Immunohistochemical profile of some neurotransmitters and neurotrophins in the seminiferous tubules of rats treated by lonidamine

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Lonidamine (LND) or [1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid] is an anticancer and antispermatogenic drug that exerts a large number of effects on tumor cells and germ cells. Sexually mature male Sprague-Dawley rats were housed at 22°C on a 12-h light/12-h dark cycle 1 week before the experiments, with free access to food and water. LND was suspended in 0.5% methylcellulose at a concentration of 10 mg/mL and administered orally at the dose of 10 mL/kg (b.w.) as a single dose. Control rats received an equal amount of vehicle. Testes were removed, fixed for 24 h in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.2 at 22°C), rinsed with the same buffer, and stored at room temperature. From each sample, a block of tissue was removed by sectioning through the organ. After dehydration in ethanol at increasing concentrations (70-100%), each block was embedded in paraffin and serial 5 µm thick sections were cut using a rotatory microtome. The immunoreactivity for NTs has been observed in spermatogonia of untreated rats, while the rats treated with LND showed an immunohistochemical localization in all the stages of germinal cells. The generally well-expressed immunoreactivity for the neurotrophins receptors in treated rats observed in our study is presumably attributable to alterations of the receptors' structure and/or expression leading to changes of the activity, affinity, localization or protein interactions that may depend on sensitization of ion channels (induced by LND). Neurotrophins (NTs) appear to be interesting proteins for the modulation of sperm maturation and motility with a prominent role for the nerve growth factor (NGF), that may exert an autocrine or paracrine role. We therefore investigated the location and distribution of immunoreactivity for some neurotransmitters (SP, VIP, CGRP, nNOS, Chat), neurotrophins (NGF, BDNF, NT-3) and their own receptors (TrKA, TrKB, TrKC, p75) in the seminiferous tubules of male rats treated by LND in the light of the literature on this topic.

Key words: neurotransmitters, neurotrophins (NTs), Seminiferous tubules, immunohistochemistry, rat, lonidamine.

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Neurotrophins, also known as neurotrophic factors, constitute a family of dimeric proteins working as polypeptidic growth factors and acting like extracellular ligands (Barbacid 1995). NTs, including nerve growth factor (NGF - discovered by Levi-Montalcini half a century ago, 1952), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and others, are involved in vertebrate neuronal cell development, differentiation, survival and functional activities (Lewing 1996). The biological role of NTs is diverse and they are expressed in neurons during development and adulthood. Neurotrophins are also involved in the modulation of adult central nervous system functions and organization, as well as in the neural control of different activities related to the vegetative innervation of several organs (Kaplan 1997; Canning 2002; Tessarollo 1998; Kannan 1996; Oelmann 1995; Kerschensteiner 1999; Moalem 2000). Recent observations have stressed the importance of neurotrophins in the reproductive organs of adult male rats (ChunMei 2005); therefore morphological and immunohistochemical analysis suggests a role of NGF/TrkA/P75 system in the physyiology and reproduction of rat testicles (Levanti 2006). On the other hand, scarce information is available concerning the simultaneous immunohistochemical detection of neurotrophins and neurotransmitters in the normal rat seminiferous tubules as well as in the tubules of rats treated by LND. The choice of LND is justified by the fact that this drug has a relevant impact on the spermatogenesis, while neurotrophins have a significant action on the maturation and mobility of the sperm cells. Our study investigates the immunohistochemical characterization and distribution of neurotrasmitters, neurotrophins and their receptors in the seminiferous tubules of rats treated by LND focussing the attention on possible relationships between the distribution of these molecules and the physiopathological mechanisms involved in sperm

maturation and motility as well as in the local modulation of the interactions between germ cells and Sertoli cells in the light of the literature on this topic.

