Expression of Glial Cell Line-Derived Neurotrophic Factor (GDNF) and the GDNF Family Receptor Alpha Subunit 1 in the Paravaginal Ganglia of Nulliparous and Primiparous Rabbits

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Purpose: To evaluate the expression of glial cell line-derived neurotrophic factor (GDNF) and its receptor, GDNF family receptor alpha subunit 1 (GFRα-1) in the pelvic (middle third) vagina and, particularly, in the paravaginal ganglia of nulliparous and primiparous rabbits.

Methods: Chinchilla-breed female rabbits were used. Primiparas were killed on postpartum day 3 and nulliparas upon reaching a similar age. The vaginal tracts were processed for histological analyses or frozen for Western blot assays. We measured the ganglionic area, the Abercrombie-corrected number of paravaginal neurons, the cross-sectional area of the neuronal soma, and the number of satellite glial cells (SGCs) per neuron. The relative expression of both GDNF and GFRα-1 were assessed by Western blotting, and the immunostaining was semiquantitated. Unpaired two-tailed Student t-test or Wilcoxon test was used to identify statistically significant differences (P≤0.05) between the groups.

Results: Our findings demonstrated that the ganglionic area, neuronal soma size, Abercrombie-corrected number of neurons, and number of SGCs per neuron were similar in nulliparas and primiparas. The relative expression of both GDNF and GFRα-1 was similar. Immunostaining for both GDNF and GFRα-1 was observed in several vaginal layers, and no differences were detected regarding GDNF and GFRα-1 immunostaining between the 2 groups. In the paravaginal ganglia, the expression of GDNF was increased in neurons, while that of GFRα-1 was augmented in the SGCs of primiparous rabbits.

Conclusions: The present findings suggest an ongoing regenerative process related to the recovery of neuronal soma size in the paravaginal ganglia, in which GDNF and GFRα-1 could be involved in cross-talk between neurons and SGCs.

Keywords: Pelvic ganglia; Nerve growth factors; Neuronal plasticity; Reproduction; Lower urogenital tract

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INTRODUCTION

The pelvic plexus of women, as well as that of some other female mammals, provides the majority of autonomic connections to the lower urogenital tract (LUT) through a network of nerve bundles and ganglia that are scattered and interwoven with the pelvic viscera [1]. Such an anatomical arrangement is clearly different in rodents, which possess a major pelvic ganglion and a few satellite ganglia [2]. The reproductive experiences of women have been proposed to be a factor influencing the organization of the pelvic plexus [3]. Laparoscopic examinations have suggested that the pelvic plexus is involved in the sensibility of the vagina and bladder, as well as in bladder tone [4].

Female rats have been used to investigate neuroplastic processes affecting the pelvic plexus [5]. However, the anatomical organization of the pelvic plexus of species such as dogs, cats, and rabbits, among others, is more similar to that of women [6-9]. In particular, in female rabbits, the pelvic plexus is innervated by the hypogastric and pelvic nerves, which send fibers innervating the urogenital tissues and rectum [6,9]. In particular, in female rabbits, the pelvic plexus is innervated by the hypogastric and pelvic nerves, which send fibers innervating the urogenital tissues and rectum [6,9].

The physiological adaptations of the female LUT in response to the hormonal milieu and reproduction are grounded in neuroplasticity [5,10-12]. Reproductive experiences have been found to be related to neuropeptide expression in neurons of the paracervical ganglia of guinea pigs, as well with changes in the morphology of pelvic neurons in rats and rabbits [13-15]. For female rabbits, data gathered from the paravaginal ganglia, a subset of pelvic ganglia located in the dorsolateral wall of the pelvic vagina [9,15], have shown that pregnancy leads to a transient decrease in the size of the neuronal somata that is reversed by 20 days after parturition [15]. Additionally, it has been hypothesized that neuroplastic modifications are linked to an apparent pruning of paravaginal neurons and modifications of the putative target tissues (i.e., smooth muscle and the content of blood vessels) in multiparous rabbits [15,16].

