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BOTH ESTROGEN RECEPTOR α AND ESTROGEN RECEPTOR β AGONISTS ENHANCE CELL PROLIFERATION IN THE DENTATE GYRUS OF ADULT FEMALE RATS

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Abstract—This study investigated the involvement of estrogen receptors α and β in estradiol-induced enhancement of hippocampal neurogenesis in the adult female rat. Subtype selective estrogen receptor agonists, propyl-pyrazole triol (estrogen receptor α agonist) and diarylpropionitrile (estrogen receptor β agonist) were examined for each receptor's contribution, individual and cooperative, for estradiol-enhanced hippocampal cell proliferation. Estradiol increases hippocampal cell proliferation within 4 h [Ormerod BK, Lee TT, Galea LA (2003) Estradiol initially enhances but subsequently suppresses (via adrenal steroids) granule cell proliferation in the dentate gyrus of adult female rats. J Neurobiol 55:247–260]. Therefore, animals received s.c. injections of estradiol (10 μg), propyl-pyrazole triol and diarylpropionitrile alone (1.25, 2.5, 5.0 mg/0.1 ml dimethylsulfoxide) or in combination (2.5 mg propyl-pyrazole triol+2.5 mg diarylpropionitrile/0.1 ml dimethylsulfoxide) and 4 h later received an i.p. injection of the cell synthesis marker, bromodeoxyuridine (200 mg/kg). Diarylpropionitrile enhanced cell proliferation at all three administered doses (1.25 mg, $P<0.008$; 2.5 mg, $P<0.003$; 5 mg, $P<0.005$), whereas propyl-pyrazole triol significantly increased cell proliferation ($P<0.0002$) only at the dose of 2.5 mg. Our results demonstrate both estrogen receptor α and estrogen receptor β are individually involved in estradiol-enhanced cell proliferation. Furthermore both estrogen receptor α and estrogen receptor β mRNA was found co-localized with Ki-67 expression in the hippocampus albeit at low levels, indicating a potential direct influence of each receptor subtype on progenitor cells and their progeny. Dual receptor activation resulted in reduced levels of cell proliferation, supporting previous studies suggesting that estrogen receptor α and estrogen receptor β may modulate each other's activity. Our results also suggest that a component of estrogen receptor–regulated cell proliferation may take place through alternative ligand and/or cell-signaling mechanisms.

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; DAB, diaminobenzidine; DCX, doublecortin; DPN, diarylpropionitrile; DSMO, dimethylsulfoxide; EB, 17β-estradiol benzoate; ER, estrogen receptor; GFAP, glial fibrillary acidic protein; IGF, insulin growth factor; ir, immunoreactivity; KPBS, potassium phosphate-buffered saline; NHS, normal horse serum; OIL, sesame oil; PB, phosphate buffer; PPT, propyl-pyrazole triol; SSC, standard saline citrate; TBS, tris-buffered saline.

Key words: estradiol, adult neurogenesis, hippocampus, estrogen receptors.

Estradiol has been shown to be neuroprotective and modulate both cognition and mood (McEwen et al., 2001). Examining the mechanisms of action behind estrogen has become complex with two identified estrogen receptors (ER), α and β (Kuiper et al., 1996) which act as ligand-inducible transcription factors. Research with selective ER modulators (SERMs) has been challenging due to their agonist/antagonist properties dependent on cell context and ER subtype involvement (Barkhem et al., 1998). This study utilized recently available ER subtype selective agonists, propyl-pyrazole triol (PPT), an ER-α agonist and diarylpropionitrile (DPN) an ER-β agonist, to examine the individual roles of each ER and their potential co-modulatory effects in estradiol-induced enhancement of cell proliferation in the dentate gyrus of adult ovariectomized female rats. PPT is a potent ER-α agonist, which does not activate ER-β (Stauffer et al., 2000), whereas DPN is a full ER-β agonist (Meyers et al., 2001). These agonists are valuable tools for elucidating the biological activity of ERs to understand estradiol's diverse effects.

