Spring 2022 – Systems Biology of Reproduction
Discussion Outline – Hypothalamus-Pituitary Development & Function
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 31, 2022
Week 12

Hypothalamus-Pituitary Development & Function

Primary Papers:

1. Belchetz et al. (1978) Science 202:631
2. Ozaki et al. (2021) Develop Growth Differ 63:154-165
3. Bhattacharya et al. (2019) Mol Reprod Dev 86 :1505-30

Discussion

Student 30: Reference 1 above

- What unique endocrine parameter was identified in the hypothalamic regulation of pituitary function?
- What physiological advantage does this have?
- How does this information fit into the understanding of Brain-Pituitary-Gonadal axis?

Student 1: Reference 2 above

- What was the experimental design and objectives of the study?
- How did the in vivo development maintain the organoid?
- What insights into organogenesis of the hypothalamus-pituitary were obtained?

Student 2: Reference 3 above

- How did the hypothalamus and pituitary induce puberty?
- What was the hypothalamus-pituitary and testis hormone axis established?
- What molecular mechanism in the hypothalamus and pituitary were involved?

scouring is less pronounced because of the lack of fracturing; (iii) the narrowness of the river allows more of the debris to be swept away; and (iv) the limestone, although resistant, appears to shed less large-size debris.

With the evidence of a consistent structural influence, we offer this generalized model for the rapid-pool-tributary sequences along the Colorado. Large faults determine zones of bedrock weakness within the Grand Canyon. Structures that run perpendicular to the river provide an advantage for side canyon drainage. The side canyon tributaries, flowing within the brecciated zones, deliver material to the main river that is too large to be carried downstream. This material forms a channel constriction, accelerated flow, and a rapid. Part of the accelerated flow at the foot of the rapids is directed downward against the bed. These high velocities, coupled with the zone of brecciation associated with the faulted bedrock, lead to deep scour below the rapids, and thus the deep pools. The hydraulic processes (autogenic) that produce regularly spaced riffles (5) on most streams, therefore, may dominate a few sections of the Colorado River in the Grand Canyon, but along most of its course these processes appear to be superimposed on, or modified by, local external (exogenic) controls.

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27 February 1978; revised 24 May 1978

Hypophysial Responses to Continuous and Intermittent Delivery of Hypothalamic Gonadotropin-Releasing Hormone

Abstract. *In rhesus monkeys with hypothalamic lesions that abolish gonadotropic hormone release by the pituitary gland, the constant infusion of exogenous gonadotropin-releasing hormone (GnRH) fails to restore sustained gonadotropin secretion. In marked contrast, intermittent administration of the synthetic decapeptide once per hour, the physiological frequency of gonadotropin release in the monkey, reestablishes pituitary gonadotropin secretion. This phenomenon is attributable to the pattern of GnRH delivery rather than to the amounts of this hormone to which the cells of the pituitary are exposed. Moreover, the initiation of continuous GnRH administration in animals with lesions and in which gonadotropin secretion is reestablished by intermittent GnRH replacement can result in a "desensitization" or "down regulation" of the processes responsible for gonadotropin release.*

Lesions induced by radio-frequency current in the medial basal hypothalamus of rhesus monkeys (*1*) abolish the secretion of the gonadotropic hormones [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] by the pituitary gland, presumably by interfering with the release of the hypothalamic gonadotropin-releasing hormone, GnRH. Attempts to restore gonadotropin secretion in such animals by the continuous infusion of synthetic GnRH succeeded only in eliciting an evanescent release of LH and FSH despite the continued administration of the decapeptide (*1*). When, on the other hand, GnRH was administered once per hour (*2*), a rate equivalent to the physiological frequency of episodic LH release in ovariectomized monkeys (*3*), sustained increases in plasma LH and FSH concentrations were achieved for the duration of the replacement regimen (up to 7 weeks). The study described here was designed to determine whether the refractoriness of the pituitary to the continuous infusion of GnRH is attributable to the pattern of hypophysiotropic hormone stimulation per se or to the quantity of the decapeptide delivered to the pituitary.

Cardiac catheters were implanted in seven ovariectomized rhesus monkeys (4.2 to 6.8 kg of body weight) in which gonadotropin secretion had been abolished or severely curtailed by placement of radio-frequency lesions in the hypothalamus (*1*). By means of an infusion-withdrawal device that permits continuous access to the venous circulation without the animal being restrained, GnRH (*4*) was infused continuously by way of the cardiac catheter at rates of 0.001, 0.01, 0.1, and 1.0 μg per minute as described (*2*). Each infusion rate was maintained for 10 days (*5*). Blood samples were taken daily by way of the catheter, or by femoral venipuncture after the animal was sedated (30 to 40 mg of sodium thiamylal per animal, intravenously), and plasma concentrations of LH and FSH were determined by use of established radioimmunoassays (*6*). The pituitary response to GnRH administered at the rate of 1 μg per minute for 6 minutes once per hour was determined in similar fashion.

The mean circulating LH and FSH concentrations during the last 5 days of each continuous GnRH infusion, which reflected the steady-state response of the pituitary to this mode of hypophysiotropic stimulation (*7*), are shown in Fig. 1A. None of the continuous infusions of releasing hormone produced a sustained increment in plasma LH and FSH concentrations. In sharp contrast, however, long-term restoration of gonadotropin secretion was achieved in the same animals by the intermittent administration of GnRH (Fig. 1B). These observations lead to the conclusion that the failure of continuous GnRH infusion, regardless of infusion rate, to initiate sustained gonadotropin secretion in ovariectomized monkeys bearing hypothalamic lesions is the consequence of the pattern of GnRH administration rather than of the total mass of the decapeptide delivered to the gonadotrophs.

The effects on gonadotropin secretion of a shift in GnRH administration from the intermittent to the continuous mode, without a change in the infusion rate, were investigated in four similarly prepared monkeys in which gonadotropin secretion had been reestablished by pulsatile hypophysiotropic stimulation. The institution of continuous GnRH administration was followed by a brief increase in plasma LH and FSH lasting approximately 5 hours. Thereafter, however, circulating gonadotropin declined, reaching a nadir within 7 to 10 days where they remained for the duration of the continuous infusion period. This inhibition was reversed when pulsatile GnRH administration was reinstituted (Fig. 2).

These influences of pattern of hypophysiotropic stimulation may be related to the phenomenon of "desensitization" or "down regulation" (*8*), whereby pro-

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longed exposure to a high circulating concentration of hormone or drug results in a decrease in the response of the target tissue. Continuous infusions of GnRH, albeit of relatively short duration, have also been reported to result in the development of pituitary refractoriness in rats and sheep (*9*). The phenomenon of "down regulation," which has been described for insulin, LH, and catecholamines, may result, in part, from a reduction in available receptors for the agonist (*10*). A decline in the number of growth hormone receptors on lymphocytes, and of thyrotropin-releasing factor receptors on a clonal strain of pituitary cells has also been reported after long-term exposure to the homologous hormone (*11*). In relating the association between receptor loss and "down regulation" to the present findings, it is tempting to speculate that an intermittent supply of GnRH permits the regeneration of its receptors, whereas the continuous mode of hypophysiotropic stimulation does not. Whatever the underlying cellular mechanism responsible for our findings may be, it appears that the intermittent mode of GnRH stimulation is optimal in eliciting gonadotropin secretion, thereby underlining the physiologic significance of the pulsatile nature of endogenous GnRH release by the hypothalamus (*3*, *12*).

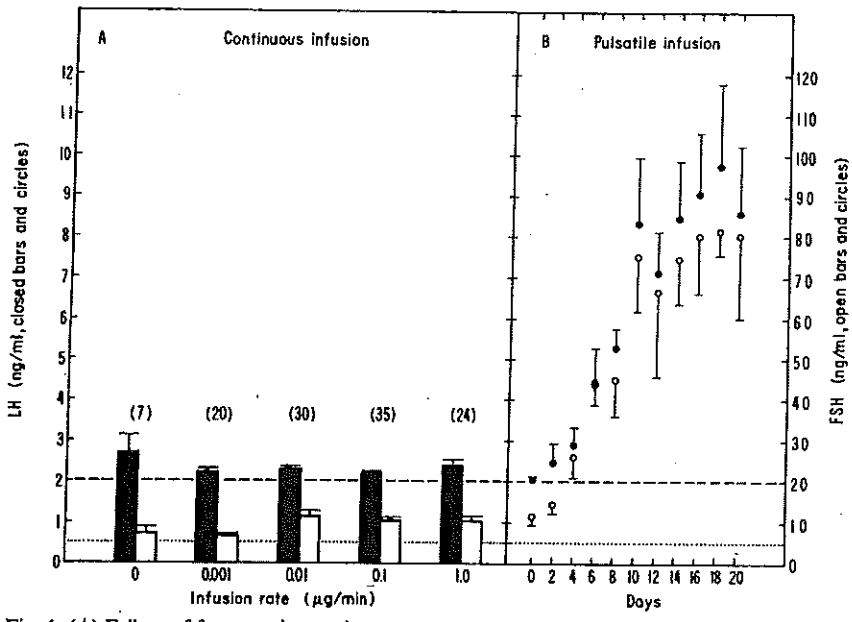

Fig. 1. (A) Failure of four continuous intravenous GnRH infusion rates to reestablish gonadotropin secretion in ovariectomized rhesus monkeys bearing hypothalamic lesions. Each bar represents the mean ± standard error (S.E.) of the number of observations in parentheses obtained during the last 5 days of the infusion period. Plasma gonadotropin concentrations during the control period were obtained just before the initiation of the GnRH infusions. (B) Effect of an intermittent GnRH infusion (1 μg/min for 6 minutes once per hour) on gonadotropin secretion in the same animals shown in (A). Each point is the mean ± S.E. of three to five observations. The horizontal dots and dashes show the sensitivity limits of the FSH and LH assays, respectively.

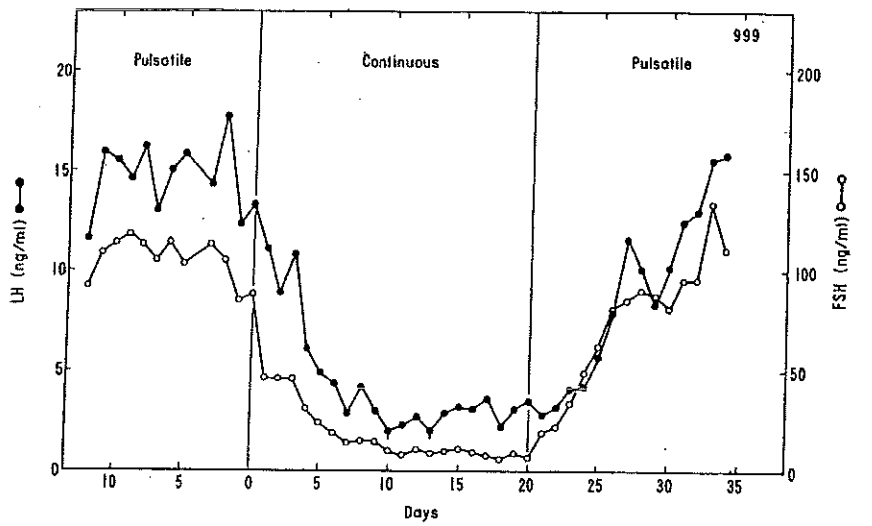

Fig. 2. Suppression of plasma LH and FSH concentrations after initiation, on day 0, of a continuous GnRH infusion (1 μg/min) in an ovariectomized rhesus monkey with a radio-frequency lesion in the hypothalamus; gonadotropin secretion had been reestablished by the intermittent (pulsatile) administration of the decapeptide (1 μg/min for 6 minutes once per hour). The inhibition of gonadotropin secretion was reversed after reinstitution of the intermittent mode of GnRH stimulation on day 20. The vertical lines beneath the LH data points on days 10 and 13 of the continuous infusion regimen indicate values below the sensitivity of the radioimmunoassay.

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4. Stock solutions of synthetic GnRH, provided by R. Guillemin (LRF, 14-200-500 and 14-136-06), Abbott Laboratories (lot-34-414AL), and the National Institutes of Health (lot 26-306AL), were prepared in 0.01*M* acetic acid in 0.9 percent NaCl and stored in small portions at −85°C. Prior to use, portions were thawed and diluted with sterile saline for infusion.
5. The GnRH was infused in both ascending (four animals) and descending (three animals) rate sequences with the same results.
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7. The transient discharge of gonadotropin described previously during the initiation of continuous GnRH administration (*1*) was also observed in this study at the highest GnRH infusion rate (1 μg/min). This sudden release of gonadotropin, which resulted in a marked increase in circulating LH and FSH concentrations 7 to 10 hours after initiation of continuous infusion followed by a decline to control levels within 2 days was only observed, however, in the three animals which received the highest GnRH infusion rate first.
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Slow Axonal Transport of Neurofilament Proteins: Impairment by β,β'-Iminodipropionitrile Administration

Abstract. *β,β'-Iminodipropionitrile (IDPN) administration prevented normal slow axonal transport of [^{35}S]methionine- or [^{3}H]leucine-labeled proteins in rat sciatic motor axons. Ultrastructural and electrophoretic studies showed that the neurofilament triplet proteins in particular were retained within the initial 5 millimeters of the axons, resulting in neurofilament-filled axonal swellings. Fast anterograde and retrograde axonal transport were not affected. The IDPN thus selectively impaired slow axonal transport. The neurofibrillary pathology in this model is the result of the defective slow transport of neurofilaments.*

The axon utilizes special systems of cytoplasmic motility to convey materials along its length. These axonal transport systems are generally distinguished, on the basis of direction and rate of movement, into fast, slow, and intermediate anterograde transport (conveying materials away from the cell body) and retrograde transport (carrying materials toward the cell body) (*1*). Neither the mechanisms of transport nor the relationships between these systems are fully defined. A unitary mechanism for all types of transport has been proposed in which the differences in rate are related to the proportion of time that various transported materials are associated with the transport mechanism (*2*). Alternatively, a mechanism for slow transport distinct from that for bidirectional rapid transport has been suggested (*3*).

Identification of selective effects of pharmacologic agents on the various transport systems provides one approach to further studies of the mechanisms and the interrelationships of the axonal transport systems. In this study, we have examined the effects on axonal transport of β,β'-iminodipropionitrile (IDPN). Previous studies (*4*, *5*) have shown that IDPN administration produces large neurofilament-filled swellings in the most proximal portion of the axon. Since neurofilaments are known to be carried by slow transport (*1*, *3*), this pathology suggested that IDPN might have an effect on slow transport. Our results show that IDPN selectively impairs slow axonal transport, without direct effects on fast or retrograde transport. This model is of special interest, since it represents the first disorder in which the pathogenesis of neurofibrillary pathology can be reconstructed.

Slow axonal transport was studied by injecting [^{3}H]leucine or [^{35}S]methionine into the lumbar ventral horns of Sprague-Dawley or Wistar rats (*6*). The animals were returned to their cages, and 1 to 8 weeks later they were killed. The sciatic nerves were rapidly removed and divided into 5-mm segments. These nerve segments were each homogenized manually in a mixture of sodium dodecyl sulfate, urea, and β-mercaptoethanol (*3*) and heated to 100°C for 4 minutes. After centrifugation, only a minute residuum remained undissolved, and essentially all the radioactivity in the segments was solubilized (*3*). To construct curves of the distribution of radioactivity along the nerve, an aliquot of each sample was counted by liquid scintillation techniques, and counts per minute for each segment were plotted against the position of the segment along the nerve (*3*, *6*).

In addition, to determine the pattern of migration of individual slowly transported proteins, portions of the samples were subjected to electrophoresis on polyacrylamide slab gels (*7*); the gels were then impregnated with 2,5-phenyloxazole and dried, and fluorograms were prepared by exposure of Kodak type RP x-ray film to the gels for 2 weeks to 4 months (*7*). [The x-ray film was preexposed to a measured light flash (*8*).] The resulting fluorograms revealed the relative amounts of individual labeled proteins in each segment of nerve.

The IDPN (Eastman Kodak, Rochester, N.Y.) was administered in one of two ways: by intraperitoneal injection of 1 or 2 g/kg, or by sustained exposure to 0.05 percent IDPN in the drinking water only (*9*). Because of the different means of administration, transport studies were performed on animals ranging from 3 to 12 months of age. Age-matched controls, purchased at the same time as experimental animals, were used in all studies.

In 14 normal animals the curves of slow transport were similar to those previously reported (*1*, *3*), with the major slow component peak moving down the nerve at 1.5 to 2 mm/day (in 200-g animals) (Fig. 1a). The fluorograms from these control animals (Fig. 2a) showed the three major groups of labeled proteins described by Hoffman and Lasek (*3*): actin (molecular weight, 46,000); proteins presumptively identified as tubulin (molecular weights, 53,000 and 57,000); and the neurofilament triplet proteins with estimated molecular weights of 68,000, 145,000, and 200,000 (*3*, *10*). In each of 11 normal rats, the rate of actin and tubulin migration ranged from 0.5 to 5 mm/day, with the density of label greatest in segments corresponding to rates of 1.0 to 3.5 mm/day (Fig. 2a). The neurofilament triplet proteins moved together at a more restricted range of rates of 1 to 2.5 mm/day, coinciding with the major slow component peak (Fig. 2a).

Similar studies were performed with rats injected with IDPN. In these studies IDPN was given either 1 to 2 days before or 1 to 2 days after microinjection of the labeled precursor into the spinal cord. Groups of animals were then killed 7, 14, or 21 days after labeling. At all times after labeling, the major slow transport peak failed to migrate beyond the initial 5 to 10 mm of the ventral roots (Fig. 1b). Gel fluorography (21 days after labeling) showed that movement of all the major slow component proteins was abnormal, with the neurofilament triplet proteins being the most strikingly affected (Fig. 2b). Most of the labeled neurofilament triplet proteins were retained in the initial 5 to 10 mm of the roots; only a small proportion were transported beyond this level. Following injection of IDPN, tubu-

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REVIEW ARTICLE

Development, Growth & Differentiation WILEY

Hypothalamic–pituitary organoid generation through the recapitulation of organogenesis

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Communicating Editor: Makoto Ikeya and Mototsugu Eiraku

Abstract

This paper overviews the development and differentiation of the hypothalamus and pituitary gland from embryonic stem (ES) and induced pluripotent stem (iPS) cells. It is important to replicate the developmental process in vivo to create specific cells/organoids from ES/iPS cells. We also introduce the latest findings and discuss future issues for clinical application. Neuroectodermal progenitors are induced from pluripotent stem cells by strictly removing exogenous patterning factors during the early differentiation period. The induced progenitors differentiate into rostral hypothalamic neurons, in particular magnocellular vasopressin^{+} neurons. In three-dimensional cultures, ES/iPS cells differentiate into hypothalamic neuroectoderm and nonneural head ectoderm adjacently. Rathke's pouch-like structures self-organize at the interface between the two layers and generate various endocrine cells, including corticotrophs and somatotrophs. Our next objective is to sophisticate our stepwise methodology to establish a novel transplantation treatment for hypopituitarism and apply it to developmental disease models.