Signaling involving neurotrophic factors is relevant for the recovery of injuries affecting autonomic innervation of the LUT [10]. Glial-cell derived neurotrophic factor (GDNF) has been found to be related to the estrogen-induced recovery of para-vaginal neuronal soma size in ovariectomized rabbits [11]. Indeed, GDNF and other ligands of the GDNF family (GFLs) influence the soma size in cultured pelvic neurons of rodents [17,18]. The analysis of GFL alpha receptor (GFRα) expression has provided relevant information about other tissues that could be influenced by GFL actions [10,17-19]. Among the four GFRα types (1-4) that can bind GFL (GDNF, neurturin, artemin, and persephin), GFRα-1 shows the greatest affinity for GDNF [20].

We hypothesized that the expression of GDNF is related to the plasticity of neuronal soma size of the paravaginal ganglia in primiparous rabbits. To test this hypothesis, we evaluated the expression of GDNF and GFRα-1 in the dorsolateral pelvic vaginal walls and, particularly, in the paravaginal ganglia of nulliparous and primiparous rabbits on day 3 postpartum.

MATERIALS AND METHODS

Animals

Twenty age-matched (approximately 7 months old) chinchilla-breed female rabbits (Oryctolagus cuniculus) were housed in individual stainless-steel cages and kept at 20°C ± 2°C under artificial lighting conditions (16 light and 8 hours dark, lights on at 06:00 hours). Rabbits are reflex ovulators that under these housing conditions are considered to be in an early proestrous phase [21]. They were provided daily with pellet food (Conejina, Purina, México) and had continuous access to water. Rabbits were allocated into 2 groups: a control group composed of nulliparous rabbits (N; n = 10) and an experimental group of primiparous rabbits (P3; n = 10). Rabbits in the P3 group were mated at 6 months of age. The rabbits in both groups were euthanized with an overdose of sodium pentobarbital (100 mg/kg of weight, intraperitoneally) when they reached the established age (N) or at 3 days postpartum (P3). The pups in the latter group were decapitated at 1-day postpartum. The Ethics Committee of the Universidad Autónoma de Tlaxcala approved all experimental procedures described below.
**Tissue Harvesting**

The vaginal tract of 20 rabbits (10 nulliparas and 10 primiparas) was excised as described elsewhere [15,16]. For 8 rabbits (4 nulliparas and 4 multiparas), the vagina was cut lengthwise in half to separate out the dorsolateral region in which the paravaginal ganglia are embedded [15]. Considering that the number of ganglion neurons is similar on the left and right sides [15], the latter group of neurons was stored at −80°C to perform Western blot assays. Otherwise, the vaginas of 6 nulliparous and 6 primiparous rabbits were harvested, and the pelvic vagina was excised, washed with saline, immersed in Bouin-Duboscq fixative for 18 hours, and embedded in Paraplast X-tra (Sigma-Aldrich, St. Louis, MO, USA).

**Histology**

Paraplast-embedded samples from the pelvic vagina were cut on a microtome (Leica, Wetzlar, Germany) to obtain 7-μm-thick transverse sections. For each animal, the slides were separated in 4 series, and 1 of them was stained with Masson trichrome, covered with mounting medium and a coverslip, and observed under light microscopy using an Axio Imager A1 microscope (Carl Zeiss AG, Oberkochen, Germany). Images were acquired with a digital camera (ProgRes CT5, Jenoptik AG, Jena, Germany) with a resolution of 5.1 megapixels.

