General and Comparative Endocrinology 176 (2012) 62–69

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Expression of nerve growth factor and its receptors TrkA and p75 in the uterus of wild female ground squirrel (*Citellus dauricus* Brandt)

Ben Li [a], Xia Sheng [a], Moshi Song [a,b], Haolin Zhang [a], Jiaju Weng [c], Mengyuan Zhang [a], Xiao Hu [a,d], Jiao Zhou [a], Meiyu Xu [a], Qiang Weng [a,e,*], Gen Watanabe [e], Kazuyoshi Taya [e]

[a] College of Biological Science and Technology, Beijing Forestry University, Beijing 100083, PR China
[b] Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm 17177, Sweden
[c] School of Basic Medical Sciences, Peking University, Beijing 100083, PR China
[d] Department of Biology, University of Ottawa, Ottawa, Canada K1N 6N5
[e] Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

ARTICLE INFO

Article history:
Received 9 April 2011
Revised 4 December 2011
Accepted 19 December 2011
Available online 28 December 2011

Keywords:
Wild ground squirrel
NGF
p75
TrkA
Uterus

ABSTRACT

In this study, we investigated the presence of nerve growth factor (NGF) and its receptors tyrosine kinase A (TrkA) and p75 in the uterus of the wild ground squirrels during the estrous period, early pregnancy and non-breeding period. In the estrous period and early pregnancy, NGF and TrkA were immunolocalized in stromal cells, luminal epithelial cells, glandular cells and smooth muscle cells whereas in the non-breeding period, both of them were detected only in luminal epithelial cells and glandular cells, but not in stromal cells or smooth muscle cells. Stronger immunostaining of NGF and TrkA was observed in luminal epithelial cells and glandular cells in the estrous period and early pregnancy as compared to the non-breeding period. p75 was immunolocalized only in luminal epithelial and glandular cells during the estrous period, early pregnancy and non-breeding period. The intensity of the immunohistochemical signals for p75 did not vary significantly in the estrous period, early pregnancy and non-breeding period. The mean mRNA levels of NGF and TrkA and p75 were significantly higher in the estrous period and early pregnancy as compared to the non-breeding period. Besides, plasma estradiol-17β and progesterone concentrations were higher in the estrous period and early pregnancy than in the non-breeding period, suggesting that the expression patterns of NGF and TrkA are correlated with changes in plasma estradiol-17β and progesterone concentrations. These results indicate that NGF and its receptor TrkA may be involved in the regulation of seasonal changes in the uterine functions of wild female ground squirrels.

1. Introduction

Nerve growth factor (NGF) belongs to a family of proteins known as neurotrophins that are important for many physiological processes such as cell survival, maintenance and development of discrete neuronal populations in the central and peripheral nervous systems [21,33]. The multiple effects of NGF are mediated via its receptors [29,37,7]. Binding of NGF to the high affinity membrane-bound receptor tyrosine kinase A (TrkA) leads to TrkA dimerization and autophosphorylation of tyrosine residues [19,18]. Phosphorylation of TrkA leads to activation of second messenger cascades, such as mitogen-activated protein kinase and phosphatidylinositol-3 kinase that are involved in essential pathways for cell survival and differentiation [42,14]. NGF also binds to the low affinity receptor p75 [7].

The density of uterine sympathetic nerves shows phases of degeneration and regeneration during the natural oestrous cycle. Even more, uterine sympathetic nerves degenerate during normal pregnancy and regenerate following delivery [3]. Pregnancy-induced uterine denervation is of physiological significance as it reduces myometrial contractility and thus prevents preterm labor [15,23]. On the other hand, much evidence has suggested that the steroid hormone-induced growth of uterine endometrial cells is mediated in an autocrine or paracrine manner by polypeptide growth factors synthesized by the uterus [6]. Previous studies have shown that in experimental and domestic animal species, NGF and its receptors TrkA and p75 are expressed in the female reproductive system and exert their biological roles in many related processes including uterine growth and proliferation [11,1,32,39–41]. Data

* Corresponding author at: Laboratory of Animal Physiology, College of Biological Science and Technology, Beijing Forestry University, Beijing 100083, PR China. Fax: +86 10 6233 6399.
E-mail addresses: liben77@126.com (B. Li), shengxia712@hotmail.com (X. Sheng), songmos@gmail.com (M. Song), zhanghl0117@hotmail.com (H. Zhang), oukaku@bjmu.edu.cn (J. Weng), zmy0428@hotmail.com (M. Zhang), xiaohucn@hotmail.com (X. Hu), xjbjzhoujiao@163.com (J. Zhou), xumeiyu@hotmail.co.jp (M. Xu), qiangweng@bjfu.edu.cn (Q. Weng), gen@tacc.ac.jp (G. Watanabe), taya@tacc.ac.jp (K. Taya).

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doi:10.1016/j.ygcen.2011.12.024

to support this concept in wild animals, however, is very limited. To study the basic mechanisms of NGF regulation of uterine function during the breeding and non-breeding season, the wild ground squirrel offers a useful model without any manipulations.

The wild ground squirrel (*Citellus dauricus* Brandt) is a typical seasonal breeder which has a strict and extremely compressed breeding period (for female individuals, it includes estrous period and pregnancy) from April to May and a long period of sexual dormancy from June to the following March including a 6-month hibernation period [28]. The wild female ground squirrel exhibit estrus immediately after emergence from hibernation in spring, and has a gestation period of 28 days [21,34]. Whether fertilized or not, all females become sexually inactive as the relatively brief breeding season ends. Although the role of NGF and its receptors on uterine function has been reported [32,38,5,22], the mechanisms through which this regulation occurs are not entirely understood. To illustrate the involvement of NGF on the regulation of uterine function changes outside the nervous system, we have investigated the expression and distribution patterns of NGF and its receptors TrkA and p75 in uteri of wild female ground squirrels during the estrous period, early pregnancy and non-breeding period.

