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MOLECULAR BASIS OF INFERTILITY–II: RELATED TO DEFECTIVE BIOSYNTHESIS, SECRETION AND ACTION OF FOLLICLE STIMULATING HORMONE

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Abstract: Infertility can be due to defective genes involved in the biosynthesis, secretion, and action of pituitary Follicle Stimulating Hormone (FSH). Brief account of these biochemical events is provided. A number of reported mutations in genes concerned with regulation of these physiological processes like biosynthesis, secretion and receptor structure and function that lead to 'infertile' state have been described.

Keywords: Infertility, Genetics, Genes and Infertility.

INTRODUCTION

The anterior pituitary gland of vertebrates synthesizes and secretes three glycoprotein hormones. Two of these i.e. Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are called gonadotropins. They exert diverse and direct effects upon the gonads (Ovary and Testes) including regulation and maintenance of essential reproductive processes such as gametogenesis, steroidogenesis, ovulation, implantation etc. Indirectly, even the third glycoprotein hormone i.e. Thyroid Stimulating Hormone (TSH) as well as Growth Hormone (GH) and Prolactin (PRL) regulate many gonadal functions.

FSH is a dimeric glycoprotein hormone comprising a hormone specific β subunit and a species specific common α subunit shared with LH and TSH. Low circulatory levels of FSH do not support follicular growth, while high levels are associated with premature ovarian failure [1]. In fact, female mice lacking FSH-β exhibit an arrest in ovarian folliculogenesis, while women with mutations in FSH-β genes are infertile [Kumar et al, 1997]. The FSH β subunit is the limiting factor for production of mature hormone and provides biological specificity. The gene for FSH-β is located on the short arm of chromosome 11 and is composed of three exons: exon 1 which encodes a 5' untranslated sequence, exon 2 with the 18-amino-acid prepeptide and residues 1-35, and exon 3 which encodes residues 36-111 of the mature peptide [2].

The ovarian tissue responds to low circulating levels of LH and FSH during the very early follicular phase of the ovulatory cycle. Gonadotropin stimulation at this time activates the mitochondrial enzymes present within specialized cells of the immature ovarian follicles (granulosa cells) to stimulate the side chain cleavage of intracellular cholesterol molecules. This conversion (of cholesterol to pregnenolone) is the rate-limiting step in steroidogenesis and provides the developing granulose cell with the substrate to manufacture androgenic steroids [3]. During this process, the androgenic precursors (i.e., andro-stenedione) are aromatized to estrogens under the influence of FSH [4]. The follicles grow as a result of this local estrogenic and FSH stimulation. Granulosa cells proliferate, thereby producing more estrogens. Ovarian estrogens travel through the general circulation to the rest of the organism. This information is perceived by estrogen-sensitive cells within the organism [5]. Following hormone-receptor interaction in the cytoplasm, this complex is translocated to the nucleus where it affects protein synthesis and biological activity. Two of the target tissues to estrogens are hypothalamus and pituitary. As the serum levels of estradiol-17β reach a certain threshold for a specific duration of time (signaling the presence of a mature follicle), a neuroendocrine event occurs within the hypothalamo-pituitary axis to promote a tremendous release of both LH and FSH from the anterior pituitary gland [6] in a surge form. This surge-like release induces a series of biochemical events within the ovary that results in follicular rupture and ultimately, ovulation [7].

FSH and LH are produced by the gonadotrophs of the anterior pituitary but they have distinctly different patterns of secretion [8,9]. There is limited pulsatile secretion of FSH, often independent of pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH) [10]. FSH appears to be continuously secreted and thus the amount released is directly related to its synthesis [61]. For the most part, the regulation of FSH is through a negative feedback of gonadal steroids like estradiol and testosterone, an also the gonadal protein inhibin which acts directly at the pituitary [11, 12].

Structural Characteristics of FSH subunits:

All glycoprotein hormones (of both pituitary and placenta) have a common α subunit. This also implies that there should be a separate control of α subunit production in the thyrotrophs, the gonadotrophs (including the subtypes for FSH and LH biosynthesis), and the trophoblastic cells of primates. If we index the homologous placement of the half-cystine residues in the molecules, highly conserved protein sequences are evident from mammals down to teleosts (Fig 1). This uniformity of the half-cystine locations implies a uniformity of the tertiary structure generated by the formation of the disulphide bonds between these residues. In these exactly homologous locations of half-cystine residues there are a few notable differences. The first observed results show a gap of four residues at position 4-7 in the human α sequence which results from a deletion in this position of the human gene. As a consequence of this there is a foreshortened *N*-terminal tail on human α subunit just prior to the first half-cystine. But this does not alter the size of any of the disulfide loops; it only shifts numbering from the human subunit. This is the only gap in the mammalian series. Thus, all of the disulfide loops in the mammalian α subunits are identical in size as determined by the amino acid residues in each loop.

There is sufficient evidence to indicate that the following assumptions may be made:

(a) The uniformity of half-cystine placements indicates a uniformity of disulfide linkages between these residues which is characteristic of the subunit (i.e., whether α or β) and regardless of the hormone (i.e. FSH, LH, or TSH).

(b) The 3-D structure will be analogous for all glycoprotein hormones. This assumption lacks direct experimental evidence at this time [2].

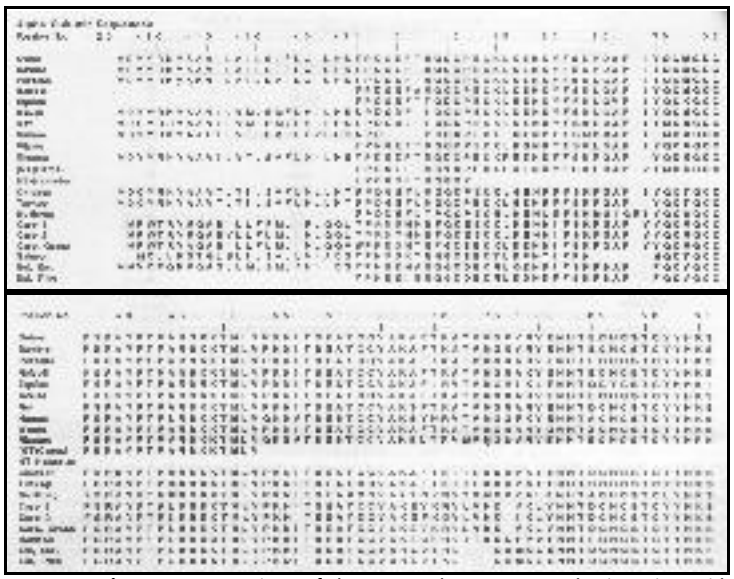


Figure.1: Comparison of the reported common α subunit amino acid sequences [Taken from 2].

Since the advent of radioimmunoassay for the glycoprotein hormones and their subunits, it has been known that α subunit and to a lesser extent free β subunits may be found in the serum and/or the secretory products of the pituitary cells or chorionic cell cultures. Kourides *et al.*,(13) observed that the majority of the "free" α subunits had molecular weight greater than "hormone-derived" α subunit, i.e., the subunit obtained from dissociation of TSH in their study or from hCG, LH or FSH in similar studies done by others subsequently. It was shown that this increased molecular weight was the result of increased glycosylation. Parson and Pierce (14) isolated free α subunit from bovine pituitaries and showed that the increased glycosylation was attributed to *O*-linked glycosylation of the threonine[43] residue and that this glycosylation prevented recombination with the bLHβ subunit. However Arundhati *et al.*,(15) showed that the naturally occurring free subunits of buffalo pituitaries are hypoglycosylated (only 27% of the fully glycosylated form) and hence do not combine with each other.

The pituitary LH-β, FSH- β and TSH-β subunits are the three types of β subunit present in all mammalian species and in the primates there is an additional β subunit type, the chorionic gonadotropin-β subunit (hCG β subunit), which is structurally very closely related to the LH-β subunit of the pituitary.

Follicle-Stimulating Hormone (Follitropin) stimulates follicular development in the ovary, particularly in the granulosa cells of the follicle. Follitropin specifically stimulates the production of the steroid hormone progesterone and the production of the enzyme system (aromatase) for converting testosterone to estrogen [16]. It also stimulates the production of the enzymes involved in plasminogen activation. In males, FSH acts on the Sertoli cells of the seminiferous tubules in the testis to stimulate the production of androgen-binding protein (ABP) and is critical for initiation of spermatogenesis.

A major problem hindering the study of FSH action has been the scarcity of highly purified hormone preparation, since most species of pituitary contain about 1/20 as much FSH (or TSH) as LH on a molar basis. Thus several investigators use a crude preparation of FSH or a substitute. The equine CG (PMSG) shows FSH-like effect in several species of laboratory animals, but not in equine [17]. There are various isoforms of FSH known with circulating half-lives that are dependent on the degree of sialylation [18]. Moreover, these half-lives are usually much shorter than that of PMSG (Pregnant Mare Serum Gonadotropin) thus; uncertainties are introduced into the dose considerations of such studies.

