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ESTROGEN-INDUCED REGION SPECIFIC DECREASE IN THE DENSITY OF 5-BROMO-2-DEOXYURIDINE-LABELED CELLS IN THE OLFACTORY BULB OF ADULT FEMALE RATS

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Abstract—Effects of chronic estrogen treatment on the survival rate of newly integrated interneurons were studied in the olfactory bulb of adult (250–300 g) female rats. Ovariectomized rats received 17-beta estradiol dissolved in sesame oil (i.p., 100 μg/100 g body weight [b.w.]) during six consecutive days, and on day 6 they were also injected with the mitotic marker 5-bromo-2-deoxyuridine (BrdU, i.p., 50 mg/kg b.w.) in every 2 hours during 8 hours. After 21 days of survival animals were killed and the density of BrdU-immunoreactive cells was analyzed in the granule cell and glomerular layer both in the main and accessory olfactory bulb.

A significant decrease was found in the density of BrdU-labeled cells in both layers examined in the accessory olfactory bulb of ovariectomized and estradiol-treated rats when compared with those of ovariectomized and vehicle-treated animals. In the main olfactory bulb, in contrast, no difference was observed in the density of BrdU-immunoreactive cells in either of the two layers.

Our results suggest that cells destined to the glomerular and granule cell layers react in the same way to chronic estrogen treatment, and the effect of estradiol is region specific, at least, within the olfactory bulb. 17-Beta estradiol reduces the density of newly generated cells in the accessory olfactory bulb, an area involved in the perception of pheromones, thus having a role in regulating sexual behavior, while the rate of integration and survival of newly born cells in the first relay station of the main olfactory pathway, i.e. the main olfactory bulb, remains unchanged.

Key words: main olfactory bulb, accessory olfactory bulb, 17-beta estradiol, 5-bromo-2-deoxyuridine, neurogenesis.

Since the early 1990s it has gained wide acceptance that neurogenesis continues in the adult mammalian brain, most importantly in the subventricular zone (SVZ) (Luskin, 1993; Lois and Alvarez-Buylla, 1993) and in the subgranular zone of the dentate gyrus (Cameron et al., 1993; Seri et al., 2004), although at a lower rate other regions, such as the olfactory bulb (Peretto et al., 2001; Vicario-Abejon et al., 2003), or in primates even the temporal lobe and the amygdala are also known to possess this activity (Bernier et al., 2002). Neurons generated in the adult SVZ migrate along the rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1993; Bonfanti et al., 1997) where they integrate into functional circuits (Lois and Alvarez-Buylla, 1993; Carleton et al., 2003; Belluzzi et al., 2003) and may be involved in olfactory discrimination (Shingo et al., 2003).

In the olfactory bulb, the vast majority of the newly born cells that originate in the SVZ differentiate into granule cells and less then 3% of them continue their way to the glomerular layer, where they become periglomerular cells (for reviews see (Lledo et al., 2004; Doetsch and Hen, 2005). It has been shown that newly added cells originating from the SVZ can be found not only in the main olfactory bulb (MOB), but also in the accessory olfactory bulb (AOB) (Peretto et al., 2001), which has a laminar organization resembling that of the MOB. Thus, both the MOB and the AOB are highly plastic areas of the CNS featuring constantly renewing neuronal circuits (Kato et al., 2000; Peretto et al., 2001; Matsuoka et al., 2002; Wilson et al., 2004). The MOB is the first relay station in the processing of olfactory information, since primary olfactory neurons located in the olfactory epithelium establish synaptic contacts with the main dendrites of mitral and tufted cells in the glomeruli of the MOB. The AOB, in turn, receives its synaptic input from the vomeronasal organ, consequently, it is the pheromonal signaling pathway and not general olfaction where AOB is essential (for review see Halasz 1990).

