Opioidergic modulation of luteinizing and growth hormone release from porcine adenohypophyses in vitro

Von dem Fachbereich Biologie der Universität Hannover zur Erlangung des Grades eines Doktors der Naturwissenschaften Dr. rer. nat.

Genehmigte Dissertation von

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geboren am 26.03.68 in Fumbe-Mamfe/ KAMERUN

2002

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Tag der Promotion : 14 .06. 2002

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ABSTRACT

Endogenous opioids are known to modify LH and GH secretion through the hypothalamo-pituitary axis. This study aimed at verifying the direct action of opioids on LH and GH secretion at the pituitary level using pituitaries (adenohypophyses) of pigs in a perifusion in vitro system. Furthermore to determine whether opioidergic modulated release of LH and GH is altered by LHRH or GHRH stimulation at the pituitary in pigs and if these effects are sex-, and age-dependent.

ß-endorphin ($5*10^{-9}$M) significantly increased the release of LH in fetuses (425% in males and 275% in females) piglets (325% in males and 175% in females) adults (245% in males and 160% in females with high (P>3.5ng/ml) progesterone concentration) as compared to saline controls. Naloxone (10^{-6}M) and dalargin (10^{-6}M) showed no effects. Surprisingly naloxone did not antagonise the ß-endorphin-induced LH release when concomitantly administered to pituitaries of adult females and males. Pretreatment with ß-endorphin attenuated LHRH-stimulated LH release in fetal and adult females but not males, leaving piglets unaffected. Naloxone and dalargin impaired LHRH-induced LH increment in both adults and fetuses. Again naloxone and dalargin preadministration had no influence on LHRH-induced LH release in piglets.

The opiate agonists, ß-endorphin and dalargin caused a significant increase in GH secretion in pituitaries of adult males but not in females thus indicating a sex-differential effect. Whereas naloxone showed no effect in both sexes, concomitant administration of ß-endorphin and naloxone showed antagonism in adult males but not in females. In piglets, naloxone elicited an increase in GH secretion in females whereas in males, a non-significant decrease was observed. ß-endorphin and dalargin showed no effects in pituitaries of both female and male of piglets. On the otherhand, ß-endorphin increased GH secretion significantly in pituitaries of fetal males but not in females. Dalargin and naloxone showed no effects in both female and male fetuses unlike in adults and piglets.

Pretreatment with ß-endorphin tended to potentiate GHRH-stimulated GH release from pituitaries of adult males. Interestingly, posttreatment with LHRH diminished GH discharge in adult females pretreated with ß-endorphin and naloxone leaving males unaffected.

These findings indicate that effects of opioids on spontaneous LH and GH secretion are evident in the pig pituitary and these effects are sex-, age- and steriod-dependent. Opioids could play a major role in modifying LHRH-induced LH release but exert minor effects in modulating GHRH-induced GH discharge from the pig pituitary.

Key words : OPIOIDS, HORMONES , PIG

ABSTRACT

Endogene Opioide modulieren die Sekretion von LH und GH hauptsächlich über die Hypothalamus-Hypophysenachse . Im Rahmen dieser Arbeit sollte der Einfluß von Opioiden auf die LH und GH Sekretion auf der hypophysären Ebene in einem Superfusionssystem untersucht werden. Weiterhin wurde untersucht, ob Opioide die LHRH induzierte LH-Ausschüttung bzw. GH Sekretion beeinflussen und ob die Effekte Alters-, und Geschlechtsabhängig sind.

Aus den Ergebnissen geht hervor, dass ß-Endorphin ($5*10^{-9}$) einen signifikanten Anstieg der hypophysären LH Sekretion in Föten (425% bei männlichen vs 275% bei weiblichen), bei Ferkeln (325% bei männlichen vs 175% bei weiblichen) und bei Adulten (245% bei männlichen vs 160% bei weiblichen, mit einer höheren Progesteron Konzentration=P>3.5ng/ml) verursachte. Naloxon (10^{-6} M) und Dalargin (10^{-6} M) dagegen wiesen keine Effekte auf. Die Vorbehandlung mit ß-Endorphin führte zu Blockaden der LHRH-stimulierende LH-Ausschüttung sowohl bei fötalen als auch bei adulten weiblichen aber nicht bei männlichen Hypophysen, während die Reaktion der Ferkel unverändert blieb. Anderseits haben Naloxon und Dalargin die LHRH induzierte LH- Ausschüttung bei adulten und fötalen Hypophysen nur leicht verdrängt. Die Vorbehandlung mit Naloxon und Dalargin zeigte dagegen keinen Effekt bei Ferkeln.

Die Opioid-Agonisten, ß-Endorphin und Dalargin verursachten einen signifikanten Anstieg der GH Sekretion bei Eber aber nicht bei Sauen. Dies deutet einen geschlechtsdifferenzierten Effekt an, wogegen Naloxon keinen Effekt bei beiden Geschlechten aufwies. Naloxon zeigte einen Antagonismus mit ß-Endorphin bei männlichen aber nicht bei weiblichen adulten Hypophysen. Bei den Ferkeln führte Naloxon zu einem Anstieg der GH Sekretion bei weiblichen, während bei männlichen nur eine nicht-signifikante Verringerung der GH Sekretion beobachtet wurde. ß-Endorphin und Dalargin wiesen keine Effekte bei männlichen und weiblichen Ferkeln auf. ß-Endorphin führte zu einem signifikanten GH Anstieg bei männlichen fötalen Hypophysen, nicht jedoch bei weiblichen. Dalargin und Naloxon dagegen zeigten keinen Effekt auf die GH Sekretion bei weiblichen und männlichen fötalen Hypophysen. Die Vorbehandlung mit ß-Endorphin hatte keine signifikante Wirkung auf die GHRH-induzierte GH Ausschüttung. Lediglich war eine relativ starke aber nicht signifikante Potenzierung der GHRH-induzierte GH Sekretion bei Ebern zu beobachten. Die Behandlung mit LHRH führte zu einer Verringerung der GH Ausschüttung bei weiblichen Hypophysen, aber nicht bei männlichen, die zuvor mit ß-Endorphin und Naloxon behandelt wurden.

Aus diesen Ergebnissen geht hervor, daß Opioide die spontane LH und GH Sekretion auf der hypophysären Ebene modulieren. Diese Effekte sind von Geschlecht, Alter und gonadalen Steriode abhängig. Opioide können eine bedeutende Rolle bei der Modulation der LHRH-induzierten LH Ausschüttung spielen, aber sie zeigen einen geringen Effekt bei der Modulation der GHRH-induzierten GH Sekretion der Schweinhypophysen.

SCHLAGWORTE : OPIOIDE , HORMONE, SCHWEIN

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1.0 INTRODUCTION

One of the systems modulating physiological events in the body is the opioid system. According to investigations carried out in the recent decades, opioids are presumed to play a vital role in the control of reproductive activity, growth and behaviour in farm animals and other species (Rahe et al., 1980, Parvizi et al., 1993, Schouten and Rushen, 1993). The effects of opioids on the secretion of different hormones are sex-, age-dependent and vary slightly in different species. Opioid modulation of gonadotrophic and somatotrophic hormone secretion also depends on intrinsic factors like the steriod hormone concentration and extrinsic factors including stress, photoperiod and seasonality. The interaction of the opioidergic system and the immunological system has recently been established. Interleukine-1ß has been shown to stimulate opioid production that is presumed to be highly functional during stress situations in cells or tissues. Available evidence suggest that endogenous opioids and their receptors comprise one of the systems that translates ovarian hormone signals into changes in LHRH and LH release. The endogenous opioid receptors have been identified, characterised and are located in different parts of the body including the brain, spinal cord, pituitary, hypothalamus, ileum, adrenal medulla, blood cells, etc. Experimental studies have also recorded that the hormonal changes in response to the opioids depend on the route of opioid administration. It was observed that actions through subcutaneous (s.c.), intramuscular (i.m.), intravenous (i.v.), intracerebroventricular (i.c.v.) were increasing in effectiveness respectively. The majority of experimental data reported has led to the general assumption that endogenous opioid modulation of luteinizing hormone release and growth hormone secretion is achieved predominantly through the hypothalamic-pituitary-axis. However direct effects on the pituitary are feasible. The feasibility of the direct effects of opioids at the pituitary level implies that the opioid actions are not necessarily brought about by changing the LHRH or GRF and /or Somatostatin imput. But could also act by changing the pituitary response to LHRH to alter LH secretion or GRF and/ or Somatostatin to influence GH release.

1.1 Literature Review

Opiates, denoted from the alkaloids of opium and its derivatives are amongst the oldest drugs or analgesics with morphine-like effects used in medicine. According to Theophrastus the use of opiates began in the year 300 BC.The probability of the existence of receptors for these opiates was first theorised by Beckett and Casy (1954). Thereafter there was increasing interest in the identification, classification and isolation of these opiate receptors by different researchers. In 1973 came the discovery of the opiate binding sites in homogenates of the brains of different mammals through opiate ligand binding assays (Pert and Synder,1973; Simon et al.,1973; Terennius, 1973; Wong and Horng,1973). Two years later it was discovered that there existed endogenous opiate peptides or ligands that bound to these receptors (Hughes et al.,1975). The isolation of two pentapeptides Leucine (Leu)-Enkephaline and Methionine (Met)-Enkephaline with morphine-like activity from pig brain marked the beginning of the opioid era (Hughes, 1975). In the same year, a long chain peptide was extracted from the pituitary of cow that showed morphine like activity. Another endogenous peptide, ß-endorphin, with morphine like activity was isolated and the amino acid chain of 31 amino acid residues was established (Cox et al., 1975 ; Li and Chung, 1976). Later on many other endogenous opioids were identified and their amino acid sequence determined as seen on table 1 (Nakanishi et al., 1979; Kakidani et al., 1982, Meunier et al., 1995).

1.2 Classification and characterization of endogenous opioids and their receptors

The term "opiate" was originally designated to narcotic drugs derived from opium, ie morphine, codeine, and many semisynthetic derivatives. Later, the word "opioid" was coined to refer in a generic sense to all drugs, natural and synthetic, which have morphine-related actions, as well as to the endogenous peptides later discovered with such actions. However many authors interchange these terms.

There are approximately 15 naturally occuring endopeptides with opiate activity called endogenous opioids. These endogenous opioids are derived from the three classical precursor proteins : proopiomelanocortin (POMC) , proenkephalin (PROENK), prodynorphin (PRODYN) and two recently discovered neuropeptide families : orphanin FQ /nociceptin and endomorphin-1 (EM-1) and endomorphin-2 (EM-2) which are derived from the precursor peptides pro-nociceptin/ orphanin FQ and pro-endomorphin (yet-to-be discovered) respectively.

The three classical families give rise to three classes of opioids namely: endorphins, enkephalins and dynorphins respectively (Noda et al.,1982; Gubler et al., 1982 ; Kakidani et al., 1982). These three families of opioids are believed to originate from a common phylogenetic gene because their precursors possess almost the same number of amino acid residues (approx. 240 a.a ; scheme 1) and the opioid primers being located at the C-terminal while the N-terminal shows a homologous chain of amino acid sequence (Simon, 1991). The N-terminals of the opioids derived from these three precursor proteins possess as amino acid sequence at positions 1- 4 : Tyr - Gly - Gly - Phe (see table 1). The difference in the various derivatives lies on the remaining amino acid sequence that constitute the C-terminal of the chain. The length of the C-terminal determines the half life of these different opioids (Hughes, 1983) and also seems to dictate the receptor selectivity (Höllt, 1986; Goldstein & Naidu, 1989).

The cloning of the POMC-mRNA from bovine cDNA revealed that POMC was a long polypeptide made up of 241 amino acids that contained ; N-terminal peptide (103a.a.), ß-LPH (93 a.a.) and ACTH (39 a.a.) with many derivatives amongst which the endogenous opioid ß-endorphin was identified (Nakanishi et al., 1979).
Although ß-endorphin contains the sequence of met-enkephalin, it has not yet been proven that this endogenous opioid could be synthesized from POMC. ß-endorphin (1-31) binds to μ and δ receptors with almost the same affinity (Kosterlitz et al., 1986; Akil et al., 1988).

The shortening of the amino acid chain of ß-endorphin reduces its affinity to its μ- and δ-receptors (Akil et al., 1981 and Ferrara & Li, 1982). On the otherhand, opioids with different intrinsic activities could arise whereby ß-endorphin (1-31) is a potent agonist, whereas ß-endorphin (1-27) a potent antagonist of opioids (Nicholas & Li, 1985). The acetylated N-terminal of the endorphins binds relatively weakly to the opioid receptor and therefore shows no opioid activity (Akil et al., 1988).

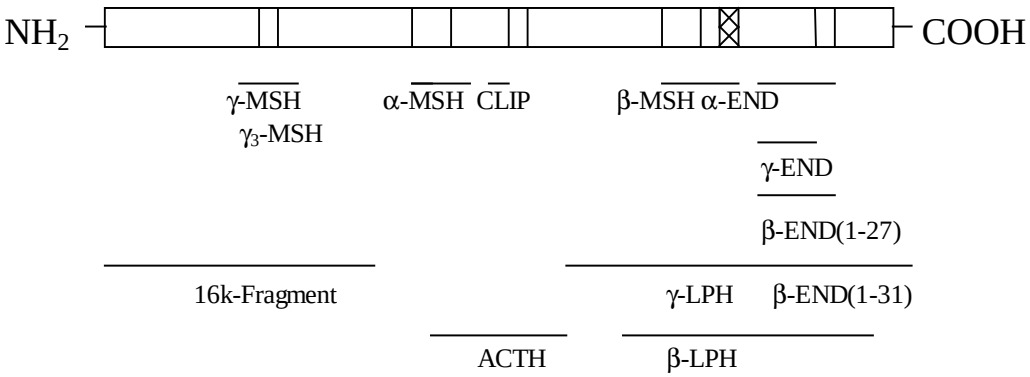
It is worthmentioning that the structure of the POMC-gene in the mouse unlike other animal species occasionally expresses pseudogenicity that results from an anomalie in the original gene. This pseudogene exhibits a 92% homology to the functional gene but two codons are not fully expressed. ß-endorphin and other derivatives like ACTH (which are cleaved by trypsin-like endopeptidases) cannot be synthesized from this pseudogene (Friedemann, 1994).

ß-endorphin was postulated to bind to another endogenous opioid receptor called epsilon "ε" (Wüster et al., 1979) but this suggestion was rejected later (Goldstein & Naidu, 1989 and Pleuvry, 1991).

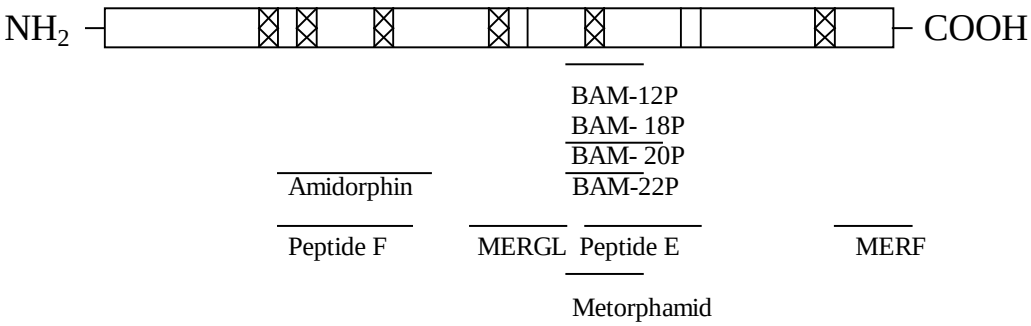
Proenkephalin, precursor peptide for the enkephalins is made up of 239 a.a. and it possesses opioid derivatives as shown on scheme 1. The enkephalins (ENK) generally have a high affinity to δ-receptors (Akil et al., 1988; Pleuvry, 1991 and Simon 1991). However metorphamid and BAM-18 show high affinities for μ and κ receptors (Corbett et al., 1982, Magnan et al., 1982, Hurblut et al., 1987).

The precursor protein, prodynorphin was cloned and sequenced from porcine adenohypophysis and contains 236 a.a (Kakidani et al., 1982). It contains dynorphin (DYN) and other opioid derivatives which show high affinity for κ-receptors (Chavkin et al., 1982; Corbett et al., 1982; Young et al., 1986). DYN (1-8) is highly selective for μ and δ-receptors.

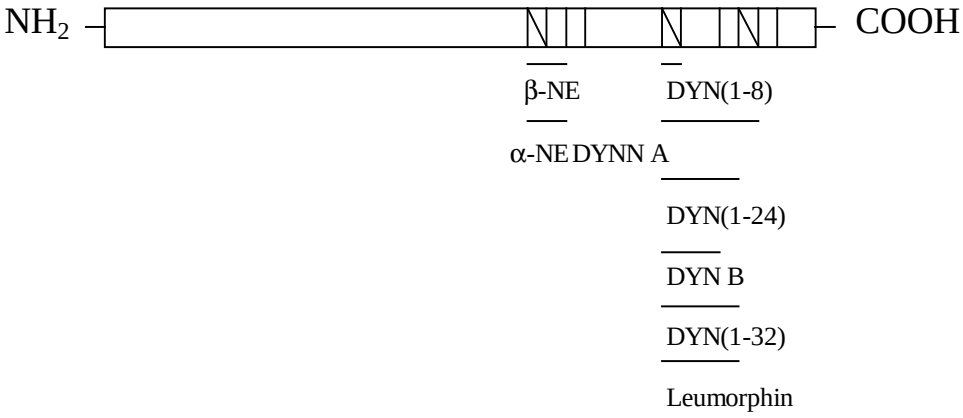
PROOPIOMELANOCORTIN (POMC): 241 AA (Porcine)



PROENKEPHALIN (PROENK) : 239 AA (Bovine)



PRODYNORPHIN (PRODYN) 236 AA (Porcine)



MET-Enkephalin : TRY-GLY-GLY-PHE-MET

LEU- Enkephalin : TRY-GLY-GLY-PHE-LEU

Scheme 1 : Schematic representation of the primary structures of the three precursor genes for the three largest opioid families and some of their opioid derivatives.
After Cox (1982), Schulz and Ehrenreich (1985) as modified by Kahle (1993).

Amongst the lately discovered endogenous opioid families is the novel heptadecapeptide, Orphanin FQ (Nociceptin) which was identified and purified from porcine brain tissue (Meunier et al., 1995). This opioid was identified as a ligand for an earlier isolated orphan heterotrimeric GTP-binding protein (G-protein)-coupled receptor called opioid receptor-like-1(ORL1), that is structurally and functionally similar to opioid receptors (Mollereau et al., 1994).

Nociceptin has a primary structure reminiscent of that of opioid peptides and is said to be processed from the precursor protein Pro-nociceptin/ OFQ (Meunier et al., 1995). Orphanin FQ binds to its receptor, opioid receptor like-1 (ORL1) with high affinity and is widely distributed in different brain regions in the guinea pig (Sinn and Childer, 1997). When injected intracerebroventricularly into mice, Orphanin FQ caused a decrease in locomotor activity but did not induce analgesia in the hot-plate test. However, application of nociceptin produced hyperanalgesia in the tail-flick assay. Thus, Orphanin FQ may act as a transmitter in the brain by modulating nociceptive and locomotor behaviour (Reinscheid, 1995). A novel synthetic peptide, [Nphe (1) nociceptin (1-13) NH (2)] has been introduced as the true selective and competitive nociceptin receptor antagonist which is devoid of any residual agonist activity (Calo' et al., 2000).

Recently two other peptides have been isolated from bovine brain that have high affinity and selectivity for the mu-opioid receptor and have been termed: endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe- NH_2). The new class of endogenous opioids are presumed to be derived from the yet-to-be discovered precursor protein pro-endomorphin (Zadina et al., 1997).

Tonini et al., (1998) revealed that the novel opioid tetrapeptides, Endomorphin-1 and Endomorphin-2 show selectivity for mu-opioid receptors located on excitatory myenteric plexus neurones in guinea pig and that they act as full agonists. The mu-opioid receptor selective peptide, endomorphin-1 has been reported to stimulate oxygen consumption in mice. This effect could be blocked by naloxone (Asakawa et al., 2000).

Endomorphin-2 based on its distribution in the CNS, could play a role in the control of neuroendocrine, cardiovascular, respiratory function, mood, sexual behaviour and pain in the rat (Pierce and Wesendorf, 2000).

Apart from the endogenous opioid peptides and derivatives from the above-mentioned precursor proteins, there exist a number of endogenous and exogenous peptides with opioid activity, whose physiological roles are not yet fully established. These include; Dermorphin, isolated from amphibian skin (Montecucchi et al., 1981) which is a potent μ-receptor agonist. Kreil et al., (1989) presented another endogenous peptide called Deltorphin with opioid activity. [D-Ala²]- Deltorphin I and [D-Ala²]-Deltorphin II (Erspamer et al., 1989) which has a high affinity for the δ-receptor.

The endogenous opioid, Kyotorphin, a dipeptide isolated from bovine brain extracts (Takagi et al., 1979), Humoral endorphin (H-endorphin), the α and β-casomorphin extracted from milk of different animal species (Henschen et al., 1979, Loukas et al., 1983; Ramabadran and Bansinath, 1989; Hazum,1991). Morphiceptin, a synthetic tetrapeptide with high affinity for the μ-receptor constitutes part of the N-terminal of β-casomorphin (1-11) as presumed by Chang et al.(1981), James and Goldstein (1984) which is perhaps a natural constituent of milk (Hazum,1991). Anodymin, is another endogenous opioid in the blood and brain, further reading is directed to Kitchen (1984) and Martinez (1989).

Table 1: The amino acid sequence for endogenous opioids and their derivatives.

Name of Opioid	Other names	Amino acid sequence
Pre-proopiomelanocortin:	Pre-POMC	ß-LPH (61-91)
ß-Endorphin	C-Fragment, ß-LPH (61-91)	^{61}Tyr-Gly-Gly-Phe-Met-Thr-Ser-
C-Fragment	ß-LPH(61-87),δ-Endorphin	Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val
γ-Endorphin	ß-LPH (61-77)	^{76}Thr-^{77}Leu-Phe-Lys-Asn-Ala-
α-Endorphin	ß-LPH (61-76)	Ile-Val-Lys-Asn-Ala-^{87}His-Lys-Lys-Gly-^{91}Gln
Pre-proenkephalin:	Pre-proenkephalin A	
Met-Enkephalin	Methionin-Enkephalin	Try-Gly-Gly-Phe-Met
Met-Enkephalyl-Arg ^{6}	Pro-Methionin-Enkephalin	Try-Gly-Gly-Phe-Met-Arg
Met-Enkephalyl-Lys ^{6}	Met-enkephalin-Lys ^{6}	Try-Gly-Gly-Phe-Met-Lys
Met-Enkephalyl-Arg ^{6}-Phe ^{7}	Met-Enkephalin-Arg-Phe, MEAP, MERF	Try-Gly-Gly-Phe-Met-Arg-Phe
Met-Enkephalyl-Arg ^{6}-Arg ^{7}	Met-Enkephalin-Arg-Arg	Tyr-Gly-Gly-Phe-Met-Arg-Arg
Met-Enkephalyl-Arg ^{6}-Gly ^{7} -Leu ^{8}	Met-Enkephalin-Arg-Gly-Leu, MERGL	Tyr-Gly-Gly-Phe-Met-Gly-Leu
Leu-Enkephalin	Leucine-Enkephalin	Tyr-Gly-Gly-Phe-Leu
Peptide B		Phe-Ala-Glu-Pro-Leu-Pro-Ser-Glu-Glu-Glu-Gly-Glu-Ser-Tyr-Ser-Lys-Glu-Val-Pro-Glu-Met-Glu-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Phe
Peptide F		Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met
Peptide I		Ser-Pro-Thr-Leu-Glu-Asp-Glu-His-Lys-Glu-Leu-Gln-Lys-Arg-^{15}Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-^{26}Glu-Trp-Met-Asp-Tyr-Gln-Lys-^{34}Arg-Tyr-^{36}Gly-Gly-Phe-^{39}Leu
Peptide E		Peptide I (15-39)
BAM-12P	Bovine Adrenal Medulla	Peptide I (15-26)

BAM-20P		Peptide I (15-34)
BAM-22P		Peptide I (15-36)
Metrophamid	Adrenorphin	Peptide E (1-8) NH_2
Synenkephalin	Pre-proenkephalin (1-70)	
Pre-prodynorphin:	Pre-proenkephalin B	
[Leu]Enkephalyl-Arg^6	Leu-Enkephalin-Arg^6	Tyr-Gly-Gly-Phe-Leu-Arg
ß-Neoendorphin		Tyr-Gly-Gly-Phe-Leu-Arg-LysTyr-Pro
α-Neoendorphin		Tyr-Gly-Gly-Phe-Leu-Arg-Tyr-Pro-Lys
Dynorphin 32		^{1}Tyr-Gly-Gly-Phe-Leu-Arg-Arg-^{8}Ile-Arg-Pro-Lys-Leu-^{13}Lys-Trp-Asp-Asn-^{17}Gln-Lys-Arg-^{20}Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-^{32}Thr
Dynorphin 1-8PH	(Porcine Hypothalamus)-8P	Sequence 1-8 (of Dynorphin32)
Dynorphin A	Dynorphin (1-17)	Sequence 1-17
Dynorphin B	Rimorphin	Sequence 20-32
Leumorphin		Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val
Nociceptin	Orphanin FQ	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln
Endomorphin-1	EM-1	Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	EM-2	Tyr-Pro-Phe-Phe-NH_2

Other Opioids:

1-) Kyotorphin		Try-Gly
2-) Dermorphin		Tyr-D-Ala-Phe-Gly-Tyr-Pro Ser-NH_2
3-) Humoral endorphin	H-Endorphin	
4-) Anodynin		
5-) ß-Casomorphin★		Tyr-Pro-Phe-Pro-Gly-Pro-Ile-NH2
6-) Morphiceptin★		Tyr-Phe-Pro-Co-NH_2

★ The acceptance of these peptides as endogenous opioids is a matter of dispute (Chiba et al.,1989).
1-) Opioid discovered in Kyoto.
2-) Opioid isolated from the skin of a South-American frog, Deltorphin.
3-) An opioid in blood, cerebrospinalfluid and brain.
4-) Opioid found in blood and in the brain.
5-) An opioid obtained from ß-caesin in milk.
6-) A fraction of the ß-caesin and potent μ-receptor-analog.