**Ganglia Morphometry**

Paravaginal ganglia and neuron profiles were analyzed in an approximately 1.4-mm-long segment of the pelvic wall of the vagina (about 20% of its entire length) as described elsewhere [15]. The urethral opening was set as the reference point to separate out the dorsal region in which the paravaginal ganglia are embedded [15]. The location of GDNF and GFRα-1 expression was assessed using immunohistochemistry, as reported elsewhere [11]. Briefly, homologous slides were incubated in a humidified chamber for 72 hours at 4°C with anti-GDNF and anti-GFRα-1 polyclonal antibodies (Santa Cruz Biotechnology; 1:100) or mouse monoclonal anti-GFRα-1 (sc-271546, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:100), followed by secondary antibodies (1:1,000, goat anti-mouse IgG-biotinylated, sc-2039, Santa Cruz Biotechnology Inc.). The sequence of epitopes used to raise anti-GDNF (human; NCBI accession version CAG46721.1) and anti-GFRα-1 (rat; NCBI accession NP_037091.1) share a homology of 100% (NCBI accession XP_008260349.1) and 96.8% (NCBI accession XP_002718804.1) with rabbit proteins. Immunoreactive polypeptides were detected using a chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate Kits, Thermo Fisher Scientific, Waltham, MA, USA) and exposed to Kodak X-OMAT film. The expression of GDNF and GFRα-1 was measured by densitometry and normalized against the signal obtained from the India ink-stained lane, which was used as a loaded control [24]. The ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for the densitometric analysis. The relative expression levels of GDNF and GFRα-1 were calculated by dividing the density of each band by the density of the matching India ink-stained lanes. Data were expressed as the percentage (%) of change.

**Western Blot Assays**

Total protein extracts were prepared from frozen vaginal tissue as described elsewhere [11]. Equal amounts of protein (100 μg) were denatured in Laemmli sample buffer and resolved through sodium dodecyl sulfate-polyacrylamide (15% for GDNF; 10% for GFRα-1) gels and electro-blotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Blots were stained with Indian ink (Pelikan Holding AG, Schindellegi, Switzerland) diluted in phosphate-buffered saline (PBS) to confirm that the protein content was equal in all lines [23]. The membranes were soaked in PBS and incubated in 5% dried skim milk diluted in PBS containing 0.2% tween-20 (PBST) for 60 minutes to block nonspecific protein binding sites. Membranes were incubated overnight at 4°C with mouse monoclonal anti-GDNF (sc-13147, Santa Cruz Biotechnology; 1:100) or mouse monoclonal anti-GFRα-1 (sc-271546, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:100), followed by secondary antibodies (1:1,000, goat anti-mouse IgG-biotinylated, sc-2039, Santa Cruz Biotechnology Inc.). The sequence of epitopes used to raise anti-GDNF (human; NCBI accession version CAG46721.1) and anti-GFRα-1 (rat; NCBI accession NP_037091.1) share a homology of 100% (NCBI accession XP_008260349.1) and 96.8% (NCBI accession XP_002718804.1) with rabbit proteins. Immunoreactive polypeptides were detected using a chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate Kits, Thermo Fisher Scientific, Waltham, MA, USA) and exposed to Kodak X-OMAT film. The expression of GDNF and GFRα-1 was measured by densitometry and normalized against the signal obtained from the India ink-stained lane, which was used as a loaded control [24]. The ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for the densitometric analysis. The relative expression levels of GDNF and GFRα-1 were calculated by dividing the density of each band by the density of the matching India ink-stained lanes. Data were expressed as the percentage (%) of change.

**GDNF and GFRα-1 Immunohistochemistry**

The location of GDNF and GFRα-1 expression was assessed using immunohistochemistry, as reported elsewhere [11]. Brieﬂy, homologous slides were incubated in a humidified chamber for 72 hours at 4°C with anti-GDNF (1:100) or anti-
GFRα-1 (1:100) antibodies, and afterwards incubated with a biotinylated secondary antibody (sc-2039, Santa Cruz Biotechnology Inc.; 1:250) for 2 hours at room temperature. Immunostaining was performed following the Vectastain ABC kit directions (Vector Laboratories Inc., Burlingame, CA, USA). Some sections were incubated with the secondary antibody alone and were considered as negative controls. Immunostained sections were counterstained with Mayer hematoxylin. The proportion of rabbits showing positive results for each immunostaining assay is indicated inside parentheses. Both GDNF and GFRα-1 immunostaining in the vaginal layers were categorized in accordance to the intensity of each marker as negative (-), weak (+), moderate (++), or strong (+++) by 2 independent observers (VGV and LGHA). These categories were then assigned numerical values instead of signs to perform a semiquantitative analysis [25]. Sections of the cranial portion of the pelvic wall of the vagina for nulliparas and multiparas were simultaneously processed to obtain a qualitative estimate of GDNF and GFRα-1 immunoreactivity based on the intensity of each marker in neurons and SGCs, as reported elsewhere [12]. To obtain representative images of vaginal GDNF and GFRα-1 immunostaining, sections were observed under light microscopy using a Nikon microscope (Nikon, Tokyo, Japan) coupled to a digital camera with a resolution of 16.25 megapixels (DS-Ri2, Nikon). Selected photographs were postprocessed using the automated color normalization function of the program GIMP (v. 2.8.6. for MacOS).