Proliferation as well as survival of newborn neurons is dependent on the influence and interaction of growth factors, neurotransmitters and several steroids as it is known from studies performed mostly in the hippocampus. Growth factors, such as BDNF (Lee et al., 2002; Mattson et al., 2004), EGF, FGF (Represa et al., 2001; Brannvall et al., 2002; Jin et al., 2003), IGF-I (Pixley et al., 1998; Perez-Martin et al., 2003) and neurotransmitters, like acetylcholine (Cooper-Kuhn et al., 2004; Mechawar et al., 2004) and serotonin (Banasr et al., 2004; for review see Djavadian 2004) are considered as factors promoting neurogenesis.

Steroid actions are more complex, since among them there are negative modulators, like glucocorticoids (Kim et al., 2004), while dehydroepiandrosterone is reported to increase cell proliferation and survival (Suzuki et al., 2004). Another interesting compound, progesterone, which also belongs to neurosteroids, is reported to exert its effects mainly through its metabolites, namely allopregnanolone, dihydroprogesterone and tetrahydroprogesterone. Allo-

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Abbreviations: AOB, accessory olfactory bulb; BrdU, 5-bromo-2-deoxyuridine; b.w., body weight; DAB, diaminobenzidine; ER, estrogen receptor; E2, 17-beta estradiol; MOB, main olfactory bulb; OVX, ovariectomized; PB, phosphate buffer; SVZ, subventricular zone.

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pregnanolone increases cell proliferation in neuroprogenitor cells derived from the rat hippocampus and also in neuroprogenitor cells of human cortical origin (Brinton and Wang, 2006), while dihydroprogesterone and tetrahydroprogesterone reduce the proliferative activity of neuroblasts and induce molecular and structural modifications of the glial compartment within the subependymal layer of the rat olfactory bulb (Giachino et al., 2003, 2004).

The most extensively studied neurosteroid, estrogen has diverse effects: it is known to increase the proliferation of embryonic neural stem cells, while in the adult, 17-beta estradiol (E2) treatment decreases the proliferation of neural stem cells stimulated by epidermal growth factor (Brannvall et al., 2002; Suzuki et al., 2004). In the dentate gyrus of adult female rats estradiol initially enhances, but subsequently suppresses granule cell proliferation, possibly by inducing adrenal steroid expression (Ormerod et al., 2003). This time-dependent effect of estrogen action can be observed under physiologic conditions in certain rodents, where reproductive status influences neurogenesis in the dentate gyrus of adult females (Ormerod and Galea, 2003).

Although estrogen action in the CNS is extensively studied, evidence is still lacking on its effect on neurogenesis in those brain areas that might be fundamental in regulating sexual behavior, such as regions related to olfaction. Thus, in the present study, we focused our attention on the olfactory bulb and aimed to examine the role of estrogen on newly born cells that proliferate, survive, migrate and integrate in the MOB and AOB, respectively.

EXPERIMENTAL PROCEDURES

Animals and treatments

Adult female Wistar albino rats (250–300 g, $n=5$ in both experimental groups) kept in a controlled environment with free access to food and water were used. Manipulation of the animals was performed according to the European Union (86/609/EEC) guidelines, which are approved by Hungary and are applicable in the Biological Research Centre, Szeged, and special care was taken to minimize the number of animals used and their suffering.

All animals were bilaterally ovariectomized (OVX) under 2,2,2-tribromoethanol (0.2 g/kg body weight [b.w.], Fluka Chemika AG, Buchs, Switzerland) anesthesia. Three weeks after ovariectomy animals were divided into two groups ($n=5$), one group received daily i.p. injections of estradiol during six consecutive days (100 μg/100 g b.w. dissolved in sesame oil, Sigma, St. Louis, MO, USA), the other group received sesame oil injections during the same days. To label all dividing cells, on day 6 all animals were injected with the S- phase marker 5-bromo-2-deoxyuridine (BrdU, i.p., 50 mg/kg b.w., Sigma) four times with a 2-hour-interval between the injections. All animals were killed 21 days after injecting BrdU (Fig. 1), since by this time point all BrdU-labeled cells had reached their final destination in the olfactory bulb.

