17beta-estradiol and predegenerated nerve graft effect on hippocampal neurogenesis in adult female rats

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Submitted: 2008-03-25 Accepted: 2008-11-25 Published online: 2009-05-10

Key words: **neurogenenesis; hippocampus; estrogen; injury; estrogen receptor; nerve graft**

Neuroendocrinol Lett 2009; **30**(2):195–203 **PMID:** 19675526 NEL300209A12

Abstract

OBJECTIVES: The aim of present work was to examine estrogen influence on neurogenesis in the model of predegenerated peripheral nerve grafts implantation into the rat hippocampal dentate gyrus.

METHODS: Experiment was carried out on female rats divided into three experimental groups: NO – non-ovariectomized, OV – ovariectomized and E – heterogeneous group with various 17-beta-estradiol substitution after ovariectomy. Proliferating cells were labeled with BrdU. Brains were subjected to immunohistochemical procedures to visualize nestin, GFAP and estrogen receptors (ERα and ERβ).

RESULTS: Proliferation rate was highest in E groups with estrogen levels resembling that in proestrus phase. Ovariectomy resulted in higher than in NO group number of new neurons, while high hyperestrogenemia worsened the results. The proportions of nestin-labeled cells correlated in similar way with different hormonal state. We found also distinct co-localization of nestin and GFAP in E group (proestrus). It may suggest the presence of radial glia, a potential source of new neurons in adult mammals. Nerve graft induced ERα expression at the site of injury in all groups. Distribution of ERβ in hippocampus was estradiol-dose-dependent and correlated with cell proliferation.

CONCLUSION: In our model, 17-beta-estradiol and predegenerated nerve graft implantation had synergistic effect on hippocampal neurogenesis.

INTRODUCTION

It has been known since 1980th that the role of estrogens is not limited to reproductive functions, but they influence also other physiological processes. For example, in young women these hormones decrease cholesterol (LDL – low density lipoprotein) levels and lead to blood vessel relaxation via induction of nitric oxide production in the endothelium. These protective effects decreasing the risk of some cardiovascular diseases are lost in postmenopausal period (Kelly, 1984), when hypoestrogenemia leads also frequently to osteoporosis (Mayes, 2007).

The pleiotropic effect of estrogens can be clearly noted in the nervous system, too. The bulk of estrogens is produced in ovaries secreting these hormones into the blood. However, probably their trophic and protective functions in the central nervous system (CNS) are related to their local syn-

ORIGINAL ARTICLE

Abbreviations

brdU	– 5-bromo-2′-deoxyuridine
CNS	– central nervous system
DAPI	– 4′,6-diamidino-2-phenylindole
DG	– dentate gyrus
DNA	– deoxyribonucleic acid
EDTA	– ethylenediaminetetraacetic acid
ER	– estrogen receptor
GFAP	– glial fibrillary acidic protein
GnRH	– gonadotropin releasing hormone
LDL	– low density lipoprotein
MAPK	– mitogen activated protein kinase
pmER	– putative membrane ER
RIA	– radioimmunological
SGL	– subgranular layer
SVZ	– subventricular zone
UV	– ultraviolet waves

thesis and secretion (Cho *et al.* 2003, Wise *et al.* 2001). In the nervous system, these hormones are secreted by glial cells: astrocytes in the CNS, and Schwann cells in the peripheral nervous system (Garcia-Segura *et al.* 2001). Experimental data obtained from animal models provided convincing evidence of positive role of 17β-estradiol in neuroprotection (Behl and Manthey, 2000), anti-apoptotic processes (Honda *et al.* 2001), neurogenesis and regeneration (Islamov *et al.* 2002). This concept has been supported by some clinical studies, showing that estrogens enhanced memory and cognition processes (Fernandez *et al.* 2003) and were efficacious in the protection of neurodegenerative diseases and in diminishing brain injury associated with a stroke or stress (Blurton-Jones and Tuszyński, 2001, Wise *et al.* 2000). The molecular mechanisms of above effects involve modulation of gene expression as a consequence of estrogen receptors stimulations. The estradiol-receptor complex regulates transcription of genes of neurotransmitters, synaptic proteins, enzymes, growth factors and cell cycle regulators (Enmark and Gustafsson, 1999). Membrane receptor responsible for non-genomic fast effects observed in estrogen-dependent neuronal plasticity is also suggested (Stefano *et al.* 2003, Toran-Allerand, 2004).

The discovery that neuronal progenitor cells are present in brains of adult rodents and other mammalian species, including humans, has changed substantially the way of thinking in neurobiology and was crucial for the development of new modern concepts. In adulthood, progenitor cells are located in subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) as well as in olfactory epithelium (Ming and Song, 2005). The intensity of cell proliferation in these areas was found to be sex-dependent. Tanapat *et al.* (1999) revealed transient increase in the hippocampal neurogenesis in proestrus phase of estrous cycle in adult female rats being the effect of elevated concentration of circulating estrogens.

Central nervous system of adult mammals, including primates and humans, has neither ability for effective spontaneous regeneration of injured neurons nor for restoration of lost functions. For decades, neuroscientists were looking for the effective method to stimulate and support regenerative processes. Numerous strategies were developed to overcome this regenerative inability. Because it was commonly accepted that CNS environment is non-permissive for axonal regrowth, there were attempts to change it for more favorable, e.g. by means of transplantation of peripheral nerve fragments.

