HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2021 December 01.

Published in final edited form as:

Mol Cell Endocrinol. 2020 December 01; 518: 110912. doi:10.1016/j.mce.2020.110912.

PACAP: a regulator of mammalian reproductive function

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an ancestral molecule that was isolated from sheep hypothalamic extracts based on its action to stimulate cAMP production by pituitary cell cultures. PACAP is one of a number of ligands that coordinate with GnRH to control reproduction. While initially viewed as a hypothalamic releasing factor, PACAP and its receptors are widely distributed, and there is growing evidence that PACAP functions as a paracrine/autocrine regulator in the CNS, pituitary, gonads and placenta, among other tissues. This review will summarize current knowledge concerning the expression and function of PACAP in the hypothalamic-pituitary-gonadal axis with special emphasis on its role in pituitary function in the fetus and newborn.

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is one of a number of ligands that coordinate with gonadotropin-releasing hormone (GnRH) to control reproduction. While initially identified as a hypophysiotropic factor, there is accumulating evidence that PACAP is primarily a paracrine/autocrine hormone in the CNS, pituitary and gonad. Overexpression of PACAP in the anterior pituitary in male mice delayed puberty and suppressed serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone levels as well as pituitary gonadotropin-releasing hormone receptor (GnRH-R) expression perhaps because pituitary folllistatin expression was markedly increased (1). On the other hand, PACAP knockout male mice are testosterone-deficient with relative LH insufficiency (2), and females are sub-fertile with lower implantation rates (3,4).

1. PACAP and its receptors.

PACAP was isolated in 1989 from sheep hypothalamic extracts based on its action to stimulate cyclic AMP production by rat pituitary cell cultures (5). PACAP is the most highly conserved member of the VIP-secretin-glucagon peptide superfamily (6). There are two

PACAP isoforms, a 38 amino acid form and a C-terminally truncated 27 amino acid form, with PACAP-38 accounting for 90% of the protein in most tissues. The human PACAP promoter contains two cAMP-response-like elements, a 12-*O*-tetradecanoylphorbol 13-acetate-response element, two sequences that are homologous to the consensus sequence for pituitary-specific factor growth hormone transactivator factor-1-binding sites, and six binding domains for the thyroid-specific transcription factor-1 (7). Given PACAP's effect to increase cAMP production, the CREs and AP1 sites in the PACAP promoter allow for a feed-forward mechanism in which PACAP transcription can be activated by PACAP itself (8) (Moore et al, Molecular and Cellular Endocrinology, in press). This mechanism, shown in Figure 1, likely mediates the change in expression that occurs abruptly between the fetal-newborn pituitary (see section 6).

Because it is found in high concentration in the hypothalamus (9), is present in the median eminence (10), and levels in portal blood exceed those of peripheral blood (at least in the rat) (11), PACAP is viewed as a hypophysiotropic factor. However, the portal:peripheral plasma ratio for PACAP was 2:4, compared to 5:120 for GnRH (12). While PACAP is found in peripheral plasma (13), PACAP in the circulation is rapidly degraded with a half-life of 2 to 10 min (14).

PACAP activates three distinct 7- transmembrane receptors: $VPAC_1$ and $VPAC_2$ receptors with relatively similar affinity for VIP and PACAP, and the highly specific PAC_1 receptor (PAC1-R; *ADCYAP1R1*) [3]. There are multiple splice variants of the PAC1-R that result from alternative splicing of two 84 bp exons in the third intracellular loop (designated hip, and hop) and were named null (neither hip nor hop), hip, hop1, hop2 (a shorter version of hop1), and hiphop1 and hiphop2 (15). PAC1-R variants that differ from the null receptor in the amino-terminal extracellular domain have also been identified [5].

VPAC-1 and -2 and all PAC1_R variants bind PACAP38 and PACAP27 with high affinity, and like other members of the group B G-protein coupled receptor family, couple with Gαs to activate adenylate cyclase and increase cAMP signaling. In some cell types, PACAP 38 >>PACAP 27 also couples with $G_{q/11}$ to stimulate IP3 production, the MAPK pathway, and increase intracellular calcium (16), as well as nitric-oxide synthase type I and cGMP (17). PAC1-R has also been shown to signal via β-arrestin1 and β-arrestin2 (18). Thus, variable expression and signaling of the PAC1 -R variants would be expected to produce different transcriptomes (7,19,20).

Consequent to the extensive distribution of the ligand and its receptors, PACAP exerts a wide array of functions including protection against neuronal apoptosis and retinal degeneration, neurotransmitter function, vaso- and broncho-dilatation, tear secretion, activation of intestinal motility, bladder pain and micturition, anti-inflammatory and antioxidant effects, immune modulation, thermogenesis, appetite suppression, and sleep and circadian rhythms, as well as effects on endocrine systems (21). There are PACAP effects in the exocrine and endocrine pancreas, hepatocytes, osteoblasts, adrenal medulla and cortex, testis and ovary, thyroid, pineal, hypothalamus, neurohypophysis and pars tuberalis as well as the anterior pituitary.

2. Functions of PACAP as a neuropeptide

Early studies by Arimura et al (9) revealed the highest concentration of immunoreactive PACAP in the hypothalamus. Further research showed that PACAP is widely distributed in the CNS (22) in various brain regions including the cerebral cortex, amygdala, and hippocampus. In situ hybridization methods revealed dense labeling in the supraoptic nucleus (SON) followed by the anterior and posterior hypothalamic regions, the dorso-ventromedial, and arcuate nuclei and in the tubero- and premammillary regions (23), the periventricular region and in the paraventricular nucleus (PVN) (24).

A series of experiment suggest that hypothalamic PACAP functions as a local activator of GnRH secretion. The intracerebroventricular (icv) injection of PACAP into adult male rats produced a small but significant increase in *GnRH* mRNA levels which was abolished by the PACAP6-38 antagonist which itself suppressed the basal hybridization signal (25). The GnRH neuronal line, GT1-7, was reported to express multiple PACAP receptor splice variants, and to respond to PACAP with an increase in cAMP production (26) and GnRH-R expression (27). The effect on GnRH may be through Kisspeptin neurons since *Kisspeptin1* mRNA levels were increased by PACAP38 in mHypoA-50 and mHypoA-55 hypothalamic cell lines although the PACAP 6-38 antagonist produced a similar effect (28).

Using in situ hybridization, we found (29) that *Pacap* mRNA expression in the PVN and pituitary vary significantly across the estrous cycle in the rat, with the greatest changes occurring on the day of proestrus (Figure 2). *Pacap* mRNA in the PVN declined significantly on the morning of diestrus. At noon of proestrus, there was a notable peak in PVN *Pacap* mRNA that occurred three hours before the gonadotropin surge, followed by a decline. Pituitary expression of *Pacap* mRNA also varied on the afternoon of proestrus with a moderate decline at the time of the gonadotropin surge and a significant increase later in the evening. Expression of the mRNA species encoding the 288 amino acid form of follistatin increased significantly following the rise in pituitary *Pacap* mRNA, at the termination of the secondary surge in *Fshb* gene expression.

