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Voluntary exercise increases the new cell formation in the hippocampus of ovariectomized mice

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ABSTRACT

Voluntary exercise, such as running, can induce dramatic increases in adult hippocampal neurogenesis and improve learning and memory function. A recent report showed that exercise also improved memory problems in postmenopausal women. In this study, we examined whether voluntary running exercise could increase new cell formation in the hippocampus under menopausal conditions, modeled with ovariectomized (OVX) mice. Voluntary running exercise for 1 week significantly increased the number of bromodeoxyuridine (BrdU)- and Ki-67-immunoreactive cells in the hippocampus of mice 2 weeks after ovariectomy. In addition, 1 week of voluntary running exercise after 2 weeks in OVX mice increased the numbers of doublecortin- and calretinin-immunoreactive cells in the hippocampus. These data demonstrate that exercise may increase the birth of new cells in the hippocampus under estrogen-deprived conditions, suggesting that exercise may be helpful in improving brain function in climacteric women.

Physical exercise has been suggested to help in preventing or relieving many menopausal symptoms in climacteric women [18], and exercise has improved the percentage of body fat and cardiorespiratory endurance in menopausal and postmenopausal women [7]. Moreover, exercise increased lumbar vertebral bone mineral content [13] and led to higher brain concentrations of serotonin, low levels of which are associated with depression [5]. Interestingly, the clinical data of Aiello and colleagues demonstrated that exercise may improve memory problems in postmenopausal woman [1].

Beneficial effects of physical exercise on brain function have been observed in numerous studies. Physical activity has been shown to increase the number of new cells in the dentate gyrus and to improve performance in spatial learning tests [20,21] and memory. In addition, accumulating data show that new cells formation is involved in improving memory.

However, the effects of exercise on new cell formation in the brain during estrogen deprivation, as in menopause, have not been investigated. In the present study, we examined whether voluntary running could increase new cell formation in ovariectomized mice using immunohistochemistry to BrdU and Ki-67, cell proliferation markers, and to DCX and calretinin, cell differentiation markers, in the dentate gyrus of the hippocampus.

Adult (9-week-old) virgin female C57/BL6 mice (Charles River Diagnostics, Wilmington, MA, USA), weighing 16–18 g at time of arrival, were used in all experiments. Mice were group housed at a temperature of 20 ± 2 °C, with free access to food and water (12-h light/dark cycle). Animals were acclimated to these housing conditions for 1 week before any experimental manipulation. During exercise, animals were housed in 48 cm × 27 cm × 20 cm polyethylene cages, equipped with running wheels (7-cm diameter).

For the experiment, mice were divided into four groups: a control sedentary (SED) group (n = 4 females), a voluntary running (RUN) group (n = 4 females), an OVX group (n = 4 females), and an OVX + RUN group (n = 4 females). The control group and running group underwent a sham operation that was identical to the ovariectomy (OVX) procedure, with the exception that the ovaries were not removed (Fig. 1A). All surgical manipulations were performed under anesthesia with sodium pentobarbital (50–60 mg/kg, i.p.). After the operation, all mice were kept in the standard (no running wheel) cages for 2 weeks. Two weeks after the operation, the running group and the OVX + RUN group were put in cages equipped with voluntary running wheels for 7 days before they were killed.

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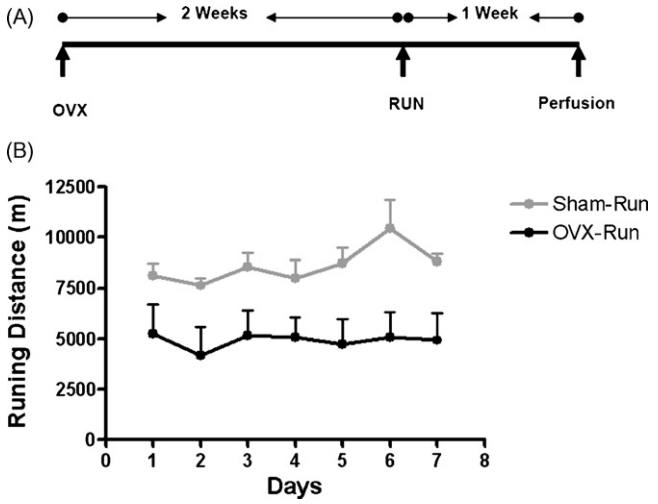

Fig. 1. Experimental design and changes in running activity after ovariectomy. (A) Two weeks after ovariectomy, animals started voluntary running for 1 week. (B) The running activity of mice decreased 2 weeks after ovariectomy.

