Foetal and neonatal development of luteinising hormone and its regulatory systems in the pig

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This review is a short summary of the "state-of-the-art" regarding the ontogeny of LH and part of its control system in the pig. The maturity of pituitary gonadotropin cells and the vascular drainage between the hypothalamus and pituitary are probably the most important steps in the developmental process of gonadotropin (LH) secretion. In the pig, these are achieved at around day 80 of foetal age, when LH cell density is comparable to that observed in adults. The hypothalamus regulates foetal pituitary LH secretion via LHRH well ahead of parturition. However, the main prerequisite of ovarian activity (ovulation), the "GnRH pulse generator", is not ready to function in the foetus. Pulsatile LH release is inducible by treatment of the foetal pituitary with LHRH, but extrahypothalamic modulating systems are not fully functioning until after birth. Likewise, there is no gonadal steroid feedback control of pituitary LH secretion up to the second week of neonatal age.

Introduction

The study of ontogenetic development of reproduction was, for decades, dominated by, and to a great extent, limited to research involving gonadal growth and onset of placental and gonadal hormone release. Gradually the emphasis was placed on sexual differentiation. This was followed by concentrated research on pituitary cell differentiation and central nervous system control of gonadotropin secretion during the foetal and neonatal periods. To date, most immunohistochemical work has been confirmed utilizing methods of molecular genetics. The latter have been helpful tools to further elucidate fine adjustments within the system.

Onset of pituitary function as well as control of the pituitary by the brain is dependent on adequate development of hypothalamic nuclei, and their afferent and efferent connections within and outside the hypothalamus, on development of the vascular drainage from the hypothalamus to the pituitary and on maturation of pituitary gonadotropin cells. This review is a short survey of the "state-of-the-art" regarding the ontogeny of LH secretion and part of its regulatory system in the pig.

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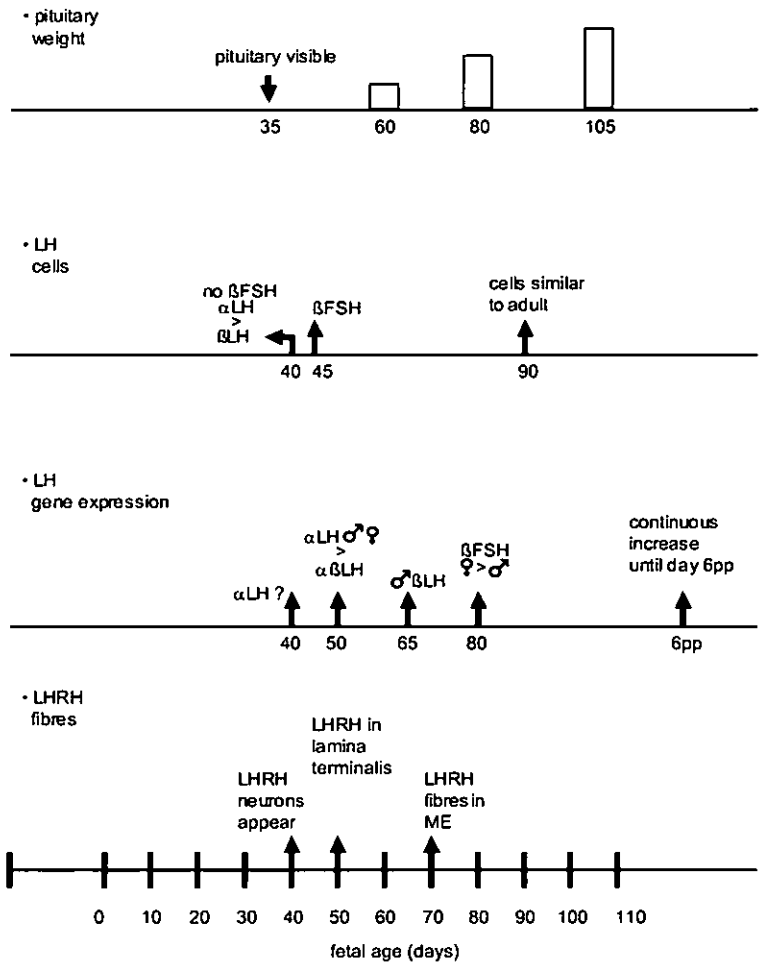
LH and LHRH producing cells

Rathke´s pouch appears around embryonic day 20. Pituitary growth is rather slow until day 60 post coitus (pc), when pituitaries weigh only 2.5 mg (Figure 1). They double in weight between day 60 pc and day 80 pc and are 5- to 6-fold heavier between day 80 and 105 pc than those at day 60 pc. The capillary loops in the median eminence are first visible at day 60 pc.



pp = days postpartum; ME = median eminence

Figure 1. Ontogeny of brain-pituitary relations

Individual, scattered LHRH perikarya were detectable in the paraolfactory and rostral precommissural area on day 40 pc (Danchin and Dubois, 1982). Immunoreactive LH, indicative of LH cells, was also present from day 40 onwards (Dacheux and Martinat, 1983; Sasaki *et al.*, 1992), and around day 80 pc, cell densities were comparable to those of adults (Dacheux, 1984). Based on histochemical techniques, the density of LH cells, relative to the glandular mass of the anterior part of the pituitary gland, was 0.12 % on day 50 pc and 0.61 % on day 90 pc. This volume increased sharply to 4.5 % between day 90 and 100 pc (Meijer *et al.*, 1985). Furthermore, a regulatory action of LHRH on pituitary gonadotropin cell development probably begins around day 70 pc.