While these observations suggest a faciliatory role for PACAP in the rat estrus cycle, in vivo experiments to examine the effects of exogenous PACAP or the PACAP 6-38 antagonist have produced variable results depending on dose and route of administration, sex and species (30). For example, intra-atrial or icv administration of PACAP38 stimulated LH release in adult male rats (31) but icv PACAP38 on the day of proestrus inhibited the release of LH and ovulation in rats (32) while in a second study, icv PACAP38 suppressed but PACAP27 enhanced the LH surge (33). Choi et al (34) reported that PAC1-R antisense oligodeoxynucleotide administered icv to immature female rats suppressed the prepubertal increases in *GnRH* and *GnRH-R* mRNA levels and delayed sexual maturation. On the other hand, Szabo et al found that the subcutaneous (sc) administration of PACAP to neonatal rats delayed vaginal opening and reduced immunoreactive GnRH in the pre-optic area in 30-day old rats (35).

The importance of PACAP to adult reproductive function has been demonstrated by studies of PACAP- and PAC-1-knockout mice which have revealed many instances of endocrine dysfunction as summarized in Table 1. PACAP knockout mice have substantial newborn

mortality. Surviving females have normal sexual maturation and estrous cycles but have disrupted mating behavior, a lower implantation rate with reduced fertility, and lower prolactin and progesterone levels (4,36). PACAP knockout males developed testosterone deficiency apparently because of LH insufficiency insofar as LH levels were normal or reduced in the setting of low testosterone. The LH activated steroidogenesis enzymes, testicular steroidogenic acute regulatory protein (StAR) and P450c17 were reduced. As these animals age (15 mo), knockout mice were protected from the germ cell depletion and vacuolization that was observed in w/t testes (37). Mice rendered deficient in PAC-R1 had increased mortality at weaning but no fertility disturbance has been noted (38,39).

PACAP is a potent suppressor of feeding behavior, by decreasing ghrelin which suppresses appetite, and by increasing leptin and GLP1 which increase satiety (40). PACAP also appears to mediate in part the effects of leptin on food intake (41) and perhaps its effect to enhance pubertal development and reproductive function (42). Ross et al (43) developed a conditional knockout model of PACAP from ventral premammillary nucleus (PMV) neurons that express the leptin receptor. They found delayed sexual maturation in females, disruption of the LH surge, and fewer pups per litter. Kisspeptin induction of LH secretion was unaffected. They also deleted PACAP from the PMV of adult female *Adcyap1*$^{fl/fl}$ mice with bilateral stereotaxic injections of an adenovirus carrying cre-recombinase, and again found dysregulation of the estrus cycle. They propose that PACAP plays a role in conveying the signal between nutrition and GnRH release.

PACAP immunoreactivity in and around magnocellular neurons and colocalization of PAC-R by in situ hybridization in arginine vasopressin (AVP) neurons in the SON suggest a role for PACAP in the control of vasopressin secretion (44).

3. PACAP and its receptors in the pituitary

Although initially identified as a hypothalamic peptide, PACAP is also produced in the pituitary. An early immunoassay detected PACAP in the adult rat pituitary, although at much lower levels than in the hypothalamus, with higher levels in the posterior than in the anterior lobe (9). No *PACAP* mRNA was found in the adult rat pituitary by Northern blotting (45). Subsequently, Koves et al (46) used dual immunohistochemistry to localize PACAP to gonadotrophs, and Jin et al (47) identified *PACAP* mRNA using RT/PCR in pituitary folliculostellate (FS) cells obtained by laser-capture microdissection from adult female rats. FS cells are agranular and star-shaped with long cytoplasmic processes (48) that intermingle with and are joined to the endocrine cells by a variety of intercellular junctions (49) allowing for intercellular communication. Thus, PACAP in gonadotrophs could have an autocrine effect, and PACAP from FS cells might be a paracrine regulator of gonadotrophs.

Each of the pituitary secretory cells, as well as FS cells, express at least one form of the PACAP receptor (19,50,51). Studies using rat (52–54) or ovine (55) pituitary cell monolayer cultures revealed, however, only small effects of added PACAP on the release of PRL, GH, and ACTH. There are some species-specific differences, as PRL synthesis and secretion are substantially stimulated by PACAP in pituitary cultures from fish (56). PACAP also effectively stimulates the release of GH and PRL from rat GH3 cells, and ACTH from the mouse pituitary tumor cell line AtT-20 (57,58).

Gonadotrophs, on the other hand, are clearly regulated by PACAP, with direct effects on gonadotropin secretion and subunit gene expression, and indirect effects by modulating the actions of GnRH. PACAP directly stimulates the release of LH and uncombined glycoprotein α-subunit from primary pituitary cell cultures (52,54) although the effect is less than that of GnRH, and desensitizes rapidly (59). Most notably, PACAP enhances LH and FSH secretion by pituitary cells that are stimulated with GnRH (52,60), an effect that is especially pronounced when pituitary cells are perifused and stimulated with pulses of GnRH as a model of the hypothalamic-pituitary unit. In this model, PACAP also increased α-subunit mRNA levels, lengthened *LHb* mRNA but suppressed *Fshb* mRNA levels (60).

Gonadotroph-derived cell lines developed by Dr. Pamela Mellon and colleagues (61) have been instrumental in understanding the intracellular signaling pathways through which PACAP regulates the gonadotropin subunit genes. αT3-1 cells, viewed as immature because they express gonadotropin-α but not β-subunits, also express PAC1-R at a high level with the hop and short variants predominating although other forms (PAC1-R hiphop and hip) were also observed (51). In these cells, PACAP stimulates cAMP production and activates inositol phosphate to increase cytosolic Ca2+(62) with the former pathway leading to an increase in *Cga* mRNA levels (62–64).

In LβT2 cells, with characteristics of differentiated gonadotrophs (65), PAC1-R expression is very low although PCR products are consistent with the short and the hop1, hop2 or hip forms (66). In this cell line, PAC1-R overexpression has been used to study PACAP signaling to *Lhb* and *Fshb* transcription (67) including experiments designed to understand differences among the various splice variants. In these cells, PACAP stimulates the *Lhb* promoter in part through cAMP-PKA (68) and increased EGR-1 (69). Stimulation of the *Lhb* promoter was more evident when cells were stimulated with intermittent PACAP pulses (67) perhaps because of desensitization with continuous PACAP. PACAP likewise activated the *Lhb* promoter in pituitary cultures from mice expressing a rat *Lhb*-luciferase transgene (68).