In the running and OVX + RUN groups, all running was monitored, regardless of the direction of wheel rotation, per 24 h by electric counter, and was converted to distance, in m (Fig. 1B).

To determine the proliferation of the neural stem cells, mice were treated with bromodeoxyuridine (BrdU; 50 mg/kg, i.p.) three times in 2 days before they were killed. Mice were anesthetized with pentobarbital sodium (50–60 mg/kg, i.p.) and perfused with 0.5 M phosphate-buffered saline (PBS), and then fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were post-fixed overnight at 4 °C in the same solution and soaked in 0.5 M PBS containing 30% sucrose for cryoprotection. Serial 30-μm-thick coronal sections were cut on a freezing microtome (Leica, Nussloch, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB, pH 7.4) at −20 °C until use.

For immunohistochemical detection of BrdU, Ki-67, doublecortin (DCX), or calretinin, every fifth brain section was taken from the region between bregma −1.34 mm and bregma −3.28 mm; this was performed for each brain [17]. Free-floating sections were preincubated with primary antibodies (rat monoclonal anti-BrdU antibody, 1:1000, Roche, Mannheim, Germany; rabbit polyclonal anti-Ki-67 antibody, 1:1000, Upstate, Lake Placid, NY, USA; rat polyclonal anti-DCX antibody, 1:1000, BD PharMingen, Los Angeles, CA, USA; rabbit polyclonal anti-calretinin antibody, 1:1000, Upstate) overnight at 25 °C in the presence of 0.3% Triton X-100 and 0.5 mg/mL bovine serum albumin. Sections for BrdU immunohistochemistry were pretreated by denaturing the DNA. All sections were the incubated with anti-rabbit or anti-rat secondary antibodies (1:200 dilution; Vector Labs, Burlingame, CA, USA) for 90 min, and with an avidin–biotin–peroxidase complex (1:100 dilution; Vector Labs) for 1 h at room temperature. Peroxidase activity was visualized by incubating sections with 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.5 M Tris–buffered saline (pH 7.6). After several rinses with PBS, sections were mounted on gelatin-coated slices, dehydrated, and coverslipped in Histomount medium.

The numbers of BrdU-, KI-67-, DCX-, and calretinin-stained cells in the inner rim of the granule cell layer of the dentate gyrus were counted for each animal. The length of the granule cell layer was measured using Stereo Investigator software (Microbrightfield, Williston, VT, USA). The cell numbers per unit length were subjected to one-way ANOVA, followed by Dunnett's *post hoc* comparisons; results are expressed as the mean ± S.E.M. Differences were considered statistically significant at $p < 0.05$.

In epifluorescence immunodetection for calretinin and NeuN, sections were washed extensively and incubated with fluorochrome-conjugated species-specific secondary antibodies (anti-rabbit Cy3 and anti-rat Cy2; 1:200; Jackson ImmunoResearch, West Grove, PA, USA). Sections were placed on gelatin-coated slices and mounted in Prolong Antifade (Molecular Probes, Eugene, OR, USA). Epifluorescence was observed and photographed under a confocal microscope (Axiovert LSM 510 META; Zeiss, Oberkochen, Germany).

Estrogen deprivation affected the voluntary running behavior of mice. Two weeks of estrogen deprivation decreased the degree of voluntary running of mice to 57% of that in the sham group (Fig. 1B).

To evaluate the effect of running on cell proliferation in the subgranular zone (SGZ), we measured the number of BrdU- and Ki-67-immunostaining cells along the innermost region of the granule cell layer, adjacent to the hilus. The number of BrdU-positive cells in the dentate gyrus of the RUN group (39.9 ± 7.6/mm) was significantly higher than in the SED group (13.2 ± 3.5/mm; $p < 0.05$). We found no significant change in the number of BrdU-positive cells (10.1 ± 2.4/mm) in the dentate gyrus of OVX animals compared to SED animals. Voluntary running significantly increased the number of BrdU-positive cells in the dentate gyrus (20.8 ± 4.9/mm; $p < 0.05$) compared to the SED and the sham operation groups (Fig. 2A–D, M). We also observed that the number of BrdU-positive cells significantly decreased in the OVX-RUN group compared to in the RUN group (Fig. 2M). The overall pattern of BrdU immunostaining was consistent with that of the Ki-67 immunoreactivity (Fig. 2E–H, N).

