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# Neural Activity Between Ovaries and the Prevertebral Celiac–Superior Mesenteric Ganglia Varies During the Estrous Cycle of the Rat

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**The ovaries' innervation arrives via the superior ovarian nerve, which originates from the celiac ganglion. Using True Blue as an antidromic marker, the present study analyzed the changes in the anatomical relation between each ovary and the prevertebral celiac–superior mesenteric ganglia during the estrous cycle. The number of labeled neurons increased from the day of diestrus 1 to the day of proestrus. The largest number of labeled cells was observed when tracer was injected into the left ovary on proestrus. The number of labeled cells was significantly higher when the tracer was injected into the left ovary on proestrus than when it was done in the right one. When tracer was injected into the left ovary, the average labeled area of cells increased significantly from diestrus 1 to proestrus, and declined at estrus. In contrast, when True Blue was injected into the right ovary, the average labeled area was similar in diestrus 1 and diestrus 2, and the values increased in proestrus and estrus. The results indicate an apparent asymmetry in the activity of neural connections between ovaries and the prevertebral celiac–superior mesenteric ganglia, and that the number of active neurons of these connections varies during the estrous cycle.**

**Key Words:** Ovaries; asymmetry; prevertebral celiac–superior mesenteric ganglia; True Blue; neurons.

## Introduction

Evidence supporting the role played by ovarian innervations in regulating ovarian functions (follicular development, ovulation, and hormones secretion) has accumulated and improved significantly during the last three decades *(1–6)*. The ovaries' innervation arrives via the superior ovarian nerve (SON), the ovarian plexus, and the vagus nerve *(7–10)*. The SON and ovarian plexus originate from the prevertebral celiac–superior mesenteric ganglia and lumbar superior splacnic nerve *(8,11,12)*.

Most of the fibers innervating the ovaries arise from the celiac ganglion, one of the main neural components controlling information on reproductive tract functions *(11)*. Two distinct classes of neurons have been described in the celiac ganglion: (1) principal neurons, characterized by their large size and norepinephrine granules, and (2) small intense fluorescent (SIF) neurons *(13)*.

In the rat, the development of the dendritic tree of pre- and postganglionic sympathetic neurons in the intermediumlateral region occurs during the first week of age and their function is similar to that of adult animals *(14–16)*.

According to Klein and Burden *(8)*, there is no difference in the number of afferent sensory neurons between the left and right sides of the pre-vertebral celiac-superior mesenteric ganglia of adult rats. On previous studies we showed that sectioning of the vagus nerve, unilaterally or bilaterally, results in fewer ova shed and in a drop of ovulation rates; and that these effects depend on both the day of the cycle when surgery was performed and on which vagus nerve was sectioned *(17,18)*. Chávez and Domínguez *(19)* showed that sectioning the SON of the left ovary results in a decrease of the number of ova shed by the left ovary and an increase in the number of ova shed by the innervated ovary (right ovary). Such differences were not observed when the right SON was sectioned.

Evidence suggesting that the ovarian innervation has considerable plasticity during the estrous cycle and pregnancy has been published, such as results indicating that peripheral nerves' activity varies along the estrous cycle *(20, 21)* and that the expression of estrogen receptors depends on estrogen plasma levels *(22,23)*. There is evidence that most of the paired endocrine organs present asymmetry. In the ovaries, asymmetry reaches its maximum expression in birds, where only the left ovary develops. In mammals, differences in ovarian performance can be observed in the bat, where ovulation occurs only in the right ovary, or in white-

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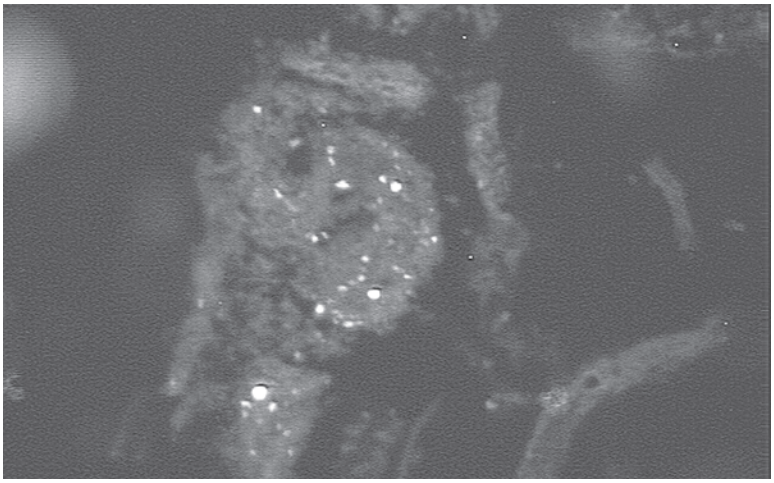