The FSH β subunit sequences are shorter, only 111 residues in length for the mammalian series and only 107 residues for bullfrog FSH. The FSH β protein is encoded by a single gene. However, Guzman *et al.*, (19) found a gene in the sheep that is 87 % homologous to FSH-β gene, indicating that there is an FSH β-like gene or pseudo gene in the sheep. The physiological significance of this finding remains to be determined.

Genetic Relationships for the Subunits:

The subunits are encoded by separate genes present on different chromosomes. In the human, the α-subunit gene is found on chromosome 6, the FSH-β gene is located on chromosome 11. The structural organization of the human genes and the corresponding mature transcript are shown in Fig.2 & 3.

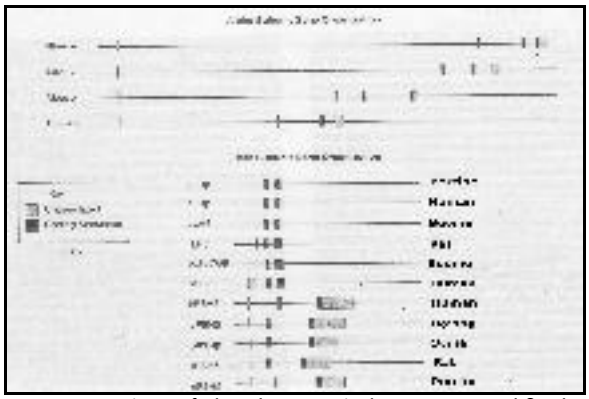


Fig. 2: Comparison of the glycoprotein hormone α and β subunit genes. The genes are drawn to the same scale to illustrate the large differences in sized between the α and β subunit genes, which are primarily accounted for by the larger introns in the α subunit gene [2].

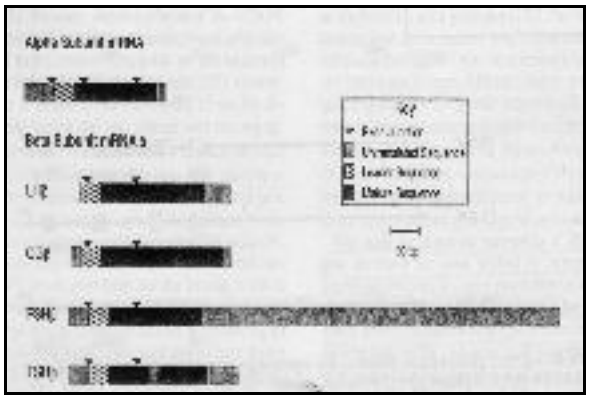
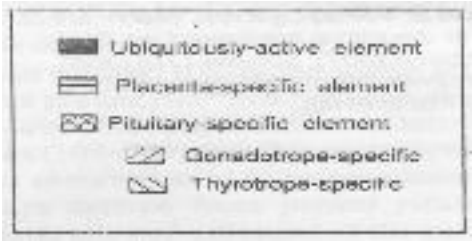


Fig.3: Comparison of the mature mRNAs for the glycoprotein hormone subunits. [Taken from 2].

An important component in the control of α subunit gene expression exists at the transcriptional level. Several groups have examined positive-acting regulatory sequences that control transcription of the human α gene in the gene transfer studies using placental cells and various reporter genes fused to α gene regulatory sequence [11, 20, and 21]. In human choriocarcinoma cells the sequences important for the reporter gene expression are confined to a 200-base-pair region spanning the α gene promoter. By analyzing the effects of specific base changes within this region on promoter activity and by examining the ability of nuclear proteins from placental and non-placental cells to interact with this region lead to the discovery of individual *cis*-acting regulatory elements. The transcriptional regulatory element of α subunit gene promoter consists of binding sites for multiple nuclear proteins. In α subunit promoter certain regulatory elements interact with widely expressed DNA-binding proteins, and others are recognized by cell-type specific factors (Fig.4). These studies demonstrate that α gene promoter has a complex organization and that the interaction of factors bound to multiple, distinct *cis*-acting elements is required for expression in the placenta and the pituitary.



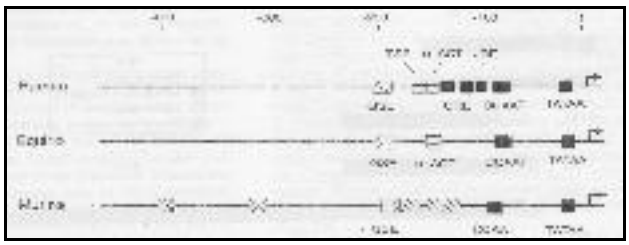


Fig.4 The α-Subunit gene promoter structure. The organization of regulatory elements in the human, equid and murine α subunit gene promoters is depicted. GSE, gonadotrope-specific element; TSE, tissue-specific element; α-ACT, α-gene promoter activation element; CRE, cAMP response element; JRE, junctional regulatory element [Taken from 2].

In CG-producing *placental cell lines*, the second messenger cyclic AMP (cAMP) stimulates α and CG-β gene expression [62]. As a result of studies into the activation of α subunit gene transcription stimulation by cAMP, the tandemly repeated cAMP response elements (CRE) in the human α gene promoter are best characterized regulatory elements that are located -146 and -111 base pairs relative to the transcription initiation site. The CRE consists of conserved palindrome sequence (TGAACGTCA) that is necessary for the cAMP response and for placental-specific transcription of α subunit promoter. The CRE consensus motif is found in many genes that are regulated by cAMP, and it binds a highly conserved nuclear factor, CREB, that is expressed in many different tissues and cell types [11, 20, 21]. Treatment of cells with cAMP activates protein kinase A, which phosphorylates CREB at serine 133 and stimulates the ability of CREB to activate gene transcription.

For maximal expression of the α promoter in placental cells the CREs cooperate with the tissue-specific upstream regulatory element (URE) and additional downstream regulatory elements. The URE consists of adjacent binding sites for two placental-specific factors, (a) *TSEB*, it requires CRE activity and binds to the trophoblast-specific element (TSE) present between -182 and -159 base pairs and; (b)*α-ACT,* lies between -161 to -142 base pairs and contributes independently to both basal expression and cAMP regulated expression of the α gene promoter and can function independently of the CREs. [11, 20].

Sex steroids and glucocorticoids can negatively regulate gonadotropin gene expression. Glucocorticoid – stimulated repression of α subunit gene expression was initially thought to be mediated by the interaction of the glucocorticoid receptor (GR) with GR-binding sites that overlap the CREs in the α subunit promoter. It was postulated that binding of GR to the α subunit promoter interfered with the binding of factors important for promoter activity by competing for promoter binding sites. [22]. Subsequent studies have shown that the glucocorticoid-mediated repression is dependent on transcriptional activation of the α-subunit promoter by CREB and independent of GR binding to the promoter [23]. Conversely, over expression of CREB interferes with GR-mediated transcriptional activation of the MMTV promoter by glucocorticoids. These results are in agreement with previous finding that inhibition of α subunit gene transcription by oestrogen is independent of oestrogen binding site in the promoter [24].

The α-subunit gene expression regulation in the *pituitary* has been studied in transgenic mice and cultured pituitary cells. Maximal transcription of the human α gene promoter in pituitary cells requires sequences extending out to approximately -450 base pairs, that includes a conserved element (termed GSE) from -223 to -200 base pair, which binds a gonadotrope-specific factor (GSEB-1); but does not require the α-ACT, or TSE motifs [21, 25, 26, 27]. Concerning the activity of the CRE motif in gonadotrope cells conflicting evidence exists, the bovine α gene promoter(lacks a CRE) is actively transcribed in the pituitary of transgenic mice, whereas the deletion of the tandem CREs from the human promoter abolishes its activity in the αT3-1 cells [21,27].

The comparative and functional analysis of α gene promoter from different species is the basis for study of placental expression [21, 28]. The differences in the nucleotide sequence of α gene promoter account for the absence of placental expression of the α subunit gene in most mammals, including bovine [21]. It has been proposed that the absence of functional CRE from the bovine, murine, and rat α gene promoters is responsible for their inactivity in placenta. This was supported by the observation that the alteration of single base pair to generate functional CRE site increased transcription of the bovine promoter in placental cell [21]. This view has been challenged by recent observation that functional CRE and TSE motifs are absent from the equine α gene promoter, which, nonetheless, is placentally expressed and responsive to cAMP [28]. Instead, these activities require the presence of α-ACT binding sites, which are absent from murine α gene promoter. These results suggest that placental α gene expression and cAMP responsiveness require the presence of either the combined CRE-URE motif (as in the human promoter) or α-ACT element (present in the human and equine promoters). It appears that independent evolutionary changes have resulted in the acquisition of distinct, yet functionally redundant, sets of regulatory elements in the α gene promoters from primates and horses.