In contrast to its activation of *Cga,* and *Lhb*, PACAP stimulation of *Fshb* is transient in primary rat pituitary cell cultures, and is followed by suppression (70,71), although not in LβT-2 cells (66,67,71). A likely explanation for this difference involves robust stimulation of follistatin expression by PACAP in the normal pituitary (70) which is essentially undetectable in LbT2 cells (66). According to this paradigm, ongoing PACAP stimulation of *Fshb* transcription is blocked in the normal pituitary when follistatin increases, binds activin, and prevents the multiplicity of activin effects including up-regulation of the *Fshb* and *Gnrhr* genes (72). How PACAP may differentially regulate the gonadotropin subunit genes is shown in Figure 3, and evidence that this mechanism is important to the ontogeny of the gonadotropins in the fetus and newborn is summarized in Section 6.

In addition to direct effects on gonadotropin subunit genes, there is substantial cross-talk between PACAP and GnRH signaling pathways in gonadotrophs. PACAP has been found to increase GnRH-R expression (73) and to enhance GnRH-R signaling (74). *Gnrhr* transcription is increased by PACAP in LβT2 cells through CREB and SF-1 [28], and *Gnrhr* mRNA levels in αT3-1 cells are increased by PKA activation in which GnRH-stimulated IP

production is increased synergistically by PACAP (62). The nitric oxide pathway also appears to contribute to the interaction between PACAP and GnRH as both ligands increase nitric oxide synthase type I protein levels in rat gonadotrophs via cAMP leading to an increase in cyclic GMP (75).

Furthermore, effects may be amplified because GnRH increases PACAP and PAC1-R expression in primary pituitary cell cultures (76) as well as in LβT2 gonadotroph cells (77). On the other hand, GnRH blunts PACAP-induced cAMP accumulation by up to 70% in both αT3-1 (78) and LβT2 cells (79) in which GnRH phosphorylates PAC1-R (79).

Pituitary PACAP may play an important role in gonadotroph function in the transition from the fetus to newborn (Section 6) as well as in the timing of pubertal development. We created a transgenic model of pituitary PACAP overexpression using the gonadotropin-α subunit promoter (1). Male transgenic mice had delayed sexual maturation based on testis weight and balano-preputial separation, with delayed spermiogenesis. LH and FSH levels were suppressed, and GnRH-receptor expression was decreased. The effect of PACAP to stimulate follistatin transcription (80) may have mediated these changes. Pituitary follistatin expression in w/t mice declines from day 10 to day 30, and then remains stable, and there is a reciprocal rise in GnRH-R expression. In the transgenic mice, however, pituitary follistatin was substantially higher while *Gnrhr* mRNA was suppressed through day 40. It is likely follistatin binding to activin blocked activin paracrine upregulation of the GnRH-R to produce gonadotropin deficiency.

4. PACAP in the testes and ovaries

In addition to regulating reproduction via hypothalamic-pituitary functioning, PACAP has direct effects on the testis and ovaries. PACAP is present in high concentration in testis (9) including the human testis (81), and is primarily found in germ cells (82,83). Accordingly, levels increase from age 20 to 60 days in the rat, and are low when spermatogenesis is disrupted, e.g. by cryptorchidism (84). A shorter PACAP mRNA is expressed in the testis from rats, mice, bovine and humans, and reflects a novel testis-specific first exon upstream from the transcriptional start-site. The gene encodes a PACAP precursor with no signal peptide suggesting a paracrine function. The level of expression across the rat seminiferous tubule varies during the spermatogenic cycle, and presumably reflects a role for PACAP in spermatogenesis (85,86).

PACAP receptors are widely distributed in testis although localization has varied by species. VPAC2 binding sites in the rat predominate in seminiferous tubules by *in situ* hybridization and are implied by ligand specificity (87,88) but localized to Leydig cells in immature mice by immunohistochemistry (89). VPAC2 knock-out mice (both males and females) were healthy and fertile young adults, and produced normal sized litters, but in older males (31 wks), VPAC2 deficiency caused diffuse seminiferous tubular degeneration with hypospermia and reduced fertility (90) supporting a role for this receptor in the effect of PACAP on spermatogenesis. Rat Sertoli cells (SC) predominantly express a unique PAC1-R splice variant (91). PAC1-R mRNA is found in rat Leydig cells (92) and in the clonal TM3 Leydig cell tumor cell line (93).

PACAP may play a role in the function of the fetal and immature testis. PACAP simulates cAMP production by SC cultures from immature rats, although less effectively than FSH, but like FSH, PACAP stimulation becomes less potent in cultures as animals mature (94). Testosterone production by fetal Leydig cells is evident by E15.5 which is thought to precede the presence of LH in the circulation. The implication is that some factor, either endocrine, autocrine or paracrine activates steroidogenesis in early fetal life. To assess this idea, El-Gehani et al (95) examined the effects of hCG and PACAP on testosterone synthesis by LC from E15.5 to E21.5 pups as well as adult rats. They found that PACAP stimulates testosterone production far more potently in fetal than in adult LC whereas the effect of hCG is sustained. The presence of PACAP mRNA, albeit at a low level, in the fetal testis supports the idea that PACAP is an autocrine stimulator of fetal LC function. Finally, LH-receptor deficient males have a normal male phenotype leading to speculation that PACAP was the sexual differentiation factor in rats; however, PACAP knock-out males likewise have a male phenotype.

As noted above, PACAP knock-out males are testosterone deficient probably because of gonadotropin deficiency, but have normal testicular morphology (37) while PAC1-R knock-out mice of both sexes had increased mortality at weaning but no fertility disturbance was noted (38,39).

Effects of PACAP on sperm have also been reported. In one study, sperm heads from PACAP-deficient mice were smaller, with more abnormal-shape, than in w/t littermates. In the same study, adding PACAP to semen samples from fertile and infertile men increased the motility of low -motility sperm (96). A study of PACAP and sperm quality in obesity was recently published (97). A high fat diet is known to reduce sperm motility, capacitation and oocyte membrane binding, and to increase intracellular reactive oxygen species in mice. PACAP i.p. daily for 4 wks partially blocked high fat diet -induced obesity in adult mice, and improved testis morphology and sperm function which was thought to be mediated through the p53 deacetylase Sirt 1 (silent information regulator 1) leading to suppression of apoptosis. Sirtl protein was found to be lower in sperm of those obese infertile men who also had decreased semen quality (97).

There is also evidence that PACAP is a paracrine regulator of ovarian function (30,98). Female gametogenesis begins with the differentiation of primordial germ cells (PGC) into oogonia and then oocytes. Akin to its role in stem cell proliferation and survival, PACAP immunoreactivity was identified on the gonadal ridge adjacent to the PGC surface in the e11-12 mouse, and PAC1-R is expressed in PGC cells in which PACAP increases cAMP production and promotes survival in culture. (99). When immature rats (100) or mice (101) are stimulated with PMSG/hCG, PACAP mRNA and protein are found in the majority of granulosa and cumulus cells from large preovulatory follicles, suggesting a role in ovulation. PACAP also inhibited the growth of preantral follicles suggesting an additional role in follicular recruitment (102).