To evaluate the effect of running on neuronal differentiation in the SGZ, we measured the number of DCX- and calretinin-positive cells along the innermost region of the granule cell layer, adjacent to the hilus. The number of DCX-positive cells in the dentate gyrus of the RUN group (118.8 ± 10.0/mm) was significantly higher than in the SED group (67.4 ± 6.3/mm; $p < 0.05$). We found no significant change in the number of DCX-positive cells (63.4 ± 10.9/mm) in the dentate gyrus of OVX animals compared to SED animals. Voluntary running significantly increased the number of DCX-positive cells in the dentate gyrus (104.4 ± 3.3/mm; $p < 0.05$), compared to the SED and sham operation groups (Fig. 2I–L, O). The number of DCX-positive cells significantly decreased in the OVX-RUN group compared to the RUN group (Fig. 2O). The immunoreactivity of calretinin was co-localized with NeuN-immunoreactive cells in the subgranular region of the dentate gyrus (Fig. 3A–D, b′ and b″). The pattern of the calretinin immunostaining was consistent with that of the DCX immunoreactivity (Fig. 3E).

To our knowledge, this is the first study to report morphological evidence of the effects of exercise on new cell formation in the hippocampus under menopausal (estrogen-deprived) conditions. This study showed that voluntary running increased cell proliferation in the hippocampus of ovariectomized mice. The number of BrdU-incorporating cells and Ki-67-immunostained cells increased after 1 week of voluntary running under estrogen-deprived conditions, with ovariectomy surgery, as also observed in the number of DCX- and calretinin-immunostained cells. Although estrogen has been reported to be involved in neurogenesis in the hippocampus [3,16,19], a recent study showed that new cell formation in the hippocampus was not dependent on estrogen [14]. Our study also suggests that exercise affect the new cell formation in the hippocampus in the estrogen-independent manner.

Numerous studies have shown that exercise increases neurogenesis and also improves learning and memory [2,6,9]. Neurogenesis is closely related to learning and memory. Interestingly, a clinical study showed the possibility that exercise may improve memory in menopausal women [1]. Although the exact mechanism underlying this was not clear, our study suggests that exercise during menopause may improve new cell formation in the hippocampus,

