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Morphology and Chemical Phenotype of the Ovarian Intrinsic Neurons in Neonate and Sexually Mature Reproductive Guinea Pig

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Abstract

Introduction: The existence of ovarian intrinsic neurons is well established. However, the morphology and chemical phenotype are not completely characterized and are even unknown for some species used in medical research. The purpose of this work was to determine the morphology and chemical phenotype of intrinsic neurons of the guinea pig ovary at two ages: neonates (0 days old) and sexually mature reproductive animals (90 days old). Materials and Methods: For the morphological analysis, we employed the modified Golgi-Cox impregnation technique. For the chemical phenotype, we used immunohistochemistry and the following antibodies; tyrosine hydroxylase (TH), calcitonin gene-related peptide (CGRP), transient receptor potential type 1 (TRPV1), neuron-specific nuclear protein (NeuN) and proto-oncogene product of the cFos gene (cFos). We also used enzyme histochemistry for NADPH-diaphorase detection. Results: The number of intrinsic neurons in the neonate ovary was low in comparison to the adult guinea pig ovary. The intrinsic neurons were located in the cortex and the ovarian medulla; some were isolated or clustered, forming ganglia, and others were interconnected and formed networks. The neurons were small, medium or large. In the cortex of neonate vs adult ovaries, the small and medium neurons comprised 23% vs 36% and 5.2% vs 11.6%, respectively. In the medulla, the percent of the same neurons was 10.1% vs 10.1% and 1.1% vs 2.2% in the neonate and adult, respectively. In both cortex and medulla < 1% were large neurons at two ages. Also, the neurons were rounded, fusiform or multipolar. In the cortex, they were 12.7% vs 20.9%, 14.9% vs 24.2% and 1.1% vs 3.0%, respectively. In the medulla, the percent of small vs medium neurons was 6% vs 7.1% and 4.1% vs 4.8% in the neonate and adult ovary, respectively, and <1% were large neurons at both ages. The chemical phenotypes were in the neonate and adult: TH/NeuN-positive neurons, 16.3% vs 26.5%; CGRP/NeuN, 13.5% vs 35.8%; TRPV1/NeuN, 10.2% vs 38.6%; and cFos/NeuN, 4.6% vs 5.4%, re-

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spectively. The percent of NADPHd-positive cells in the cortex was 9.5% vs 25.1% and 3.2% vs 62.2% in the medulla in the neonate and adult, respectively. Conclusion: Altogether, these data showed that the number of ovarian intrinsic neurons was low at birth and increased in the sexually mature reproductive guinea pig. The chemical phenotype was rich and peptidergic, catecholaminergic and nitrergic in nature and positive for cFos immunoreactivity. Therefore, intrinsic neurons can be chemical sensors inside of the gonad and transmit signal to the central nervous system.

Keywords

1. Introduction

It has been established that the mammalian ovary is supplied by sympathetic and sensory nerve fibers [1]-[3] and enclosed intrinsic neurons [4]-[8]. The sympathetic fibers predominantly release noradrenaline (NA) and vasoactive intestinal peptide (VIP), and the sensory fibers release peptides such as CGRP, substance P (SP) and galanin, among others [9]-[12]. Under physiological conditions, nerve fibers are involved in early follicular development and ovarian steroidogenesis [2] [13]-[15], *i.e.*, NA stimulates androgen and progesterone secretion [16] [17], VIP stimulates estrogen production [18] [19] and CGRP and SP are involved in ovarian vascular fluid [9] [12]. Ovarian intrinsic neurons have been studied in several mammals (rats, monkeys and humans) [5] [7] and birds (hen and emu) [18] [19] and modulate steroidogenesis and local vascular fluid [2] [6] [14]. In the rat ovary, intrinsic neurons are located in the hilum and medulla of the ovary, first appear in the cortex during the juvenile period [5], and increase significantly during prepubertal development, reaching maximum values before puberty [6]. Using specific antibodies, neuronal cell bodies were recognized in the adult ovary of primates that contain the low-affinity receptor for nerve growth factor (NGFR), and some intrinsic neurons also tested positive for tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis [4]. In addition, the catecholaminergic nature of these neurons was demonstrated by the detection of mRNAs encoding both tyrosine hydroxylase (TH) and dopamine β-hydroxylase in the perikarya [6] [7]. Moreover, the peptidergic nature of these neurons were tested by immunohistochemistry and shown to be neuropeptide Y (NPY) positive [6]. Employing histological and histochemical criteria, isolated neurons and ganglia have been visualized in the ovary of prepubertal adult Wistar rats [5]; however, they were absent in the ovaries of Sprague-Dawley rats [6]. Despite these advances in ovarian intrinsic innervation, the information on these issues is incomplete and even unknown in several species frequently used in medical research. To increase the knowledge on this matter, the present study was designed to examine the distribution, morphology and chemical phenotype of the guinea pig ovarian intrinsic neurons at two ages: neonates (0 days old) and adult animals (90 days old).