RT-PCR revealed PAC1-R and VPAC2-R in rat granulosa cells whereas only VPAC1-R and VPAC2-R were found in thecae-interstitial cells (103). PACAP is a potent activator of cAMP production and estradiol and progesterone secretion by immature rat granulosa cells (104) in

which PACAP inhibited cell apoptosis (103). Human granulosa-luteal cells obtained at the time of IVF also express PACAP and VPAC1-R and VPAC2-R mRNAs that are increased by LH/FSH (105)

PACAP and PACAP type I receptor mRNAs are also expressed in the rat corpus luteum (106) in which PACAP mRNA gradually increases in pregnancy. Finally, PACAP and its receptors are present in human placenta (107) where it may play a role in cell proliferation and angiogenesis.

5. PACAP, stress and reproduction.

Stress is a major cause of reproductive dysfunction (108), and there is considerable evidence linking PACAP to the stress response (109). PACAP and PAC1-R are highly expressed in the amygdala and the bed nucleus of the stria terminalis (BNST), together with corticotropin-releasing hormone (CRH) and other stress-related peptides (110). When adult male rats were exposed to a 7-day variate stress paradigm, PACAP mRNA was markedly increased in the BNST (111). PACAP produces stresslike effects in rats when injected icv or specifically into the paraventricular nucleus (PVN) or the central amygdala, and potentiates acoustic startle when injected into the BNST (111–114). In a comprehensive series of experiments, Eiden et al have shown that PACAP functions in the response to stress as a neurotransmitter to CRF-ACTH (115), and is an activator of catecholamine synthesis and secretion (116). Restraint stress increased hypothalamic CRH mRNA as well as fos and Egr1 in hypothalamic sections from wild-type mice but not in mice deficient in PACAP (117) in which the corticosterone response was also partially attenuated [31]. Many lines of evidence indicate that stress suppresses GnRH secretion by activating the CRF system as well as sympathoadrenal pathways (118,119) which may be linked to effects of PACAP. For example, the combination of a CRF antagonist and naloxone partially restored LH secretion in female rats in which LH was suppressed by PACAP on proestrus (120).

PACAP may also play a role in stress-associated reproductive dysfunction through it effect to suppress food intake (121,122), which may indicate a role for PACAP in eating disorders, anxiety and depression, as well as the risk for drug and alcohol abuse (123). Furthermore, a SNP in a putative estrogen response element in PAC1-R has been associated with posttraumatic stress disorder in heavily traumatized humans (124).

6. Pituitary PACAP and the control of gonadotrophs in the fetus and newborn

Targeted pituitary PACAP overexpression in male mice (1) delayed sexual maturation associated with a suppression of gonadotropin subunit and *Gnrhr* mRNAs, and a large increase in follistatin expression. In male rats, there is a significant decline in pituitary PACAP mRNA expression between postnatal days 17 and 21 (125). This decline is paralleled by a significant decline in pituitary follistatin expression and a reciprocal and preferential rise in circulating FSH and pituitary *Fshb* mRNA levels. These data, summarized in Figure 4, support the notion that alterations in pituitary PACAP expression are involved in developmental changes in gonadotropin synthesis during key periods of sexual maturation.

A more substantial change in pituitary PACAP expression is observed during the perinatal period (Figure 5). During rodent embryonic development, pituitary LH immunoreactivity is detectable as early as embryonic day 12 (E12) while FSH is not found until E19 or E21 (126,127). We identified a significant and abrupt decline in pituitary PACAP expression at or around the time of birth (128). A coincident decrease in pituitary follistatin expression and reciprocal increase in FSHb and GnRH-R expression were also observed. Furthermore, in rat pituitary cell cultures, exposure to a potent PACAP antagonist decreased basal LH secretion in both pre- and postnatal cultures, decreased aGSU expression, but selectively increased *Fshb* mRNA levels only in cultures from prenatal animals. These results suggest that high levels of PACAP expression in the fetal pituitary preferentially suppress FSH synthesis through stimulation of follistatin expression. This hypothesis is supported further by the observation that pituitary PACAP expression is significantly higher in postnatal day 1 females when compared to males, and their post-natal rise in FSH secretion (129) and FSH expression is delayed.

In subsequent experiments designed to identify potential factors that could inhibit pituitary PACAP expression during the perinatal period, we identified activation of dopamine-2 receptor (Drd2) signaling as a mechanism that may explain the pronounced and abrupt decline in PACAP expression in the perinatal pituitary (130). Drd2 is present on the membranes of rat gonadotrophs, and Drd2 mRNA was detected in individual gonadotrophs from postnatal day 1 rats (130,131). In cultures of E19 rat pituitaries, adding the Drd2 agonist, bromocriptine, significantly reduced PACAP mRNA expression in a dose-dependent manner. Conversely, daily subcutaneous injections of the dopamine antagonist, haloperidol from postnatal day 1 to 3 in rats, partially reversed the early postnatal decline in pituitary PACAP expression. These data suggest that the perinatal decline in pituitary PACAP may be mediated by the dramatic increases in brain dopamine levels which occur just prior to the time of birth (132). This sequence of events is summarized in Figure 5.

Additional studies using αT3-1 and LβT2 gonadotroph cells were performed to address the mechanism by which Drd2 signaling may suppress PACAP expression. Drd2 mRNA and peptide are present in αT3-1 and LβT2 cells (133,134). Furthermore, αT3-1 cells express high levels of PACAP and PAC1-R mRNA whereas LβT-2 cells express low levels of PAC1-R and PACAP is nearly undetectable (51,66,135,136) mimicking the differences in PACAP expression observed in late fetal and early postnatal pituitaries (128). In αT3–1 cell cultures, the Drd2 agonist, bromocriptine, produced a dose- and time-dependent decrease in PACAP mRNA levels and suppressed the activity of a transiently transfected PACAP promoter-reporter construct (130). Arimura and colleagues discovered PACAP based on its ability to stimulate cAMP production in the pituitary, and PACAP is known to stimulate its own production through a cAMP response element in the PACAP promoter (Moore et al., in press). Bromocriptine also decreased PACAP-stimulated cAMP production in αT3–1 cells suggesting that Drd2 coupling to Gαl, and the resultant inhibition of adenyl cyclase activity, may be the signal that interrupts a feed-forward mechanism in which PACAP will self-regulate and maintain high levels of pituitary PACAP expression until interrupted by Drd2 signaling at the time of birth. More studies are needed to confirm this hypothesis.