Visualization of LH and FSH peptides by immunohistochemistry as well as monitoring of LH and FSH gene expression by in situ-hybridisation indicated that the α-subunit is present before the ß- subunits, and LH precedes FSH. The first signals for both LH-ß and FSH-ß subunits were clearly located in the area close to the intermediate lobe (Ma *et al.*, 1996). More recent studies, utilizing the northern blot technique to quantify development of LH and FSH mRNA, confirmed that ontogeny of the foetal pituitary is characterised by a marked increase in LH-ß mRNA from day 50 pc until day 110 pc in female foetuses (Fig. 2) while the increase during this period is less obvious in male foetuses (Granz *et al.*, 1997). It is appropriate to note that the LH receptor peptide and LH receptor gene-expression were present as early as day 30 pc in both ovarian and testicular tissue (Goxe *et al.*, 1993; Derecka *et al.*, 1999). However, neither immunohistochemical nor in situ hybridisation nor northern blot hybridisation studies could prove that LH production started in the pituitary before day 40-50 pc (Dacheux, 1984; Ma *et al.*, 1996; Granz *et al.*, 1997).

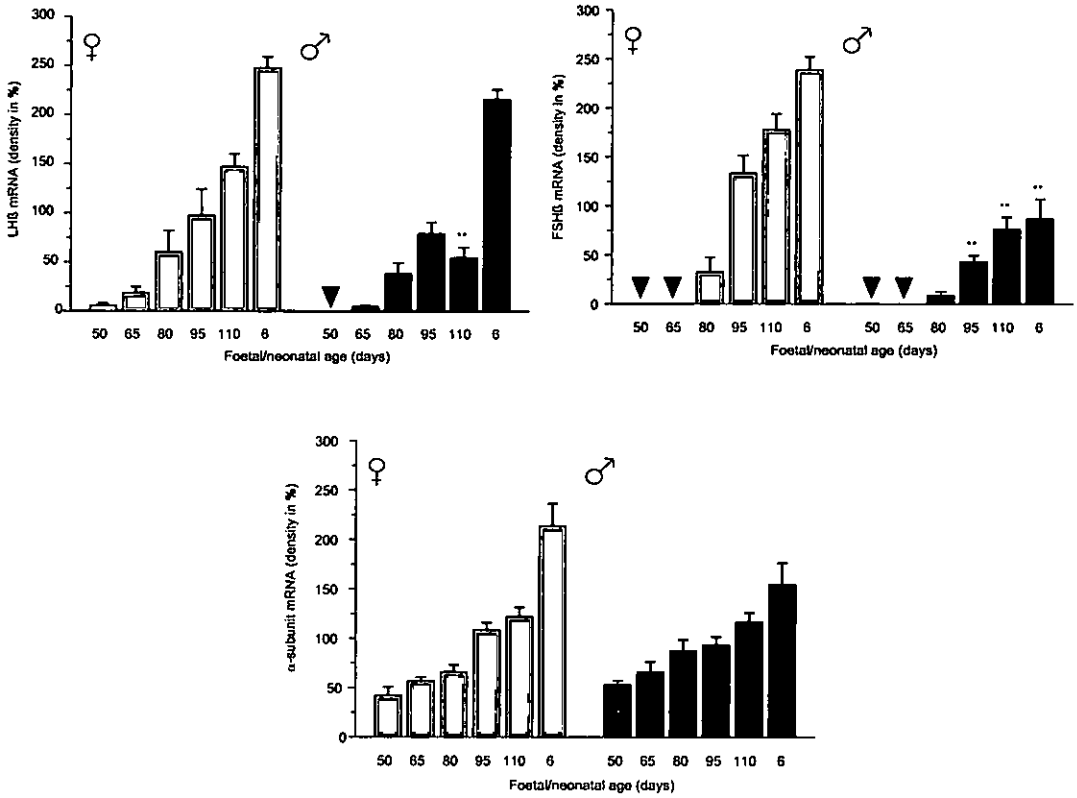


Figure 2. Levels of LH-ß mRNA, FSH-ß mRNA and α-subunit mRNA in male and female foetal (day 5 to 110) and neonatal (day 6) pigs. ▼ = not detectable; ** = $P \leq 0.05$ male vs. female (Data adapted from Granz *et al.*, 1997).

Neuronatin, a peptide consisting of an α- and ß-form, with 81 and 54 amino acids, respectively, was originally found to be expressed during hind brain, Rathke´s pouch and adenohypophysial development in the rat and human (Joseph *et al.*, 1994, 1995; Wijnholdeset *al.*, 1995; Usui *et al.*, 1997). Its expression declined in adulthood, thus neuronatin is most likely involved in brain and pituitary development (Dou and Joseph, 1998). Strong support for this postulation

comes from results showing that neuronatin is under control of the pituitary transcription factor gene, prop1, in mice (Sornson *et al.*, 1996). Both forms of neuronatin were expressed at day 40 pc in the foetal pig pituitary. The density of expression remained constant throughout foetal life, but increased up to 2-fold shortly after birth. ß-neuronatin seems to be the dominant form in foetuses. However, around day 8 post partum (pp), expression of the α-form exceeded that of the ß-form (Aikawa *et al.*, 2003).