Our early studies revealed that peripheral nerve grafts predegeneration prior to transplantation in the CNS increases their growth-promoting activity and this effect was time-dependent and reached maximum at 7th day of nerve in situ predegeneration (Lewin-Kowalik *et al.* 1992). We did not examine, however, whether axons we found grew from newly originating cells or from already present that had survived the lesion.

The aim of the present work was to examine whether the implantation of predegenerated peripheral nerve graft stimulates neurogenesis in DG and to analyze the influence of different plasma levels of 17β-estradiol on neurogenesis in this experimental model in adult female rats. We examined also the distribution of estrogen receptors alpha and beta in DG as well as in implanted graft, to look into the mechanisms involved in the processes of cell proliferation and differentiation.

MATERIAL AND METHODS

Animals

Young adult female Wistar C rats (mean b.w. 230 g) bred at the Medical University of Silesia Animal Farm were used in the experiment. The animals were kept in individual cages with free access to standard rodent food and water, in the temperature 20–22 ^{0}C with 12/12 h light/dark cycle. Only females with normal estrous cycles, as verified by vaginal smears (Marcondes *et al.* 2002), were used in the study. All experiments were carried out in accordance with European Council Directives regarding care and use of laboratory animals and they were approved by the local Ethics Committee.

The animals were randomly divided into three groups:

1. Non ovariectomized reference group without ovariectomy, without hormonal substitution (**NO**, n=5)
2. Ovariectomized group, with no hormonal substitution (**OV**, n=16)
3. Ovariectomized group with 17β-estradiol substitution (**E**, n=14), divided into three subgroups:
   - $\mathbf{E_{C.25}}$ (n=5) – crystalline estradiol closed in the 0.25 cm capsule
   - $\mathbf{E_{C.50}}$ (n=5) – crystalline estradiol closed in the 0.5 cm capsule
   - $\mathbf{E_I}$ (n=4) – 10 μL of estradiol oil solution (10mg/mL)

Animals were anesthetized i.p. with chloral hydrate (420 mg/kg b.w.; Fluka, USA).

Surgical and laboratory procedures

Ovariectomy and sciatic nerve transection

Anesthetized animals were immobilized in the prone position. In the ovariectomized (OV and E) groups, the ovariectomy was performed (Waynforth and Flecknell, 1992). Briefly, through single incision in the mid-line of the back at the Th12-L3 level, going to the left and subsequently to the right side, after blunt preparation of abdominal muscles, the appropriate ovary was exposed, ligated and dissected, and the wound was sutured (thread 4/0, Ethicon, USA). In OV and E groups, complete transection of right sciatic nerve near hip joint and ovariectomy *via* the dorsal flank were done. In NO group, only right sciatic nerve transection was performed in proestrus phase of estrous cycle.

Grafting and estrogen substitution

Seven days following the nerve transection, in anesthetized animal, 5-mm-long distal stump of degenerating sciatic nerve was dissected (being a 7-day-predegenerated graft). This fragment was then implanted stereotaxically into the injured right hippocampus, according to the method described previously by Lewin-Kowalik *et al.* (1990). Subsequently, in E subgroups, we performed as follows:

- $E_{C.25}$, $E_{C.50}$ animals were implanted subcutaneously with 1.5 cm-long piece of silastic tubing (Silastic Medical Grade Tubing, ID 1.587mm, OD 3.175 mm; Dow Corning, USA) containing, respectively, 0.25 cm or 0.50 cm capsule with crystalline 17β-estradiol (Sigma, USA). The capsules were prepared and generously provided by Dr. Victor Viau. Prior to implantation, the capsules were incubated 2×24 h in PBS (pH 7.2) in temperature 37°C to enhance hormone penetration through the capsule wall (Goodman 1978)
- E_I rats received subcutaneous injection of 10 μL of an oil solution Estradiol Dep. (10mg/mL; Schering AG, Poland) to produce the intended overdose in estrogen level.

Determination of estradiol concentration

Blood samples (about 1 mL) were taken from the retro-orbital plexus into ice-cold collection tubes with EDTA (3,75g/100 mL D.W.; Sigma, Germany). 17β-estradiol concentrations in plasma were determined by radioimmunoassay (RIA kit; MP Biomedicals, USA) at the beginning of the experiment, the grafting day and at the 7th day of estrogen substitution.

BrdU labeling

Six days after graft implantation to the hippocampus, all animals received intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma, Germany) (10mg/mL in 0.007M NaOH solution in 0.9% NaCl; dose 50mg/kg b.w.). The injection was repeated after 24h (Nowakowski *et al.* 1989).

Tissue collecting

One hour after second BrdU injection, animals were deeply anesthetized and perfused transcardially with PBS and 4% formaldehyde in PBS. Whole brains together with the graft were carefully collected, post-fixed, cryoprotected and embedded in TissueTek (Sakura, Japan).

