical staining for DCX allowed us to determine the number of cells differentiating into new neurons born during approximately the last 14 days preceding sacrifice, the time period when newborn cells show this early neuronal marker [2,10]. It is likely that proliferation contributes to the number of DCX-positive cells, as well as the survival and differentiation of cells generated earlier. The number of DCX-positive cells in the GCL of animals exposed to a combination of fluoxetine treatment and stimulation in the water maze was increased when compared to animals exposed to WM acquisition alone or treatment with fluoxetine alone, but was similar to control animals. Gould et al. showed that 4 days of acquisition in the water maze increased the survival of already labeled newborn cells [8]. Our results are not in discrepancy with this experiment in view of the fact that the cells were labeled before exposure to the potentially stressful experience in the water maze. Stress has been shown to suppress the proliferation of granule cells [9,11,12]. The water maze training in our study did not result in decreased proliferation in the granular cell layer when the stress was reduced by the administration of fluoxetine.

Sub-granular zone astrocytes are considered to be the primary precursors of the D cells that differentiate into neurons [1]. Astrocytes that extend their basal processes under the blades of the granular cell layer and their apical processes through the layer, together with endothelial cells from apposed blood vessels, form a niche for developing newborn neurons [4]. Photomicrographs (Fig. 1F–L) acquired on a confocal microscope show GFAP-positive processes forming a basket around only some of the cells in a cluster of BrdU-positive cells. The survival of newborn cells is likely to be successful when stimulating substances diffusing from the blood stream as well as impulses from the functional neuronal network are delivered through the astrocytic syncitium. We have shown previously [21] that learning deficits in aged rats are related to the disorganization of astrocytic processes and the loss of extracellular matrix in the hippocampus. Similar changes were observed after postnatal hypobaric hypoxia and were also related to learning deficits [20].

In our study we have not examined the survival of newborn cells for an extended period of time. It would be of interest to see if the combination of fluoxetine, which increases the proliferation rate, and hippocampus-dependent learning in the water maze, which increases survival, would dramatically change the number of surviving cells 4 weeks after labeling. Moreover, determining the survival of cells stimulated by fluoxetine would also be clinically relevant. A further question to be addressed is the mechanism of action of stressful learning combined with fluoxetine on the rate of neurogenesis. Our study revealed the negative effect of long-term exposure to the conditions of the Morris water maze on the proliferation of new neurons that are believed to be a potential substrate for new learning capacity. This should be considered for future behavioral studies using the Morris water maze test.

Acknowledgements

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