LH secretion

In foetal pigs, circulating levels of LH were detectable as early as day 60 pc (Fig. 2). Plasma LH levels doubled between day 60 and 80 pc, and increased further until day 110 pc (Bruhn *et al.*, 1983; Behrens-Herrler and Parvizi, 1993). This developmental secretory pattern follows LH-ß subunit gene expression in the pituitary (Fig. 3).

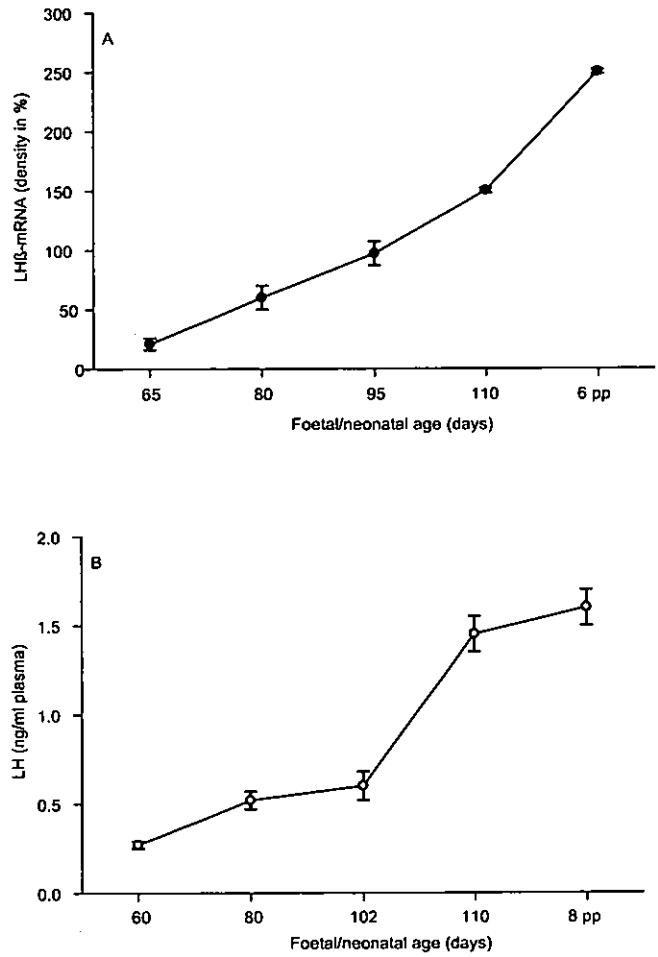


Figure 3. Foetal (day 60 to 110) and neonatal (day 6 or 8) levels of LH-ß mRNA (A) and plasma LH concentrations (B) (Data adapted from Ponzillius *et al.*, 1986 and Granz *et al.*, 1997).

Apparently, there is a slight sex difference in the concentration of plasma LH towards the end of gestation. Plasma LH levels were higher in female foetuses than in males when single samples were withdrawn under so called acute experimental conditions (Colenbrander *et al.*, 1977; Goxe *et al.*, 1993). This difference could not be fully demonstrated using the chronically catheterised foetal pig model (Colenbrander *et al.*, 1982; Ponzillius *et al.*, 1986; Behrens-Herrler and Parvizi 1993). This controversy can be attributed to differences in experimental conditions and blood sampling procedures, or just to the method used for statistical verification of data; e.g. the difference in plasma LH values between male and female foetuses was 20 % in the paper of Goxe *et al.* (1993) and 17 - 35 % in the work published by Behrens-Herrler and Parvizi (1993). In general, plasma LH values measured in acute studies were higher in both female and male foetuses than LH levels obtained in chronically catheterised foetuses. On the other hand, the pituitary of female foetuses produces and stores much larger amounts of LH than the male foetal pituitary (Elsaesser *et al.*, 1988; Granz *et al.*, 1997). This deposit was easily released when pituitary cells were cultured *in vitro* (Elsaesser *et al.*, 1988). Acute experimental condition could also induce such a LH discharge.

Mechanisms controlling pulsatile release of LH are obviously not fully developed in the prenatal pig. The few individual pulses, which occurred between day 80 to 110 pc, disappeared entirely on day 113 pc (gestation = 114 ± 1 days), but reappeared with a marked increase in frequency on day 4 postpartum (pp) (Ponzillius *et al.*, 1983). Brain control of pituitary function starts when the prerequisites of releasable LHRH, LHRH fibres and capillary loops near the median eminence and infundibulum are established around days 70 to 80 pc. At this age, intravenous (iv) injections of LHRH, as well as electrical or electrochemical stimulation of the hypothalamus, resulted in an incremental increase in plasma LH concentrations. The response increased as foetuses got older (Colenbrander *et al.*, 1982; Bruhn *et al.*, 1983). Stimulation of extrahypothalamic brain areas, such as the amygdala and hippocampus, had no effect on foetal pig LH secretion. Hence, extrahypothalamic control of LH seems to develop at the end of foetal life or later. LH release was provoked in pituitary cell cultures obtained from 50 or 60 day-old female, but not male, foetuses by LHRH challenges (Elsaesser *et al.*, 1988; Zeng *et al.*, 2005). This LHRH effect was mimicked by cAMP and the adenylcyclase activator, forskolin, whereas the protein kinase C activator, ATP, enhanced LH secretion in both sexes. Similar treatments on day 80 pc evoked massive LH discharges in female and male foetuses (Zeng *et al.*, 2005).