Immunohistochemistry

Ten μm frontal frozen sections through the entire dentate gyrus were cut (Cryotom; Anglia Scientific, UK). Sections in three series (each series contained every-third section, ordered consecutively) were mounted on Superfrost Plus slides (Menzel Glaeser, Germany) and coverslipped in Vectashield with DAPI (Vector, UK). Slides from single series were subjected to immunohistochemical labeling:

1. for **BrdU** built in nuclei in S phase of cell cycle (Nowakowski *et al.* 1989) using antibodies against BrdU conjugated with fluorochrome (anti-BrdU AlexaFluor 488 conjugate; Molecular Probes, USA) [DNA denaturation was provided by incubating slides in 2N HCl in 30 min. at 37°C, and then neutralized by immersion in 0.1M borate buffer (all reagents POCH, Poland)]
2. for neuroepithelial line marker – **nestin** (anti-nestin antibody) and for glial marker – **GFAP** (anti-glial fibrillary acidic protein antibody) (both Chemicon, USA) with secondary antibodies conjugated with AlexaFluor 568 or 488, respectively (Molecular Probes, USA)
3. for **estrogen receptors** alpha and beta (Abcam, UK) with secondary antibodies conjugated with AlexaFluor 488 or 568, respectively (Molecular Probes, USA).

Standard antibody-labeling procedure was used, with overnight incubation in 4°C for the primary antibody (1–4) and 60 min. incubation in room temperature for the secondary antibody (2–4).

Slides examination

All analyses were performed both on grafted and non-grafted side of the brains and compared to estimate the local consequences of peripheral nerve implantation.

Slides were examined under confocal laser scanning microscope (Olympus IX70, Japan) with krypton-argon laser, with FluoView software and digitally recorded. For double-labeling studies, separate images of AlexaFluor 568 and AlexaFluor 488 fluorescence were captured from the same section and then digital overlay was generated. Nuclei labeled with DAPI were examined in UV light and recorded by digital camera (Camedia; Olympus, Japan).

BrdU- and nestin-labeled cells were counted in 5 consecutive brain slices in appropriate series (i.e. 5

every third slices), in 5 squares (100μm × 100μm) on each slice. Cell counting was performed independently by two scientists, unaware of experimental groups. The average cell number per slice was then re-calculated per mm^2.

Cells co-localized for nestin and GFAP were given in percentage of total labeled cells number in this area – i.e. sum of cells expressing GFAP only, nestin only and nestin with GFAP was considered as 100%.

Estrogen receptors were analyzed qualitatively and their subcellular localization was described.

RESULTS

In all slides we found lesion in grafted hippocampus and intact hippocampus on the contralateral side.

17β-estradiol concentration in plasma

Hormone level in OV group systematically decreased after surgery, confirming the effectiveness of total ovariectomy. In subgroups $E_{C.25}$ and $E_{C.50}$, 17β-estradiol plasma level was maintained in physiological range imitating proestrus phase (the highest in estrous cycle) by capsules implantation (Viau and Meaney, 1991). The highest results in E_I group were over the detection limit of the essay (3000 pg/mL – maximum standard). All results are presented in Table 1.

In NO group, we did not check estrogen concentration, because female rats with normal estrous cycle were used. In all ovariectomized groups, the reference range was the estrogen level before ovariectomy (Tab. 1, results [A]).

Markers

I) The number of cells labeled with **bromodeoxyuridin** (**BrdU**) (Fig. 1A) was considered as so-called proliferation rate, i.e. number of dividing cells per 24 hours (first injection 24 hours before brain dissection). Figure 2 shows all the results.

In reference group (NO), no significant differences between grafted and the opposite side were observed (454±27 and 455±78, respectively).

In ovariectomized group without hormone substitution (OV), mean number of BrdU-positive cells on grafted side was significantly higher (896±206) as compared with contralateral side (531±178) ($p<0,01$), and exceeded the number of cells found in the NO group on the same side ($p<0.0001$). In the non-grafted side, no significant difference between OV and NO group was found.

In both subgroups with hormonal substitution with crystalline 17β-estradiol – $E_{C.25}$ and $E_{C.50}$ – proliferation efficacy expressed as cell number per mm^2 was similar – respectively, 1146±140 and 1150±52 on the grafted side, and 720±163 and 772±12 cells on the contralateral side. Differences between grafted and non-grafted sides in each group were significant: $E_{C.25}$ – $p<0.001$, $E_{C.50}$ – $p<0.05$. On the opposite side, the results were higher than in NO as well as OV groups, and these differences were statistically significant ($p<0.05$, $p<0.01$, respectively).

Examination of BrdU immunolabeling in subgroup of animals treated with 17β-estradiol injection (E_I) revealed that in DG on both sides, number of cells was similar to corresponding sides in NO group (562±31 and 300±42, respectively).

II) The number of **nestin**-positive cells (Fig. 1B) in all groups was significantly higher on grafted side of the brain than on non-grafted one (Fig. 3).

No significant differences in nestin-positive cells number on corresponding sides of the brain between NO and E_I groups were found: (NO) – 593±11 vs. 419±27 ($p<0.05$), (E_I) – 562±31 vs. 300±52 ($p<0.05$). In ovariectomized group (OV), results were as follows: ipsilateral – 803±140 and contralateral – 471±103 ($p<0.0001$). The highest numbers of nestin-positive cells were found on the grafted side in subgroups $E_{C.25}$ – 938±68 and $E_{C.50}$ – 1080±71 cells per mm^2. On the non-grafted side in these groups, respectively, 628±97 and 723±52 labeled cells/mm^2 were present.