Immunohistochemistry

Animals were perfused intracardially with 4% of paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4), brains were removed and the olfactory bulbs were dissected and postfixed overnight at 4 °C in the same fixative. The whole olfactory bulb was cut into 30-μm-thick serial sagittal sections using a Vibratome (Oxford Laboratories, St. Louis, MO, USA) and the sections were stored in 0.1 M PB at 4 °C until use. Immunostaining was performed on free floating sections. Endogenous peroxidase activity was quenched by incubating the sections for 30 min at room temperature with 10% methanol and 3% hydrogen peroxide. To ensure access of antibody to BrdU containing DNA chains, sections were incubated for 15 min at 37 °C with 2 N HCl. To restore the pH required for immunostaining, sections were rinsed in 0.1 M borate buffer (pH 8.5), than transferred to 0.1 M PB. Aspecific binding sites were blocked by incubating the sections at room temperature for 1 h with 5% normal goat serum (Sigma) and 0.3% Triton X-100 (Sigma) in 0.1 M PBS. Sections were then incubated with mouse anti-BrdU antibody (1:50,000, mAb G3G4, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) overnight at 4 °C. Sections were rinsed in PBS, than incubated for 2 h at room temperature with biotinylated anti-mouse IgG (1:400, Jackson ImmunoResearch Europe, Cambridgeshire, UK). Following another series of washing, sections were incubated with ABC (1:500, Vector Laboratories, Burlingame, CA, USA) for 2 h and the immunoreaction was visualized using diaminobenzidine (DAB) (Sigma) as chromogen. The sections were mounted on gelatin-coated slides and counterstained with Methylene Blue.

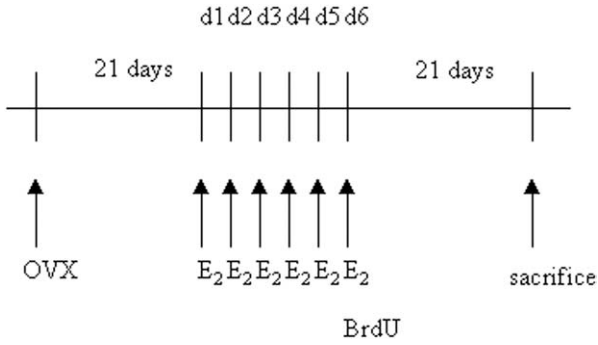

Fig. 1. Experimental design for the treatment and killing of the animals. Two-month-old female rats were OVX and after 21 days of recovery half of them were injected with E2 dissolved in sesame oil (i.p. 100 μg/100 g b.w.) during six consecutive days (OVX+E2). On day 6 each animal received BrdU (i.p. 50 mg/kg b.w.) four times with a 2-hour interval between the injections. All animals were killed by intracardial perfusion 21 days following BrdU treatment.

Quantification of BrdU-labeled cells

The density of BrdU-immunoreactive cells was analyzed on coded sections. Every 6th section containing both the MOB and AOB was used, so that possible volume changes that might have occurred during hormone treatment or fixation procedure would affect cell densities in exactly the same way in both parts of the OB. All nuclei that were either completely filled with DAB reaction product or contained only patches of staining were considered for counting. Cell counting was performed using the optical dissector method. The area of analysis comprised the entire granule cell layer and glomerular layer of the AOB, while in the MOB BrdU-immunoreactive cells in the granule cell layer were counted in squares of 100 μm^2 according to standard stereological principles (Howard and Reed, 1998) and around each glomerulus in the glomerular layer. Measurement of the areas in the AOB and in the glomerular layer of the MOB as well as the densitometric analysis were carried out using the Image Pro Plus software.

Statistical analysis

Data were analyzed using the one-way ANOVA. Cell densities obtained in each section from the two olfactory bulbs of each animal were pooled, consequently, n for the statistical analysis was the number of animals used. $P<0.05$ was considered statistically significant.