Development of pituitary gonadotropin cells has been assumed to be LHRH independent. However, during the late foetal stage, from day 70 pc onwards, exogenous LHRH disturbed the normal development of gonadotropic cells (Meijer *et al.*, 1985).

Gonadal steroid feedback

The ability of foetal testes, but not foetal ovaries, to secrete steroids is very high at around days 32 to 36 of gestation (Raeside and Middleton, 1979; Ford *et al.*, 1980). This corroborates data showing increased steroidogenic enzyme gene expression in testes of embryonic and foetal pigs (day 30 pc) (Conley *et al.*, 1994). Testicular descent starts at around day 70 pc and is completed at around day 90 pc. Mean plasma testosterone levels are quite high before testicular descent. Testicular content of testosterone, as well as its metabolite, dihydrotestosterone, increased between day 60 pc and 90 pc (Visser and Heyns 1994). Sexual differentiation of the brain leading to the failure of males to produce LH surges comparable to those in the preovulatory stage of the oestrous cycle in females occurs around day 30 pc, a time of high testicular testosterone activity (Elsaesser and Parvizi, 1979; Petric *et al.*, 2004). Both LH-ß gene expres-

sion and plasma LH concentrations were attenuated in 80 day-old male foetuses when their mothers received testosterone at 30 days of gestation. Intrauterine testosterone treatments affected female foetuses on day 40 pc, while treatment of sows on gestational day 30 had no effect on LH-ß mRNA or plasma LH levels in their female offspring (Fig. 4; Petric *et al.*, 2004). Therefore, the critical period of sexual differentiation of the control of LH release seems to be situated between days 30 and 40 of gestation as previously suggested by Elsaesser and Parvizi (1979). Regardless of these effects of testosterone, foetal gonadectomy on day 105 pc had no effect on LH secretion (Fig. 5 A) for up to 4 days thereafter (Ponzilius *et al.*, 1986). In the male foetus, castration led to a significant reduction in circulating testosterone levels. Yet, there were no changes in plasma progesterone or 17-ß oestradiol concentrations (Fig. 5 A) after gonadectomy in female or male foetuses at day 105 pc (Ponzillius *et al.*, 1986). This supports the proposal of a non-gonadal origin for these prenatal steroids. Interestingly, ovariectomy of 4 day-old neonates also failed to alter circulating levels of these steroids (Fig. 5 B), indicating that the pig ovary is quiescent at this age. The absence of gonadal steroid feedback on LH secretion in late stages of foetal life was also shown in experiments in which chronically catheterised foetuses were treated with oestradiol. In these studies (Parvizi, 1986), iv injections of 17 ß-oestradiol had no effect on LH secretion in male or female foetuses on day 105 to 108 of gestation while administration of 2-hydroxy-oestradiol, a catecholoestrogen, resulted in a rapid decline in plasma LH levels. A non-oestrogenic action of 2-hydroxy-oestradiol on LH secretion may explain differences in the LH response to these two steroids. Several possible pathways for a non-oestrogenic action of catecholoestrogen include: a) interfering with metabolism of catecholamines by competition with catecholamines for catechol-o-methyltransferase; b) inhibition of tyrosine hydroxylase, the rate limiting enzyme of catecholamine biosynthesis; and c) binding of catecholoestrogen to brain and pituitary dopamine and adrenoceptors (Paden *et al.*, 1982). Catecholoestrogen, now known as a brain steroid, inhibited LH secretion when microinjected into the brain in neonates and adults (Parvizi and Ellendorff, 1975, 1980, 1983).

Opioids and their actions

Opioids have been postulated to be important regulators of neuronal growth (Hauser *et al.*, 1989), therefore, one should anticipate their early appearance in the brain. The opioid peptide synthesizing system functions in the early stages of foetal life. Immunoreactive α- and ß-endorphin was visible as early as day 30 to 34 pc (Dacheux, 1984) and POMC-mRNA, visualised by in situ hybridisation, was present on day 30 pc (Ma *et al.*, 1994) in the primordial hypophysis. A POMC signal was measurable on day 40 pc in the intermediate lobe of the hypophysis and in structures of the diencephalon, e.g. arcuate nucleus and medial nucleus of thalamus, and 10 days later the signal was extended to fasciculus tegmenti. POMC mRNA in the intermediate lobe increased during gestation and reached adult levels around day 80 pc (Ma *et al.*, 1994; Kineman *et al.*, 1989). Northern blot analysis demonstrated a linear increase in both proenkephalin A (PENK A) and proenkephalin B (PENK B, prodynorphin) mRNA in the foetal hippocampus during development (Fig. 6 A). However, the increase in mRNA coding for PENK B in the hippocampus occurred faster than mRNA coding for PENK A. In contrast, a maximum was observed in the striatum. PENK A and B mRNA levels continued to increase up to around mid-gestation and then declined (Pittius *et al.*, 1987) and the ratio of PENK B mRNA to PENK A mRNA remained constant during foetal development.