Table 1. Comparison of 17β-estradiol plasma concentration before and after ovariectomy in ovariectomized female rats without (OV group) or with (E groups) estrogen substitution.

	Group			
	OV	**$E_{C.25}$**	**$E_{C.50}$**	**E_I**
[A] – before ov.	104.41 ± 16.23	99.24 ± 23.11	100.42 ± 14.15	113.58 ± 11.44
[B] – 7 days after ov.	59.01 ± 21.34	77.38 ± 24.45	77.38 ± 5.38	82.7 ± 17.09
[C] – 14 days after ov.	61.98 ± 23.9	97.23 ± 29.8	153.75 ± 54.49	>3000
[A] vs. [B]	$p<0.05$	NS	$p<0.05$	$p<0.05$
[B] vs. [C]	NS	NS	$p<0.05$	$p<0.05$
[C]E vs.[C]OV	–	$p<0.05$	$p<0.05$	$p<0.05$

17β-estradiol concentration given in pg/mL (mean ± SD)
Estrogen substitution: $C._{25}$ – capsule 0.25 cm, $C._{50}$ – capsule 0.50 cm, I – injection
Abbreviations: ov. – ovariectomy; NS – not significant

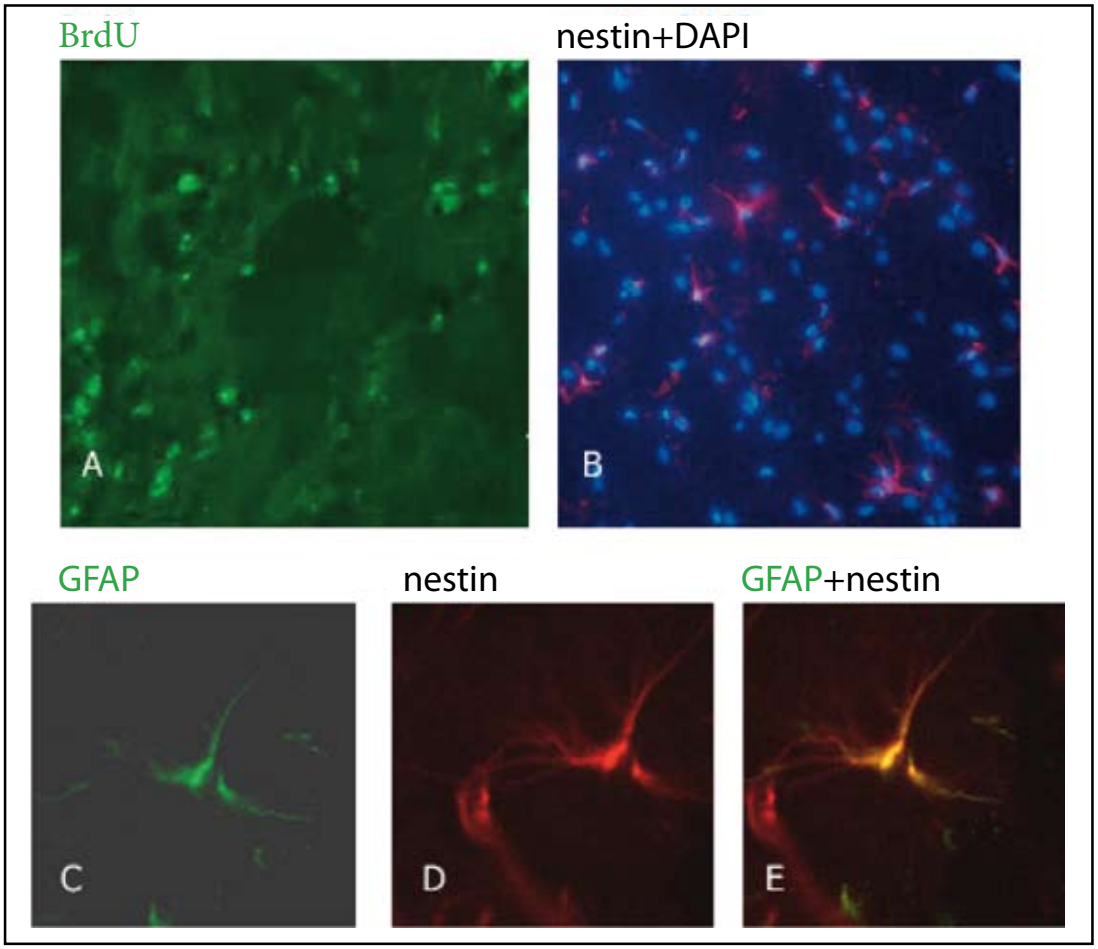

Fig. 1. Microphotographs of hippocampal dentate gyrus a week after 7-day-predegenerated nerve graft implantation. A – BrdU labeled (green) cell nuclei in $E_{C.50}$ group (400x); B – nestin-positive cells (red), nuclei stained with DAPI (blue) in $E_{C.25}$ group (400x); C – GFAP-positive cell; D – nestin-positive cell; E – GFAP/nestin colocalization in the cell considered as radial glia; C, D, E in $E_{C.50}$ group (1000x)