**Fig. 1.** Small ganglion localized in the angle between the aorta and renal arteries of animals injected with True Blue on P, and sacrificed 4 d after treatment. The individual markers correspond to positive cells for the fluorescent tracer (10×).

toothed shrews, where the left ovary plays a dominant role over the right one. In humans, the right ovary receives more innervation than the left one, the incidence of endometriotic ovarian cysts is significantly higher in the left than in the right ovary, and the incidence of rhabdomyosarcoma is significantly higher in the right than in the left ovary *(24)*.

Asymmetry in paired endocrine organs is also found in golden hamsters, where on d 9 of pregnancy, the number of corpora lutea in the right ovary was greater than that in the left. In mice the right ovary produces more eggs than the left, and this relation is reversed in iv/iv *situs inversus* animal. In the rat, the left ovary releases an average of six ova, while the right one releases only four. Furthermore, gerbil females that had the position of their ovaries exchanged gestated more male fetuses in the left uterine horns than in the right. These data support the hypotheses that lateral asymmetries exist in the gerbil ovaries rather than in gerbil uterine horns *(24)*.

Taken together, the asymmetric characteristics of the reproductive system described above cannot be explained only by endocrine mechanisms and, thus, we postulate that the ovarian innervation has a series of regulatory circuits; that the presence of these circuits are reflected in the autonomous ganglion cells; and that in the adult animal, the number of functional celiac ganglion cells related to the ovaries varies according to both the day of the estrous cycle and the ovary they are connected to.

The aim of the present study was to analyze the changes in the anatomical relation between each ovary and the prevertebral celiac–superior mesenteric ganglia along the estrous cycle. For this purpose, adult female rats were injected with the fluorescent retrograde tracer, True Blue, directly into the ovarian bursa, to localize postganglionic perikarya projecting to the ovaries.

## Results

True Blue positive neurons were localized in the prevertebral celiac–superior mesenteric ganglia and in a small ganglion localized in the angle between the aorta and the renal arteries (Fig. 1). Most of the labeled cells were present in the prevertebral celiac–superior mesenteric ganglia.

When tracer was injected into the right ovary, labeled neurons were observed only in the ipsilateral prevertebral celiac–superior mesenteric ganglia (Fig. 2). In turn, when True Blue was injected into the left ovary, labeled neurons were observed in the ipsilateral and contralateral prevertebral celiac-superior mesenteric ganglia.

- Experiment 1. When True Blue was injected into the right or left ovary on P and sacrificed, after two consecutive estrous cycles, on estrus day, animals injected in the left ovary showed higher numbers of True Blue–labeled cells than animals injected in the right ovary (65 ± 3.1 vs 38 ± 4.7, $p < 0.05$ Student's $t$ test). Such differences were not observed when the animals were treated on E and sacrificed after two consecutive estrous cycles, on E (64 ± 6.3 vs 50 ± 5.7, nonsignificant).
- Experiment 2. Animals injected with True Blue into the ovarian bursa of the right or left ovary, on each day of the estrous cycle, sacrificed on the following cycle the same day of the treatment, the number of labeled cells in the prevertebral celiac–superior mesenteric ganglia varied according to both the ovary treated and the day of the estrous cycle when the tracer was injected.

The number of labeled neurons increased from D 1 to P, with a small drop in animals injected on E. The highest number of labeled cells was observed when tracer was injected into the left ovary on P. A significant difference in the number or labeled cells was observed between the right and left ovaries of animals injected on P (Fig. 3).