Note: On the table above the amino acid (a.a.) sequence of porcine ß-endorphin is given. In other animal species this sequence is slightly altered e.g In bovine, camel and ovine ß-endorphin, Val at position 83 is replaced by Ile. Human ß-endorphin possesses in position 87 and 91 Tyr and Glu respectively (Friedemann,1994; Martin-Schild et al.,1999; Reinscheid et al., 1995).

As earlier mentioned, Beckett and Casy (1954) suggested the possible existence of a biochemical substance at the tissue level or enzyme system with complementary stereochemical configuration of an opiate that mediated it's function in the cell. Yet, the existence of a single receptor for different opiates was questionable until Martin et al., (1976) owing to their observation from behavioral and neurophysiological studies on dogs postulated that there existed three different types of opiate receptors. Martin et al., (1976) observed that morphine and morphine-like substances interacted with specific receptors which they denoted as μ (mu)-receptors ; other synthetic opiates, ketocyclazocine and ethylketocyclazocine interacted with κ (kappa)-receptors and lastly SKF 10047 (N-allyl-norcyclazocine) interacted with the σ (sigma)-receptors. The cloning and sequencing of the opioid receptors (μ-398, δ-372, κ-380 a.a.) of the rat revealed 60% homogenity in their amino acid chain thus indicating a close structural and pharmacological relationship to one another (Satoh and Minami, 1995).

However, the application of mono and polyclonal antibodies against μ and δ-receptors revealed that endogenous opioid receptors possess different immunogenetic characteristics and therefore could represent molecules of separate entities (Carr et al.,1990).

The isolation of Met-enkephalin and Leu-enkephalin from the vas deferens of the mouse led to the discovery of the δ (delta)-receptor that showed very high affinity to these enkephalins (Hughes et al.,1975; Lord et al., 1977). However, there exist other receptors binding to different endogenous and exogenous opioid ligands (see table 2).

The opioid receptor ε (epsilon) showed high affinity for ß-endorphin and the λ (lambda)-receptor showed high affinity for naloxone and epoxymorphine (Grevel et al., 1985). The putative epsilon receptor is not a pecularity of rat brain but is readily measurable in forebrain of guinea pig, cow, chicken and pig brain as well. It is more abundant than mu, delta or kappa-1 receptor, representing 38 to 55% of the total opioid population in all species examined (Nock et al.,1993).

Zagon et al.,(1991) named the ζ-(Zeta) receptor after the Greek word for life Zoe and postulated that the interaction of Met-enkephalin with ζ-receptor provoked growth of nerve cells.

Numerous studies have revealed that μ, δ and κ- receptors possess different subtypes. The existence of these subtypes denoted μ_1 and μ_2 for μ (Pasternak, 1982 ; Pasternak and Wood, 1986, Childers, 1993) and κ_1 and κ_2 for κ (Attali et al., 1982; Zukin et al.,1988) has been established. Furthermore, Clark et al., (1989) proposed the existence of a κ_3 site, with high affinity to naloxone benzoylhydrazone but no affinity for U50,488H.

The existence of δ1 and δ2-opioid receptor subtypes has been suggested through behavioural and binding studies (Portoghese et al.,1992; Armstead, 1995).

Pharmacological studies of ligands binding to sigma receptors using brain tissue from the guinea pig revealed two subtypes of sigma receptors designated sigma 1 (σ1) and sigma (σ2) (Gonzalez and Werling, 1997).

Recently, Schlicker et al., (1998) cloned the opioid receptor-like-1 (ORL-1) that binds with the lately identified endogenous opioid peptide, nociceptin (orphanin FQ). The existence of a subtype of this receptor has not yet been established.

Table 2 : Some common selective synthetic exogenous and endogenous ligands for different endogenous opioid receptors (Calo's et al., 2000).

Receptor Type	Enogenous Ligand	Exogenous (specific) Ligand
μ	ß-endorphin, Enkephalin,	Morphin, Naloxone, DAGO
	Dynorphin, Endomorphine 1& 2	BIT, DAMGO, Methadone, Etorphine, Buprenorphine
δ	Enkephalin,Endorphin	Morphin, Naloxone, Dalargin Buprenorphine,DPDPE, BNTX SUPERFIT, SNC80, Natrindole
κ	Dynorphin	Morphin, Naloxone, U69593, Buprenorphine, nor-binaltorphine U-50.488H, Ketocyclazocine
ε	ß-endorphin	?
σ	?	SKF 10.047, PCP,Buprenorphine Dizocilpine
λ	?	Naloxone, 4,5 Epoxymorphinan
ORL-1	Nociceptin (OrphaninFQ)	[Phe1psi(CH_2-NH)Gly2]NC (1-13) NH_2 [Nphe(1) nociceptin (1-13) NH2]

Having highlighted the different opioid receptors in different animal species, the development of opioid receptors in the porcine species will be elaborated hereafter. In the pig, opioid receptors in the pituitary and CNS are not detectable until day 50 post coitus (p.c). The fetal pig shows a sex-differential expression of opioid receptors regarding their number and affinity to opioid ligands as fetal development proceeds until birth (Parvizi, 1988). During gestation the number of binding sites increases dramatically in female fetuses such that shortly before birth, the number is almost twice as much as that of male fetuses (Parvizi, 1988). However at the end of pregnancy, receptors of the male fetuses show a higher affinity to their endogenous ligands than the females (Parvizi, 1988). Amongst the endogenous opioid receptors μ and κ are predominant during the fetal stage whereas the δ is virtually absent (Kahle and Parvizi, 1993; Parvizi et al., 1995).

Around puberty females have less binding sites than males in the striatum and hippocampus. But in adult animals, the females have significantly more binding sites in the hypothalamus and amygdala than the males. In cyclic females it was also suggested that opioid binding sites do not fluctuate during the oestrous cycle (Kahle and Parvizi, 1993).

Results of the study of μ and δ opioid receptor densities in brainstems of piglets between 2 and 21days old show that the binding characteristics of each receptor remained unchanged over the age-range. Delta opioid receptor density was minimal in young (2-7 days old), and increased over the age-range studied. Mu opioid receptor density exceeded the delta opioid density in young (2-7days old) and old (20-21days old) piglets. It was concluded that the development of delta opioid receptors in swine lags behind that of the mu opioid receptors (Laferriere et al., 1999). The putative epsilon (ε) receptor has also been identified in forebrain of the pig. It is proposed that the epsilon (ε) receptor is more abundant than the μ, δ, κ opioid receptor types in the forebrain of pig (Nock et al., 1993).

Although endogenous ligands for the σ-opioid receptor have not yet been isolated, results of binding studies with exogenous ligands of σ receptors revealed their existence in the gastric fundic mucosa in pigs (Harada et al., 1994). Recently, Osinski et al., (1999) showed that the opioid receptor-like-1 (ORL-1) for the endogenous ligand nociceptin is expressed in the porcine cerebral cortex, kidney and the ileum. Other opioid receptors like the gamma and Zeta opioid receptors are rather rare or absent in the pig.

1.3 The mechanism of Opioid Ligand-Receptor interaction in modification of cell function.

Opioid receptors are allosteric proteins (ligand binding sites) interacting with extracellular physiological signals (opioid ligands) and converting them into intracellular effects leading to modification of cell function (Barnard and Simon, 1993). Hucho (1993) proposed the triune receptor model in which three functional moieties interacted to modify cell activity. The three functional moieties included : a signal receiving "R" (receptor or binding site) part, an effector "E" (ion channel or enzyme), and a transducer "T" (G-protein) coupling "R" and "E" and transducing the signal from binding site to the effectors. The transmitter (opioid ligand) is recognised and bound at the extracellular surface of the receptor at the cell membrane. The transducers are mostly G-proteins which in turn transduce stimulatory (Gs) or inhibitory (Gi or Go^{-}) effects to the effectors. The G-proteins are known to couple specific receptors and possess α, β and γ subunits (Childers, 1991). The effector system is either an ion channel (Na^{+}, Ca^{2+}, K^{+}) and/ or an enzyme (phospholipase C or adenylate cyclase). The activation of opioid receptors by agonist ligands generally inhibits neuronal excitability through inhibiting Ca^{2+} channels and activating k^{+} channels (Satoh and Minami, 1995). The affinity of opioid receptors for opioid agonists is decreased by sodium ions, whereas that for antagonists is rather enhanced (Pert and Synder, 1974). The binding of opioid agonists to G-proteins coupled receptors cause stimulation (Federmann et al., 1992, Kaneko et al., 1994) and inhibition (Childers, 1993) of adenylate cyclase.

1.4.0 Endogenous opioids and luteinizing hormone secretion

It has been established that endogenous opioids play a significant role in modulating gonadotropin secretion (for review see, Brooks et al., 1986a). The first evidence suggesting that opioids inhibit LH secretion was derived from experiments in which the effects of opioid receptor agonists and antagonists on LH secretion were evaluated. Bruni et al., (1977) showed that naloxone, an opioid receptor antagonist, increased release of LH and FSH in male rats and this effect was prevented by concomitant administration of met-enkephalin.

Effects of opioids on LH are sex-, age-, and species-dependent. Furthermore other intrinsic and extrinsic factors like steriod hormone concentration, nutrition, photoperiod and stressors may interplay to modify opioid effects on LH secretion (Britt et al., 1993).
Intravenous administration of naloxone increased, whereas morphine reduced LH pulse frequency in sheep (Ebling et al., 1989). Similarly, intracerebral administration of naloxone enhanced LH secretion (Malven et al., 1990), whereas ß-endorphin injected into the cerebroventricular system decreased LH secretion in sheep (Horton et al., 1990). Opioid modulation of LH is predominantly probable via the hypothalamic-pituitary-axis. Data supporting this presumption showed that naloxone increased LHRH in hypothalamo-hypophysial portal blood in sheep (Horton et al., 1989). In addition morphine failed to decrease LH secretion in sheep bearing hypothalamo-pituitary disconnections (Horton et al., 1990).

In the prepubertal ewe lamb, naloxone had no influence on LH secretion. Interestingly, as ewes approached puberty, naloxone could increase LH pulse frequency and mean serum LH concentrations (Rawlings et al., 1993). Whereas in the prepubertal male lamb, naloxone treatment resulted in an increase in LH pulse amplitude at age from 5-10 weeks. Surprisingly, at 10 weeks of age, naloxone caused a decrease in LH secretion. Nevertheless, at 25 weeks of age naloxone increased LH pulse frequency (Rawlings et al., 1991).

Similarly, prepubertal bulls and heifers responded to naloxone treatment with increases in LH pulse frequency (heifers) and LH pulse amplitude and basal serum LH concentrations (bulls) (Evans et al., 1991; Rawlings et al., 1993). However, Gazal and Anderson, (1995) reported that injection of naloxone had no effects on plasma LH secretion in prepubertal heifers. The difference in results obtained in both studies is related to age at which treatment was given. While, Rawlings et al. (1993) gave naloxone between 4-18 weeks, Gasal and Anderson (1995) administered naloxone at 32 weeks of age. At 32 weeks of age, sudden rises in progesterone concentration prior to first oestrus may act negatively to reduce LH secretion from the pituitary (Rawlings et al., 1993).

Cicero et al., (1993) reported that naloxone did not reverse the supressive-effects of morphine on serum LH levels in the prepubescent rat whereas it fully reversed this effect in the adults. They propounded that the effects of opiates on the hypothalamic-pituitary-axis in pre- and postpubertal male rats could be age-dependent. They suggested that the effects of morphine and naloxone on LH secretion could be mediated by different receptors at the pubertal stage, but in adults they act at the same receptors. It was concluded that morphine suppresses LH secretion at very early stages of development well before puberty, whereas naloxone does not increase LH secretion until after puberty (Cicero et al., 1993).

A specific delta-opioid receptor agonist, [D-Pen^{2}, D-Pen^{5}] enkephaline: DPDPE, was found to abolish the LH surge in the hypothalamus of the female rat. Naloxone reversed the inhibitory effect of DPDPE. It was concluded that the activation of δ-opioid receptors may exert an inhibitory effect on LH release probably via monoaminergic systems as they are parallely suppressed by DPDPE in all the hypothalamic regions investigated (Yilmaz et al., 1998).

Application of opioids at different periods of the oestrus cycle in female animals has shown different responses of LH secretion indicating a role of steriod hormone concentration. During the follicular phase of the oestrus cycle, the opioid agonist, morphine and the met-enkephalin analogue, FK 33-824 inhibited LH secretion in heifers (Brooks et al., 1986a; Armstrong and Johnson, 1989). Similarly, intracerebroventricular administration of ß-endorphin to ovariectomized or intact ewes in the follicular phase of the oestrus cycle also caused inhibition of LH secretion (Horton et al., 1989). Likewise in the male castrated sheep, the opioid agonist, FK 33-824 decreased both average and episodic LH secretion in both acute and chronic treatments (Armstrong and Spears, 1991). These results suggest that opioid agonists exert inhibitory effects on LH secretion independent of the steriod hormone concentration during the follicular phase of the oestrus cycle in the cow and sheep.

On the otherhand, the opioid antagonist, naloxone increased LH concentrations in ewes with luteal phase concentrations of progesterone, but not in ewes with low concentrations of progesterone. These results led to the assumption that the negative feedback actions of progesterone on tonic LH secretion may be mediated by endogenous opioids (Malven et al., 1984). In another study, intravenous infusion of naloxone resulted in an increase in LH pulse frequency in ovariectomized ewes that were not pretreated with steriods and in those pretreated with progesterone and oestradiol. However, when oestradiol was given alone, no effect of naloxone was seen (Currie et al., 1991).

In the cyclic intact ewe, naloxone treatment increased basal LH secretion and LH pulse amplitude during the luteal phase of the oestrus cycle. During the follicular phase, naloxone increased LH pulse amplitude but decreased LH pulse frequency (Rawlings et al., 1993). It seems that opioid antagonists elevated LH concentrations in both the luteal and follicular phases of the oestrus cycle in intact cyclic ewe. However, LH parameters (LH pulse frequency and LH pulse amplitude) are continously fluctuating at different periods of the oestrus cycle.

Results of Rawlings et al., (1993) also revealed that in the ram, naloxone increases LH secretion in an age-dependent manner. A higher level of testosterone in adult males inhibits LH secretion, whereas in the young ram lamb, opioidergic effects are steriod-independent.

The LH response to opioid agonists or antagonists in postpartum suckled cows probably depends on the stage of postpartum and whether calves are suckling during the treatment period. Peck et al., (1988) reported that an injection of morphine (1mg/kg) or a 7-hour infusion of morphine ($15mg.kg.^{-1}h^{-1}$) suppressed LH in cows whose calves had been weaned 36 hours before treatment. This is because the endogenous opioid tone caused by suckling is lowered after weaning. Thus, one would expect opioid agonists to cause suppression in LH secretion in suckled cows only if the suckling was not fully suppressive already. On the otherhand, when naloxone was given 48 hours after transient weaning on day 35 postpartum, the LH response differed between cows nursing calves and those from which calves had been weaned (Whisnant et al., 1986). In cows being nursed, basal LH was already suppressed due to the suckling-induced effect and naloxone induced a threefold increase in LH secretion. In cows whose calves were weaned 48 hours before treatment, naloxone had no effect because weaning already increased basal LH threefold and no further increase could be achieved. In contrast, however, Rund et al., (1989) reported that naloxone increased LH concentrations in both suckled and nonsuckled cows. It can be concluded that opioid agonistic inhibitory effects and opioid antagonistic stimulatory effects on LH secretion in the postpartum cow is suckling-dependent. The opioidergic inhibition of LH release in suckled cows depends on the time post partum that drug is given. This inhibition seems to decrease slowly with time post partum, whereas opioid antagonistic stimulatory effects on LH secretion probably increases with time post partum.

1.4.1 The effects of endogenous opioids on LH secretion from fetal to prepubertal stages in the pig.

In the fetal pig, LH gene expression starts at around day 45-50 p.c. (Ma, 1991). Endogenous opioid genes switch on as early as day 35 p.c.(Pittius et al.,1987) and opioid receptors can be detected as from around day 50 p.c. (Kahle and Parvizi, 1993). The hypothalamus takes over control of LH around day 80. Electrical or electrochemical stimulation of the hypothalamus causes a LH surge around day 80. Stimulation of the hypothalamus around day 60 was ineffective (Bruhn et al., 1983). As development of the fetus continues, the pituitary becomes more responsive to hypothalamic releasing factors (Colenbrander et al., 1982). In the fetal pig, regulation of LH is more or less not affected by fetal steriods. Hence, fetal gonadectomy (Ponzillius et al. 1986) or estradiol-17ß treatment (Parvizi et al., 1986) do not alter the LH secretory pattern. Opioidergic control of LH begins as soon as the opioid receptors become functional.

The predominant opioid receptors are of the kappa and mu-subtypes. Unlike in the fetal sheep (Yang and Challis, 1991) where the delta-opioid receptor is more important, the fetal pig has virtually no δ-opioid receptor (Kahle and Parvizi, 1993). It has been observed that towards the end of pregnancy, morphine acutely inhibits the secretion of LH in male and female fetuses. Daily treatment of fetuses with morphine over a period of 14 days before birth caused an inhibition of basal LH in females, while male fetuses were unaffected (Behrens-Herrler and Parvizi, 1992). Co-administration of naloxone and morphine reversed the long term morphine-induced inhibitory effect on LH secretion in females. Administration of a single injection of naloxone had no effects on LH secretion in both female and male fetuses. However repeated injections of naloxone over a 7 day period surprisingly decreased LH secretion in males whereas females remained unaffected. The paradoxical long-term inhibitory effect of naloxone on LH secretion in male fetuses indicates the age-dependent function of opioid receptors. The authors suggested that the link between opioids and the luteinizing hormone system is functional in the pig from at least 2 weeks before birth (Behrens-Herrler and Parvizi, 1992).

Trudeau et al., (1988) observed no influence of morphine and /or naloxone on the secretion of LH in intact and castrated immature 6 weeks old male pigs. Naloxone also failed to alter LH secretion in intact prepubertal gilts and in ovariectomized, progesterone-treated prepubertal gilts (Barb et al.,1988). However, LH secretion increased when gilts were ovariectomized prepubertally and treated with progesterone and naloxone at a chronological age when puberty occurred in intact gilts (Barb et al., 1988).

In contrast to the above-mentioned work of Barb et al.,(1988), intracerebroventricular administration of morphine (Estienne et al., 1990) in ovariectomized gilts resulted in a decrease in serum LH concentration and serum LH pulse frequency. However, the serum LH pulse amplitude did not change.

Prunier et al., (1990) reported that administration of naloxone to male and female piglets (8-10 days) caused a reduction in the concentration of circulating LH levels in both sexes. Leu-enkephalin showed no effect. Further investigation with 42- 47days old male piglets showed inhibitory effects of naloxone on LH secretion in intact and castrated testosterone-substituted animals. In contrast, Leu-enkephalin was only effective when the animals were castrated and received no steriod (Kahle et al., 1989).

1.4.2 Endogenous opioids and luteinizing hormone secretion in the adult pig

In the adult pig, opioid agonists generally inhibit LH secretion whereas opioid antagonists enhance LH release. However, slight variations in LH response to opioids are observed in the adult female depending on the reproductive stages. On the otherhand, LH response to opioids in adult males has a more consistent pattern but may differ in castrated or intact males. In the adult female pig, intracerebroventricular administration of the opiate agonist morphine reduced LH secretion in both ovariectomized mature and prepubertal gilts. Treatment with naloxone stimulated luteinizing hormone release in mature but not in prepubertal gilts (Barb et al., 1989).

As mentioned above, the modulation of LH secretion by endogenous opioids is strongly related to gender, age and level of steriod hormones in the pig. The steriod hormone concentration in female animals influences the opioidergic modulation of the release of luteinizing hormone. The application of microinjections of met-enkephalin into the mediobasal hypothalamus showed no effect on LH secretion in pig. However pre-application of oestradiol-17ß and testosterone resulted in a significant decrease in LH concentration (Parvizi, 1989).

The microinjection of ß-endorphin (30ng) into the hypothalamus or amygdala of ovariectomized miniature pigs revealed that it is not the hypothalamus but the amygdala in which ß-endorphin becomes effective. However simultaneous micro-injection into the hypothalamus and amygdala showed that inhibition of pituitary LH secretion was exacerbated as compared with the inhibitory effect observed when microinjections were carried out into the amygdala alone (Parvizi and Ellendorff, 1980). Naloxone enhances plasma LH levels when injected into the mediobasal hypothalamus of cyclic sows (during dioestrus) but not in ovariectomized sows (Parvizi, 1988). ß-endorphin may modulate LH release independently from gonadal steriods in male and female pigs, whereas leu-enkephalin and met-enkephalin actions are most probably steriod-dependent (Parvizi, 1989). These results demonstrate that opioid actions are not only sex-, steriod hormone concentration-dependent but also vary with route or site of administration of drug.

The administration of naloxone and met-enkephalin analogue (FK 33-824) injections did not affect LH secretion during the early or late follicular phase in gilts. However, continous infusion of FK 33-824 for 4 hours decreased LH secretion during the late follicular phase of the oestrus cycle (Okrasa et al.,1992). On the otherhand, a single injection of FK 33-824 decreased the number of LH pulses over a 3 hour period in the luteal phase. Morphine exerted an inhibitory effect on the estradiol benzoate (EB) - induced LH surge during the positive feedback phase, 60-64hours after EB administration, in ovariectomized gilts. Administration of naloxone in ovariectomized

gilts during the negative feedback phase (30-34 hours after EB administration) did not alter LH secretion. These results indicate that endogenous opioid petides participate in the regulation of LH in the oestrus cycle in the gilt (Okrasa et al. 1992). On the otherhand, naloxone was found to stimulate LH secretion in the luteal phase but not in the early and late follicular phases in gilts (Barb et al., 1986a ; Okrasa et al., 1990).

During pregnancy, luteinizing hormone secretion is relatively high but towards the last month of pregnancy the LH secretion is depressed (Quesnel and Prunier, 1995). At the end of gestation, Willis et al., (1996) reported that naloxone increased LH concentration in sows. After partuirition, LH secretion increases but is again inhibited by the establishment of lactation (Quesnel and Prunier, 1995).

Mattioli et al., (1986) injected naloxone through indwelling vena cava canulla in lactating sows on day 15 postpartum. Naloxone induced an increase in LH secretion within 20-50 minutes. The authors concluded that endogenous opioids are involved in modulating the endocrine patterns occuring during lactation, that is, down regulation of LH secretion. Similarly, infusion with naloxone or transient weaning elicited a threefold increase in the frequency of LH pulses although neither pulse amplitude nor average concentration of LH was significantly altered in lactating sows (Britt et al.,1993). The authors assumed that the infusion of naloxone accelerated the GnRH pulse generator but the amount of GnRH released during each pulse was not altered. After weaning, naloxone was found to increase mean LH concentrations when administered into sows (De Rensis and Foxcroft., 1999a).

On the otherhand, Britt et al., (1993) reported that infusion of morphine into sows nursing piglets tended to reduce LH pulse frequency. When morphine administration was combined with transient weaning, LH pulsatility was depressed further. Similarly, morphine administration resulted in a decrease in LH secretion in suckling sows and in zero-weaned (piglets removed immediately after farrowing) sows (De Rensis et al., 1999b). The authors concluded that the suckling stimulus is associated with suppression of LH secretion immediately after birth. However, chronic naloxone

treatment on lactating sows was found to reverse the suckling-induced inhibition of LH secretion in the early post partum period. The naloxone-induced increase in LH secretion was observed on day 10-11 of lactation (De Rensis et al., 1993, 1998). It can be suggested that opioid antagonist effects on LH secretion are dependent on the steriod environment, stress (suckling) but LH response to opioid agonist effects are apparently steriod hormone or suckling-independent.

1.4.3 In vitro effects of opioids on luteinizing hormone secretion

There is very little information on opioidergic effects at the pituitary level in vitro in the pig. For this reason, the literature has been reviewed on other animal species.

A number of controversial reports and observations of paradoxical effects of opioids suggest that a direct action at the pituitary level cannot be ruled out (Blank et al. 1986, Cacidedo and Sanchez-Franco, 1986, Chao et al. 1986, Barb et al. 1990, Dragatsis et al. 1995). Interestingly, the administration of ovine ß-endorphin and human ß-endorphin to the ovine pituitary cells caused stimulation of LH (Matteri and Moberg, 1985). The LH secretion induced by hß-endorphin was found to be dose-dependent. However, naloxone pretreatment did not reduce the hß-endorphin-induced LH secretion (Matteri and Moberg, 1985).

The direct effect of human ß-endorphin on LH secretion at the pituitary in the rat seems to be different from that of the sheep. Cacicedo and Franco Sanchez (1985) showed that treatment with human ß-endorphin inhibited LH secretion from rat pituitary cells after 24 hours. The inhibition was dose-dependent and more evident after 48 hours.

Interestingly, naloxone enhanced LH secretion and also blocked the human ß-endorphin-induced inhibitory effects. The administration of met-enkephalin and D-ala^2-met-enkephalinamide (DALA) also produced a significant decrease in LH secretion. (Cacicedo and Franco Sanchez, 1985).

Morphine sulfate was found to have a direct inhibitory effect on both basal and GnRH-stimulated LH release by cultured female rat pituitary cells. The inhibitory effect was prevented by the opiate antagonist, naltrexone. Treatment of cells with naltrexone or ß-endorphin antiserum significantly increased basal LH release (Blank et al., 1986).

The administration of naloxone in bovine pituitary cell culture increased basal secretion of LH after 2 hours but not after 24 hours of culture. Addition of met-enkephalin into the culture suppressed basal LH release (Chao et al.,1986).

Naloxone increased LH release from porcine pituitaries after 4hours and 24hours of cell culture. When GnRH was simultaneously given with naloxone, the increase was greater than after GnRH alone after 4hours but not after 24hours. Beta-endorphin failed to alter basal LH secretion after 4hours but decreased secretion after 24hours (Barb et al., 1990).

The µ-opioid agonist DAGO, inhibited the release of LH from rat pituitary in a concentration-dependent manner. When naloxone and DAGO were concomitantly administered, the inhibitory effect of DAGO on LH release was abolished. The release of LH induced by D-Ala^6-LHRH was also inhibited by DAGO, although this was not effective at low concentrations. These results demonstrate a mu-opioid receptor-mediated effect on the spontaneous and GnRH-induced release of LH at the pituitary level in vitro (Dragatsis et al., 1995). On the otherhand, the delta-opioid peptide agonist DSLET did not modify the spontaneous release of LH. Surprisingly, GnRH analog, D-Ala^6 LHRH-induced LH release was inhibited by subsequent administration of DSLET. The delta-opioid antagonist, diallyl-G blocked this inhibition (Dragatsis et al., 1995).