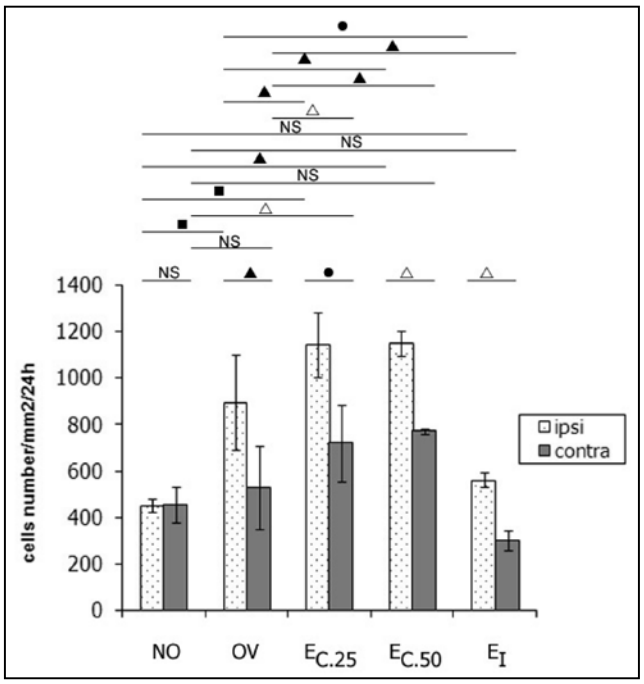

Fig. 2. The number of BrdU-positive cells in all groups in grafted (ipsi) and contralateral (contra) side of the brain. NS – not significant, △ p<0.05, ▲ p<0.01, ● p<0.001, ■ p<0.0001

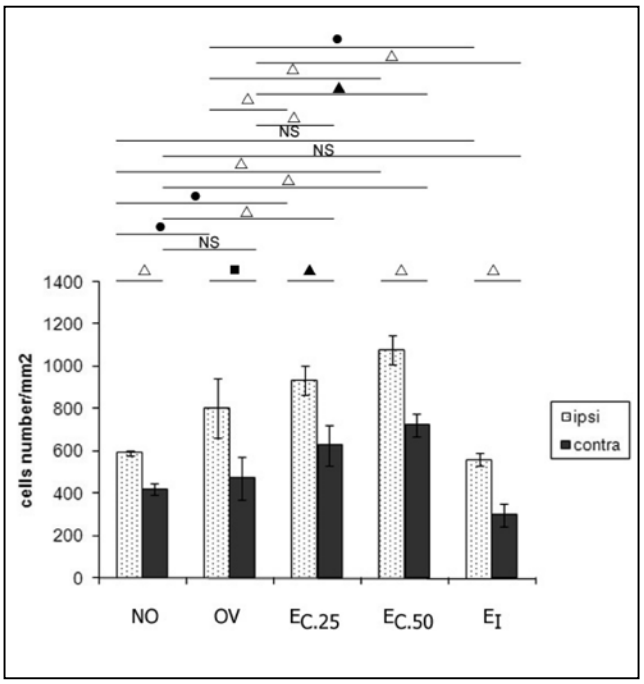

Fig. 3. The number of nestin-positive cells in all groups in grafted (ipsi) and contralateral (contra) side of the brain. NS – not significant, △ p<0.05, ▲ p<0.01, ● p<0.001, ■ p<0.0001

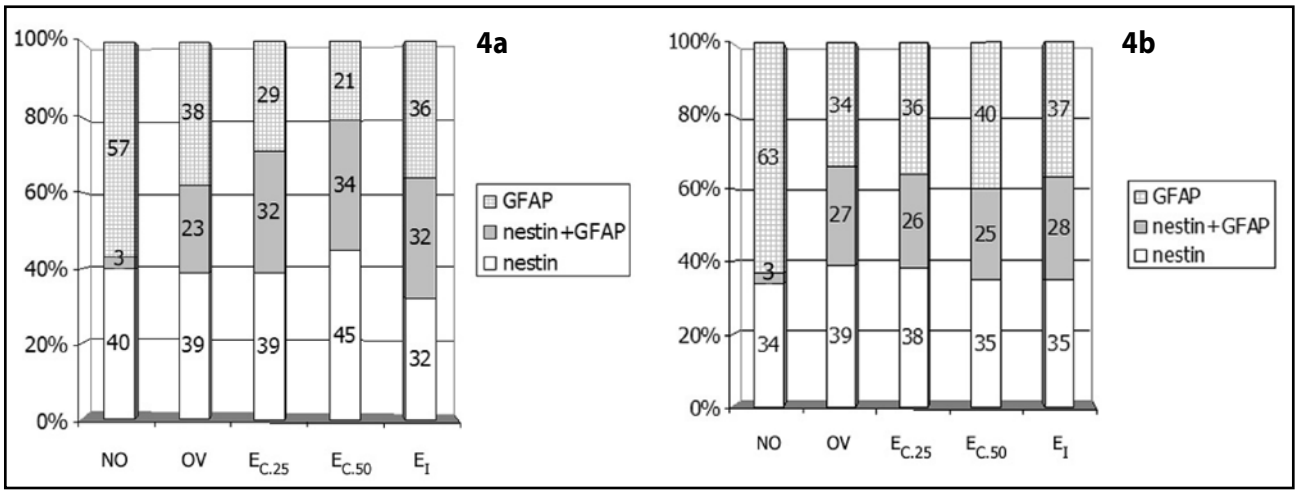

Fig. 4. The percentage distribution of GFAP-positive, nestin-positive and double-labeled cells in the hippocampus 7 days following the surgery in all groups. A – 7-day-predegenerated nerve graft side; B – intact side.