7. Summary and future considerations

PACAP is an ancestral protein which functions as a paracrine regulator in the CNS, pituitary and gonads, and may be a hypophysiotropic factor. Many intriguing findings have been summarized in this chapter, yet there are substantial knowledge gaps in understanding PACAP's role in reproduction. While many studies demonstrate in vitro effects, some observations are inconsistent or contradictory, and there are few in vivo studies in which there are sex- and species-differences. The diversity in PACAP actions is partly due to its multiple receptors and signaling pathways which extend its therapeutic potential but raise suspicion about the impact of lack of specificity. Moreover, the wide distribution and actions of the peptide and its receptors can limit the interpretation of results. Specifically, neonatal mortality and sensitivity to stress in global PACAP knock-out mice may have mediated many of the reported negative effects on reproduction, and tissue-specific knock-out models are needed. The role of PACAP in the estrus cycle remains to be defined. Our results suggest that PACAP may be especially important in the fetus. Perhaps the strongest evidence identifies a PACAP feed-forward mechanism that controls gonadotropin subunit and *Gnrhr* gene expression through follistatin and activin signaling in the fetal-newborn transition, at least in rodents. While PACAP may be involved in a wide array of human diseases from neurodegenerative disorders to cancer, there are presently no human disease conditions that are clearly linked to PACAP or its receptors. Research on this important molecule continues in a wide variety of biological systems.

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Highlights

1. While originally identified as a potential hypophysiotropic factor, PACAP and its receptors are co-expressed in the pituitary and gonads, implying a paracrine role in reproductive functioning.
2. PACAP stimulates expression of each of the gonadotropin subunit genes while pronounced stimulation of follistatin blocks activin signaling to suppress FSHb-and GnRH-R expression.
3. PACAP stimulates its own promoter through a cAMP mechanism establishing a feed forward mechanism allowing for rapid changes in expression levels.
4. Pituitary PACAP and follistatin expression decline rapidly at or around the time of birth in rodents allowing GnRH-R and FSH-b expression to increase and initiate sexual maturation.

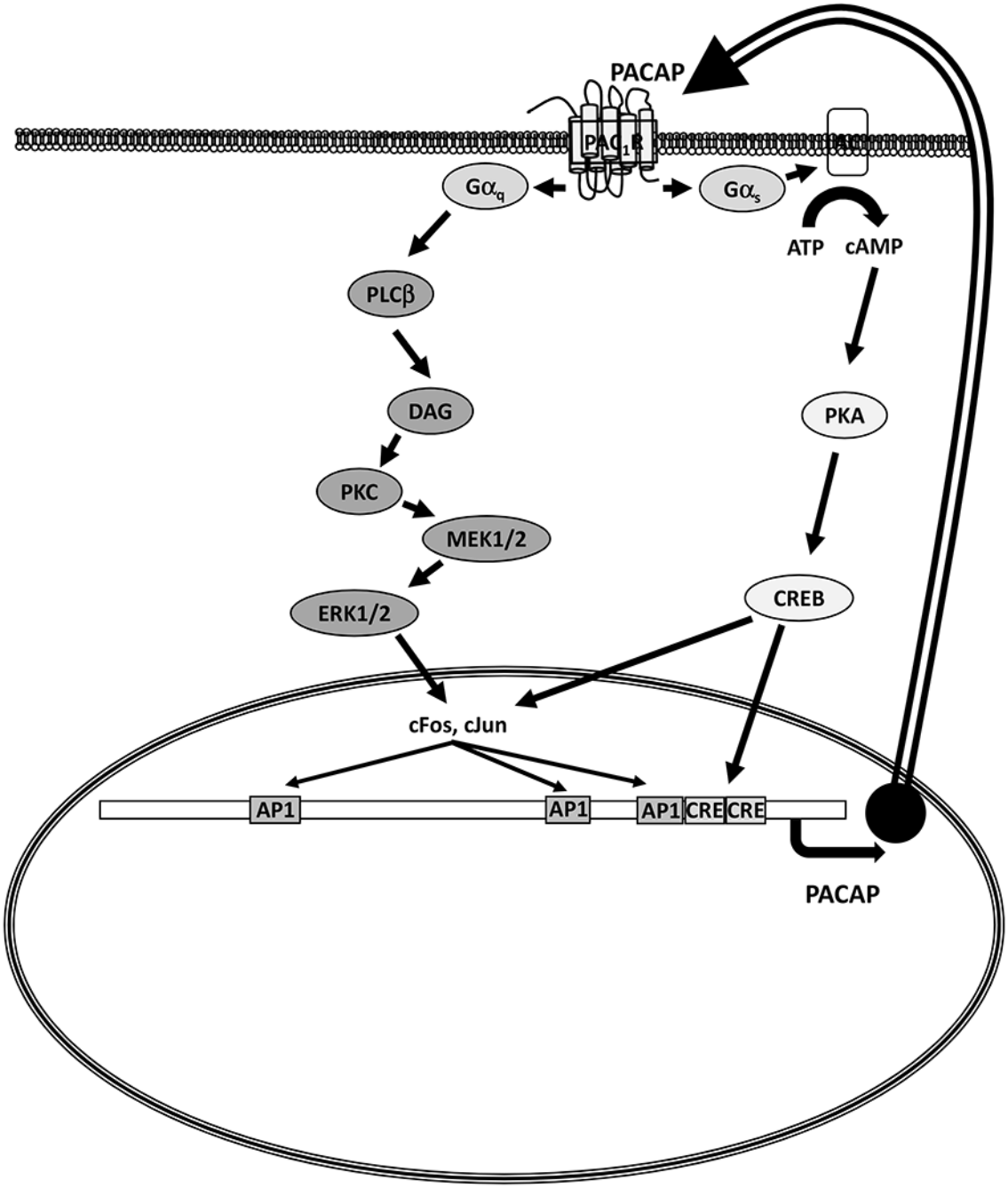

Figure 1.

PACAP receptor second messenger signaling in pituitary cells. The schema describes a paracrine/autocrine feed-forward mechanism to maintain a high level of PACAP in the fetal pituitary. PACAP and PAC1-R in gonadotrophs and folliculostellate cells likely contribute to the autocrine/paracrine system. The mouse PACAP promoter is depicted with three AP1 sites (−948, −448, −275) and two CRE sites (−205, −179).