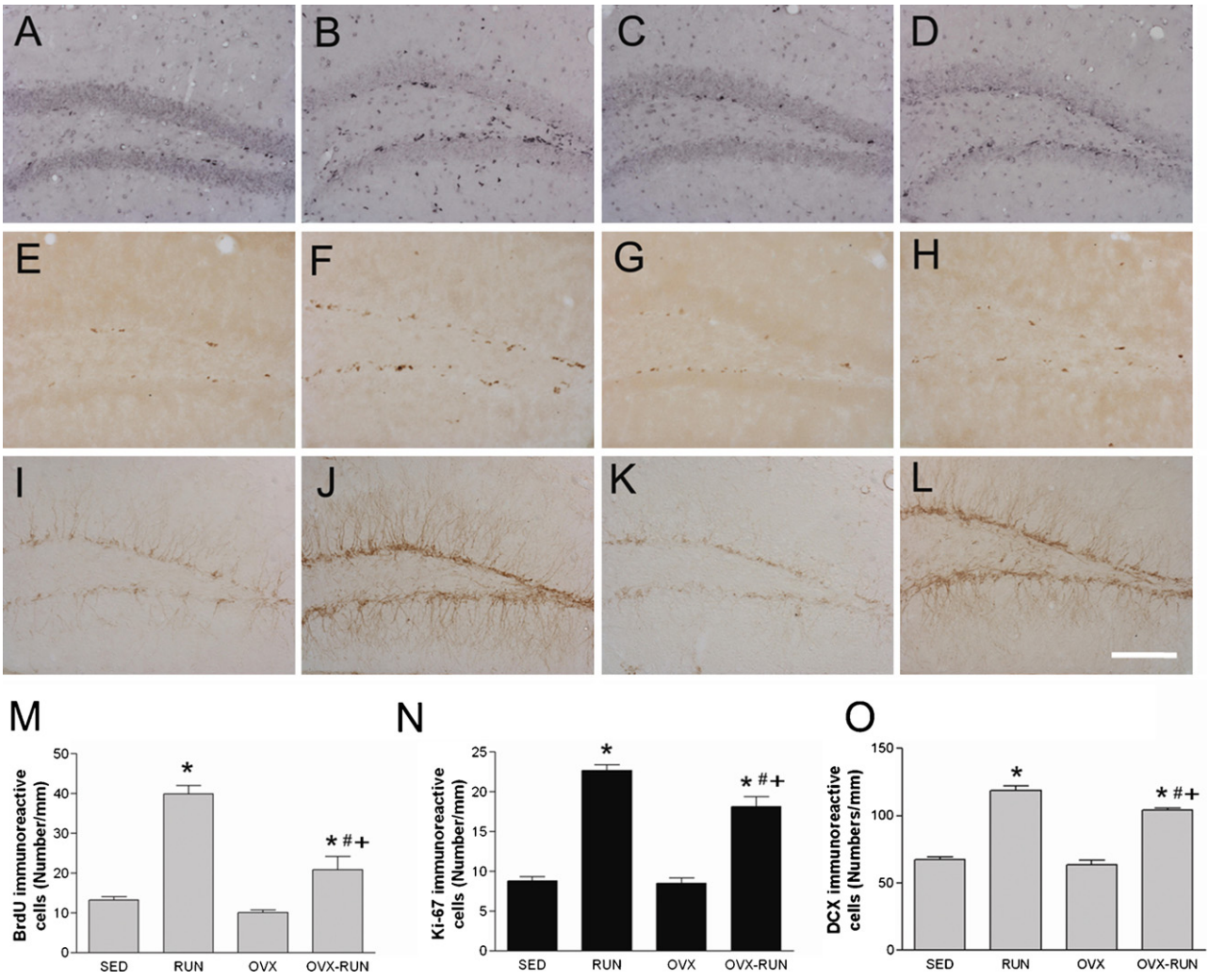

Fig. 2. Effects of voluntary running on stem cells in the dentate gyrus of ovariectomized mice. (A–D, M) Changes in BrdU-immunoreactive cells. (E–H, N) Changes in Ki-67-immunoreactive cells. (I–L, O) Changes in DCX-immunoreactive cells. Compared to the control, increases in the number of BrdU-, Ki67-, and DCX-labeled cells in the subgranular zone of the dentate gyrus were observed in the Run and OVX + RUN groups. In the OVX group, we found no effect on the changes in the number of BrdU-, Ki-67-, or DCX-labeled cells. (M–O) Values indicate the mean number of immunoreactive cells ± S.E.M. (*) indicates a statistically significant difference ($p < 0.05$) from the control group based on Dunnet's C *post hoc* comparison; (#) from the OVX group; (+) from the RUN group. Scale bar = 100 μm.

which would be one possible explanation consistent with the clinical study.

We examined the expression of doublecortin and calretinin to evaluate the degree of new neural cell formation in the dentate gyrus of animals. Doublecortin (DCX), a protein that promotes microtubule polymerization, is present in migrating neuroblasts and young neurons [10,11], and can serve as a marker of adult neurogenesis in the hippocampus. Calretinin is known as a marker for specific nonpyramidal γ-aminobutyric acid (GABA)-ergic neurons within the adult hippocampus [12]. A subpopulation of calretinin-positive neurons in the DG was described as being localized at the interface with the hilus; these cells were immunoreactive for PSA-NCAM and BrdU (7 days postinjection) [8,15] and were thus considered to be newly generated post-mitotic neurons. At the late phases of neurogenesis, new neurons expressed calretinin and doublecortin or NeuN [4].

In the present study, estrogen deprivation had no obvious effect on cell proliferation or differentiation in the dentate gyrus of the mouse hippocampus. Tanapat et al. [19] demonstrated that the new cell production appeared to be mediated by circulating estrogen during adulthood; 6 days after ovariectomy, a significant decrease in cell proliferation was seen in the adult rat dentate gyrus. Furthermore, Banasr et al. [3] reported that estrogen deprivation for 6 days induced decreases in the proliferation of stem cells in the dentate gyrus. Changes in estrogen levels over 21 days, however, had no effect on cell proliferation in the rat dentate gyrus. Interestingly, a recent study showed that the production of new neurons in the mice hippocampus was not influenced at 3 weeks after ovariectomy [14], which is consistent to our results. Thus, although the detail mechanism is not yet clear, the effects of estrogen deprivation on cell proliferation and differentiation may be somewhat transient and adult neural stem cells in the hippocampus may adapt to the long-term (≥about 3 weeks) estrogen deprivation.