Materials and Methods

Sexually mature (80- to 90-day old, 300-325 g) male Sprague-Dawley rats were obtained from Harlan (Milan, Italy). All animals were treated in agreement with the Helsinki Convention on the use of animals in research approved by the Institutional Review Board. The animals were housed at 22°C on a 12-h light/12-h dark cycle 1 week before the experiments, with free access to food and water. LND was suspended in 0.5% methylcellulose at a concentration of 10 mg/mL and administered orally at the single dose of 100 mg/kg b.w. (Gatto 2002). Control rats received an equal amount of vehicle. The animals (ten per group) were killed by exsanguination under ether anesthesia 24 h after the treatment. Testes were removed, fixed for 24 h in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.2 at 22°C), rinsed with the same buffer, and stored at room temperature. From each sample, a block of tissue was removed by sectioning through the organ. After dehydration in ethanol at increasing concentrations (70-100%), each block was embedded in paraffin and serial 5 μm thick sections were cut using a rotatory microtome (Reichert-Jung Autocut model 1150, Wien, Austria), mounted on gelatin-coated slides and processed for immunohistochemistry as described elsewhere (Bronzetti 1995).

Immunohistochemical analysis

The following molecules were investigated: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), tyrosine kinase A (TrKA), tyrosine kinase B (TrKB), tyrosine kinase C (TrKC) and protein 75 (p75), calcitonin gene-related peptide (CGRP), choline acetyltransferase (ChAT), , substance P (SP), vasointestinal peptide (VIP), neuronal nitric oxide synthase (nNOS).

To study the immunolocalization of neurotrophins and their own receptors, the antibodies used were: i) rabbit anti nerve growth factor (anti NGF) polyclonal antibody (Santa Cruz) which displayed less than 1% cross-reactivity against recombinant human NT-3, NT-4 and BDNF; ii) rabbit anti tyrosine kinase A (anti TrKA) polyclonal antibody (Santa Cruz, CA, USA), which recognized an epitope corresponding to aminoacids 763 to 777, mapping adjacent to the carboxy terminus of human TrKA p140; iii) goat polyclonal antibody to human p75 NT receptor (Santa Cruz), which recognized the amino acid sequence mapping the carboxy terminus of the p75 NT receptor precursor of human origin; iv) rabbit anti brain derived neurotrophic factor (anti-BDNF) polyclonal antibody (Santa Cruz), which recognized the amino-terminal of mouse BDNF; v) rabbit anti tyrosine kinase B (anti TrKB) polyclonal antibody (Santa Cruz), which recognized an epitope corresponding to aminoacids 794 to 808 of mouse TrKB p145; vi) rabbit anti neurotrophin 3 (anti NT-3) polyclonal antibody (Santa Cruz), which was raised against the amino-terminal of mouse NT-3; vii) rabbit polyclonal anti tyrosine kinase C (anti TrKC) antibody (Santa Cruz), which recognized an epitope corresponding to aminoacids 798 to 812 of porcine TrKC p140.

For analysis of neurotransmitters, the following antibodies were used: i) rabbit anti-vasoactive intestinal peptide (anti VIP) polyclonal antibody (Chemicon International); ii) rabbit anti-substance P polyclonal antibody (anti SP) (Chemicon International); iii) mouse anti-calcitonin related peptide (anti CGRP) monoclonal antibody (Chemicon International); iv) rabbit anti-nNOS polyclonal antibody (Chemicon International); and v) goat anti choline acetyltransferase (anti ChAT) polyclonal antibody (Chemicon International). Incubation with primary antibodies was performed overnight at 4°C at a final concentration of 2-5 ng/mL. Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After exposure to the primary antibodies, slides were rinsed twice in phosphate buffer and incubated (1 h and 30 min at room temperature) with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (final dilution 1:100). The secondary antibody-HRP linked against rabbit immunoglobulins was purchased from Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany), while secondary antibodies-HRP linked against mouse and goat immunoglobulins were from Sigma (Sigma Chemicals Co, St Louis, MO, USA). After a further wash with phosphate buffer, slides were treated with 0.05% 3,3-diaminobenzidine and 0.1% H_2O_2. Finally, sections were counterstained with Mayer's hematoxylin and

observed using a light microscope. To block endogenous peroxidase activity, slides were pretreated with 3% H_2O_2, whereas the non-specific binding of immunoglobulins was prevented by adding 3% fetal calf serum to the incubation medium. Negative control experiments were done: i) by omitting the primary antibody; ii) by substituting the primary antibody with equivalent amount of non specific immunoglobulins; iii) by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to customer's instructions). In preliminary experiments, immunohistochemistry was also performed on frozen sections of rat testis tissue. No differences were found in the intensity or distribution of immunostaining using the two types of sections, but microanatomical details were better preserved in paraffin-embedded material.