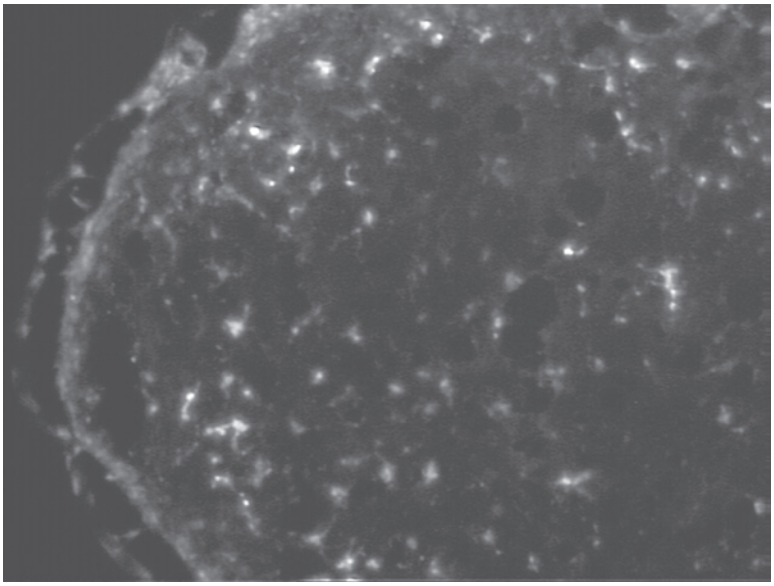

**Fig. 2.** Left prevertebral celiac–superior mesenteric ganglia in animals injected with True Blue on E and sacrificed 4 d after treatment (10×).

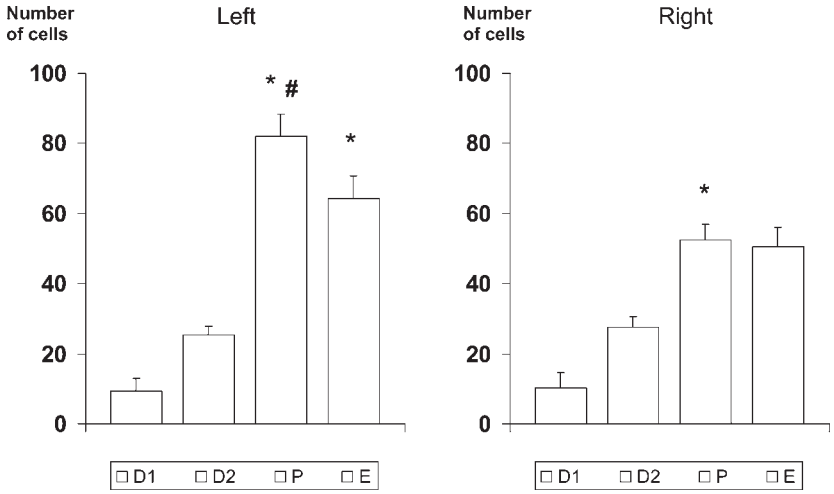


**Fig. 3.** Mean ± SEM of the number of labeled cells in the prevertebral celiac–superior mesenteric ganglia of animals injected with True Blue on D1, D2, P, or E, and sacrificed 4 d after True Blue treatment. $^{*}p < 0.05$ vs diestrus days, $^{\#}p < 0.05$ vs right side in the same day.

The total labeled area extent in the prevertebral celiac–superior mesenteric ganglia cells presented asymmetry and varied along the estrous cycle. When tracer was injected into the left ovary, the extent of labeled area increased significantly from D 1 to P, and declined on E. In contrast, when True Blue was injected into the right ovary, the extent of labeled area was similar in D 1 and D 2, and increased in P and E (Fig. 4).

The mean surface area of each labeled neuron was also different between the right and the left side of the prevertebral celiac-superior mesenteric ganglia, and the changes on this parameter along the estrous cycle presented a mirror image (Fig. 5).

## Discussion

The results obtained in the present study indicate an apparent asymmetry in the neural connections between ovaries and the prevertebral celiac–superior mesenteric ganglia, and suggests that the number of active connections varies along the estrous cycle.

In a previous study, we showed that ovulation rates of cyclic rats with unilateral sectioning of the SON depend on which ovary was denervated *(19)*. The largest difference in ovulation rates, between the right and left ovary, was observed in animals with the SON sectioned on D2. Similarly, compensatory ovulation and compensatory ovarian hypertrophy by the left and right ovaries are different, and these