The FSH β subunit is encoded by a single gene as isolated from the human, bovine, pigs and rats. In sheep there appear to be two distinct FSH-β genes; however, it is not known if both genes are expressed [19]. The genomic FSH-β genes in each species, like the other β subunit gene, are evolutionarily conserved and contain three exons and two introns. Among the glycoprotein hormone β subunit genes, the FSH-β gene is unique for its rather long 3' untranslated region (Fig. 4). It was recently shown that a 10-kilobase region containing 4 kilobases and 2 kilobases of 5' and 3' flanking sequences, respectively, were sufficient for pituitary-specific expression of the human FSH β gene in transgenic mice [30, 31]. Ciccone *et al.,*(31) characterized a major GnRH-responsive element within the proximal FSH-β gene, which contains a partial cAMP response element (CRE)/AP1 site in LβT2 cell lines. GnRH responsive element appears promiscuous with the ability to bind the bZIP transcription family member, CREB, as well as members of the AP1 family, such as c-Fos and c-Jun. GnRH stimulates FSH-β transcription by inducing phosphorylation of promoter-bound CREB, leading to the recruitment of the histone acetyltransferase CREB binding protein (CBP) and allowing for the tethering of the basal transcriptional machinery. However, the role of this GnRH-responsive site in the differential stimulation of FSH-β transcription by GnRH pulse frequencies still remains to be determined. A possible mechanism is the increased activation of repressor(s) of CREB/AP1 transcription factors at high GnRH pulse frequencies that cause a reduction in FSH-β gene expression (Fig. 7). Potential transcriptional repressors that may fulfil such a role include CCAAT/enhancer-binding protein β and inducible cAMP early repressor (ICER).

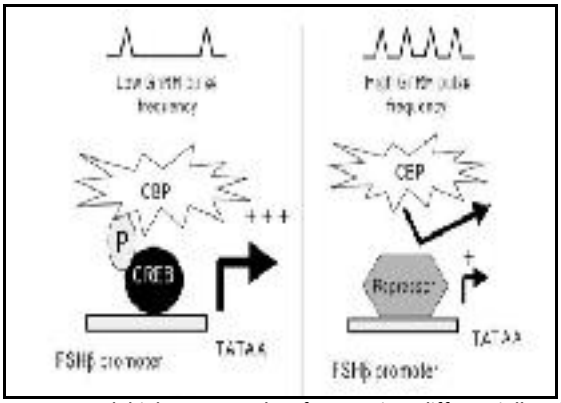


Fig.7: Low and high GnRH pulse frequencies differentially alter gonadotropin β-subunit transcription through recruitment of histone acetyl transferases (HATs) and induction or modification of transcription factors [Taken from 31].

Biochemical Aspects:

To study the FSH molecule, it is first necessary to obtain a purified preparation with which all FSH activity may be compared. Many techniques have been employed to separate the FSH molecule from other anterior pituitary proteins. Those techniques generally include an initial ethanol, acetone or ammonium sulphate precipitation step, followed by ion exchange chromatography (separation of proteins by overall charge) and gel filtration (separation by molecular weight) [32]. The intact hormone has a molecular weight of approximately 30,000. Following purification, the bonds that connect the FSH α and β subunits may be broken by treatment with the denaturant, urea. The free subunits may be isolated by ion exchange chromatography.

Once the genetic controls of the pituitary hormones initiate transcription of the required mRNA, protein translation occurs on the polysomes bound to the rough endoplasmic reticulum (RER). The completed linear subunit from this synthetic step carries a 'signal peptide' and has no formed disulphide bonds. The signal peptide (also called the leader sequence) at the N-terminus of the subunit precursor is characteristic of the particular subunit. For example, the α-subunits currently known all have a 24-residue leader sequence (Fig. 1), with a high homology among the species. The known FSH-β leader sequences have 18 residues for the known cases (Fig.2) but these have sometimes been reported as 19 or 20 residues because of uncertainties about the exact cleavage point of the signal protease. The correct cleavage site is shown to be between cysteine and asparagine, arginine or histidine (see Fig.2 for the FSH-β subunit leader sequences).

The signal peptide which may range from 15 to 40 amino acid residues, characteristically has a very hydrophobic cluster of amino acids 5 to 20 residues toward the N-terminus from the site of cleavage (processing) by the signal peptidase. This step of the protein processing probably occurs co-translationally at about the time the C-terminal portion of the peptide chain coded by the mRNA is being completed. Thus, the signal peptide removal marks the removal of the completed protein chain from the ribosomes and release of the protein into the cisternae space of RER.

The signal peptide removal in the RER is probably involved in the transfer mechanism from the RER cisternae along the membrane to the Golgi apparatus (GA).This signal peptide removal is probably the final step in the co-translational processing that occurs in RER. However, for the glycoprotein hormones, there is another important co-translational event (*N*-glycosylation) that occurs in the RER. *N*-Glycosylation takes place at two sites on the α-subunit and one or two sites on the β-subunit (Fig.8).

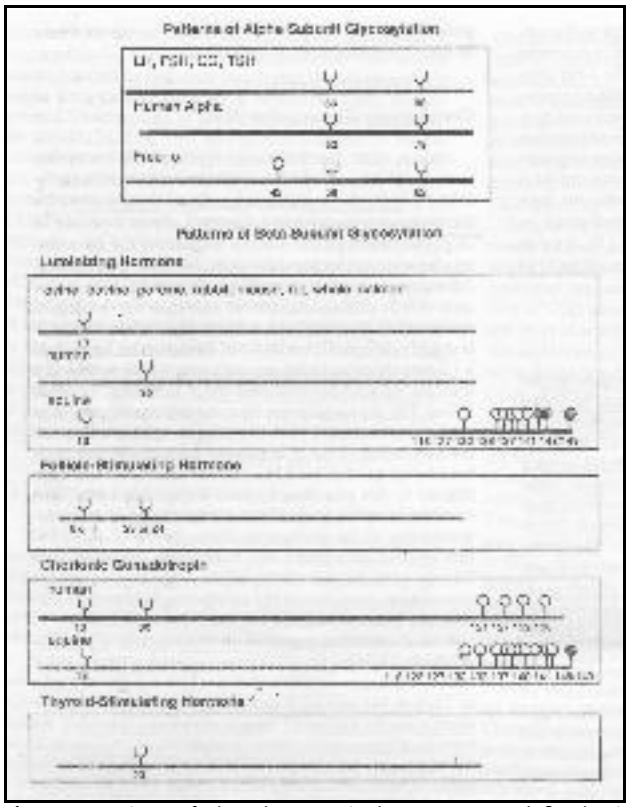


Fig. 8. Locations of the glycoprotein hormone α and β subunit glycosylation sites. The subunit proteins are indicated by solid bars. The *N*-linked oligosaccharides are indicated with dibranched symbol, and the "lollipop" indicates *O*-linked oligosaccharides. The number under each glycosylation site indicates the amino acid residue to which the oligosaccharide is attached [2].

N-glycosylation (Fig. 10) is mediated through a "high-mannose" dolichol phosphate intermediate, enzymatically targeted to specific *N*-glycosylation sites. Not all sites are glycosylated in those proteins that contain this structural feature, but in the glycoprotein hormones all potential *N*-glycosylation sites are usually glycosylated. Exception includes partial glycosylation of some FSH-β subunits.

The Carbohydrate moiety transferred from the dolichol phosphate intermediate has the general composition glucose$_3$ mannose$_9$ *N*-acetylglucosamine$_2$. The two *N*-acetylglucosamine residues form the link to the asparagine amine nitrogen in the *N*-glycosylation site. On the nonreducing end of this characteristic disaccharide attachment to the asparagines nitrogen amide is a mannose residue linked through position 1 to position 4 of the last *N*-acetylglucosamine residue. This mannose residue, in turn, is linked (through positions 3 and 6) to the two additional mannose residues. This generates a "biantennary" structure. In some of the more complex carbohydrate structures associated with the glycoprotein hormones, a "triantennary" branching is required. Whether a biantennary or triantennary carbohydrate will be produced in the "mature" glycoprotein will be determined by the Golgi apparatus, but a structure that provides for this possibility is contained in the high-mannose form or precursor form generated in the RER. Characteristically the three glucose residues in a linear array are attached to the longest mannose chain on one arm of the trianternnary high-mannose precursor from dolichol phosphate. While the glycoprotein is still in the RER the three glucose residues are removed; α-glucosidase I removes the terminal glucose residues, then α-glucosidase II removes the remaining two glucose residues.

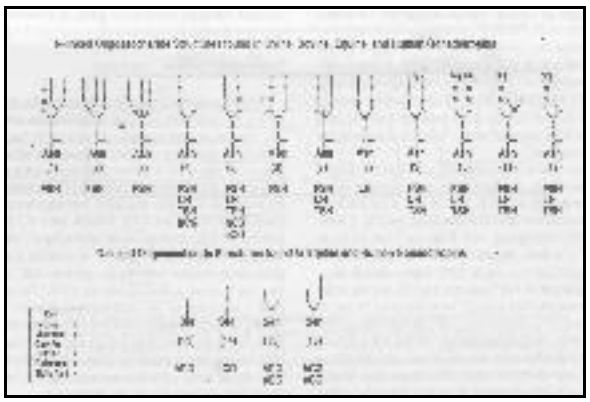


Fig.9: Oligosaccharide structures determined for the glycoprotein hormones. Variations in the structure shown here have been characterized and result from the absence of fucose residues or from the absence of one or more of the sulphate or sialic acid residues [2].

