Journal of Animal and Veterinary Advances 2 (3): 119-125, 2003
© Grace Publications Network, 2003

Effects of Putative Neurotransmitters on Testosterone Production from *in vitro* Mice Interstitial Cells Culture

[1]Krishna Kaphle, [1]Leang-Shin Wu, [2]Yuan Feen Tsai, [3]Nai-Yen Jack Yang and [1]Jen Hsou Lin
[1]Department of Animal Science, [2]Department of Physiology, College of Medical Science
National Taiwan University, [3]China Institute of Technology, Taipei, Taiwan

Abstract: Testosterone is the main androgen in the male, mainly synthesized by the Leydig cells in the interstitial tissues of the testes. It is a pro-hormone for biologically active 5-αreduced androgen and estrogens and is produced by the action of luiteinizing hormone (LH) through cyclic adenosine monophosphate (cAMP) pathway. This classical pathway of steroidogenesis is a major but not sole player in testosterone synthesis in the Leydig cells. Other intracellular signaling systems also play important role in Leydig cells via which several endogenous bio-chemicals have been found to actively participate either in stimulation or inhibition of testosterone production. One of the factors that have been poorly investigated for their autocrine and paracrine action in the testosterone production by the Ledyig cells are the neurotransmitters. In this experiment we investigate some putative neurotransmitters (NTs) namely dopamine (DA), 3, 4-dihydrophenylacetic acid (DOPAC), epinephrine (E), norepinephrine (NE), 5-hydroxytryptamine (5-HT), homovanillic acid (HVA), 3-methoxytyramine (3-MTA), 5-hydrooxyindoleacetic acid (5-HIAA) for their direct effect on the testosterone production by isolated interstitial cells from mice. The finding reveal that DA, 5-HT at lower doses can acutely stimulate while DOPAC, E, 3-MTA inhibits testosterone production significantly over the basal level ($p<0.05$). At longer duration of incubation the stimulation and inhibition of either NTs is not significantly different then the basal except for high dose of mg/ml 5-HT which showed significant inhibition both at 4 and 24 hours of incubation over the basal level. The exact mechanism how some of these putative NTs can acutely stimulate testosterone production while others can inhibit is a matter of further investigation. It is postulated that enzymatic metabolization of 5-HT to 5-HIAA and DA to 3-MTA is partly responsible for the significant inhibition of testosterone production acutely and at longer duration of incubation as seen with higher dose. Further investigation is necessary before we can come to any conclusive decision regarding their exact mechanism but NT's local role in testosterone production from Leydig cells is an crucial clue to unfold the unique mystery of testosterone synthesis.

Key words: Neurotransmitters, Testosterone, Interstitial cells, Mice

Introduction

Testosterone, the main androgen in the male is produced in the Leydig cells located in the interstitial tissue of the testes. Two generations of Leydig cells are said to have developed one at the prenatal stage to achieve masculanity of the urogenital system and the other at puberty to initiate spermatogenesis and functional reproductive physiology. Three periods in the life of a normal male are found to have relatively higher concentration of testosterone in the plasma; phase of embryonic development, the neonatal period, and throughout sexual life. The Leydig cells are thought to be differentiated from mesenchyma like stem cells originating first in the mesonephros and migrating to the interstitial tissues (Wartenberg, 1978; Byskow, 1986). However, the theory of neural crest derivation of Leydig cells is also put forward (Davidoff, *et al.*, 1993). The evidence of this neural origin is based in the expression of several neural specific proteins such as neural cell adhesion molecule, neurofilament protein 200, microtubular associated proteins in the Leydig cells (Davidoff *et al.*, 1993; Middendorff *et al.*, 1993; Mayerhofer *et al.*, 1992, 1996). Even though adult Leydig cells are not derived from pre-existing fetal Leydig cells they do possess their nervous relationship. Nerve terminals and their neurotransmitters are distributed in the testicular interstitium (Celuch and Sloley, 1988; Campos and Chiocchio, 1990; Zhu *et al.*, 1995), while sympathetic nerves involved in testicular steroidogenesis was reported by Carvalho *et al.*, (1996). The testis innervation is by superior spermatic nerve (SSN) and inferior spermatic nerve (ISN) with major contribution from the latter (Zhu *et al.*, 1995). Even though major contributor to the steroidogenesis ability of Leydig cells is dependent on the pituitary synthesized gonadotropins, locally controlled subtle regulation is equally important if not more