KEYWORDS

ES/iPS cells, hypothalamus, pituitary, self-organization, three-dimensional culture

1 | INTRODUCTION

The hypothalamic–pituitary system is essential for maintaining physiological homeostasis by controlling systemic hormones, but its regeneration remains mostly unclear. Therefore, attention is focused on the regeneration of the hypothalamus and pituitary from stem cells.

Somatic stem cells have drawn attention for their potential for use in regenerative medicine. In the adenohypophysis, the existence of somatic stem cells was reported (Chen et al., 2005), and their functions were discussed at three phases: during early postnatal pituitary maturation (Chen et al., 2009; Fauquier et al., 2001; Gremeaux et al., 2012; Kikuchi et al., 2007; Mollard et al., 2012), after pituitary damage (Fu et al., 2012; Langlais et al., 2013; Luque et al., 2011), and in pituitary tumorigenesis (Andoniadou et al., 2012; Gaston-Massuet et al., 2011; Garcia-Lavandeira et al., 2012; Li, Collado, et al., 2012).

In addition to somatic stem cells, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are also attracting attention. These pluripotent stem cells have self-renewal properties and pluripotent differentiation abilities that make them attractive cell sources for differentiated tissues in clinical applications. Differentiated tissues from ES/iPS cells are expected for regenerative medicine and as a model of development or disease. In this review, we mainly describe the differentiation of hypothalamic and pituitary tissues from mouse and human ES/iPS cells and their applications.

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2 | THREE-DIMENSIONAL DIFFERENTIATION CULTURE METHOD FROM ES CELL TO CENTRAL NERVE TISSUE

In the first step, mouse ES cells are used rather than human ES cells. The development duration of fetal mouse and humans is about 20 days and 300 days, respectively. Because of the short fetal development duration, mouse ES cells have a short developmental period. Mouse ES cells differentiate into hypothalamus and pituitary in ~30 days (Suga et al., 2011; Wataya et al., 2008), whereas human ES cells differentiate into these tissues in ~90 days to150 days (Ogawa et al., 2018; Ozone et al., 2016). Mouse ES cells are therefore suitable for establishing novel differentiation methods with numerous trial-and-error processes.

Organ formation occurs during embryogenesis with local interactions between different tissues. An organogenesis model using a three-dimensional culture for ES cells was called "serum-free culture of embryoid body-like aggregates with quick re-aggregation (SFEBq)" (Eiraku et al., 2008). The most critical process in this method is to maintain undifferentiation of ES cells. These ES cells under maintenance are dissociated to single cells, and the cells are quickly aggregated in differentiation medium using low-cell adhesion 96-well plates.

This culture method exhibits self-organization (Sasai et al., 2012) and spontaneous formation of highly ordered structures or patterns and is suitable for the induction of various derivatives. The retinal differentiation method from human ES cells (Nakano et al., 2012) is based on that from mouse ES cells (Eiraku et al., 2011). Although there are differences between these two differentiation methods, they are generally similar. The essential principles of mouse ES cells can apply to human ES cells, which shows that mouse ES cells provide the pioneering work for a human model.

On the basis of developmental information, several other neural tissues were induced by SFEBq method under a medium suitable for each tissue: cerebral cortex (Danjo et al., 2011; Eiraku et al., 2008; Kadoshima et al., 2013), cerebellum (Muguruma et al., 2010), hippocampus (Sakaguchi et al., 2015), thalamus (Shiraishi et al., 2017), and spinal cord (Ogura et al., 2018).

3 | NEEDS FOR HYPOTHALAMUS REGENERATIVE MEDICINE

Diabetes insipidus is a disease characterized by the excretion of an abnormally large amount of diluted urine, resulting in polyuria and polydipsia (Fujiwara & Bichet, 2005). Central diabetes insipidus (CDI) is due to a deficiency of the antidiuretic nonapeptide hormone arginine vasopressin (AVP) in the blood. Many somatic and central disorders cause CDI, including central nervous system tumors, metastases, inflammation, neurosurgical interventions, and congenital disorders.

Desmopressin (DDAVP) is an AVP analog used in the treatment of CDI. A deficiency or excess of DDAVP can lead to life-threatening hypernatremia or hyponatremia. Some patients with CDI also exhibit loss of thirst (adipsia) due to dysfunction of hypothalamic osmoreceptors, and these patients dehydrate easily unless they are treated properly (Di Iorgi et al., 2012; Fenske & Allolio, 2012). Patients with adipsic CDI cannot feel thirsty, thus failing to consume adequate amounts of water, even with severe hypernatremia and dehydration. Although most patients with adipsic CDI determine their daily water intake based on their body weight measured every day and adjust their DDAVP dose, their risk of death is still higher than those without adipsia (Arima et al., 2014). When regenerated AVP neurons can be transplanted, they are expected to produce hormones more naturally than DDAVP administration by the patients themselves. Regenerative medicine of the hypothalamus, including AVP neurons, can overcome these limitations of current DDAVP therapy.

3.1 | Differentiation of hypothalamic neurons from mouse ES cells

Hypothalamic neurons including AVP^+ neurons are differentiated from mouse ES cells with SFEBq cultures (Wataya et al., 2008). Cultured in a growth-factor-free, chemically defined medium (gfCDM), the ES cell aggregates differentiate efficiently to hypothalamic neurons. It is suggested that the hypothalamus is the default-like region for the SFEBq method. When removing strictly exogenous growth factors during early differential steps, the ES cells generated rostral hypothalamic-like progenitors ($Rax^+/Six3^+/Vax1^+$) (Figure 1). It is important to minimize exogenous growth factors; insulin, widely used in cell culture, strongly inhibited differentiation via the Akt-dependent pathway. Rostral–ventral hypothalamic precursors ($Rax^+/Pax6^-/Nkx2.1^+$) were induced by sonic hedgehog (SHH) action. SHH is actively expressed in the ventral region of the embryonic hypothalamus (Markakis, 2002). Without SHH treatment, rostral–dorsal hypothalamic progenitors ($Rax^+/Pax6^+/Nkx2.1^-$) were induced (Figure 1).

The $Pax6^+$ progenitors generated $Otp^+/Brn2^+$ neuronal precursors (rostral–dorsal hypothalamic neurons). The neuronal precursors induced magnocellular AVP neurons releasing AVP upon KCl stimulation. In the presence of exogenous SHH, Rax^+ hypothalamic progenitor cells differentiated into $SF1^+/VGLUT2^+$ ventral hypothalamic neurons, $TH^+/$

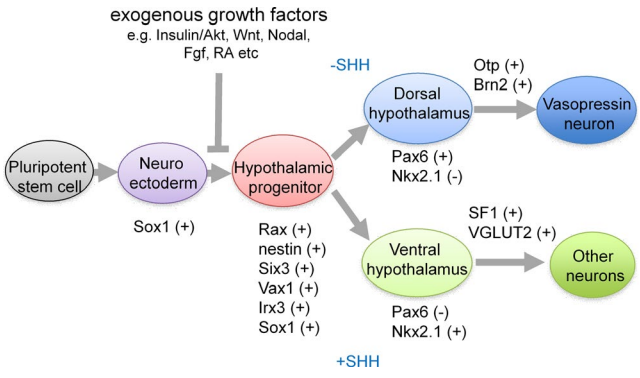


FIGURE 1 Schema of hypothalamic differentiation

Nkx2.1$^+$ dopaminergic neurons, and AGRP$^+$/NPY$^+$ neurons, which are found in the arcuate nucleus of the hypothalamus (Figure 1).

Therefore, when exogenous growth factors were minimized, mouse ES cells differentiated into rostral hypothalamic progenitors and subsequently characteristic hypothalamic neuroendocrine tissue in a stepwise fashion as observed in vivo. This showed that minimizing exogenous patterning signal plays a key role in rostral hypothalamic specification, although adding inductive signals was one of the methods for organogenesis.

We improved the differentiating methods of hypothalamic AVP neurons with mouse iPS cells (Mitsumoto et al., 2019). Three-dimensional floating culture was also effective for hypothalamic differentiation from mouse iPS cells. We integrated fibroblast growth factor (FGF)8b treatment to increase the numbers of Otp$^+$ precursors and subsequently AVP$^+$ neurons, as FGF8 was reported to be necessary to increase Otp expression (Tsai et al., 2011) and FGF8-hypomorphic mice showed an AVP reduction (McCabe et al., 2011).

3.2 | Differentiation of hypothalamic neurons from human pluripotent stem cells

Both Wang et al. and Merkle et al. established methods for differentiating human hypothalamic neurons in two-dimensional culture. In human ES and iPS cells, the combination of early activation of SHH signaling followed by Notch inhibition efficiently induced ventral hypothalamic Nkx2.1$^+$ precursors (Wang et al., 2015). Subsequently, the application of a Notch inhibitor and brain-derived neurotrophic factors induced arcuate nucleus hypothalamic neurons that express hypothalamic neuron markers proopiomelanocortin (POMC), NPY, AGRP, somatostatin, and dopamine. Merkle et al. differentiated human pluripotent stem cells into neuroectoderm by inhibiting bone morphogenetic protein (BMP), TGFβ, NODAL, and activin signal, and induced ventral neural characteristics using Wnt inhibitors and SHH signaling (Merkle et al., 2015).

These reports by Wang et al. and Merkle et al. did not demonstrate the secretion of AVP. We recently achieved improved efficiency of hypothalamic neuron induction from human ES cells using three-dimensional culture (Ogawa et al., 2018). When adding a small amount of knockout serum replacement into gfCDM, human ESCs aggregated successfully. With the combination of both SAG and BMP4, these aggregates differentiated into Rax$^+$ hypothalamic progenitors. Combined with Akt inhibitor, these progenitors induced AVP$^+$ neurons and their numbers increased. AVP was secreted spontaneously into the culture medium, and its concentration increased when stimulated with KCl.

4 | GENERATION OF HYPOTHALAMIC NEURAL STEM/PROGENITOR CELLS

Some reports say that neural stem/progenitor cells exist in the floor and lateral walls of the hypothalamic third ventricle (Haan et al., 2013; Lee et al., 2012; Lee & Blackshaw, 2012; Li, Tang et al., 2012; Robins et al., 2013; Zhang et al., 2017). These cells are called tanycytes, which play essential roles in adult neurogenesis in the hypothalamus. Tanycytes are said to regulate energy balance, feeding, and weight. Disruption of tanycytes can lead to impaired neuronal differentiation, which can result in obesity and prediabetes. These hypothalamic neural stem/progenitor cells are abundant at the neonatal stage, but they gradually diminish with increasing age. Rax widely expresses in hypothalamic progenitors in the early development period. By the mature stage of hypothalamic development, Rax expression is limited in tanycytes and absent from all other hypothalamic regions. Therefore, tanycytes are considered a part of the adult hypothalamus expressing Rax.

On the basis of this idea, we isolated Rax$^+$ cells from mouse ES cell aggregates in vitro at the mature phase of hypothalamic differentiation. These cells self-renewed and formed neurospheres. All three neuronal lineages (neurons, astrocytes, and oligodendrocytes) also differentiated from these Rax$^+$ neurospheres. Thus, Rax$^+$ residual cells in the late stage of hypothalamic differentiation culture were multipotent neural stem/progenitor cells (Kano et al., 2019). These tanycyte-like neural stem/progenitor cells from pluripotent stem cells could represent a new treatment approach for the dysfunction of hypothalamic energy regulation or homeostatic mechanism caused by disease and aging.

5 | NEEDS FOR ADENOHYPOPHYSIS REGENERATIVE MEDICINE

Hypopituitarism is one of the most severe pituitary dysfunctions and can cause life-threatening complications such as adrenal crisis due to glucocorticoid hormone insufficiency. Hypopituitarism is divided into congenital disorders and acquired diseases, and postoperative hypopituitarism is known as one of the most frequent acquired disorders. As hormone-secreting cells rarely improve, patients need to supplement their deficient hormones for life.

Hormone replacement therapy is currently the only effective treatment, but it has limitations. For instance, exogenous hormone replacement cannot mimic the drastic physiologic changes in the secretion of endogenous hormones in response to various physical and psychological stressors. One clinical research study showed that even well-educated patients with chronic adrenal insufficiency had a high incidence of adrenal crisis and that approximately 6% of patients had deaths associated with adrenal crisis (Hahner et al., 2015). Therefore, human pluripotent stem cell-derived pituitary cells with the ability to respond to both releasing and feedback signals can contribute to better treatment alternatives to current hormone replacement therapy.

5.1 | Differentiation of pituitary placode based on in vivo development

The adenohypophysis, also known as the anterior pituitary gland, consists of several types of endocrine cells secreting hormones such

as adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The posterior pituitary gland contains axons and terminals of hypothalamic neurons such as vasopressin and oxytocin neurons.

The development process of the adenohypophysis is complex. During the early stage, the adenohypophysis anlage arises as a placode from the nonneural ectoderm adjacent to the anterior neural plate (Figure 2a). The adenohypophyseal placode and the hypothalamic anlage interact with each other. The thickened placode invaginates and then separates from the oral ectoderm to develop a hollowed vesicle called "Rathke's pouch" (Zhu et al., 2007) (Figure 2b).

There are intensive studies about the molecular mechanism of this local inductive interaction during the initial phase of pituitary development. SHH, BMP, and FGF signals appear to play important roles (Brinkmeier et al., 2007; Takuma et al., 1998). Lhx3^{+} pituitary progenitors induce several hormone-type-specific lineages (Figure 2c). A lineage that expresses transcription factor Tbx19 differentiates into the ACTH-producing corticotroph. Another lineage absent of Tbx19 expression generates Pit1^{+} intermediate precursors, which subsequently differentiates into GH-, PRL-, and TSH-producing cell lineages. The third lineage involved in GATA2 differentiates LH- and FSH-producing cells.

Suga et al. (2011) established a differentiation method of the anterior pituitary from mouse ES cells, using interactions between the hypothalamic progenitors and oral-ectoderm-formed Rathke's pouch and pituitary anlage (Zhu et al., 2007). In this method, these two tissues within one aggregate were coinduced to recapitulate embryonic pituitary developmental processes.

Wataya et al. (2008) reported hypothalamic differentiation from mouse ES cells. Cultured with the SFEBq method in gfCDM, mouse ES cells differentiated into hypothalamic cells. Based on this report, some technical changes were made to slightly shift the positional information and to coinduce oral ectoderm and hypothalamic progenitors (Suga et al., 2011). In mouse embryo, the oral ectoderm developed from the rostral and midline region adjacent to the hypothalamic region (Figure 2a). The signal of rostral and midline shift was theoretically appropriate for mouse ES cell culture.

Suga et al. researched many culture conditions to identify two conditions that efficiently induced oral ectoderm and hypothalamic progenitors. Firstly, adding BMP4 was important. Treatment with 0.5 μM BMP4 induced oral ectodermal differentiation but strongly inhibited hypothalamic neuron differentiation. Secondly,

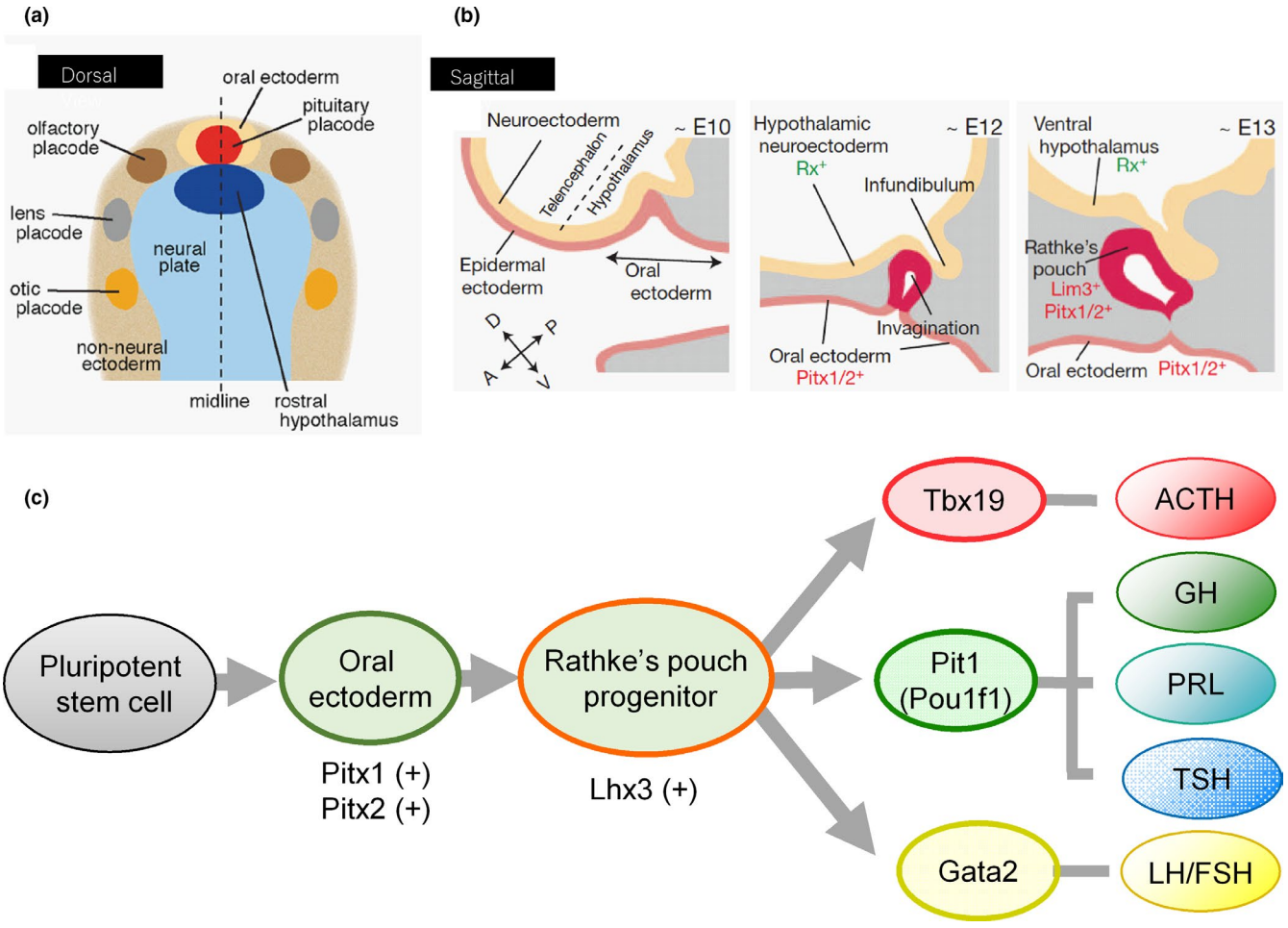


FIGURE 2 Pituitary development. (a, b) Dorsal and sagittal view of pituitary embryogenesis. (c) Schema of pituitary differentiation

high-density cell aggregation (10,000 cells per aggregate instead of 3,000 in SFEBq culture) was also important. This condition is called large cell aggregation (LCA) (Figure 3a). In the LCA culture, both the oral ectoderm (Pitx1/2$^+$) and hypothalamic progenitors (Rax$^+$) were coinduced within one aggregate (Figure 3b). When cells derived from the three germ layers of the embryo were aggregated in various combinations, it became clear that the reaggregated cells were spatially separated depending on the cell type. (Townes & Holtfreter, 1955). Our two-layer formation is consistent with this principle.