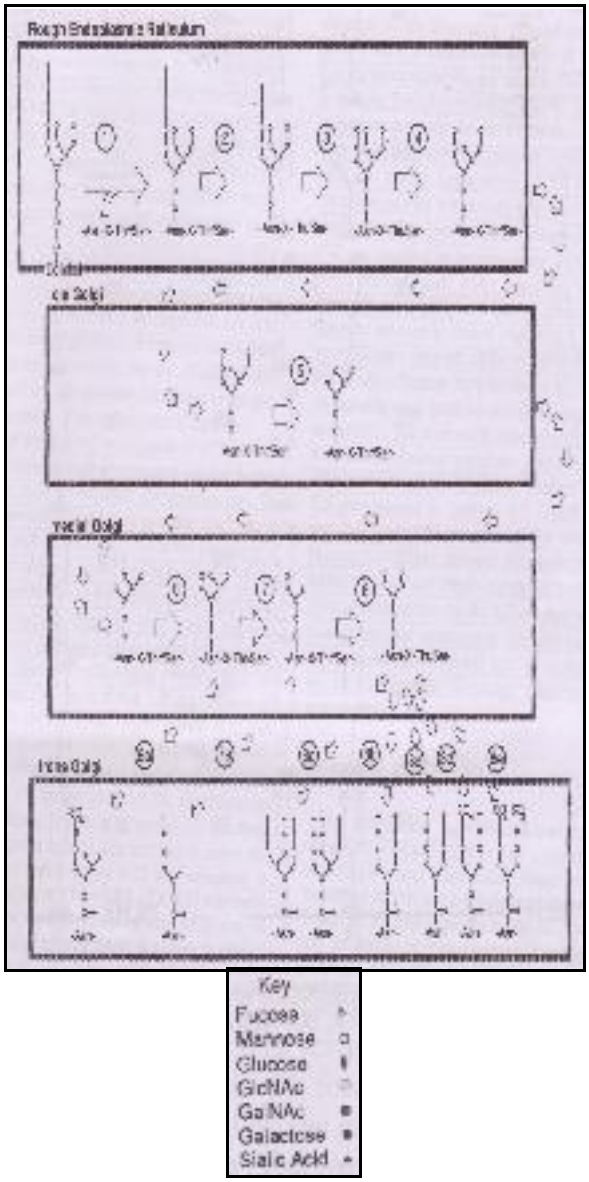


Fig.10: Biosynthetic pathway for *N*-linked oligosaccharides leading to completed *N*-linked oligosaccharide structures shown in Fig. 9. This is based on the pathways for N-linked glycosylation and pathways suggested by structures on glycoprotein hormones. **1:** Oligosaccharidase-catalyzed transfer of dolichol-linked $Glc_3Man_9GlcNA_2$ oligosaccharide to asparagines at glycosylation site: -Asn-Xaa-Ser/Thr-. **2:** α-Glucosidase I removes terminal glucose. **3:** α-Glucosidase II removes remaining two glucose residues. **4:** Endoplasmic reticulum α-1, 2-mannosidase removes one mannose residue. **5:** Golgi α-mannosidase I removes another three mannose residues, producing the precursor for *N*-acetylglucosamine transferase I. **6:** *N*-acetylglucosamine transferase I adds *N*-acetylglucosamine, creating precursor for S-1 type having only one completed branch or leading to continued processing. (**a**) Addition of *N*-acetylglucosamine diverts processing to complex bi- or trianternnary oligosaccharide, resulting in this single-branch sulphated structure. Signal for GalNAc transferase recognition appears to be –Pro-Xaa-Arg/Lys-. **7:** Golgi α-mannosidase II removes two mannose residues. (**a**) Addition of *N*-acetylglucosamine could lead to this structure. **8:** *N*-acetylglucosamine transferases II addsa a second *N*-acetylglucosamine, leading to further processing, which produces the following oligosaccharide structure: (**a**) triantenarry sialylated; (**b**) biantennary sialylated; (**c**) disialylated, as a result of α2-6GalNAcβ1-aGlcNAc sialyltransferase in human pituitaries; (**d**) hybrid sialylated-sulfated; (**e**) disulfated.

Post-translational Events:

Another major development that occurs in the RER is the formation of disulphide bonds in the α and β subunits. Ruddon and colleagues have demonstrated that formation of all the α-subunit disulfide bonds and formation of most of the β-subunit disulfide bonds precedes subunit association. This is consistent with the Pierce-Bahl model [33,34] for α subunit disulfides in which most of the disulfide bonds stabilize local interactions in either the *N*-terminal domain (11-35 and 14-36) or the *C*-terminal domain (63-91 and 86-88) and therefore, probably form co-translationally. Only one disulfide bond (32-64) connects these two domains. In contrast, the Ward-Bahl model for β-subunit disulfides proposes that four of the six disulfide bonds connect Cys residues that are in opposite halves of the subunit (9-90, 23-72,26-100 and 34-88), whereas only two disulfide bonds stabilize local regions of the subunit (38-57, 93-100) [35, 36].

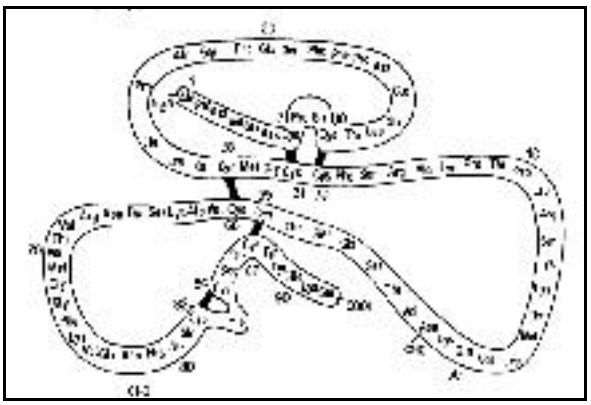


Fig.11. Primary sequence of hCG-α showing locations of the disulfide bonds [34].

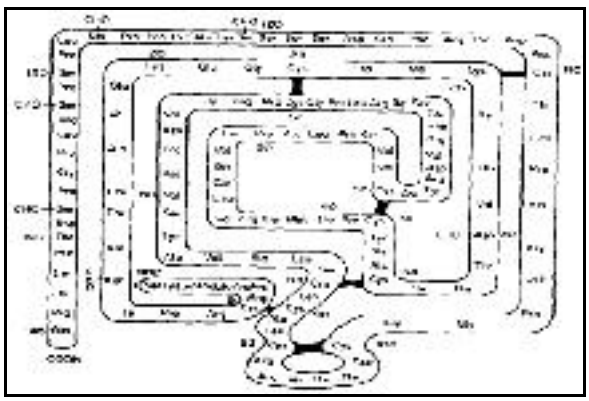


Fig.12. The amino acid sequence of hCG-β showing location of disulfide bond [36].

The combination of α and β subunits is initiated in the RER with the high mannose forms of the subunits. It is not yet clear whether all of the β subunit disulfide bonds are formed here or if they are completed in the Golgi. Ruddon and co-workers have detected discrete intermediates in this process. The final step in the RER processing of the glycoprotein hormone subunit is probably one of loading (via the signal peptidase and its postulated chain of events) into transport vesicle involved in the movement of the partially processed "high-mannose" carbohydrate-bearing subunit.

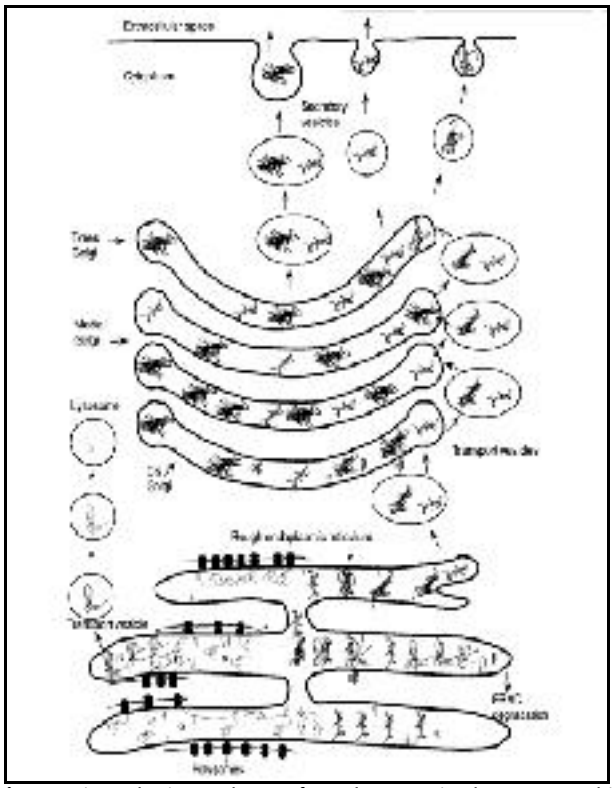


Fig.13: Biosynthetic pathway for glycoprotein hormones. This pathway is depicted, since most of the available data has been obtained from hCG in which only the constitutive secretory pathway is operative. The subunits are synthesized in the RER, where *N*-glycosylation occurs co-transcriptionally. Folding of the α-subunit probably occurs co-translationally as well. Folding of the β-subunit occurs post-translationally and appears to be completed after association with the α-subunit. Improperly or partially folded β-subunit is retained in the RER and eventually shunted to lysosomes, where it is degraded. Hormone dimer, properly folded free β subunit are transferred to the Golgi, where their oligosachharides are processed before secretion [2].