2. Materials and Methods

2.1. Animals and Samples

During the experimental part of this study, we followed the principles of animal care approved by the BUAP Animal Care Committee and the national laws on animal protection (Mexican Council for Animal Care, Norma Oficial Mexicana NOM-062-ZOO-1999). The local guidelines were also in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the USA. All efforts were made to minimize animal suffering and to reduce the number of animals used. The immature guinea pigs (*Caviaporcellus*) were obtained at birth from pregnant animals. The adult guinea pigs were housed in groups (3 animals per cage) under a dark-light cycle of 12 - 12 hours with a room temperature of 22 ± 2 degrees Celsius. Additionally, the animals had free access to food and potable water, which was supplemented with Vitamin C. We used thirty female guinea pigs, fifteen immature animals (at birth day, P0) and another fifteen sexually mature reproductive animals (90 days old, P90), randomly divided by ages into six groups (n = 5). The animals of the first and second group (P0 or P90, respectively) were used for ovary collection and modified Golgi-Cox impregnation. The ovaries of the animals of the third and fourth group were used for immunocytochemistry and evaluation of the co-expres-

sion of CGRP/NeuN-, TRPV1/NeuN-, TH/NeuN-, and cFos/NeuN-positive cells, and the ovaries of the animals of the fifth and sixth groups were used for enzyme histochemistry and evaluation of NADPHd-positive cells. All experimental guinea pigs were sacrificed with CO_2.

2.2. Morphological Evaluation of the Ovarian Intrinsic Neurons by the Golgi-Cox Impregnation Technique

The ovaries were treated by the modified Golgi-Cox technique [20] [21]. Previously, we tested different impregnation protocols (data not shown here) and the best results were obtained when the impregnation time was six weeks in the dark and a room temperature of 22 ± 2 degree Celsius. During this time, the impregnation solution was replaced with a fresh solution every seven days. When the impregnation time was complete, the ovaries were immersed in a 30% sucrose solution for one week and kept in the dark at room temperature. After that, the ovaries were serially sectioned at a thickness of 30 µm at a freezing temperature on a microtome Leica (VT1000). All sections were placed onto gelatin-coated glass slides and exposed to NH_4OH and fixed with Kodak solution (Kodak Co., USA). Subsequently, the histological sections were treated with alcohol and xylene and mounted on microscope slides with synthetic resin. The slides were stored in the dark before visualization under light microscopy.

2.3. Antibodies

The following antibodies were used: rabbit polyclonal and mouse monoclonal anti-CGRP (Calcitonin Gene-Related Peptide, Life Science); mouse monoclonal anti-TRPV1 receptor (Transient Receptor Potential Vanilloid type 1, Millipore); anti-TH (Tyrosine Hydroxylase, Abcam); rabbit polyclonal anti-cFos (Santa Cruz Biotechnology); rabbit polyclonal anti-FOX3/NeuN (FOX3, Abcam); and secondary antibodies FITC (IgG-free from goat, Millipore) and Texas Red (IgG-free from goat, Millipore).

2.4. Chemical Phenotype Evaluation of the Ovarian Intrinsic Neurons by Immunohistochemistry

The animals from each experimental group were sacrificed with CO_2 and perfused with intracardiac isotonic saline solution (NaCl, 0.9%) followed by paraformaldehyde (4%) in PBS, pH 7.4 (PF-PBS solution). The ovaries were removed and post-fixed in PF-PBS solution, embedded in paraffin and sectioned with a Leica SM2010R microtome (5 µm). The sections were treated with xylene and ethanol and washed with PBS solution. The sections were blocked with bovine albumin (IgG-free), treated with Triton X-100 and washed with PBS at 24 degrees Celsius. The samples were incubated with the anti-CGRP (1:500), anti-TRPV1 receptor (1:50), anti-TH (1:800), FOX3/NeuN (1:250) or cFos (1:500) antibody overnight at 4 degrees Celsius and then incubated with a secondary antibody (FITC) (IgG-free from goat; 1:250) or Texas Red (IgG-free from goat; 1:400) for 2 hours at room temperature. The specificity of the antibodies was confirmed in separate experiments with additional negative controls, including tissue sections incubated in the absence of primary antibody. All sections were mounted on microscope slides with mounting fluid (Millipore, USA) and observed under a Leica fluorescence microscope.

We analyzed ovary sections from five different animals with three repeat analyses for each gonad. The images were captured with a Leica-DFC325 camera, and the data were stored on the PC hardware. For five different ovaries, we counted the positive cells in 10 representative fields of each tissue section using the cell counter tool from NIH ImageJ software. From a qualitative point of view, the following three levels of fluorescence intensity were identified: light, medium and high. The cells were considered positive if they had a strong color signal and negative if they had a light or medium color signal. The final quality score was assessed using the software measure tool; the cells were positive when the measure was ≥50 arbitrary units, and the cells were negative when the measure was ≤49 arbitrary units.

2.5. Identification of NADPH-d Ovarian Intrinsic Neurons by Enzyme Histochemistry

For NADPH-d staining, whole mounts of the ovary serial sections (30 µm) through five ovary segments from different animals were used. The samples were incubated for one hour at 37 degrees Celsius with 1 mg/mL βNADPH-d (Sigma-Aldrich, USA), 0.2 mg/mL nitro-blue tetrazolium chloride (Sigma-Aldrich, USA), and 0.5% tri-

ton X-100 PBS in 10 mM malic acid. After incubation, the whole mounts were washed in 0.1 M PBS, and the sections were mounted with synthetic resin and examined by light microscope (Zeiss, Germany). In each experiment and for negative controls, we used samples with the same protocol in the absence of the βNADPH-d enzyme.