Opioid binding sites were present in the brain at day 45 pc (Fig. 6 B), which is about 10 to 15 days after the appearance of opioid peptides in the brain. In the rat, a species which is less mature at birth, opioid receptors appeared in the brain by the second trimester of gestation (Kent *et al.*,

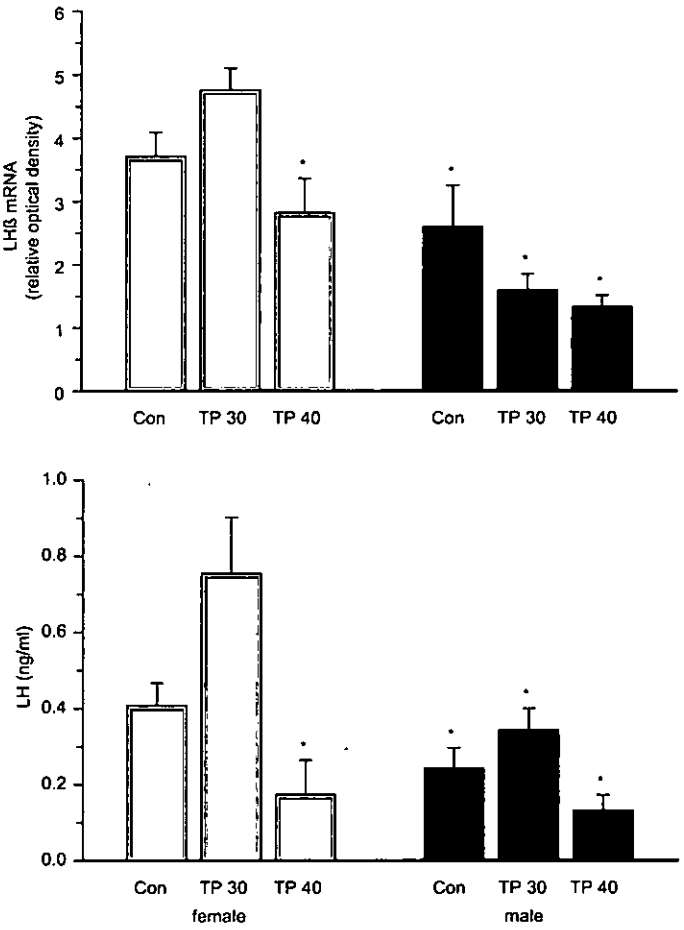


Figure 4. Contents of pituitary LH-ß mRNA and plasma LH concentrations in 80 day-old male and female foetuses. Mothers were treated three times at 2 day intervals with testosterone propionate (TP) starting at day 30 (TP30) or on day 40 (TP40) of gestation. * = different from TP30 female $P \leq 0.05$; con = control (data adapted from Petric *et al.*, 2004).

1982), while opioid peptides first appeared in small amounts after birth (Bayon *et al.*, 1989). Opioid binding sites increase during gestation, culminating in a sex difference around day 110 pc (Fig. 6 B). Opioid receptor concentrations, measured as B max of [^{3} H]-diprenorphine binding to brain tissue homogenates, decreased one day after birth and were comparable to those on day 90 pc with no sex difference (Fig. 6 B). A more detailed study of opioid receptors revealed that the sex difference in opioid receptor in whole brain homogenates was not reflected in specific brain areas, such as the striatum, hypothalamus, amygdala and hippocampus (Fig. 7). Another interesting, and to some extent, unexpected finding was that the delta-receptor type was apparently not fully developed in the foetus (Kahle, 1993). The mu-type seemed to be the predominant opioid receptor in the pig foetus (Fig. 7). Hence, the actions of opioids are most probably brought about by mu-receptors in the foetus. This is in contrast to the sheep foetus, in which delta and kappa receptors are the predominant ones (Yang and Chalis, 1991; Taylor *et al.*, 1996).