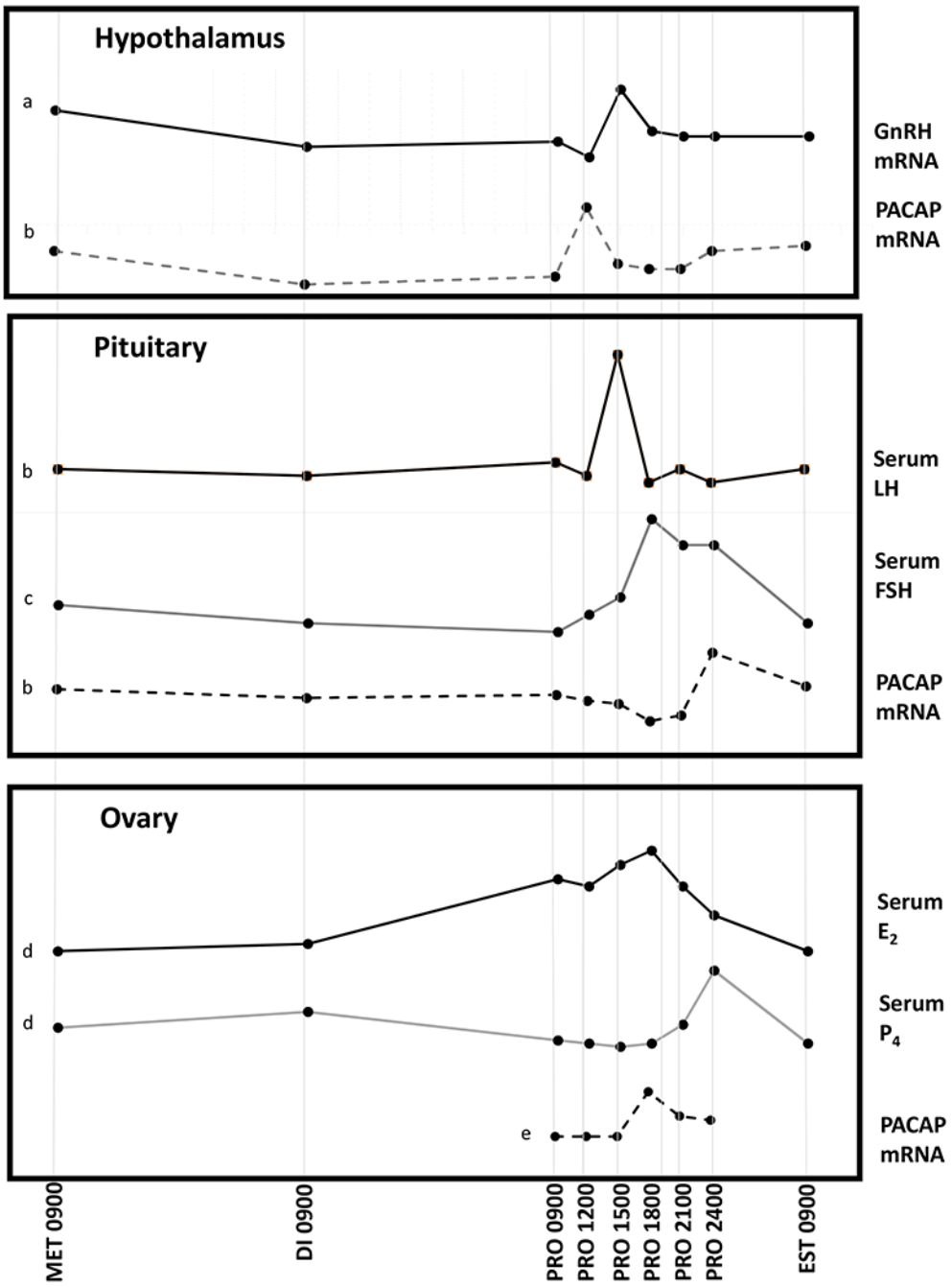

Figure 2.

Pattern of PACAP expression in the hypothalamus (PVN), anterior pituitary, and ovaries during the rat estrus cycle. PACAP expression increases in the PVN just prior to the rise in GnRH in the preoptic area which initiates the surge in serum LH and FSH. PACAP expression in the pituitary rises prior to a decline in FSH secretion. PACAP expression in the ovary rises in response to the proestrus surge in circulating gonadotropins, and soon thereafter serum progesterone increases. Abbreviations: *MET* Metestrus; *DI* Diestrus; *PRO*

Proestrus; *EST* Estrus; E_2 estradiol; P_4 progesterone. a: Schirman-Hildesheim, 2005 (150), b: Moore, 2003 (151) c: Ozawa, 2005 (152), d: Smith, 1975 (153), e: Ko, 1999 (154).