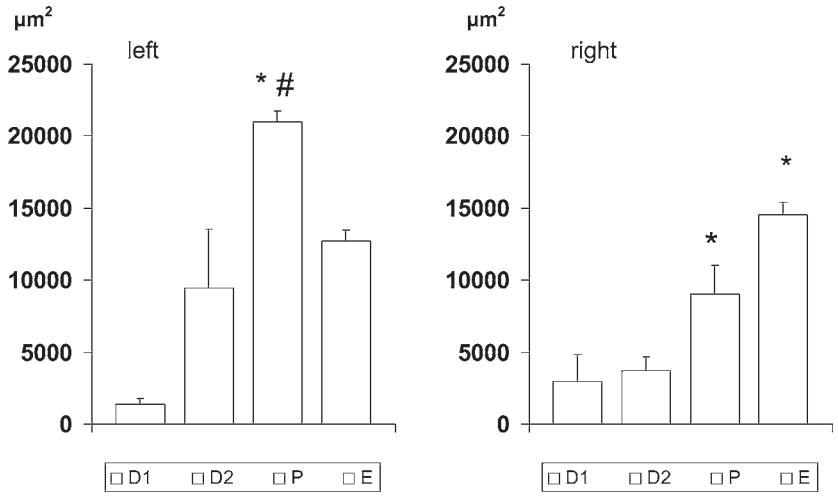


**Fig. 4.** Mean ± SEM of labeled cellular area surface (µm$^2$) in the prevertebral celiac–superior mesenteric ganglia of animals injected with True Blue on D1, D2, P, or E and sacrificed 4 d after True Blue treatment. *$p < 0.05$ vs diestrus days, $^{\#}p < 0.05$ vs right side in the same day.

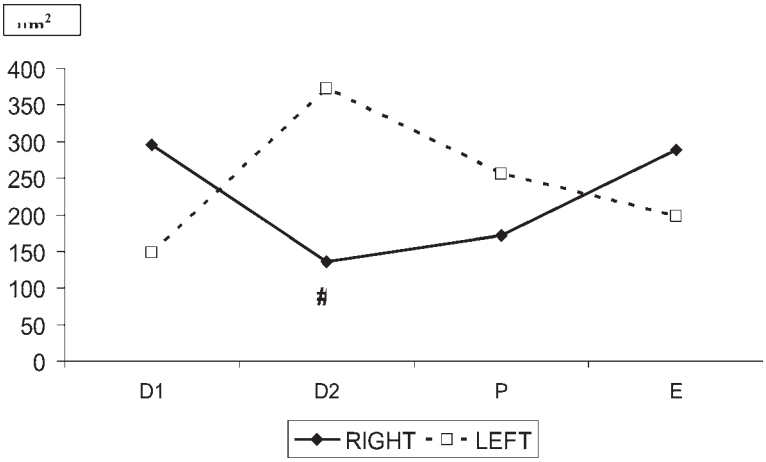


**Fig. 5.** Mean of the neuron surface (total surface area/number of neurons labeled) in the prevertebral celiac-superior mesenteric ganglia of rats injected with True Blue on D1, D2, P, or E, and sacrificed 4 d after treatment. $^{\#}p < 0.05$ vs right side.

differences depend on the day of the estrous cycle when hemiovariectomy was performed *(19)*.

Such differences have been interpreted as indicating asymmetry between the ovaries regulating systems and on the way they interact with their ovarian innervation. The difference in the number of labeled neurons between the right and left ovaries along the estrous cycle, as well as the changes in the neurons' labeled area observed in this study, suggest that the number of active neurons in the celiac ganglion varies along the estrous cycle. Such changes could be related to oscillating estradiol plasma levels along the estrous cycle and to changes in the neurons' ability to be stimulated by estrogens. If this is the correct explanation, injecting estradiol to rats in D1 must result in an increase in the number of labeled neurons. Another possibility is that the production of estrogen by the left and right ovaries is different, and thus affects the intrinsic ovarian neurons and neural fibers at differential strengths.

Previously, we postulated that the ovarian innervation modulates the response of the follicles and the interstitial gland to gonadotropins via the release of neurotransmitters and by acting on the ovary's neurons. The loop is closed when some of the neurons on the ovary register changes in several parameters of the ovarian function, and such changes are registered through modifications in the release of local molecules. One ovary sends a signal to the other ovary via the innervation arising in the neurons present in the ovary, traveling through the intermediolateral column, via sympathetic nerves, and arriving at the other ovary via the superior ovarian nerve. The ovary also sends neural signals to the central nervous system via the vagus nerve, which works as an efferent pathway from the ovary to the central nervous system.

According to Papka et al. *(26)* the neurons' connections in female reproductive organs have estrogen receptors. The observed bilateral labeling in the celiac ganglion when the

tracer was injected into the left ovary supports the idea of a neural communication between the ovaries *(6,11,26–29)*. Such communication could partially explain the differences observed in the right and left ovaries response to peripheral denervation.