2.6. Samples and the Assessment of the Number of Neurons in the Ovaries

Given that the ovaries are different sizes because they were from animals at two different ages, immature (0 days old, P0) and sexual mature reproductive guinea pigs (90 days old, P90), we standardized the number of samples using 10 histological sections per ovary symmetrically distributed along the entire gonad. After that, the neuron bodies were identified using light microscopy in 5 fields for each histological section, and the images were captured with a Cannon S80, stored in PC hardware and analyzed with the software Zoom Browser program, EX.

2.7. Statistical Analysis

Data represent the number of neurons identified in 50 microscopy fields examined from 10 sections and 5 fields per section per ovary. All data are expressed as the mean and SEM and were compared using the Student t-test. A probability of less than 5% was considered significant.

3. Results

### 3.1. Amount, Location and Cell Body Size of the Ovarian Intrinsic Neurons Recognized by Golgi-Cox Impregnation

In neonate ovaries, the number of intrinsic neurons was low (10 ± 0.4) but increased 62% over neonates in sexually mature guinea pig ovaries (16.1 ± 0.5). These neurons were differentially distributed; in the cortex of the neonate ovary there were 7.7 ± 0.5 intrinsic neurons (28.9%), and in the cortex of the adult ovary there were 12.9 ± 0.4 neurons (48.5%) ($^{*}p < 0.05$, Student T-test). In the medulla of the neonate, the number of neurons was 2.8 ± 0.2 (10.5%), and in medulla of the adult ovary there were 3.2 ± 0.3 neurons (12.1%) which was not statically different from the neonate (Student t-test).

Using the size of the cell body, the ovarian intrinsic neurons were classified as small [7 - 15 µm (33.1% vs 46.3% for the neonate and adult, respectively)], medium [15 - 16 µm (6.2% vs 13.6% for the neonate and adult, respectively)] or large [26 - 40 µm < 1% at both ages], located both in the cortex and in medulla of the ovary. In the ovarian cortex, the number of small neurons in the neonate ovary was 6.2 (23%) and in the adult ovary the number was 9.7 (36%), 0.6 times higher than the neonate ovary (**Figure 1(A)**) ($^{*}p < 0.05$, Student t-test). The number of medium neurons was 1.4 (5.2%) and 3.1 (11.6%) in the neonate and adult, respectively, and the number of large neurons was <1% at both ages (**Figure 1(A)**), the number of neurons was not statistically significant (**Figure 1(B)**) (Student t-test). In the medulla of the ovaries, the number of neurons was similar in the neonate and adult: small neurons 2.7 (10.1%) and 2.7 (10.1%), respectively; medium neurons 0.3 (1.1%) and 0.6 (2.2%), respectively and the number of large neurons was <1% at both ages. There was no statistical significance between any of these values (**Figure 1(B)**) (Student t-test).