We observed the estrogen deprivation affected the voluntary running behavior of mice. Two weeks of estrogen deprivation decreased the degree of voluntary running of mice. We also observed that the number of BrdU, Ki-67, DCX or calretinin-positive cells significantly decreased in the OVX-RUN group compared to in the RUN group. The present study suggests that the decreased running activity after ovariectomy is sufficient to significantly increase the new cell formation in the dentate gyrus. However, further study should be performed to elucidate whether the decrease of new cell formation in the OVX-RUN group is due to hormonal change or decreased-degree of running.

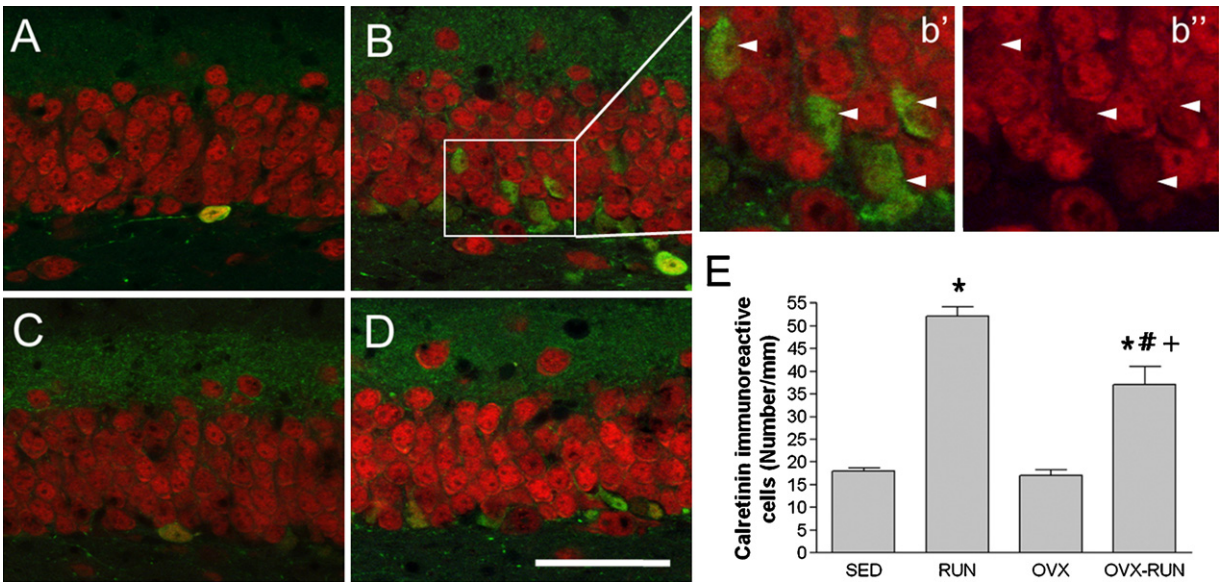

Fig. 3. The increase in calretinin immunoreactivity and co-localization with NeuN immunoreactivity in the hippocampus after voluntary running in ovariectomized mice. (A–D) Changes in calretinin-immunoreactive cells; compared to the control, the increase in the number of calretinin-labeled cells (green) was observed in the subgranular zone of the dentate gyrus in the RUN and OVX + RUN groups. (E) Values indicate the mean numbers of immunoreactive cells ± S.E.M. (*) indicates a statistically significant difference ($p < 0.05$) from the control group based on Dunnet's C *post hoc* comparison; (#) from the OVX group; (+) from the RUN group. (b′ and b″) Calretinin- (green) and NeuN-labeled cells (red) were co-localized in the dentate gyrus (arrowhead). (A) SED; (B) RUN; (C) OVX; (D) OVX + RUN. Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

We examined the effects of voluntary running exercise on new cell formation in the hippocampus under estrogen-deprived conditions. In the present study, voluntary running exercise after OVX increased the numbers of BrdU- and Ki-67-positive cells (markers of proliferation), and DCX- and calretinin-immunoreactive cells (markers of differentiation) in the hippocampus of ovariectomized mice. These data demonstrate that exercise may increase the birth of new neurons in the hippocampus under estrogen-deprived conditions, suggesting that exercise may be useful in improving brain function in climacteric women.

