Prenatal programming of the female reproductive neuroendocrine system by androgens

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Abstract

It has been clear for several decades that the areas of the brain that control reproductive function are sexually dimorphic and that the ‘programming actions’ of the male gonadal steroids are responsible for sex-specific release of the gonadotrophins from the pituitary gland. The administration of exogenous steroids to fetal/neonatal animals has pinpointed windows of time in an animal’s development when the reproductive neuroendocrine axis is responsive to the organisational influences of androgens. These ‘critical’ periods for sexual differentiation of the brain are trait- and species-specific. The neural network regulating the activity of the gonadotrophin releasing hormone (GnRH) neurones is vital to the control of reproductive function. It appears that early exposure to androgens does not influence the migratory pathway of the GnRH neurone from the olfactory placode or the size of the population of neurones that colonise the postnatal hypothalamus. However, androgens do influence the number and the nature of connections that these neurones make with other neural phenotypes. Gonadal steroid hormones play key roles in the regulation of GnRH release acting largely via steroid-sensitive intermediary neurones that impinge on the GnRH cells. Certain populations of hormonally responsive neurones have been identified that are sexually dimorphic and project from hypothalamic areas known to be involved in the regulation of GnRH release. These neurones are excellent candidates for the programming actions of male hormones in the reproductive neuroendocrine axis of the developing female.

Introduction

Just over a decade ago, David Barker proposed that the environment of the developing fetus could programme adult physiological function (Barker 1990). His hypothesis suggested that certain factors, if experienced for a discrete period during early development, could lead to dysfunctional conditions many months or even years later. Although originally focused on the effects of nutrition on the development of adult disease, it is becoming clear that other factors that impinge on the fetus may play key roles in the onset of disease states or abnormal physiological function in adulthood. Among these factors are the gonadal steroid hormones. It is now acknowledged that a short exposure of developing females to abnormally high concentrations of androgenic steroids before and/or immediately after birth may have long-lasting effects that are now regarded as ‘programming’ the female neuroendocrine axis to malfunction in adulthood. Such steroid exposure may occur when female fetuses (or their mothers) produce excessive concentrations of androgens (such as in congenital adrenal hyperplasia; Barnes et al. 1994), are exposed to androgens via a male sibling (as in the freemartin; Parkinson et al. 2001), are positioned adjacent to a male sibling in the uterus of a species that produces multiple offspring, as in the rodent (Meisel & Ward 1981, Galdelman 1986) and pig (vom Saal 1989) or via an exogenous source, including endocrine disruptive agents (Colborn & Clement 1992) and certain medical interventions (Wilkins 1960, Jacobson 1962). Although the signs of in utero masculinisation at the level of the external genitalia are well described (Burns 1961) and abnormalities at higher levels of the reproductive axis have been acknowledged for many years (Pfeiffer 1936), the latter have received much less attention. Most importantly, the neural mechanisms underlying the programming of the reproductive neuroendocrine axis, which at time may lead to sub-fertile states, are unclear. The mechanisms by which prenatal testosterone programmes the female reproductive system, especially the gonadotrophin releasing hormone (GnRH) neuronal network to malfunction in adulthood, are the focus of this review.
A window of opportunity for programming

The idea that the brain is organised along gender-specific lines and that these organisational actions are caused by some event in early fetal life is certainly not a new idea, but it was articulated several thousand years ago by Hippocrates (460–377 BC) and Aristotle (384–322 BC). The window during which the developing human brain was given its ‘soul’ was estimated by both to be between about days 30 and 50 of gestation (Swaab & Hofman 1984). Relative to the reproductive axis, this idea was initially given scientific flesh with studies in the guinea pig which explored the hypothesis that male hormones were important in early development for the sexual differentiation of the brain (Phoenix et al. 1959). The species specific ‘window’ in development when the male steroid hormones are able to exert an organisational action on the GnRH neuronal network has now been defined in several mammalian species. In small rodents, this ‘critical period’ spans the late gestation/early postnatal period (Gorski 1971, 1985). However, in mammals that are more precocious at birth, this window falls within in utero life. Specifically, in the guinea pig with a gestational length of about 65 days, the period of sensitivity has been determined to be between days 30 and 37 of gestation (Goy et al. 1964); in the rhesus monkey, it is in the first trimester as it is in the human (Goy & Robinson 1982, Herman et al. 2000) while in the sheep, it extends from about 30–90 days of a 147 day pregnancy (Short 1974, Clarke et al. 1976, Wood et al. 1995). However, recent data obtained from sheep have led us to question whether this very precise delineation of the ‘critical period’ for sexual differentiation of the GnRH system should be more flexible. Such a doubt has arisen following the observation that chronic exposure to steroids after birth (for example, if the ewe lamb is androgenised ewes from puberty to at least 18 months of age (Wood et al. 1997)) and extended to larger mammalian species including the pig (Elsaesser & Parvizi 1979) and the sheep. In the ewe, studies in several laboratories across continents have clearly demonstrated that elevated testosterone concentrations, if present during the in utero critical period for sexual differentiation of the brain, severely compromise the positive feedback actions of oestrogen (Fabre-Nys & Venier 1991, Herbosa et al. 1996, Robinson et al. 2002, Sharma et al. 2002, Unsworth et al. 2005) and that this action impairs the ability of this steroid to stimulate the GnRH surge that is needed to trigger the luteinising hormone (LH) surge and subsequent ovulation (Fig. 1). As dihydrotestosterone (DHT) does not have this action, this strongly suggests that testosterone must be aromatised to oestrogen to have this defeminising effect (Masek et al. 1999).