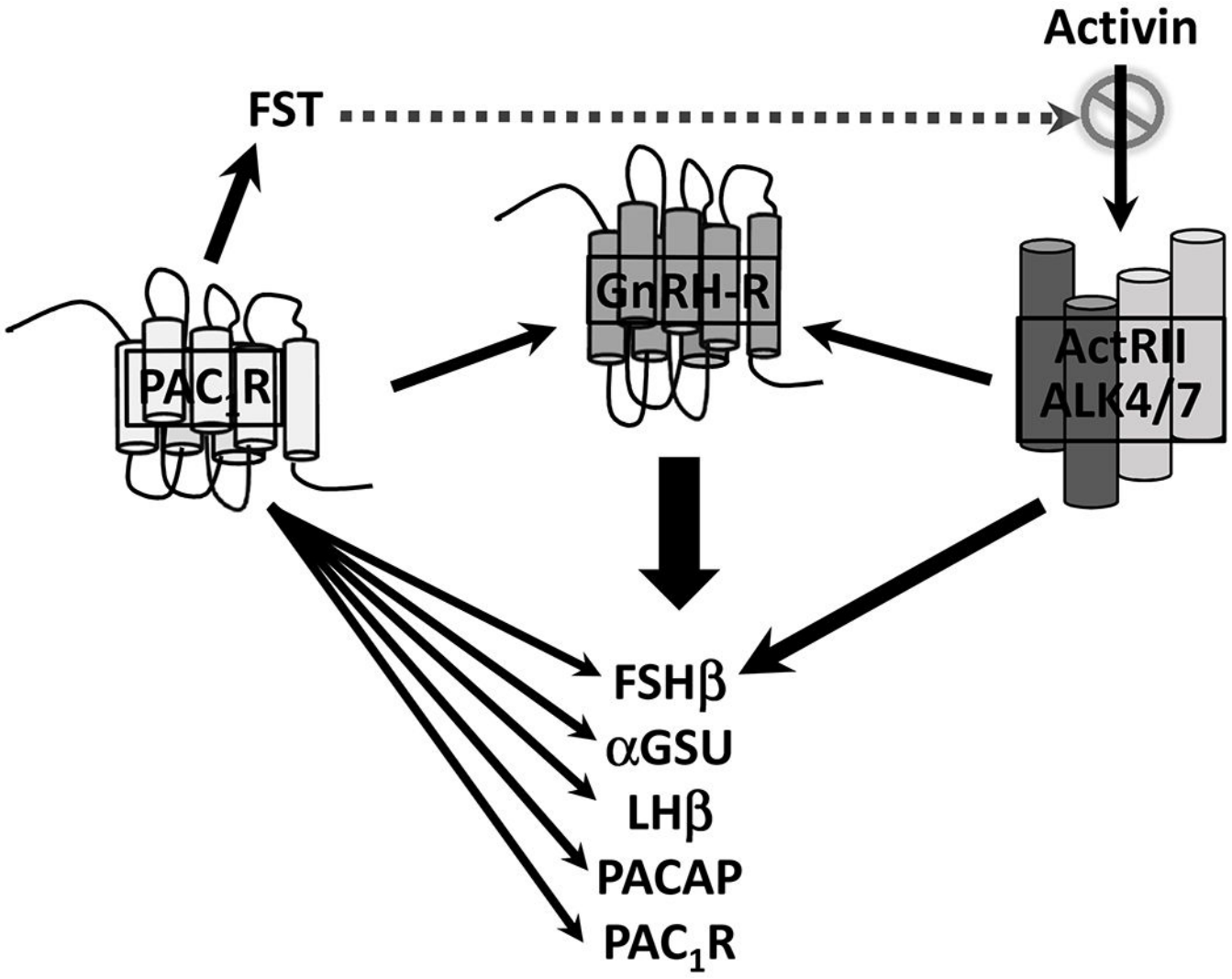

Figure 3.

A model for the mechanism by which PACAP differentially regulates gonadotropin expression. Experiments using primary pituitary cell cultures and/or immortalized gonadotroph cell lines have shown that PACAP directly stimulates transcription of each of the gonadotropin subunits, enhances GnRH-stimulated expression of α-subunit and LHb as well as expression of PACAP and the PAC_1 receptor. PACAP also stimulates expression of GnRH-R and enhances GnRH signaling. On the other hand, PACAP suppresses *Fshb* and *GnRH-R* expression indirectly by stimulating follistatin (FST) expression in gonadotrophs and folliculo-stellate cells, which neutralizes stimulation of *Fshb* and *GnRH-R* by activin.

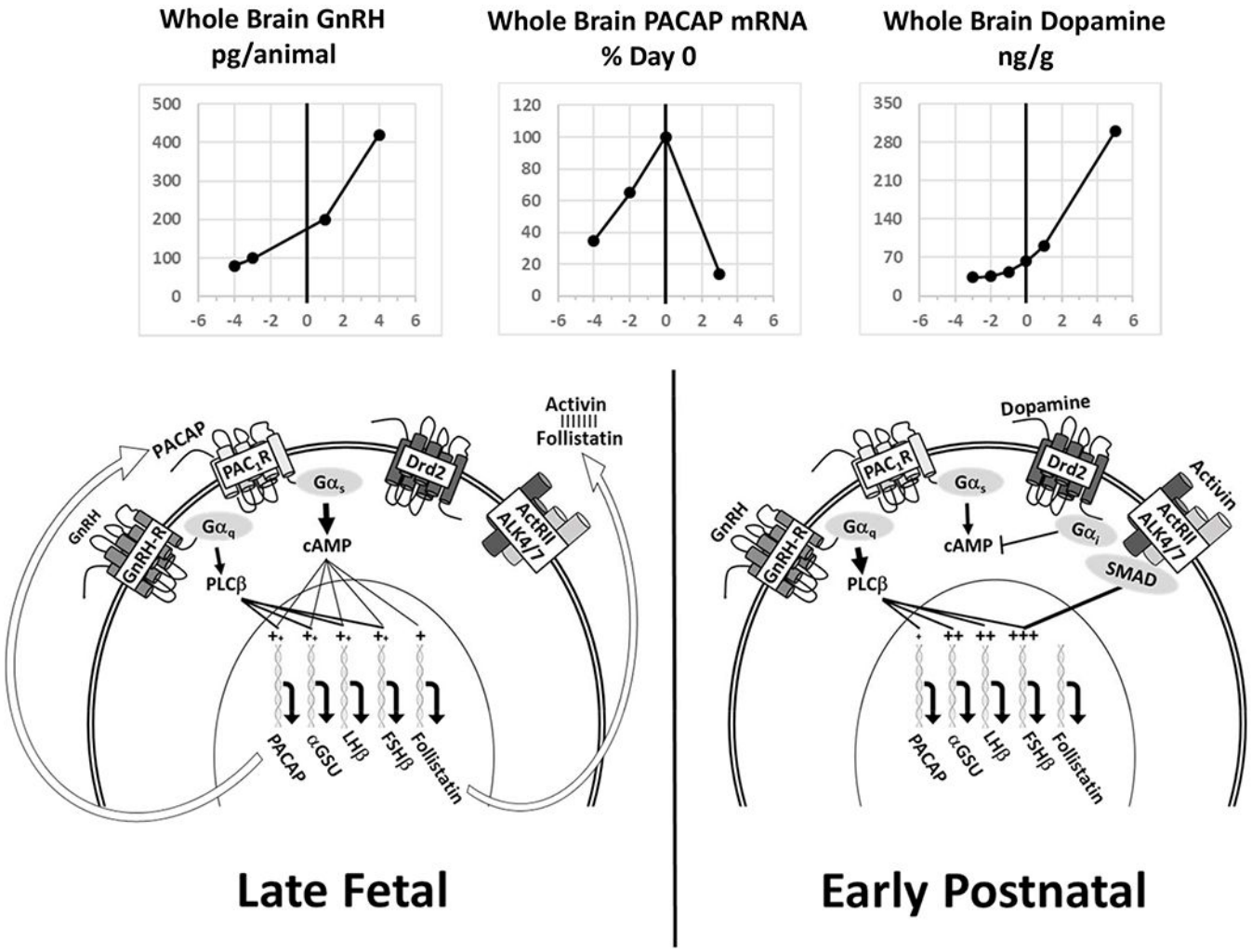

Figure 4.

Hypothalamic (PVN) and pituitary PACAP expression in the male rat during early maturation. PACAP expression in the PVN and pituitary decrease significantly between postnatal days 17 and 29. There is parallel decrease in pituitary follistatin (FST) expression and a reciprocal rise for the gonadotropin subunits (*LHb* and *Fshb*) and GnRH-R.