Treatment with a BMP4 antagonist inhibited the differentiation into oral ectoderm. In the LCA aggregates, quantitative polymerase chain reaction analyses showed that internal BMP4 expression was significantly higher.

In embryo, Rathke's pouch develops at the midline of the head ectoderm. SHH expresses in the oral ectoderm and ventral diencephalon but not in the invaginating Rathke's pouch (Wang et al., 2010; Zhu et al., 2007). Rathke's pouch appears to receive SHH signals from neighboring tissues in vivo. SHH signals provide positional information to adjust toward the midline (Zhu et al., 2007).

Therefore, based on developmental information, Suga et al. added a strong SHH signal, that is, a smoothened agonist (SAG), to the differentiation medium in vitro, which resulted in the formation of multiple oval structures in the SAG-treated LCA aggregates (Figure 3c). These structures were located between the oral ectoderm and hypothalamic progenitors and expressed Lhx3, which suggested they had similar characteristics to Rathke's pouch. These Lhx3$^+$ cells produced thick epithelium on the surface of aggregates, then invaginated and finally formed hollowed vesicles. The vesicle's major axis was about 200 μm long, similar to the embryonic Rathke's pouch. Both in vitro and in vivo, the size of Rathke's pouch seems to be fixed by a specific order.

Lhx3$^+$ vesicles were not formed from isolated oral ectoderm alone or hypothalamic tissues alone. Lhx3 expression recovered only when the two isolated parts were reassembled. The interactions between oral ectoderm and hypothalamic progenitors appeared to be essential for in vitro induction of Rathke's pouch. In vitro, several pouches were often formed in a single aggregate, whereas in vivo there was usually only one pouch in the embryo.

5.2 | Differentiation from Lhx3$^+$ progenitors into hormone-producing cells

When Lhx3 is knocked down in mice, subsequent differentiation into hormone-producing cells was inhibited (Sheng et al., 1996). Lhx3$^+$ progenitors are involved in several lineages, including

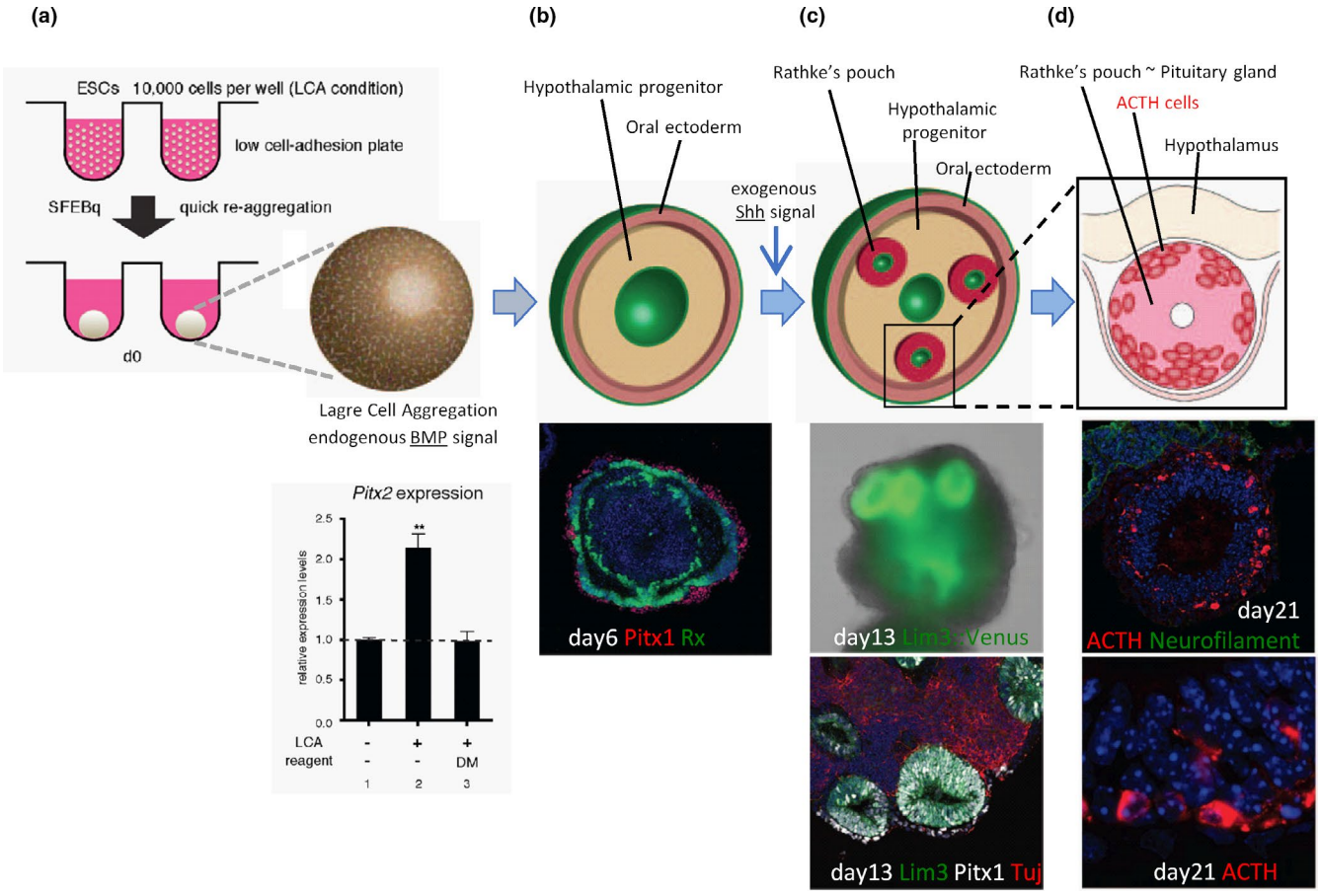


FIGURE 3 Differentiation from mouse ES cells into anterior pituitary. (a) Schema of SFEBq. (b) Two-layer formation in LCA aggregates. (c) Self-formation of Rathke's pouches. (d) Subsequent generation of ACTH$^+$ cells

corticotroph, somatotroph, lactotroph, thyrotroph, gonadotroph, and melanotroph (Davis et al., 2011).

The corticotroph lineage, which produces ACTH, expresses the transcription factor Tbx19 before ACTH expression. Notch signaling inhibits Tbx19 expression (Kita et al., 2007; Lamolet et al., 2001; Zhu et al., 2006). We examined the effect of DAPT as a Notch inhibitor. DAPT treatment increased Tbx19 expression in $Lhx3^+$ aggregates. Subsequently, a significant number of $ACTH^+$ cells were developed in the $Tbx19^+$ lesion (Figure 3d).

Canonical Wnt signaling promotes Pit1 expression (DiMattia et al., 1997; Olson et al., 2006; Sornson et al., 1996). Consistent with this finding, the Wnt agonist BIO treatment increased Pit1 expression, resulting in GH^+ and PRL^+ cell differentiation in our experiment. In other conditions, the induction into LH^+, FSH^+, and TSH^+ cells was achieved successfully.

These findings suggest the ability of mouse ES cell-derived $Lhx3^+$ pituitary progenitors to develop multiple endocrine lineages in vitro (Suga et al., 2011).

5.3 | Functionality of induced $ACTH^+$ cells

Endocrine cells are regulated by positive and negative feedback of the exogenous stimulus. When differentiated aggregates in vitro were stimulated by corticotropin-releasing hormone (CRH, upstream stimulator of ACTH cells), ACTH secretion at levels as high as in mouse peripheral blood was observed (Figure 4a). The downstream glucocorticoid hormone negatively controls ACTH secretion from the pituitary gland in vivo. Consistent with this, glucocorticoid pretreatment suppressed in vitro ACTH secretion (Figure 4b). Similar to in vivo, mouse ES cell-derived $ACTH^+$ cells had both positive and negative feedback systems. Maintaining homeostasis, they were indispensable for regulation of hormonal systems by the surrounding stimulus.

5.4 | Rescue for hypopituitary animals by transplantation

Finally, the effect of the induced $ACTH^+$ cell transplantation was evaluated. Because of technical difficulties, we chose ectopic transplantation into the kidney subcapsule (Figure 4c) rather than orthotopic transplantation into the sella turcica. We used hypophysectomized severe combined immunodeficient (SCID) mice. This type of pituitary resection is known to be lethal within weeks in mice, mainly because of insufficiency of adrenocortical functions caused by the lack of ACTH. After the induced $ACTH^+$ cell transplantation into hypopituitary model mice, the basal levels of ACTH in the blood even without CRH loading were significantly higher. The level of ACTH in the blood was elevated substantially with CRH loading (Figure 4d). The levels of corticosterone were also significantly higher, indicating that the downstream hormones were sufficiently induced by ACTH from the graft (Figure 4d). Accordingly, the transplanted hypopituitary mice locomoted more spontaneously and survived longer (Figure 4e). These findings proved that induced $ACTH^+$ cells derived from mouse ES cells function as endocrine tissue and that they have potential for use in regenerative medicine.

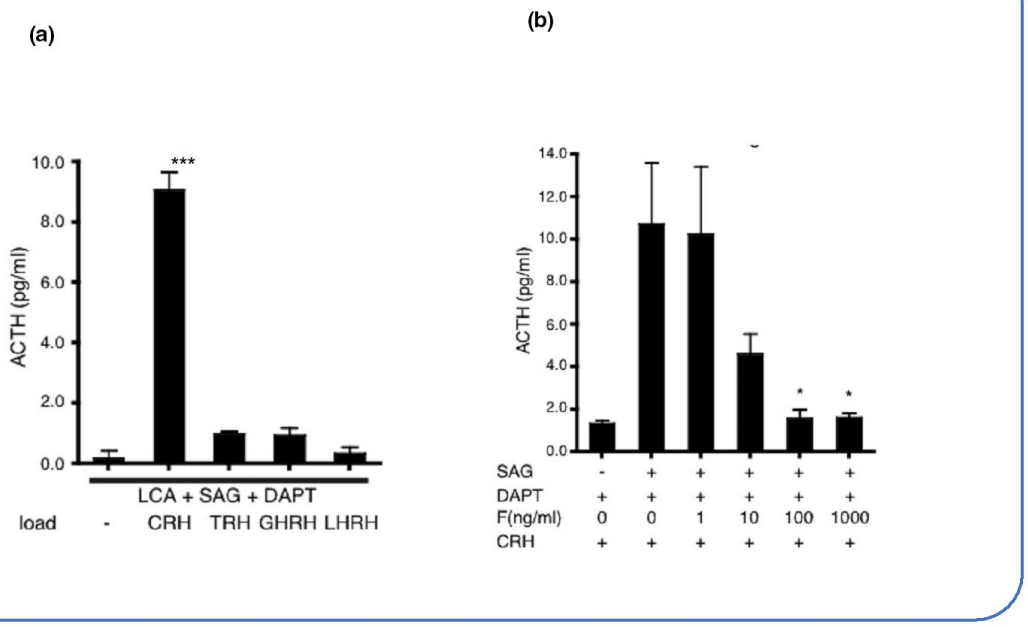
6 | APPLICATION TO HUMAN ES/iPS CELL CULTURE

Recovery of lost pituitary function is an important medical challenge because the anterior pituitary gland is unlikely to regenerate. As some pituitary dysfunctions cannot be treated with drugs alone (Arima et al., 2014; Hahner et al., 2015; Sherlock et al., 2009), regenerative medicine with stem cells is expected as a new form of therapeutic intervention. Hence, mouse ES cell culture method (Suga et al., 2011) has to be applied to human ES cells. Ozone et al. adapted this pituitary differentiation method to human ES cells (Ozone et al., 2016). This method also had some beneficial features for regenerative medicine.

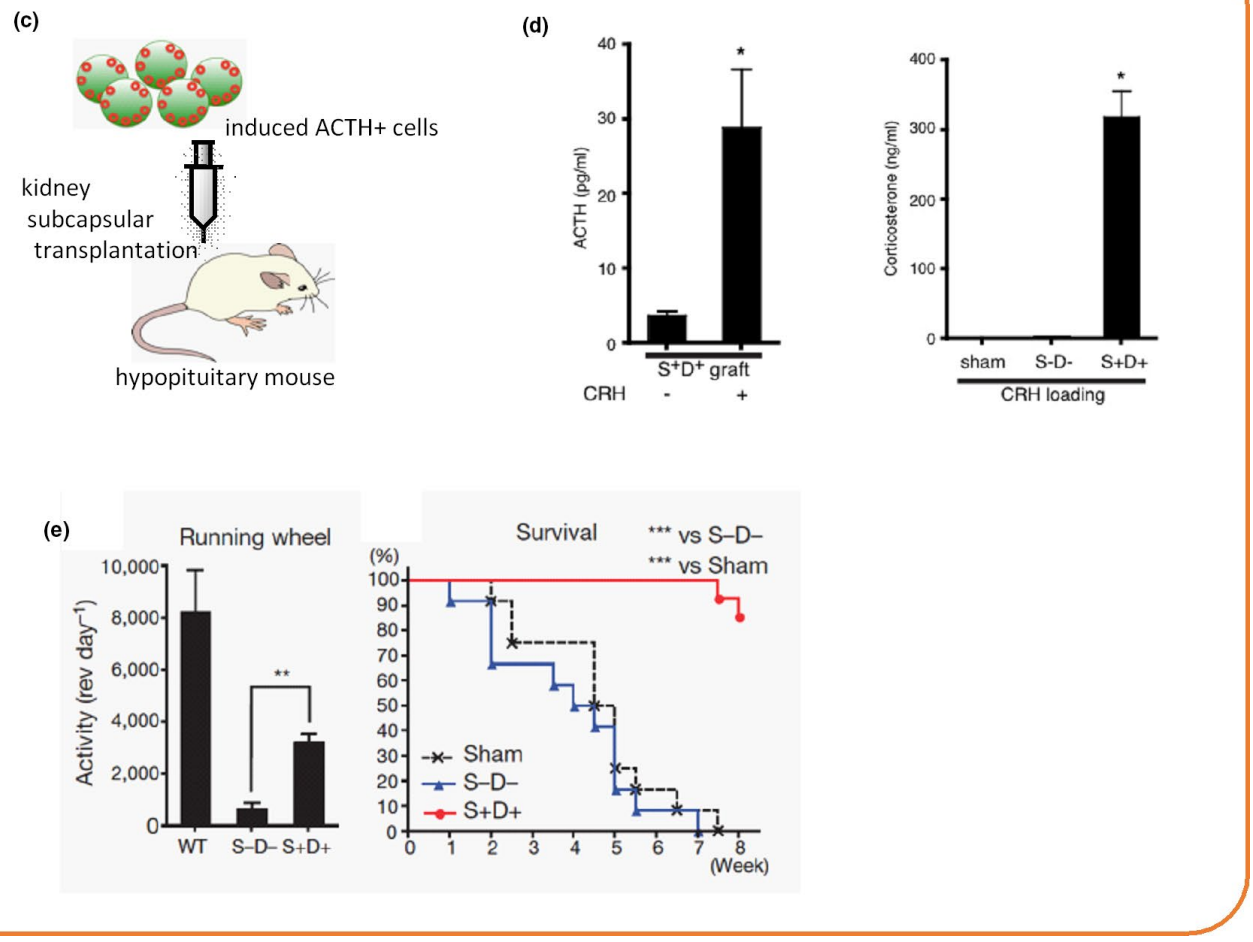
This culture for human ES cells kept important principles of the differentiation method into pituitary. Hypothalamic neuroectoderm and oral ectoderm were coinduced in vitro and subsequently invaginated (Figure 5a). The adjacency of these tissues promoted the formation of the $Lhx3^+$ pituitary placode, the features of which were consistent with those of Rathke's pouch in vivo. The $Lhx3^+$ pituitary placode derived from mouse ES cells efficiently invaginated to form pouches, while the rest of the oral ectoderm remaining on the surface did not express pituitary-specific markers even at a later stage. In contrast, human ES cell-cultured tissues also formed $Lhx3^+$ pouches, but the rest of the oral ectoderm remaining on the surface also became $Lhx3^+$ pituitary placode after long-term culture. Subsequently, these pituitary placodes differentiated into pituitary hormone-producing cells, in which we identified all six types of pituitary hormone-producing cells (Figure 5b). Among them, a normal response to releasing and feedback signals of human ES cell-derived corticotroph was confirmed. Accumulation of secretory granules in the cytoplasm of these cells was observed with electron microscopy (Figure 5c).

