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Innervation of amphibian reproductive system. Histological and ultrastructural studies

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ABSTRACT

In the present study we describe for the first time in anuran amphibians the histological and ultrastructural characteristics of innervation in the female reproductive organs. The observations in *Rhinella arenarum* revealed the presence of nerve fibers located predominantly in the ovarian hilium and in the oviduct wall. In both organs the nerves fibers are placed near blood vessels and smooth muscles fibers. In the present study the histological observations were confirmed using antibodies against peripherin and neurofilament 200 proteins. Ultrastructural analyses demonstrated that the innervation of the reproductive organs is constituted by unmyelinated nerve fibers surrounded by Schwann cells. Axon terminals contain a population of small, clear, translucent vesicles that coexist with a few dense cored vesicles.
The ultrastructural characteristics together with the immunopositive reaction to tyrosine hydroxylase of the nerve fibers and the type of synaptic vesicles present in the axon terminal would indicate that the reproductive organs of *R. arenarum* females are innervated by the sympathetic division of the autonomic nervous system.

1. Introduction

The reproductive system of adult amphibian females comprises ovaries and oviducts (Uribe Aranzabal, 2011). Ovaries lie on both sides of the midline of the body cavity and are attached to the kidneys by the mesentery (Fig. 1), the site where blood vessels run (Rasar and Hammes, 2006; Uribe Aranzabal, 2011). Ovaries are constituted by multiple lobes, each lobe having a cortical zone with follicles at different stages of folliculogenesis and a hollow medulla (Dumont and Brummett, 1978; Lofts, 1974). The size of the gonad varies according to the stage of oogenesis and reaches its maximum level during the preovulatory period, which is characterized by the predominance of fully grown oocytes.

After ovulation, oocytes accumulate in the coelomic cavity; then they enter the oviduct and pass through it to be finally released in the external medium during oviposition. With the exception of its initial (pars recta) and final (ovisac or uterus) zones, the rest of the oviduct, called pars convoluta (PC), is characterized by numerous convolutions (Uribe Aranzabal, 2011; Winik et al., 1999), thus remarkably increasing its length (Fig. 1). The oviductal wall is made up of three layers arranged concentrically. The innermost layer is composed of epithelial cells that secrete toward the duct lumen the components that form jelly coats surrounding the oocytes at the moment of deposition. These components (glycoproteins, proteins, cations) are indispensable for fertilization (Crespo et al., 2009, 2012; Medina et al., 2000; Winik et al., 1999). The next layer, made up of smooth muscle, is surrounded by a serous envelope or peritoneum that lines the outer surface. The peritoneum becomes continuous with the mesentery, which attaches the oviduct to the coelomic wall and where the blood vessels that provide irrigation to the organ are found (Moreno, 1972).

In most amphibian species reproductive activity is cyclic and seasonal. In the cycle two periods can be identified: A — a breeding period and B — a post reproductive period. During the breeding period, in the spring–summer months, both ovary and oviduct reach their maximum growth and functionality (Hedrick and Nishihara, 1991; Valdez Toledo and Pisanó, 1980; Winik et al., 1999). In fact, the most important events involved in reproduction such as oocyte maturation, ovulation and oviposition and oviductal synthesis and secretion take place during this period, in which environmental conditions, especially humidity, temperature, photoperiod and food availability (Paniagua et al., 1990) are optimal for the survival of the new individuals (Fernández and Ramos, 2003; Rastogi et al., 2002). The post-reproductive period, during the fall–winter months, is characterized by the recovery of the organs involved in reproduction (Fernández and Ramos, 2003).

It is known that numerous physiological processes of the organism are sensitive to the variations of external factors. In fact, in this species

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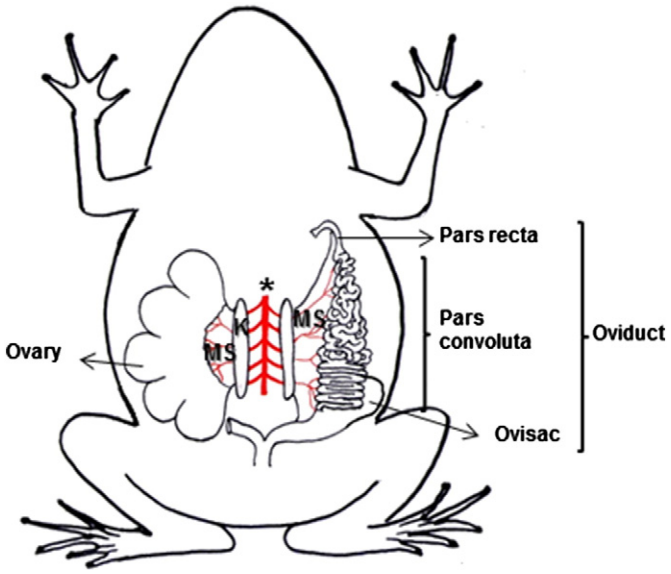

Fig. 1. Diagram of the anatomical organization of *Rhinella arenarum* reproductive system. The position of the ovary is shown on one side of the body axis and the arrangement of the oviduct on the other. Kidney (K), mesentery (MS), urogenital vessels (*).

the environmental variations have a remarkable incidence on the determination of the periods of the reproductive cycle (Fernández and Ramos, 2003). Bearing this in mind, although it is well known that the reproductive function of amphibians is under the hormonal control of the hypothalamic–hypophyseal–gonadal axis (Kim et al., 1998; Polzonetti-Magni et al., 1998), the influence of the neural control on the reproductive activity cannot be ignored.