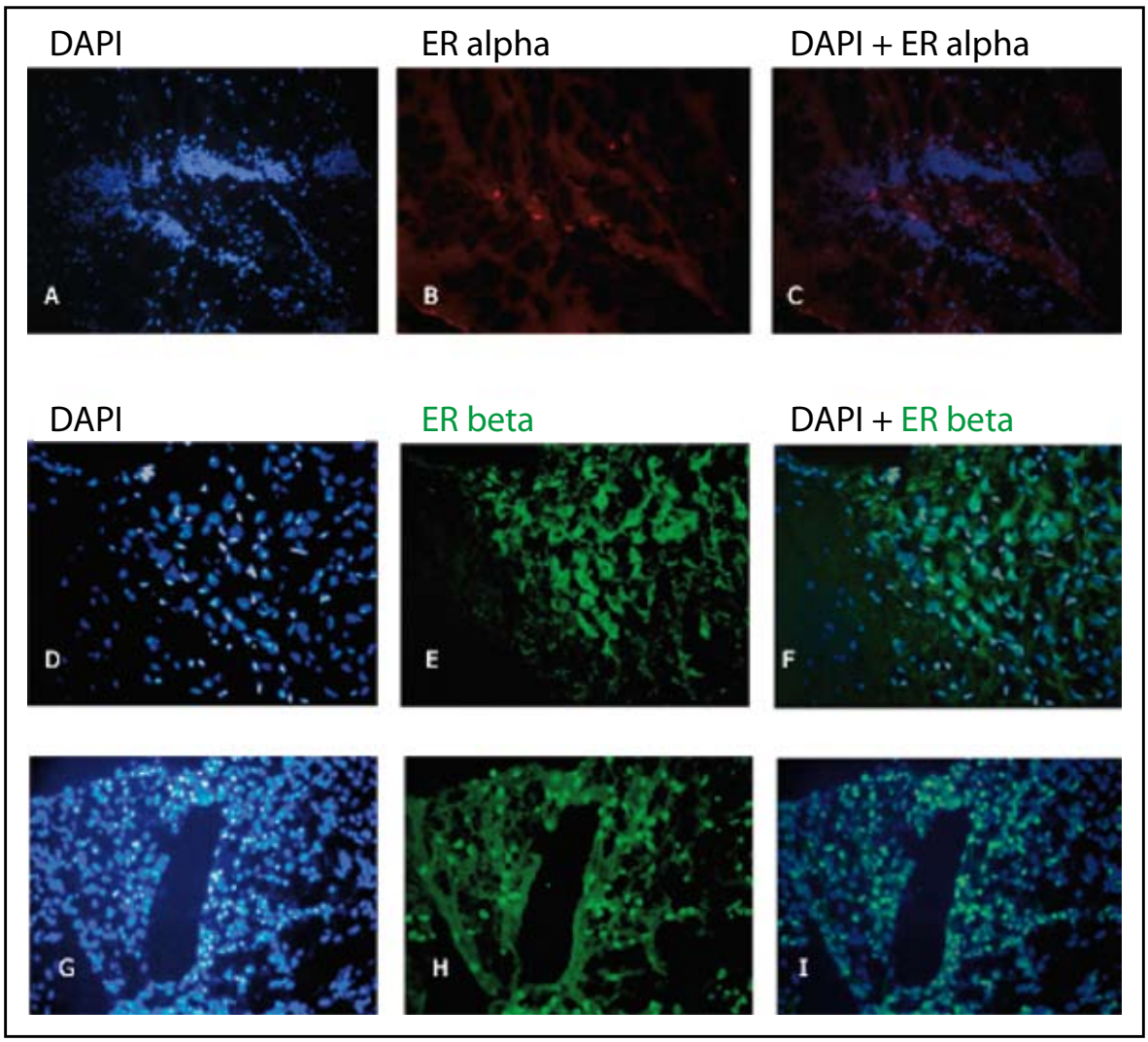

Fig. 5. Microphotographs of immunofluorescent staining of estrogen receptors in hippocampal dentate gyrus a week after 7-day-predegenerated nerve graft implantation.
A – estrogen receptors alpha (red) in cytoplasmic localization in DG of hippocampus, B – nuclei stained with DAPI (blue), C – merged images ERalpha/DAPI, D – nuclei stained with DAPI (blue), E – estrogen receptors beta (green) in cytoplasmic localization in hippocampus, F – merged images ERbeta/DAPI, G – nuclei stained with DAPI (blue), H – estrogen receptors beta (green) in nuclear localization in nerve graft, I – merged images ERbeta/DAPI.
A, B, C in group NO (200x); D, E, F in group $E_{C.25}$ (400x); G, H, I in group $E_{C.50}$ (400x)