FIGURE 4 Functional tests in vitro and in vivo of mouse ES cell-derived $ACTH^+$ cells. (a) In vitro ACTH release for the releasing factors. Among the releasing factors, CRH most efficiently induces ACTH secretion. (b) Negative feedback test. F, glucocorticoid pretreatment. Pretreatment with glucocorticoid suppresses CRH-stimulated ACTH secretion. (c) Schema of ectopic transplantation to mouse. All mice, except for the WT mice, underwent hypophysectomy; hypopituitarism was confirmed by CRH loading. (d) In vivo blood ACTH and subsequent release of corticosterone. S^+, D^+, SAG- and DAPT-treated aggregates, respectively. S^-, D^-, no SAG or DAPT treatment, respectively. (e) Improvement of activity and survival by transplantation. The values shown on graphs represent mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (from Suga et al., 2011, modified)

in vitro functionality



in vivo functionality



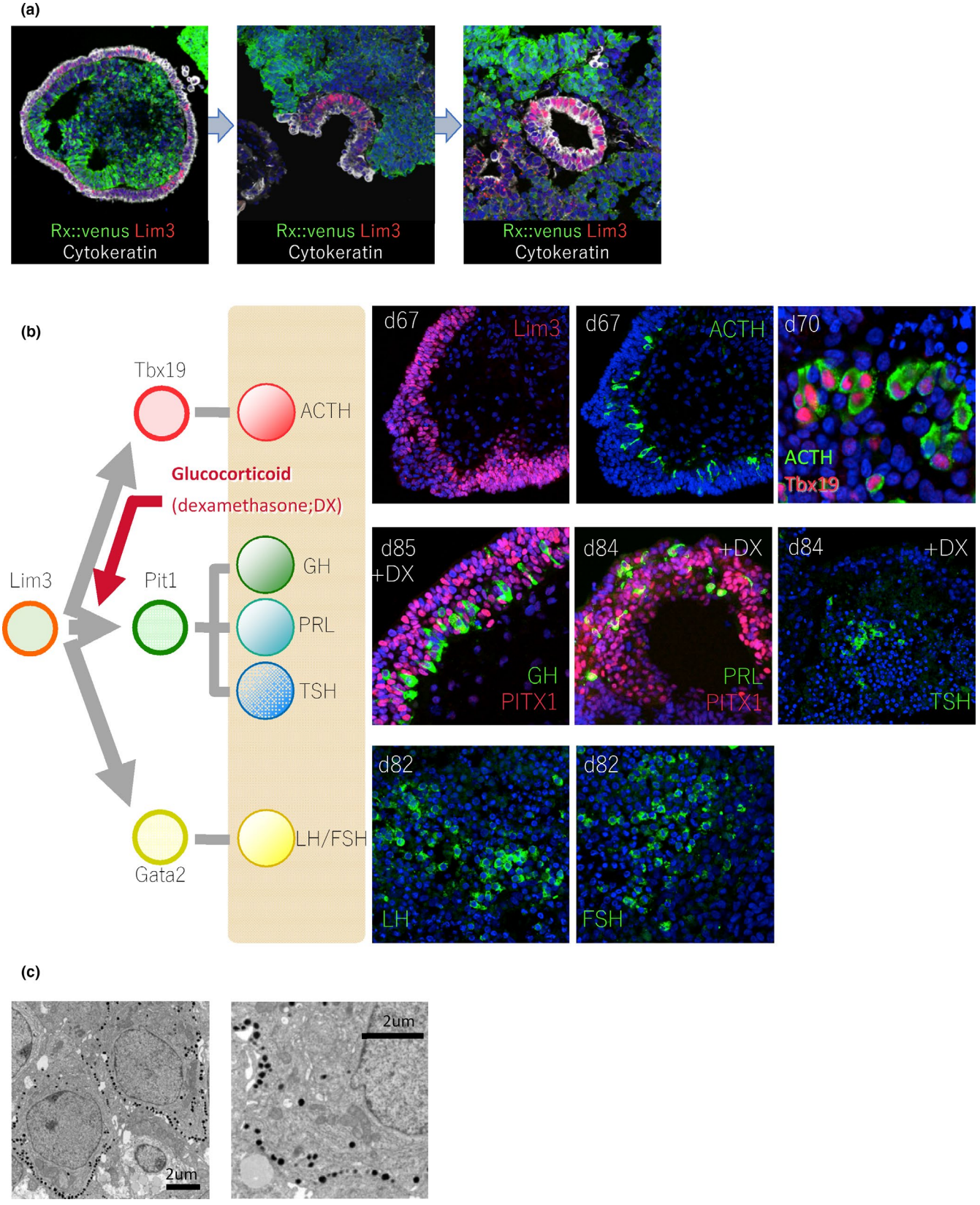


FIGURE 5 Differentiation from human ES cells into anterior pituitary. (a) Two-layer formation and subsequent self-formation of Rathke's pouch. (b) Lim3^{+} cells differentiation into multiple lineages. (c) Secretory granules of endocrine cells with electron microscopy

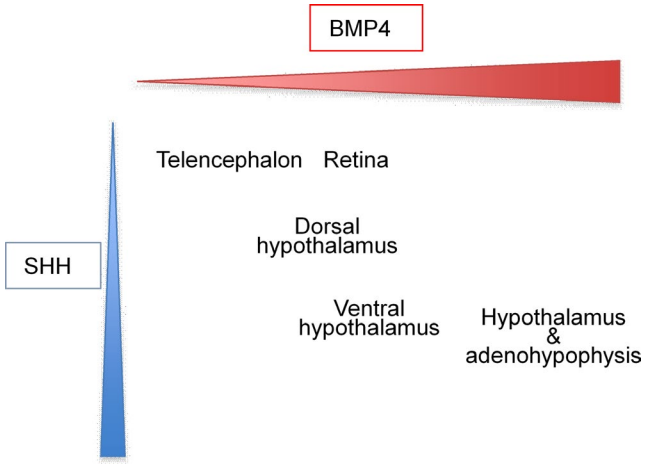


FIGURE 6 Relationship between BMP4 and SHH signals and induced areas in human pluripotent stem cell culture

7 | HYPOTHALAMIC CONTRIBUTION TO PITUITARY FUNCTIONS RECAPITULATED IN VITRO

Several human iPS cell lines differentiated into pituitary cells using the same methods (Kasai et al., 2020). In these aggregates, after long-term maturation culture, inner hypothalamic tissues matured and expressed hypothalamic neuron markers such as CRH, and outer ACTH$^+$ cells expressed CRH receptors. Pituitary ACTH was secreted through hypothalamic CRH neurons from the combined aggregates under hypoglycemic stimulation. A functional hypothalamic–pituitary axis seemed to recapitulate within one aggregate as seen in vitro. A series of differentiation methods using BMP4 and SHH suggest the central neuron region around the hypothalamus–pituitary gland is determined as shown in Figure 6.

8 | FUTURE PERSPECTIVES

Human ES/iPS cell-derived pituitary cells have two primary uses. Their first use is as a human model of development or disease. Our studies show that the current culture methods recapitulating embryogenesis can be used in the field of developmental biology. Tissues derived from disease-specific iPS cells can be useful as a human disease model for therapeutic screening of diseases caused by genetic mutations.

We recently established an iPSC-based congenital pituitary hypoplasia model (Matsumoto et al., 2019). These were disease-specific iPS cells from a patient with panhypopituitarism who had a heterozygous mutation in OTX2, which determined the development of the forebrain, eye, and pituitary. We examined the differentiation of the OTX2 mutant iPS cells into pituitary. The patient's iPS cells showed increased apoptosis in pituitary progenitor cells, and the differentiation into pituitary hormone-producing cells was severely impaired. This hypopituitary organoid revealed underlying mechanisms of congenital pituitary hypoplasia involving an OTX2 mutation.

The second primary use for human ES/iPS cell-derived endocrine cells is for regenerative medicine. Although stem cell-based therapeutics are highly anticipated for the treatment of diabetes mellitus, the use of regenerative medicine for hypothalamus–pituitary dysfunctions has received little attention. The generation of anterior pituitary tissues with regulatory hormonal control in vitro is essential for transplantation therapies. Our mouse ES cell-derived ACTH$^+$ cells had recapitulated both positive and negative feedback systems. Since our studies revealed that ES/iPS cell-derived ACTH-producing cells function after transplantation, this finding is expected to be applied to the grafting of ES/iPS cell-derived artificial pituitary tissue for novel treatments. Our differentiation methods into endocrine organoids in this three-dimensional culture might also be a better way to recapitulate the in vivo microenvironment, which is essential for improving the efficiency of differentiation in vitro. Therefore, our approaches can be applied to differentiation into other functionally mature endocrine tissues.

Currently, we are investigating the purification of pituitary cells for transplantation into humans. Further research is needed to translate pituitary regenerative medicine directly to clinical practice.

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REVIEW ARTICLE

Pubertal orchestration of hormones and testis in primates

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Abstract

The term "*Puberty*", socially known as "Adolescence" is the transitional period from juvenile life to adulthood with functional maturation of gonads and genital organs. In this process, some remarkable developmental changes occur in morphology, physiology, and behavior leading to reproductive competence. Despite sufficient levels of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]), robust spermatogenesis is not initiated during infancy in primates due to the immaturity of testicular Sertoli cells. Recent studies suggest that developmental competence augmenting functional activities of receptors for androgen and FSH is acquired by Sertoli cells somewhere during the prolonged hypo-gonadotropic juvenile period. This juvenile phase is terminated with the re-awakening of hypothalamic Kisspeptin/Neurokinin B/Dynorphin neurons which induce the release of the gonadotropin-releasing hormone leading to reactivation of the hypothalamo-pituitary-testicular axis at puberty. During this period of pubertal development, FSH and LH facilitate further maturation of testicular cells (Sertoli cells and Leydig cells) triggering robust differentiation of the spermatogonial cells, ensuing the spermatogenic onset. This review aims to precisely address the evolving concepts of the pubertal regulation of hormone production with the corresponding cooperation of testicular cells for the initiation of robust spermatogenesis, which can be truly called "*testicular puberty*."

KEYWORDS
gonadotropins, hypothalamus, Leydig cells, puberty, Sertoli cells, spermatogenesis, testis

1 | INTRODUCTION

Reproduction is essential for the survival and perpetuation of species. Sexual development in higher primates is tri-phasic as it is divided into-infancy, juvenile period, and adulthood (Plant, Terasawa, & Witchel, 2014). The term "Puberty" is defined as the transition from the juvenile period to adulthood when an individual achieves the ability to sexually reproduce. The maturation of the gonads as well as genital organs and the secondary sexual characteristics manifest during this period (Kaprara & Huhtaniemi, 2017; Plant, 2015a). The initiation of puberty is under the control of the neuro-endocrine system (Plant, 2015b). Group of neurons in the hypothalamus release a decapeptide hormone called gonadotropin-releasing hormone (GnRH) in a pulsatile manner to stimulate the synthesis and secretion of gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Kaprara & Huhtaniemi, 2017; Plant, 2015a). The hypothalamic network, generally called as the GnRH "pulse generator," is active and responsible for the release of GnRH thereby elevating the circulating levels of gonadotropins (FSH and LH) and testosterone (T) during early infancy (2–3 months in monkeys and 3–6 months of postnatal age in human) in male primates (Plant, 2015b). However, the transition from infancy to the juvenile period (at around 4–6 months of postnatal age in monkeys) is associated with a neuro-biological switch that turns off, arresting the pulsatile release of GnRH (Plant, 2015b). The GnRH pulse generator is re-triggered, after the protracted hypo-gonadotropic phase of juvenile development (3–4 years of

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age in male rhesus monkeys and 10–12 years of age in boys) causing resurgence of pulsatile GnRH secretion leading to elevated levels of gonadotropins which act on testis, which has maturated by that time, inducing onset of prolific spermatogenesis (Herbison, 2016; Plant, 2015b). Failure of this neuroendocrine event or an appropriate testicular maturation under elevated hormonal milieu at puberty may lead to infertility indicating the critical necessity of maturation of both systems at this phase of life.

The alarming decline in sperm count during the past few decades has become a global concern (Levine et al., 2017). Therefore, there is an urgent need for an in-depth study of the hormone-induced molecular events in pubertal testes which are critical for establishing normal spermatogenesis and male fertility at puberty (Hai et al., 2014). This review will address the current concepts of the hormonal regulation of pubertal development leading to the spermatogenic onset in primate testis.

2 | THE NEURO-ENDOCRINE REGULATORY CIRCUIT

The hypothalamo-pituitary-testicular axis (HPT axis) is the classic example of a neuro-endocrine regulatory circuit, with hierarchical cascades of regulatory feedback (both positive and negative) loops (Kaprara & Huhtaniemi, 2017; Plant, 2015a). Specific hypothalamic nuclei, like mediobasal preoptic area (POA) and infundibular (in humans) or arcuate nucleus (ARC, in monkeys) synthesize the decapeptide GnRH, which stimulates the gonadotrophs present in the anterior pituitary gland through a G-protein-coupled receptor (GPCR that signals via G_q and or G_{11} to activate phospholipase-C inducing the mobilization of Ca^{+2} by inositol phosphate 3) leading to the secretion of gonadotropins (LH and FSH). In the median eminence, the axon terminals of GnRH neurons make contact with the hypophysial portal vessels which transport this releasing hormone, secreted in a pulsatile manner, to the anterior pituitary (Kaprara & Huhtaniemi, 2017; Plant, 2015a). However, recent studies have elucidated the involvement of several other hypothalamic hormones in fine-tuning GnRH secretion (Kaprara & Huhtaniemi, 2017; Plant, 2015a). These include classical neurotransmitters such as Noradrenaline, Dopamine, Glutamate and γ-aminobutyric acid (GABA), Neuropeptide Y, Galanin-like peptide, Opioid peptides and Orexins (Kaprara & Huhtaniemi, 2017; Plant, 2015a). The hypothalamic GnRH pulse generator drives both basal (pulsatile) and tonic (surge) release of GnRH thereby regulating the gonadal functions (Herbison, 2016; Witchel & Plant, 2013). There are two concepts proposed so far for the pulsatility of the hypothalamic GnRH pulse generator. The first one suggests a role of the intrinsic pulsatility of the GnRH neuron (Plant, 2015a). The second theory suggests that the neurons in the ARC nucleus are responsible for the pulse generation (Herbison, 2016; Plant, 2015a). In 2003, this latter notion has gained acceptance following the establishment of the role of hypothalamic kisspeptin in regulating the HPT axis.

3 | KISSPEPTIN/NEUROKININ B/DYNORPHIN NEURONS: THE KEY REGULATOR OF GnRH PULSE GENERATOR

Kisspeptins, a group of neuropeptides encoded by the *Kiss1* gene is broadly recognized as an essential gatekeeper of puberty and fertility. Loss of *Gpr54* and/or *Kiss1* genes in humans and mice results in severe *hypogonadotropic hypogonadism* (Lippincott, True, & Seminara, 2014). Kisspeptin acts via the KISS1-Receptor, a G protein-coupled receptor 54 (GPR-54). Kisspeptin is an exceptionally potent inducer of GnRH secretion. GnRH neurons express KISS1-Receptor, and kisspeptin fibers project to GnRH cell bodies as well as GnRH fibers. However, many neurons in the ARC also express, two other peptides, namely, stimulatory neurokinin B, a tachykinin and inhibitory dynorphin, an endogenous opioid. Such neurons expressing three peptides are referred to as "KNDy (Kisspeptin-Neurokinin B and Dynorphin) neurons" (Lehman, Coolen, & Goodman, 2010). Interestingly, loss of function mutations in man in either *Neurokinin B* or its receptor (*Tac3-Receptor*) is associated with *hypogonadotropic hypogonadism* manifesting delayed puberty or sometimes loss of pubertal onset of spermatogenesis, cause being the lack of sufficient gonadotropins (Topaloğlu, 2017). In male rhesus monkeys, neurokinin B has been reported to induce GnRH release, indirectly via the production of kisspeptin (Ramaswamy et al., 2010). Therefore, it is proposed that the GnRH pulse generation is achieved by the KNDy neurons present in the ARC via complex crosstalk between the stimulatory neurokinin B and inhibitory dynorphin signals to eventually release kisspeptin which stimulates the GnRH neurons (Kaprara & Huhtaniemi, 2017; Plant, 2015a, b). However, these GnRH neuronal networks are also associated with other neighboring excitatory glutamatergic, inhibitory GABAergic, pre-pro-enkephalinergic neurons, astroglial and ependymal cells that collectively evoke the discharge of GnRH into the hypophysial-portal circulation (Herbison, 2016; Plant, 2015a). The differential regulation of FSH and LH secretion is achieved by low and high-frequency pulses of GnRH respectively (Thompson & Kaiser, 2014). Figure 1a,b shows the regulatory network of the GnRH pulse generator in higher primates.

4 | DEVELOPMENTAL REGULATION OF GnRH SECRETION

The onset of puberty is considered to be timed by two postnatal regulators, the first is the "arrest" or "restraint" that holds the GnRH pulse generator in a blockade mode during the infantile–juvenile transition and finally the "switch" that removes such arrest at the termination of juvenile development leading to the re-awakening of GnRH pulse generation (Plant, 2015b).

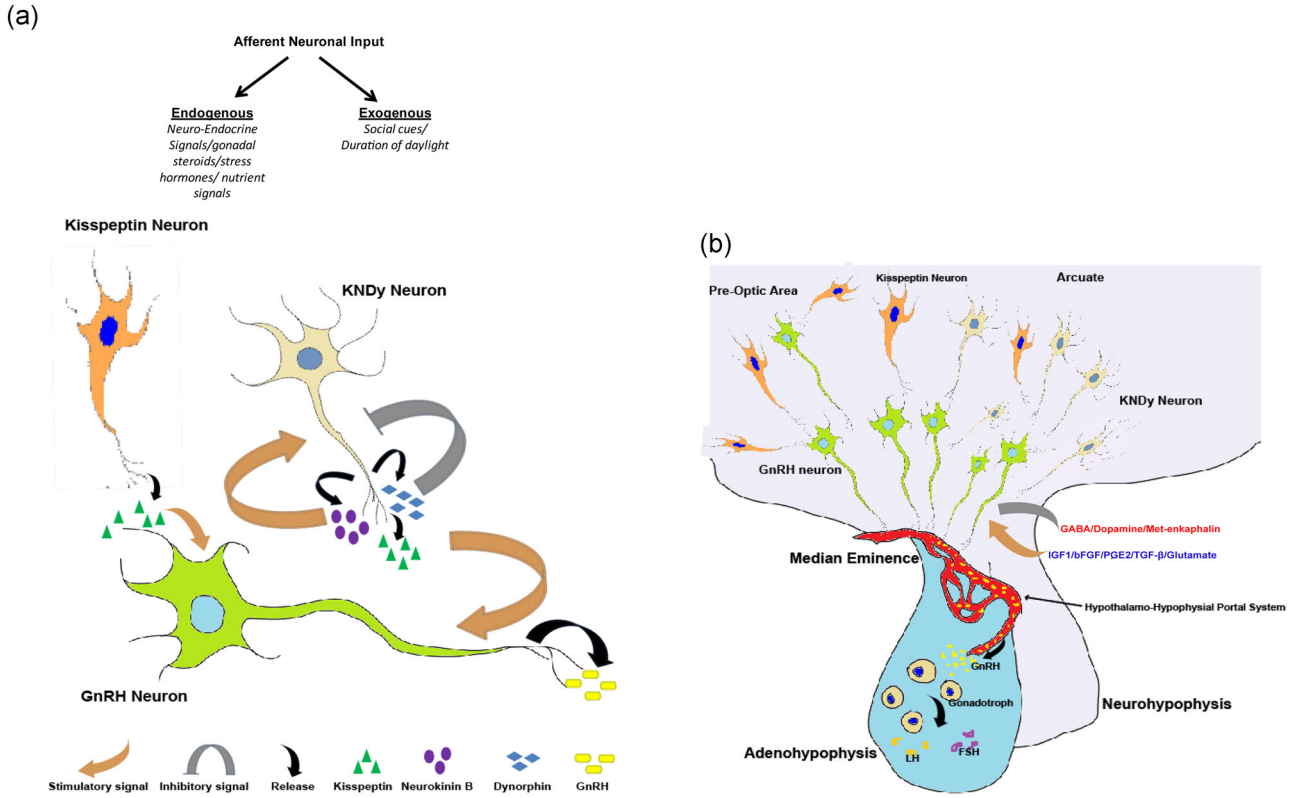

FIGURE 1 The GnRH neuronal network. (a) Typical GnRH neuron with its afferent neuronal inputs and functional output. (b) The GnRH pulse generator, showing feedback loops regulating functions of the neuronal network. GnRH, gonadotropin-releasing hormone [Color figure can be viewed at wileyonlinelibrary.com]

4.1 | Infancy

During early embryonic development, GnRH neurons, which differentiate outside the brain from the olfactory placode, migrate through the forebrain to the hypothalamus (Herbison, 2016). In primates, the fetal hypothalamus at mid-gestation is developed with an adult number of GnRH neurons, distributed diffusely in both the POA and medial basal hypothalamus with extensive projections to the median eminence (Herbison, 2014). Time course studies of the gonadotropin secretion during gestation and infantile period of primates have indicated that the hypothalamic GnRH pulse generator is functional by the second trimester of pregnancy (Plant, 2015b). Gonadotropin secretion during infancy is dependent upon GnRH, as evidenced by suppression of LH and FSH levels when animals were treated with a GnRH receptor-specific antagonist (Plant et al., 2014). Although testes are exposed to adult-like hormonal milieu of LH and FSH during infancy, spermatogenesis is not initiated (Plant, 2015b). It is reasonable to believe that unlike the GnRH pulse generator at the hypothalamic level, the testicular activity is suboptimal during infancy, limiting it from producing sperm.