Estradiol has also been shown to dynamically influence neurogenesis (both cell proliferation and survival) in the adult rodent hippocampus (Tanapat et al., 1999, 2005; Ormerod and Galea, 2001; Ormerod et al., 2003). Estradiol exposure for 4 h enhances, whereas estradiol exposure for 48 h, suppresses cell proliferation in the dentate gyrus of adult female rodents (Ormerod and Galea, 2001; Ormerod et al., 2003). Estradiol increases cell proliferation through a serotonin mechanism (Banasr et al., 2001) and suppresses cell proliferation by stimulating adrenal activity (Ormerod et al., 2003). ER-α and ER-β mRNA have been detected both isolated (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001) and co-localized (Su et al., 2001) in the adult hippocampus suggesting that both receptors may directly influence progenitor cells. However, there are inconsistent findings in the literature with some studies finding colocalization of both ER subtypes (mRNA or protein) in cells expressing endogenous or exogenous cell proliferation markers (Perez-Martin et al., 2003; Isgor and Watson, 2005) or no colocalization (Tanapat et al., 2005).

The aim of these experiments was to determine which ER subtype (if any) mediates the enhancement in cell proliferation at the 4 h time interval and whether different exposure to ER agonists influenced colocalization of ER-α

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and ER-β mRNA with dividing cells. As mentioned above, both ER subtypes (ER-α and ER-β) are found either in isolation (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001) or coexpressed (Su et al., 2001) in the hippocampus. These two subtypes have a differential distribution and also vary quantitatively, with ER-β being more abundant in the hippocampus (Shughrue et al., 1997). There are also regional differences of receptor expression in the rat hippocampus, ER-α (mRNA and protein expression) has been identified in the CA1 pyramidal layer, CA3 pyramidal layer and in the hilus of the dentate gyrus (Weiland et al., 1997; Orikasa et al., 2000; Blurton-Jones et al., 2004), whereas ER-β protein expression has been identified in the CA2 pyramidal layer, CA3 pyramidal layer, dentate gyrus granule cell layer and subiculum (Blurton-Jones et al., 2004). Additionally, non-nuclear ER-α and -β protein expression has been identified in the same corresponding areas as their nuclear counterparts in the hippocampus of the adult female rat (Kalita et al., 2005; Milner et al., 2005). Thus, both ER-α and ER-β are located in areas that could influence estradiol's effects on cell proliferation. Therefore, the aim of experiment 1 was to investigate, with novel ER subtype specific ligands, which subtypes were mediating estradiol-induced enhancement of cell proliferation and the aim for experiment 2 was to investigate whether both ER subtypes were colocalized with dividing cells after treatment with ER specific agonists.

EXPERIMENTAL PROCEDURES

All experiments were conducted in accordance with the policies established by the University of British Columbia, the Canadian Council on Animal Care and National Institutes of Health Guide for the Care and Use of Laboratory Animals regarding the ethical treatment of animals used for research. All efforts were made to reduce the number and the suffering of animals.

Subjects

Adult female rats from the University of British Columbia Animal Care Centre (Vancouver, BC, Canada), weighing between 225 and 250 g, received food and water *ad libitum*. The rats (n=95, experiment 1=69, experiment 2=26) were maintained on a 12-h light/dark cycle with lights on at 07:00 h with controlled colony room temperature to correspond with previous literature investigating estradiol's effects on neurogenesis (Tanapat et al., 2001; Ormerod et al., 2003).

Surgery

Approximately 1–2 weeks after arrival, all females were bi-laterally ovariectomized. Rats were placed in a chamber to which halothane was delivered at an induction flow rate of 3% (flow rate of O_2 was 2%) and maintained on a flow rate of 1–3% to maintain a stable respiratory rate. The rats were given 7 days to recover prior to the commencement of experimental manipulation.