After processing the carbohydrate of the α-β dimer in the Golgi to the several forms of complex carbohydrate (mature hormone), the hormone is transported to storage vacuoles in the cytoplasm (pituitary cells) or directly to the cellular exterior (human chorion cells). The degree of sulfation among the pituitary hormones varies widely depending on the species of the pituitary cell donor and the type of hormone [2].

Regulation of Biosynthesis and Secretion:

The intracellular processes involved in the biosynthesis, described above, lead to secretion of FSH. However the secretory pattern of FSH is not uniform. Hence some of these biochemical events must be regulated to alter the secretory pattern. Details are not known. However many endocrine interactions occur at the level of the hypothalamus and anterior

pituitary gland to influence the rate of FSH biosynthesis and/or secretion. A few are examined below.

Pro-estrus gondatoropin surge:

A four-day cycling rodent exhibit one LH surge and a biphasic release of FSH. During the first 2 days following ovulation the serum concentrations of both gonadotropins remain at low levels, and exhibit only minor diurnal variations (day 2 and 3 in Fig.14). Follicular growth and serum estradiol increases, during this period. Around early afternoon of the 4th day (proestrus), a dramatic increase in the serum concentration of both LH and FSH occurs. Surge-like release of hypothalamic GnRH into the pituitary portal circulation induces pre-ovulatory surgesof LH and FSH. GnRH binds to specific receptors on the surface of gonadotroph of the anterior pituitary gland and stimulates the secretion of LH and FSH.

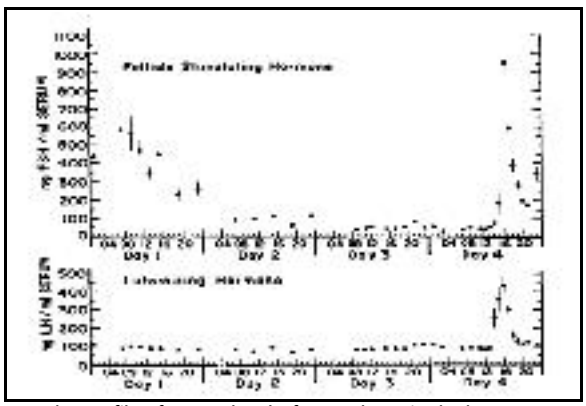


Fig.14: The profile of serum level of LH and FSH in the hamster throughout the estrous cycle [taken from 18].

There is a direct neural connection between the optic nerves and the suprachiasmatic nucleus of the hypothalamus. Thus, a "neural clock", located in this hypothalamic nucleus seems to be responsible for the timed releases of GnRH. A daily neural signal is transmitted to the GnRH-containing neurons whose cell bodies are located in the preoptoc area, anterior to the hypothalamus. Following activation, these neurons release GnRH from their terminals located in the median eminence. A specialized capillary network located around this circumventricular organ picks up and delivers GnRH to the pituitary gland [37]. It is well established that estrogen plays a critical role in the cyclic discharge of hypothalamic GnRH and therefore gonadotropins. As ovarian follicles mature during diestrous day 1 and 2, estrogen is secreted into the general circulation. This steroid interacts with estrogen-receptor bearing neurons within the medial preoptic-suprachiasmatic area (POA-SchN). After a period of exposure to a critical level of estradiol, an increase in the multiunit firing rate of these neurons is observed [18].

Feedback action of gonadal steroids and Inhibin:

Preoptic-suprachiasmatic area (POA-SchN) has been termed the cyclic centre, due to its role in the cyclic, estrogen induced gonadotropin surge. It is well known that electrical stimulation of the POA-SchN region of the hypothalamus will elicit a proestrus-like surge of LH and FSH (Fig. 15). It has also been demonstrated that during proestrus, electrical stimulation of the dorsal anterior hypothalamic area (DAHA) will elicit a release of FSH from the anterior pituitary gland. There is reduction in serum FSH levels during in animals with bilateral lesions of the DAHA. These findings suggest that both the POA-SchN region and the DAHA are responsible for full expression of the LH and FSH proestrus surge in rodents [18].

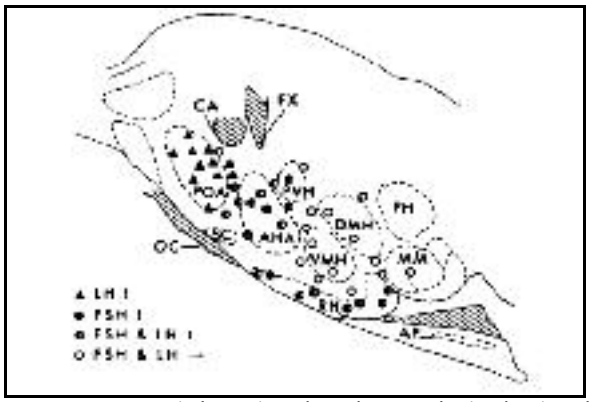


Figure.15: A parasagital section through a rat brain showing the location of areas which, when electrochemically activated, stimulated the release of one or both gonadotropins [18].

Many times during the cycle (except proestrus after noon), estrogens act at the level of the hypothalamus and pituitary to suppress LH and FSH release. This negative feedback action involves the neurons in medial basal hypothalamus that regulate the basal "tonic" secretion of GnRH and therefore gonadotropins. This tonic gonadotropin release is indispensible for the normal development of ovarian follicles. The negative feedback actions of estradiol may be explained through a rapid membrane phenomenon: influencing perhaps neuronal ion fluxes to hyperpolarize neurons or by decreasing neurotransmission by the inhibition of tyrosine hydroxylase activity. Conversion of estrogen to catecholestrogens may be responsible for this event. Negative feedback occurs rapidly and doesn't require protein synthesis [18].

Progestrone also induces pre-ovulatory gonadotropin surge. It is well established that progestrone's effectiveness depends upon prior exposure to estrogen and that the mode of action of estrogen in this CNS priming mechanism involves the induction of progesterone receptors in the hypothalamus. Estrogen treatment of ovariectomized rats is effective in inducing a recurring pattern of

gonadotropin releases every 24 hr for approximately 6 days [6]. If progesterone is administered in the 24 hrs later, a temporal advancement inand an increase in the surge magnitude occurs. At the same time, subsequent daily gonadotropin surges are abolished. So, Progestrone appears to play both a positive, organizing role, with respect to the pre-ovulatory endocrine events as well as an inhibiting one with respect to the hypothalamic "memory" induced by estrogen. These observations have led to the proposal that progesterone might be the endocrine factor that limits the LH and FSH pre-ovulatory surges to 1 day of the cycle [38].

GnRH plays *no* important role in the second (estrus) phase of FSH release in the rat. Removal of all GnRH stimulation of the pituitary gland of ovariectomized rats by administration of a potent GnRH antagonist reduced LH secretion by 90% but FSH secretion by only 60%. It appears that the pituitary gland, following initial activation, may autonomously secrete FSH in the presence or absence of hypothalamic LHRH (Fig.16). Thereafter, FSH secretion is apparently regulated only by inhibitory input arising from the developing ovarian follicle. Thus an interesting level of control may be envisioned: FSH secretion during estrus begins as a result of neuroendocrine (GnRH-dependent) events that occur during proestrus. The stimulus for the extended FSH release during estrus appears to involve the exposure of animals to proestrus levels of gonadotropins [39, 40]. After an initial stimulation during proestrus, FSH secretion may become GnRH independent during estrus [41]. As FSH secreted during estrus stimulates ovarian secretions, these factors (estradiol, inhibin) may feedback upon the pituitary to reduce future FSH release. Thus follicular maturation is assured.

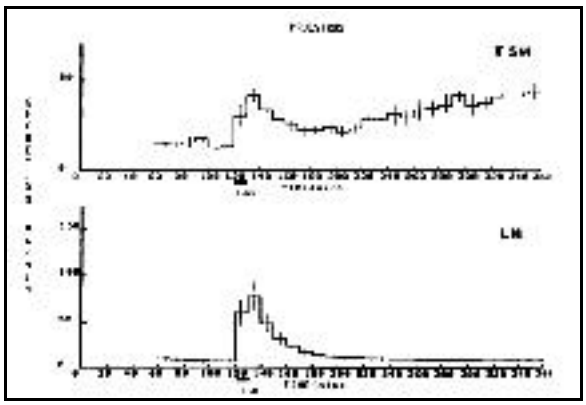


Fig.16: Release of LH and FSH from pituitaries superfused *in vitro* following exposure to one 10 min pulse of LHRH. Note the continuous release of FSH as LH secretory rates fall [41].