The control of episodic GnRH release is also influenced by the prenatal steroid environment such that the suppressive actions of both oestrogen (Steiner et al. 1976, Dumesic et al. 1997, Sharma et al. 2005) and progesterone (Robinson et al. 1999, Moenter et al. 2005) on gonadotrophin secretion are substantially reduced (Fig. 2). An elegant series of studies performed in the laboratory of Douglas Foster have demonstrated that the agonalad ewe lamb, treated chronically with oestradiol, is highly sensitive to the inhibitory actions of this steroid on LH release until she reaches about 30 weeks of age. However, the ram lamb and the androgen-treated ewe lamb escape from such oestrogen negative feedback some 20 weeks earlier when they are about 10 weeks of age (Wood et al. 1991). In contrast to the observation that aromatisation of androgens is necessary to defeminise the LH surge mechanisms, the rise in LH secretion at the time of neuroendocrine puberty seems to be an effect of testosterone acting via the androgen receptor (Masek et al. 1999).

As the negative feedback influences of steroids on GnRH secretion are impaired, it is probable that the frequency of GnRH pulses will be elevated. Relative to this, we have noted elevated LH pulse frequency in androgenised ewes from puberty to at least 18 months of age (Robinson et al. 1999). Such enhanced releasing hormone secretion that, as mentioned above, may be for a protracted period is likely to have simple negative feedback mechanism mainly based on testicular androgens. Pioneering studies performed in rodent species (Gorski 1985) demonstrated that the oestrogen-stimulated GnRH surge is present in many mammals unless it has been given a signal within a specific developmental window that obviates the positive feedback mechanism. These observations have more recently been reinforced in the rodent (Connolly & Resko 1994, Rhees et al. 1997) and extended to larger mammalian species including the pig (Elsaesser & Parvizi 1979) and the sheep. In the ewe, studies in several laboratories across continents have clearly demonstrated that elevated testosterone concentrations, if present during the in utero critical period for sexual differentiation of the brain, severely compromise the positive feedback actions of oestrogen (Fabre-Nys & Venier 1991, Herbosa et al. 1996, Robinson et al. 2002, Sharma et al. 2002, Unsworth et al. 2005) and that this action impairs the ability of this steroid to stimulate the GnRH surge that is needed to trigger the luteinising hormone (LH) surge and subsequent ovulation (Fig. 1). As dihydrotestosterone (DHT) does not have this action, this strongly suggests that testosterone must be aromatised to oestrogen to have this defeminising effect (Masek et al. 1999).