III) In double-labeling for **nestin and GFAP** (Fig. 1C-E) we calculated the percentage of co-localization of these two markers in merged scanned pictures of the same brain section. We used following interpretation key:

- nestin only – young cells of neuronal line
- GFAP only – astrocytes
- nestin and GFAP together – progenitor cells or radial glia that may differentiate into neurons

The results are shown in Figure 4. We found the most intensive neurogenesis in 17β-estradiol substitution groups (imitating proestrus) $E_{C.25}$ and $E_{C.50}$, where most of labeled cells contain nestin or nestin+GFAP (both together reach 70–80%). It may suggest the presence of radial glia. In groups NO, OV and E_I, the percentage of GFAP(+) cells increases on both sides, and the ratio of colocalization with nestin decreases.

IV) Estrogen receptors immunoreactivity presented a specific pattern of distribution in the area around the graft tip as well as different cellular localization.

In all groups, in DG cells on grafted side **ERs-alfa** were observed in the cytoplasm (Fig. 5A-C). They were also found in OV and $EC._{C.25}$ groups inside the implanted nerve fragment, in cytoplasm as well.

However, **ERs-beta** were present in cytoplasm of hippocampal cells (Fig. 5D-F) in groups $E_{C.25}$ and $E_{C.50}$, and inside graft in cell nuclei (Fig. 5G-I) in all groups.

Very little ERs-positive cells were found on the non-grafted side of the brain in all groups.

We did not found cells with co-localization of ER-alfa and ER-beta, though both types of receptors were present in the same structure (hippocampus, graft).

DISCUSSION

In the non-ovariectomized animals, with continuous ovarian cycle, the cell proliferation rate (as measured by BrdU labeling) is almost the same on the injured side with the nerve implant as on the intact one. On the other hand, nestin-expressing new neuronal-line cells are more numerous on the injured side. It suggests that predegenerated peripheral nerve implantation into the hippocampus rather affects cell differentiation than influences the proliferation of progenitors in the subgranular layer.

In ovariectomized groups we observed similar effect, but the difference between injured and non-injured side was bigger, probably because of simultaneous action of both graft and hormone. Surprising were the results found in the ovariectomized rats without 17β-estradiol substitution, where number of proliferating cells on the grafted side of the brain was higher than in control group. One possible explanation of the enhanced neurogenesis in this group may be the fact that following the ovaries removal, both estrogens and gestagens (progesterone) were eliminated. The latter are known to inhibit neurogenesis (Galea, 2007, Giachino *et al.* 2004). There is also one recent hypothesis about local synthesis of estradiol in the hippocampus that could be involved in cell proliferation. Fester *et al.* (2006) observed an increased level of the aromatase – the main enzyme transforming testosterone to estradiol – in ovariectomized animals as well as in cultured hippocampal granule cells. They also claim that the plasticity (proliferation vs. apoptosis) as well as synaptic density in the hippocampus depend on local 17β-estradiol production (Kretz *et al.* 2004). The cyclicity of 17β-estradiol production in the hippocampus is suggested to be related to GnRH fluctuations and its action on GnRH receptors found here. It was found *in vitro*, that aromatase inhibitor letrozol neutralized this effect and produced up-regulation of GnRH receptors (Prange-Kiel *et al.* 2006). The ovariectomy ceases the negative feedback between gonads and hypothalamus, and the following excessive GnRH release could initiate the synthesis of 17β-estradiol in the hippocampus. This may be the reason of better results of proliferation and higher number of nestin-positive cells found in OV group in our experiment. No difference found on the non-injured side of brain between OV and NO group indicates that ovariectomy may stimulate neurogenesis in hippocampal dentate gyrus, but only in the presence of some additional stimulating factor – e.g. predegenerated nerve graft, being a source of various growth-promoting factors (Stoll and Mueller, 1999).

Our previous studies revealed time-dependent neurotrophic activity of the predegenerated nerve grafts while implanted into the CNS, with two peaks – 7 and 28 days following nerve transection (Lewin-Kowalik *et al.* 1992). In the present study we examined whether 7-day-predegenerated implant stimulated not only axon outgrowth but also neurogenesis. Presented results confirm this hypothesis. It seems, that in the same model of injury and grafting, most of neurons labeled with retrograde markers (FITC-HRP) applied into the graft end (Lewin-Kowalik *et al.* 1990, 1992) were new cells from proliferating population in subgranular zone of the hippocampus.

The hormonal substitution imitating proestrus peak of 17β-estradiol influenced positively the neurogenesis in the rat hippocampus. Some authors emphasize that during normal estrous cycle, this phenomenon is transient, and new neurons die in apoptotic process during the rest of the cycle (Tanapat *et al.* 1999). One should remember that in our experiment whole estrogen administration lasted only 7 days; at longer time-lapse negative effects of hormonal replacement cannot be excluded (Ormerod *et al.* 2001, Galea, 2007). Tanapat *et al.* (2005) revealed no positive influence on hippocampal neurogenesis during chronic (3 weeks) estrogen administration in ovariectomized female rats. They also found negative effect of both high- and low-dose hormonal substitution.