Results

Sections of rat testis samples exposed to the primary/secondary antibodies developed a dark-brown (intense), yellow-brown (slight) immunostaining or no staining (as shown in Figures 1-2). No immunostaining developed in sections incubated with antibodies previously adsorbed with peptides used for raising them or with a pre-immune serum. Immunostaining was located in germ cells (with different degrees of expression).

The testicular immunoreactivity expression of the neurotrophins, their receptors and the neurotransmitters evaluated in control and LND treated rats are described in detail in Figure 1 and Figure 2.

Neurotrophins and receptors

The seminiferous tubules of control and treated rats expressed a strong immunoreactivity for NGF but not for BDNF. A strong immunoreactivity was evidenced for NT3 in all the germ cells of treated animals, but it appeared moderate in the controls (Figure 1 a,b,e,f,i,l). With regard to the expression of their receptors (Figure 1c,d,g,h,m,n,o,p) while a strong immunoreactivity was observed for TrKA, TrKB and TrKC both in tubules of treated and untreated rats; a distinct immunoreactivity was observed for p75, being its expression weak in all the germ cells of the treated rats and marked in the basal germ cells of the untreated rats.

Neurotransmitters

In the untreated rats the immunoreactivity was not detected for the neurotransmitters SP, CGRP, neural NOS and ChAT (Figure 2 a, e, g, i) and was weakly expressed for VIP (Figure 2 c); in the tubules of the LND treated rats an increase of the immunoreactivity was observed for the neurotransmitters, being marked for VIP (Figure 2 d), moderate for nNOS (Figure 2 h) and weak for SP and ChAT (Figure 2 b,l). No immunoreactivity was instead observed for CGRP in the tubules of treated rats (Figure 2 f).

Discussion

In addition to their well-known role within the nervous system, the NTs and their receptors regulate some functions in non-nervous tissues (Ricci 2001; Ricci 2004; Seidl 1996).

It is well known that NGF has an inflammatory role and its increase is directly related to inflammation, allergies and diseases of the immune system (Otten 2000; Stanisz 2000). The most interesting aspect of this link is represented by the prominent role of NGF in inflammatory hyperalgesia (Mendell 1999; Shu 1999). The latter effect is probably due to the direct action of NGF on mast cells and sensory neurones, as proposed by Woolf *et al.*, 1996). A local production of NGF by immune cells (depending upon the stimulus of IL-1β and TNFα) is the probable source of NGF (Ricci 2000; Bronzetti 2005; Bronzetti 2006). NGF levels are also increased in asthma and in other allergic diseases (Bonini 1996; Lambiase 1997; Renz 2001; de Vries 2002).

Moreover it is interesting to note that NGF seems to be capable of exerting some specific actions in the reproductive organs of adult male rats (Levanti 2006). In fact, according to ChunMei *et al.,* 2005, NGF may play a role in the regulation of sperm maturation and motility: moreover, the expression of NGF and both receptors TrKA and p75 in the efferent duct of the reproductive system may lead to the hypothesis that NGF participates in the regulation of fluid reabsorption in the rat reproductive tract. Our data concerning the NGF and its receptor TrKA demonstrate that the immunoreactivity is markedly positive in tubules of untreated and treated rats; p75-like immunoreactivity was moderately expressed in the tubules of our untreated animals and weakly expressed in all the germ cells of treated rats. Concerning the role of the other neurotrophins we can only affirm that, in the lack of clear and comparable experimental data in the