In contrast to the results obtained using mu and delta agonists, the kappa opioid agonist, U-50488H increased the spontaneous LH release in a concentration-

dependent fashion. Simultaneous addition of the specific kappa-opioid antagonist, MR-2266 and U-50488H attenuated the stimulatory effect. Interestingly, the kappa opioid agonist U-50488H did not show any effect on the GnRH analog-induced LH release (Dragatsis et al., 1995).

According to several results obtained so far, opioidergic control of LH is not solely via the hypothalamo-pituitary axis. Direct effects of opioids can be seen at the anterior pituitary level. These opioid effects depend on type of opioid peptide, opioid receptors and the animal species.

1.5.0 Endogenous opioids and growth hormone secretion

Growth hormone is mainly regulated by two hypothalamic peptides, namely: Growth hormone releasing hormone (GHRH or GRF) which stimulates GH secretion (River et al., 1982 ; Guillemin et al., 1982) and Somatotrophic releasing inhibiting hormone (SRIH) also known as somatostatin, that inhibits GH release (Brazeau et al., 1973). Supportive data indicate that hypophysectomy or active immunization against growth hormone releasing factor retards normal growth in cattle and pigs (Simpson et al., 1991).

However, other peptides including insulin growth factors (IGFs), neuroactive amino acids and endogenous opioid peptides are also involved in the direct or indirect modification of growth hormone release from the pituitary gland (Simpkins et al., 1993).

Several reports have documented the involvement of endogenous opioid peptides in the regulation of growth hormone in farm animals and humans. In addition, most studies indicate that opioids stimulate growth hormone via a stimulatory tone on GRF (Wehrenberg et al., 1985, Armstrong et al., 1990) and an inhibitory tone on somatostatin (Drouva et al., 1981, Villa et al., 1997). Nevertheless, a direct

opioidergic effect on the anterior pituitary secretion of growth hormone cannot be excluded (Armstrong et al., 1990; Villa et al.,1997).

Opioids generally elevate circulating GH in farm animals (Armstrong et al., 1993) and in human (Grossman, 1983, Schulte et al., 1993). Opioid activation of GH probably involves the delta binding sites (Koenig et al., 1984) and μ but not k-receptors (Krulich et al., 1986). It is well known that endogenous opioids play an important role in the growth of developing, neoplastic, renewing and healing tissues, both in prokaryotes and eukaryotes (Zagon and Mclaughlin, 1989) in addition to serving as neuroregulators (Zagon et al.,1996).

A synthethic analog of leucine enkephaline-hexapeptide, dalargin was shown to stimulate healing of skin wounds in rats, dogs and mini-pigs at local and parenteral administration. The proliferation rate of mitotic division of fibroblast was greatly increased. Early and active growth of vascular elements, rapid maturation of granulation tissue and accelerated epithelization were also observed. The authors suggested that the opiate agonist could increase GH secretion and thus growth activity in animals (Il'inskii, et al., 1987).

The opioidergic modulation of GH secretion also depends on the age-, sex-, steriod hormone level, stress (food restriction etc.) and photoperiod in seasonal variation (Rushen et al., 1993, Aurich et al., 1999). It is interesting to note that opioid-induced effects on GH secretion apparently vary slightly with the different routes of administration of treatment and animal species investigated (Zhai et al.,1995).

1.5.1 The effects of endogenous opioids on growth hormone secretion from fetal to pubertal stages.

Owing to lack of a broad scope of literature on the effects of endogenous opioids on GH secretion in the pig, the literature for other animal species is also reviewed

hereafter. In the neonatal female and male rat, the mu-agonist, morphine and kappa agonist U50-488H caused a stimulation and an inhibition of GH secretion on postnatal day 10 respectively. It was observed that kappa-inhibition was already effective as early as postnatal day 2, but mu-stimulation was not substantial until postnatal day 10. The intracerebroventricular (i.c.v.) administration of the mu-selective peptide [D-Ala2-Nmet-Phe4-Gly-ol]- enkephalin (DAMGO) elicited a marked rise in GH secretion, while administration of the delta-agonists [D-pen2D-pen5]-enkephalin (DPDPE) or deltorphin II caused only a minor and non-dose-dependent increase in GH secretion in the neonatal rats (Eason et al., 1996). When older pups were administered DAMGO intracerebro-ventricularly on postnatal day 20, GH secretion was stimulated. On the otherhand, neither DPDPE nor deltorphin II consistently increased GH secretion on postnatal day 20 in female and male rats. Furthermore, peripheral administration of either morphine or the highly selective mu-agonist, sufentanil elicited marked GH secretion on postnatal day 20, but only combined administration of the μ-opioid receptor antagonist beta-funaltrexamine (beta-FNA) and the delta-opioid receptor antagonist natrindole substantially diminished GH responses to morphine and sufentanil. These results suggest that both mu-and kappa-opioid receptors are involved in the regulation of GH secretion in neonatal rats. While delta-receptors do not play a significant independent role in this response, they may act synergistically with mu-receptors in producing stimulation (Eason et al., 1996).

In prepubertal heifers at 8-9 months of age, the opioid agonist FK 33-824 caused a 4-5 fold increase in GH secretion (Simpson et al., 1991). Interestingly, the response of GH to the opioid agonist, FK 33-824 was greater at 8 months than at 9 months of age (Armstrong et al., 1993). The authors suggested a possible desensitization of growth hormone to opioid effects with increasing age.

Serum concentration of GH was elevated in 4-10 months old heifers after i.c.v. morphine injections. An equimolar dose of naloxone reverted the morphine-induced increase in GH secretion. Application of naloxone showed no dose-related effects. All doses of naloxone decreased serum GH concentration in older (234 ±6days) heifers

but proved ineffective in younger (86 ±11days) heifers. These results suggest an age-related naloxone effect on GH secretion in heifers (Leshin et al., 1990).

In contrast to results observed in rats (Eason et al., 1996) in heifers (Simpson et al., 1991) and in pigs (Barb et al.,1992, Parvizi et al.,1995) plasma concentrations of growth hormone were found to decrease following intravenous administration of morphine sulfate in young chickens. The opiate antagonist naloxone had no stimulatory effect on basal GH concentrations but attenuated the GH response to morphine (Harvey & Scanes, 1987). These results indicate that opioid effects in control of GH release in young chickens are probably inhibitory.

In the fetal pig, growth hormone gene expression can be seen in the pituitary as early as day 45 p.c (Ma, 1991). Opioidergic control of pituitary GH release could be estimated to become effective as from day 55 onwards in the fetal life (Ma, 1994). During fetal development, the fetuses display higher plasma GH and IGF-I levels than their sows. It was observed that the levels of GH fall within a few days after birth (Bauer and Parvizi, 1996). Dispite these high levels of GH in fetuses, GRF (10µg/kg, GRF 1-29NH_2) induced a dramatic GH surge as from day 80 p.c. and later in gestation, as well as in neonates. The GRF-induced GH peak reached 134 ± 12µg/ml in fetuses and 43 ± 4µg/ml in neonates. On the otherhand, somatostatin (1-14 cyclic) given as a bolus injection (50µg/kg) followed by 5 subsequent injections (25µg/kg each) at 5 minutes interval neither inhibited basal nor GRF-induced GH release in fetal as well as neonatal pigs.

The somatostatin inhibitory pathway is still premature in the fetal pig (Parvizi et al., 1995). Inhibition of GH secretion could only be achieved by passive immunization with GRF antibody. Surprisingly an enhancement of plasma GH concentration occured during and after somatostatin application in some piglets (Parvizi et al., 1995).

When naltrexone and morphine were administered to chronically catheterized fetal (1mg/kg/daily) and neonatal (1mg/kg/single shot) pigs, naltrexone showed no effects

whereas morphine exhibited remarkable changes on GH secretion. In fetuses daily injections of morphine over 3-4 days were essential to prime the brain/ pituitary to initiate a GH response. A single injection of morphine was ineffective before day 16 p.p. but stimulated GH secretion in piglets older than 16 days. The authors concluded that GH stimulatory systems seem to be functioning prior to birth, whereas the somatostatin inhibitory axis is apparently far from maturation at parturition (Parvizi et al., 1995).

In the immature 6 weeks old male pig, morphine treatment resulted to an increase in GH levels. Although naloxone alone did not affect GH levels, it's administration immediately after morphine delayed the GH increment seen in morphine treated pigs (Trudeau et al., 1988). Similarly, in prepubertal gilts, intracerebroventricular injection of morphine and intravenous administration of FK 33-824 stimulated a rapid increase in serum growth hormone secretion (Barb et al., 1992; Armstrong et al., 1993).

On the otherhand, intravenous injections of naloxone to prepubertal gilts under stress condition inhibited stress-induced GH increase. It was concluded that endogenous opioids enhance GH response to stress in pigs (Rushen et al., 1993). It could be concluded that opioids participate in the control of GH release in the immature animal and GH response becomes more effective with increasing age.

1.5.2 The effects of endogenous opioids on growth hormone secretion in adults.

The opioid modulatory effects on GH parameters has been studied in the females at different periods of the oestrous cycle, gestation, lactation and postpartum. Interestingly, results in most species studied so far showed sex-and species-differential effects at different physiological states of the female animal. Whereas male animals showed a more consistent pattern of GH release in response to opioid treatments.

Plasma concentrations of growth hormone (GH) were found to increase following intravenous administration of the mu-agonist morphine in male and ovariectomized female rats (Terry et al., 1982; Koenig et al., 1983 and Krulich et al., 1986). The opiate antagonist, naloxone had no stimulatory effect on basal GH concentrations but attenuated the stimulatory effect of morphine on GH secretion. On the otherhand, bremazocine and U-50-488, two selective kappa receptor agonists suppressed GH release at high doses (Krulich et al., 1986).

Simpkins et al., (1993) reported that chronic morphine exposure of male rats caused a 12-fold increase in basal GH levels and a modest rise in GH pulse frequency. The mean concentration of GH secretion increased 3-fold over the 6 hours experimental period. On the contrary, in female rats, administration of chronic morphine reduced GH pulse amplitudes but did not significantly affect other parameters of GH secretion. A sex-differentiated effect of morphine on GH secretion can be seen in the adult rat.

Zhai et al., (1995) continuously infused male sprague-dawley rats with morphine through subcutaneous implanted mini-osmotic pumps over a period of 5 days. They found that acute administration of morphine significantly decreased the density of growth hormone binding sites in choriod plexus and hypothalamus. The plasma levels of GH correlated negatively with the density of binding sites in these regions of the rat brain. The same authors administered intermittent injections of morphine intracerebro-ventricularly (icv) and subcutaneously (s.c) and observed a similar decrease in rGH binding. They noticed a differential time course of response between the two routes of administration, with intracerebroventricular (i.c.v.) injections achieving faster response than subcutaneous (s.c) treatment.

In an attempt to determine if the growth hormone response to morphine sulphate could be affected during steriod-induced LH surges, Singh et al., (1992) provided evidence that gonadal steriod treatment which induce a surge in LH secretion in female rats suppressed opiate-induced increase of GH secretion. This dampening of opiate-induced GH secretion is confined to the time of the steriod-induced LH surge. The

authors concluded that ovarian steriod-induced increase in LH do not only blunt morphine modulation of LH secretion but also affects opioid-induced GH release in rats (Singh et al., 1992).

The endogenous opioid, met-enkephalin, has been found to interact with zeta (ζ) opioid receptors to influence growth both *in vivo* and *in vitro* in rats. Unlike most opioids, met-enkephalin also termed “Opioid Growth Factor” (OGF) serves as a negative modulatory agent in cell proliferation, migration, differentiation and survival. It is presumed here that met-enkephalin maintains a continuous tonic effect on growth events since suppression of met-enkephalinergic tone with naltrexone results in a stimulatory response in growth. Furthermore, developing and adult rats exposed prenatally to naltrexone were not analgesic after morphine challenge and exhibited a marked decrease in μ-opioid receptors (Zagon et al., 1998).

In a study, steers (castrated bulls) were administered two i.v injections of FK 33-824 (2.3μg/kg) 1 hour apart. The first but not the second, injection of FK 33-824 caused a four- to six-fold increase in GH concentration (Armstrong and Spears, 1991). In a subsequent study by the same authors, wethers (castrated rams) were administered FK 33-824 via two s.c. or i.v. injections (1 hour apart), or wethers were infused (i.v.) with FK 33-824 (8μg/kg/h) for 2 hours. In contrast to the results in steers, both injections of FK 33-824, either i.v. or s.c. elicited an increment in GH release in wethers. Furthermore, acute i.v. infusion of FK 33-824 (8μg/kg/h) stimulated GH during the 2 hours experimental period in wethers. When wethers were chronically administered (s.c.) a low (3μg/kg/h) or high (12μg/kg/h) dose of FK 33-824 for 7 days, the high dose but not the low one stimulated GH secretion during the first 4 hours. However, by 48 hours after initiation of treatments, concentrations of GH were similar in control, low and high-dose wethers. The authors speculated that neurones mediating the effects of endogenous opioid peptides on GRF became tolerant to continous exposure to FK 33-824 (Armstrong and Spears, 1991).

During the follicular phase in heifers, morphine and the met-enkephalin analogue FK 33-824 stimulated GH secretion within 60 minutes after injection (Armstrong et al., 1993).
At the beginning of gestation, plasma GH concentrations remain high and these high levels are maintained throughout pregnancy. Surprisingly, naloxone induced a significant increase in GH around day 280 but not at an earlier or a later period in ovary-intact and pregnant mares (Aurich et al., 1999). In contrast, naloxone decreases GH secretion during pregnancy in rats (Miki et al., 1981) as well as in cattles and pigs (Armstrong et al., 1993). These results indicate that GH release at gestation is partly influenced by an opioid tone that may be stimulatory or inhibitory at particular periods during pregnancy depending on the animal species.

During lactation, serum concentrations of growth hormone is elevated in ruminants and swine. Nevertheless, activation of opioid receptors with FK 33-824 stimulated growth hormone secretion in cows (Armstrong et al.,1991).
In the postpartum cow, administration of FK33-824 significantly elevated GH secretion on day 47 and 121, but not on day 19 postpartum. This result indicates that a shift in endogenous opioid tone on GH secretion is apparent in the postpartum cow (Armstrong et al., 1991). Surprisingly, when naloxone was administered on days 19, 47 and 121 postpartum, a decrease in GH secretion was only observed on day 47 p.p. (Armstrong et al., 1991). In another study, 3 injections of naloxone were administered to lactating beef cows on day 27, 51, 91 and 110 postpartum. Serum growth hormone levels were unaffected by naloxone (Moore et al., 1992). Although, opioid agonists clearly stimulated release of GH, the response to naloxone showed that elevated growth hormone during lactation in the cow is not likely solely the result of elevated endogenous opioids. Nevertheless, the possibility exists that receptor subtypes not affected by naloxone are involved in the regulation of growth hormone in the cow post partum.
The role of opioid peptides on GH response to stress in nursing sows was investigated. Results disclosed that piglet removal and restraint (nose-snare prohibition) decreased GH secretion in sows. Treatment of sows with naloxone injections (i.v. 2mg/Kg) was

found to decrease GH concentration. But return of piglets to sows augmented the GH release again. The rise in GH release following piglets' return was abolished by the combination of restraint and naloxone. This indicates that endogenous opioids may comprise one of the stimulatory pathway leading to GH increment during stress in sows (Rushen et al., 1995).

1.5.3 Interaction of opioids and GHRH in the control of growth hormone secretion

Majority of the studies performed to investigate the role of opioids on GHRH or GRF-induced GH secretion have been carried out on humans and rats. Information on the interaction of opioids and the hypothalamic releasing hormones in the pig is scarse.

Several studies indicate that endogenous opioids participate in the regulation of growth hormone release through down- and up-regulatory mechanisms of the two hypothalamic neuropeptides: growth hormone releasing hormone (Barbarino et al., 1987, Delitala et al., 1989, Armstrong et al.,1990, Vaccarino and Taube, 1997, Tomasi et al., 1998) and somatostatin (Wehrenberg et al., 1985, Armstrong et al., 1990).

When adult rats were passively immunized against GRF, a complete inhibition of GH stimulatory response to morphine and ß-endorphin occured. From these results, the authors suggested that opioid peptide stimulation of pituitary GH secretion is mediated through hypothalamic GRF (Wehrenberg et al., 1985). Furthermore, administration of morphine appeared to increase the GRF-induced GH release in male rats (Vaccarino and Taube, 1997).

In human, the endogenous opioid, ß-endorphin was found to enhance GHRH-induced GH secretion in prepubertal children but not in pubertal children (Pugliese et al., 1992). Similarly, ß-endorphin infusion into adult human males enhanced GH response to GHRH (Schulte et al., 1993). The effect of the met-enkephalin analogue (G-DAMME) on GH response to GHRH in normal men was studied. Simultaneous

administration of the opioid agonist G-DAMME and GHRH caused a higher GH response than when G-DAMME or GHRH was given alone. Since the GHRH-(1-29)NH_2 dose (100µg) used was a maximally stimulatory one, the authors suggested that the enhancing effect of G-DAMME on GHRH-induced GH release may be mediated through inhibition of somatostatin release (Delitala et al., 1989).

The effect of chronic naltrexone treatment on GH secretion in normal human females (25-38yrs old) revealed that long term opioid receptor blockade has an inhibitory role on GHRH-induced GH secretion but showed no effect on basal GH release. The inhibition of GHRH-induced GH release without interference with GH basal level could indicate that opioids regulate only GH dynamics. The authors presumed that an opioid-induced enhancement of somatostatin activity or secretion could be responsible for the differential effect of naltrexone on GHRH/GH release and GH basal secretion. Nevertheless, a direct involvement of opioid control at the pituitary cannot be excluded (Villa et al., 1997). Similarly, opioid receptor blocade with naloxone significantly blunted the GH response to GHRH in postpubertal human males. These findings indicate that there exists an opioid stimulatory tone on GH secretion (Tomasi et al., 1998).

In the adult pig, opioid control of GH secretion was studied in primiparous sows that had been immunized against GRF (1-29) conjugated to human serum albumin (HSA) or only HSA, to determine changes in GH after naloxone treatment. Naloxone suppressed GH levels in all sows. In HSA sows, naloxone abolished episodic release of GH and decreased average but not basal (lowest levels) concentrations of GH. In sows immunized against GRF-HSA, naloxone decreased average and basal GH levels but failed to decrease frequency of GH pulses. The authors concluded that opioids alter concentrations of GH release not only through GRF-dependent but GRF-independent pathways are also involved (Armstrong et al., 1990).

2.0 AIMS AND OBJECTIVES

As the literature review indicates, most of the opioid effects are brought about via the action of opioids on the central nervous system. Peripheral opioid effects or a direct effect on pituitary are however also probable. Thus the present project aimed to evaluate the direct effects of opioids on pituitary LH and GH secretion. To achieve this, a series of experiments were conducted with the following questions using a pig pituitary perifusion system.

- Do opioids affect spontaneous LH and GH secretion ?
- Do opioids affect LHRH-induced LH secretion ?
- Do opioids affect GHRH-induced GH secretion ?
- Are opioid effects on GH secretion modified during LHRH surge ?
- Are these opioid effects gender- and/or age-dependent ?

3.0 MATERIALS AND METHODS

3.1 Experimental animals

A total number of 147 female and male Göttingen miniature pigs with ages 254 ± 3.7days (adults), 14 ± 2.4days (piglets) and 109 ± 3days post coitus (fetuses) were taken from the institute's experimental farm. The adults were fed twice daily with a commercial diet (≅ 1.5kg/day). Pregnant animals received a standard diet for pregnant sows. The piglets were kept with their sows for suckling until slaughter. The fetuses were obtained from the sows immediately after caesarian section. The experimental animals were kept under natural lighting condition. The room temperature was maintained at approximately 22°C and relative humidity of 60%. Data presented on table 3 indicates the number of animals in the different groups according to age and gender irrespective of the replicates of the different treatments.

Table 3 : Number of experimental animals according to age and gender

Gender / Group	Adults	Piglets	Fetuses
Males	46	17	13
Females	39[1]	19	13
Total	85	36	26

1= 31 adult females with P_4 > 3.5ng/ml (luteal phase) and 8 with P_4 <3.5ng/ml

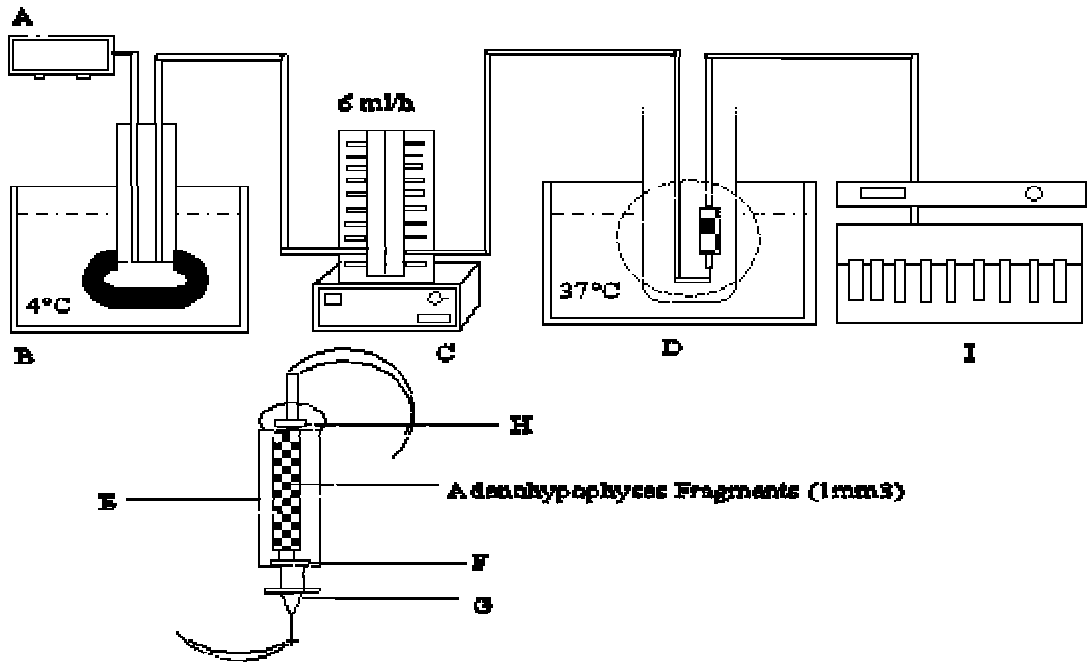
3.2 Removal of anterior pituitary tissue

To obtain adenohypophyses (anterior pituitary, AP) from the fetuses, caesarian sectioning of pregnant sows was carried out under general anaesthesia (Methomidate-HCL, 500mg/sow, Janssen, Düsseldorf, Germany) and strict hygienic conditions. Anterior pituitary tissues were removed from the adults and piglets immediately after slaughter in the slaughter house of the institute.

Pituitary glands were aseptically removed and immediately introduced into a petri dish containing solution 2 (see 3.4) within 5-7 minutes of death. The anterior and posterior lobes were carefully separated after washing with solution 2. The anterior lobe was cut into small fragments of ≅1mm^3 using sterile scalpel blades. The fragments of adenohypophyses were further washed with solution 2 and transfered into the perifusion chambers with pasteur pipettes. The weights of the pituitaries were measured before beginning the perifusion.

3.3 Perifusion system

The perifusion system was set up as described by Porter (1985) and Porter and Licht (1985) and as modified by Gracia-Navarro et al., (1991) see scheme 2. The adenohypophyses tissues to be perifused were placed in small columns fashioned from 1ml disposable plastic syringes (Henke-Sass-Wolf, Tuttlingen, Germany). The effective volume of each column was 100µl. These columns were placed in 25ml tubes that were inserted in a warm water bath maintained at 37°C. Each perifusion chamber (column) contained only fragments from one animal, with ½ adenohypophysis (AP) tissue for adults and piglets and 1 AP for the fetuses. 12 chambers were run simultaneously in parallel at each experiment. All 12 chambers with AP tissues were attached to a peristaltic pump (Ismatic type IPN 12, Zürich-Switzerland) that delivered the minimum essential medium (MEM) (solution 1, see 3.4) at a flow rate of 0.1ml/min (6ml/h) and fraction samples were collected at 15 minutes or 5 minutes intervals. Before begin of treatment, tissues were perifused for about 22-24 hours with medium only to acclimatize the cells and stabilize the basal secretion of hormones.



Scheme 2 : Schematic diagram of perifusion system modified after Gracia-Navarro et al., (1991).

A = Air pump
B = Cold water bath (4°C)
C = Peristaltic pump
D = Warm water bath (37°C)
E = Chamber for pituitary tissue
F = Nylon filter platform
G = Hypodermic needle
H = Rubber stopper
I = Fraction collector

3.4 Reagents

Solution 1 (perifusion medium)

- Minimum Essential Medium (MEM) eagle (Sigma , Munich, cat n° M0643) composed of 2.2g $NaHCO_3$ and 2.383g HEPES was dissolved in autoclaved bidest to obtain 1 litre of perifusion solution with pH 7.3 .The medium was filtered into autoclaved bottles.
- Antibiotics : 100 UI/ml penicillin, 0.25µg/ml amphotericin B, 100µg/ml streptomycin (Antibiotic/Antimycotic drugs, Sigma n° A 7292) were also dissolved in 20 ml autoclaved bidest. After filtration with membrane filters (milipore type 0.22um), 10 ml of the antibiotic solution was transfered into the 1 litre MEM perifusion solution and later conserved at 4°c in a refrigerator. Before anterior pituitaries were perifused, 1g of bovine serum albumin (BSA) was diluted and given into the perifusion medium.

Solution 2 (extraction and washing medium)

- 100 ml of perifusion solution (solution 1) was measured and 0.5 ml antibiotic solution was added into it. 10% BSA was diluted in phosphate buffer saline (PBS) composed of 0.56g $NaH_2PO_4 .2H_2O$ and 0.85g NaCl at pH 7.4. The solutions were mixed and stirred with a magnetic stirrer, then covered with parafilm and stored at 4°c. Both the perifusion and extraction/ washing solutions were used within 2 weeks.