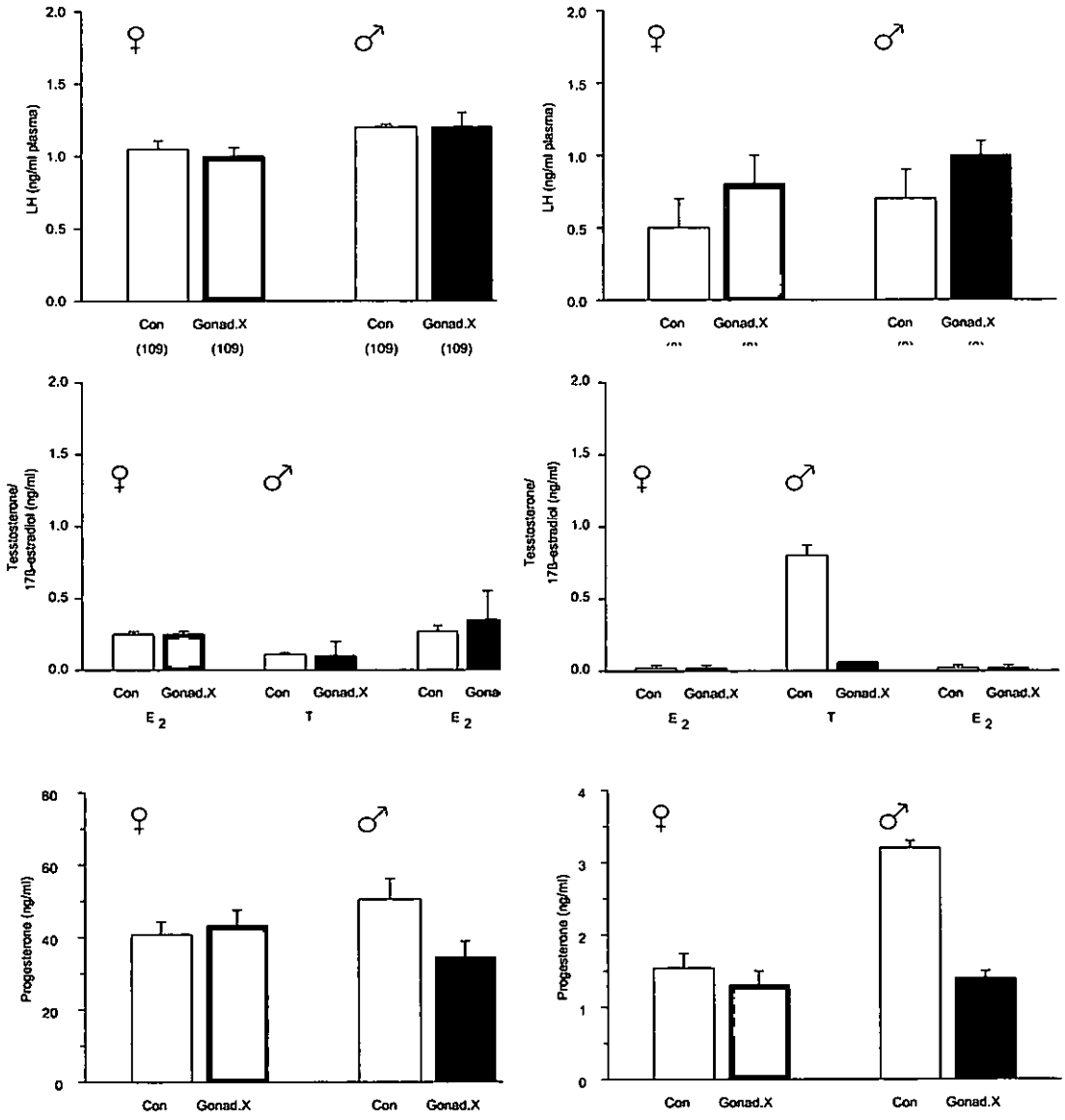


Figure 5. (A and B) Effects of gonadectomy (Gonad.X) on plasma LH, testosterone (T), 17-ß oestradiol (E_2) and progesterone concentrations in male and female foetal (day 109, A) and neonatal (day 8, B) pigs; con = control.

Daily iv treatments with morphine reduced LH release in female, but not male foetuses (Fig. 8). In the male foetus, attenuation of LH levels was induced by repeated iv applications of the opioid antagonist, naloxone (Fig. 8), whereas, single injections of naloxone had no effect (Behrens-Herrler and Parvizi, 1993). Several reasons have been postulated for the paradoxical long-term effects of naloxone (Dingledine *et al.*, 1978; Cicero *et al.*, 1989; Behrens-Herrler and Parvizi, 1993). Naloxone in low doses occasionally blocked auto-inhibition of opioid neurons at synapses (Rasmussen *et al.*, 1988), which increased opioids, and thus, inhibited gonadotropin release. Also, under certain circumstances, naloxone does not act as a pure opioid antagonist.