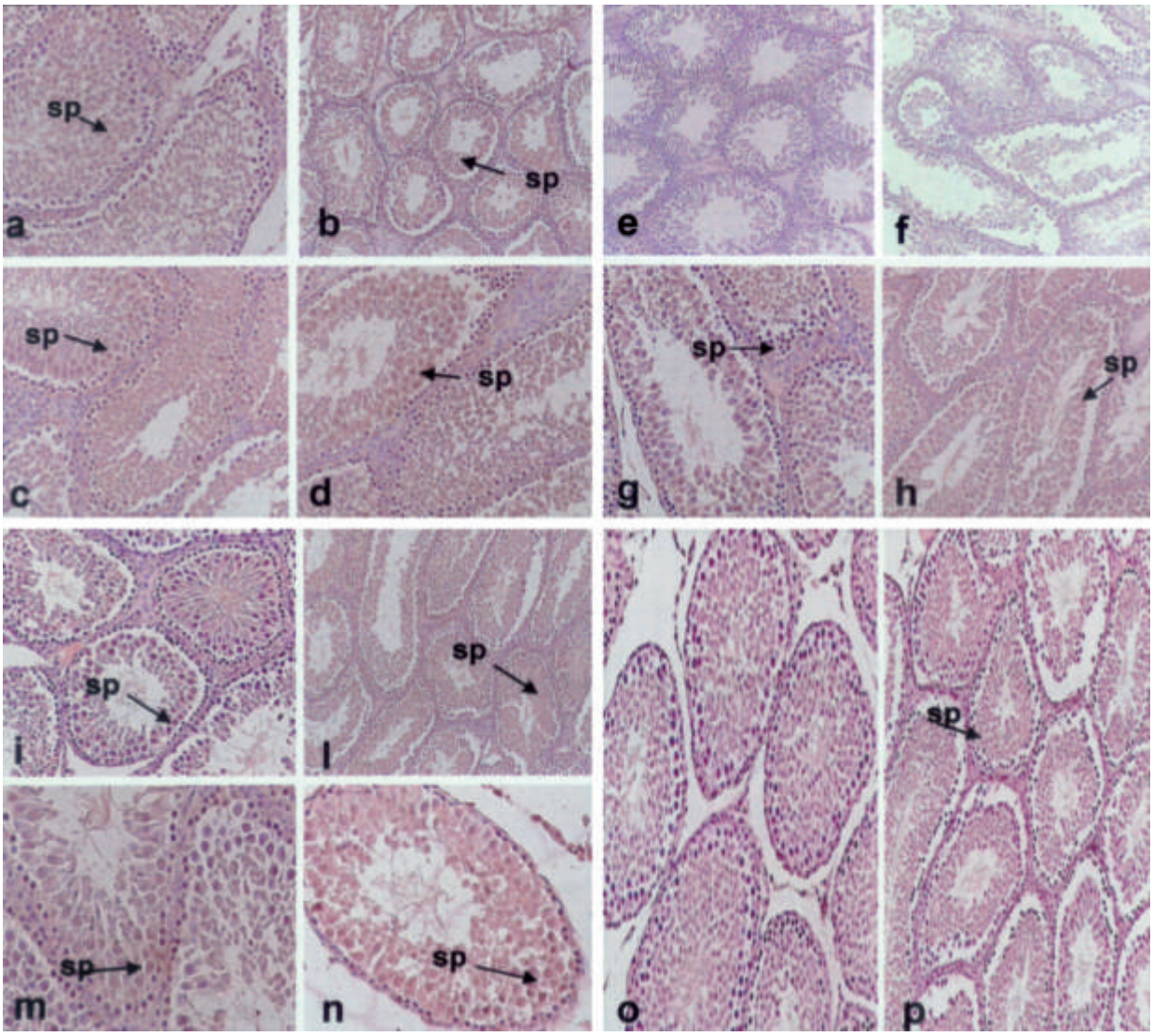

Figure 1. NGF (a,b): a strong immunoreactivity for NGF was observed in samples from treated and untreated (control) rats. TrKA (c,d): strong immunoreactivity for the TrKA receptor has been detected in the spermatogonia and in the interstitial connective tissue of control rats. in treated rats a strong immunoreactivity was expressed also in the spermatocytes. BDNF (e,f): no immunoreactivity for BDNF is expressed in the seminiferous tubules of treated and untreated rats. TrKB (g,h): an evident immunoreactivity for the TrKB receptor has been detected in the tubules from treated and untreated rats. NT3 (i,l): a moderate immunoreactivity has been observed in the tubules of untreated rats. A strong immunoreactivity (l) was found in all germ cells of treated animals. TrKC (m,n): a strong immunoreactivity of TrKC has been observed both in tubules of treated and untreated rats with marked expression in all the germ cells. p75 (o,p): a distinct immunoreactivity for the p75 has been observed in the tubules of untreated rats (o) with a marked positivity for the basal germ cells. The immunoreactivity for p75 is weak in the tubules of the treated rats (p).
sp= spermatocytes (a, b, c, d, g, h, l, l, m, n, p.)

available literature, the expression of BDNF- and NT3-like immunoreactivity is substantially absent in the seminiferous tubules of our untreated rats, while the same immunoreactivity is well-expressed in the seminiferous tubules of treated animals. TrKB- and TrKC-like immunoreactivity is evident in the same specimens. The immunoreactivity for NTs has been observed in spermatogonia of untreated rats, while the rats treated with LND showed an immunohistochemical localization in all the stages of germinal cells. The generally well-expressed immunoreactivity for the neurotrophins receptors in treated rats observed in our study is presumably attributable to alterations of the receptors' structure and/or expression leading to changes of the activity, affinity, localization or protein interactions that may depend on sensitization of ion channels (induced by LND). The latter is one of the mechanisms attributable to the effect of LND on the complex interaction with normal and neoplastic tissues.