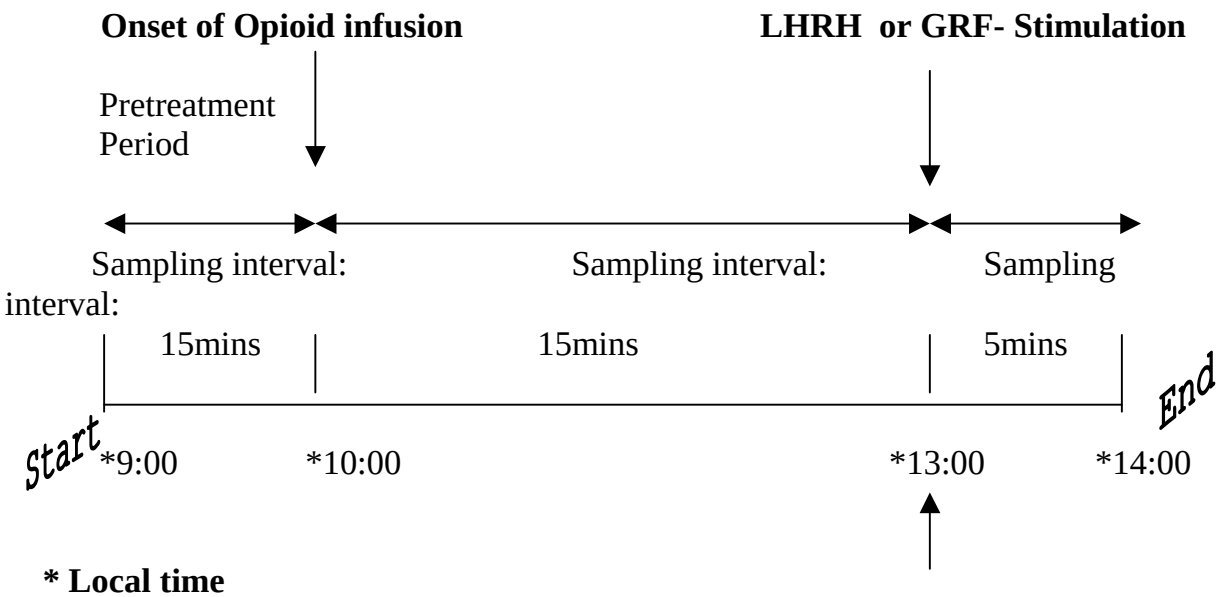
3.5 Experimental design

Following stabilization, the pituitary tissues were perifused for 5 hours. Samples were collected every 15 minutes for 60 minutes before the onset of opioid treatment. Immediately thereafter the infusion with one of the following opioids began: ß-endorphin 5 x 10^{-9}M (ß-endorphin, E0637, Sigma-Aldrich Chemie GmbH, Steinheim-Germany) ; dalargin 10^{-6}M (D-Ala^2-Leu-enkephalin-arg, Bachem,

Biochemica GmbH; Heidelberg-Germany); naloxone 10^{-6} M (Naloxone Hydrochloride, Sigma-Aldrich Co., München-Germany) and concomittant infusion of naloxone 10^{-6}M and ß-endorphin 10^{-9}M. The perifusion with opioids lasted for180 minutes during which the collection of samples occurred in 15 minutes interval.
At the end of opioid infusion, some pituitaries were stimulated with 10^{-9}M luteinizing hormone releasing hormone (LHRH, Bachem, Biochemica GmbH, Heidelberg-Germany) or 10^{-9} M GRF (GHRH, Bachem, Biochemica GmbH, Heidelberg-Germany) for 5 minutes. After application of LHRH or GRF, samples were collected for 60 minutes at 5 minutes intervals (see scheme 3). Each sample of 1ml was centrifuged at 3000g for 10 minutes at 4°c to discard the cellular debris. The supernatant was then transfered into labelled test tubes and immediately stored at -20°c until hormone analysis were carried out. Control samples were obtained in which saline treatment was given into perifusion medium after 1 hour pretreatment period and later LHRH or GHRH treatments were administered after 3 hours of sampling. Data on table 4 show number of animals used for the different treatments as well as controls.

Table 4: Number of animals for the different treatments.

	Adults		Piglets		Fetuses	
Treatments	Females	Males	Females	Males	Females	Males
ß-endorphin + LHRH	12	8	5	4	4	3
Dalargin +LHRH	5	6	3	5	2	3
Naloxone + LHRH	10	8	4	4	4	3
ß-end + Nal + LHRH	7	6	-	-	-	-
Saline + LHRH	5	8	7	4	3	4
ß-end + GHRH	-	5	-	-	-	-
Saline + GHRH	-	5	-	-	-	-
Total	39	46	19	17	13	13



Scheme 3: Schematic representation of experimental design

3.6.0 Hormone Analysis

3.6.1 Luteinizing Hormone (LH) Analysis

The concentration of LH in the samples was evaluated through a homologous double-antibody radioimmunoassay (RIA) as described by Pomerantz et al., (1974) and Ponzilius et al., (1986). The specific antiserum (polyclonal) for porcine LH extracted from a rabbit was got from UCB-bioproducts, Brüssel, Belgium) and was used in a dilution of 1: 80000. 100µl of this solution can bind to 30% of the ^{125}I- labelled porcine LH (tracer). The cross-reactivity of this antiserum with other porcine hypophyseal hormones is less than 1%. The standards were evaluated in triplicate whereas the samples underwent double analysis. Pure porcine LH (UCB-bioproducts, Brüssels, Belgium) with a biological activity of 0.85IU/mg x NIH st. LH S19 was used in the standards and the radiolabelling with ^{125}I. The minimum detectable concentration of LH in the assay was at 0.2ng/ml. The intra- and interassay coefficients of variation were 3.5 and 6.5 % respectively. The CPM in the standards

and samples was estimated by an automatic γ-Counter (LKB- Wallace, 1277 Gamma-master) and the concentration of LH was done by a RIA calc. computer programm.

3.6.2 Growth Hormone (GH) Analysis

Growth hormone concentration was also measured with a homologous double-antibody radioimmunoassay that has been modified and validated in our laboratory (Bauer and Parvizi, 1996). Pure porcine GH utilised for the standards and tracer was obtained from (UCB-bioproducts, Brüssels, Belgium). The first antibody (Rabbit antiserum pGH, final dilution 1:100000) and the second antibody (Goat-antiserum-antirabbit gammaglobulin, final dilution 1:100) were extracted and prepared in our laboratory. These antibodies (polyclonal) do not show any cross-reactivity with other pituitary hormones. The intra- and interassay coefficients of variations were 6.5 and 11.5 % respectively.

3.6.3 Progesterone Analysis

Plasma samples obtained from adult females were evaluated for progesterone (P4) level using an enzymimmunoassay validated and modified in our laboratory (Herrler et al., 1991).The specific antiserum (polyclonal) solution utilised was obtained from goat and showed high affinity in a concentration of 3µg/ml. The enzyme solution was made of 2.5% caesin in a buffer solution with pH 7.2 and given at a proportion of 300-350 µl/ well.The tracer used was HRP-6-progesterone in a concentration of 4µg/ml.The intra- and interassay coefficients of variation were 12.2 and 15.5% respectively. The cross-reactivity with 17α-hydroxyprogesterone, estradiol and cortisol were less than 0.1%. Females with high (P_4 >3.5ng/ml) and low (P_4 <3.5ng/ml) plasma progesterone levels were grouped and tested separately.

3.7 Statistical Analysis

The data obtained from the measurements of LH and GH concentrations were statistically analysed using the "SAS" statistic programm (version 6.08). Firstly, the absolute mean concentrations of the control samples for the different age and sex groups were determined. Data were converted to percentage of basal secretion before averaging to minimize differences between experiments. The absolute mean concentration of basal secretion for control samples obtained was considered here as 100%. The net percentage increase or decrease of LH and GH release were calculated by : $\frac{ECS - CMCc}{CMCc} * 100\%$

Where ECS = Evaluated concentration of Sample

CMCc = Calculated Mean Concentration of control

A positive net percentage value indicated an increase, therefore added to 100%.
A negative net percentage value indicated a decrease, therefore substracted from 100%.

The area under the curves (AUC) for GH and LH profiles were calculated by substracting basal GH and LH concentrations (mean value) at the pretreatment period from the posttreatment values. The net values were then summed up to obtain the AUC. The Students' t-test was then employed to compare the means of GH and LH concentrations in opioid treated and in saline (controls) in the same age and sex groups at probability level of 5%. To obtain variations in different age groups with the same treatment, the analysis of variance (ANOVA) one-way was used. P-value less than 0.05 ($P < 0.05$) was taken as significantly different from controls. Comparison in different age groups with different treatments was calculated using the one way ANOVA. The standard errors of means (SEM) are not indicated on the figures to enable visual clarity of plots.

4.0 RESULTS

The spontaneous LH and GH release, calculated by the means of samples before the beginning of treatments in different age groups and gender are shown in table 5.

Table 5 : Mean ± SEM of basal secretion of LH and GH before treatment.

	LH (ng/mg/AP)		GH (ng/mg/AP)	
Age / Gender	Males	Females	Males	Females
Adults	12.92 ±4.2	18.42 ±3.65**a** **(1)**	28.4 ±6.3	32.02 ±5.6
Piglets	6.81 ±2.3	8.05 ±3.4	25.7 ±3.4	18.6 ±2.1 **b**
Fetuses	4.05 ±1.35	5.61 ±1.9	63.4 ±15.2	58.3 ±8.7 **c** **(2)**

a indicates a statistical significant difference (P<0.05) in basal secretion of LH from adult females compared with males.
b indicates a statistical significant difference (P<0.05) in basal secretion of GH between female and male piglets.
c indicates a statistical significant difference (P<0.05) in basal secretion of GH from fetuses compared with piglets and adults.
(1) indicates a statistical significant difference (P<0.05) in basal secretion of LH from adults compared with piglets and fetuses.
(2) indicates a statistical significant difference (P<0.05) in basal secretion of GH from fetuses compared with piglets and adults.

The mean basal secretion of LH was significantly higher in adult females (18.42 ±3.65ng/mg/AP) than in adult males (12.92 ±4.2ng/mg/AP). There was no significant difference in basal secretion of LH between male (6.81 ±2.32ng/mg/AP) and female piglets (8.05 ±3.4ng/mg/AP) as well as between male (4.05 ±1.35ng/mg/AP) and female (5.61 ±1.9ng/mg/AP) fetuses. As expected, basal secretion of LH was significantly higher in the adults as compared with the piglets and fetuses. There was no significant difference in LH secretion from pituitaries of sows with low (P4< 3.5ng/ml) and high (P4> 3.5ng/ml) plasma progesterone concentrations. Data on basal secretion of progesterone is not indicated on the above table because the assays were evaluated only for adult females.

Growth hormone basal secretion was different in the 3 age groups, with fetuses showing the highest levels in males (63.4 ±15.2ng/mg/AP) and in females (58.3 ±8.7ng/mg/AP). The basal secretion from piglet (25.7 ±3.4 ng/mg/AP in males and

18.6 ±2.1ng/mg/AP in females) and adult pituitaries (28.4 ±6.3ng/mg/AP in males and 32.02 ±5.6ng/mg/AP in females) differed only slightly. A significant gender-difference in GH basal secretion was only seen in the piglets, with the males showing higher levels (25.7 ±3.4ng/mg/AP) than females (18.6 ± 2.1ng/mg/AP).

4.1.0 Opioidergic effects on LH release

4.1.1 Opioidergic effects on LH release in fetuses

The opioid agonist ß-endorphin caused a significant increase ($p<0.05$) in LH secretion from both male and female fetal pituitaries (adenohypophyses) when compared to controls (fig. 1). ß-endorphin-induced increment in LH secretion started 15 minutes after onset of opioid treatment and lasted for 165 minutes. There was a significant sex-difference ($p<0.05$) in LH secretion from fetal pituitaries, with males showing a greater response (425 ±20%) than females (275 ±15%) see (table 6 on page 53). Naloxone and dalargin did not affect LH secretion from both pituitaries of fetal females and males (fig. 2 & 3). The LH release in response to naloxone reached only 150 ±10% in females and 130 ±15% in male pituitaries. The dalargin-induced LH release was also marginal reaching 130 ±15% in females and 120 ±10% in male pituitaries (table 6 on page 53).

4.1.2 Opioidergic effects on LH release in piglets

A high individual variation in response to ß-endorphin was a characteristic reaction in piglets. The ß-endorphin-induced LH discharge was seen in pituitaries of 3 out of 5 females and 3 out of 4 males. ß-endorphin was found to significantly increase ($P<0.05$) LH secretion from both female and male pituitaries of piglets when compared to controls (fig 4). Increase in LH secretion began 15 minutes after opioid treatment and lasted for 160 minutes in both pituitaries of females and males. The ß-endorphin-induced LH secretion attained peak levels of 325 ±35% in males and 175 ±15% in females (tab. 6).

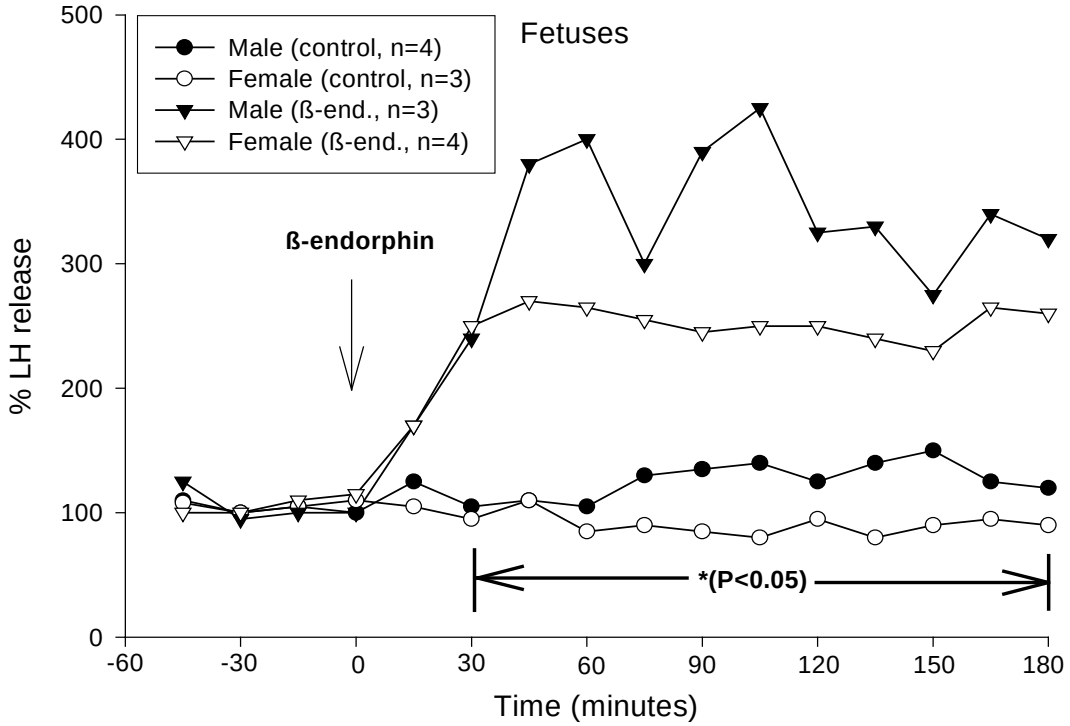


Figure 1: In vitro release of LH from perifused pituitaries of fetuses treated with or without beta-endorphin.
***P<0.05** shows a statistically significant difference between treated males and females and controls at period indicated with arrows.

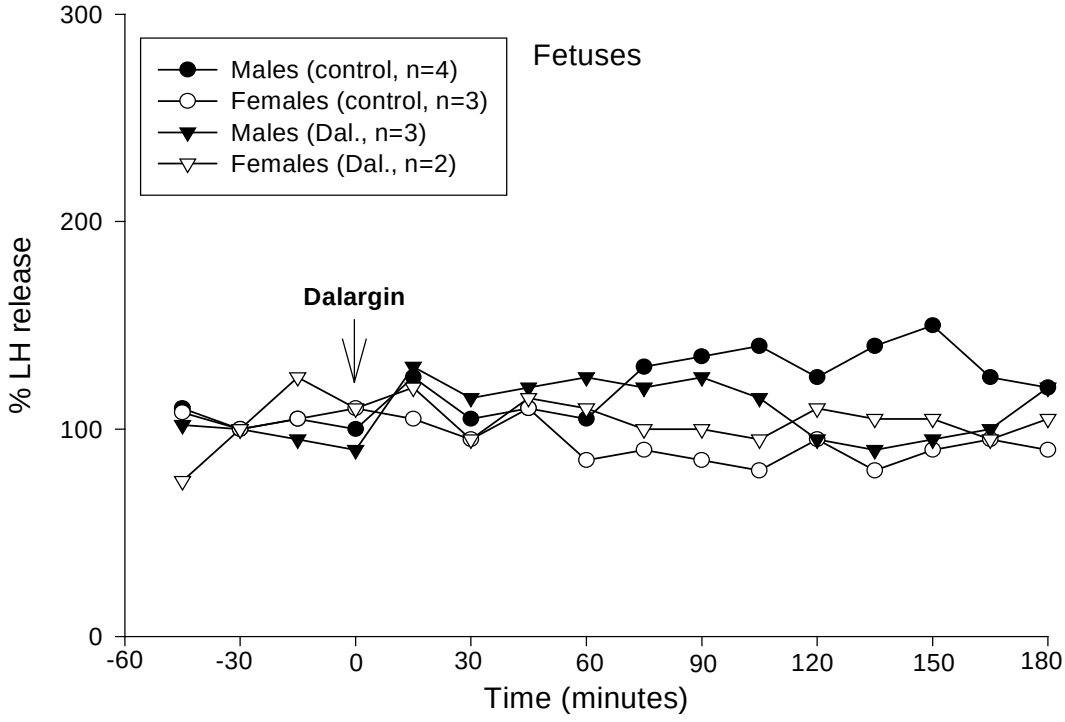


Figure 2: In vitro release of LH from perifused pituitaries of fetuses treated with or without dalargin.

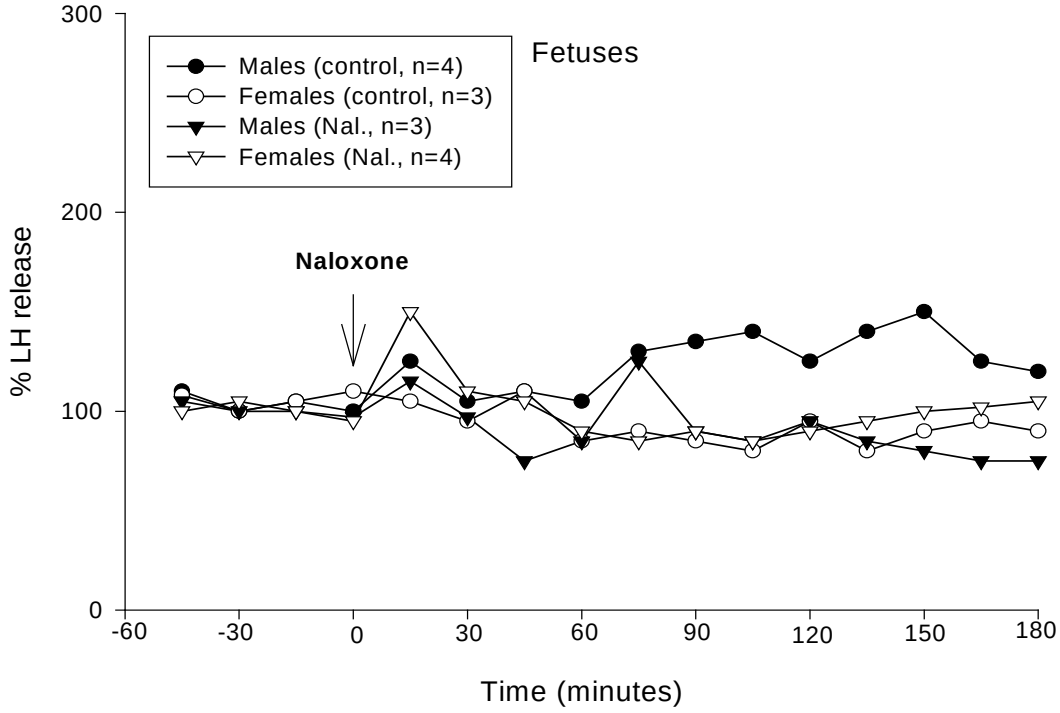


Figure 3: In vitro release of LH from perifused pituitaries of fetuses treated with or without naloxone.

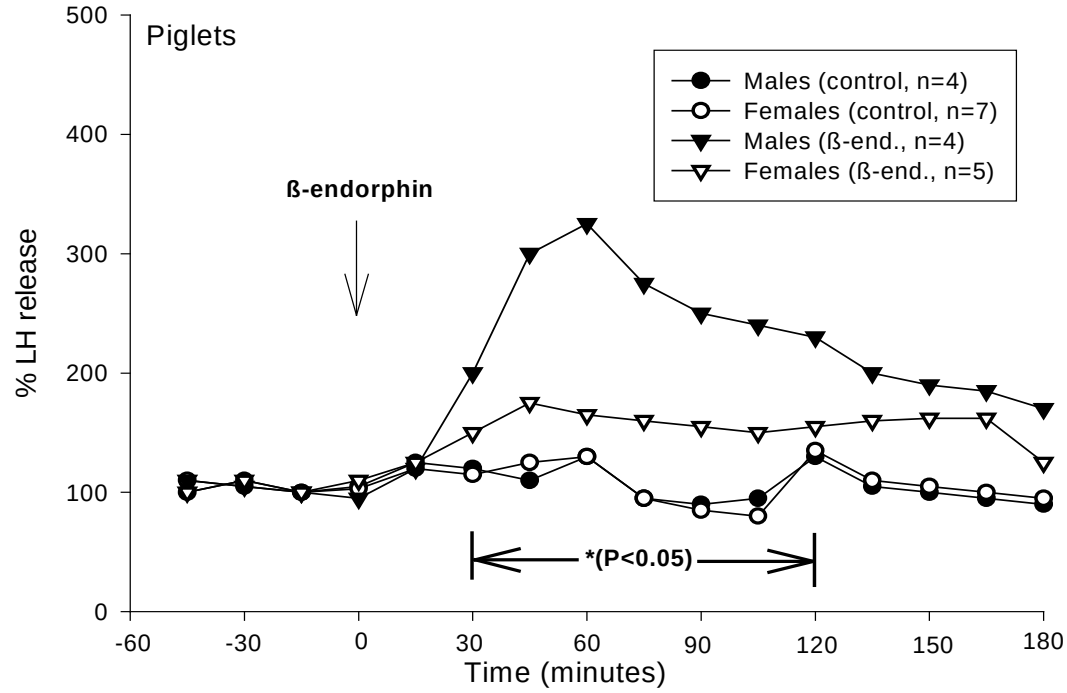


Figure 4: In vitro release of LH from perifused pituitaries of piglets treated with or without beta-endorphin. ***P<0.05** shows a statistically significant difference between treated males and females and controls at period indicated with arrows.

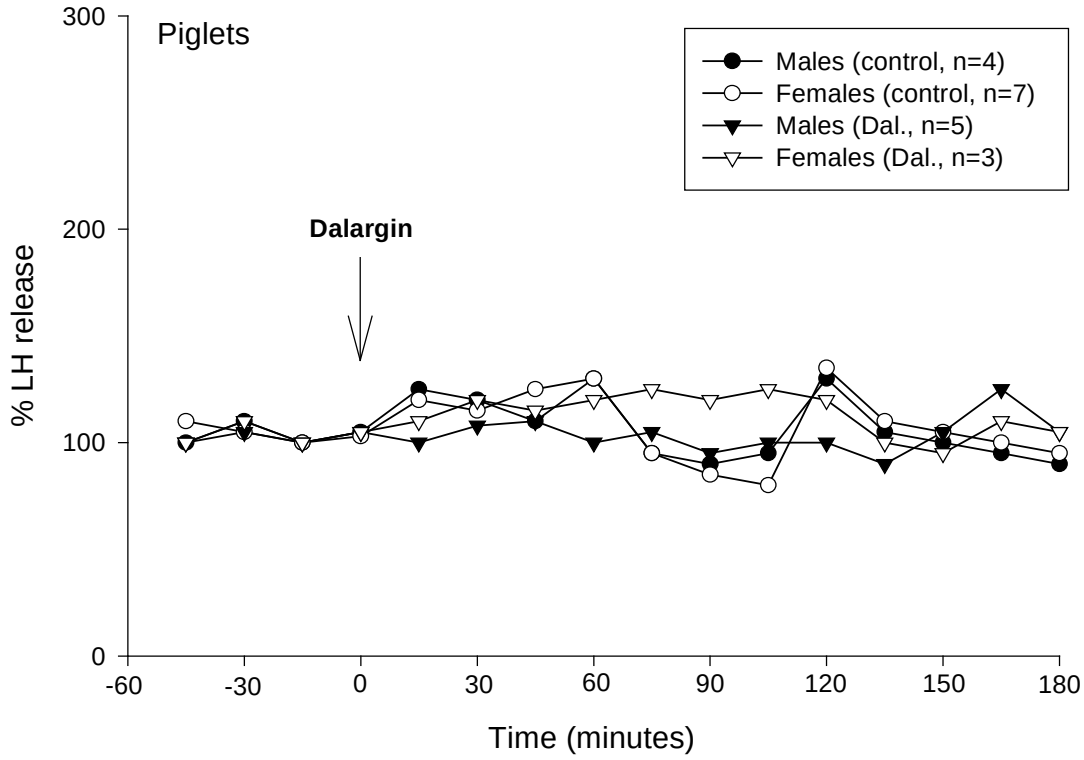


Figure 5: In vitro release of LH from perifused pituitaries of piglets treated with or without dalargin.