Another factor that has been implicated in the selective regulation of FSH secretion is inhibin. Inhibin (which is found in follicular fluid) exposure causes a reduction in pituitary FSH but not LH secretion. Although the sites of action appears to be the anterior pituitary gland as assessed by both *in vivo* and *in vitro* studies. It has been suggested that decreasing inhibin titer during proestrus provide the proper hormonal milieu for the induction of the selective increase in serum FSH during proestrus evening and estrus. This suggestion is strengthened by experimental observations of DePaolo *et al.*,(42) who demonstrated a reciprocal relationship between ovarian plasma inhibin levels (as assayed by bioassay) and serum FSH concentration (Fig. 17). Since the source of inhibin within the ovary appears to be the granulosa cells and the number of these cells increases as the follicle matures, and interesting level of control may be postulated. Rising levels of inhibin (and perhaps estradiol) may signal the maturation of the next cohort of follicles late during estrus and act the level of the pituitary to reduce FSH secretion.

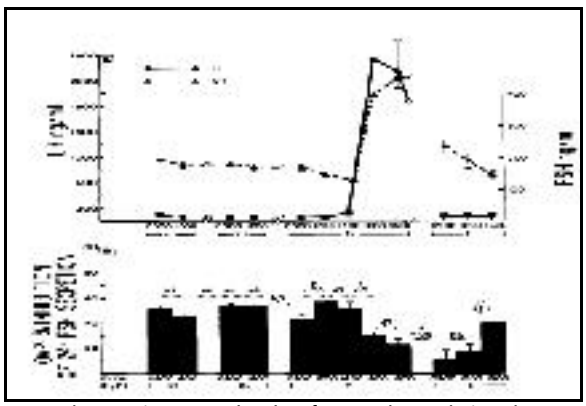


Fig. 17. Changes in serum levels of LH and FSH during the estrous cycle of the rat (Panel A). Also shown (Panel B) is the change in the inhibin activity of ovarian venous plasma (OVP) during the same period. Note the reduction in inhibin activity late during proestrus and during the morning of estrus, when serum FSH levels are elevated [Taken from 42].

Other Regulators:

Hypothalamic-hypophysiotropic peptides are the proximate regulators of pituitary cells, but they cannot fully account for the complex functioning of these cells. There are arrays of peptides produced in the pituitary that exert paracrine/autocrine functions. One such peptide, pituitary adenylate cyclise-activating polypeptide (PACAP), was originally identified as a hypothalamic activator of cAMP production in pituitary cells. Gonadotrophs and folliculostellate cells are the main source of pituitary PACAP, and each pituitary cell types expresses a PACAP receptor. PACAP increases alpha-subunit (*Cga*) and *Lhb* mRNAs, and it stimulates the transcription of follistatin (*Fst*) that, in turn, restrains activate signaling to repress Fshb and gonadotropin-releasing hormone-receptor (*Gnrhr*) expression as well as other activin-responsive genes. The PACAP (*Adcyap1*) promoter is activated by cAMP, and pituitary cells may communicate by a feed-forward, cAMP-dependent mechanism to maintain a high level of PACAP in the fetal pituitary. At birth, pituitary PACAP

declines and pituitary follistatin levels decrease, which together with increased gonadotropin-releasing hormone secretion allow *Gnrhr* and *Fshb* to increase and facilitate activation of the newborn gonads. Changes in Adcyap1 expression levels in the adult pituitary may contribute to the selective rise in FSH from age 20-30 days to the midcycle surge and to secondary increase in FSH that occurs before estrus. These results provide further support for the notion that PACAP is a key player in reproduction through its action as a pituitary autocrine/paracrine hormone [43].

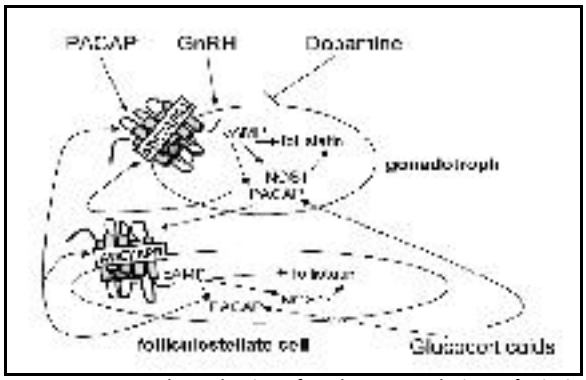


Fig. 18. A proposed mechanism for the up-regulation of pituitary *Adcyap1* expression through cAMP signaling in gonadotrophs and folliculostellate cells. PACAP stimulates ADCYAP1R1 to increase cAMP production, which induces *Adcyap1* and *Adcyap1r1* as well as *Nos1* and *Fst* gene expression. PACAP secreted by gonadotrophs may activate folliculostellate cells, which likewise secrete PACAP that stimulates gonadotrophs. Dopamine, GnRH, central nervous system PACAP, and glucocorticoids may influence *Adcyap1* expression. nNOS, neuronal NOS [43].

We know that the FSH-β subunit is limiting factor for production of mature hormone and provides biological specificity. Activin dramatically induces FSH-β transcription, and the secondary rise in FSH, important for follicular development, is dependent on this induction. Thus, regulation of FSH-β levels by activin is critical for female reproductive fitness [44].

Activin is a dimer of two subunits. There are multiple β isoforms: βA, βB, βC and βE. βA and βB, which share 65% sequence identity but are differentially espressed, form dimmers known to have physiological roles. Thus, activin A is a homodimer of βA subunits and activin B contains two βB subunits. βA and βB can also form heterodimers. Both activin A and B can induce FSH-β gene expression in immortalized gonadotrope cells [45]. Activin binds to thr type II receptor A (ActRIIA) on the surface of gonadotrope cells, which in turn results in the recruitment and phosphorylation of the type I receptior, ALK4. Activin B binds with higher affinity to ActRIIB receptors, which selectively recruit and activate ALK7 [46, 47].. All of these receptors are expressed in pituitary gonadotrope cells. ALK4 and ALK7 are likely to be functionally interchangeable in the gonadotrope cells, since both receptors phosphorylate Smad2 and Smad3 [48].

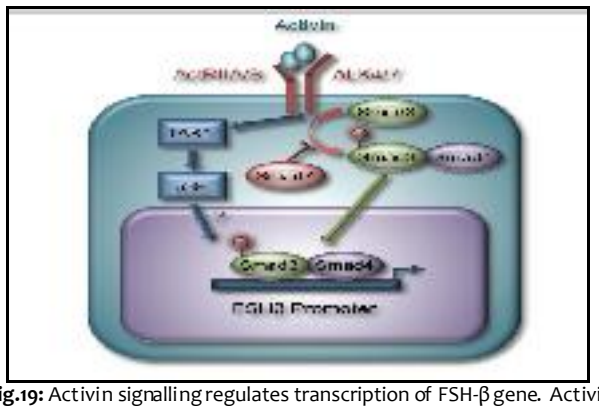


Fig.19: Activin signalling regulates transcription of FSH-β gene. Activin signalling, via ActRIIA/B and ALK4/7 receptors, results in the phosphorylation of Smad2 and 3, which then dimerize with the common Smad4 and translocate into the nucleus of the cell. Activated Smad3, and potentially Smad2, regulate transcription of FSH-β and other specific target genes. The inhibitory Smad7 prevents the phosphorylation of Smad3 [44].

Follicle-Stimulating Hormone Receptor (FSHR):

The human FSH receptor (FSHR) is a glycosylated, heptohelical G-protein coupled receptor, with a large extracellular domain, a seven transmembrane domain, three intracellular and three extracellular loops and an intracellular tail (Fig. 20) [49]. This 695 amino acid glycoprotein is encoded by a 54 kb gene on chromosome 2q p21, which contains ten exons [50]. The first nine exons, ranging 69-251 bp, encode the extracellular domain, while the last exon of 1234 bp encodes the entire transmembrane domain and intracellular domain of the receptor. The FSHR is expressed in the granulose cells in females and sertoli cells in males.

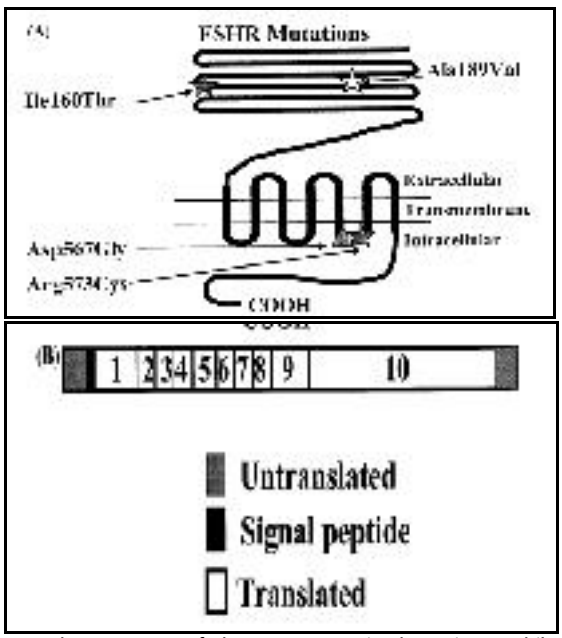


Fig. 20. The structure of the FSHR gene is shown in A. While the protein with location of reported human mutations is shown in B [51].