By describing the morphology of the cell body the ovarian neurons could be considered rounded, fusiform and multipolar (**Figure 2(A)**). The rounded and fusiform neurons exhibited two processes arising from opposite poles of the cell body, whereas multipolar neurons had a triangular or rounded cell body and multiple processes emerging from the cell body (**Figure 2(A)**). In the cortex of the neonate ovary, the number of rounded neurons was 3.4 ± 0.6 (12.7%), while in the adult ovary it was 5.6 ± 0.5 (20.9%), 0.6 times higher than the neonate ovary (**Figure 2(B)**) ($^{*}p < 0.05$, Student t-test). The number of fusiform neurons in the neonate and adult were 4.0 ± 0.6 (14.9%) and 6.5 ± 0.8 (24.2%), respectively; fusiform neurons were 0.6 times higher in the adult than in the neonate ovary (**Figure 2(B)**) ($^{*}p < 0.05$, Student-test). The number of multipolar neurons in the neonate and adult was 0.3 ± 0.1 (1.1%) and 0.8 ± 0.2 (3.0%), respectively, (**Figure 1(B)**) and no statistical significance was found between them (Student t-test). In the ovarian medulla and in comparison to the ovarian cortex, the number of neurons was lower and similar for the neonate and adult, rounded neurons 1.7 (6%) and 1.9 (7.1%), fusiform neurons, 1.1 (4.1%) and 1.3 (4.8%) and multipolar neurons 0.2 (0.7%) and 0.05 (0.2%), and no statistical significance was observed (**Figure 2(B)**) (Student t-test).
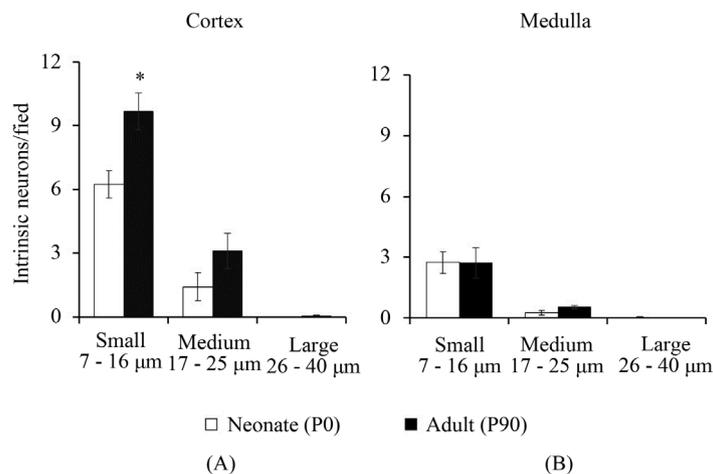
Figure 1. Distribution and cell body size of the intrinsic neurons in the guinea pig ovary. Graphic illustration of intrinsic neurons in the cortex (A) and medulla (B) of the neonate and adult ovary. The bars show mean and EEM, $^*p < 0.05$, Student t-test.
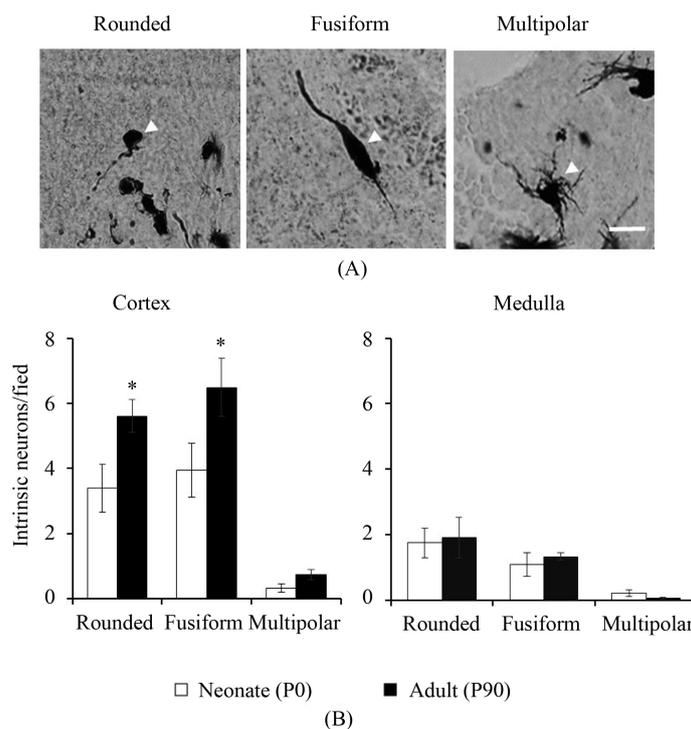

Figure 2. Morphology and distribution of intrinsic neurons in the guinea pig ovary visualized by Golgi-Cox impregnation. Rounded, fusiform and multipolar intrinsic neurons (A). Graphic illustration of intrinsic neurons via the morphology of the cell body in the cortex and medulla of the neonate and adult ovary (B). The bars show mean and EEM, $^*p < 0.05$, Student t-test. Scale bar, 20 µm.