4.2 | Juvenile–puberty transition

After 4–6 months of postnatal life in human infants, the GnRH pulse generator activity declines to ensure the hypo-gonadotropic state of the juvenile phase, known as "*childhood*" in humans (Plant, 2015b). Recent finding suggests that low amplitude pulsatile LH release is present in boys and girls before the onset of puberty (Witchel & Plant, 2013). This indicates that the activity of this neuro-endocrine system is diminished rather than its severance from the GnRH neuronal network during juvenile development (Plant, 2015b). This notion is further supported by two recent reports in the male rhesus monkey. First, the number of neurons in the ARC nucleus expressing kisspeptin is reduced during the transition from the infantile to juvenile stage of development (Ramaswamy, Dwarki, Ali, the Gibbs, & Plant, 2013) and second, the expression of *Kiss1* gene that encodes kisspeptin peptide increases remarkably in the ARC of male rhesus monkeys during the transition from the juvenile to pubertal stage of development (Plant, Ramaswamy, & DiPietro, 2006; Shahab et al., 2005). However, it is interesting to note here that the dependence of GnRH secretion on kisspeptin mediated signaling is reported to be predominant during infancy as compared to that of puberty (Shahab et al., 2018). Three possible mechanisms can be proposed to define the pubertal onset of such neuronal activity. The first is the inherent pubertal clock resident in the primate brain. The second, pubertal change in the plasticity of GnRH neurons via remodeling of their dendritic trees with increased spine density leading to the attainment of a bipolar arrangement with elevated neuro-secretory capacity (Perera, Lagenaur, & Plant, 1993; Plant, 2007). Finally, the growth tracking sensor in the brain, termed a "*somato-metabometer*" monitors the circulating signal of somato-skeletal development and thereby co-ordinates the reactivation of the GnRH pulse generator with the impending attainment of optimal body size (Plant, 2015b). Figure 2a–c is a schematic

representation of the neuroendocrine regulation of the developmental changes in HPT axis of male primates.

5 | INDUCTION OF PRECOCIOUS PUBERTY DURING JUVENILE PERIOD

The intermittent stimulation of the hypothalamus with neuro-chemical N-methyl-D-aspartate (NMDA, an agonist of Glutamate) in male juvenile rhesus monkeys was first reported to drive the pituitary-testicular axis into the pubertal mode in a GnRH dependent manner (Plant, Gay, Marshall, & Arslan, 1989). A recent study has provided support to this by demonstrating the role of microRNA Mir-664-2 in precocious puberty pathogenesis by regulating the expression of NMDA receptor-1 in rats (Ju et al., 2019). Pulsatile GnRH infusion (0.1–0.3 μg GnRH pulse over 2 min/3 hr) to juvenile monkeys for 4–5 weeks causes the onset of puberty-like maturation in the HPT axis with the precocious onset of spermatogenesis (Majumdar, Sarda, Bhattacharya, & Plant, 2012; Majumdar et al., 1995). Also, in the case of humans, a number of gene mutations have been associated with the precocious onset of puberty. For instance, mutations in Makorin Ring Finger Protein 3 (*MKRN3*) and *DLK1* gene have been reported to be associated with precocious puberty (Gomes et al., 2019; Yi et al., 2018). While going through the history of such cases, one must recognize that unless testis has matured during mid to late juvenile phase, onset of hypothalamic pulse generator prepubertally would never manifest through precocious puberty at testicular levels. This means testis matures and is ready to be responsive to hormones much ahead of so-called hypothalamic reactivation of pulse generator which is a phenomenon displaying puberty at the level of the neuroendocrine system. Pulsatile injection of GnRH to subhuman primates at mid-juvenile stage (1.5 years) has been shown to induce adult levels of testosterone (T) production and onset of robust spermatogenesis in GnRH driven monkeys, supporting the notion that testicular maturation happens ahead of the onset of neuro-endocrine puberty (Devi, Sarda, Stephen, Nagarajan, & Majumdar, 2006; Majumdar et al., 1995).

Although it is difficult to determine whether such testicular maturation is independent of hormonal stimulation as very low levels of gonadotropins may be present during juvenile phase of development, but failure of testis to respond to elevated gonadotropins at puberty in certain infertile individuals, suggests the attainment of puberty by two systems (neuro-endocrine and testicular consortium) may be independent of each other.

Factors involved in re-awakening the GnRH pulse generator setting during transition to puberty are described in Table 1.

6 | TESTICULAR DEVELOPMENT LEADING TO PUBERTAL ONSET OF SPERMATOGENESIS

Gonadotropins-FSH and LH released from the pituitary in response to pulsatile GnRH, act on specific testicular cell types to regulate the initiation and maintenance of spermatogenesis. FSH and LH bind to their specific receptors present on Sertoli cells and Leydig cells, respectively. The gonadotropins act on these cells to regulate processes critical for the robust initiation of spermatogenesis during the onset of puberty (Ramaswamy & Weinbauer, 2014). In the following section, we discuss the basic aspects of testicular development with special emphasis on the neuroendocrine regulation of Sertoli cell and Leydig cell function.

6.1 | Fetal testicular morphogenesis: role of FSH and LH

Testicular development during early embryogenesis is independent of pituitary gonadotropins. A complex gene expression program initiated in the fetal Sertoli cells orchestrates testicular morphogenesis during fetal life *in utero* (O'Shaughnessy & Fowler, 2014; Svingen & Koopman, 2013). The coordinated actions of fetal Sertoli cells and fetal Leydig cells regulate (a) the formation of testicular cords, which are precursors of seminiferous tubules, (b) differentiation of the male reproductive tract, (c) differentiation of primordial germ cells into male germ cells and (d) testosterone (T) production.

Sertoli cells are the major somatic component in testis which directly regulates the division and differentiation of germ cells. In fetal testis, Sertoli cells originate from the neighboring coelomic epithelium (Karl & Capel, 1998). One of the key events in the development of the male phenotype involves the degeneration of the para-mesonephric ducts, also known as Mullerian ducts. Anti-Mullerian Hormone (AMH) produced by fetal Sertoli cells induces degeneration of Mullerian ducts which are precursors of the female reproductive tract. Fetal AMH expression is initially independent of gonadotropins, however, induced by several transcription factors (Edelsztein, Grinspon, Schteingart, & Rey, 2016). For example, SOX9, a direct downstream molecule of Sex-Determining Region Y gene (*Sry*), binds to the *Amh* promoter to initiate its expression, subsequently, other transcription factors like SF1, GATA4, WT1, NFκB, and AP2 further increase AMH production (Edelsztein et al., 2016). Although the basal level of AMH expression is hormone-independent, later on by 17–18th week of gestation in humans, FSH via proliferation of fetal Sertoli cells by increasing their numbers or by directly upregulating AMH transcription per Sertoli cell (Edelsztein et al., 2016; O'Shaughnessy & Fowler, 2014) contributes to the overall rise in AMH concentration in fetal testes. It is interesting to note here that unlike females, germ cells do not enter into meiosis but remain in G_0 arrest as gonocytes due to the breakdown of retinoic acid in the fetal testis (Bowles, 2006).

Fetal Leydig cells originate from mesenchymal-like stromal cells present in the testicular interstitium by the 8th week of gestation in humans (Rotgers, Jørgensen, & Yao, 2018; Shima & Morohashi, 2017) and remain active in terms of steroidogenesis, independent of gonadotropins. However, during 10th week of fetal age, placental hCG reaches at its peak subsequently inducing the production of T by

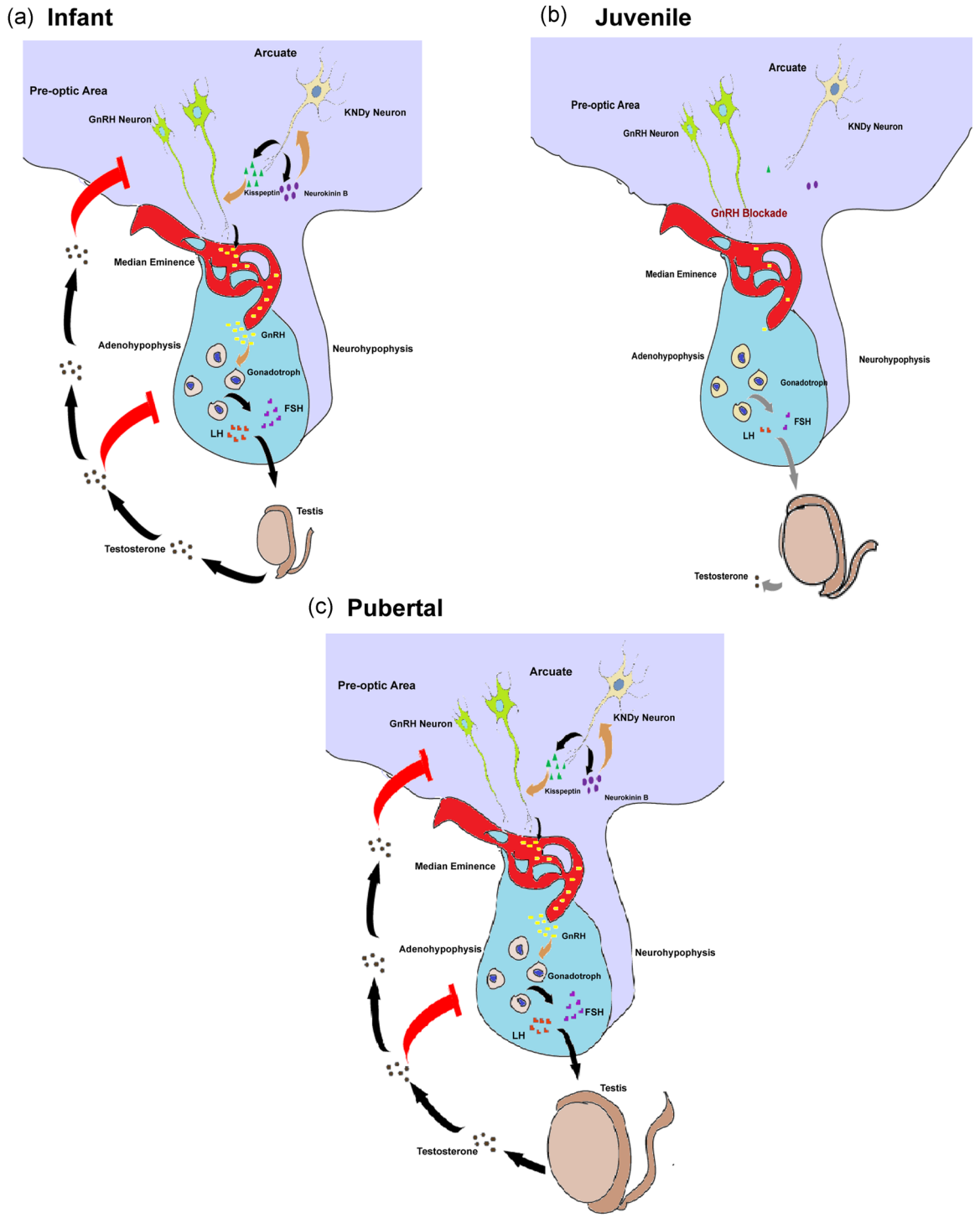

FIGURE 2 Regulation of HPT axis in male primates (a–c). Developmental changes in the neuroendocrine control of GnRH pulse generator activity. GnRH pulse generating mechanism resides in the arcuate and the output of this signaling is relayed to GnRH terminals in the median eminence by Kisspeptin projections arising from perikarya in the arcuate. During infancy (a), arcuate GnRH pulse generating activity is robust leading to the intermittent release of Kisspeptin in the median eminence, resulting in a corresponding pattern of GnRH release into the portal circulation. This drives pulsatile LH and FSH secretion. During the transition from infancy to the juvenile phase of development (b), a neurobiological blockade holds the arcuate GnRH pulse generating mechanism in check and pulsatile release of Kisspeptin in the median eminence is markedly suppressed. This leads to reduced GnRH release and to a hypogonadotropic state in the juvenile period. The onset of puberty (c) is initiated when such check is removed and GnRH pulse generation with the robust intermittent release of Kisspeptin in the median eminence is reactivated. Red arrow indicates mediated negative feedback (-) by the testis. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HPT, hypothalamo-pituitary-testicular; LH, luteinizing hormone [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Factors involved in reawakening the GnRH pulse generator setting the pubertal timing

Name of the factor	Description	Reference
Photoperiod	Melatonin is secreted from the pineal gland to relay the signal of the duration of daylight to the hypothalamus and plays a critical role in seasonal breeders. It is considered to have anti-gonadotropic properties. However, in monkeys, the impact of pinealectomy during juvenile development on the pubertal reactivation of pulsatile GnRH release has been demonstrated to show a negative result.	Plant (2015b)
γ-aminobutyric acid (GABA)	GABA is the major inhibitory neurotransmitter. GABA plays an important role in the timing of the onset of puberty in female rhesus monkeys. Although, during the infantile–juvenile transition the expression of its synthesizing enzyme, glutamic acid decarboxylase GAD 65 or GAD67 increases, however, the pubertal reactivation of GnRH pulse generator activity in the male hypothalamus independent of such expression.	El Majdoubi, Sahu, and Plant (2000), Mitsushima, Hei, and Terasawa (1994), and Urbanski, Rodrigues, Garyfallou, and Kohama (1998)
Glutamate	Intermittent activation of the NMDA receptor (one of the receptor subtypes which transduce glutamate signals) has been shown to mimic the action of intermittent kisspeptin stimulation in inducing a precocious pubertal pattern of GnRH release from the hypothalamus of the castrated juvenile male rhesus monkeys.	Gay and Plant (1988) and Plant et al. (1989)
Neuropeptide Y (NPY)	The pattern of NPY expression from birth to puberty in the castrated male monkey is found to be inversely related to that of the pulsatile GnRH release, as reflected by circulating LH concentrations. However, the pharmacological inhibition of NPY signaling in the hypothalamus of the male juvenile monkeys does not result in GnRH release.	El Majdoubi, Sahu, Ramaswamy, and Plant (2000)
Norepinephrine and dopamine	These two neurotransmitters are reported to have a negative effect on GnRH neuronal network in pubertal or adult mice.	Han and Herbison (2007) and Liu and Herbison (2013)
Leptin	Adipose tissue hormone Leptin does not function as a somatic trigger the onset of pubertal timing by reactivating the robust GnRH secretion. This is supported by the finding that in young children with leptin deficiency, treatment of hormones does not induce puberty immediately but rather this occurs only after prolonged exposure of hormones with optimal age.	Allison and Myers (2014)
Thyroid hormone	Thyroid hormones (thyroxine [T_4] and triiodothyronine [T_3]) sets the "*metabometer*" for the pubertal timing. The male rhesus monkeys have been used as the experimental model and thyroid hormone activity is manipulated by surgical or chemical thyroidectomy and by replacement on the other. Results indicate that the resurgence in pulsatile GnRH release at the termination of the juvenile phase of development is dependent on these hormones. However, whether the action is mediated directly on hypothalamic centers regulating the pulsatile release of GnRH or indirectly on somatic development remains to be determined.	Mann and Plant (2010)
Growth	Development of bones sets the '*somatometer*' in the hypothalamus that acts as a sensor of growth and metabolism and sends a cue to the GnRH pulse generator when a pubertal like age is attained.	Zofkova (2015)
Genetic	The high correlation in pubertal onset is observed within racial/ethnic groups, members within families, and between monozygotic twins indicating a strong genetic basis of the regulation of pubertal timing. Very recently, it has been	Abreu et al. (2013), Lomniczi et al. (2015), Ojeda et al. (2006), and Yi et al. (2018)

(Continues)

TABLE 1 (Continued)

Name of the factor	Description	Reference
	shown that the hypothalamic expression of several Zinc finger protein (ZNFs) like Kruppel-associated box (KRAB) domain, GATA zinc finger domain-containing protein 1 (GATAD1), makorin RING finger protein 3 (MKRN3) are decreased in castrated male monkeys in association with the pubertal reactivation of gonadotropin secretion. In addition, a study on single nucleotide polymorphisms in MKRN3 gene in Korean boys and girls has been suggested to be associated with precocious puberty. Furthermore, the expression of GATAD1 was found to be increased with the suppression of gonadotropins during late infancy. GATAD1 has been reported to repress the transcription of two key puberty-related genes, *Kiss1* and *Tac3*, directly, and reduces the activating histone mark H3K4me2 at each promoter via the recruitment of histone demethylase KDM1A. On the other hand, the loss of function mutations in *Mkrn3*, is associated with GnRH dependent precocious puberty.	
Sex	Puberty in primates is initiated earlier in females than in males. This sex difference may be regulated by the action of testicular Testosterone (T) on the fetal hypothalamus. Interestingly, this observation is consistent with the following findings, first, loss of function mutation in the *Androgen Receptor* (*AR*) resulting in "androgen insensitivity" syndrome is associated with the peak growth rate at puberty occurring at an age earlier than that observed in normal boys and secondly, administration of the anti-androgen flutamide accelerates the onset of puberty in the male monkeys.	Herman, Zehr, and Wallen (2006) and Zachmann et al. (1986)
Nutrition and environmental	The timing of pubertal onset may also be delayed by malnutrition and the presence of social or environmental stress factors.	Plant (2015b) and Soliman, Sanctis, and Elalaily (2014)

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; NMDA, N-methyl-D-aspartate.

fetal Leydig cells in adult range (Scott, Mason, & Sharpe, 2009). During the first trimester, T induces the differentiation of the Wolffian duct into the epididymis, seminal vesicle, vas deferens, and external genitalia. By the end of the second and third trimester, LH from the fetal pituitary gland becomes the predominant stimulus for Insulin-like Factor 3 (INSL3) and androgen secretion which promotes testicular descent and penile growth (Hughes & Acerini, 2008; O'Shaughnessy & Fowler, 2014).

The developmental time schedule of testicular morphogenesis is described in Table 2.