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substantial consequences for downstream levels of the reproductive axis, including the pituitary gland and the ovary. It is also possible that androgens have separate, but potentially cumulative, actions directly at the levels of these tissues. Recently, we have noted that the pituitaries of in utero testosterone treated ewes are significantly heavier than those of control ewes, even when we express hypophysial weight as a proportion of body weight. This suggests that either hypothalamic hyperstimulation or a direct action of androgens has resulted in hypertrophy of this tissue. Recently, preliminary investigations have shown that the pituitaries of in utero androgenised ewes are significantly heavier than those of control ewes, even when we express hypophysial weight as a proportion of body weight. This suggests that either hypothalamic hyperstimulation or a direct action of androgens has resulted in hypertrophy of this tissue. Recently, we have noted that the pituitaries of in utero testosterone treated ewes are significantly heavier than those of control ewes, even when we express hypophysial weight as a proportion of body weight. This suggests that either hypothalamic hyperstimulation or a direct action of androgens has resulted in hypertrophy of this tissue. Recently, preliminary investigations have shown that the pituitaries of in utero androgenised ewes are significantly heavier than those of control ewes. However, since the weight of the androgenised pituitary is on average 40% greater than that of controls, these animals have a larger complement of gonadotrophs. In relation to the oestrogenic control of LH secretion, we have also determined that the percentage of oestrogen-responsive gonadotrophs is higher in the control animals (that respond to oestrogen positive feedback) than the androgenised animals (that do not exhibit oestrogen positive feedback; Fig. 1). These findings may be important for the actions of oestrogen at the level of the hypophysis and consequently for the feedback mechanisms that modulate the activity of the ovary. At the level of the gonad, fetal androgen excess leads to abnormalities in ovarian morphology and function (Abbott et al. 1997, West et al. 2001). Specifically, the ovary of the androgen-treated ewe is significantly larger and tends to be multifollicular or contains large fluid-filled ‘cysts’ when compared with the control animals (Fig. 1). These ovarian abnormalities are apparent from at least as early as 3 weeks of age (West et al. 2001). This action requires the aromatisation of testosterone to oestrogen, because when pregnant ewes are treated with DHT there is no ovarian hypertrophy in the offspring ewe lambs and the increase in follicular number is not observed. It is currently unknown whether these morphological differences are a result of androgens actions directly on the ability of the ovary to produce normal cycles in hormone secretion (Birch et al. 2003), the response to abnormal gonadotrophic stimulation, or a combination of the two.

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**Figure 1** Schematic illustration of both the organisational and the activational effects that prenatal androgen exposure exerts on the reproductive axis of the ewe. At the level of the GnRH neuronal network (top of figure), prenatal androgens neither alter the migratory route of the GnRH cells nor the number that arrive in the preoptic area. However, androgen-exposed ewes form about half the number of synaptic connections (yellow rectangles) on the GnRH cell body (purple/red) when compared to normal ewes. Neurotransmitter systems that impinge on the GnRH neurone are also organised depending on the animals’ prenatal steroid environment (pale blue, dark blue and green irregular shapes) as well as the percentage of neurones in a specific populations that are steroid sensitive (black dots). Pituitaries of androgenised animals are heavier than control tissue, while the control animals have a higher percentage of oestrogen-receptive gonadotrophs. Androgen exposure severely disrupts the steroid feedback mechanisms that modulate GnRH and LH secretion. In addition, the ovaries of the androgenised animals are significantly larger and folliculogenesis is disrupted. The ovaries shown in this figure are from 3-week-old ewe lambs (West et al. 2001).
GnRH neurones and sexual dimorphism in their distribution

As the GnRH neuronal network is programmed to function in a sexually differentiated manner, a major question to be resolved is what neural substrate is responsible for these differences. The first port of investigation must be the GnRH neurone itself. As this neural phenotype originates outside the brain and migrates, in early embryonic life, from the developing olfactory placode into the hypothalamus (Schwanzel-Fucuda 1999), it is possible that prenatal androgens disrupt some aspect of the migratory pathway. However, despite early embryonic androgen exposure, it appears that GnRH neurones are not substantially perturbed on their migratory trek, but colonise the hypothalamus/preoptic area in similar numbers and locations to control animals in the sheep (Wood et al. 1992), rat (Wray & Hoffman 1986) and rhesus monkey (Goldsmith & Song 1987). Therefore, this suggests that the neural mechanisms that impinge on the GnRH neurone may be altered by prenatal androgens and by the implication that the androgens act on the area to which the GnRH neurones are travelling and/or the neural connections that they form when they arrive at their destination rather than on their migratory route or the number of cells that successfully complete the journey (Fig. 1). It has previously been proposed that steroidal influences on the migration of certain neurotransmitter-synthesising cells in the developing hypothalamus could contribute to the sexually differentiated functioning of the brain (Tobet 2002).