Mutations in FSH beta-subunit and receptor genes:

Our knowledge of FSH-receptor signalling system has been enhanced by the identification of several human mutations and the generation of genetically engineered mice with various alterations of the endogenous genes for the FSH ligand and the FSH receptor (FSHR).

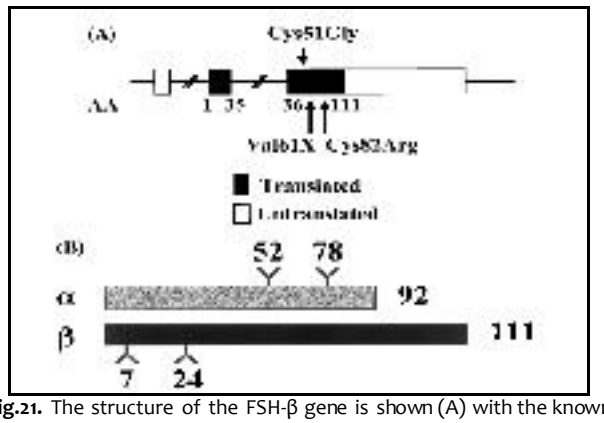


Fig.21. The structure of the FSH-β gene is shown (A) with the known human mutations indicated; (B) the FSH dimer is hown, with the location of *N*-linked glycosylation sites (Y). The α-subunit is 92 a.a. and the β subunit is 111 a. a. [51].

FSH-β knock-out mouse:

a) Female (fshβ -/ fshβ -)

Using standard embryonic stem (ES) cell methods, fsh-β knock-out mice were generated using an inbred starin of mice (129SvEv) (table 1) [30]. Mice heterozygous (fshβ+/fshβ-) for the loss of FSH-β ligand had normal estrous cycle and fertility, similar to homozygous normal mice (fshβ+/fshβ+). Homozygous knock-out female (fshβ-/fshβ-) demonstrate lack of estrous cycle, sterility, and low levels of FSH.

b) Male (fshβ -/ fshβ -)

FSHβ deficient male mice had normal sexual development (Table1) [30]. The serum levels of LH in fshβ-/fshβ- mice were similar to control males. The principle functional difference between male and female knock-oout mice is fertility. The FSH-β deficient male is fertile, in spite of a decrease in testicular size, and a 75% reduction in sperm number. Microscopic examination of the testes reveals a relative diminution in all testicular.

FSHR knock-out mouse:

The FSHR knock-out mouse displays similar characteristics to the FSH-β knock-out mouse with sterility in females and oligospermia with reduced fertility in males (Table1). FSH-β knock-out mice (both sexes) have very low FSH levels, but in contrast to this, female FSHR knock-out mice have (15-fold) elevated FSH levels, while the male FSHR knock-out mice have (3-fold) elevated FSH (52). Interestingly, the expression of FSH-responsive genes, cyclin D2 in females and cAMP responsive element modulator (CREM) in males (necessary for spermiogenesis) is unaltered.

a) Female homozygous FSHR knock-out mice demonstrated primordial, primary and secondary follicles, but no mature follicles were seen, indicating that impaired follicular maturation causes the infertility. And thus, the decreased size of ovary in these FSHR knock-out female is caused by the lack of large Graafian follicle and by absence of corpora lutea caused by ovulatory failure.

b) Males have small seminiferous tubules and normal accessory structure similar to the male FSH-β knock-out mice. Sperm analysis indicates significant decrease in cell number and motility and a percent increase of aberrant spermatozoa (Table 2). Affected sperm had abnormal and bent tails with cytoplasmic droplets. Also it was seen that lack of FSHR may influence the structural and functional organization of the basement membrane, leading to disarray in spermatogenesis (Fig. 22) [53].

Table.1: Summary of FSH-β and FSHR knock-out mice and transgenic mice [Taken from 51]

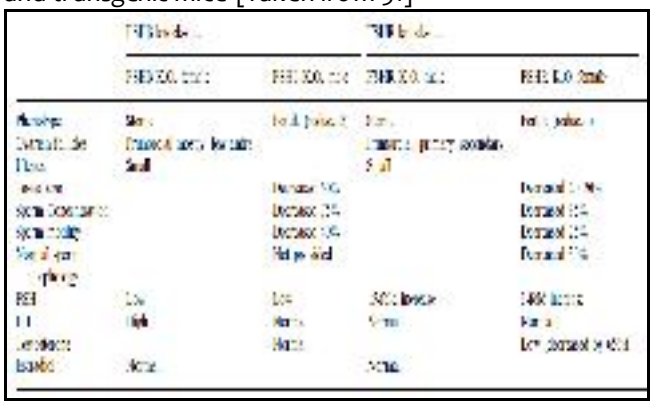


Table 2: Sperm count and motility [53]. Sperm were collected from epididymis and counted with haemocytometer.

	+/+	−/−
Spermatozoa/mouse	3.6×10^8	3.6×10^8
Motility, % moving spermatozoa	62	47
Aberrant structure, %	18	47

Human FSH-β gene mutation:

Only a small number of human patients with isolated FSH deficiency have been confirmed at the molecular level (Table 3). The first patient was a 27-year-old female, who had primary amenorrhea, low FSH, high LH [54]. Her breast development was absent at age 13, but she had previously been on a hormone replacement and at age 27 her breast development was described as 'poor'. She did conceive with exogenous gonadotropin therapy. Upon sequencing of the FSH-β gene, the proband was found to be homozygous for two base pair (GC) deletion at codon 61 (Val61X). Although not studied in vitro, this frameshift mutation

was predicted to change amino acids 61 to 86, then produce a stop codon, resulting in a truncated FSH-β protein lacking AA 86-111. Her heterozygous parents had a history of infertility prior to her conception, suggesting that perhaps heterozygous FSH-β gene mutations might produce infertility [54].

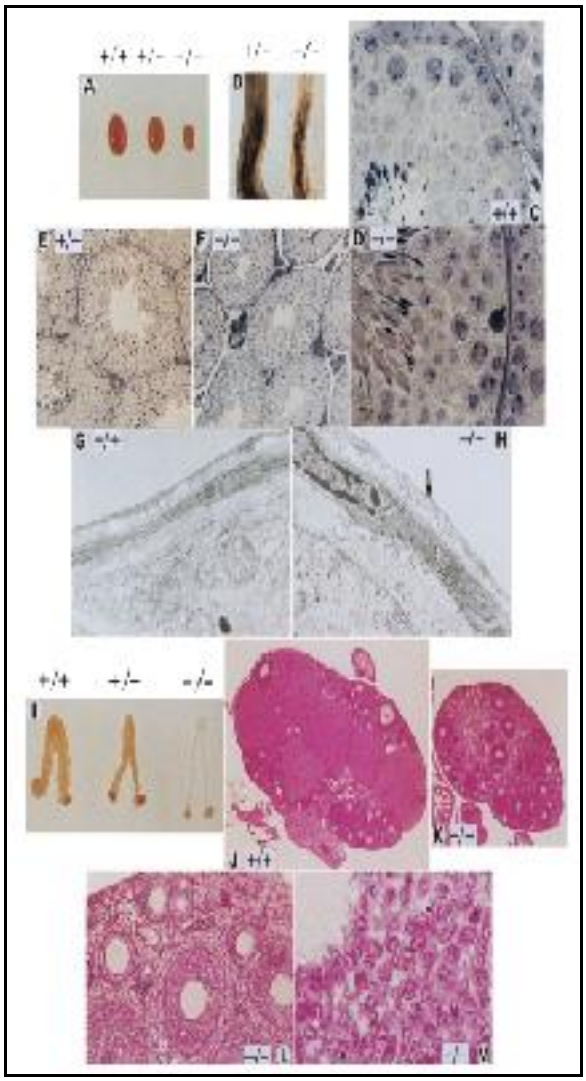


Fig. 22. Analysis of testis and ovary from FSH-R mutant mice. **A:** Testes comparision of 8 weel-old wild-type, heterozygous and homozygous littermates. **B:** Transilluminated seminiferous tubules (stage VII-VIII of the seminiferous epithelium cycle) dissected from wild-type and homozygous mice; note the smaller tubule diameter in the mutant as compared with the wild type. Mutant mice conserve a normal spermatogenic wave. Histological section at lower (**E**) and higher (**C**) magnification of seminiferous tubuli from a wild-type and mutant (**D** and **F**) mouse. (**G** and **H**) Electron micrographs of the basal membrane; note the altered organization of the collagen fibrils in mutant mouse tubules (arrow). (**I**) Reproductive tracts from 8 week-old littermates; ovaries and uteri from FSH-R mutant mice are smaller and thinner as compared with the wild type; a reduction in size is also evident in the heterozygous. (**J-M**) Histological sections of the ovary from wild type (**J**) and homozygous (**K**) mice [53].