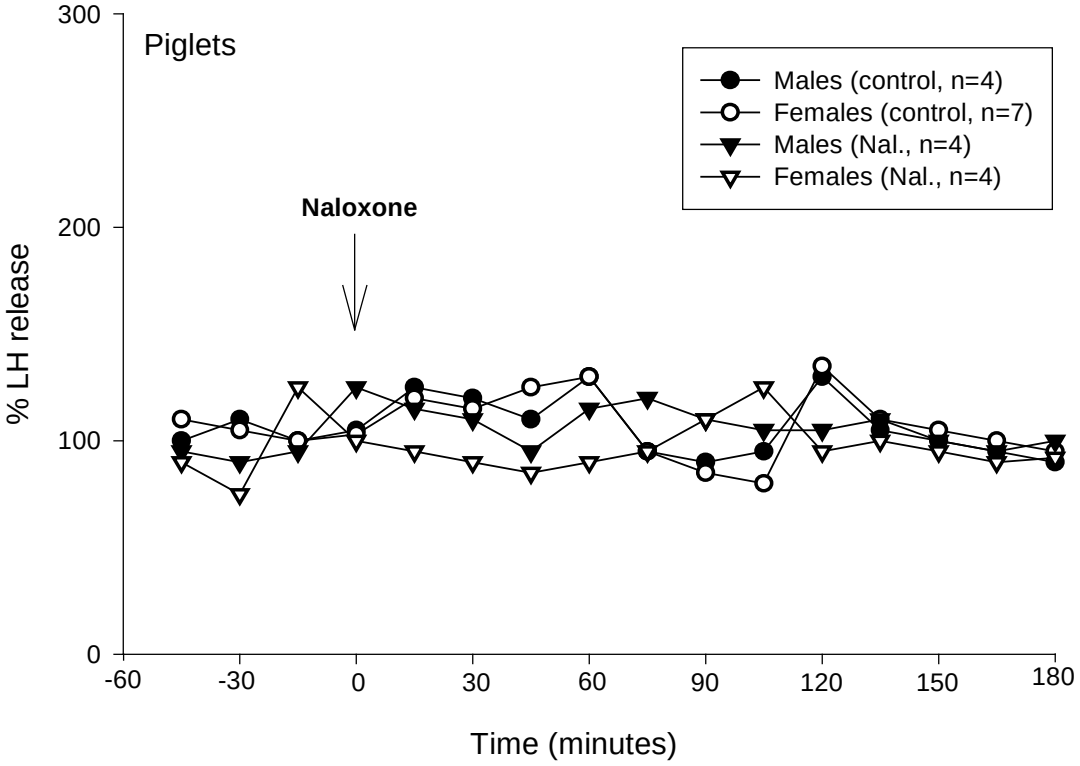


Figure 6: In vitro release of LH from perifused pituitaries of piglets treated with or without naloxone.

However, the total LH increase found in piglets was significantly lower than that seen in fetal pituitaries.

Naloxone and dalargin did not show any effects on LH secretion in pituitaries of both female and male piglets. The naloxone-induced LH release reached 125 ±20% in pituitaries of females and 125 ±40% in males. The LH release in response to dalargin attained 135 ±15% in pituitaries of males and 115 ±10% in females (table 6).

4.1.3 Opioidergic effects on LH release in adults

ß-endorphin elicited a significant increase ($p<0.05$) in LH release in pituitaries of adult females and males as compared to controls. In adult male pituitaries LH secretion reached a peak level (highest concentration of LH measured after the administration of opioid) of 245 ±45% recorded 90 minutes after opioid treatment (fig. 7). LH secretion in adult female pituitaries attained a peak level of 160 ±15% recorded 60 minutes after opioid treatment in females with plasma progesterone level higher than 3.5ng/ml (see figure 9). The LH discharge in response to ß-endorphin treatment was significantly higher ($p<0.05$) in adult males than in adult females. The net increase in LH secretion was higher in adult pituitaries than in fetuses and piglets when their area under the curves (AUC) were compared.

Again, naloxone and dalargin exerted no significant actions on LH secretion in both pituitaries of adult females and males (fig. 8, 10 & 11). However, a slight but not significant decline in LH release occurred towards the end of the sampling period in response to naloxone in females with low levels ($P < 3.5$ng/ml) of circulating progesterone (fig.11, page 52). Interestingly, neither in adult males nor in adult females did naloxone antagonize ß-endorphin-mediated LH release (fig.12, page 52).

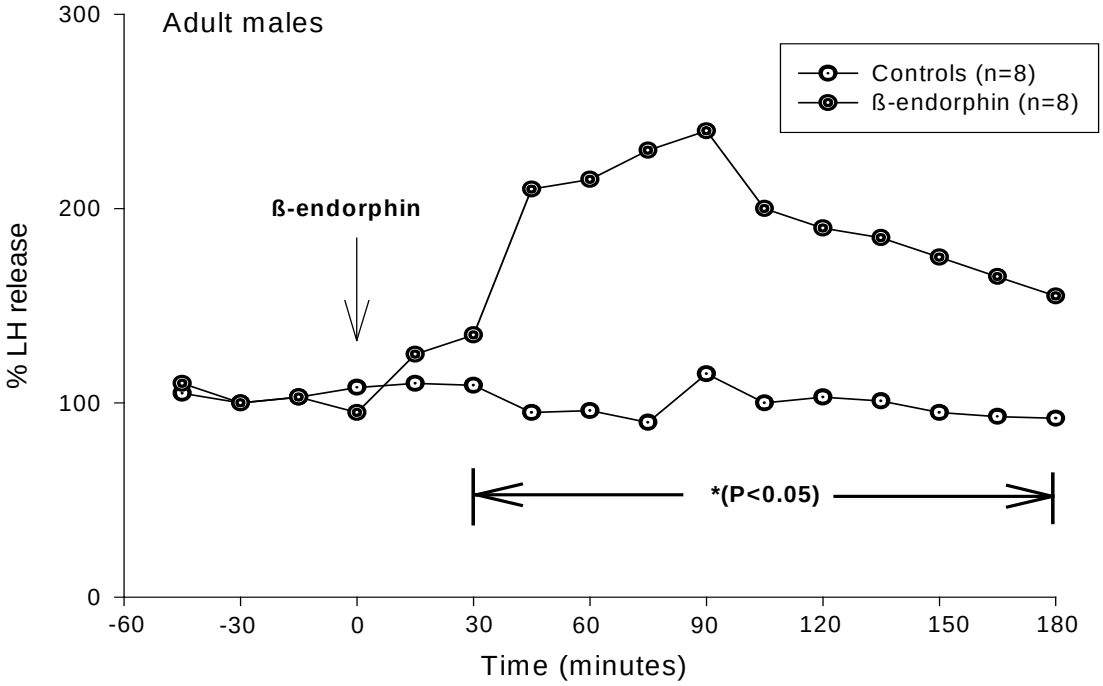


Figure 7 : In vitro release of LH from perifused pituitaries of adult males treated with or without beta-endorphin.
***P<0.05** shows a statistically significant difference between treated males and controls at period indicated with arrows.

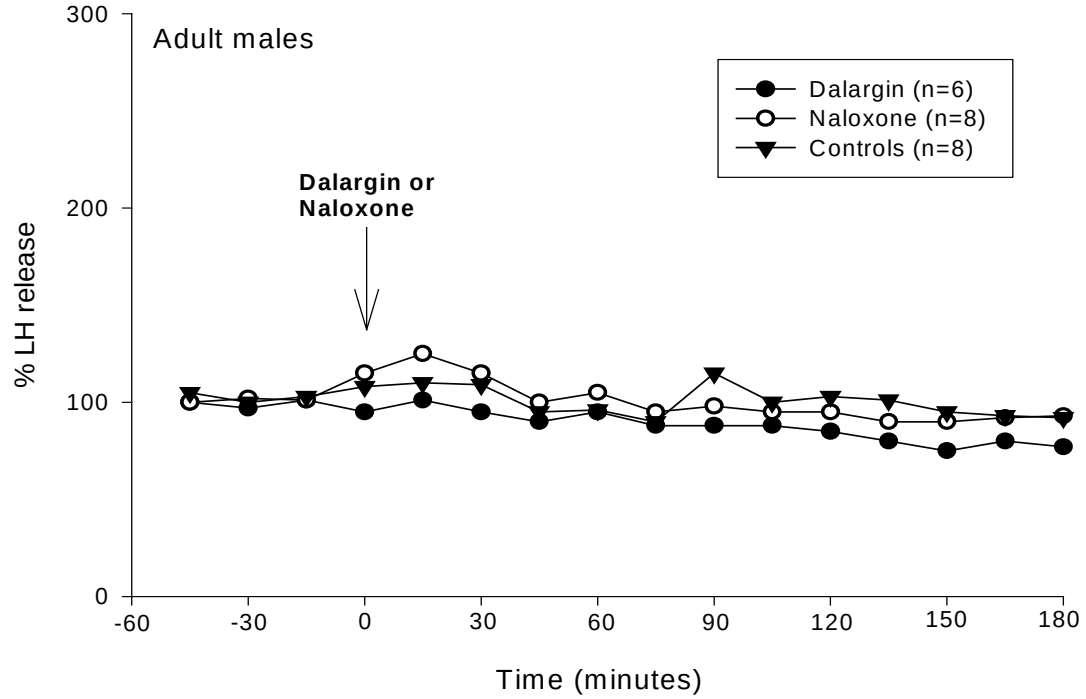


Figure 8 : In vitro release of LH from perifused pituitaries of adult males treated with or without dalargin or naloxone.

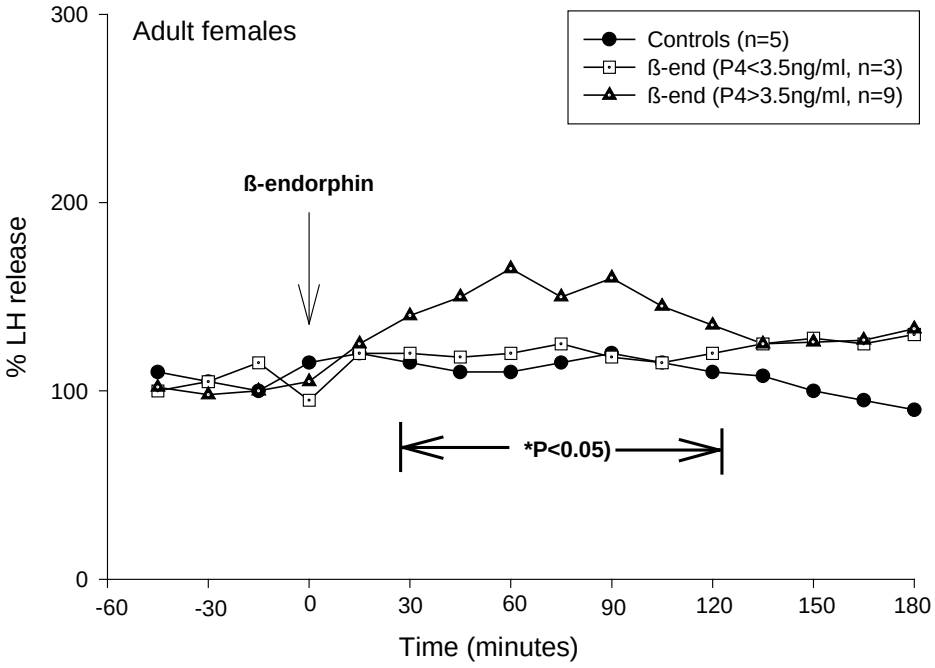


Figure 9 : In vitro release of LH from perifused pituitaries of adult females treated with or without beta-endorphin. ***P<0.05** shows a statistically significant difference between treated females with high (P4>3.5ng/ml) and low (P<3.5ng/ml) or controls (P=3.5ng/ml) progesterone levels at period indicated with arrows.

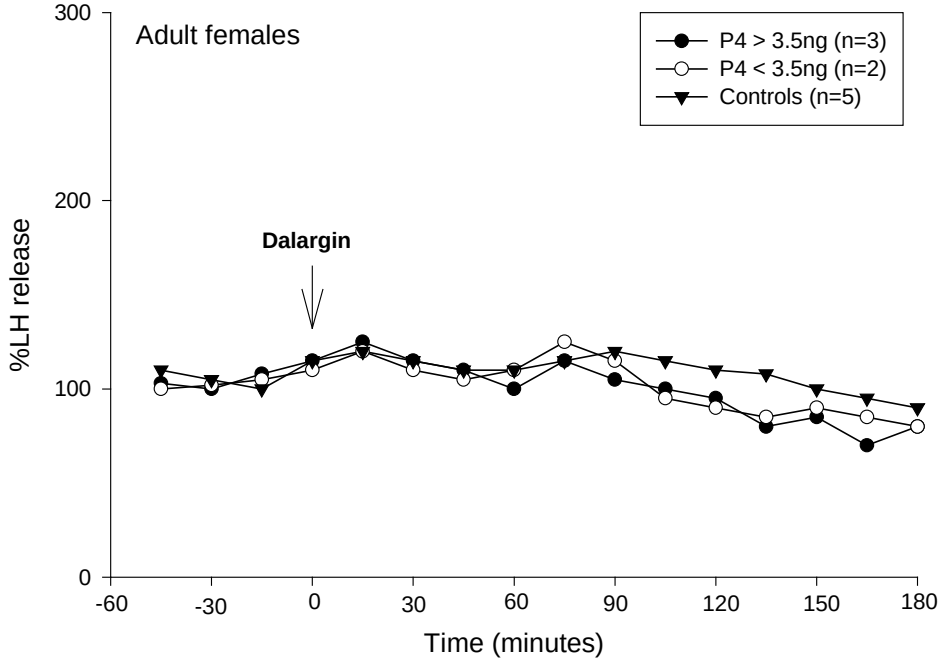


Figure 10 : In vitro release of LH from perifused pituitaries of adult females treated with or without dalargin.

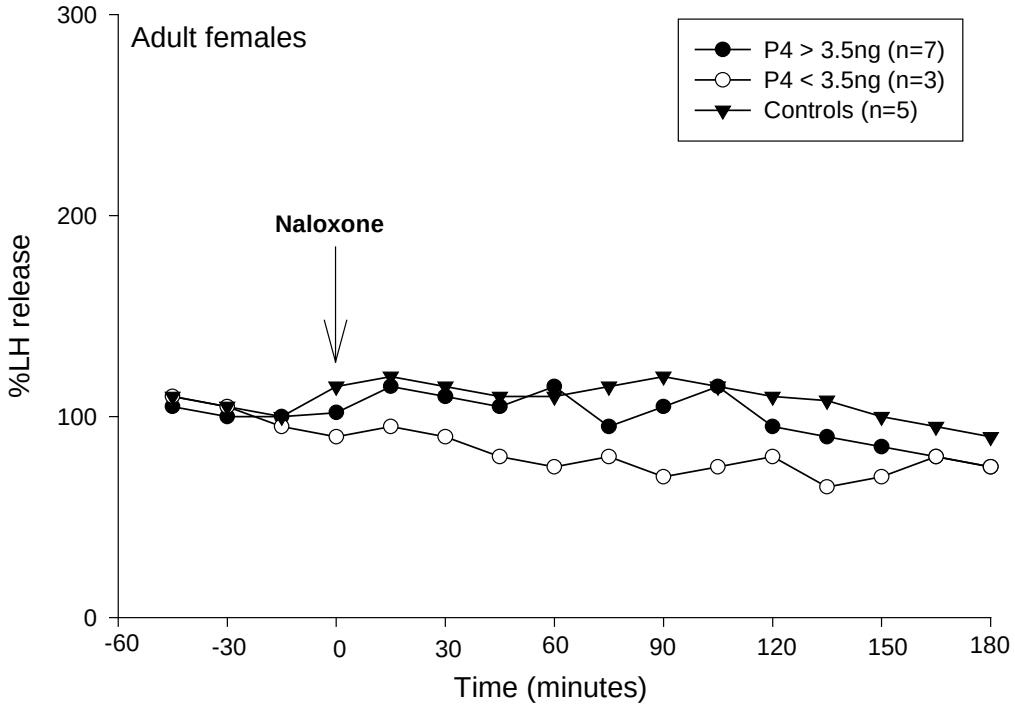


Figure 11 : In vitro release of LH from perifused pituitaries of adult females treated with or without naloxone.

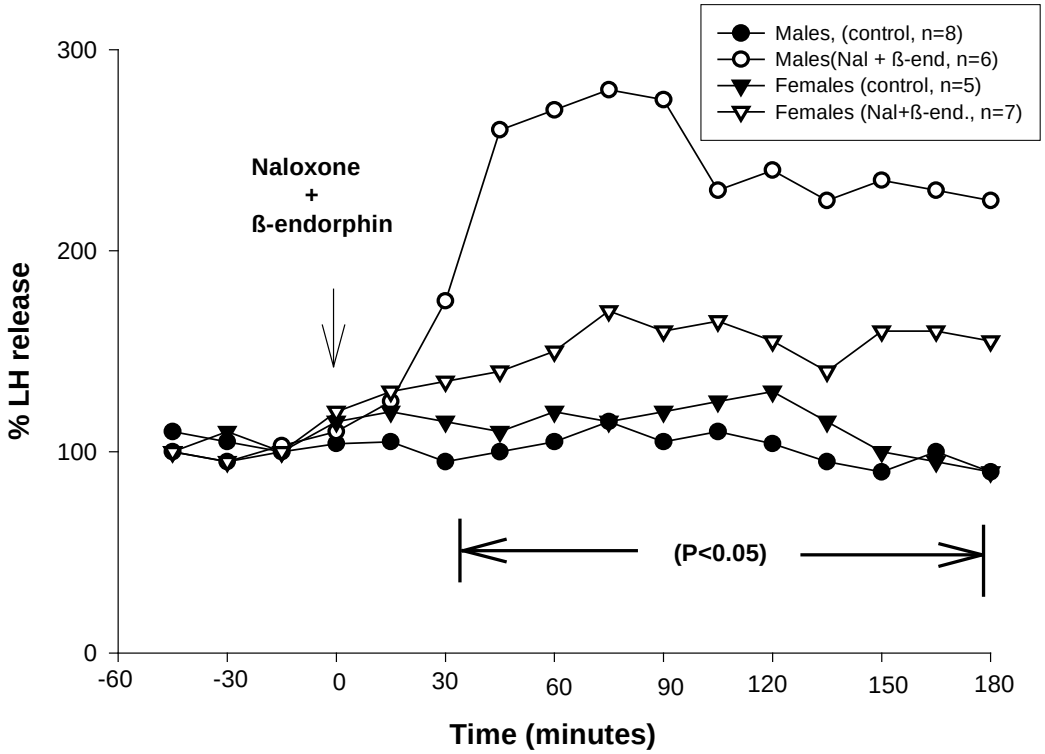


Figure 12 : In vitro release of LH from perifused pituitaries of adult males and females treated with or without naloxone + ß-endorphin.***P<0.05** shows a statistically significant difference in treated males and females and controls at period indicated with arrows.

Table 6 : Opioid-induced LH release from pituitaries of females and males in all age groups.

Age group	Fetuses		Piglets		Adults	
Gender/ Parameter	Females	Males	Females	Males	Females	Males
% Peak level of ß-endorphin-induced LH release	* 275 ±15% (n=4)	* **(a)** 425 ± 20 % (n=3)	* 175 ± 15% (n=5)	* **(b)** 325 ±35% (n=4)	* 160 ±15% (n=9)	*(c) 245 ±45% (n=8)
% Peak level of naloxone-induced LH release	150 ±10% (n=4)	130 ±15% (n=3)	125 ±20% (n=4)	125 ±40% (n=4)	135 ±10% (n=7)	130 ±25% (n=8)
% Peak level of dalargin-induced LH release	130 ±15% (n=2)	120 ±10% (n=3)	115 ±10% (n=3)	135 ±15% (n=5)	105 ±15% (n=3)	135 ±% (n=6)

* indicates a statistical significant (P<0.05) difference compared to corresponding controls considered as 100%

a, b and **c** indicate a statistical significant (P<0.05) difference between ß-endorphin treated males vs. females.

4.2.0 Opioid-LHRH interaction and LH release

The opioidergic interaction with LHRH on LH release was measured in pituitaries from female and male (fetuses and piglets) and adult females. Pituitaries received LHRH (10^{-9}M) stimulation for a period of 5 minutes beginning 180 minutes after opioid treatment and samples were collected at 5 minutes interval for an experimental period of one hour.

4.2.1 Fetuses

Fetal female but not male pituitaries pretreated with ß-endorphin showed a reduction in LHRH-induced LH release that lasted for 10 minutes after LHRH-stimulation when compared to time and age-matched saline pretreated controls see (fig. 13). There was an impairment of LHRH-induced LH release in naloxone and dalargin pretreated pituitaries of fetal females and males beginning 30 minutes after LHRH-stimulation (fig. 14 & 15). These effects were however not statistically significant when compared to controls.

4.2.2 Piglets

There was a 300 % and 205 % enhancement in LH secretion 30 minutes after the start of LHRH challenge in pituitaries of female and male control piglets respectively (fig. 16).

It is interesting to note that the peak level of LH release from pituitaries of male piglets pretreated with ß-endorphin was significantly ($p<0.05$) higher than that caused by LHRH stimulation in the control group (325 % vs 225 %) respectively. Unlike in fetal and adult female pituitaries where ß-endorphin pretreatment resulted to a blockade in LHRH-induced LH release, female piglets showed no effect in the LHRH-induced LH release in ß-endorphin pretreated pituitaries compared to saline pretreated controls.

Pretreatments with naloxone and dalargin also did not modify the LHRH-induced LH release in both pituitaries of female and male piglets (figure 17 & 18).

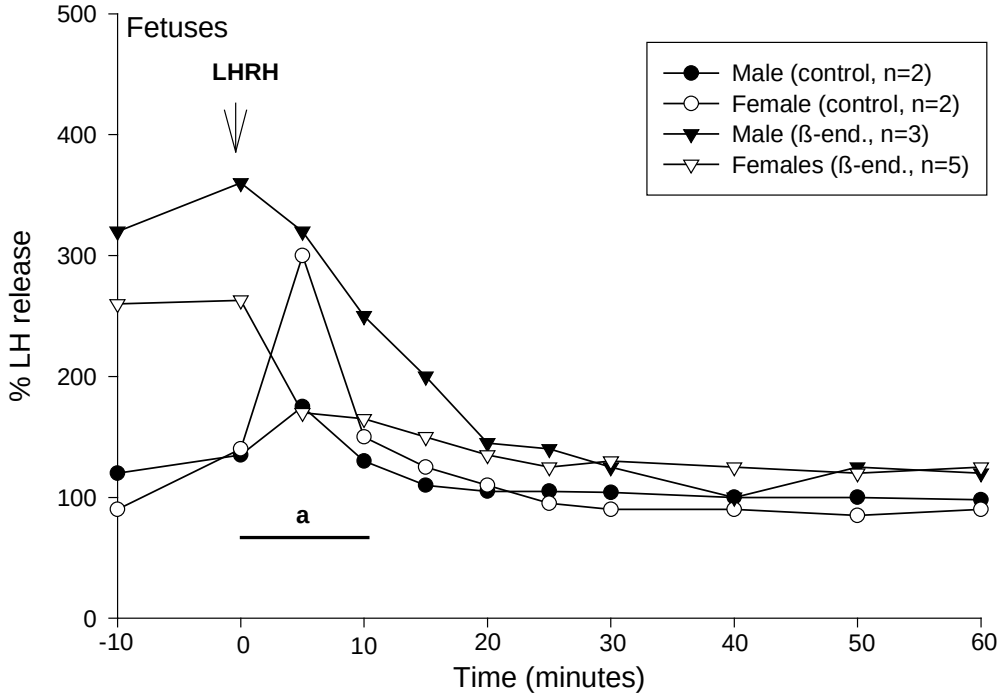


Figure 13: In vitro release of LH from perifused pituitaries of fetuses after beta-endorphin pretreatment and later LHRH administration. **a** shows a statistically significant difference between treated female and control at period indicated with line.

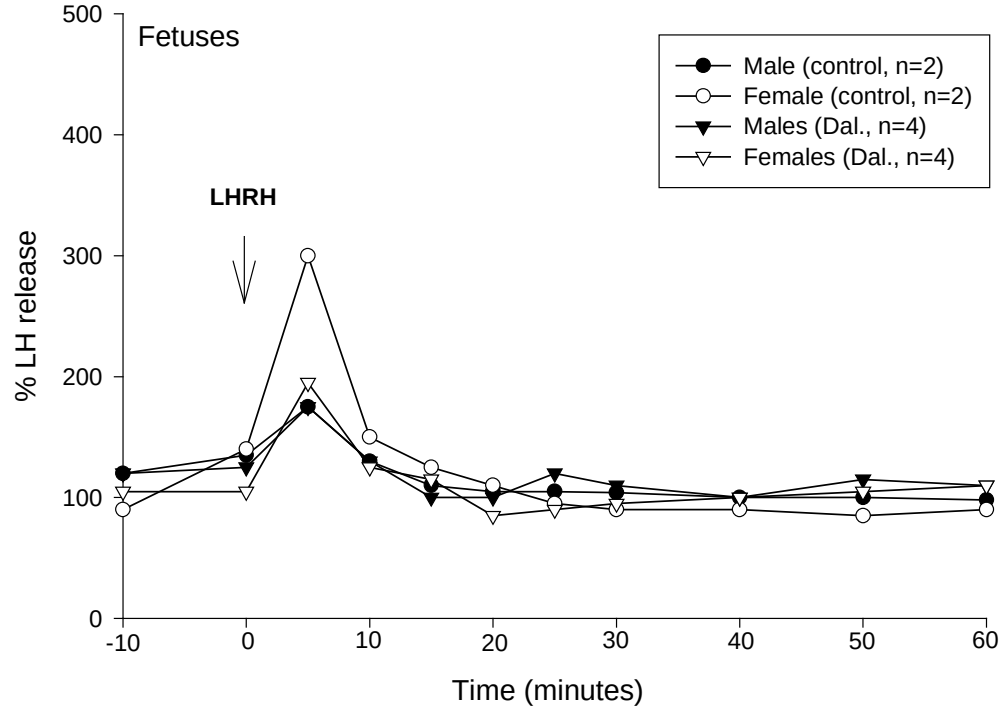


Figure 14: In vitro release of LH from perifused pituitaries of fetuses after dalargin pretreatment and later LHRH administration.