Statistical Analysis

The data for morphometric variables are presented as means ± standard error of the mean. Significant differences (P ≤ 0.05) were identified using the unpaired 2-tailed Student t-test. Data from western blot assays and immunohistochemistry for GDNF and GFRα-1 are presented as medians ± maximum and minimum values. Significant differences (P ≤ 0.05) were identified using the Wilcoxon matched-pairs signed-rank test. Statistical analyses were done with the program Prism 4c for PCs (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Ganglia Morphometry

The morphology of the paravaginal ganglia was inspected in Masson trichrome sections (Fig. 1A, B). The area covered by the ganglia in primiparous rabbits and nulliparous rabbits was similar (Fig. 1C). The same was true for the average number of ganglionic neurons (Fig. 1D) and the cross-sectional area (CSA) of the neuronal somata (Fig. 1E). When the CSA was separated into 3 bins, a significant reduction in the percentage of neurons with larger (650–1,400 μm²) soma CSA was measured in primiparous rabbits (Fig. 1F). The percentages of small (50–350 μm²) and medium (351–650 μm²) neuronal soma CSA were similar between nulliparas and primiparas. The average number of SGCs per neuron soma was also similar for nulliparous and primiparous rabbits (Fig. 1E). The same was true for the percentage of paravaginal neurons associated with SGCs (Fig. 1F).

Vaginal GDNF Expression

Western blot assays showed the presence of a single GDNF immunoreactive band with an approximate molecular weight of 17 kDa in nulliparas and primiparas (Fig. 2A). The GDNF expression measured in the pelvic vagina in the N and P3 groups was highly similar (Fig. 2B).

In the vaginal sections of nulliparous rabbits, GDNF immunostaining was observed in the epithelium (4 of 6; Fig. 2C), submucosa (2 of 6), smooth muscle cells of the muscle layer (4 of 6; Fig. 2F); and in the skeletal myofibers of the external layer (5 of 6; Fig. 2I). A highly similar pattern of GDNF staining was seen in the epithelium (5 of 6; Fig. 2D), submucosa (1 of 6; Fig. 2G), smooth muscle (5 of 6; Fig. 2F), and skeletal muscle (5 of 6; Fig. 2J) of primiparous rabbits. Such staining was highly specific in comparison to the negative control results (Fig. 2E, H, K). In a semiquantitative analysis of the intensity of GDNF immunostaining, there were no significant differences between the nulliparous and primiparous rabbits for any of the inspected vaginal regions (Fig. 2L–O).

Vaginal GFRα-1 Expression

Two major GFRα-1 immunoreactive bands were detected between 53 and 79 kDa in the total protein extracts from the pelvic vagina of nulliparas and primiparas (Fig. 3A). Both bands were considered to determine the relative expression of GFRα-1. The relative GFRα-1 expression was similar between the N and P3 groups (Fig. 3B).

GFRα-1 immunostaining in the nulliparous rabbits (Fig. 3) was seen in the epithelium (5 of 6; Fig. 3C), submucosa (2 of 6), smooth muscle of the muscle layer (4 of 6; Fig. 3E), and skeletal myofibers of the external layer (6 of 6; Fig. 3G). The distribution was highly similar in the primiparous rabbits, with GFRα-1 immunostaining observed in the epithelium (6 of 6; Fig. 3D),

![Image]

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submucosa (2 of 6; Fig. 3D), smooth muscle (5 of 6; Fig. 3F), and skeletal muscle (5 of 6; Fig. 3H). In a semiquantitative analysis, no significant differences between the nulliparous and primiparous rabbits were found (Fig. 3I–L).