In this sense, it has been postulated that in vertebrates such as birds and mammals with seasonal reproduction the nervous system would act as a receptor and transducer of environmental stimuli, modulating and regulating the activity of the hypothalamic–hypophyseal–gonadal axis (Bellingham and Foster, 2002; Ubuka et al., 2013).

A relevant aspect to be considered in the study of neural control is the morpho-functional characteristics of the innervation of the effector organs. In this regard, only in birds and mammals is there a wide and detailed description of the innervation of the reproductive system, it having been demonstrated that both the ovary and the oviduct receive autonomic innervation with predominance of the sympathetic division (Czaja et al., 2001; Gerendai et al., 2005; Liu et al., 2007; Spicer, 1986; Sporrong et al., 1991). The adrenergic nerve terminals are located mainly near the blood vessels and smooth muscle fibers in the gonadal stroma and on the oviductal wall (Gerendai et al., 2005; Spicer, 1986; Sporrong et al., 1991). With respect to neurotransmitters, in birds and mammals, catecholamines have been implicated in the regulatory action on the vasomotor control of blood vessels as well as in the modulatory effect of steroidogenic activity (Aguado and Ojeda, 1986; Gerendai et al., 2005; Müller-Marschhausen et al., 1988) and in the control of oviductal secretion (Einspanier et al., 1999). In addition, the presence of intragonadal catecholamine-producing neurons has been described in the ovary of some mammals (Dees et al., 2006). Moreover, it was also reported that other neurotransmitters such as neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), ATP and galanin are also present in the fibers that innervate the urogenital tract in most vertebrate classes (Jobling, 2011).

However, it is important to note that up to now there are no data concerning either the innervation or the importance of neural control in the reproductive function of anuran amphibians.

On the basis of these data, the aim of this work was to determine and analyze the innervation of the ovary and oviduct of *Rhinella arenarum* from the histological and ultrastructural points of view.

2. Materials and methods

2.1. Animals

Sexually mature adult females of *R. arenarum* were collected in the neighborhood of San Miguel de Tucumán, Argentina, during the reproductive (N = 5) and post-reproductive (N = 5) periods of the sexual cycle. The specimens were used immediately after capture or kept in captivity for short periods (2–3 days) at appropriate temperature and humidity.

Animal maintenance and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (European Communities Council Directive, 1986).

2.2. Obtainment of histological samples

Ovaries and oviducts were obtained from ether-anesthetized specimens that were then sacrificed by pithing.

For the histological analysis of innervation at the ovarian level, gonads were carefully washed in amphibian Ringer's solution, pH 7.4 (113.0 mM NaCl, 1.3 mM $CaCl_2$, 2.0 mM KCl) and then fixed in Ancel and Vitemberger (10% formaldehyde, 0.5% acetic acid and 0.5% NaCl) for 48 h at 4 °C. Since oocytes present high yolk contents, samples were treated with celloidin for 60 min at 25 °C and then incubated with chloroform for 30 min, before their embedding in Paraplast® according to the technique of Manes and Nieto (1983).

For the study of the oviduct, samples of the organ were removed at the level of the pars convoluta (PC) and of the ovisac or uterus, washed in amphibian Ringer's solution and fixed in 10% formol in phosphate buffer saline (PBS) 0.1 M, pH 7.4 for 48 h at 4 °C.

Then they were dehydrated through an ascending ethanol series (70%, 95%, 100%) for 1 h each and finally embedded in Paraplast®.

The transverse histological slices, obtained with a Leitz 1208 microtome, were 5–6 μm thick. The preparations were then deparaffinized with xylene.

For immunohistochemical detection of tyrosine hydroxylase (TH) ovary and oviduct samples were fixed in 4% in PBS 0.1 M, pH 7.4 for 48 h at 4 °C, washed in the same buffer, and then transferred to 30% solutions of sucrose in PBS for 12 h at 4 °C. Then the samples were embedded in Bright Cryo M Bed compound (Bright Instrument Company Limited, England). 6-μ-thick cryosectioned slices were obtained with a Bright Clinicut 3020 cryostat.

All sections obtained (15 per animal/per structure) for histological or immunohistochemical studies were hydrated first with a decreasing ethanol series (100%, 95%, 70%, 50%) for 1 min each and finally with PBS 0.1 M, pH 7.4 for 2 min.

2.3. Histological and immunohistochemical studies

Sections of both organs under study were stained either with Ehrlich's hematoxylin–eosin solution or with methylene blue for 1 min, dehydrated with progressively increasing concentrations of ethanol and mounted in Canada balsam.

Histological sections of ovary and oviduct were subjected to the indirect immunohistochemical technique developed with extravidin–biotin that amplifies the reaction of the antigen–antibody (Ag–Ab) interaction (Vacca, 1982). Unless indicated otherwise, the whole technique was carried out at room temperature.

In order to eliminate endogenous peroxidase activity the samples were incubated with 0.3% hydrogen peroxide and 10% methanol in PBS for 30 min. Then they were washed twice in PBS for 10 min and incubated for 40 min with 0.1 M bovine serum albumin (BSA) in PBS/Tween 20 to block non-specific sites.

After washing two times with PBS for 10 min sections were incubated overnight in a humid chamber at 4 °C with primary antibodies against: neurofilament 200 (N4142, rabbit polyclonal, Sigma, St Louis,