In our experiment, estradiol overdose (E_I group) decreased the neurogenesis. The proliferation rate and

number of nestin-positive cells were even worse than in the NO group. Nerve graft presence exerted only a very little influence on the origin of new cells. This reduced neurogenesis may be the result of a neurotoxic effect of 17β-estradiol metabolite 2-methoxyestradiol produced in hyperestrogenemia and counterbalancing the neuroprotective influence of estrogens and their another metabolite, 2-hydroxyestrogen (Picazo *et al.* 2003).

There are the new ideas considering radial glia as neuronal progenitors (Gregg and Weiss, 2003; Imura *et al.* 2003; Kempermann *et al.* 2004; Ming and Song, 2005). Recently, many scientists pay attention to the radial glia expressing both neuronal and glial markers and differentiating into two lines – neurons or astrocytes (Ihrie and Alvarez-Buylla, 2008). There is a strong evidence for the positive role of radial glia that can stimulate neurogenesis and form a scaffold for migration of progenitor cells during embryonic development of the nervous system (Nowakowski and Hayes, 1999, Gregg and Weiss, 2003). Numerous papers indicate expression of both GFAP and nestin as a marker of early neuronal precursors (Kempermann *et al.* 2004, Ming and Song, 2005). In adult organism, a stress or brain injury can be the signal activating progenitor cells (Gregg and Weiss, 2003). We considered both nestin-positive and nestin+GFAP-positive cells as evidences for neurogenesis, but GFAP expressing cells as developing into the glial cells line. The best results were found in proestrus-imitating groups (neuronal progenitors made over 80% of cells), especially on the grafted side. On the contralateral side, cell percentage was similar in all ovariectomized groups. Thus, one can suspect that the nerve graft is not only the factor inducing more dynamic cell proliferation. The nerve implant together with estrogens are also able to stimulate these new cells to differentiate into neurons. Animals in the NO group had normal hormonal cycle and we found only about 3% of double-labeled cells in hippocampi on each side.

Analyzing ERs distribution, we found that on the intact side of the brain, both alpha and beta receptors were very sparse in all groups.

On the grafted side in all animals, ERs-α were present in cytoplasmic localization in the hippocampus. It is interesting phenomenon, confirming pioneer research of Dominique Toran-Allerand on non-genomic actions of estrogens. She described the receptor binding antibodies against C-end region of ER-α in ER-α-knockout mice, present both in cytoplasm and plasma membrane (named ER-X) (Toran-Allerand, 2004). Other authors found in neurons ER-α related to MAPK (mitogen activated protein kinase), which regulated intracellular pathway involved in cell divisions and apoptosis in young rats (Shughrue *et al.* 1997). Thus, we may suspect that the enhanced neurogenesis in the hippocampus following peripheral nerve implantation is related to this action of estrogen receptors.

We found ER-β only in the cytoplasm of hippocampal cells in groups with hormone substitution – the same with the best results of proliferation as well as the presence of young neurons marker – nestin – and probably is the result of the activating effect of implanted nerve fragment. In the implanted grafts, the same ER-β receptors were localized inside the nuclei. It seems possible, that in our model, nuclear receptors do not participate in the transmission of hormonal signal in the hippocampus but they have genomic effects in gene transcription in the graft. Mature and active cells like Schwann cells, macrophages or mast cells are present in the distal stump of the degenerating nerve (Stoll and Muller, 1999), responding to this genomic action.

It is known that both types of estrogen receptors produced in cell bodies of spinal motoneurons can be transported along the sciatic nerve axons (Islamov *et al.* 2003; Xu *et al.* 2003). Therefore, in the predegenerated nerve they can be present as free proteins or absorbed by phagocyte cells, what we observed as ER-alfa-positive cytoplasm of the cells in the graft.

Our study showed positive influence of 17-beta-estradiol on neurogenesis in the hippocampus, especially following the implantation of 7-day-predegenerated nerve graft. However, this was effective only in physiological concentration (imitating proestrus phase), and in overdose it decreased the neurogenesis. Process of ovariectomy can activate neurogenesis in the hippocampus only when other stimulus, as peripheral nerve graft, is present. Both factors – estradiol and implant – are involved in expression of estrogen receptors in various subcellular localizations, reflecting their different functions in processes of cell survival, proliferation and differentiation.

STATISTICS

Statistical analysis of data was completed using ANOVA. All data are presented as mean±SD. Statistical significance was set at $p<0.05$.

ACKNOWLEDGEMENTS

We would like to thank Dr. Victor Viau (BSc, MSc, PhD, Assistant Professor of The Department of Anatomy & Cell Biology, Faculty of Medicine, The University of British Columbia, Vancouver, Canada) for the estrogen substitution instructions and help in capsules preparation, and Dr. Richard Nowakowski (PhD, Associate Professor of The Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, The University of Medicine and Dentistry of New Jersey, Piscataway, USA) for the BrdU labeling instructions. This work was supported with Medical University of Silesia grants №: NN-2-048/06 and NN-2-121/07.

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