**GDNF and GFRα-1 Expression in the Paravaginal Ganglia**

Weak GDNF immunostaining was observed in the paravaginal ganglia, particularly in paravaginal neurons; notably, such immunostaining was seen as discrete clusters that were restricted to the cytoplasm near the nuclear perimeter (Fig. 4A). GDNF immunostaining was also observed in SGCs (Fig. 4A). Stronger GDNF immunostaining was noted in the cytoplasm of both the neurons and SGCs of primiparous rabbits (Fig. 4B).

GFRα-1 immunostaining was also noted in the cytoplasm of paravaginal neurons and SGCs (Fig. 4C). Such labeling was stronger in the neurons and SGCs of primiparous rabbits (Fig. 4C).
4D). No immunostaining was seen in sections incubated with the secondary antibody alone (Fig. 4E).

**DISCUSSION**

The ganglionic area, Abercrombie-corrected number of neurons, and the number of SGCs per neuron were similar in nulliparous and primiparous rabbits. The findings regarding the first 2 variables are consistent with those reported for primiparous rabbits when evaluated on postpartum day 20 [15]. Indeed, the number of ganglionic neurons also remained unchanged in rats subjected to vaginal distention [26]. The SGC-to-neuron ratio was somewhat unexpected, as the estradiol-related recovery of neuronal soma size has been linked to a high...
SGC-to-neuron ratio in ovariectomized rabbits [11]. Overall, the fact that none of these morphometric variables were modified leads us to believe that primiparity did not induce morphometric alterations in the paravaginal ganglia of rabbits. This conclusion is in accord with another study conducted in late-pregnant guinea pigs [14].

Unlike guinea pigs, there is a reduction in the soma size of paravaginal neurons in late-pregnancy (day 30) rabbits, with recovery by 20 days postpartum [15]. We expected that the paravaginal neuronal somata would still be reduced in size on day 3 postpartum. However, the present findings suggest that the recovery of a great proportion of the paravaginal soma size may have occurred earlier, by 3 days postpartum, suggesting that the recovery of size could start during pregnancy. It may be the case that the reduction in neuronal soma size at 30 days of pregnancy is part of an ongoing recovery process that is likely associated with fluctuations in sex steroids [27]. In this regard, it is important to bear in mind the neuroprotective role of es-

**Fig. 3.** Vaginal glial cell line-derived neurotrophic factor (GDNF) family receptor alpha subunit 1 (GFRα-1) expression in nulliparous (N) and primiparous rabbits (P3). A representative immunoblot showing GFRα-1 expression in the dorsolateral pelvic vagina of N and P3 rabbits and matched India ink-stained lanes (A). Relative expression of GFRα-1 in nulliparous and primiparous rabbits (B); data are medians ± minimum to maximum values (n = 4 rabbits per group) and individual values. GFRα-1 immunostaining in the vagina of nulliparous (C, E, G) and primiparous rabbits (D, F, H). Fields of mucose and submucose (C, D), smooth muscle (E, F), and external (G, H) layers are shown. The intensity of immunostaining was semiquantitated for the mucose (I), submucose (J), smooth muscle (K), and external (L) layers; data are medians ± maximum and minimum values and individual values. GFRα-1-ir, GFRα-1-immunoreactivity. Arrows, positive cells; asterisks, blood vessels. Scale bar, 100 μm.
trogens in the nervous system and their relationship with neurotrophic actions [28]. Indeed, some neurotrophic factors are regulated by estrogens [11,29].

Reproduction affects the content of neurotrophic factors in the serum and peripheral organs [12,30,31]. In particular, the actions of GFLs are relevant for understanding the plasticity of LUT tissues in males and females [10,32]. The expression of GDNF was analyzed in this study, in light of its recently reported relationship with the morphometric plasticity of paravaginal neurons in ovariectomized rabbits [11].