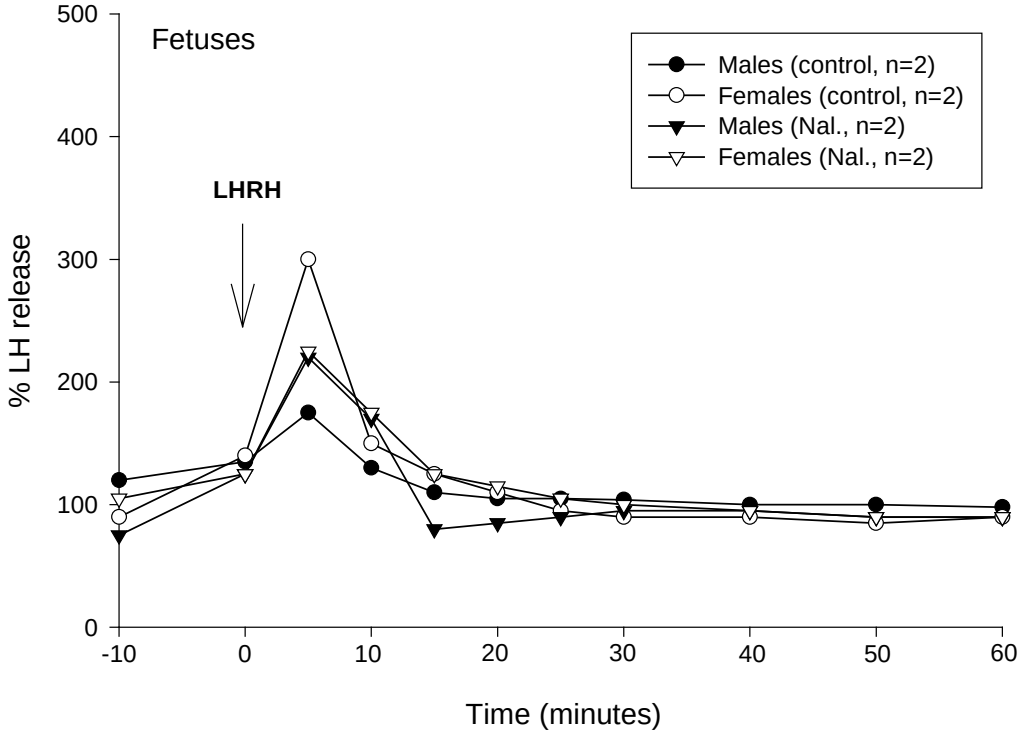


Figure 15: In vitro release of LH from perifused pituitaries of fetuses after naloxone pretreatment and later LHRH administration.

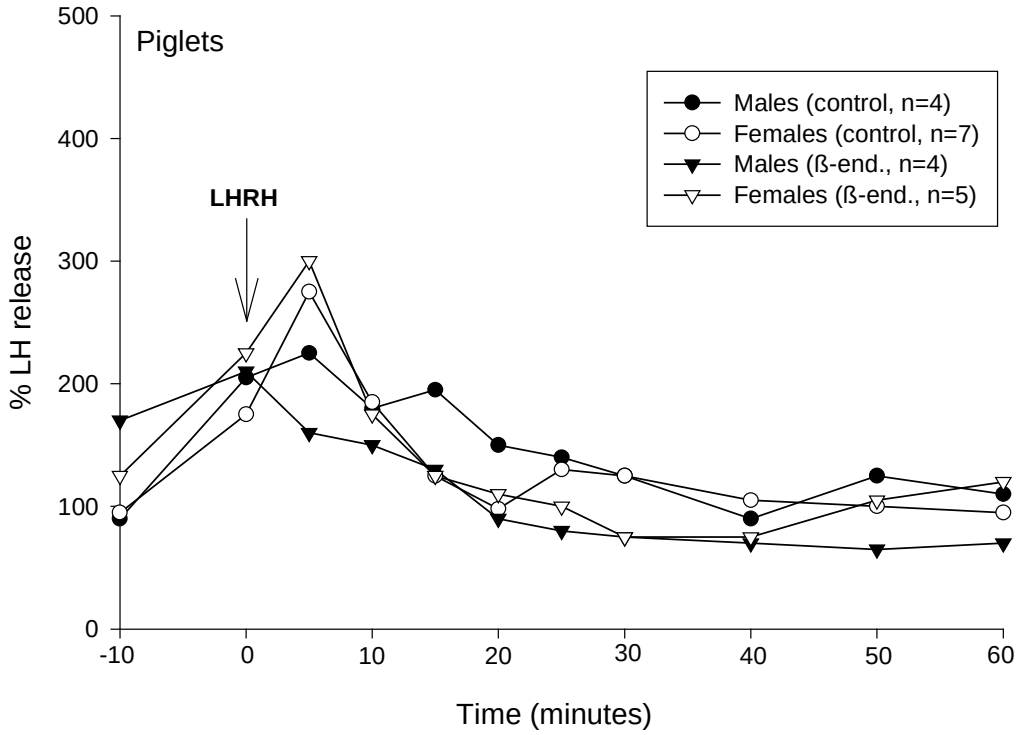


Figure 16: In vitro release of LH from perifused pituitaries of piglets after beta-endorphin pretreatment and later LHRH administration.

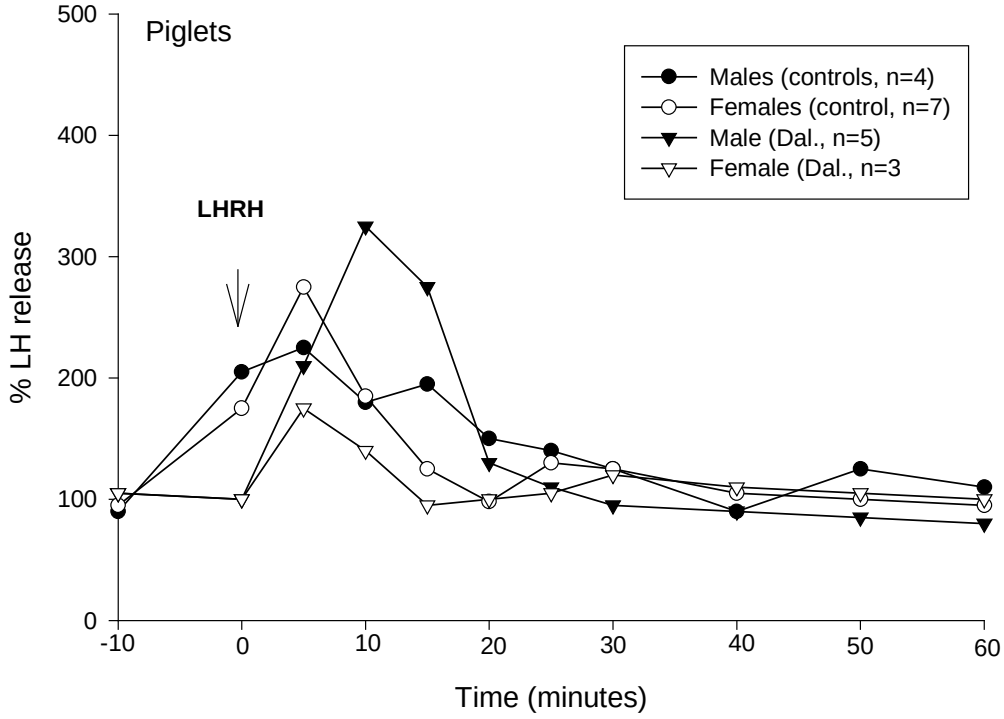


Figure 17: In vitro release of LH from perifused pituitaries of piglets after dalargin pretreatment and later LHRH administration.

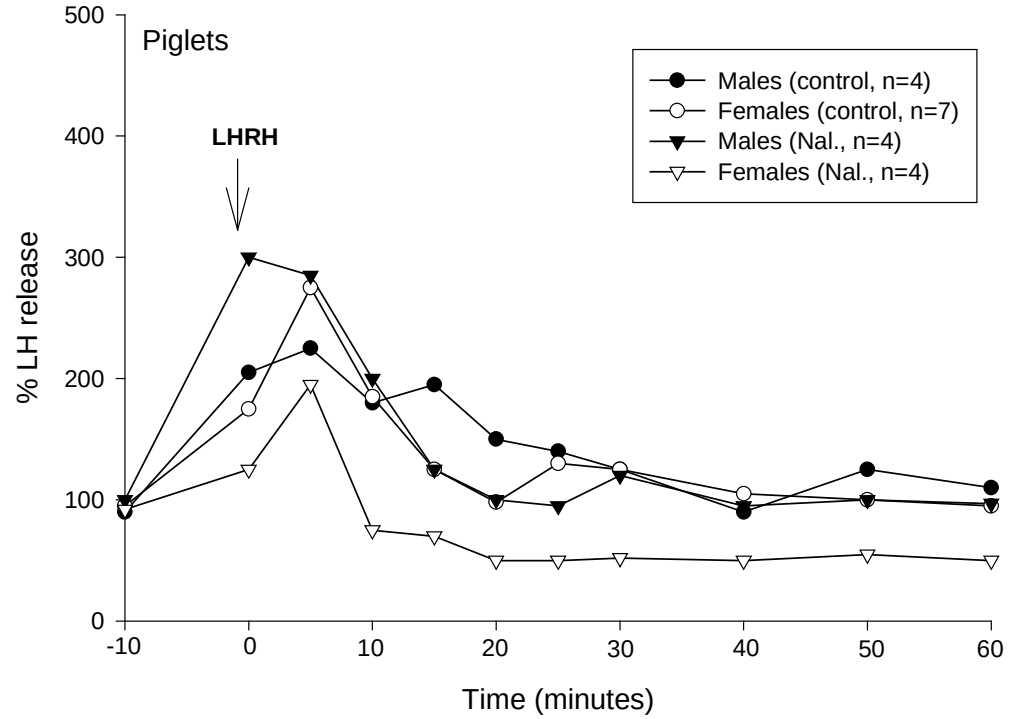


Figure 18: In vitro release of LH from perifused pituitaries of piglets after naloxone pretreatment and later LHRH administration.

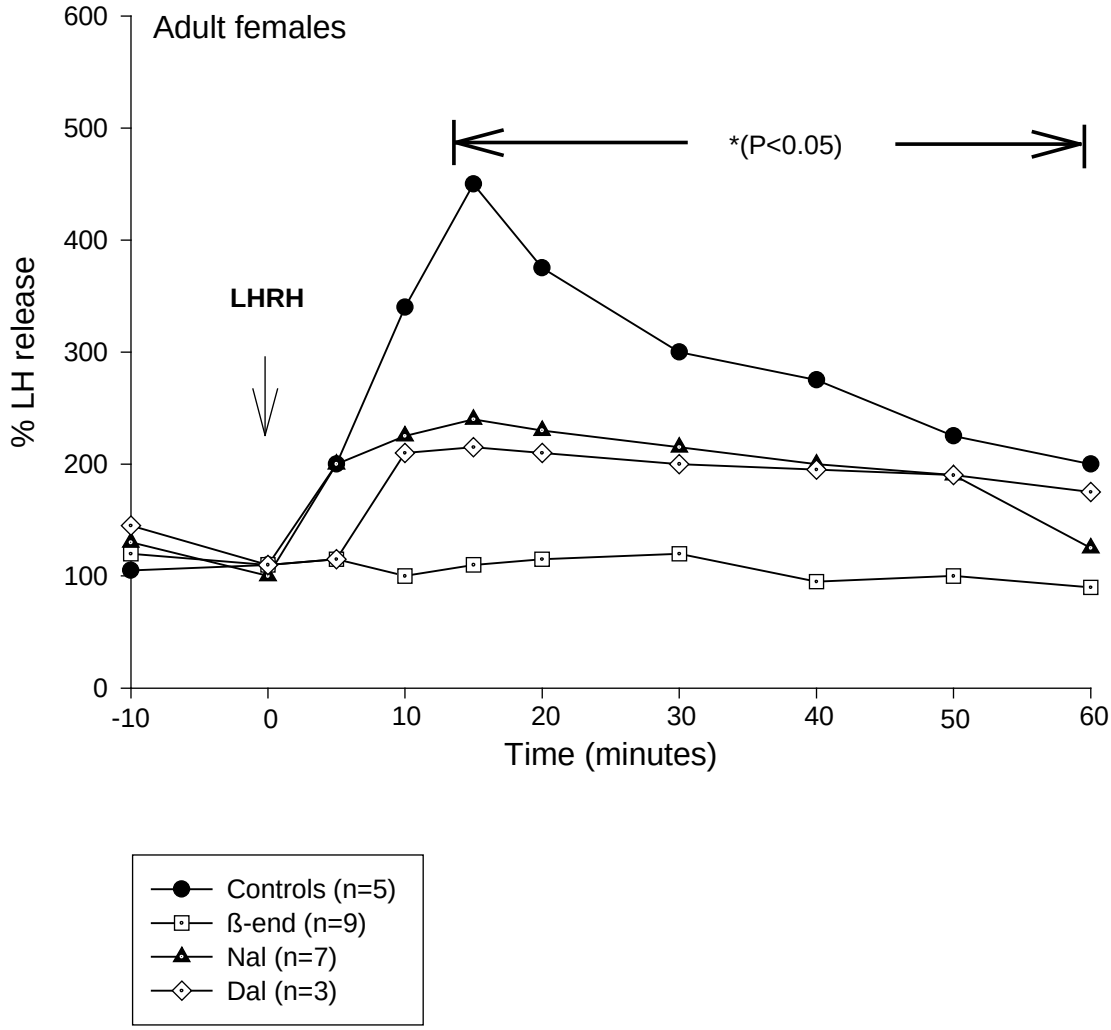


Figure 19 : In vitro release LH from perifused pituitaries of adult females after pretreatment with different opioids and later LHRH administration.
***P<0.05** shows a statistically significant difference between beta-endorphin pretreated females compared to saline pretreated controls at period indicated with arrows.

4.2.3 Adult females

In adult females, LHRH stimulation caused as expected a significant increase ($p< 0.05$) in LH release in adult female pituitaries. The LH peak level reached 455 % of basal levels within 20 minutes after LHRH stimulation in saline pretreated controls (fig.19, page 58). On the otherhand ß-endorphin pretreated pituitaries did not show any stimulatory tone on LH secretion after LHRH application. The peak level of LHRH-induced LH release attained only 120% in ß-endorphin pretreated pituitaries whereas the corresponding control level at the same time was 300% compared to ß-endorphin treated pituitaries.
Pituitaries pretreated with dalargin and naloxone tended to show an impairment of LHRH-induced LH release as compared to saline controls. But this impairment was not statistically significant ($P>0.05$).

4.3.0 Opioidergic effects on GH secretion

4.3.1 Opioidergic effects on GH secretion in fetuses

Spontaneous GH secretion from pituitaries of female and male fetuses can be seen on fig 20. Treatment with naloxone did not appear to influence the basal GH secretion from pituitaries of fetal females and males. Growth hormone secretion from pituitaries of fetal males was slightly higher (but not significant) when compared to fetal females as calculated from AUC (fig. 20).

The opiate agonist ß-endorphin caused a significant increase ($p< 0.05$) in GH secretion from pituitaries of male fetuses beginning 30 minutes after treatment and lasting for 90 minutes (fig. 21). Although, there was an apparent increase in GH concentration from pituitaries of fetal females , this was not statistically significant ($p>0.05$) thus indicating a sex differential effect of ß-endorphin on GH secretion in fetuses. The opioid agonist, dalargin showed no effect on GH release from pituitaries of fetal females and males when compared to saline controls (fig. 22).

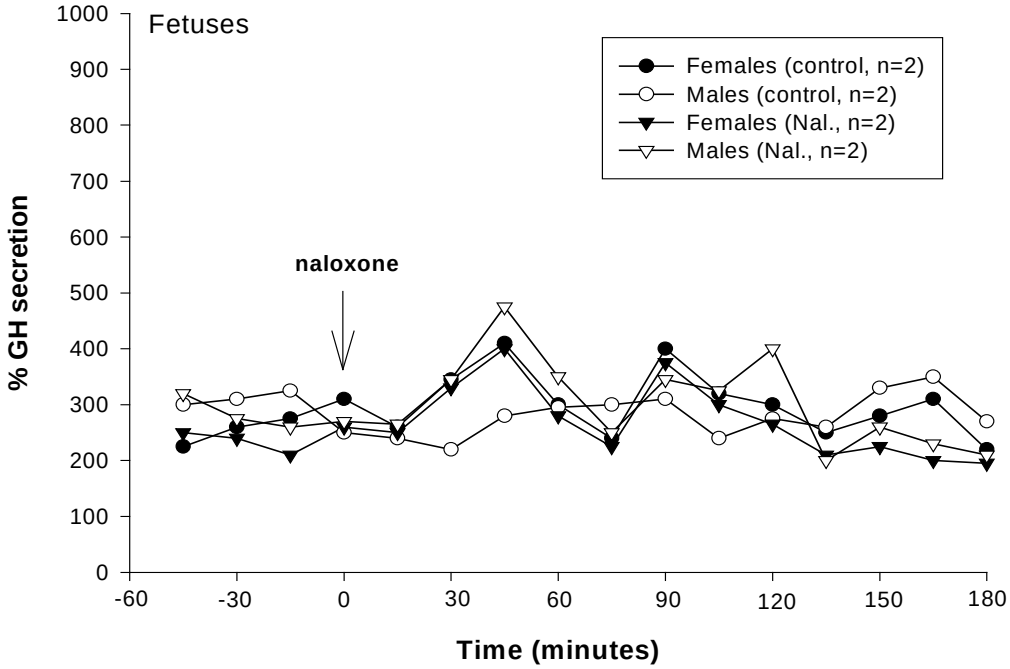


Figure 20 : In vitro secretion of GH from perifused pituitaries of fetuses with or without naloxone treatment.

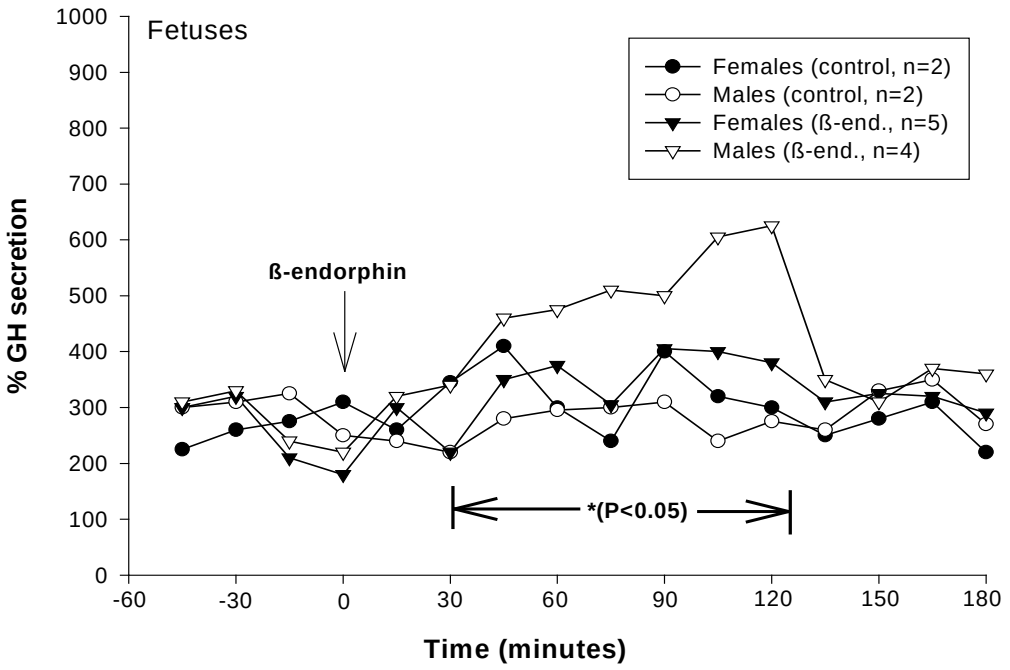


Figure 21 : In vitro secretion of GH from perifused pituitaries of fetuses with or without beta-endorphin treatment.
***P<0.05** shows a statistically significant difference between treated males and controls at period indicated with arrows.

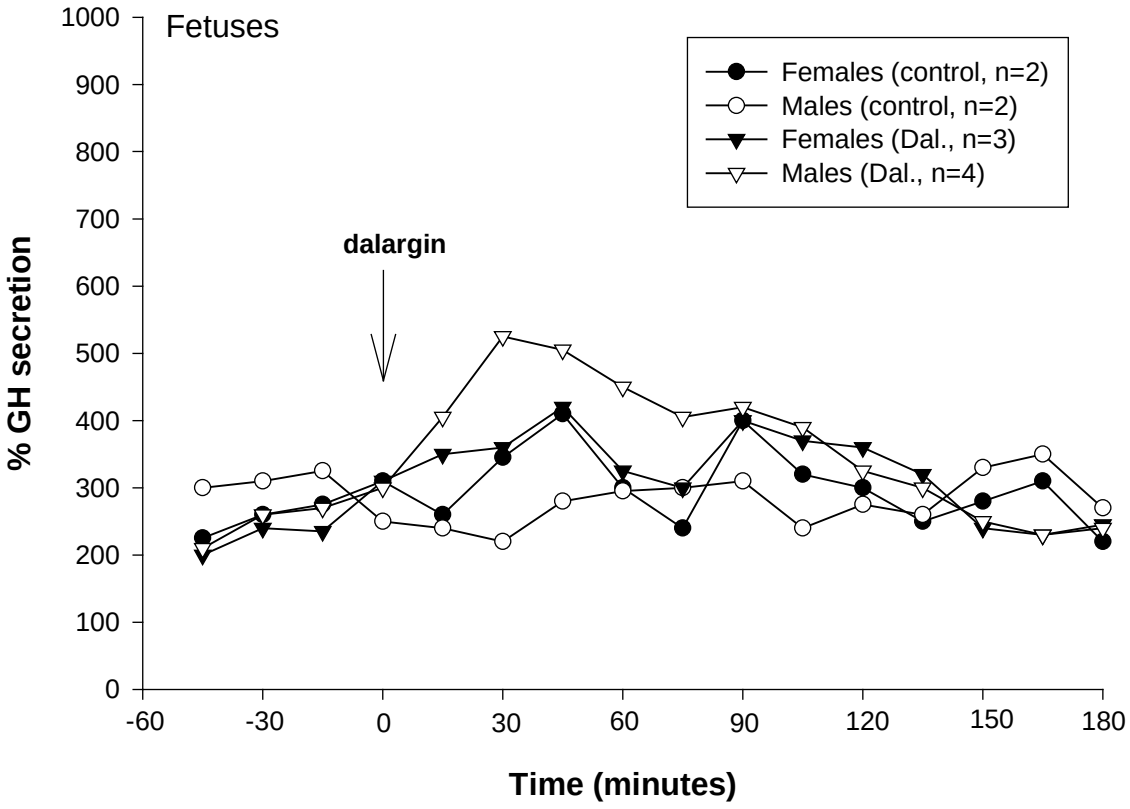


Figure 22 : In vitro secretion of GH from perifused pituitaries of fetuses with or without dalargin treatment.

4.3.2 Opioidergic effects on GH secretion in piglets

In male piglets, the opiate antagonist naloxone elicited a decrease in spontaneous GH secretion after one hour of treatment, but this decline was not statistically significant. On the otherhand, female pituitaries showed a significant ($p<0.05$) increase in GH secretion (680 ±75%) see (table 7, page 67) after two hours of naloxone treatment (fig. 23). ß-endorphin and dalargin showed no effects on the spontaneous GH release from pituitaries of both female and male piglets throughout the treatment period (fig. 24 & 25).

4.3.3 Opioidergic effects on GH secretion in adults

Spontaneous GH secretion from pituitaries of adult females and males is illustrated in figure 26. Treatment of both pituitaries of adult females and males with the opiate antagonist naloxone did not show any significant effects on the spontaneous secretion of GH as compared to controls (fig. 26). The sporadic secretory pattern of GH was maintained in both sexes. The concentration of GH secreted by adult males was higher than in females as seen from AUC.

The opiate agonists, dalargin and beta-endorphin both significantly ($p<0.05$) increased the secretion of GH from adult male adenohypophyses from 10 minutes after treatment until 90 minutes thereafter. Dalargin and ß-endorphin caused an increment in spontaneous GH secretion reaching 610 % and 660 % respectively (fig. 27 & 28). Contrary, the pituitaries from adult females were not affected after dalargin and ß-endorphin administration.

The concomitant administration of naloxone and ß-endorphin to adult male pituitaries showed a drastic decline in GH levels indicating that naloxone could antagonize ß-endorphin-mediated GH increase in adult males (fig. 29). Whereas in adult female pituitaries, naloxone apparently does not exhibit effect.

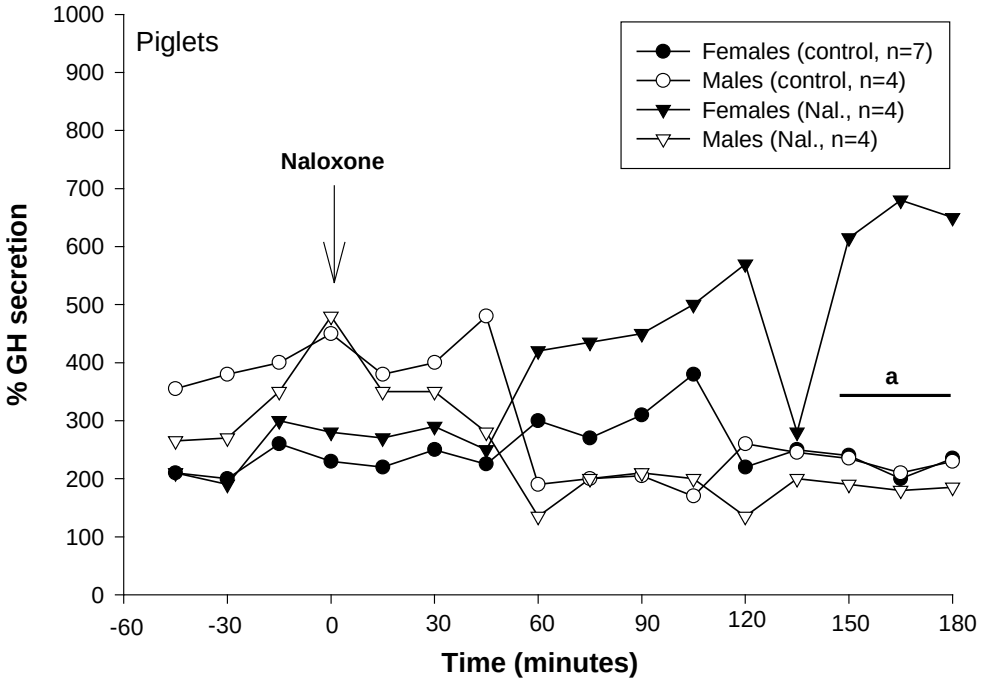


Figure 23 : In vitro secretion of GH from perifused pituitaries of piglets treated with or without naloxone.
a shows a statistically significant difference between treated females and controls at period indicated with line.