(Skinner, 1991; Saez, 1994). Luteinizing hormone (LH) can acutely stimulate steroidogenesis in the testis via their seven transmembrane-G protein coupled receptors which interact with adenylyl cyclase to form the second messenger 3′, 5′-cyclic adenosine monophosphate (cAMP). Besides the predominant LH/hCG stimulated cAMP pathway involving the rate limiting factor steroid acute regulatory (StAR) protein, several other pathways have been identified capable of steroidogeneis in the Leydig cells. Many endogenous bio-active factors are linked with testosterone production from Leydig cells; lipophilic factor from macrophages (Lukyanenko *et al.*, 1998), TGF-α (Kulin *et al.*, 1981), TGF-β, FGFs (Saez and Lejeune, 1996), activin, inhibin (Risbridger, 1996), EGF/TGFα (Ackland *et al.*, 1992), oxytocin (Tahri-Joutei and Pointis, 1989), IL-1 (Verhoeven *et al.*, 1988), angiotensin II, vasopressin, endothelin (Tomic *et al.*, 1995). There is a long list of locally produced factors in the testes that are able to regulate Leydig function but majority of them does not play a major regulatory role (Lejeune *et al.*, 1993). NTs are one among them but that do not rule out their contribution in the overall functioning of the complex tissues in the testes in general and Leydig cells in particular which have background of neural origin. However with difficulties in distinguishing the effect of NTs in an *in vivo* experiments some *in vitro* works in ovary of female rats (Casais *et al.*, 2001) and whole testis of male rats (Zhu *et al.*, 2002) are reported. The fact that catecholamines can stimulate testosterone secretion in an *in vitro* Leydig cell preparation (Cooke and Hunter, 1982) and the presence of adrenergic (Anakwe *et al.*, 1985), 5-HT_2 subtype (Tinajero *et al.*, 1992) receptors in the Leydig cells. Potent neurotransmitter serotonin or 5-HT and monoaminoxidase enzyme that metabolizes it are present in the testes (Ellis *et al*, 1972, Tinajero *et al.*, 1993), they are synthesized in the Leydig cells, mast cells, testicular nerves and systemic sources. Intratesticular administration of 5-HT is reported to exert stimulatory steroidogeic action in immature but suppressive action in mature rats (Csaba *et al.*, 1998). Dopamine agonist Mesulergine (N-(1-6,dimethylergolin-8a-y1)-N′, N-dimethylsulfamide hydrochloride) administrater directly to the rat Leydig cells did not inhibit cAMP production or testosterone production except at high concentrations (Dirami and Cooke, 1998).

NTs are the chemical messengers present in the presynaptic terminal and discharged when neurons are appropriately stimulated. More than 50 different chemicals are at present recognized as NTs or neurotransmitter candidate, (Marieb, 1998). NT's action may vary with the context of the ongoing synaptic events-enhancing excitation or inhibition, rather than operating to impose direct excitation or inhibition (Bourne and Nicoll, 1993). They have a great role to play initiating from the early developmental sex differentiation as contributed by dopamine to the complex neuro-endocrinological function that covers all aspect of physiology of life. The effect of normal and pathologic level of endogenous and externally administered NTs on testosterone production is an important area of investigation as more and more drugs aimed at altering NT status in patients are being employed. The high incidences of Leydig cells hyperplasia/adenoma in users of new synthetic NTs agonist and antagonists demand urgent need for clear understanding of the physiology involved.