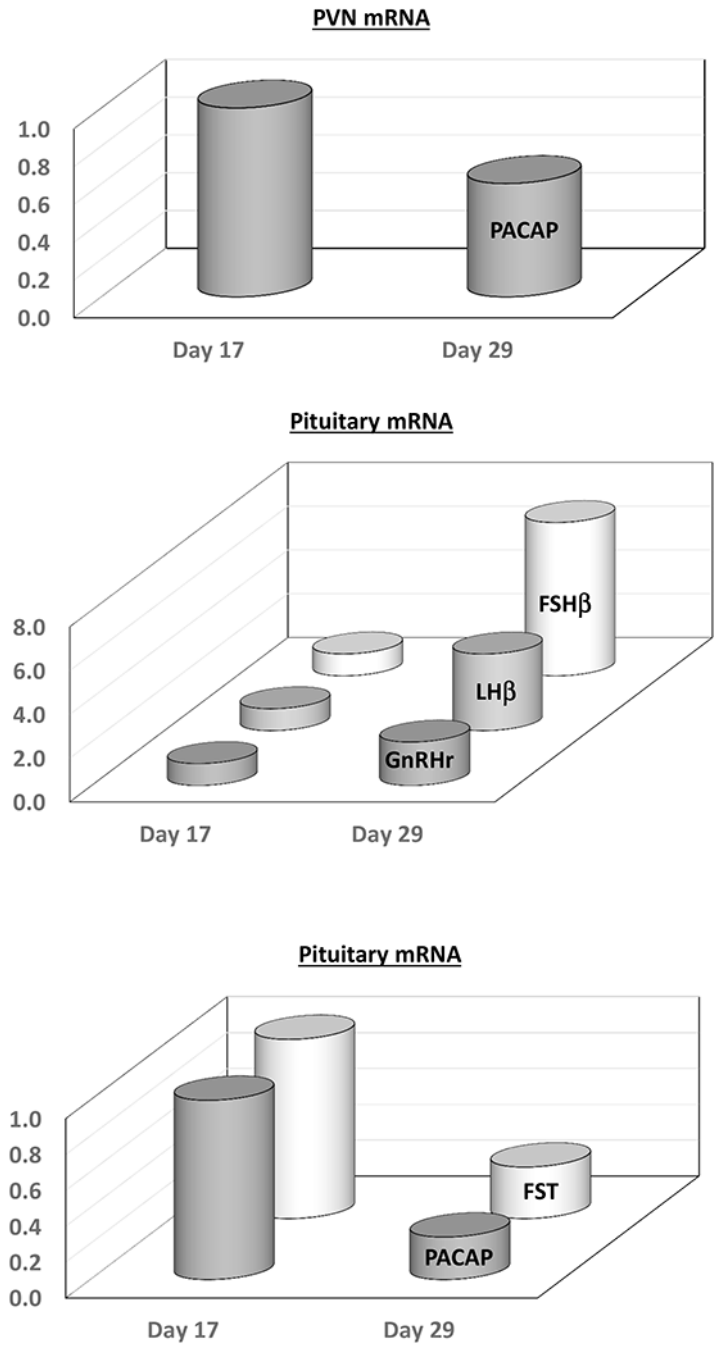

Figure 5.

Schematic representation of a model elucidating the possible role of pituitary PACAP expression in the ontogeny of sexual development in the perinatal period. In late fetal life, pituitary PACAP expression is very high and stimulates expression of αGSU and LHb. PACAP stimulation also produces high levels of follistatin that effectively block activin signaling to *GnRH-R* and *fshb*. As the hypothalamic dopaminergic system develops, increased dopamine exposure activates type 2 dopamine receptors (Drd2) and thereby $G\alpha_i$ which suppresses cAMP production and block the PACAP-stimulated cAMP feed-forward

mechanism. As PACAP signaling declines, follistatin production is reduced, and activin is freed to stimulate GnRH-R and FSH synthesis, among other genes. The top graphs depict the rep orted changes in brain levels of GnRH (155), PACAP (156) and dopamine (132).

Table 1.

Endocrine changes with PACAC genetic manipulation

Reproductive Function		
PAC1R-KO	Disrupted estrus cycle	Jamen, 2000 (38)
PACAP-KO	Testosterone deficient with LH insufficiency	Lacombe, 2006 (37)
	Delayed testicular aging	Lacombe, 2006 (37)
	Abnormal sperm shape and size	Brubel, 2012 (96)
	Sub-fertile females	Isaac, 2008 (36)
	Decreased progesterone and prolactin levels during pregnancy	Isaac, 2008 (36)
	Reduced uterine implantation rates	Shintani, 2002 (4)
Lpr+ PMV neurons	Delayed female sexual maturation	Ross, 2018 (43)
	Disruption of LH surge	Ross, 2018 (43)
	Low pup number per litter	Ross, 2018 (43)
αGSU-PACAP	Delayed male sexual maturation	Moore, 2012 (1)
	Lifelong suppressed gonadotropins	Moore, 2012 (1)
glucose homeostasis		
PAC1R-KO	Glucose intolerance	Winzell, 2007 (137) Persson, 2002(138)
	impaired insulin secretion	Winzell, 2007 (137)
	Decreased glucagon secretion	Winzell, 2007 (137) Persson, 2002(138)
	Less body wt & white adipose tissue Hypoinsulinemia	Tomimoto, 2008 (139)
	Increased insulin sensitivity	Tomimoto, 2008 (139)
PACAP-KO	Microvesicular fat accumulation in	Gray, 2001 (140)
	liver, skeletal muscle and heart increased triglycerides and cholesterol	Gray, 2001 (140)
	increased insulin and hypoglycemia	Gray, 2001 (140)
	impaired thermoregulation	Gray, 2002 (140)
	Susceptibility to insulin-induced hypoglycemia, and related death	Hamelink, 2002 (141)
β-cell overexpression	Increased insulin secretion	Yamamoto, 2003 (142)
	Improved insulin sensitivity in high fat diet-induced diabetes	Tomimoto, 2004 (143) Sakuri, 2012 (144)
Stress Response		
PAC1R-KO	Reduced contextual fear conditioning	Otto, 2001 (145)
	Impaired hippocampal long-term potentiation	Otto 2001 (146)
PACAP-KO	Lack of stress-induced CRH upregulation	Agarwal, 2005 (112)
	Disrupted stress-induced catecholamine release from splanchnic nerves.	Stroth, 2010 (147)
	Impaired stress-induced ACTH secretion	Stroth, 2010 (147)
	Reduced stress-induced corticosterone secretion	Stroth, 2010 (147)
	Impaired stress-induced adrenal steroidogenesis	Stroth, 2010 (147)
Feeding Behavior		
PAC1R-KO	Reduced POMC expression in the arcuate nucleus	Mounien, 2009 (148)

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Reproductive Function		
	Increased postprandial ghrelin	Vu, 2015 (40)
	Decreased postprandial GLP-1, insulin and leptin	Vu, 2015 (40)
	Loss of PACAP-induced reduction in food intake	Vu, 2015 (40)
PACAP-KO	Reduced carbohydrate intake	Nakata, 2004 (149)
	Impaired leptin-stimulated adipose tissue sympathetic nerve activity	Tanida. 2013 (41)
	reduced food intake and body weight	Nakata, 2004 (149)
	reduced body weight	Gray, 2001 (140)