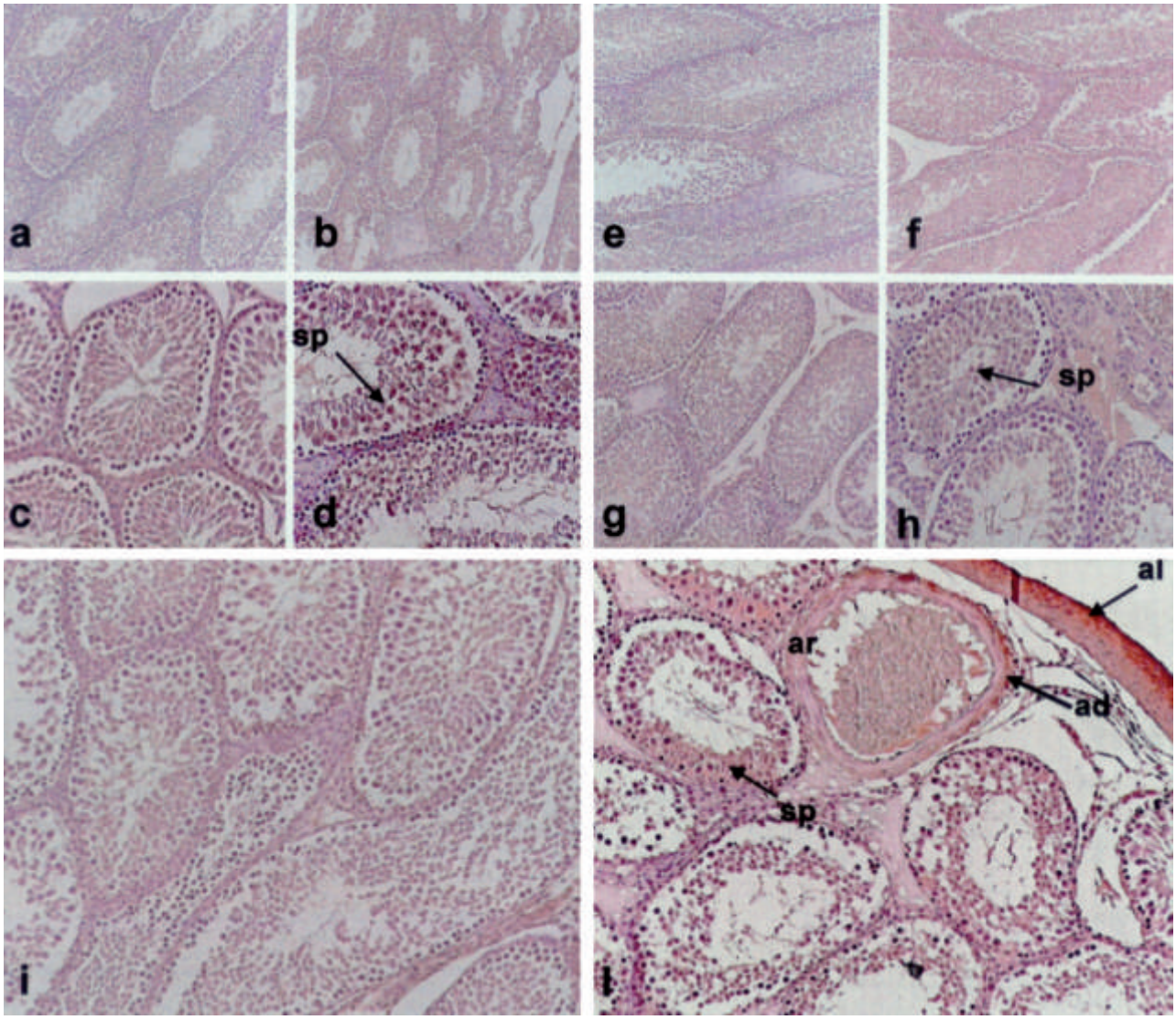

Figure 2. SP (a,b): no immunoreactivity for the SP has been detected in all the tubules of the untreated rats (a); weak immunoreactivity for the SP was present in the tubules of treated rats (b). VIP (c,d): weakly expressed immunoreactivity for the VIP has been detected in the tubules of the control rats (c); marked immunoreactivity for the VIP has been observed in the tubules of treated rats (d). CGRP(e,f): no immunoreactivity for the CGRP is evident in the tubules of untreated and treated rats. nNOS (g,h): no immunoreactivity for the neural NOS has been detected in the tubules of untreated rats (g); a moderate immunoreactivity for the neural NOS is visible in the seminiferous tubules of treated rats (h). ChAT(i,l): no immunoreactivity for the ChAT has been observed in the tubules of control rats (i); a weak immunoreactivity for the ChAT was detected in the germ cells of the treated rats (l). sp= spermatocytes (h,l); ar= artery; ad= adventitia; al= albuginea (l).

The blockade of spermatogenesis induced by LND might lead to a *reactive* increase of the levels of neurotrophins whose action is mediated by their receptors with an evident local increase of the immunohistochemical reactivity in all the germinal cytotypes of treated rats. The morphological profile of testis treated by LND could be compared with alterations occurring in some testicular diseases. We may suppose a trophic effect of NTs, mainly NT3, that are able to stimulate the cellular proliferation (Cordon-Cardo 1991; Skinner 2005). Neurotrophins may play a role of modulation of the development, trophism and differentiation of rat testis cytotypes by means of an autocrine and/or paracrine mechanism (Cupp 2002; Li 2005).

Concerning the immunoreactivity for the investigated neurotransmitters we can affirm that SP-like and CGRP-like immunoreactivity are substantially absent in germinal cells of both the treated and untreated rat groups, because these fibres run

mainly along surface of testis. This finding may be consistent with a relatively secondary role of these neurotransmitters in a compartment (the testis) in which sensitive (SP) and trigeminovascular (CGRP) patterns of innervation do not prevail. On the contrary, the well-expressed immunoreactivity for VIP and nNOS (more evident in the tubules of the rats treated by LND) may be interpreted as a reactive recruitment of these neurotransmitters whose traditional role in the testicular compartment is well-ascertained. The function and the immunohistochemical profile of the ChAT appears to be more controversial. The immunoreactivity for the ChAT appears to be more evident in the seminiferous tubules of the treated rats and this findings confirms a general *reactivity* in the expression and distribution of the functionally relevant neurotransmitters after the treatment by LND. It is presumable that the administration of LND, owing to the various effects on the cell cycle, the metabolism and the survival of the different testis cells, may induce a general *rearrangement* of the physiopathological local conditions leading to a new modulation and assessment of the neural transmission in the testis and related structures.

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