Materials and Methods

The NTs and their metabolites used in this experiment were freshly prepared in the Physiology Department, College of Medical Science, National Taiwan University (NTU). The oLH used in this experiment was obtained as a gift from NIDDK (I-3). The chemicals and equipment used in this experiment were purchased from Sigma, Gibco, Merck, Worthington, Boehrington Mannheim, Costar and other companies. The mice were procured from the Animal Center of Medical College, National Taiwan University and housed in the Physiology laboratory of Animal Science Department, NTU.

Adult male mice of ICR strain were kept in the laboratory animal's room in a controlled environment with a 14: 10 light: dark schedule. The experimental procedures for the animals used was accomplished in line with the approval by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine. The technique employed is slight modification from our previous experiments (Yang *et al.*, 2002) briefly introduced here. The mice of 8-12 weeks were killed by decapitation on the morning of the day of experiment and the testes collected in a sterilized way, two mice provided one gram of testes enough for each experiment. The testes were then placed in a tube containing medium 199 (M199) insulated by ice during transport to culture room. After careful decapsulation and mincing into smaller parts with the aid of forceps it was placed into a tube containing Hanks balanced salt solution (HBSS). The HBSS solution was added with collagenase,

bovine serum albumin 1% and N-2-Hydroxyethylpiperazine-N1-2- ethanesulfonic acid (HEPES). The tube was then covered by an aluminum foil and oscillated in room temperature for twenty minutes at 50 rounds per minute (rpm). After which it was then taken back to the culture room and in the sterile condition inside the laminar flow the contents sieved through a mesh net into a new tube. The tube was then centrifuged for five minutes at 2000 rpm and the supernatant discarded. Fresh solution of M199 blank were then drawn and after mixing the packed cells in the bottom of the tube by drawing and releasing the solution up and down and it was then re-centrifuged. The procedure was repeated for four times to discard the germ cells and the spermatogonia. After which 50µl of the medium containing uniform distribution of the cell was taken in an eppendorf and mixed properly with equal amount of trypan blue. This was used to estimate the number of cells in the medium by aid of haemocytometer under a compound microscope. Blank M199 was then added to achieve a uniform distribution of 10^5cells/ ml of the culture medium. The medium was then placed in a 48-well culture plates (Costar) with each well receiving 500µl of the medium and 10µl of NTs at 1, 10 µg and 1mg/ml and oLH at 1, 10 ng /ml doses according to the prearranged pattern. The culture plate was incubated at 37°C, 95% relative humidity and 5 % carbon dioxide level. Medium was collected at 4 and 24 hours and kept in an ependorf, numbered and frozen at -20°C until assayed for testosterone. The collected medium was then assayed by EIA method and the result obtained by automatic reader using optical density variation. The obtained data was compared with the basal as control, stimulation with oLH as positive control, and various doses of the NTs relative to their testosterone producing ability. Each test was repeated several times and on being convinced of similar results on each trial, a set of triplicate data among those was chosen. The data was statistically evaluated by Duncan's Multiple Range Test (DMRT) using Statistical Analysis System (SAS) application software program. All the results were expressed as mean ± SEM and the level of significance for comparisons set at $p<0.05$.

Results

The data obtained by assaying 4 hours medium collected from the culture of interstitial cells of mice for the testosterone production is presented in Figs. 1, 2, 5 and 6. The Figs. indicate that in lower dose of 10 µg DA, 5-HT can

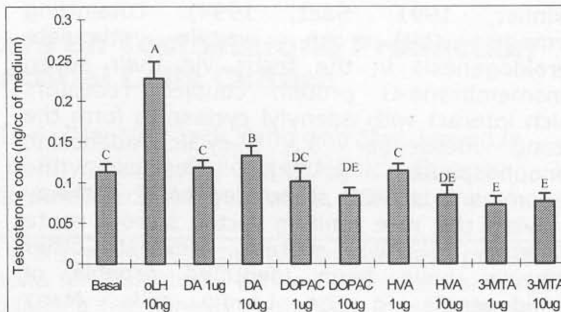

Fig. 1: Four hours testosterone production by different neurotransmitters at variable doses compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different ($p<0.05$).