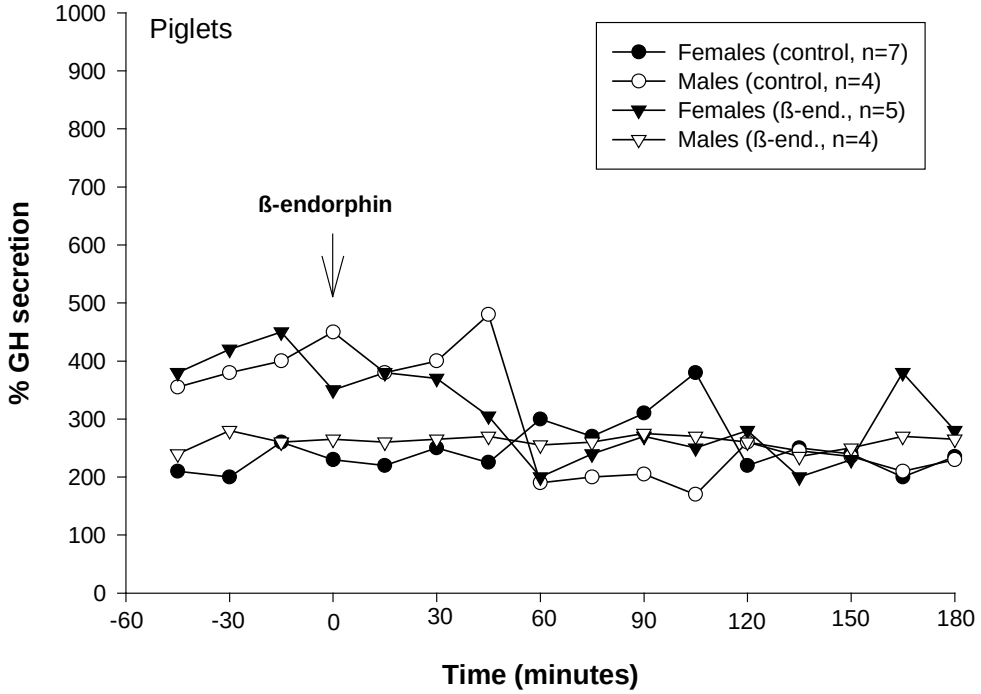


Figure 24 : In vitro secretion of GH from perifused pituitaries of piglets with or without beta-endorphin treatment.

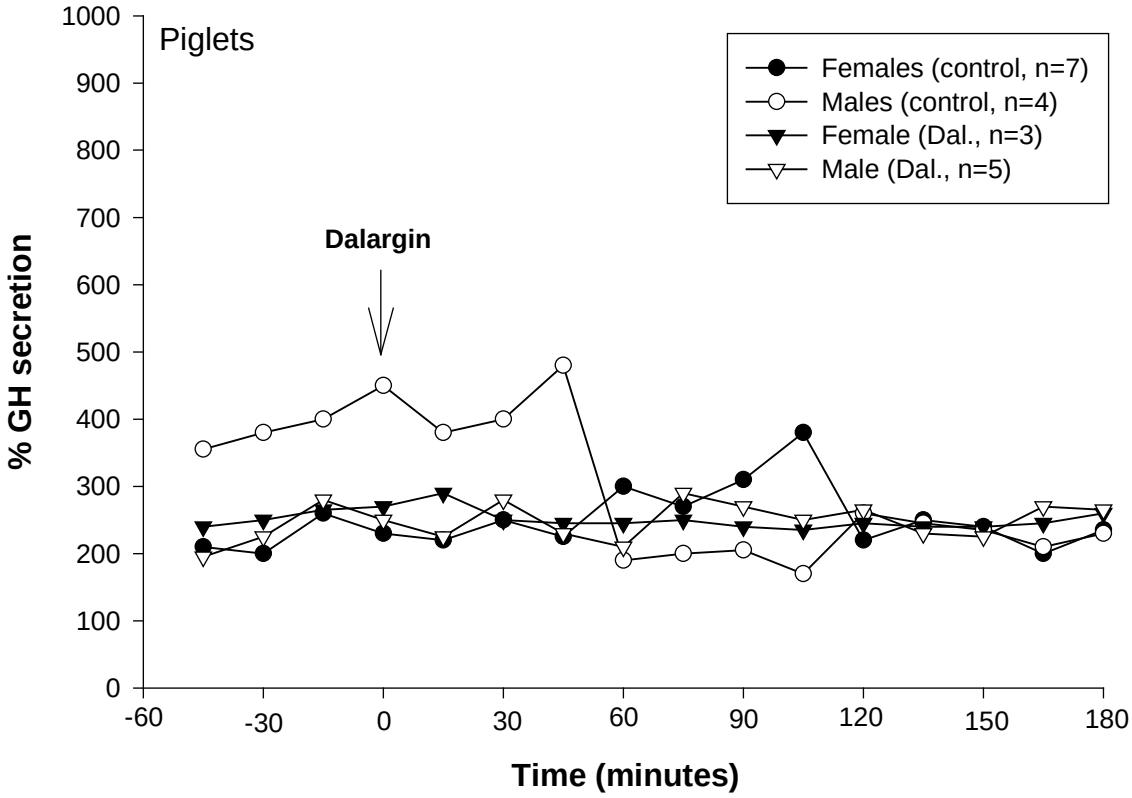


Figure 25 : In vitro secretion of GH from perifused pituitaries of piglets with or without dalargin treatment.

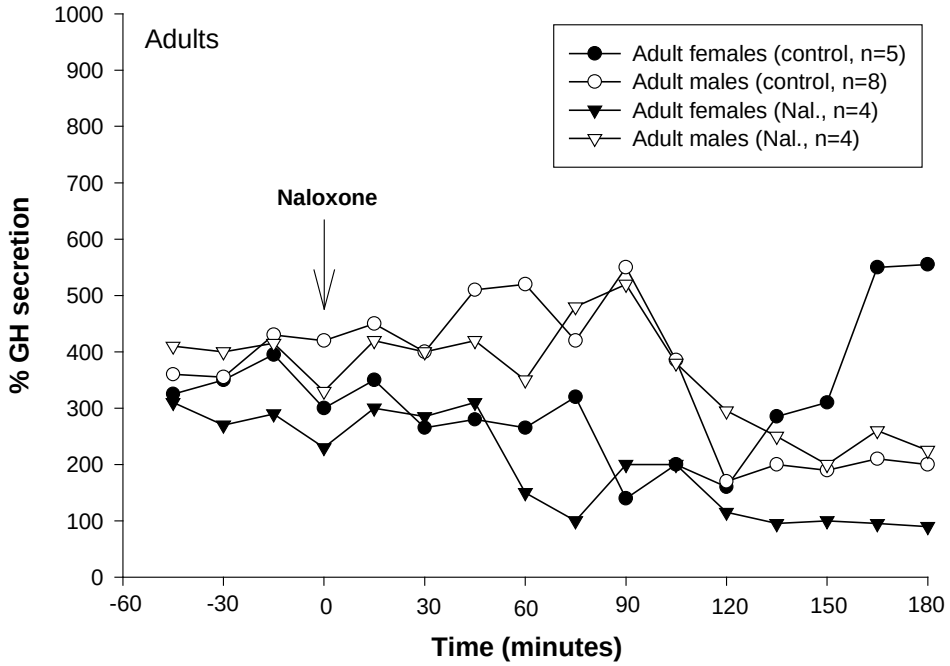


Figure 26 : In vitro secretion of GH from perifused pituitaries of adults with or without naloxone treatment.

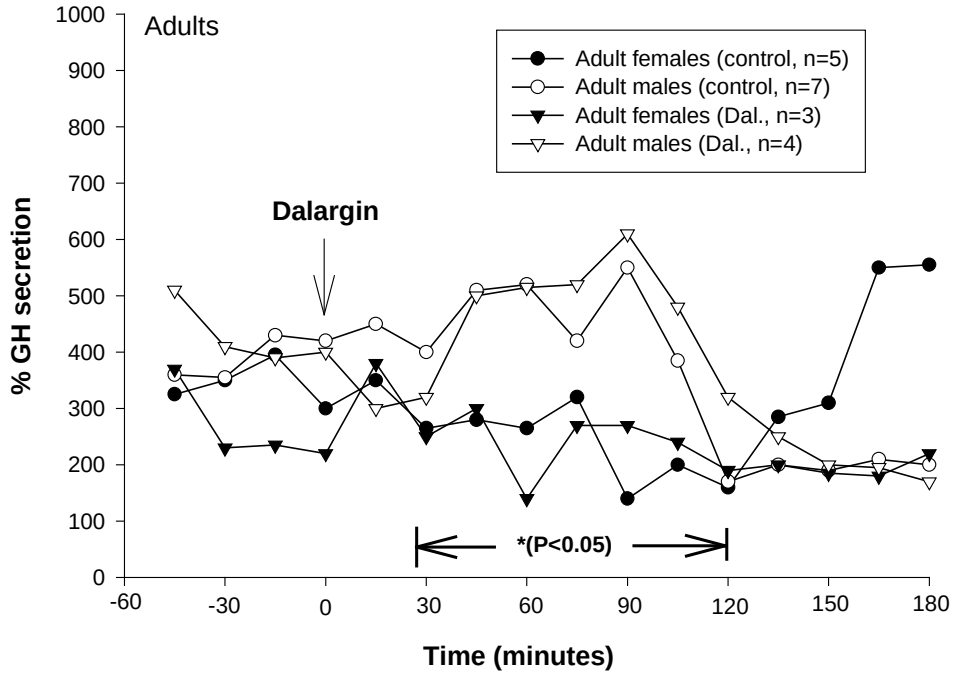


Figure 27 : In vitro secretion of GH from perifused pituitaries of adults with or without dalargin treatment. ***(P<0.05)** shows a statistically significant difference between adult males and controls at period indicated with arrows.

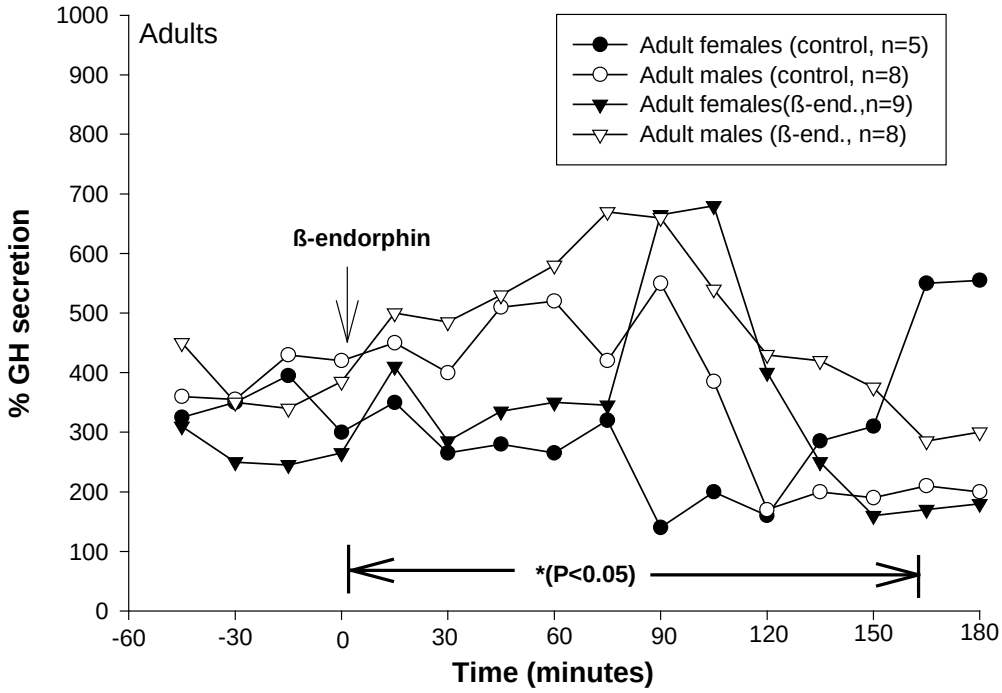


Figure 28 : In vitro secretion of GH from perifused pituitaries of adults with or without beta-endorphin treatment. *** P<0.05)** shows a statistically significant difference between adult males and controls at period indicated with arrows.

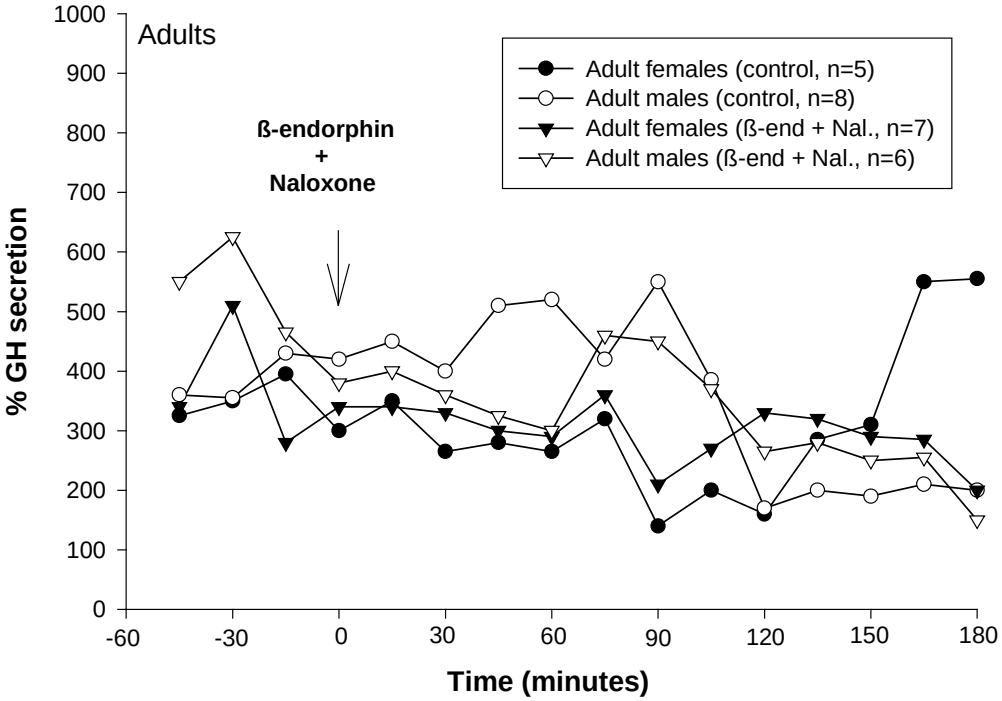


Figure 29 : In vitro secretion of GH from perifused pituitaries of adults with or without beta-endorphin + naloxone treatment.

Table 7 : Opioid-induced GH release in both sexes of all age groups

Age group	Fetuses		Piglets		Adults	
Gender/ Parameter	Females (n=9)	Males (n=10)	Females (n=12)	Males (n=13)	Females (n=19)	Males (n=22)
%Peak level of ß-endorphin-induced GH secretion	405 ±110%	**a** 625 ±125%	380 ±65%	275 ±35%	680 ±75%	**b** 660 ±65%
%Peak level of naloxone-induced GH secretion	400 ±45%	475 ±55%	**c** 680 ±120%	350 ±50%	310 ±45%	520 ±105%
%Peak level of dalargin-induced GH secretion	420 ±20%	420 ±25%	250 ±35%	290 ±35%	380 ±20%	**d** 610 ±95%

a indicates a statistical significant ($P<0.05$) difference between ß-endorphin treated pituitaries of fetal males and controls.

b indicates a statistical significant ($P<0.05$) difference between ß-endorphin treated pituitaries of adult males and controls.

c indicates a statistical significant ($P<0.05$) difference between naloxone treated pituitaries of female piglets and controls.

d indicates a statistical significant ($P<0.05$) difference between dalargin treated pituitaries of adult males and controls.

4.4 Opioid-LHRH interaction on GH secretion

Interaction between opioids and LHRH on the secretion of GH was measured in adult females and males exclusively. GH secretion from pituitaries of adult females (both high and low plasma concentrations of progesterone) pretreated with naloxone decreased significantly ($p<0.05$) after LHRH application. Whereas there was no alteration in GH levels after LHRH application in pituitaries of adult males pretreated with naloxone compared to controls (fig. 30).

The dalargin-induced GH increment in adult males was attenuated after LHRH stimulation when compared with saline pretreated controls. Pretreatment with dalargin did not alter GH secretion after LHRH administration in pituitaries of adult females (figure 31).

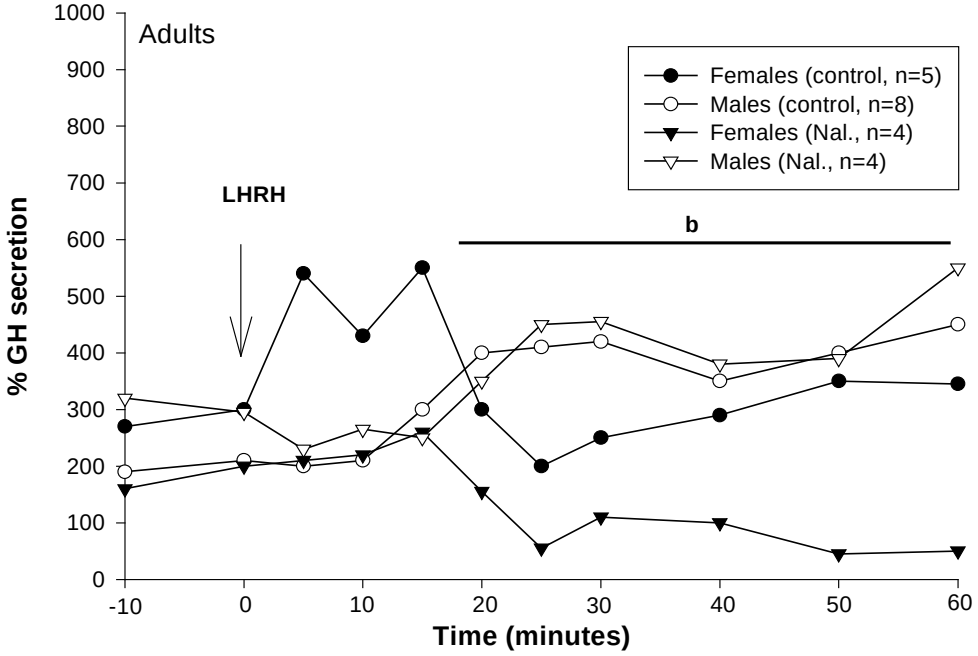


Figure 30: In vitro secretion of GH from perifused pituitaries of adults after naloxone pretreatment and later LHRH administration.
b shows a statistically significant difference **(P<0.05)** between adult females and control at period indicated with line.

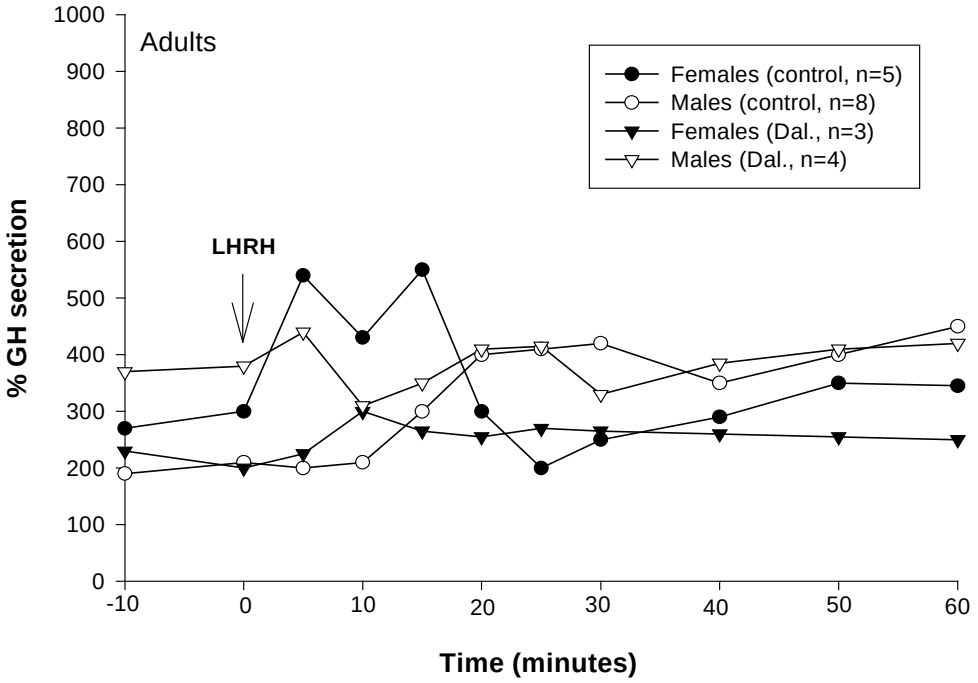


Figure 31: In vitro secretion of GH from perifused pituitaries of adults after dalargin pretreatment and later LHRH administration.

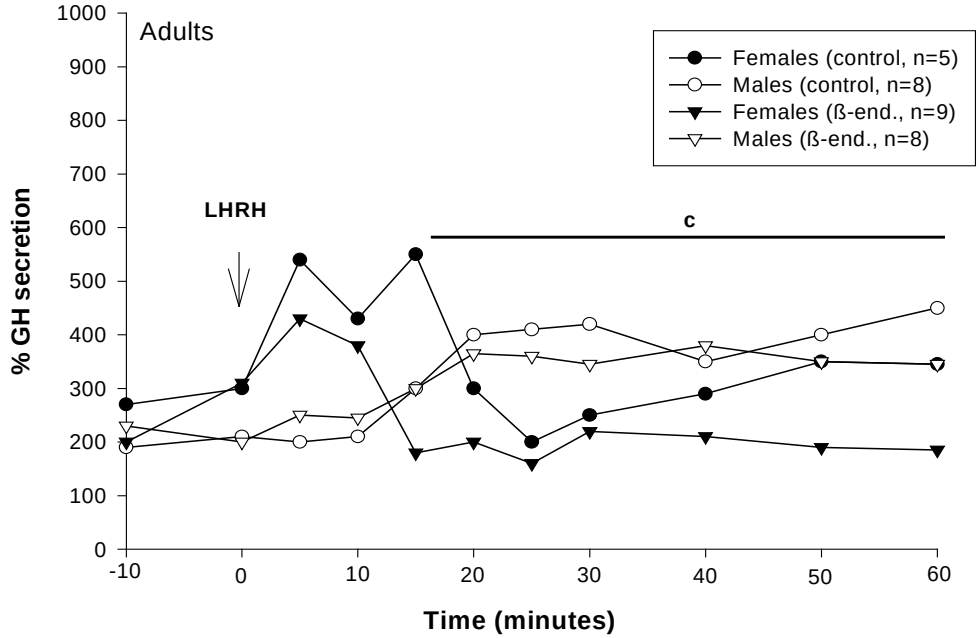


Figure 32: In vitro secretion of GH from perifused pituitaries of adults after beta-endorphin pretreatment and later LHRH administration.
c shows a statistically significant difference **(P<0.05)** between adult females and control at period indicated with the line

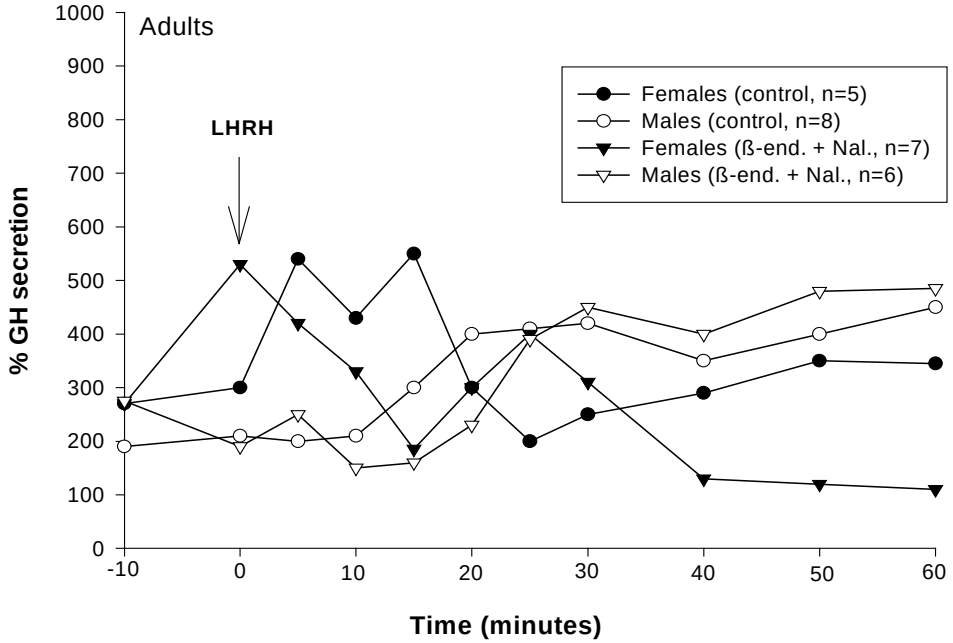


Figure 33: In vitro secretion of GH from perifused pituitaries of adults after beta-endorphin and naloxone pretreatment and later LHRH administration.

It was observed that GH secretion decreased significantly ($p<0.05$) after LHRH administration in pituitaries of adult females pretreated with ß-endorphin. The decline in GH secretion occurred approximately 30 minutes after stimulation. Whereas GH levels in pituitaries of adult males pretreated with ß-endorphin were unaffected by LHRH stimulation (fig. 32). Simultaneous pre-administration of naloxone and beta-endorphin did not appear to change the pattern of GH secretion observed after LHRH challenge in beta-endorphin pretreated pituitaries of females and males (fig. 33).

4.5 Opioid-GHRH interaction on GH secretion

The interaction of opioids (ß-endorphin) and GHRH was tested only in pituitaries of adult males. The pretreatment with ß-endorphin did not appear to influence GHRH-induced GH secretion. There was no significant difference ($p> 0.05$) in GH secretion after GHRH-stimulation in pituitaries pretreated with ß-endorphin compared with the saline pretreated controls. There was no interaction between GHRH and opioids at the pituitary (fig. 34).