6.2 | Testicular development during infancy

At the time of birth, the seminiferous tubules of primate testes have no lumen, Sertoli cells remain immature and keep on proliferating in response to FSH (O'Shaughnessy & Fowler, 2014), Fetal Leydig cells are fully replaced by neonatal Leydig cells (Prince, 2001) and germ cells are represented by gonocytes and undifferentiated spermatogonia (Edelsztein et al., 2016; R. A. Rey, 2014). Level of gonadotropins is low in the first week of postnatal life then increases by the second week and remains high upto 4–6 months (Plant, 2008). The GnRH pulse generator activity in infant boys and male monkeys drives the LH and FSH secretion in an adult manner (Plant, 2008), which in turn leads to the secretion of testicular T and AMH from neonatal Leydig cells and Sertoli cells, respectively (R. A. Rey, 2014). Studies suggest that such initial exposure of testes to gonadotropins is critical for male fertility. It is noted that there is a massive expansion of Sertoli cell population during this period (Sharpe et al., 2000; Simorangkir, Marshall, & Plant, 2003) which is dependent on FSH and becomes the major determinant of the maximal output of sperm production in adulthood (Oatley, Racicot, & Oatley, 2011). It is also demonstrated that the treatment with GnRH-antagonist during infancy leads to suppression of gonadotropins causing delayed onset of puberty in male monkeys (Mann, Gould, Collins, & Wallen, 1989; Sharpe et al., 2000). Surprisingly, despite sufficient circulating levels of gonadotropins and T produced by Leydig cells during infancy, robust onset of spermatogenesis is not discernible in the testis at this period of development (Plant, 2015b; Plant et al., 2014). The Sertoli cell is the target for the action of FSH and T as it is the only cell bearing receptors for both hormones. Findings from our laboratory have revealed that

TABLE 2 Developmental events during human fetal testicular morphogenesis

Age	Remarks	Events
6–8 weeks of gestation	Fetal pituitary independent stage	Sex determination by XY/XX system (*Sry* gene in Y chromosome), Testicular differentiation and formation of testicular cords, Differentiation of Sertoli cells, Fetal Leydig cells from coelomic epithelium and interstitial mesenchymal-like stromal cells respectively, Differentiation of male gonocytes from primordial germ cells.
10 weeks of gestation		Placental hCG is at its peak, The proliferation of Fetal Leydig cells and production of T and Insl3 by hCG, Gonadotropin independent proliferation of Sertoli cells and gonocytes, Fetal Sertoli cells produce AMH, Sox9, Dmrt1 etc.
11–13 weeks of gestation		Testicular T in the adult range and male masculization programming.
17–18 weeks of gestation onwards	Fetal pituitary dependent stage	Fetal pituitary starts producing LH and FSH.
24 weeks of gestation onwards		Numbers of Fetal Leydig cells and Testosterone (T) production starts declining, LH mediated INSL3 leads to penile growth and testicular descent.
End of 2nd trimester to 8 months of postnatal age		Gonocytes do not enter into meiosis but a migration to the basement membrane of seminiferous tubules to establish the spermatogonial stem cell population.

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone.
Source: O'Shaughnessy and Fowler (2014).

insufficient T and FSH signaling in testicular Sertoli cells is responsible for such spermatogenic quiescence in testes of infants (Majumdar et al., 2012). During infancy, FSH signaling in Sertoli cells results in poor cAMP response due to restricted expression and activity of stimulatory Gα subunit ($G\alpha_S$) associated with the FSH-Receptor (FSH-R) limiting Sertoli cells from inducing robust differentiation of the spermatogonial cells (Bhattacharya et al., 2015). Also, Sertoli cells of infant primates are immature at this time as they poorly express functional Androgen Receptor (Majumdar et al., 2012; R. A. Rey, Musse, Venara, & Chemes, 2009).

6.3 | Testicular quiescence during the juvenile period

In humans, after first 4–6 months of postnatal life, neonatal Leydig cells gradually start disappearing from the testicular interstitium with the declining gonadotropin levels (Defalco, Saraswathula, Briot, Iruela-Arispe, & Capel, 2013; Prince, 2001), ensuring a protracted juvenile phase of childhood where testicular activity is restricted only to gonadotropin independent slow proliferation and survival of spermatogonial cells (Simorangkir, Marshall, Ehmcke, Schlatt, & Plant, 2005). This phase which lasts for 3–4 years in male monkeys and 10–12 years in boys, though quiescent spermatogenically, may be involved in intrinsic maturation of the testicular cells over this period of time so as to become capable for efficiently reading the signals from gonadotropins at so-called hormonal onset of puberty which sets in at the end of this phase.

6.4 | Pubertal maturation of testis leading to the spermatogenic onset

After a prolonged juvenile period, the HPT axis gets reactivated due to the reawakening of "GnRH pulse generator" (Plant, 2008). This reinitiates the robust secretion of FSH and LH from the pituitary gland leading to the stimulation of testicular Sertoli cells and Leydig cells, respectively (Plant, 2015b). The initial phase of this event is mainly associated with the Sertoli cell proliferation in response to FSH (Avendaño, Vazquez, & Tena-Sempere, 2017; Marshall & Plant, 1996) and maturation of Adult Leydig cells in the testis (Shima & Morohashi, 2017; Tena-Sempere, 2017). Adult Leydig cells start producing T and eventually, the intra-testicular T concentration rises up to the adult range. Since, by this time, Sertoli cells have already acquired the necessary developmental competence to express functional Androgen Receptor sufficiently (Boukari et al., 2009; Majumdar et al., 2012; McKinnell et al., 2001; R. A. Rey et al., 2009), T promotes the "*functional maturation*" of these cells (R. M. Sharpe, McKinnell et al., 2003). This process includes some remarkable changes in this cell population. For instance, proliferation of Sertoli cells ceases, AMH production is downregulated, and finally Sertoli cell-Sertoli cell tight junctions are formed to establish the Blood-testes-barrier (R. A. Rey, 2014). Unlike that found in rhesus monkeys, circulating levels of inhibin during infancy are comparable to that during puberty in humans (Andersson et al., 1998; Chada et al., 2003). However, the negative correlation between inhibin and FSH proving the inhibitory ability of circulating FSH by inhibin is attained only at puberty.

It is essential to note here that, during infancy, FSH action is restricted to the proliferation of testicular Sertoli cells, whereas in pubertal testis, FSH shows pleiotropic effects from Sertoli cell proliferation to induction of robust germ cell differentiation (Plant and Marshall, 2001). Our recent data suggested that, in pubertal Sertoli cells, T augments FSH signaling by upregulating the expression of FSH-R and $G\alpha_S$ (Bhattacharya et al., 2018, 2015). This augmentation in FSH signaling leads to the upregulation of Stem cell factor and glial derived neurotropic factor expression in pubertal Sertoli cells necessary for inducing spermatogonial differentiation (Bhattacharya et al., 2018, 2015). Finally, "*testicular puberty*" is triggered with the exaggerated meiotic entry of differentiated spermatogonia B to form the primary spermatocytes,

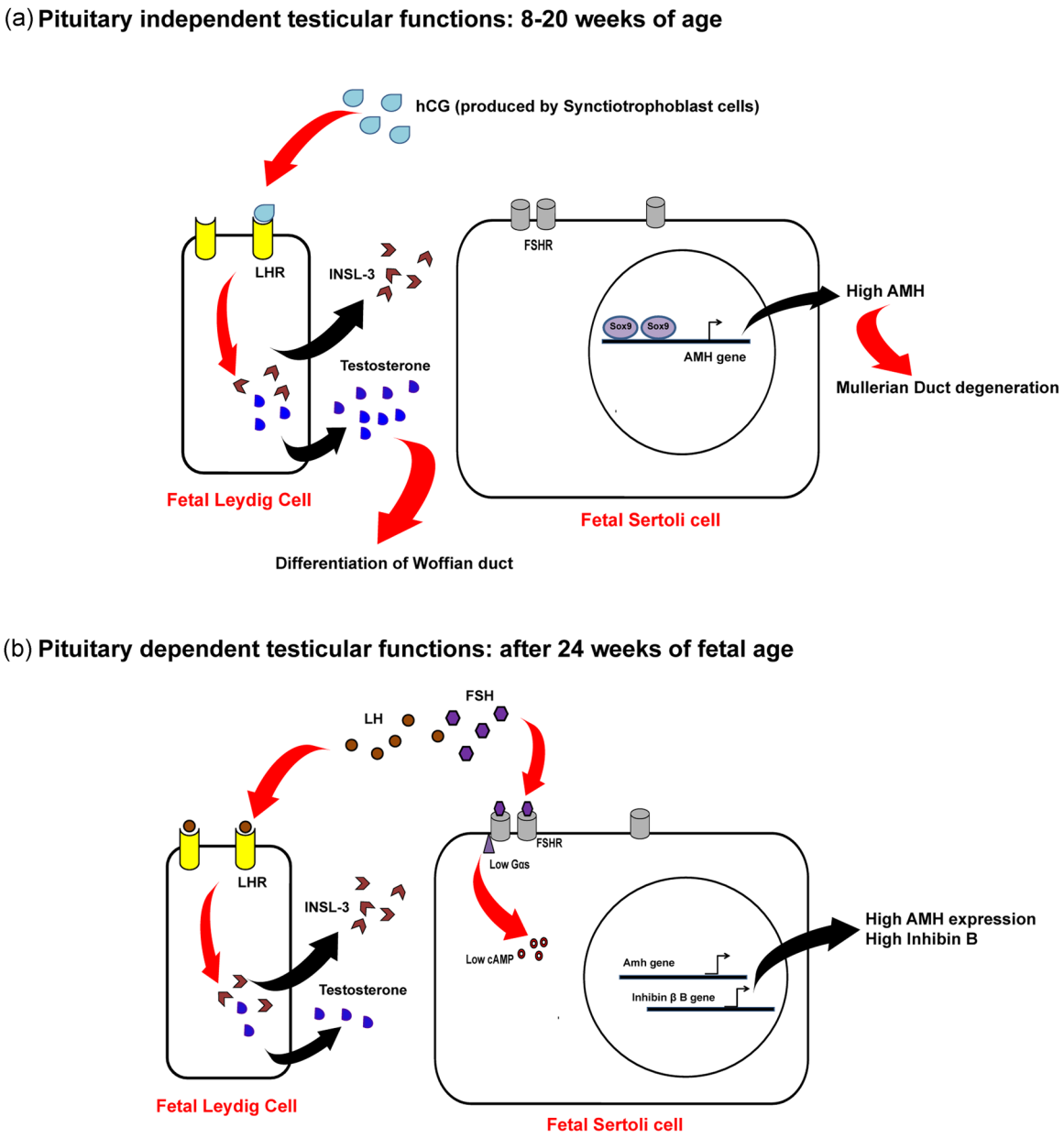

FIGURE 3 Molecular events in the testis during fetal development. (a) Fetal testicular differentiation in human during 6–8 weeks of age. (b) Pituitary independent fetal testicular development in human during 8–20 weeks of age [Color figure can be viewed at wileyonlinelibrary.com]

followed by their subsequent maturation into spermatozoa (R. A. Rey, 2014). This suggests that testicular maturation is essential to manifest testicular puberty.

6.5 | Pubertal timing of functional maturation of Sertoli cells

Many in vivo and in vitro studies have demonstrated that FSH or T (produced by Leydig cells in response to LH) responsiveness in the monkey Sertoli cells are spontaneously acquired somewhere around 12–22 months of age (Arslan, Weinbauer, Schlatt, Shahab, & Nieschlag, 1993; Bhattacharya et al., 2015; Majumdar et al., 2012; Marshall, Wickings, & Nieschlag, 1984; Ramaswamy, Plant, & Marshall, 2000; Schlatt, Arslan, Weinbauer, Behre, & Nieschlag, 1995). Clinical reports of infant boys with congenital *hypogonadotropic hypogonadism* have demonstrated a robust rise in inhibin B and AMH secretion after long term treatment by rhFSH and rhLH (Bougnères et al., 2008; Rohayem et al., 2017). Furthermore, it has been demonstrated that when gonadotropins stimulation of the infant monkey testis was sustained beyond 3 months of age by xenografting infant monkey testis into recipient adult mice, Sertoli cells maturation in the grafted testis was initiated before 10 months of age postbirth (Arregui & Dobrinski, 2014; Rathi et al., 2008). Studies in rodents have shown the potential role of thyroid hormone (T3/T4) and retinoic acid in

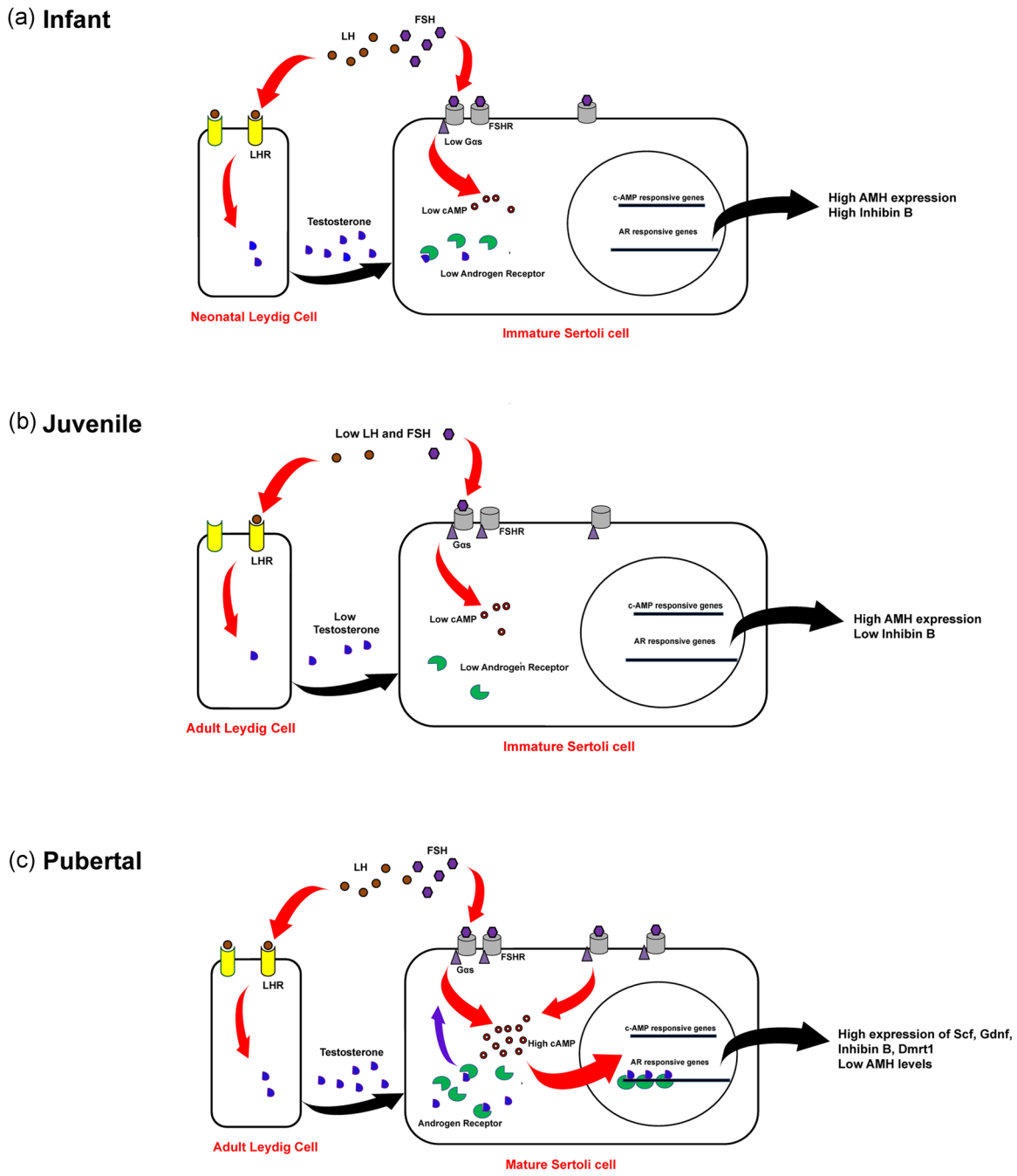

FIGURE 4 Molecular events in the testis during postnatal development leading to the pubertal onset of spermatogenesis in male primates. The action of testicular cells (a) during human infant boys upto 3–4 months of postnatal age, (b) during juvenile phase of life in human boys (6 months to 10–12 years of age), (c) during pubertal onset leading to robust initiation of first spermatogenic wave. Note: Blue arrow (c) indicates the nongenomic action of testosterone in augmentation of FSH-R mediated signaling in pubertal Sertoli cells. FSH, follicle stimulating hormone-receptor [Color figure can be viewed at wileyonlinelibrary.com]

Sertoli cell maturation (Buzzard, Wreford, & Morrison, 2003; Holsberger, Jirawatnotai & Hiroaki Kiyokawa, 2003; R. M. Sharpe, McKinnell et al., 2003; Wood & Walker, 2009). A recent study on primates has also suggested that a gonadotropin induced a selective increase in thyroid hormone responsiveness during the juvenile phase of testicular development may be involved in the functional maturation of Sertoli cells (Aliberti et al., 2018). This study also demonstrated that gonadotropin regulated miRNAs derived from the DLK1-DIO3 locus may play a critical role in regulating Sertoli cell proliferation in juvenile monkeys (Aliberti et al., 2018).

A schematic representation of events related to testicular maturation during fetal, juvenile, and postnatal life regulating pubertal spermatogenic onset is shown in Figures 3a,b and 4a–c, respectively.

Although it is well understood that proper responsiveness of Sertoli cells towards hormones (FSH and T) is essential during the pubertal

elevation of gonadotropins for the induction of spermatogenesis (Bhattacharya et al., 2015), the accurate time point when such developmental switch for testicular responsiveness towards gonadotropins occurs, remains to be determined. Another intriguing question that still remains unanswered is how this switch gets established during the juvenile period when the levels of circulating gonadotropins are barely minimal. It is also reasonable to consider that the compromised maturation of Sertoli cells may lead to impaired functioning of these cells with insufficient expression of genes important for regulation of spermatogenesis, as has been shown by us (Das et al., 2013; Mandal et al., 2017). Such a situation may not allow the Sertoli cells to efficiently augment germ cell proliferation and differentiation, even in the phase of elevated hormones, which manifest at puberty. Spermatogenesis may not be evident despite discernible hormonal puberty if testicular maturation is suboptimal by that time as shown in Figure 5.

It is important to note here that such *idiopathic male infertility* is noticed in pubertal boys which are untreatable by conventional external hormonal supplementations by the clinicians (Calogero, Condorelli, Russo, & Vignera, 2017). Recent research from our lab suggests that infant Sertoli cells can potentially function like mature pubertal cells, if post hormone receptor downstream signaling cascade is activated intracellularly, bypassing the receptors using forskolin or c-AMP (Bhattacharya et al., 2015). This generates a tremendous scope for using in vitro mode of treatment to biopsy tissue for overcoming compromised receptors (using testicular tissue culture, postbiopsy) for such infertile males who have persistent immature Sertoli cells with poor FSH-Receptor and Androgen Receptor activity even in adulthood (Hai et al., 2014).