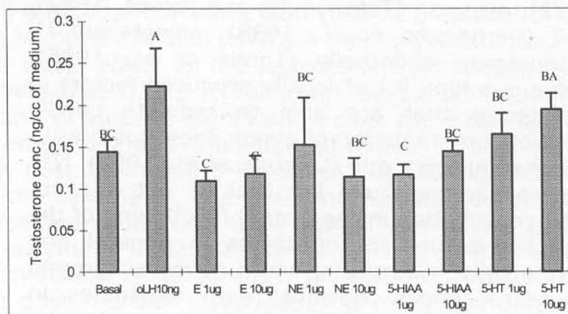

Fig. 2: Four hours testosterone production by different neurotransmitters at variable doses compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different ($p<0.05$).

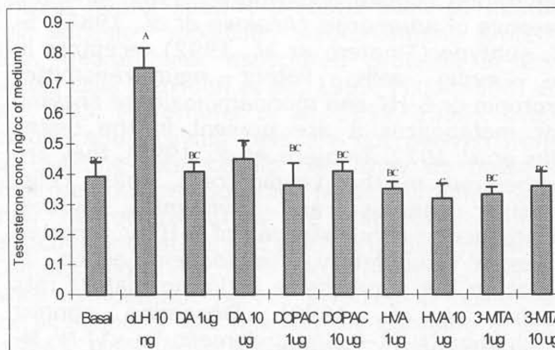

Fig. 3 Twenty-four hours testosterone production by different neurotransmitters at variable doses compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different ($p<0.05$).

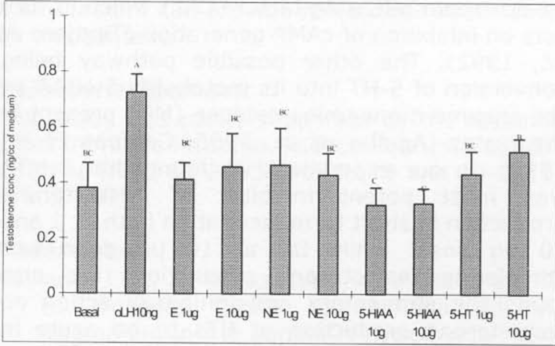

Fig. 4: Twenty-four hours testosterone production by different neurotransmitters at variable doses compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different (p<0.05).

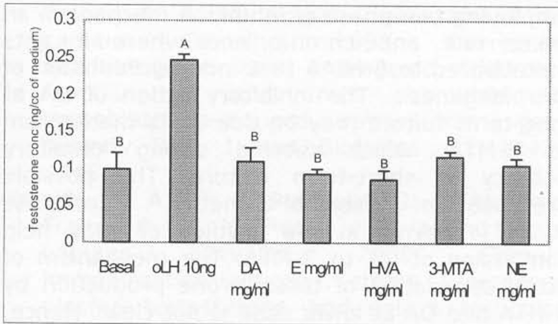

Fig. 5: Four hours testosterone production by different neurotransmitters at higher dose of mg/ml compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different (p<0.05)

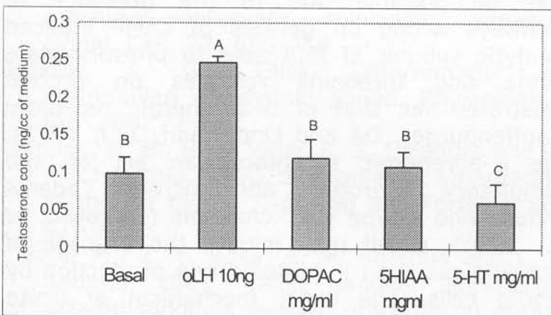

Fig. 6 Four hours testosterone production by different neurotransmitters at higher dose of mg/ml compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different (p<0.05).