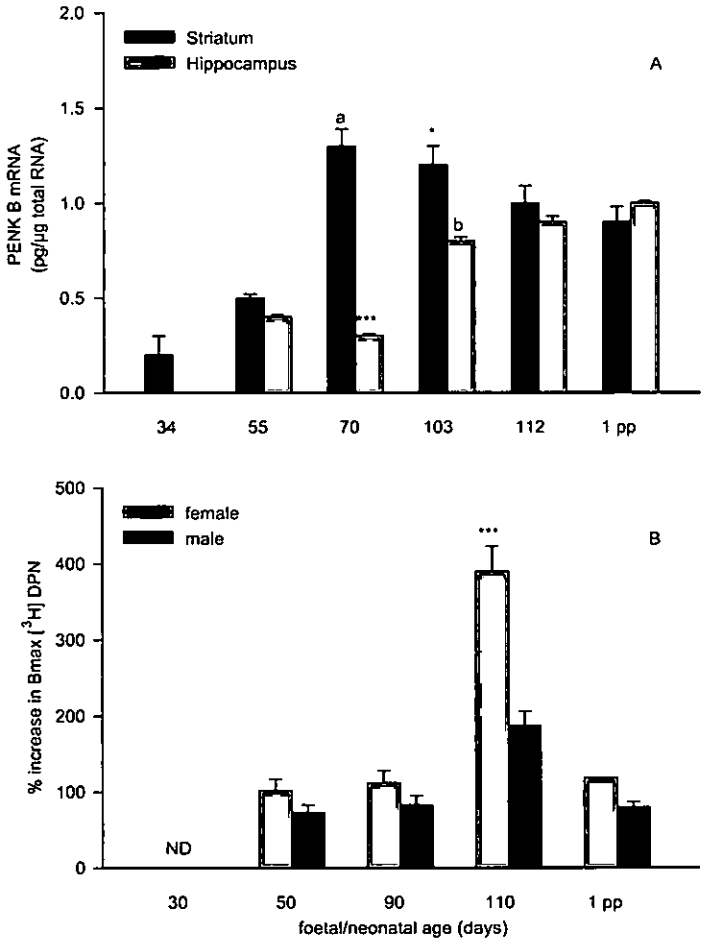


Figure 6. (A) Foetal (day 34 to 112) and neonatal (PP; day 1) ontogeny of proenkephalin B (PENK B; prodynorphin precursor) in striatum and hippocampus. For each tissue, four northern blots were scanned using a laser densitometer; solution hybridisation allowed determination of absolute PENK B mRNA levels. * = $P < 0.05$; *** = $P < 0.01$ striatum vs. hippocampus; a = significantly (P at least = 0.05) different from all other values in striatum. b = significantly (P at least = 0.05) different from preceding values; 34 = whole brain. (B) Development of the maximum binding capacity (B_{max}) of ^{3}H-diprenorphine (DPN) in foetal and neonatal pig brain. *** $P < 0.01$ male vs. female; ND = not detectable.

Further, naloxone given chronically in the absence of opioids may act on sites other than opioid receptors (Grevel *et al.*, 1985). Dingledine *et al.* (1978) demonstrated that naloxone acts as an antagonist when bound to gamma-amino butyric acid (GABA) receptors. Under most experimental conditions, GABA stimulated LH release, hence, blockade of GABA receptors by naloxone would lead to a decline in LH secretion. However, naloxone, in combination with morphine, acted as an opioid antagonist and abolished the long-term inhibitory effect of morphine on LH secretion in female foetuses (Fig. 8). Other foetal endocrine systems, such as the hypothalamo-pituitary-adrenal axis are much more sensitive to naloxone than the LH secretory system. Single iv injections of naloxone provoked a significant increase in foetal male and female plasma concentrations of cortisol (Li and Parvizi, unpublished).

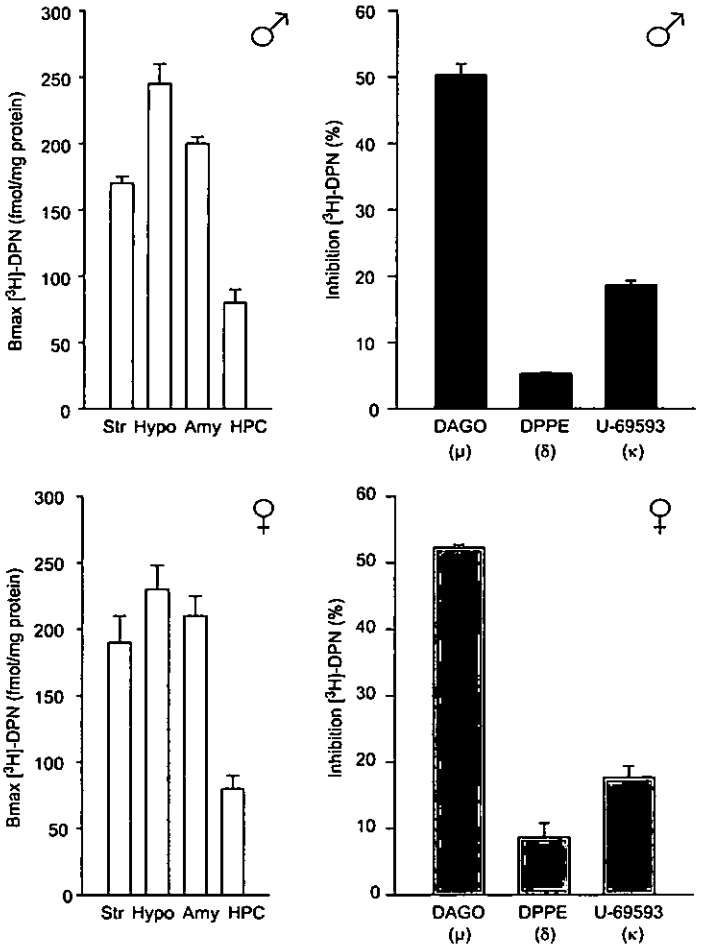


Figure 7. Left side: maximum binding capacity (B_{max}) of ^{3}H-diprenorphine (DPN) in different brain areas of male and female pig foetuses (day 110). Str. = striatum; hypo = hypothalamus; amy = amygdala; HPC = hippocampus. Right side: Percentage inhibition of ^{3}H-diprenorphine binding in foetal male and female (day 110) hypothalamus using specific ligands of μ - (DAGO); δ- (DPPE) and K- (u-69593) opioid receptor types.

Other than the possible critical involvement of cannabinoid receptors in survival of the newborn (Fride, 2002), nothing is known about the effects of endocannabinoids in the foetal pig. Cannabinoids are compounds found in marijuana. The first endogenous cannabinoid ligand, 'endocannabinoid', was isolated from the pig brain in 1992 (for review see Fride, 2002) and named anandamide (arachidonoyl ethanol amide). This lipid neurotransmitter binds to the three cannabinoid receptor subtypes CB1, CB2 and CB1A. A general inhibitory action of anandamide on LH secretion was described by de Miguel *et al.* (1998) and Wenger *et al.* (2001).