7 | MODELS IN MALE PUBERTY RESEARCH "RODENTS TO PRIMATES"

Puberty in primates fundamentally differs from the laboratory rodents, by the occurrence of an infantile period of active HPT axis followed by an extended hypo-gonadotropic juvenile phase of testicular quiescence (childhood in boys). In mice, GnRH mRNA levels are low during the first week of life but progressively elevated by 15–25 days of age and the kisspeptin projections to GnRH neurons markedly increase between Day 25–31 leading to the rise in LH and thereby T secretion (Terasawa, Guerriero, & Plant, 2013). In rodents, kisspeptin neurons are distributed in the anteroventral periventricular nucleus (AVPV) and rostral

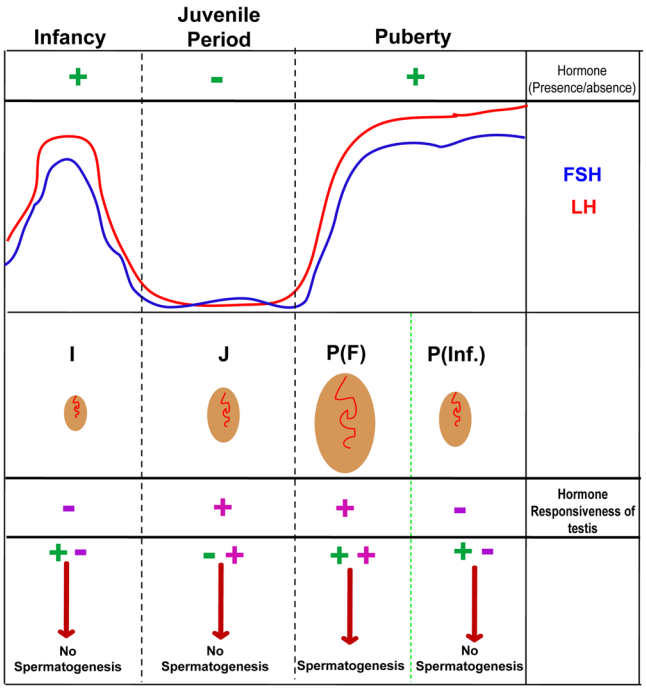

FIGURE 5 Cartoon diagram showing insufficient testicular maturation may lead to infertility in the phase of hormonal puberty whereas matching testicular puberty may lead to spermatogenesis, hence fertility. Infant testes (I): gonadotropins present (+) but the testis does not respond to the hormones (−), as a consequence spermatogenesis is not evident in the infant testis. Juvenile testis (J): absence of sperm production due to low levels of circulating gonadotropins (−). Pubertal testis [P(F)]: Prolific spermatogenesis occurs because of elevated levels of circulating gonadotropins (+) and attainment of testicular puberty (+). Infertile testis [P(Inf.)]: no spermatogenesis occurs in spite of sufficient levels of circulating gonadotropins due to impaired testicular puberty [Color figure can be viewed at wileyonlinelibrary.com]

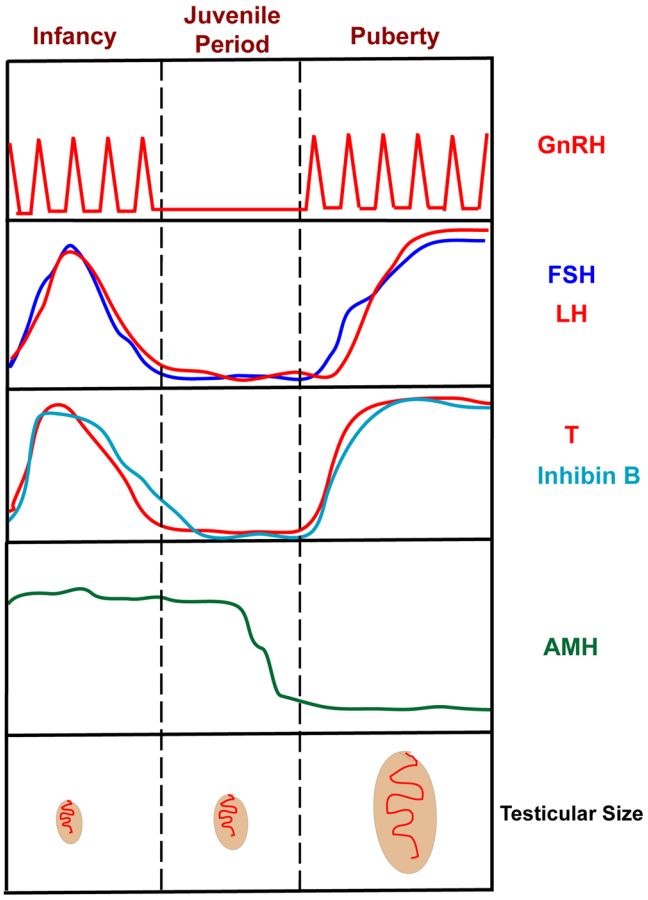

FIGURE 6 Cartoon diagram showing changes in the levels of GnRH, pituitary and gonadal hormones during infancy, juvenile, and pubertal phases of development. Information taken from: Aksglaede et al. (2010), Edelzstein et al. (2016), and Plant et al. (2014) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Chronological summary of some of the pioneering studies on the regulation of Hypothalamo-Pituitary-Testicular (HPT) axis for the onset of puberty in males

A. GnRH pulse generator/KNDy neurons			
Year	**Species studied**	**Major outcome**	**Reference**
1951	Human	Neural mechanisms controlling the pituitary–gonadal axis.	Harris (1951)
1971	Sheep and pig	Purification of master regulator decapeptide GnRH (LHRH earlier name) in ovine and porcine species respectively from Guillemin and Schally's laboratory independently. This study has been awarded by Noble Prize in 1977.	Amoss et al. (1971) and Matsuo, Baba, Nair, Arimura, and Schally (1971)
1975	Rhesus monkey and rat	Unlike rodents continued androgen stimulation from the fetal stage to postnatal life does not affect the cyclic pattern of GnRH release in monkeys.	Karsch, Dierschke, and Knobil (1973)
1978	Rhesus monkey	Intermittent GnRH stimulation of the pituitary found to be essential for the sustained secretion of both LH and FSH.	Belchetz, Plant, Nakai, Keogh, and Knobil (1978)
1979	Rhesus monkey	Stimulation of pituitary and testicular function by GnRH in fetal and infant males.	Huhtaniemi, Korenbrot, Lautala, and Jaffe (1979)
1984	Rhesus monkey	Gonadotropin secretion during infancy is dependent upon GnRH, as LH and FSH levels were experimentally suppressed by postnatal treatment with a GnRH receptor antagonist.	Mann et al. (1984)
1988	Rhesus monkey	Intermittent activation of the NMDA receptor (one of the receptor subtypes which transduce glutamate signals) has been shown to induce a precocious pubertal pattern of GnRH release from the hypothalamus of the castrated juvenile males.	Gay and Plant (1988)
1989	Rhesus monkey	Prolonged intermittent NMDA stimulation of GnRH neurons within the hypothalamus of the juvenile males (15–16 months of age) for 16–30 weeks results the onset of precocious puberty with full activation of the HPT axis with the initiation of spermatogenesis.	Plant et al. (1989)
1989	Rhesus monkey	Blockade of HPT axis using GnRH antagonist in neonatal males (10–13 days of age) for 112 days showed a delayed pubertal onset. This is the first study to indicate that the neonatal activation of HPT axis is critical in the process of pubertal development.	Mann et al. (1989)
1989	Rhesus monkey	Hypo-gonadotropic state in juvenile males is not associated with a decline in hypothalamic GnRH content.	Fraser, Pohl, and Plant (1989)
1989	Human	Impaired migration of GnRH neuron in Kallmann Syndrome.	Schwanzel-Fukuda, Bick, and Pfaff (1989)
1991	Cynomolgus monkey	The suppression of HPT axis via a long-acting GnRH analog, D-Trp6, Pro9-NEt-LHRH, during fetal and early infancy leads to in markedly stunted penile and testicular growth without affecting general somato-skeletal growth.	Liu et al. (1991)
1993	Rhesus monkey	Pubertal modulation of GnRH neural network plasticity by elevated expression of polysialic acid neural cell adhesion molecule (PSA-NCAM), a plasma membrane-associated glycoprotein.	Perera et al. (1993)
1998	Rhesus monkey	Pubertal onset can be assessed by nocturnal GnRH pulse frequency acceleration (from <1 pulse/7 hr to approximately 4 pulses/7 hr) at the termination of the juvenile phase of development.	Suter, Pohl, and Plant (1998)
1999	Rhesus monkey	Fetal GnRH neurons obtained from the embryonic olfactory placode shows the pulsatile release of GnRH in vitro.	Terasawa, Keen, Mogi, and Claude (1999)
2000	Marmoset	Long-acting GnRH antagonist treatment in neonatal males leads to impaired Sertoli cell proliferation.	Sharpe et al. (2000)
2002	Rhesus monkey	Elevating circulating leptin in prepubertal males does not elicit precocious GnRH release.	Barker-Gibb, Sahu, Pohl, and Plant (2002)
2003	Human	Loss-of function mutations in Kisspeptin-Receptor a GPCR, known as *Gpr54*, are associated with *hypogonadotropic hypogonadism* in men and women.	de Roux et al. (2003) and Seminara et al. (2003)
2003	Marmoset	Suppression of HPT axis by GnRH antagonist leads to no significant change in spermatogonial survival, division or differentiation in neonatals.	R M Sharpe, Fraser et al. (2003)

(Continues)

TABLE 3 (Continued)

A. GnRH pulse generator/KNDy neurons			
Year	**Species studied**	**Major outcome**	**Reference**
2005	Rhesus monkey	Repetitive administration of the GPR54-Receptor agonist kisspeptin-10 (2 μg as a brief 1-min infusion once every hr for 2 days) to the juvenile male rhesus monkeys induces a GnRH-dependent discharge of LH and FSH suggesting that the transition from the juvenile (attenuated GnRH release) to pubertal (robust GnRH release) state is controlled by the activation of GPR54 signaling resulting from increased expression of hypothalamic *Kiss-1*, the gene that encodes for kisspeptin.	Shahab et al. (2005)
2006	Rhesus monkey	The transition of the juvenile phase to pubertal onset is regulated by GPR54 signaling due to increased expression of hypothalamic *KiSS-1* and the release of kisspeptin.	Plant et al. (2006)
2006	Rhesus monkey	Desensitization of GPCR-54 induced LH secretion via metastin 45–54 (analog of Kisspeptin 112–121) administration in juvenile males.	Seminara, DiPietro, Ramaswamy, Crowley, and Plant (2006)
2006	Human	Homozygous *R262q* mutation in GnRH-Receptor leads to a constitutional delay of puberty with subsequent *Oligozoospermia*.	Lin et al. (2006)
2006	Mice	Remodeling of the dendritic tree with increased spine density in GnRH neurons leads to bipolar arrangement during pubertal development.	Cottrell, Campbell, Han, and Herbison (2006)
2007	Ewe	GnRH expression neurons in the arcuate nucleus (ARC) co-express, three peptides, namely, Kisspeptin, Neurokinin B and Dynorphin.	Goodman et al. (2007)
2007	Mice	Both *Kiss1* and *Gpr54* knockout male mice are viable but infertile having abnormal sexual maturation; the majority of males lack preputial separation, significantly smaller testes.	Lapatto et al. (2007)
2008	Rhesus monkey	Non-synaptic intercommunication in the median eminence considered to be of kisspeptin regulation of GnRH release.	Ramaswamy, Guerriero, Gibbs, and Plant (2008)
2008	Rat	RFRP-1/3, ortholog of hypophysiotrophic gonadotropin release-inhibiting hormone (discovered in birds) has suppressive action on the GnRH induced pulsatile LH production.	Rizwan, Porteous, Herbison, and Anderson (2008)
2009	Human	Loss of function mutations in man in either *neurokinin B* or its receptor (*Tac3-Receptor*) is associated with. *hypogonadotropic hypogonadism* and delayed or absent puberty.	Guran et al. (2009) and Topaloglu et al. (2009)
2009	Human	Dynorphin fibers form intimate contacts with GnRH neurons suggesting a probable role in GnRH pulse regulation.	Dahl, Amstalden, Coolen, Fitzgerald, and Lehman (2009)
2010	Rhesus monkey	Neurokinin B is colocalized with kisspeptin in the arcuate nucleus (ARC) stimulates GnRH release.	Ramaswamy et al. (2010)
2010	Human	Sexually dimorphic neuronal connections between kisspeptin-immuno-reactive fibers and GnRH cells, with co-expression of kisspeptins and neurokinin B in the ARC.	Hrabovszky et al. (2010)
2011	Mice	Unlike males, pubertal onset in females mice is found to be unaffected by selective ablation of kisspeptin neurons. Male mice are infertile with small testes.	Mayer and Boehm (2011)
2011	Rhesus monkey	The stimulation of neurokinin B for GnRH release is upstream from KISS1-Receptor.	Ramaswamy, Seminara, and Plant (2011)
2012	Human	*Kiss-1* mutation leads to *hypogonadotropic hypogonadism*.	Topaloglu et al. (2012)
2012	Human	Majority neurokinin B neurons in the ARC do not synthesize detectable amounts of kisspeptin and dynorphin in young (less than 37 years) male individuals.	Hrabovszky et al. (2012)
2012	Human	Age-related progressive elevation in neurokinin B and kisspeptin signaling inputs to GnRH neurons.	Molnár et al. (2012)
2013	Mice	GnRH neuron-specific deletion of *Gpr54* leads to infertility.	Kirilov et al. (2013)
2013	Rhesus monkey		Ramaswamy et al. (2013)

(Continues)

TABLE 3 (Continued)

A. GnRH pulse generator/KNDy neurons			
Year	**Species studied**	**Major outcome**	**Reference**
		The number of neurons in the arcuate nucleus (ARC) expressing kisspeptin is reduced in association with the transition from the infantile to the juvenile stage of development.	
2016	Human	Immunoglobulin superfamily member 10 (IGSF10) is essential for the migration of GnRH neurons and two pathogenic mutations in IGSF10 lead to delayed puberty in six unrelated families.	Howard et al. (2016)
2017	Mice	Selective optogenetic activation of kisspeptin neurons in ARC leads to a pulsatile LH release confirming that these cells are critical for the GnRH pulse generator.	Clarkson et al. (2017)
2017	Rhesus monkey	Expression of kisspeptin in the Preoptic area (POA) requires E_2 via the aromatization of T at the hypothalamus.	Vargas Trujillo, Kalil, Ramaswamy, and Plant (2017)
2018	Human	Biallelic loss-of-function mutations in *Kiss1-Recoptor* have no impact on the timing of adolescent puberty, indicating that the infantile mini puberty predominantly depends on kisspeptin induced, GnRH secretion rather than that of the adolescent puberty.	Shahab et al. (2018)
B. Pituitary responsiveness towards LH/FSH release and testicular feedback by T/E_2/inhibin			
Year	**Species studied**	**Major outcome**	**Reference**
1972	Human	The nocturnal sleep-dependent rise in pulsatile LH secretion as a physiological indicator of puberty.	Boyar et al. (1972)
1974	Human	The study of 46 infant boys aged between 27 and 348 days showed no correlation between age and gonadotropins or testosterone (T).	Forest, Sizonenko, Cathiard, and Bertrand (1974)
1975	Rhesus monkey	Surgical disconnection of the medial basal hypothalamus does not affect pituitary function in terms of pulsatile LH secretion.	Krey, Butler, and Knobil (1975)
1975	Human	Prepubertal gonadotropin secretion is not dependent on gonadal functions.	Conte, Grumbach, and Kaplan (1975)
1977	Rhesus monkey	Synergistic effect of testicular estradiol-17 (E_2) with T as the negative feedback controlling of gonadotropins.	Resko, Kaleem Quadri, and Spies (1977)
1977	Rhesus monkey	Fetal hypophysectomy (on 111 and 116 days of gestation) leads to a critical decline in Leydig cells and spermatogonial populations in the fetal testes suggesting the HPT axis is active by this stage of development.	Gulyas, Hodgen, Tullner, and Ross (1977)
1980	Rhesus monkey	Testes do not play a major role in determining the pattern of gonadotropin secretion throughout neonatal and prepubertal development.	Plant (1980)
1987	Human	The gonadotropin secretory responses in terms of FSH are decreased at higher frequencies of GnRH administration with a fixed-dose.	Spratt, Finkelstein, Butler, Badger, and Crowley (1987)
1987	Human	The pulse frequency of GnRH stimulation can differentially control FSH and LH secretion by the pituitary gland, low frequency of GnRH favors high FSH release.	Gross, Matsumoto, and Bremner (1987)
1988	Human	Pituitary responsiveness to GnRH increases at slower frequencies of GnRH stimulation and such changes in pituitary responsiveness occurs independently of the gonadal steroid secretion.	Finkelstein, Badger, O'Dea, Spratt, and Crowley (1988)
1988	Rhesus monkey	T negatively modulates the differential release of LH and FSH occurring due to the changing pulse frequency of GnRH.	Adams, Clifton, Bremner, and Steiner (1988)
1989	Human	Direct inhibitory effect of T on the pituitary for gonadotropin secretion.	Sheckter, Matsumoto, and Bremner (1989)
1989	Rhesus monkey	T replacement prevents the post-castration hypersecretion of FSH with an intact central nervous system but not in hypothalamic-lesioned, GnRH-replaced animals, indicating that the hypothalamus site of negative feedback of T.	Abeyawardene and Plant (1989)
1990	Human	In male babies, a hypophysial LH discharge was observed leading to the secretion of T by the neonatal testes probably due to the termination of placental steroid mediated negative feedback.	Corbier et al. (1990)

(Continues)

TABLE 3 (Continued)

B. Pituitary responsiveness towards LH/FSH release and testicular feedback by T/E_2/inhibin			
Year	**Species studied**	**Major outcome**	**Reference**
1991	Human	T inhibits gonadotropin secretion at both pituitary and hypothalamic levels.	Finkelstein J. S., Whitcomb, R.W. et al. (1991)
1991	Human	E_2 inhibits gonadotropin secretion at the pituitary level.	Finkelstein J. S., O'dea L. S. T, Whitcomb, and Crowley (1991)
1992	Human	Inactivating mutation in *LH-β* subunit leads to hypogonadism.	Weiss et al. (1992)
1995	Rhesus monkey	Major role of testicular inhibin B ($\alpha\beta_B$ dimer) is to regulate the secretion of FSH by a direct negative feedback action at the level of the pituitary.	Majumdar et al. (1995)
1996	Human	Inhibin B is responsive to FSH stimulation and is not detectable in the sera of orchidectomized men.	Illingworth et al. (1996)
1998	Rhesus monkey	Although inhibin is the major regulator of FSH secretion at the pituitary level, the pituitary is not the target organ for the negative feedback action of T in the male.	Fingscheidt, Weinbauer, Fehm, and Nieschlag (1998)
1998	Human	Inactivating mutation in FS*H-β* subunit leads to hypogonadism.	Phillip, Arbelle, Segev, and Parvari (1998)
1999	Rhesus monkey and Rat	The primary mode of androgen-mediated negative feedback in monkeys is to restrain GnRH pulse generator, whereas in rats T directly decreases LH synthesis and secretion from the pituitary. In monkeys, E_2 suppresses GnRH-stimulated LH secretion, but E_2 amplifies the action of GnRH in the rats.	Kawakami and Winters (1999)
2000	Human	E_2 has dual sites of negative feedback, acting at the hypothalamus to decrease GnRH pulse frequency and at the pituitary to decrease responsiveness to GnRH.	Hayes, Seminara, DeCruz, Boepple, and Crowley (2000)
2000	Human	LH and FSH were detected in mid-pregnancy male fetuses but were suppressed at term, whereas, inhibin B remained uniform from mid-gestation to term pregnancy in male fetuses.	Debiève, Beerlandt, Hubinont, and Thomas (2000)
2001	Human	T exerts both direct and indirect feedback on LH secretion, whereas its effects on FSH appear to be mediated largely by via E_2due to the aromatization of T.	Hayes, DeCruz, Seminara, Boepple, and Crowley (2001)
2001	Human	The pituitary is the site of the direct action of endogenous E_2 as evident by rise in gonadotropin secretion after inhibition of P450 aromatase enzyme in early and mid pubertal boys.	Wickman and Dunkel (2001)
2003	Rhesus monkey	LH and FSH mediated inhibition and stimulation respectively were observed in the production of testicular inhibin B in juvenile or pubertal males.	Ramaswamy, Marshall, Pohl, Friedman, and Plant (2003)
2009	Human	The fetal concentration of hCG is 10 fold higher than that of the LH during the second trimester of gestation (11 hr to 19th weeks) and a further decline in fetal hCG is resulted in decline in T.	Fowler, Bhattacharya, Gromoll, Monteiro, and O'Shaughnessy (2009)
2014	Human	Accumulated deep sleep in children is a powerful predictor of pulsatile LH secretion during pubertal onset.	Shaw et al. (2015)
2015	Rhesus monkey/ human	Expression of GATAD1, a Zinc finger protein increases with suppression of gonadotropin secretion during late infancy and further represses the transcription of two key puberty-related genes, *Kiss1* and *Tac3* directly.	Lomniczi et al. (2015)