2. Materials and methods

2.1. Animals

All the procedures on animals were carried out in accordance with the Policy on the Care and Use of Animals by the Ethical Committee, Beijing Forestry University and approved by the Department of Agriculture of Hebei province, PR China (JNZF11/2007). Wild female ground squirrels that were regarded as adults according to their body weights (242–412 g) were captured on April 13 (10.2 h of daylight) after emergence from hibernation in the breeding period ($n = 10$) and on June 9 (12.6 h of daylight) in the non-breeding period ($n = 5$) of 2009 in Hebei Province, PR China. The uterus, ovary and brain of each animal were quickly removed and dissected. The ovarian and part of the uterine tissues were fixed in 0.05 M phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde (Sigma, St. Louis, MO, USA) for histological and immunohistochemical observation; brain and the rest of the uterine tissues were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and Western blotting detection.

2.2. Histology

Uterine and ovarian samples were dehydrated through ethanol series and embedded in paraffin wax. Serial sections (4 μm) were mounted on poly-L-lysine (Sigma, St. Louis, MO, USA) coated slides. The sections were stained with hematoxylin–eosin (HE) for general histological observations. The number of uterine glandular nuclei was assessed with NIH ImageJ software, with the method described by Kirby et al. [20].

2.3. Immunohistochemistry

Uterine sections were blocked with 10% normal goat serum to prevent the non-specific binding of the second antibody. The sections were then incubated with polyclonal primary antibody against NGF (0.4 μg/ml, M-20), TrkA (2 μg/ml, 763) or p75 (2 μg/ml, H-92) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h at 4 °C, and incubated with the second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin for 1 h at room temperature. The sections were visualized using a rabbit ExtrAvidin™ staining kit (Sigma, St. Louis, MO, USA) in 150 ml of 0.05 M Tris–HCl buffer containing 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) plus 30 μl H_2O_2. Finally, the sections were counterstained with hematoxylin (Merck, Tokyo, Japan) and NGF, TrkA and p75 were detected, respectively. Control sections were treated with normal rabbit serum (Sigma, St. Louis, MO, USA) instead of the primary antisera. The immunostained slides were scanned using the software Image-Pro Plus 4.5 (Media Cybernetics, MD, USA) at 20× magnification. The background of each section was used as the internal control during evaluating the intensity of the immunostaining. Similar with a previous study [32], immunoreactivity was showed as − for negative staining, + for the positive staining, ++ for the strong positive staining and +++ for the very strong positive staining.

2.4. Western blotting

Uterine tissues were weighed and dissected into small pieces using a clean razor blade. The tissues were homogenized in a tissue homogenizer containing 300 μl of 10 mg/ml PMSF and incubated for 30 min on ice. Homogenates were centrifuged at 12,000*g* for 10 min at 4 °C. Protein extracts (25 μg) were mixed with equal volumes of 2× Laemmli sample buffer. Equal amounts of proteins from each sample were loaded onto a 12% SDS–PAGE gel and electrophoretically separated at 18 V/cm and transferred to nitrocellulose membrane using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membrane was blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membrane was carried out using NGF, TrkA or p75 antibody (1:1000 dilution) for 1 h at room temperature. Secondary incubation of the membrane was then carried out using an IRDye (1:5000 dilution, Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. Finally, the membrane was washed in 25 ml Tris-buffered saline with Tween 20 (TBST wash buffer, 0.02 M Tris, 0.137 M NaCl and 0.1% Tween 20, pH 7.6) plus 3 μl H_2O_2 and visualized with Odyssey infrared imaging system. Brain tissue of wild ground squirrel was used as a positive control and water, instead of primary antisera, was used as a negative control. β-Actin was selected as the endogenous control. The intensities of the bands were quantified using Quantity One software (Version 4.5, Bio-Rad Laboratories) and expression ratios were calculated.

2.5. RNA isolation

Total RNA from each sample was extracted using ISOGEN (Nippon Gene, Toyama, Japan). Approximately 1 g of uterine tissues were thawed and immediately homogenized in 10 ml of ISOGEN™. The homogenate was incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes. After the addition of 2 ml of chloroform, the mixture was vigorously shaken for 3 min at room temperature and centrifuged at 12,000*g* for 10 min at 4 °C. The aqueous phase was then transferred to a fresh tube and washed with an equal volume of chloroform. An equal volume of isopropanol was added, and the sample was kept for 10 min at room temperature. RNA was precipitated by centrifugation at 12,000*g* for 10 min at 4 °C. The RNA pellet was washed twice with 75% ethanol, briefly dried under air, and dissolved in 100 μl of diethylprocarbonate-treated water.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The first-strand cDNA from total RNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo $(dT)_{12-18}$ according to the manufacturer's protocol. The 20 μl of reaction mixture contained 4 μg of total RNA, 0.5 μg of oligo $(dT)_{12-18}$, 2.5 mM $MgCl_2$, 0.5 mM dNTP, 10 mM dithiothreitol, 20 mM Tris–HCl (pH 8.4) and 200 U of Superscript II enzyme. The