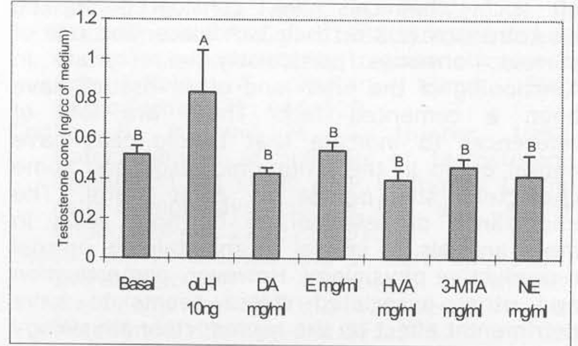

Fig. 7: Twenty-four hours testosterone production by different neurotransmitters at higher dose of mg/ml compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different (p<0.05).

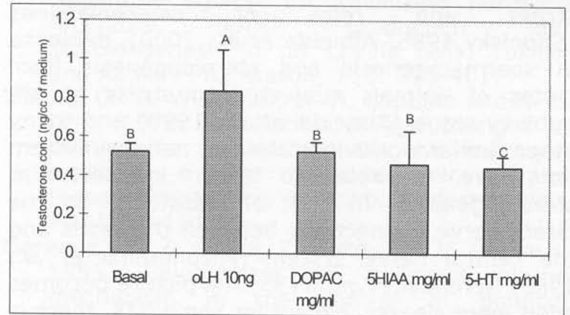

Fig. 8: Twenty-four hours testosterone production by different neurotransmitters at higher dose of mg/ml compared with that of basal and oLH as positive control (n=3). The values are expressed as mean ±SEM, means with same letter are not significantly different (p<0.05).

stimulate testosterone production significantly over basal level (p<0.05). DOPAC, HVA, 3-MTA can inhibit testosterone production significantly over the basal level. 3-MTA can also inhibit testosterone production compared to basal level at 1 mg dose the pattern not seen with other NTs used, while at the higher dose of mg/ml, 5-HT inhibited testosterone production. In 24 hours sample there was no significant stimulation or inhibition compared with the basal level except for mg/ml of 5-HT, Figs. 3, 4, 7 and 8.

Discussion

Neurotransmitters are crucial players of the nervous system and they achieve so by acting on specific receptors located at the post-synaptic junctions. Their role in regulating neuroendocrine function is well documented and several scores