According to Brauer et al. *(29,30)* and Zoubina et al. *(26)*, along the estrous cycle the uterus presents changes on its peripheral innervation, density of axon terminals, and activity of dopamine β hydroxylase.

Taken together, the present results add structural bases for the previously postulated asymmetry between the ovaries and its relation to ovarian innervation *(31,32)*.

## Materials and Methods

The study was conducted with virgin adult female rats of the CIIZ-V strain from our own stock. Animals were kept under controlled lighting conditions (lights on from 05:00 to 19:00 h), with free access to food (Purina S.A., Mexico) and tap water, following NIH Guide parameters for care of laboratory animals. The Neuroethology PhD Committee of the Universidad Veracruzana at Xalapa and the FES Zaragoza approved the protocols of this study.

Estrous cycles were monitored by daily vaginal smears. Only rats showing at least two consecutive 4-d cycles were used in the experiment. All surgeries were performed under ether anesthesia, between 13:00–13.15 h on diestrus 1 (D1), diestrus 2 (D2), proestrus (P), or estrus (E). Rats were randomly allotted to one of the following experimental group (four rats by group):

- *Experiment 1*. Groups of rats were injected with the fluorescent tracer on either the day of proestrus or estrus. Animals were sacrificed on the day of estrus, two cycles after treatment.
- *Experiment 2*. In order to analyze if the difference in the number of labeled cells depends on the day of the estrous cycle when fluorescent tracer treatment was performed, groups of rats were injected on each day of the estrous cycle (D1, D2, P, and E) and sacrificed the following estrous cycle on the same day of treatment, i.e., 4 d after True Blue treatment.

### *Injection of the Fluorescent Retrograde Tracer True Blue into the Ovaries*

A unilateral incision was performed 2 cm below the last rib, affecting skin, muscle, and peritoneum. The left or right ovary was exposed and 5–8 µL of True Blue (Sigma, St. Louis, MO, USA) solution at 4%, diluted in distilled water, was injected into the ovarian bursa, following a previously described methodology *(33)*. The needle was kept in the bursa for 10 min after injection treatment to prevent the leakage of the tracer. Subsequently, the ovary was carefully cleaned, dried, and returned to the abdominal cavity.

### *Autopsy Procedures*

The rats were anesthetized with sodium pentobarbital (40 mg/kg body weight), laparotomized, and intracardiac perfused with 250 mL of cold saline solution, followed by the injection of 150 mL solution of 4% paraformaldehyde in phosphate buffer at pH 7.3.

After perfusion of the fixative solution, the prevertebral celiac–superior mesenteric ganglia, ovaries, and uterus were dissected and kept in the fixative solution overnight (approx 18 h). The ganglia were cryoprotected in sucrose solutions of increased concentration, and washed with a phosphate buffer afterward. The following day, each ganglion was embedded in Cryo-embedding compound (MICROM International GmbH). Twenty micrometer sections of tissue were cut serially with the aid of a cryostat kept at −20°C, and placed onto clean microscope glass slides. All sections were viewed with a Carl Zeiss Axioplan II microscope equipped with a vertical fluorescence illuminator and a 340–380 nm excitation filter for True Blue visualization. The tracer only marks nervous fibers, and, thus, only principal neurons were labeled with True Blue; small fluorescent neurons were not labeled with the antidromic tracer.

The possibility that True Blue leaked into the abdominal cavity was assessed by exposing the cavity to a fluorescent light. Only animals where fluorescence in the abdominal cavity was absent were included in the study. Eight animals were discarded.

### *Image Analysis*

Ten to fifteen images of the right or left celiac ganglia, from the ovaries of animals injected with True Blue, were used to count the number of positively labeled cells. Positive True Blue cells are defined as cells in which fluorescence is present when the sections were exposed to UV light.

The pictures were obtained with a Digital Camera (Optronics 60300, USA) and analyzed with a KS-300 Imaging System 3.0 (Carl Zeiss Vision GmbH, Germany). The imaging system was programmed to generate binary regions, automatically measure the stained areas (µm$^2$), count and integrate them, and produce a single fluorescent stain measurement value per cell region.

### *Statistical Analysis*

The mean number of True Blue positive cells and the mean fluorescent area of all the positive cells were analyzed by a multivariate analysis of variance (MANOVA), followed by Tukey's test. A $p < 0.05$ was assumed as significant.

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