C. Fetal/neonatal/infant testis development			
Year	**Species studied**	**Major outcome**	**Reference**
1953	Rabbit	The existence of Anti-müllerian hormone (AMH) has been postulated as the fetal testicular hormone guiding the regression of the Müllerian ducts in the male fetus.	Jost (1953)
1975	Human	Comparable serum LH levels with that of the adolescent range by 1 week of age, which peaks at 1 month and further declines to the usual childhood range by 4 months, whereas FSH in early postnatal life, peaks levels up between 1 week and 3 months, followed by a decline by 4 months.	Winter, Faiman, Hobson, Prasad, and Reyes (1975)
1976	Human	The concentration of hCG peaks at 11–14 weeks whereas LH levels begin to rise at 12 weeks, when hCG levels start to decline, suggesting that hCG is the primary stimulus	Clements, Reyes, Winter, and Faiman (1976)

(Continues)

TABLE 3 (Continued)

C. Fetal/neonatal/infant testis development			
Year	**Species studied**	**Major outcome**	**Reference**
		to the fetal Leydig cell which results in T secretion (peak 11–17 weeks) and masculine differentiation of the genital tract.	
1979	Human	Direct evidence for the regulatory action of the placenta on fetal testes and hCG-stimulated in situ steroidogenesis in Fetal Leydig cells.	Huhtaniemi and Lautala (1979)
1980	Human	The total absence of Leydig cells and a low number of gonocytes in the anencephalic human fetus.	Baker and Scrimgeour (1980)
1981	Human	Testicular T production is maximal between 8 and 11 weeks of gestation which further decreases in the beginning of the second trimester of pregnancy.	Tapanainen, Kellokumpu-Lehtinen, Pelliniemi, and Huhtaniemi (1981)
1983	Rhesus monkey	First in vitro demonstration revealing that FSH signaling may not be fully operational in the infant Sertoli cells.	Lee, Pineda, Spiliotis, Brown, and Bercu (1983)
1986	Human	Four types of Leydig cells : (a) fetal- from birth to 1 year of age with round nuclei, abundant smooth endoplasmic reticulum and mitochondria with tubular cristae; (b) infantile- from birth to 8–10 years of age, showing a multilobed nucleus, moderately abundant smooth endoplasmic reticulum, some lipid droplets and mitochondria with parallel cristae; (c) prepubertal, from 6 years of age onwards with regularly-outlined round nuclei, abundant smooth endoplasmic reticulum, mitochondria with tubular cristae, and some lipid droplets and lipofuscin granules; and (d) mature and fully differentiated from 8 to 10 years of age onwards.	Nistal, Paniagua, Regadera, Santamaria, and Amat (1986)
1987	Rhesus monkey	The first evidence of specific high-affinity binding of radiolabeled FSH in homogenates of near term fetal testes.	Huhtaniemi, Yamamoto, Ranta, Jalkanen, and Jaffe (1987)
1990	Human	The number of Fetal Leydig cells is found to be decreased progressively from the 24th week of gestation up to birth to the second month of postnatal life.	Codesal, Regadera, Nistal, Regadera-Sejas, and Paniagua (1990)
1990	Human	The development of Leydig cells is a tri-phasic event, i.e. fetal, neonatal, and pubertal.	Prince (1990)
1990	Mice	Discovery of *Sry* gene for testis differentiation in XY embryo.	Koopman, Münsterberg, Capel, Vivian, and Lovell-Badge (1990)
1998	Mice	Coelomic epithelium gives rise to the Sertoli cell population in fetal testes.	Karl and Capel (1998)
2000	Human	Supplementations of rhLH or rhFSH in six patients with acquired complete *hypogonadotropic hypogonadism* showed Leydig cells is the major source of LH mediated T and E_2 production, whereas Sertoli cells are the major source of FSH induced inhibin B.	Young et al. (2000)
2001	Marmoset	Androgen receptor immuno-expression in testicular Sertoli cells increases with age.	McKinnell et al. (2001)
2002	Human	Postnatal T and LH surge occurs in neonates with Partial Androgen insensitivity syndromes (AIS) but is absent in those with Complete AIS.	(Bouvattier et al., 2002)
2003	Rhesus monkey	In infant the rate of Sertoli cells proliferation is maximal with robust gonadotropin secretion.	Simorangkir et al. (2003)
2003	Human	The number of male germ cells per testis was found to be increased from approximately 3,000 by 6th week to approximately 30,000 by the 9th week.	Bendsen et al. (2003)
2005	Rhesus monkey	Mitotic expansion of A_d and A_p spermatogonia in infant and juvenile testes is gonadotropin independent.	Simorangkir et al. (2005)
2006	Human	Poor expression of Androgen Receptor in early pre-pubertal testis.	Berensztein et al. (2006)
2006	Rat	Adult Leydig cell progenitor/stem cell has been characterized as 3-βHSD^{-ve}, LH-Receptor^{-ve}, platelet-derived growth factor receptor^{+ve} , GATA4^{+ve}, c-kit-Receptor^{+ve}, and leukemia inhibitory factor Receptor^{+ve} spindle-shaped cells in 7 days old rat testicular interstitium.	Ge et al. (2006)
2006	Human	Retinoic acid is a potential regulator of both male germ cell differentiation and steroidogenesis in fetal (6–12 weeks of age) testis.	Lambrot et al. (2006)
2007	Human	During 11–19 weeks of gestation, numbers of fetal Sertoli cells and gonocytes were found to be increased exponentially, whereas, Fetal Leydig cells slowed towards the end of 19 weeks. Sertoli cell-specific (KIT-L, FGF9, SOX9, FSH-R, WT1) and Germ	O'Shaughnessy et al. (2007)

(Continues)

TABLE 3 (Continued)

C. Fetal/neonatal/infant testis development			
Year	**Species studied**	**Major outcome**	**Reference**
		cell-specific (c-KIT, TFAP2C) transcripts were increased per testis through the second trimester, whereas Fetal Leydig cells markers (HSD17B3, CYP11A1, PTC1, CYP17, LH-R, INSL3) remained unaltered. By 11th Week LHCGR were expressed in Fetal Leydig cells. However, the expression of GATA4, GATA6, CXORF6, WNT2B, WNT4, WNT5A were also increased significantly per testis during this time period (11–19 weeks of gestation).	
2008	Human	Two infant boys at 7 and 10 months of age with congenital *hypogonadotropic hypogonadism* showed a robust rise in inhibin B and AMH secretion after long term treatment by a supra-physiological dose of rh-FSH and rh-LH.	Bougnères et al. (2008)
2008	Rhesus monkey	Sustained gonadotropin stimulation in the infant monkey testis beyond 3 months of age by xenografting into recipient adult mice, showed Sertoli cell maturation in the grafted testis within 10 months.	Rathi et al. (2008)
2008	Human	Compromised expression of Androgen Receptor in Sertoli cells leads to physiological androgen insensitivity of the fetal, neonatal and infantile testis.	Chemes et al. (2008)
2008	Rat	Identification of a masculinization programming window in between days 16–20 of fetal age of rats and the experimental blocking of the androgen action during this time induce birth defects in the male reproductive tract like *hypospadias*, *cryptorchidism*, and altered penile length and anogenital distance (AGD).	Welsh et al. (2008)
2009	Human	Androgen receptor expression is progressively increased in Sertoli cells with postnatal maturation of testes.	R. A. Rey et al. (2009)
2009	Human	FSH-receptor mRNA is expressed from 28 weeks of gestational age and its level of expression remains unaltered up to adulthood and lack of Androgen Receptor expression in Sertoli cells leads to the absence of AMH repression during infant testis.	Boukari et al. (2009)
2011	Human	Conversion of T to 5α- di-hydro-testosterone (DHT) is critical for the virilization of the fetus.	Flück et al. (2011)
2011	Mice	The coelomic epithelium and specialized cells along the gonad-mesonephros border are the two distinct progenitor lineages that give rise to the mesenchymal precursor stromal cells present in the interstitium that differentiate into fetal Leydig cells.	DeFalco, Takahashi, and Capel (2011)
2011	Human	Consistent high levels of LH and T is associated with accelerated testicular and penile growth in pre-term infant boys as compared to full-term infant boys.	Kuiri-Hänninen et al. (2011)
2011	Human	Male germ cells do not enter into meiosis by some unknown Cyp26b1 independent Retinoic Acid resistance pathway.	Childs, Cowan, Kinnell, Anderson, and Saunders (2011)
2011	Human	The number of somatic cells and germ cells were increased from 154,000 to 2,035,000 and 3,700 to 1,417,000 respectively in fetal human testes during 5–19 weeks of gestation.	Mamsen, Lutterodt, Andersen, Byskov, and Andersen (2011)
2012	Rhesus monkey	Inadequate T binding to Androgen Receptor and FSH induced cAMP response in testicular Sertoli cells are responsible for the spermatogenic quiescence in infants.	Majumdar et al. (2012)
2012	Human	During 8–20 weeks of gestation, DMRT1 expression was predominantly found to be Sertoli cell-specific, whereas from later 22–40 weeks of gestation to infancy and puberty DMRT1 was most abundant in spermatogonia, except in the A-dark type. Furthermore, putative meiosis inhibitors, CYP26B1 and NANOS2, were primarily expressed in Leydig cells and spermatocytes, respectively.	Jørgensen, Nielsen, Blomberg Jensen, Græm, and Rajpert-De Meyts (2012)
2013	Human	Unlike rats, in utero exposure (15–19 weeks) to exogenous estrogens including diethylstilboestrol (DES) shows minimal risk to masculinization of the human fetus.	Mitchell et al. (2013)
2014	Rat/Mice	Adult Leydig cells are originated from chicken ovalbumin upstream promoter transcription Factor II (COUP-TFII or Nr2f2) expressing interstitial stem/progenitor cells and experimental reduction of fetal androgen production results into a decline in these cell populations during puberty ultimately leads to Adult Leydig cell deficiency in adult testes.	Kilcoyne et al. (2014)
2015	Rhesus monkey	Poor expression of $G_{\alpha}s$ and *Ric8b* in infant Sertoli cells accounts for the compromised FSH signaling at this stage of testicular development.	Bhattacharya et al. (2015)

(Continues)

TABLE 3 (Continued)

C. Fetal/neonatal/infant testis development			
Year	**Species studied**	**Major outcome**	**Reference**
2017	Rat	Gestational exposure of dibutyl phthalate (DBP) during the male programming window of rat embryo suppressing the action of T leads to reduced Anogenital distance (AGD), *Testicular dysgenesis syndrome* (TDS) with pubertal defects at a later stage of life.	van den Driesche et al. (2017)
D. Juvenile/pubertal testis development			
Year	**Species studied**	**Major outcome**	**Reference**
1963	Human	The duration of human spermatogenesis has been estimated to be around 64 days.	Heller and Clermont (1963)
1973	Human	Early spermatid stage has been found in the Leydig cell tumor bearing testis of a 6-year-old boy.	Steinberger, Root, Ficher, and Smith (1973)
1984	Monkey	Spermatogenesis with motile sperm has been induced in 12 months old male monkeys via T treatment (first 21 weeks of testosterone capsules; thereafter weekly injections of 125 mg testosterone enanthate for next 1 year).	Marshall et al. (1984)
1986	Human	The onset of sperm production i.e. *spermarche* occurs in boys with limited or no development of pubic hair.	Nielsen et al. (1986)
1993	Human	Progressive decline and rise in AMH and Androgen respectively are observed in boys either during normal or precocious pubertal development.	R. A. Rey, Campo, Bedecarrás, Nagle, and Chemes (1993)
1993	Rhesus monkey	Juvenile male monkeys of 12–18 months of age were stimulated with either FSH or T alone or in combination for 12 weeks showing induction of spermatogenic initiation, via expansion of A_{pale} (not A_{dark}) spermatogonia. However, inhibin concentrations reached adult levels only in FSH-treated groups (not by T).	Arslan et al. (1993)
1994	Mice	AMH deficient male mice show fully descended testes and Leydig cell hyperplasia with mature sperm but fail to copulate due to interference from the well-developed female reproductive tract.	Behringer, Finegold, and Cate (1994)
1994	Human	Persistent AMH expression in patients with androgen insensitivity.	R. Rey et al. (1994)
1995	Human	Homozygous *LH-Receptor* mutation underlies autosomal recessive congenital Leydig cell hypoplasia.	Kremer et al. (1995)
1995	Rhesus monkey	Juvenile males aged 14–20 months were stimulated with either FSH or hCG alone or in combination for 4 weeks demonstrated the positive synergistic effect of FSH and hCG in terms of morphological and functional differentiation of Sertoli cells, peritubular cells, Leydig cells, and spermatogonia.	Schlatt et al. (1995)
1996	Rhesus monkey	Puberty occurring either spontaneously or induced precociously in monkeys is associated with the proliferation of Sertoli cells.	Marshall and Plant (1996)
1997	Rhesus monkey	FSH (but not LH) induces immuno-active inhibin B secretion from the juvenile testis.	Majumdar, Winters, and Plant (1997)
1997	Mice	FSH induces AMH production in immature Sertoli cells, and pubertal downregulation of AMH is caused by T and meiotic Germ cells.	Al-Attar et al. (1997)
1999	Mice	Genetic ablation of *Insl3* leads to failure in testicular descent.	Nef and Parada (1999)
2000	Rhesus monkey	Pulsatile treatments of LH or FSH either alone or in combination to juvenile males for 11days has showed that- i) LH, in combination with FSH, plays a critical role in the initiation of the pubertal proliferation of Sertoli cell in primates, ii) combined gonadotropin treatment led to the appearance of early primary spermatocytes, indicating the robust initiation of spermatogenesis.	Ramaswamy, Plant, and Marshall (2000)
2006	Rhesus monkey	Pubertal Sertoli cells produce sufficient levels of lactate and E_2 in an FSH independent manner.	Devi et al. (2006)
2011	Rhesus monkey	Juvenile males treated with methylphenidate hydrochloride (MPH) showed delayed pubertal progression with impaired testicular descent and reduced testicular volume.	Mattison et al. (2011)
2017	Rhesus monkey	Investigation of Global testicular gene expression from juvenile monkeys stimulated by recombinant monkey LH and FSH showed the repression of GFRA1 (glial cell line-derived	Ramaswamy et al. (2017)

(Continues)

TABLE 3 (Continued)

D. Juvenile/pubertal testis development			
Year	**Species studied**	**Major outcome**	**Reference**
		neurotrophic factor family receptor α 1) and NANOS2 (nanos C2HC-type zinc finger 2) that favors permatogonial stem cell renewal.	
2018	Rhesus monkey	Analysis of non-protein coding transcripts in juvenile monkey transcripts revealed a potential role of thyroid hormone in Sertoli cell maturation.	Aliberti et al. (2018)
2018	Rhesus monkey	The critical of T in augmenting FSH mediated signaling via up-regulating the activity and expression of FSH-Receptor in pubertal primate Sertoli cells.	Bhattacharya et al. (2019)

periventricular region of the third ventricle (RP3V), whereas in primates, the KNDy neurons are located either in POA or in infundibular/ARC nucleus (Clarkson, d'Anglemont de Tassigny, Colledge, Caraty, & Herbison, 2009). In humans, the infundibular KNDy neurons generate the negative feedback to the GnRH pulse generator, whereas in rodents such feedback operates via the kisspeptin neurons located in the arcuate nucleus (Hrabovszky et al., 2010). It is important to note here that there is also a species-specific difference in the site of androgen-mediated downregulation of GnRH induced LH secretion. Unlike male monkeys, T directly suppresses GnRH induced LH secretion from the pituitary gland in male rats. However, when T gets aromatized to E_2, it further augments GnRH action in rat pituitary whereas, E_2 inhibits GnRH action in primate pituitary (Kawakami & Winters, 1999). In male rhesus monkeys, the appearance of differentiating spermatogonia B, which is an early event of testicular puberty initiating the first spermatogenic wave, is observed only after 36 months of age (Hermann et al., 2012). On the other hand, in mice or rats the differentiating spermatogonia appear in the testis as early as postnatal Day 3-5 (Plant, Zeleznik, & Prevot, 2015).

Figure 6 describes the changes in the levels of GnRH, gonadotropins and testicular hormones during various stages of development in primates.

Table 3 summarizes some of the pioneering studies in the present field revealing the regulation of HPT axis during pubertal onset, in chronological order.

8 | CONCLUDING REMARKS AND FUTURE DIRECTIONS

In summary, sincere efforts for more than 60 years from various laboratories across the globe have concluded that a successful orchestration between the neuro-endocrinological network and developmental maturation of testicular cells leads to the timely onset of pubertal spermatogenesis. However, the major challenges that remain to be addressed are in understanding the neurobiological mechanisms that evoke the GnRH pulse generator and also in delineating the neuronal control that regulates the timing of pubertal onset at the hypothalamic level. Along with the onset of hormonal puberty, it is crucial to have a pubertal testis that is capable of responding to these hormones. It is necessary to undertake studies for divulging the molecular basis by which the testicular cells achieve such developmental competence during the juvenile period to initiate the robust germ cell differentiation at puberty, in response to elevated hormones. Future investigations need to be conducted in an integrative approach involving high through-put genomic (differential omics) and genetic (gene knock out/down models/Optogenetics) tools to reveal these unanswered questions (Majumdar & Bhattacharya, 2013; Ojeda et al., 2006). To this end, our lab has used differential DNA microarray between infant and pubertal Sertoli cells, followed by determination of transcription factor binding sites in promoters of differentially expressed genes using TRANSFAC. This was coupled with high throughput proteomics (like SWATH analysis etc.) to identify factors in infant and pubertal Sertoli cells which are crucial for spermatogenesis (Mandal et al., 2017). The new information will significantly improve our current understanding of the etiology of some forms of pubertal defects leading to idiopathic male infertility.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

AUTHOR CONTRIBUTIONS

S. S. M. and I. B. conceived the concept. I. B. wrote the first draft of this manuscript, Tables, and Figures. S. S. M. contributed in

manuscript drafting and the final Figures. S. S. M., I. B., and S. S. S edited the manuscript to produce the final draft.

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