Cytokines

Among the regulatory mechanisms of LH secretion, cytokines and nitric oxide (NO) are still newcomers. Although a body of evidence illustrates the predominantly excitatory role of NO

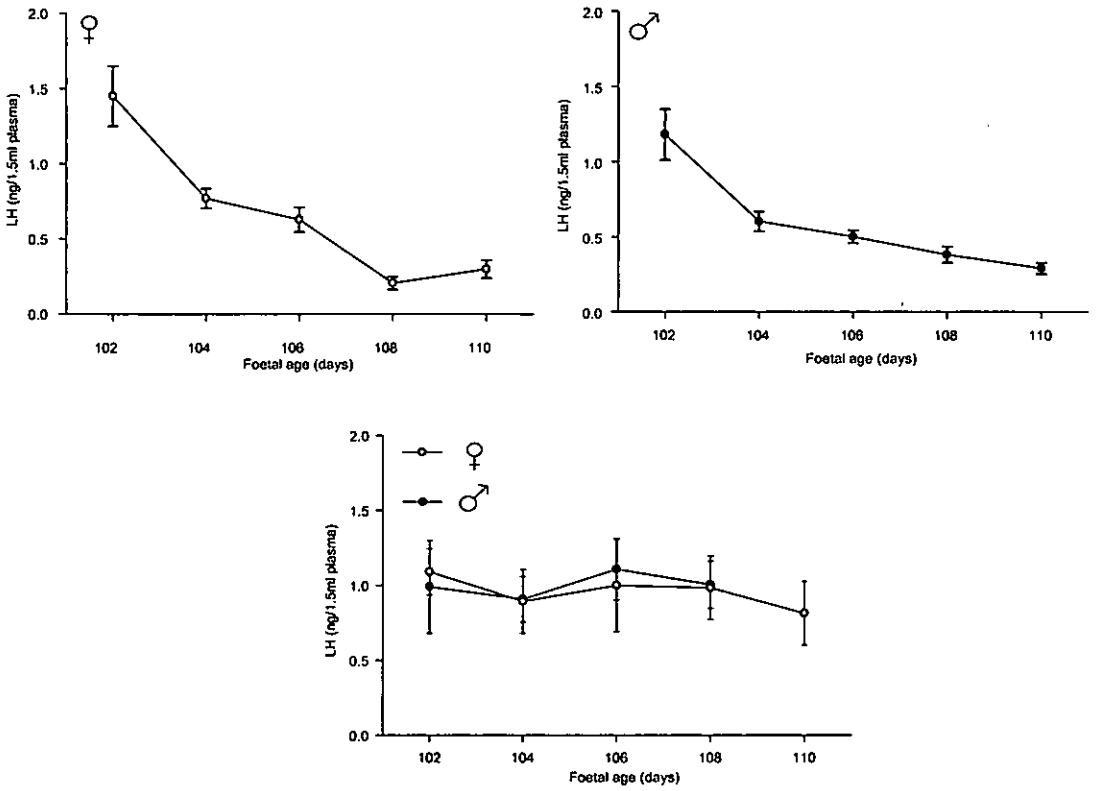


Figure 8. Plasma LH concentrations in foetal pigs receiving daily injections of morphine (0.1 mg/kg, left panel), naloxone (0.1 mg/kg; right panel) or morphine + naloxone (lower panel).

in regulation of LH secretion (McCann *et al.*, 2001; Gouveiaand Fronci, 2004; Moreno and Franci, 2004) and LH action (Nishida *et al.*, 2000), there are no data supporting the role of NO in the ontogeny of LH secretion. Cytokines have been postulated to be mediators of immuno-endocrine interactions (Besedovsky and Rey, 1996), but the significance of cytokines in development, maturation and function of the hypothalamo-pituitary system has yet to be established. In general, interleukins, particularly interleukin-1 ß, inhibit LH release (Kalraet *al.*, 1990; Bonavera *et al.*, 1994). There has also been an occasional report that interleukin-1 stimulated LH secretion. Our studies showed that both iv administration and microinjections of human recombinant interleukin-1 ß into the hypothalamic area of foetal pigs induced a sexually dimorphic response. Such microinjections caused a rapid rise in plasma LH levels in 103 to 107 day-old female foetuses, while male foetuses remained unaffected. It is noteworthy that a close relationship between opioids and interleukins exists at different levels. Interleukin-1 and interleukin-2 bind to brain opioid receptors (Jiang *et al.*, 1995). Furthermore, interleukins stimulated opioid production in the brain. Both opioids and interleukins are highly active during situations of so-called tissue stress, such as phases of rapid growth. Whether these two systems interact to regulate neuronal growth is not yet known.

In conclusion, development of the "regulatory phenotype" of pituitary LH secretion commences around day 30 pc and continues throughout pregnancy. Hypothalamic control mechanisms are fully functioning before birth. In contrast, little is known about the development of

extrahypothalamic mechanisms. Consequences of malfunction of steroids during foetal ontogeny are well documented. On the other hand, not much information is available concerning foetal development of such systems as opioids, catecholamines, cytokines etc. which are regarded as secondary regulatory systems.

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