In general, we found that fusiform and rounded neurons were isolated while other neurons came together forming compact ganglia (**Figures 3(A)-(D)**). The multipolar neurons were persistently placed in the ovarian cortex, and this observation reached statistical significance (**Figure 4(A)**). In addition, we recognized nerve fibers close to neurons, follicles or blood vessels (data not shown here).
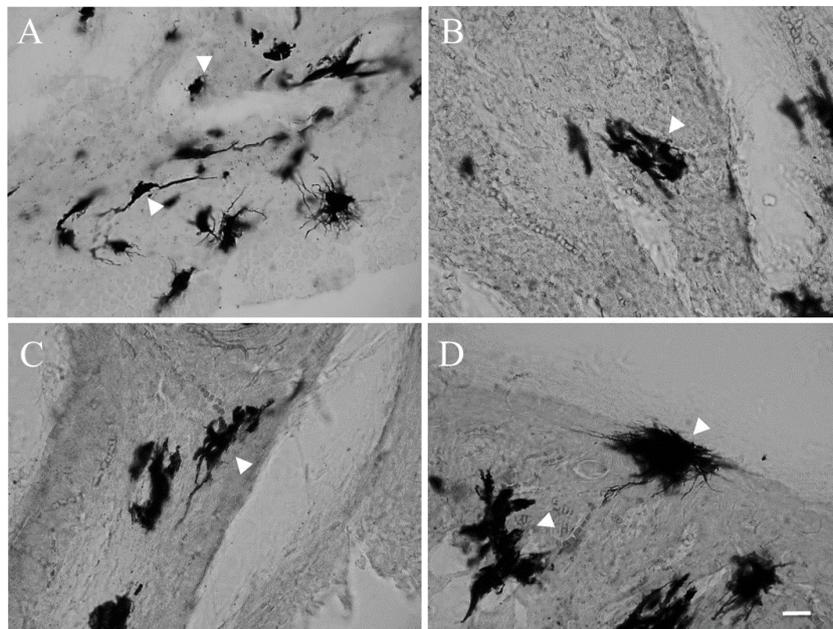
Figure 3. Neurons and ganglia in the adult guinea pig ovary visualized by Golgi-Cox impregnation. In the ganglia, the neurons had fusiform and rounded cell bodies. The neurons or ganglia are marked with arrowheads. Scale bar, 20 µm.
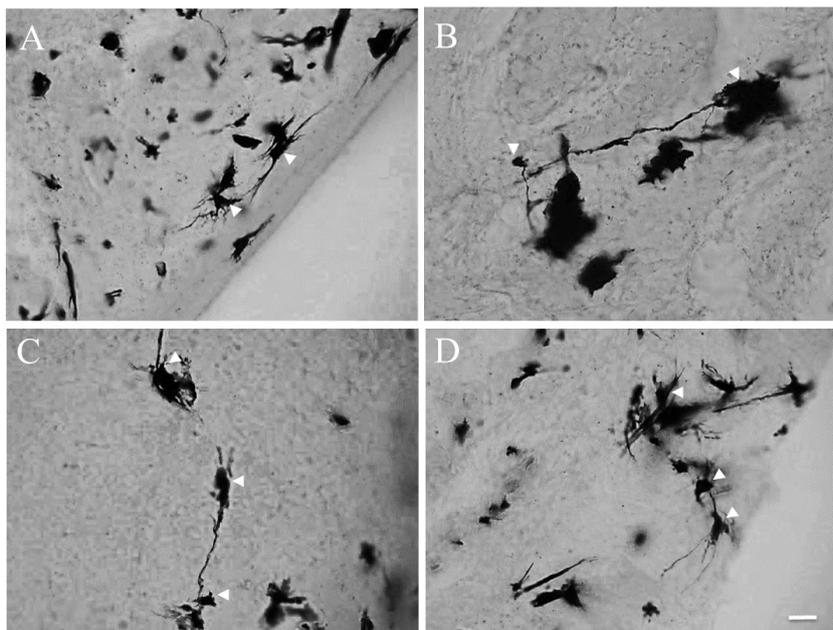
Figure 4. Neuron networks in the adult guinea pig ovary visualized by Golgi-Cox impregnation. The neuron networks were in the cortex and formed by bipolar or multipolar neurons. The cell bodies are marked with arrowheads. Scale bar, 20 µm.

3.2. Ovarian Intrinsic Neuronal Networks

Distinct from neurons in neonate ovaries, the adult guinea pig ovary had neurons making networks (**Figure 4**). The neuron networks were delimited to the cortex of the ovary and were structured by two neurons (**Figures 4(A)-(B)**), three neurons (**Figure 4(C)**) or several neurons (**Figure 4(D)**). The neuronal networks were primarily organized by multipolar cells. However, in some cases there were fusiform and rounded intrinsic neurons (**Figure 4(C)**).

3.3. Chemical Phenotype of the Ovarian Intrinsic Neurons

#### 3.3.1. Tyrosine Hydroxylase Positive Cells (TH-Positive Cells)

In the neonate ovary, the number of NeuN-positive cells, a specific marker for neurons, was 5.1 (23.7%), and in the adult ovary there were 7.2 (33.5%), 0.4 times higher than in the neonate ovary, whereas the number of neurons that co-expressed two neuronal markers (TH/NeuN-positive cells) was 3.5 (16.3%) and 5.7 (26.5%). In the adult ovary, the number of TH/NeuN-positive neurons increased 0.6 times in comparison to the neonate ovary and was statistically significant (**Figures 5(A)-(B)**) ($^{*}p < 0.05$, Student t-test).

3.3.2. Calcitonin Gene-Related Peptide Positive Cells (CGRP-Positive Cells)

In the neonate ovary, the number of NeuN-positive cells was 4.6 (14.1%), and in the adult guinea pig, it was 11.9 (36.6%). Therefore, the number of neurons that co-expressed two cell markers (CGRP/NeuN-positive cells) were 4.4 (13.5%) and 11.6 (35.8%), respectively, numbers that show an increase in neurons 1.6 times in comparison to neonate ovaries (**Figures 6(A)-(B)**) ($^{*}p < 0.05$, Student-test).

(A)   (B)
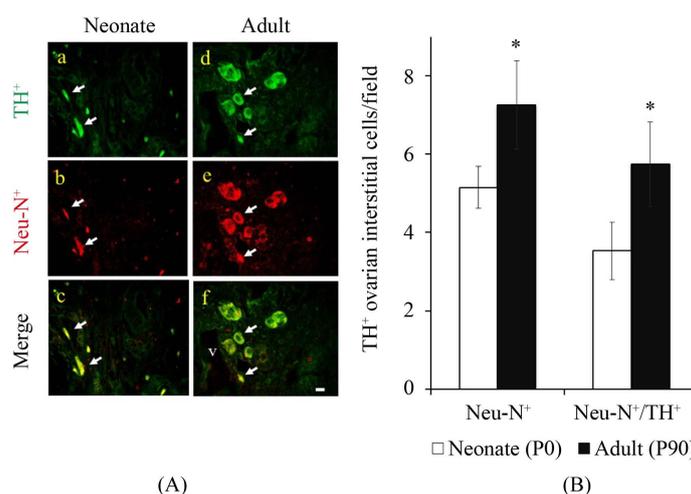
Figure 5. Immunohistochemistry of TH-positive neurons in the guinea pig ovary. The TH-positive neurons are in green marked with FITC for anti-TH ((A)a and (A)d), neuron bodies are in red, marked with Texas red and the monoclonal NeuN antibody (A(b) and A(e)) and merge ((A)c and A(f)), blood vessel (v). Graphic illustration of the number of intrinsic neurons in the neonate and adult guinea pig ovary (B). The bars show mean and EEM (n = 5), $^{*}p < 0.05$, Student t-test. Scale bar, 20 µm.