A second female patient presented with absent breast development, primary amenorrhea, low FSH, undetectable estradiol and elevated LH level [55]. She was a compound heterozygote for two different FSH-β gene mutations, one being the same VAL61X, identified in the first patient inherited from her mother and the other a missense mutation in which Cys at codon 51 was changed to a Gly (Cys51Gly) (Fig.23).

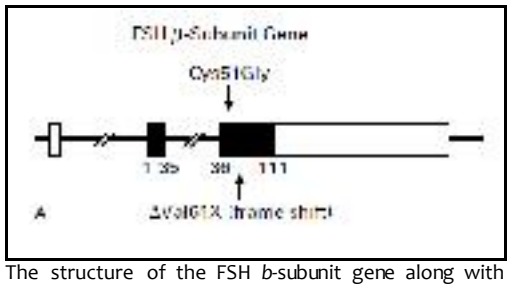


Fig. 23. The structure of the FSH *b*-subunit gene along with the locations of the mutations in exon 3 (arrows at codons 51 and 61). The amino acid numbers appear below the gene. The three exons are shown as rectangular boxes. The shaded regions refer to portions of the exons that are translated into protein, whereas the unshaded regions represent untranslated exonic sequences [Taken from 55].

Table.3: A summary of human FSH-β and FSHR gene mutation in males [taken from 51].

When mutants were studied *in vitro* in Chinese hamster ovary cells, both the Val61X and Cys51Gl resulted in immeasurable immunoreactive and bioreactive FSH level (Fig. 24) [55]. These in vitro data confirmed that phenotypic findings of the patient and indicated that FSH is necessary for pubertal development and fertility in females. Since five heterozygotes within the family were fertile, it seems likely that homozygous mutations only interfere with fertility [55]. These findings are in contrast with first described family [54].

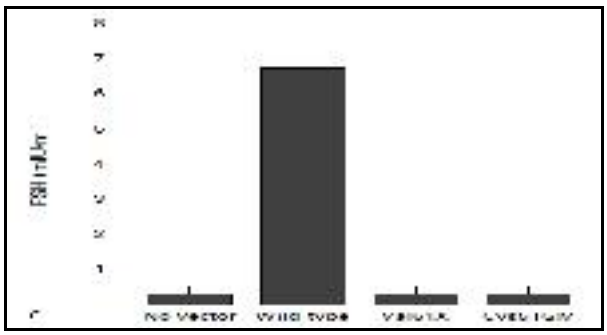


Fig. 24. This shows the FSH concentrations measured by immunoradiometric assay of the medium from cultures of Chinese-hamster-ovary cells stably transfected with each of the mutant FSH *b*-subunit genes (Val61X and Cys51Gly) and the wild-type gene [55].

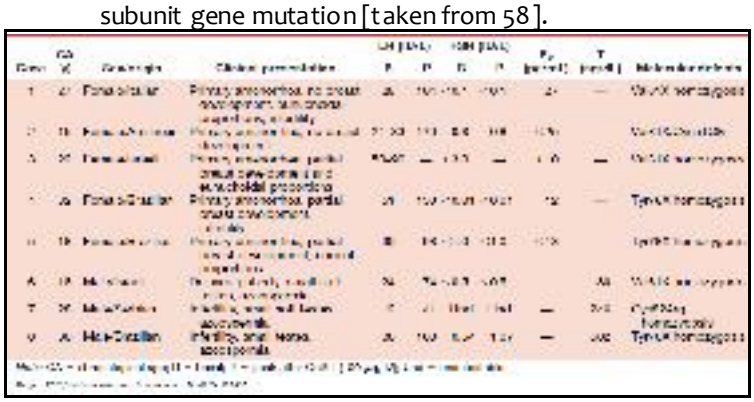
FSH-β mutation in males would be expected to result in a phenotype of normal pituitary (since LH-induced testosterone production should be normal), but there could be varying degrees of spermatogenic failure (Table 4). The first reported male with an FSH-β

gene mutation had exactly this presentation, with normal pubertal development with small testes (3 and 6 cc volume; normal 15-25 cc), a normal adult testosterone level, high LH and low FSH [56]. He was homozygous for a Cys82Arg missense mutation, which was predicted to interfere with dimer formation, but was not studied *in vitro*.

Table.4: A summary of human FSHβ and FSHR gene mutation in males [taken from Layman *et al.*, 2000].

A second case in male showed the phenotypic effects of a more severe FSH-β gene mutation [57]. Unexpectedly, this male had very small testes (1 or 2 cc testes) with no pubertal development, low testosterone, elevated LH and low FSH. He was homozygous for the Val61X [57] previously shown to be completely devoid of immune and bioactivity [55]. Severe mutations of FSH-β gene (Val61X) [57] are associated with delayed puberty and azoospermia, while less severe mutations (Cys82Arg) [56] do not affect puberty, but do result in azoospermia. These findings suggest that FSH is necessary in men for androgen and gamete formation [51].

Table.5: Clinical and hormonal data of five women and three men with selective FSH deficiency due to FSH β-subunit gene mutation [taken from 58].



Human FSH Receptor mutation:

The first reported FSHR gene mutations were identified in Finnish kindreds with 46, XX ovarian failure (XXOF) (Table 3). In Finland the church were investigated for families containing women with primary amenorrhea, elevated gonadotropins and normal chromosomes [59]. Positional cloning was performed and strong linkage with chromosome 2p was observed [60]. The FSHR gene was known to reside on chromosome 2p and was found to be in linkage disequilibrium with XXOF [60]. In about one-third of all the Finnish XXOF patients studied, a homozygous Ala189Val missense mutation was identified [60].

When the mutation was created by site directed mutagenesis and transfected with MSC-1 Sertoli cells (Mouse Sertoli Cell), the binding affinity was similar to the wild type receptor (K_a=6.7 x 10^9 mol/L for wild type and 4.8 x 10^9 mol/L for the mutant), but the binding capacity was markedly suppressed, being only 3% of those cells with the WT [60]. In addition, cAMP level was drastically reduced in immortalized MSC-1 Sertoli cells, in response to recombinant FSH. Cells expressing the WT receptor caused 3-4 folds increase in cAMP above baseline, while the cells expressing the mutant failed to increase cAMP. These studies demonstrated that receptor number is drastically reduced and that signal transduction of the receptor is impaired

SUMMARY

Follicle-Stimulating Hormone (FSH) is a glycoprotein synthesized and secreted by the anterior pituitary gland. It consists of two dissimilar, glycosylated, non-covalently linked polypeptide chains. These chains are attached by several bonds which stabilize the molecule's tertiary structure. The structure of alpha (α) subunit of FSH is common to all three pituitary glycoproteins, as well as the placental glycoprotein, human chorionic gonadotropin. The beta (β) subunit, however, endows each molecule with its biological specificity. FSH is essential for normal gametogenesis. In females FSH is required for ovarian development and follicular maturation whereas in males FSH determines Sertoli Cell number and quantitatively and qualitatively normal spermatogenesis.

The secretion of FSH is known to be known to be regulated by an array of factors. One of the most important factor is GnRH (Gonadotropin Releasing Hormone). GnRH binds to specific receptors on the surface of the gonadotroph cells of the anterior pituitary gland and stimulates the secretion of LH and FSH. It is believed that there is a 'neural clock' in the brain which is responsible for timed release of GnRH. The electrical stimulation of the Preoptic-suprachiasmatic area (POA-SchN) of the hypothalamus elicits a proestrus-like surge of LH and FSH (Fig. 15). It has also been demonstrated that electrical stimulation of the dorsal anterior hypothalamic area (DAHA) will elicit a release of FSH from the anterior pituitary gland during proestrus. Progestrone is also known to regulate the FSH secretion. Inhibin (which is found in follicular fluid) exposure causes a reduction in pituitary FSH but not LH secretion. Other regulators include pituitary adenylate cyclase-activating polypeptide (PACAP), which was originally identified as a hypothalamic activator of cAMP production in pituitary cells, and Activin which dramatically induces FSH-β

transcription, and is also responsible for the secondary rise in FSH, important for follicular development.

The pituitary gonadotropin FSH interacts with its membrane-bound receptor, to produce biologic effect. The FSH-receptor (FSHR) gene is localized on chromosome 2p21 and spans a region of 54 kb. It consists of ten exons: exon one to nine encode the large extracellular domain and the transmembrance domain is comprised of exon ten. Mutations of both FSH-β and FSHR genes have been characterized in humans, although phenotypic effects of the ligand appear to be more profound than those of its receptor. FSH is essential for normal puberty and fertility in females, particularly ovarian follicular development beyond than antral stage. In males, FSH is necessary for normal spermatogenesis and when FSH function is completely absent, infertility occurs. With partial FSH deficiency in males, spermatogenesis is affected, but fertility may be possible, FSH may also be necessary for normal androgen synthesis in males and females.

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