of such chemicals are considered to be neurotransmitters or their candidate. The role of steroid hormones particularly testosterone in functioning of the brain and other tissues have been a cemented fact. There are lots of references to indicate that Leydig cells have neural origin in the embryonic stage and some characters still persist in adult stage. The importance of testosterone hormone level in male animals is crucial in maintaining normal reproductive physiology. However, domestication and other associated stress seems to have detrimental effect on the reproductive physiology of these animals. The evidences for stress and disruption of normal endocrine function at higher level of the brain related to sexual physiology are plenty. Stress induced hyposensitivity of testis to gonadotropins (Charpenet, *et al.*, 1982), elevated testosterone concentration in stress and roles of catecholamines (Sapoisky,1985; Almeida *et al.*, 2000), decrease in spermatogenesis and steroidogenesis from testes of animals subjected to stress at pre puberty stage (Almeida *et al.*, 1998) and many other similar works indicate that nervous system do have a role to play in testicular steroidogenesis. In light of knowledge on the direct nerve connections between the testis and the central nerve system (Mizunumma *et al.*, 1983; Gerendai *et al.*, 1995) the picture becomes even more clearer. Except for some NTs, there is no such information on the role of transmitters directly involved in testosterone production from the Leydig cells. In an *in vitro* experiments with acetylcholine administration in the spermatic nerve plexus of adult rats, there is report of inhibited testosterone secretion (Zhu *et al.*, 2002). With increasing evidence indicating several biologically active substances synthesized and crucial in steroidogenesis the role of NTs cannot be underestimated. NTs mostly β-adrenergic, serotoninergic are widely reported to influence steroidogenesis of Leydig cells. The presence of receptors and enzymes for metabolization of NTs at the site of testosterone production is not without a purpose. Our *in vitro* experiment revealed that DA and 5-HT have significant stimulation at lower doses. This finding confirms earlier reporting about the presence of both stimulatory and inhibitory steroidogenic activity of 5-HT (Csaba *et al.*, 1998), and the non inhibition of testosterone producing ability of Leydig cells at lower dose by DA agonist (Dirami and Cooke, 1998). The inhibition of testosterone production by 5-HT at higher dose is possibly by release of corticotropin releasing factor (CRF) which in turn acts on inhibition of cAMP generation (Tinajero *et al.*, 1992). The other possible pathway being conversion of 5-HT into its metabolite 5-HIAA by the enzyme monoamine oxidase (MO) present in the testes (Aguilar *et al.*, 1995, Campos *et al.*, 1990). In our experiment we found that 3-MTA was most potent inhibitor of testosterone production in short term incubation both at 1 and 10 µg dose, while DA at 10 µg dose was stimulating testosterone production. We also found the stimulatory and inhibitory action on testosterone production of NTs to be acute in nature, as with increase in duration of incubation there was no significant difference among them and the basal level. However, 5-HT in higher dose could inhibit testosterone production both at 4 and 24 hours of incubation significantly below basal level. This can be possible by employing two phase of inhibition mechanism an acute one and chronic one where it gets metabolized to 5-HIAA that induces inhibition of steroidogenesis. The inhibitory action of DA at long-term culture may be due to its metabolism to 3-MTA, which showed strong inhibitory potency in short-term culture. The possible presence of catechol-O-methyl transferase (COMT) enzyme in the Leydig cell may help conversion of DA to 3-MTA. The mechanism of acute stimulation of testosterone production by 5-HTA and DA at lower dose is not clear. Hence, it can be concluded that NTs have different effect on testosterone production by isolated mice interstitial cells culture. The inhibitory effect of 5-HT at higher dose both at acute and long-term culture may be because of metabolization to HIAA. The acute stimulatory action of some NTs may be possible due to the presence of pathways acting on genesis of cAMP induced catalytic subunit of PKA able to phosphorylate serine and threonine residues on protein substrates like that of α-adrenergic receptors (Lauffenburger, DA and Linderman, J. J, 1993). The α-adrenergic receptors can act on the stimulatory G-protein and increase adenyl cyclase and L-type Ca^{2+} channels (Lefkowitz *et al.*, 1996), which may initiate the cascade of signaling resulting in testosterone production by Leydig cells. The exact mechanism of acute stimulation by some NTs and inhibition of some others for the testosterone production ability of isolated mice Leydig cells culture in an *in vitro* experiment needs further investigation. The physiological action of NTs on testosterone production in intact tissue condition where the amount available is very low and influenced by

many factors can provide more clear information on this context.

Acknowledgement

This study was partly supported by the research grant from The National Science Council, Republic of China (NSC 89-2313-B-002-179 and 90-2313-B-002-301).

References

Ackland, J. F., N. B. Schwartz, K. E. Mayo and R. E. Dodson, 1992. Nonsteroidal signals originating in the gonads. Physiological Rev., 72: 731-788.

Aguilar, R., F. Anton, C. Bellido, E. Aguilar and F. Gaytan, 1995. Testicular serotonin is related to mast cells but not to Leydig cells in the rat. J. Endocrinology, 146: 15-21.

Almeida, S. A., S. O. Petenusci, J. A. Anselmo Franci, A. A. M. Rossa e silva and T. L. Lamano Carvalho, 1998. Decreased spermatogenic and androgenic testicular function in adult rats submitted to immobilization-induced stress from pre-puberty. Braz. J. Med. Biol. Res., 32: 1443-1448.

Almeida, S. A., S. O. Petenusci, J. A. Anselmo Franci, A. A. M. Rossa e silva and T. L. Lamano Carvalho, 2000. Chronic immobilization-induced stress increases plasma testosterone and delays testicular maturation in pubertal rats. Andrologia, 32: 7-11.