(A)   (B)
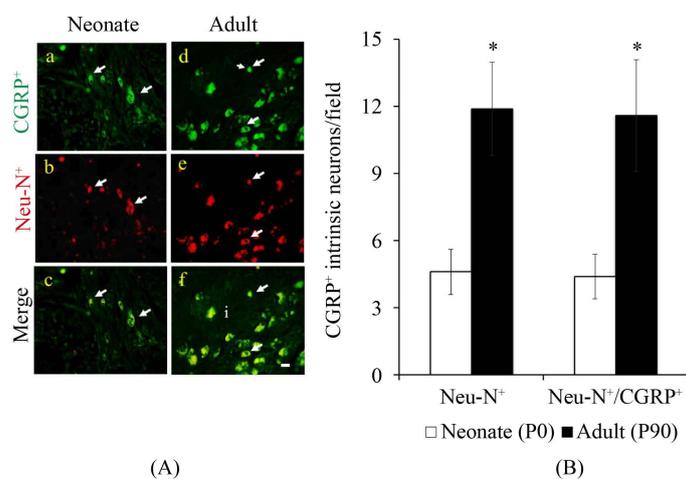
Figure 6. Immunohistochemistry of CGRP neurons in the guinea pig ovary. The CGRP-positive intrinsic neurons are in green marked with FITC for anti-CGRP ((A)a, A(d)), and the NeuN-positive neurons are in red marked with Texas red for the NeuN monoclonal antibody ((A)b, (A)d) and merge ((A)c, (A)f). Graphic illustration of the number of intrinsic neurons in the neonate and adult guinea pig ovary (B). The bars show mean and EEM (n = 5), $^{*}p < 0.05$, Student t-test. Scale bar, 20 µm.

3.3.3. Transient Receptor Potential Vanilloid Type 1-Positive Cells (TRPV1-Positive Cells)

In both the neonate and adult guinea pig ovaries, the NeuN-positive cells were 5 (11.6%) and 17.1 (39.6%), respectively. The number of neurons that co-expressed two cell markers (TRPV/NeuN-positive cells) was 4.4 (10.2%) in the neonate ovary and 16.7 (38.6%) in the adult guinea pig. In the adult, the number of TRPV1/NeuN-positive neurons increased 2.8 times in comparison to the neonate ovary (**Figures 7(A)-(B)**) ($^*p < 0.05$, Student t-test).
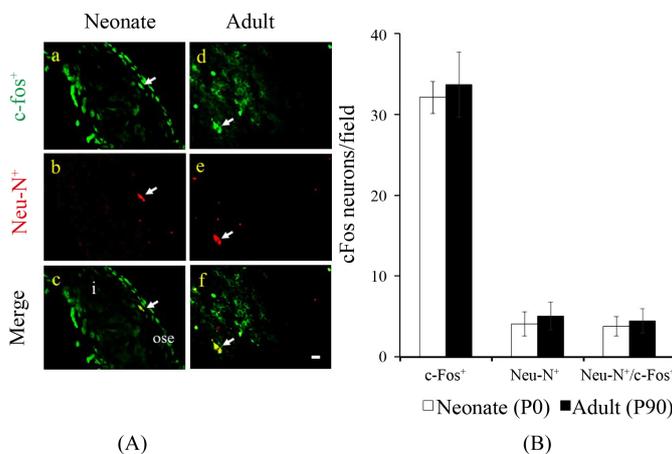
3.3.4. cFos Positive Cells

In both the neonate and adult ovary, the number of cFos-positive cells was similar, with 32.1 (38.6%) vs 33.6 (40.4%) positive cells, respectively. However, the number of NeuN-positive cells was 4.1 (4.9%) vs 5.1 (6.1%). Finally, the number of cells that co-expressed the two cell markers (NeuN/cFos) was 3.8 (4.6%) vs 4.5 (5.4%). No statistical significance was observed for any of the cases (**Figure 8(B)**) (Student t-test).