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References

[1] E.J. Aiello, Y. Yasui, S.S. Tworoger, C.M. Ulrich, M.L. Irwin, D. Bowen, R.S. Schwartz, C. Kumai, J.D. Potter, A. McTiernan, Effect of a yearlong, moderate-intensity exercise intervention on the occurrence and severity of menopause symptoms in postmenopausal women, Menopause 11 (2004) 382–388.
[2] J.B. Aimone, J. Wiles, F.H. Gage, Potential role for adult neurogenesis in the encoding of time in new memories, Nat. Neurosci. 9 (2006) 723–727.
[3] M. Banasr, M. Hery, J.M. Brezun, A. Daszuta, Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus, Eur. J. Neurosci. 14 (2001) 1417–1424.
[4] M.D. Brandt, S. Jessberger, B. Steiner, G. Kronenberg, K. Reuter, A. Bick-Sander, W. von der Behrens, G. Kempermann, Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice, Mol. Cell. Neurosci. 24 (2003) 603–613.
[5] B.S. Brown, T. Payne, C. Kim, G. Moore, P. Krebs, W. Martin, Chronic response of rat brain norepinephrine and serotonin levels to endurance training, J. Appl. Physiol. 46 (1979) 19–23.
[6] E. Bruel-Jungerman, C. Rampon, S. Laroche, 1. Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses, Rev. Neurosci. 18 (2007) 93–114.
[7] M.M. Cowan, L.W. Gregory, Responses of pre- and post-menopausal females to aerobic conditioning, Med. Sci. Sports Exerc. 17 (1985) 138–143.
[8] M.I. Dominguez, J.M. Blasco-Ibanez, C. Crespo, A.I. Marques-Mari, F.J. Martinez-Guijarro, Calretinin/PSA-NCAM immunoreactive granule cells after hippocampal damage produced by kainic acid and DEDTC treatment in mouse, Brain Res. 996 (2003) 206–217.
[9] E. Drapeau, W. Mayo, C. Aurousseau, M. Le Moal, P.V. Piazza, D.N. Abrous, Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 14385–14390.
[10] F. Francis, A. Koulakoff, D. Boucher, P. Chafey, B. Schaar, M.C. Vinet, G. Friocourt, N. McDonnell, O. Reiner, A. Kahn, S.K. McConnell, Y. Berwald-Netter, P. Denoulet, J. Chelly, Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons, Neuron 23 (1999) 247–256.
[11] J.G. Gleeson, P.T. Lin, L.A. Flanagan, C.A. Walsh, Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons, Neuron 23 (1999) 257–271.
[12] A.I. Gulyas, R. Miettinen, D.M. Jacobowitz, T.F. Freund, Calretinin is present in non-pyramidal cells of the rat hippocampus. I. A new type of neuron specifically associated with the mossy fibre system, Neuroscience 48 (1992) 1–27.
[13] B. Krolner, B. Toft, S. Pors Nielsen, E. Tondevold, Physical exercise as prophylaxis against involutional vertebral bone loss: a controlled trial, Clin. Sci. (Lond.) 64 (1983) 541–546.
[14] D.C. Lagace, S.J. Fischer, A.J. Eisch, Gender and endogenous levels of estradiol do not influence adult hippocampal neurogenesis in mice, Hippocampus 17 (2007) 175–180.
[15] Y. Liu, N. Fujise, T. Kosaka, Distribution of calretinin immunoreactivity in the mouse dentate gyrus. I. General description, Exp. Brain Res. 108 (1996) 389–403.
[16] B.K. Ormerod, T.T. Lee, L.A. Galea, Estradiol enhances neurogenesis in the dentate gyri of adult male meadow voles by increasing the survival of young granule neurons, Neuroscience 128 (2004) 645–654.
[17] G. Paxinos, D. Waston, The Mouse Brain in Stereotaxic Coordiates, 1st ed., Academic Press, San Diego, CA, 1997.
[18] M.M. Shangold, Exercise in the menopausal woman, Obstet. Gynecol. 75 (1990) 53S–58S.
[19] P. Tanapat, N.B. Hastings, A.J. Reeves, E. Gould, Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat, J. Neurosci. 19 (1999) 5792–5801.
[20] H. van Praag, B.R. Christie, T.J. Sejnowski, F.H. Gage, Running enhances neurogenesis, learning, and long-term potentiation in mice, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 13427–13431.
[21] H. van Praag, G. Kempermann, F.H. Gage, Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus, Nat. Neurosci. 2 (1999) 266–270.