Anakwe, O. O., P. R. Murphy and W. H. Moger, 1985. Characterization of beta adrenergic binding sites on rodent Leydig cells. Biol. Rep., 33: 815-826.

Bourne, H. R and R. Nicoll, 1993. Molecular machines integrate coincident synaptic signal. Cell, 72: 65S-75S.

Byskow, A. G., 1986. Differentiation of mammalian embryonic gonad. Physiological Rev., 66: 77-112.

Campos, M. B., M. L. Vitali, R. S. Calandra and S. R. Chiocchio, 1990. Serotonergic innervation of the rat testis. J. of Rep and Fertility, 88: 475-479.

Casais, M., Z. Y. Sosa, A. M. Rastrilla and L. I. Aguado, 2001. Celiac ganglion adrenergic activity modifies ovarian progesteron during pregnancy; its inter-relationship with LH. J. of Endocrinology, 170: 575-584.

Celuch, S. M. and B. D. Sloley, 1998. Regional distribution of dopamine, 5-hydroxytryptamine and noradrenaline in the rat vas deferens. Neurochemical Res., 13: 967-972.

Charpenet, G., Y. Tache, M. Bernier, J. R. Ducharme and R. Collu, 1982. Stress-induced testicular hyposensitivity to gonadotropins in rats. Role of pituitary gland. Biol. Reproduction, 32: 616-623.

Cooke, B. A. and M. G. Hunter, 1982. Catecholamine stimulation of testosterone production via cyclic AMP in mouse Leydig cells in monolayer culture. Mol. and Cellular Endocrinology, 27: 221-231.

Csaba, Z., V. Csernus and I. Gerendai, 1998. Intratesticular serotonin affects steroidogenesis in the rat testis. J. of Neuroendocrinology, 10: 371-376.

Davidoff, M. S., W. Schulze, R. Middendorff and A. F. Holstein, 1993. The Leydig cell of the human testis. A new member of the diffuse neuroendocrine system. Cell and Tissue Res., 271: 429-439.

Dirami, G. and B. A. Cooke, 1998. Effect of a Dopamine agonist on luteinizing hormone receptors, cAMP production and steroidogenesis in rat Leydig cells. Toxicol and Applied Pharmacol., 150: 393-401.

Ellis, L. G., A. W. Jaussi, M. H. Baptista and R. L. Urry, 1972. Correlation of age changes in monoamine oxidase activity and androgen synthesis by rat testicular minced and teased preparation *in vitro*. Endocrinology, 90: 1610-1618.

Gerendai, I., Z. Csaba, Z. Voko, and V. Csernus, 1995. Involvement of a direct neural mechanism in the control of gonadal functions. J. of Steroid Biochemistry and Mol. Biology, 53: 299-305.

Kulin, H. E., E. Samdjlike, R. Santen and S. Santner, 1981. The effects of growth hormone on the Leydig cell response to chorionic gonadotropin in boys with hypopituitarism. Clinical Endocrinology, 45: 468-472.

Lefkowitz, R. J., B. B. Hoffman and P. Taylor, 1996. In Joel. G. Hardman and Lee E. Limbird edited Goodman and Gilman's "The Pharmacological Basis of Therapeutics", Ninth edition, McGraw-Hill Companies, USA, 105-139.

Lejeune, H., M. Skalli, P. Sanchez, O. Avallet, and J. M. Saez, 1993. Enhancement of testosterone secretion by normal adult human Leydig cells by co-culture with enriched preparations of normal adult human Sertoli cells. International J. of Andrology, 16: 27-34.

Lukyanenko, Y. O., A. M. Carpenter, D. E. Brigham, D. M. Stocco and J. C. Huston, 1998. Regulation of Leydig cells through a steroidogenic acute regulatory protein independent pathway by a lipophilic factor from macrophages. J. of Endocrinology, 158: 267-275.

Marieb R. N. Elaine, 1998. Human Anatomy and Physiology. Addision Wesley Longman, Benjamin/Cummings Science Publishing, California US, 389-395.