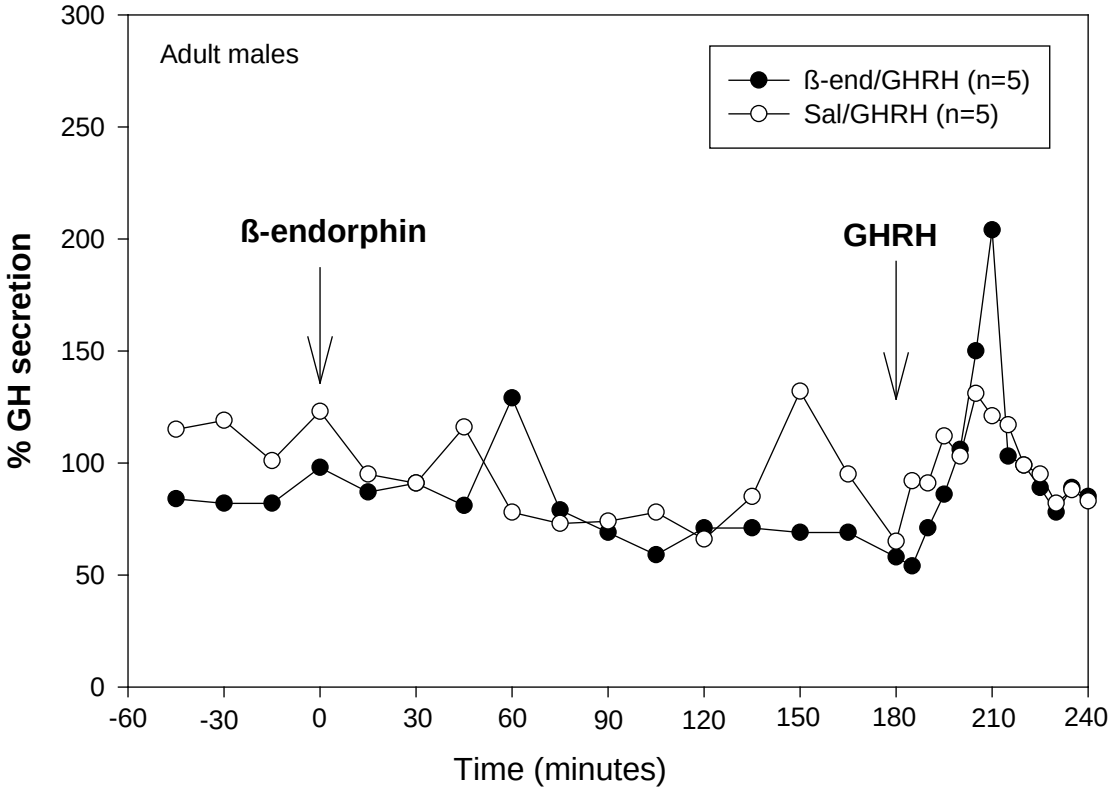


Fig. 34: In vitro release of GH from perifused pituitaries of adult males after beta-endorphin treatment and subsequent GHRH administration.

5.0 DISCUSSION

There are several routes through which endogenous opioids might act to exert their effects. Regarding the regulation of hormone secretion the route via the CNS seems to be the major one. But there is considerable evidence that opiates can exert direct effects on the pituitary to modify the regulation of LH and GH. This route is however less explored.

The present study was therefore geared at verifying the effects of opioids on LH and GH release activity at the pituitary level in male and female pigs at different stages of development.

ß-endorphin enhanced LH release in both pituitaries of fetal females and males in vitro. Interestingly, LH secretion from pituitaries of fetal males was higher than from female ones. Although the basal pituitary LH release has been shown to be significantly lower in male than in female fetuses (Elsaesser et al., 1988), this difference was evident in the present study tendentiously. This indicates that pituitaries of fetal males are presumably more sensitive to ß-endorphin than adenohypophyses of fetal females to secrete LH. The ß-endorphin-mediated stimulatory effect on LH secretion in the pituitary of the fetal pig has not yet been mentioned before. Comparative studies with other species at the fetal stage are lacking. But related in vivo studies in the fetal pig by Behrens-Herrler and Parvizi (1992) revealed that single and repeated administrations of the opioid agonist, morphine decreased LH in both female and male fetal pigs.

The opioid antagonist naloxone did not alter LH secretion in fetal pituitaries of both females and males. Supportive data has been reported by Behrens-Herrler and Parvizi (1992), where a single injection of naloxone was not effective in modifying LH release from female and male fetal pigs. However, an inhibitory naloxone effect could be induced with daily naloxone injections in male fetuses but not in females. In contrast to the above findings, in the chronic catheterized fetal lamb, naloxone induced a prompt increment in plasma LH levels (Cuttler et al., 1985). The naloxone effector mechanism on LH release in the fetal pig is assumed to be quiescent and probably becomes operative in the pig weeks after birth (Parvizi et al., 1995).

The leu-enkephalin opioid agonist, dalargin did not modify LH release from fetal pituitaries. This opioid analogue was used because it has been shown to have effects on local peripheral target tissues and its effects are not necessarily brought about through the CNS (Il'inskii et al., 1987). Dalargin has a higher affinity to the δ-opioid receptor (Pencheva, 1996) but the δ-receptor is virtually absent in the fetal pig (Kahle and Parvizi, 1993). The ineffectiveness of dalargin to influence LH secretion could be a result of absence of delta receptors in the fetal pig. The absence of δ-opioid receptors, however, cannot be solely responsible for the lack of dalargin effect, since dalargin has no effects in piglets (see below) where δ-opioid receptors are detectable (Kahle, 1993).

Similarly in the piglets, ß-endorphin treatment evoked an increase in LH secretion from pituitaries of both females and males. Again, naloxone and dalargin showed no effects. Naloxone also failed to influence LH secretion in intact and castrated immature male pigs (Trudeau et al., 1988), in heifers (Gazal and Anderson, 1995) and in prepubescent male rats (Cicero et al., 1993). These observations led to the suggestion that naloxone does not alter LH secretion until after puberty. It is worthy to mention that heifers are not necessarily a good example for immature female animals, because naloxone is also not effective in inducing any changes in LH release in adult cattles with exception of suckled cows.

Interestingly, the response of adult pituitaries to ß-endorphin treatments was an increment in LH discharge into the medium. The effect of ß-endorphin in females was only significant when the pituitaries were removed at a period of cycle with high progesterone (P_4>3.5 ng/ml) levels. It is certainly accepted that female animals with plasma progesterone concentrations greater than 3.5 ng/ml are well in the luteal phase of the cycle. However, animals with P_4<3.5 ng/ml cannot be considered to be homogenous concerning their pituitary LH release and ovarian steroid hormone background. They could be in a phase of the cycle immediately after luteolysis, before, in or during the LH peak or shortly after ovulation. Again the pituitaries of males exhibited a more profound response than females, despite the significantly higher basal LH secretion from pituitaries of females. This more sensitive reaction of pituitaries of males throughout the different stages of development has not been

studied and thus not described previously. It could be a testosterone-dependent event. However, it seems to be a yes or no response and not neccessarily a dose-dependent one. ß-endorphin stimulation of pituitary LH release in vitro has been also documented in the sheep where human ß-endorphin and γ-endorphin elevated LH secretion in perifused pituitaries 30 minutes after exposure (Matteri and Moberg 1985).

A report by Barb et al., (1990) also disclosed that 10^{-9}M ß-endorphin significantly augmented LH secretion in mature gilts pituitary cell cultures. Whereas lower 10^{-10}M or higher 10^{-7}M doses of ß-endorphin failed to alter LH release after 4 hours exposure. In contrast, ß-endorphin caused a reduction of LH secretion when applied to murine (Lucinda and Franco, 1985) and bovine (Chao et al., 1986) pituitary cells.

The opioid antagonist, naloxone failed to modify LH secretion. This is somehow to be expected because naloxone was given in the absence of opioid agonist and naloxone as a pure opioid antagonist should not have any intrinsic effect. Strange is that naloxone could not inhibit the effect of ß-endorphin. This could be due to the mode of application. In the present study, naloxone and ß-endorphin were applied concomitantly. The better mode would have been a naloxone treatment preceding the ß-endorphin challenge. Nevertheless, these findings are confirmed by Matteri and Moberg, 1985 and Cicero et al., 1979 who also could not show any effects of naloxone on sheep and rat pituitary cultures, respectively . Controversially, Barb et al., 1990 reported a stimulatory action of naloxone on pig pituitary cells. The discrepancy between this and the present results could be due to differences in the methodological approach. Barb et al. used a static cell culture and incubated the cells for a period of over 24 hrs with naloxone. This long-term exposure could have evoked some naloxone effects not attributable to the opioids (Kahle, 1993).

Dalargin, the leu-enkephalin analogue used in the present study did not alter LH secretion from pituitaries of females and males. However, it does decrease LH secretion when microinjected into the pig brain in vivo (Anderheiden & Parvizi, 1996). The endogenous opioid, met-enkephalin and the synthetic D-Phe^2-met-enkephalin inhibited LH release from cultured bovine pituitary cells (Chao et al.,

1986) and rat pituitaries (Dragatsis et al., 1995) respectively. Met-enkephalin binds to the μ and δ-receptors with almost the same affinity (Akil et al., 1988) but its analogue dalargin, seems to have a higher affinity to delta receptors and may only weakly bind to μ-receptors (Pencheva, 1996). At the adenohypophyses, mu-opioid receptors are more abundant than the delta receptors (Mansour et al., 1988). The inability of dalargin to act directly at the pituitary level suggests that opioidergic control of LH at the adenohypophysis may be predominantly mediated through mu-opioid receptors. This study was also aimed at determining if opioids could modify LHRH-induced LH secretion at the pituitary level. Generally, LHRH has a stimulatory effect on LH release that may reach several folds compared to the basal secretion. However the LHRH-induced LH increment depends on the dose applied and other physiological conditions of the animal or tissue utilised for the experimental study. Barb et al., (1990) revealed that 10^{-9}, 10^{-8}, 10^{-7} M GnRH increased LH secretion 140%, 210% and 250% respectively in pituitary cell culture in vitro. Results of this study indicated that ß-endorphin pretreatment caused an attenuation of LHRH-induced release of LH in pituitaries of fetal and adult females with higher peripheral progesterone (P4) concentrations. Whereas the LHRH-induced LH secretion was rather impaired but not significantly in pituitaries of female and male piglets pretreated with ß-endorphin. The difference seen in LH response of piglets to ß-endorphin pretreatment and later LHRH stimulation when compared to fetal and adult pituitaries, could be related to lower progesterone concentration. It is well known that before puberty, neonates and prepubescents possess relatively low concentrations of circulating steriod hormones, whereas fetuses receive highly rich steriodal blood from their mothers through the placental vascular complex into the fetal system (Ponzilius et al., 1986). Results of in vitro studies carried out by Sanchez-Franco and Cacicedo (1983), indicated that gonadotropin releasing hormone stimulatory activity on LH release was blocked by beta-endorphin in male rat pituitary cells. The intra-pituitary physiological concentration of beta-endorphin in the pig is estimated to be $3\text{-}5 \times 10^{-9}$ M (Symth and Zarkarian, 1980) and this corresponds to the experimental dose employed thus rejecting the possibility of using an inappropriate dose.

A plausible explanation of LHRH-induced LH secretion observed after ß-endorphin could be attributable to a pituitary maximal secretory capacity. It is possible that once the gonadotrophs responded to beta-endorphin pretreatment with maximal secretory LH levels, no further release could be achieved with any other LH stimulant, thus lowered LH response was observed. And of course another possibility is that ß-endorphin counteracts with LHRH through so far unexplored routes, to control LH release. It is here to emphasize that the inhibitory action of ß-endorphin on LH secretion is the most often observed effect of the opioids on gonadotropin secretion in vivo (Parvizi et al., 1993).

Pretreatments with naloxone and dalargin manifested no effects on LHRH-induced LH discharge in fetuses and piglets. Comparative studies on the interaction of opioids and LHRH on LH release at the pituitary in fetuses and postnatals are not available. Having in mind that both naloxone and dalargin did not alter spontaneous LH secretion from pituitaries of fetuses and piglets, one might carefully claim that the opioid receptor activation mechanism mediating these effects is not fully developed or depends on other non-opioid physiological factors involved in the regulation of LH secretion in fetal and postnatal pig pituitary. However, the lack of an opioidergic tonus on LH secretion at the pituitary level in the young animal is most feasible. On the otherhand, pretreatment with naloxone and dalargin impaired the LHRH induced LH release from pituitaries of adult females but this was not statistically significant. A comparative study conducted by Chao et al., (1986) demonstrated that simultaneous addition of naloxone and LHRH in bovine pituitary cell culture resulted to a non-significant decrease in LH secretion when compared to LH levels after LHRH alone. The authors concluded that naloxone does not affect LHRH-induced LH discharge from bovine pituitary cells. Furthermore, Delitala et al., (1981) also reported that naloxone showed no effects on LHRH-induced LH release in man. As mentioned above, the pretreatment with dalargin, an enkephalin analogue only slightly decreased LHRH-induced LH release. It is worthy to note that dalargin was not effective in altering spontaneous LH secretion in this study. Similarly, met-enkephalin did not

affect basal in vitro LH secretion in ovine pituitary cell culture (Matteri and Moberg, 1985). In another in vitro study, leu-enkephalin enhanced gonadotropin responsiveness to LHRH (May et al., 1979). The discrepancies between the data could be due to the species difference and specificity of the substances used. Leu- and met-enkephalin have been shown to have different effects on LH secretion in the pig (Parvizi, 1989).

This study concurrently soughted to determine if opiates could influence GH secretion directly at the pituitary level. It is hypothesized that opioid peptide stimulation of pituitary GH secretion is mediated through hypothalamic GHRH (Wehrenberg et al., 1985; Armstrong et al.,1990) and an opioidergic inhibition of somatostatin is also possible (Delitala et al., 1989). Results of the present study indicate that, ß-endorphin caused a significant increase in GH secretion from male but not from female fetal pituitaries. Comparative in vivo studies were carried out on chronically catheterized fetuses using morphine. Chronic treatment over 3-4 days with daily injections of morphine were necessary to prime the brain/ pituitary to response to GH, while single injection (one day of treatment) had no effects (Parvizi et al., 1995). In the fetal pig like other species, somatotrophs secrete extremely high concentrations of GH, (see Bauer, 1992). The reason for a sex-differentiated GH response to ß-endorphin is not fully understood. It is, however, worthmentioning that male fetuses have significantly higher plasma GH levels (Bauer and Parvizi, 1996) and pituitary GH gene expression (Granz et al., 1997) than their age-matched females. But blocking opioid receptors with naloxone showed no effects on GH secretion from both female and male fetal pituitaries in this study. This is in line with results of Parvizi et al., (1995) who revealed that the opioid antagonist naltrexone had no effects on GH secretion in fetal pigs. This indicates once more that the opioidergic system controling pituitary hormone secretion is not fully functioning before birth.

In piglets, naloxone treatment caused a sex-differentiated response. The females showed a significant increase in basal GH secretion after 2 hours of exposure to naloxone. An earlier report of Trudeau et al.,(1988) disclosed that treatment with naloxone did not alter GH secretion in 6 weeks old male pigs. Similarly, blocking

opioid receptors with naltrexone did not alter GH secretion in piglets (Parvizi et al., 1995).

Both dalargin and beta-endorphin showed no effects on GH secretion from pituitaries of both female and male piglets. But Trudeau et al.(1988) reported an increment in GH secretion in 6 weeks old male pigs after morphine treatment, while Armstrong et al.,(1993) also revealed an elevation in serum GH release in hypogonadal gilts. Similarly, morphine caused GH increments in postnatal pigs after 16 days but not before (Parvizi et al., 1995). It must be noted here that the experimental animals of the above-mentioned studies and the present one were not age-matched. The present study used two weeks old piglets whereas the previous studies used older animals. The failure of these opioids to exert effects on pituitary GH may not only be due to the difference in experimental methods but also the age difference of experimental animals. The age-dependency of opioidergic GH secretion seems to be crucial (for review see Rettmer, 1994).

In adults, the opiate antagonist naloxone had no effect on basal secretion of GH from the pituitaries of both female and male pigs. These findings are in conformity with earlier reports by Pfeiffer and Herz (1984), Trudeau et al., (1988) and later Armstrong et al., (1993) who observed no opioidergic control on basal secretion of GH using an opioid antagonist. In contrast to the above findings, naloxone attenuated GH concentrations in mature pigs (Barb et al., 1992). In addition, naloxone also reduced GH levels in lactating sows that were either immunized against GRF or non-immunized sows (Armstrong et al., 1990a). Because naloxone decreased GH secretion in both GRF-immunized and non-immunized sows, immunization against GRF cannot be the sole reason for the decrement in GH levels in these sows. This suggest that other intrinsic factors associated to the physiological state (lactation) of the sows were involved in decreasing GH levels after naloxone treatment. The elevated levels of GH during lactation is presumed to be mediated by endogenous opioids (Armstrong et al., 1990b). Thus the inability of naloxone to alter GH secretion in the present study could be attributable to absence of these physiological intrinsic factors in vitro.

On the otherhand, dalargin and ß-endorphin treated pituitaries of adult males but not female pituitaries showed a marked increase in GH basal levels. This observation is in line with several reports suggesting that opioid agonists cause increases in GH secretion in heifers (Leshin et al., 1990; Johnson et al.,1993) in wethers (Armstrong and Spears,1991) in rats (Eason et al.,1996) in pigs (Barb et al., 1992) and in human (Schulte et al.,1993). The sex-differentiated effects of opioids on GH secretion has not been studied in other species. The effect is probably steriod-dependent, however, the present study does not present any direct evidence for this dependency.

GHRH induced a GH discharge from pituitaries of males comparable to that published previously (Elsaesser et al., 1996). Interestingly, ß-endorphin pretreatment potentiated, although not significantly, the effect of GHRH on GH secretion from pituitaries of adult males. Most interesting is the observation that, application of LHRH to pituitaries pretreated with ß-endorphin and naloxone showed a significant decrement in GH secretion in females but not in males. Earlier studies have shown that treatment with gonadal steriods that cause a surge in LH blunt the stimulatory effect of morphine on GH release in female rats (Singh et al., 1992). Looking at the experimental design, one might assume that, challenge of pituitaries with LHRH mimicks the physiological condition seen around a preovulatory surge in LH mediated by LHRH in vivo. Several laboratories have revealed that the opioid receptor number and naloxone binding are reduced at periods of steriod-induced LH hypersecretion (Jacobsen et al., 1989; Weiland et al., 1990). Hence the decrement in GH release is presumed to be a result of suppression of opioid receptor number and activity.

The findings of the present study indicate that effects of opioids on spontaneous LH and GH could be seen at the pituitary level and these effects are sex-, age- and steroid-dependent. Furthermore, LHRH-induced LH secretion could be opioid-dependent. But endogenous opioids could exert only minor effects on GHRH-induced GH secretion at the adenohypophyses in the pig.

6.0 SUMMARY AND CONCLUSION

- **Opioidergic effects on LH secretion**

(i) ß-endorphin treatment showed a significant increase in LH release from pituitaries of fetal, neonatal and adult pigs. However, in adult females significant levels were measured only from pituitaries of sows with high progesterone levels. Interestingly, pituitaries of males released significantly higher LH levels in response to ß-endorphin than females in all age groups.

(ii) Naloxone and Dalargin treatments showed no effects on LH secretion from pituitaries of both male and female fetal, neonatal and adult pigs. Blocking opioid receptors with naloxone to increase LH secretion is particularly conventional in *in vivo* studies in adults but not in *in vitro* studies. In fetal and neonatal pigs, opioid blockade is less effective in altering LH release. The lack of delta opioid receptors and prematurity of the opioid system in fetal and neonatal pigs partly accounts for the inability of dalargin and naloxone to alter LH secretion.

(iii) Naloxone did not antagonize the ß-endorphin-induced LH release neither from adenohypophyses of adult males nor adult females when administered concomitantly. Antagonism might have been observed if naloxone treatment preceded ß-endorphin treatment.

- **Interaction of opioid and LHRH in LH release**

(i) ß-endorphin blocked significantly the LHRH-induced LH release in pituitaries of adult females with high progesterone (P>3.5ng/ml) concentration. Similar results were obtained in fetal pituitaries but in piglets, ß-endorphin only impaired (not significant) the LHRH- induced LH discharge. This observation suggests that the steriodal milieu plays a fundamental role in gonadotropic releasing hormonal influence on opioidergic modulated release of luteinizing hormone in the pig.

(ii) Naloxone and Dalargin impaired (not significant) the LHRH-induced LH release in pituitaries of both adult females and males. Meanwhile pretreatment with

naloxone and dalargin had no effects on LHRH-induced LH release in piglets and fetuses. The similarity in fetal and adult pituitaries response to ß-endorphin pretreatment and subsequent stimulation with LHRH on LH release suggest a steriod hormonal influence of luteinizing hormone at the pig pituitary.

- **Opioidergic effects on GH Secretion**

(i) ß-endorphin and dalargin treatment caused marked increases in GH secretion in adenohypophyses of fetal and adult males but not females, whereas both pituitaries of female and male piglets showed no effects on GH release.
(ii) Naloxone had no gender-related effect on GH secretion in pituitaries of fetuses and adults, whereas in piglets there was a sex-differentiated response with males showing a non-significant decrease and females a significant increase in GH secretion. This observation suggest that a sex-differentiated opioidergic tonus participates in the control mechanism of GH secretion at the pituitary level.
(iii) Concomitant administration of naloxone and ß-endorphin attenuated the ß-endorphinergic-induced increment of GH secretion seen in adult males.
It could be concluded that opioid control of GH secretion is sex-, age- and possibly steroid-dependent.

- **Interaction of opioid and LHRH in GH secretion**

(i) Pretreatment of adenohypophyses with naloxone and ß-endorphin showed a marked decrease in GH secretion after LHRH treatment in females (unlike in their male counterparts that responded with increased GH secretion after ß-endorphin administration) as compared to saline pretreated controls.
(ii) Dalargin pretreated adenohypophyses did not show any effect on GH secretion after LHRH administration in female but attenuated GH secretion in adult male pituitaries. The interaction of opioids and LHRH in GH secretion does not show any clear cut pattern in pituitaries of females and males. It rather suggests a probable dependency on the specific substance in action.

- **Interaction of opioid and GHRH in GH secretion**

Preadministration of ß-endorphin and subsequent stimulation with GHRH showed a non-significant increase in GHRH-induced GH secretion when compared to saline pretreated controls. These results indicate that opioids exert a stimulatory tone on basal GH secretion but play a minor role in GHRH-induced GH release in the pig pituitary.

The discordance in findings of this present study as compared to previous reports could be attributed to differences in methodological approaches, animal species and physiological state of the experimental animal. However, direct opioid control of LH and GH at the pituitary level is evident and the response is age-, sex- and steroid-dependent. Furthermore, LHRH-induced LH secretion could be opioid dependent but GHRH-induced GH discharge may only be slightly opioid-dependent in the pig pituitary.

7.0 LIST OF SCHEMES

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a.a.	Amino acid
ACTH	Adrenocorticothyriod hormone.
Ala	Alanine
AP	Adenohypophysis
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BAM	Bovine Adrenal Medulla Peptide.
BIT	2(P-ethoxybenzyl)-1diethyl-5-isothiocyanatobenzinidazol.
CNS	Central Nervous System.
cDNA	complemetary Dioxiribonucleic acid.
cAMP	cyclic adenosine 3‘-5‘monophosphate.
CHAP	(3-chloamidopropyldimethylamonio-1-propanesulphate)
CPM	Counts per minutes
DAGO	D-Ala^2,N-Met-Phe^4,Gly^5-OL-Enkephalin.
DSLET	Tyr-D-Ser-Gly-Phe-Leu-Thr-Enkephalin.
DPDPE	D-Pen^3-D-Pen^5-Enkephalin
d	day
DHPG	dihydroxyphenolglycol
EM-1	Endomorphine-1
EM-2	Endomorphine-2
Fig	Figure
G-DAMME	Guanyl-[D-Ala2, MePhe4-Met-enkephalin-(o)-ol, FK 33-824]
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
g	Unit of centifugal force
G-DAMME	Guanyl-[D-Ala2, MetPhe4-Met-enkephalin-(0)-ol, FK 33-824]
GnRH	Gonadotropic releasing hormone
GTP	Guanine Triphosphate
G-proteins	Guanine proteins
GH	Growth hormone
GHRH	Growth hormone releasing hormone.
5-HT	5-hydroxyhyptamine
5-HIAA	5-hydroxyindoleacetic acid
H-endorphin	Humoral endorphin
hrs	hours
^{125}I	Isotopic Iodine for labelling.
icv	intracerebroventricular
iv	intra venus
im	intra muscular
ip	intra peritoneal
IGFs	Insulin-like-growth factors
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone

LPH	Lipotropic hormone or Lipotropin
Leu-Enkephalin	Leucine enkephalin
Lys	lysine
min	minutes
M	molar
MEM	Maximum Essential Medium
Met-enkephalin	Methionine enkephalin
mRNA	messenger Ribonucleic acid.
mm	millimeter
NaCl	Sodium Chloride
ORL-1	Opioid receptor like-1
PBS	Phosphate Buffer Saline
PCP	Phencyclidin
p.p	Post partum
p.c	post coitus
pGH	porcine Growth Hormone
Phe	Phenylalanine
POMC	proopiomelanocortin
PROENK	Proenkephalin
PRODYN	Prodynorphin
Pro	Proline
α	alpha
β	beta
δ	delta
ε	epsilon
γ	gamma
κ	kappa
μ	mu
σ	sigma
ζ	Zeta
SRIH	Somatotrophic releasing inhibiting hormone or Somatostatin

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12.0 ACKNOWLEDGEMENT

I am very grateful to my surpervisor, Prof. Dr. Dr. N. Parvizi for proposing the research topic and for her useful suggestions and criticisms . Above all I wish to thank her for being so understanding and tolerant.

I extend my gratitute to Prof. Dr. Dr. Dr. F. Ellendorf, head of the institute, for giving me the opportunity to learn and work in the Federal Research Centre for Agriculture, Institute for Animal Science and Animal Behaviour, Mariensee-Neustadt, Germany.

I also express sincere thanks to Prof. Dr. C. Aurich at the Veternary Medical University in Vienna-Austria and Prof. Dr. S. Steinlechner at the Veternary Medical School, Hannover, for their painstaking scrutiny and amelioration of the dissertation and for being my Co-supervisors.

My immense thanks also goes to the laboratory technicians and colleagues of the department whose cooperation and discussions facilitated my work.

I wish to express my profound gratitute to my family, wife and friends for their inestimable moral and/ or financial support during this period.

Lastly, my thanks goes to the Almighty God for his guidance and blessings.

15.0 CERTIFICATION

This is to certify that this research project was carried out at the institute for Animal breeding and Animal behaviour, FAL, Mariensee-31535 Neustadt-Germany by Enowmpey Enowtambong (M.Sc.) as partial fulfillment of the requirements for the award of a doctorate degree in natural sciences (Dr. rer. nat.) and submitted to the faculty of Biological Sciences at the University of Hannover-Germany.