### 3.3.5. NADPHd Positive Cells

Neonate as adult guinea pig ovaries had NADPHd-positive neurons (**Figures 9(a)-(b)**). In the cortex there were
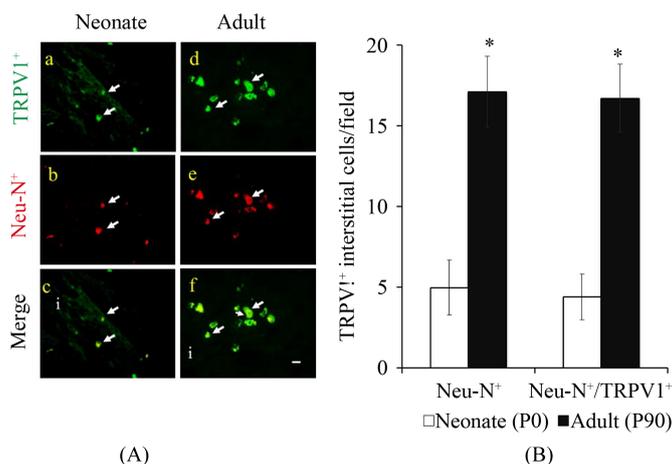
Figure 7. Immunohistochemistry of TRPV1-positive neurons in the guinea pig ovary. The TRPV1-positive intrinsic neurons are in green marked with FITC for the anti-TRPV1 receptor ((A)a and (A)d), and the NeuN-positive neurons are in red marked with Texas red for the NeuN monoclonal antibody ((A)b and (A)e) and merge ((A)c and (A)f). Graphic illustration of the number of ovarian intrinsic neurons (B). The bars show mean and EEM (n = 5), $^*p < 0.05$, Student t-test. Scale bar, 20 μm.

Figure 8. Immunohistochemistry for cFos/NeuN-positive cells (cFos$^+$) in the guinea pig ovary. The cFos+ cells are in green marked with FITC for anti-cFos ((A)a, (A)c). NeuN-positive neurons are in red ((A)b, (A)d) and merge ((A)c, (A)f), ovarian surface epithelium, ose. Graphic illustration of the number of ovarian intrinsic neurons (B). The bars show mean and EEM (n = 5), $^*p < 0.05$, Student t-test. Scale bar, 20 μm.
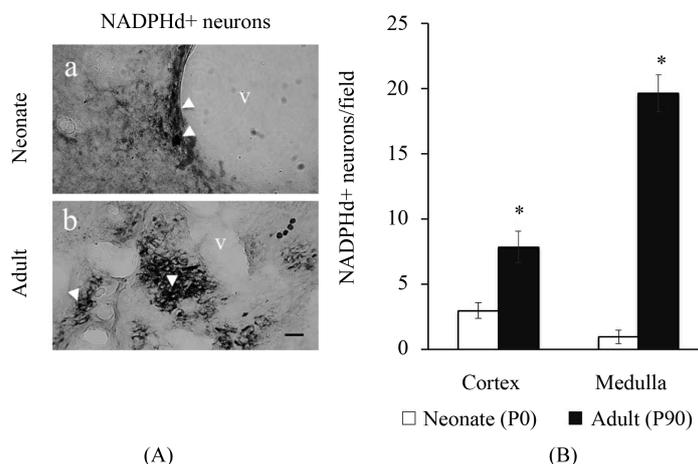
Figure 9. NADPHd labeled neurons in the guinea pig ovary. NADPHd positive neurons in the neonate (A)a and adult guinea pig ovary (A)b. Graphic illustration with the number of NADPHd neurons in the cortex and ovarian medulla (B). The bars show mean and EEM, $^*p < 0.05$, Student t-test. Scale bars, 20 µm.

3.0 (9.5%) and 7.9 (25.1%) NADPHd-positive neurons for the neonate and adult, respectively, and in the medulla there was 1.0 (3.2%) and 19.6 (62.2%) positive cells, respectively (**Figure 9(B)**) ($^*p \leq 0.5$ Student t-test).

4. Discussion

This is the first report to indicate the distribution, morphology and chemical phenotype of intrinsic neurons in the guinea pig ovary. The high number of neurons found in the adult ovary in comparison to the neonate ovary, and the higher amount in the cortex in comparison to the ovarian medulla adds to the information on ovarian intrinsic innervation in mammals. Additionally, these results are in concordance with other studies previously reported in rats [5] [6], monkeys and humans [7] [8] that reported ovarian intrinsic neurons increasing at sexual maturation [4] [8].

The small number of TH, CGRP and TRPV1 neurons in the neonate ovary (16.3%, 13.5% and 13.5%, respectively) that increased in the adult ovary (26.5%, 35.8% and 38.6%, respectively) correlates with the high rate of steroidogenesis and high vascular blood fluid displayed in sexually mature reproductive ovaries [2] [3] [17]. In the primate ovary, catecholaminergic neurons are developed at the onset of puberty and are modulated by the ovarian hormones [4] [8]. Specifically, the long-term estradiol treatment of adult gilts down-regulates the population of noradrenergic neurons in the sympathetic chain gangliaprojecting to the ovary of adult pigs [22] [23]. Additionally, in cystic ovaries induced by dexamethasone, there are decreased CGRP and SP nerve fibers in the vicinity of the blood vessels in the gilt cystic ovaries [22]. Although controversial, another possibility is neurogenesis *in situ*. This is because, *in vitro*, a mixture of testosterone and progesterone after estradiol pretreatment converts the majority of ovarian epithelial cells of the porcine ovary into neural stem cells thereby differentiating them to neuronal cells [24] [25]. Also, ovarian stem cells show a high degree of plasticity and are able to differentiate into various types of somatic cells, including neural stem cells [24]-[26]. Ovarian stem cells express several markers recognized as pluripotent markers (SSEA-4, OCT4, SOX-2, among others) or multipotent markers (M-CAM/CD146, Thy-1/CD90, STRO-1, among others), which show support for the neural differentiation linage [26]-[28]. However, this hypothesis needs to be tested.