Drug treatment

Ovariectomized female Sprague–Dawley rats were randomly assigned to one of 10 treatment groups (n=4/5 per group) and received a single s.c. injection of either: sesame oil (OIL), dimethylsulfoxide (DMSO), 17β-estradiol benzoate (EB; 10 μg/0.1 ml OIL), one of three doses of the ER-α agonist PPT (1.25 mg, 2.5 mg or 5.0 mg/0.1 ml DMSO), one of three doses of the ER-β agonist DPN (1.25 mg, 2.5 mg or 5.0 mg/0.1 ml DMSO) or a PPT and DPN combination dose (2.5 mg PPT+2.5 mg DPN/0.1 ml DMSO). These doses of PPT and DPN were chosen based on a prior studies investigating female sexual behavior (Mazzucco et al., submitted for pulication). Four hours following the treatment injection, each animal received an i.p. injection of the cell synthesis marker bromodeoxyuridine (BrdU) in a volume of 1.0 ml/100 g body weight (200 mg BrdU/kg). Changes in blood–brain barrier permeability may alter BrdU availability; however, blood–brain barrier permeability is only altered by estradiol after at least 3 weeks of exposure in rats (Ziylan et al., 1990). The animals were perfused 24 h or 4 days after BrdU administration in order to examine cell proliferation (Cameron and McKay, 2001) or cell phenotype, respectively. For experiment 2, animals were divided into five groups: Oil (n=5), EB (n=5, 10 μg), DMSO (n=5), PPT (n=5, 2.5mg), DPN (n=6, 2.5 mg).

Drug preparation

The ER α agonist, PPT (Tocris Bioscience, Ellisville, MO, USA) and the ER β agonist, DPN (Tocris Bioscience) were dissolved in DMSO (Sigma Aldrich, St. Louis, MO, USA). EB was combined with OIL (Sigma Aldrich) over low heat in a light insensitive container to obtain a concentration of 10 μg EB per 0.1 ml OIL (Ormerod et al., 2003). BrdU (Sigma Aldrich), a marker of dividing cells, was prepared just prior to injection. BrdU was dissolved to a concentration of 20 mg/ml in warm freshly prepared 0.9% saline (buffered with 7 μl 2 N NaOH/ml saline).

Experiment 1

Histology. Rats were anesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde within 24 h (to assess cell proliferation) or 4 days (to assess cell phenotype) after injection of BrdU. A 24-hour survival time post-BrdU injection was followed to allow for one mitotic division (Cameron and McKay, 2001). The phenotype of BrdU-ir cells was determined in the 4 day survival group using confocal microscopy and counting the number of BrdU cells co-labeled with doublecortin (DCX, a microtubule-associated protein expressed by migrating and differentiating granule neurons (Francis et al., 1999; Gleeson et al., 1999; Jin et al., 2001) or glial fibrillary acidic protein (GFAP; an astroglia marker)) immunoreactivity (ir) (Cameron et al., 1993; Gould et al., 1999; Smith et al., 2001). Following extraction, brains were stored in 4% paraformaldehyde for 48 h, before transfer into a solution of tris-buffered saline (TBS) for a minimum of 24 h (all at 4 °C). Brains were sliced through the entire extent of the dentate gyrus (18–20 sections per rat) in a bath of TBS (PH 7.5) using a vibratome (Leica VT1000S; Leica Microsystems, Inc., Richmond Hill, ON, Canada). The 40 μm sections were stored in sterile culture plates filled with TBS prior to BrdU immunohistochemistry processing. BrdU-labeled cells were counted on peroxidase-treated tissue and the phenotype of new cells was determined on fluorescent probe–treated tissue.

BrdU immunohistochemistry. Tissue was processed to reveal BrdU labeling by applying solutions to the free-floating tissue sections. The sections were rinsed repeatedly between steps in TBS (0.1 M tris-phosphate buffer in 0.9% saline; pH 7.4) unless stated otherwise. DNA was denatured by applying 2 N HCl for 30 min at 37 °C. Sections were blocked with 3.0% normal horse serum (NHS) for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:200+3% NHS+10% Triton-X; Boehringer Mannheim, Laval, QC, Canada) at room temperature. The following day, sections were incubated in mouse secondary antisera (1:129+3% NHS; Vector Laboratories, Burlington, ON, Canada) for 4 h. Sections were incubated in avidin–biotin horseradish peroxidase complex (ABC Elite Kit; 1:50; Vector Laboratories) for 120 min. Sections were reacted for approximately 10 min in 0.02% diaminobenzidine (DAB; Sigma Aldrich)