The expression of GDNF was observed in dorsolateral segments of the pelvic vagina by Western blotting and immunohistochemistry assays. The former assays showed that GDNF was similarly expressed in total protein extracts from the pelvic vagina of nulliparas and primiparas. The latter assays showed that primiparity did not alter the intensity or distribution of GDNF immunoreactivity in the epithelial, intermediate, and external layers of the vagina. Overall, these data support the notion that the wide distribution of GDNF in vaginal tissue can mask changes in its expression as evaluated by Western blots, which implies a methodological limitation, since paravaginal ganglia were not isolated to obtain protein extracts. Additional-ly, immunostaining showed cell types (e.g., smooth muscle, epithelial, and endothelial cells) that are potential targets for autonomic innervation and are therefore able to synthesize GDNF. Primiparity, however, did increase the GDNF immunostaining in the somata of paravaginal neurons. Such a response is in agreement with the increase in the GDNF immunoreactivity of paravaginal neurons found in ovariectomized rabbits showing a reduction in soma size [11]. Taking into account the data gathered for sensory and motor neurons from aged female rats and the pelvic neurons of male rats subjected to a cavernous nerve injury, it is also plausible to propose that GDNF immunoreactivity may be an indicator of the ongoing neuroregenerative process in paravaginal neurons [33,34]. GDNF immunostaining in SGCs was rather similar between nulliparous and primiparous rabbits, resembling the behavior of these cells in chronically ovariectomized rabbits [11].

In this study, we also estimated the expression of GFRα-1, which is the receptor with the greatest affinity for GDNF [20].

![Fig. 4. Expression of glial cell line-derived neurotrophic factor (GDNF) (A, B) and GDNF family receptor alpha subunit 1 (GFRα-1) (C, D) in the paravaginal ganglia of nulliparous (A, C) and primiparous (B, D) rabbits. Representative photomicrographs showing GDNF and GFRα-1 immunostaining in neurons (asterisks) and satellite glial cells (arrows); counterstaining, Meyer hematoxylin. Immunostaining when vaginal sections were incubated with the secondary antibody alone (negative control) (E). Scale bar, 20 μm.](image-url)
Similarly to GDNF expression, no significant differences were seen between GFRα-1 expression in nulliparas and primiparas as measured by Western blot assays. The same was true for GFRα-1 immunostaining in the different layers of the vagina, and particularly in smooth muscle, epithelial, and endothelial cells. This localization, highly coincident with that of GDNF, suggests paracrine and/or autocrine actions of GDNF/GFRα-1 in the vagina of rabbits. This possibility should be further investigated.

In contrast, GFRα-1 immunostaining in the neuronal somata of primiparas was stronger than in nulliparas. This finding is in agreement with the increased expression of this receptor in response to stroke in the forebrain of rats [35] and in the sensory and autonomic pathways of the spinal cord after peripheral injury of the pelvic visceral sensory nerves [19]. In addition, an increase in GFRα-1 immunostaining has been observed in the paravaginal neurons of chronically ovariectomized rabbits [11]. Taking these studies into account, it is reasonably likely that high GFRα-1 expression is related to neuroprotective effects on paravaginal neurons in primiparous rabbits. Additionally, GFRα-1 immunostaining in the SGCs of primiparous rabbits was stronger than in the SGCs of nulliparous rabbits. Such a finding agrees with data from chronically ovariectomized rabbits [11]. This information leads to the hypothesis that cross-talk between neurons and SGCs through GDNF/GFRα-1 may be involved in neuroprotective effects, possibly through the modulation of neuronal soma size in the paravaginal ganglia during the early postpartum period [15].

Our study suggests an ongoing regenerative process related to the recovery of neuronal soma size in paravaginal ganglia, in which GDNF and GFRα-1 may be involved in cross-talk signaling between neurons and SGCs. Further experimental approaches should address the present findings to characterize the underlying mechanisms, as well as functional outcomes.

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