Mayerhofer, A., G. Seidl, K. Lahr, D. Bitter-Suermann, A. Christoph, D.Barthels, W. Willie and M. Gartzl, 1992. Leydig cells express neural cell adhesion molecule *in vivo* and *in vitro*. Biol of Rep., 47: 656-664.

Mayerhofer, A., G. Lahr, K. Seidl, B. Eusterschulte, A. Christoph and M. Gartzl, 1996. The neural adhesion molecule (NCAM) provides clues to the development of testicular Leydig cells. J. of Andrology, 17: 223-230.

Middendorff, R., M. Davidoff and A. F. Holstein, 1993. Neuroendocrine marker substances in human Leydig cells. Changes by disturbances of testicular function. Andrologia, 25: 257-262.

Mizunuma, H., L. Palatis and S. M. McCann, 1983. Effect of unilateral orchidectomy on plasma FSH concentration: evidence for a direct neural connection between testes and CNS. Neuroendocrinology, 37: 291-296.

Risbridger, G. P. 1996. Regulation of Leydig cells by inhibins and activins. In the A.H Payne, M.P Hardy and L. D. Russel edited Leydig cell, Cache River press, Vienna, 493-506.

Saez, J. M. and H, Lejeune, 1996. Regulation of Leydig cell function by hormones and growth factors other than LH and IGF-I. In the A.H Payne, M.P Hardy and L. D. Russel edited Leydig cell, Cache River press, Vienna, 383-406.

Sapolsky, R. M., 1986. Stress induced elevation of testosterone concentration in high ranking baboons: role of catecholamines. Endocrinology, 32: 1630-1635.

Skinner, M. K., 1991. Cell-cell interactions in the testis. Endocrine Rev. 12: 45-77.

Saez, J. M., 1994. Leydig cells: endocrine, paracrine and autocrine regulation. Endocrine Rev., 15: 574-626.

Tahri-Joutei, A. and G. Pointis, 1989. Developmental changes in arginine vasopressin receptors and testosterone stimulation in Leydig cells. Endocrinology, 125: 605-611.

Tinajero, J. C., A. Fabbri and M. I. Dufau, 1992. Regulation of corticotropin-releasing factor secretions from Leydig cells by serotonin. Endocrinology, 130: 1780-1788.

Tinajero, J. C., A. Fabbri, D. R. Chiocca and M. I. Dufau, 1993. Serotonin secretion from rat Leydig cells. Endocrinology, 133: 3026-3029.

Tomic, M., M. L. Dufau, K. J. Catt and S. S. Stojilkovic, 1995. Calcium signaling in single rat Leydig cells. Endocrinology, 136: 3422-3429.

Verhoeven, G., J. Cailleau, J. V. Van Damme and A. Billiau, 1988. Interleukin-1 stimulates steroidogenesis in cultured rat Leydig cells. Mol. and Cellular Endocrinology, 57: 51-60.

Wartenberg, H., 1978. Human testicular development and the role of the mesonephros in the origin of a dual Sertoli cell system. Andrologia, 10: 1-21.

Yang, N. Y. J., C. W. Chang, K. Kaphle, L. S. Wu, T. F. Kuo and J. H. Lin, 2002. A preliminary study of *Lepidium meyenii* on its steroidigenic capability of dispersed Leydig cells from mice. J Animal Vet Advances, 1(3): 129-133.

Zhu, B. C., R. S. Chiocchio, A. M. Suburo and J. H. Tramezzani, 1995. Monoaminergic and peptidergic contributions of the superior and inferior spermatic nerves to the innervation of the testis in the rat. J. of Andrology, 16: 248-258.

Zhu, B. C., M. N. Palmada, L. I. Aguado and C. Carlos, 2002. Administration of acetylcholine to the spermatic nerve plexus inhibits testosterone secretion in an in vitro isolated rat testis-nerve plexus system. Int. J. of Andrology, 25: 134-138.