A novel result reported herein was the identification of multipolar neurons in the ovarian cortex, cells with several processes emerging from round or oval cell bodies. The multipolar ovarian neuron morphology was similar to Dogiel type II neurons described in the small intestine of several species [29]-[31], also called intrinsic primary afferent neurons [29] [30]. This comparison is supported by their chemical phenotype because guinea pig ovarian intrinsic neurons tested positive in response to TRPV1/NeuN antibodies, similar to Dogiel type II neurons that express TRPV1 receptors [32]. In these neurons, CB1 antagonists augment CGRP release via stimulation of the TRPV1 receptor and enhancement of the peristaltic reflex [32].

The neuron networks occur in the adult ovary and not in the immature ovary of the guinea pig, suggesting that they are developed along with the maturation of the reproductive system. In the sexually mature ovary, neuron

molding is possible because long-term 17β-estradiol treatment reduces the population of neurons in the sympathetic chain ganglia connected to the ovary [33]. Furthermore, testosterone administration reduces the number of neurons in the caudal mesenteric ganglion innervating the ovary in sexually mature gilts [22] [23]. The high molding of the intrinsic neurons of the guinea pig ovary is supported by the widespread chemical phenotype expressed; TH/NeuN co-expression is indicative of catecholaminergic neurons [6] [8], CGRP/NeuN and TRPV1/NeuN co-expression are markers for sensory neurons [34] [35] and NADPHd positivity is suggestive of nitrergic neurons and nitric oxide production [36] [37]. In addition, the fact that intrinsic neurons of the guinea pig ovary co-express cFos/NeuN proteins and cFos is a marker for early transcriptional activity [38] supports the notion that these neurons are functionally active. Moreover, TRPV1 and CGRP peptides are characteristic of sensory neurons that are abundant in pelvic organs. The CGRP neurons have two or more long processes emerging from an oval or round cell body, a characteristic of the intestine intrinsic primary afferent neuron [32] [39]. Therefore, the inhibition of the CGRP intrinsic sensory neurons modifies physiological actions [32].

The neuron networks and chemical phenotype of the intrinsic neurons in the guinea pig ovary suggest an integrative role. The presence of TH-positive neurons are in agreement with numerous networks formed by catecholaminergic neurons in the primate ovary, which are highly developed at puberty [4] and remodeled by local conditions such as hypoxia [40] [41]. Our results are the first to demonstrate that TRPV1-positive intrinsic neurons increase in population in the sexually mature reproductive guinea pig ovary. In sensory nerve fibers, the stimulation of the TRPV1 receptor induces CGRP release and vasodilation [42] [43], protecting the visceral tissues during ischemic processes [42]. The CGRP released from nerve perivascular terminals causes relaxation of the ovarian arteryin the sexually mature pig [44] and produces hypotension during pregnancy in ovariectomized rats treated with estradiol and progesterone [44]. In guinea pig ovaries, TRPV1/NeuN- and CGRP/NeuN-positive neurons were predominantly present in the interstitial compartment of the ovarian cortex, a site with a high number of follicles at a different stage of the development of sexually mature ovaries. Previously, it has been reported that CGRP immunoreactive nerve fibers were distributed in the medullar stroma in close contact with blood vessels [45]. In addition, in the guinea pig ovary, nitrergic neurons, identified by NADPHd positivity, are indicative of NOS production, and agree with previous results reported in rat ovaries [46] and monkeys [8]. In the guinea pig, NADPHd-positive neurons were more abundant in the medulla and less abundant in the cortex, suggesting regional differences in oxide nitric production. A differential distribution of NADPHd neurons has been reported in other reproductive organs, such as the urethra and penis of the rat, and is related to the different innervation pattern [47]; this is also the case in the rat dorsal root ganglia (L4) where the number of NADPHd-positive neuronsincreased after treatment of the LPS-treated sciatic nerve [48].

5. Conclusion

The ovary contained round, fusiform and multipolar-shape neurons, isolated or grouped in ganglia, making networks in the adult ovary. The neurons had profuse chemical phenotypes (CGRP/NeuN-, TRPV1/NeuN-, TH/NeuN- and NADPHd-positive) and active considering its cFos immunoreactivity, supporting the notion that ovarian intrinsic neurons can be sensors in the gonad and transmit signals to the central nervous system.

Acknowledgements

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors read and approved the final manuscript.

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List of Abbreviations

Calcitonin Gene Relate Peptide (CGRP);
Transient Receptor Potential Vanilloid Type 1 (TRPV1);
Tyrosine Hydroxylase (TH);
NADPH-Diaphorase (NADPHd);
Proto-Oncogene Product of the cFos Gene (cFos);
Neuron-Specific Nuclear Protein (NeuN).