Sexually dimorphic input to GnRH neurones

It is clear that the GnRH neurone is influenced not only by its steroidal environment, but also by other sensory and endocrine cues that include the length of the day, level of stress, metabolic factors, ageing and the presence of a mate. Information about the animals’ internal and external environment is relayed to the GnRH cell by various neural inputs to influence its activity. However, determining the chemical and structural nature of such inputs is technically extremely challenging in part because such information transduction may take place at various points of the neurone from the cell body to the web of neural processes and the terminal field where GnRH is released into the portal vessels. Although this is a largely unexplored field, there are reports of sexually dimorphic inputs to GnRH neurones at the level of the cell body (rat; Chen et al. 1990: sheep; Kim et al. 1999). In the latter case, the GnRH perikarya and proximate dendrites of female ovine GnRH neurones received twice the number of
synaptic contacts as those of male neurones. Significantly, this input is influenced by the prenatal androgen environment of the developing animal, as the number of synapses on the androgenised ewe lambs was similar to that of males but significantly fewer than that of non-androgen-exposed ewe lambs (Fig. 1). Recent studies in mice using biocytin to fill the GnRH neurones of adult mice have suggested that the number of synaptic inputs to GnRH neurones may have been substantially underestimated in these earlier studies. Specifically, the long dendritic processes and cell bodies of these biocytin-filled neurones possess many spiny processes which, in the mouse, were not sexually differentiated (Campbell et al. 2005). Whether a similar situation is present in the ovine preoptic area has not yet been explored. Although the GnRH neurones and proximal dendrites of some species may not show sexually differentiated inputs, the activity of the GnRH neurone can potentially be altered at other locations. In relation to this, two areas of the brain that play critical roles in the control of gonadotrophin secretion and female sexual behaviour in the rodent (the arcuate nucleus (ARC) and the ventromedial nucleus (VMN)) have been shown to have sexually differentiated patterning of synaptic spines. This is highly region-specific, with the female exhibiting more axodendritic spine synapses than the male in the ARC, while the opposite sex dominance is observed in the ventrolateral VMN. Importantly, this spine pattern is altered by steroid exposure during the critical period for sexual differentiation (Matusomoto & Arai 1980, 1986).

A continued focus on the sexually differentiated synaptic inputs to GnRH neurones at whatever level must attempt to determine the chemical nature of these inputs and identify those conveying steroidal information to the reproductive neuroendocrine axis. However, this undertaking is technically difficult and labour intensive and so many researchers have embarked on an alternative approach. This involves identification of the location and chemical nature of steroid-sensitive, sexually dimorphic neuronal populations that project from areas of the brain known to be involved in the hormonal control of GnRH release to the hypothalamic site of the GnRH neurones themselves.

Steroid hormone receptors in the brain

As the steroid control of the GnRH neurone is clearly sexually differentiated in several species because of the prenatal experience of the fetus, an understanding of how this is achieved is obviously paramount. A major (but not exclusive) mechanism by which steroids alter GnRH neuronal activity is by altering the activity of steroid sensitive inputs. This is because GnRH neurones themselves are largely devoid of progesterone, androgen and oestrogen (z) receptors (Herbison 1995, Skinner et al. 2001). Sex differences in the distribution of steroid receptive neurones in brain areas that influence reproductive function have been described in several species including the human (Kruijver et al. 2003), rodent (Orikasa & Sakuma 2004, Foecing et al. 2005), bird (Gahr 2001) and sheep (Scott et al. 2000, 2004, Robinson et al. 2003). However, sex steroids are involved in physiological functions other than the control of GnRH release and so the identification of specific neurones that convey information to the reproductive neuroendocrine axis is inherently complicated.

Sexually dimorphic neurotransmitter input

The identification of the specific steroid-responsive neurotransmitter systems that modulate the activity of the GnRH neurone has been a quest for reproductive neuroendocrinologists for several decades. The fact that steroid-sensitive inputs to GnRH neurones may be indirect and mediated by one or more intermediate neural population adds further complication. In addition, other neural cells, for example glia, may be extremely important in altering the ability of input neurones to form synapses. It has been shown that glial morphology is modified by steroid hormones and this alters the ensheathment of the GnRH neurone (Mong et al. 1999).

A major leap forward has been made in the identification of specific brain sites that appear to be key in the feedback mechanisms of both oestrogen and progesterone and are sexually dimorphic. In rodents, the brain regions that are gender-specific and implicated in the steroidal control of GnRH are found in the preoptic area/anterior hypothalamic areas, including the anteroventral periventricular nucleus (AVPv) and the medial preoptic nucleus (Gorski et al. 1980, Hammer 1984, Simler et al. 1984, 1985, Sumida et al. 1993, Davis et al. 1996). Sex differences have been reported in the existence/position and/or size of specific cell groups (Bleier et al. 1982, Henderson et al. 1999), the dimensions of cell perikarya (Brown et al. 1999) and the chemical phenotype of these cells (De Vries 1990, Park et al. 1997, McCarthy & Auger 2002, Simler 2002, Otten et al. 2004, Wolfe et al. 2005). However, one limitation of many of these studies in the context of this review is the confirmation that these sexually dimorphic features of the hypothalamus are a consequence of steroidal programming of the brain.

In an attempt to identify specific neural populations that might have sex-specific steroid inputs to GnRH neurones to induce the GnRH surge, we have focused our efforts on the ARC/VMN area of the ovine hypothalamus. This is because microimplants of oestrogen placed in this region have been shown to induce the preovulatory GnRH surge (Caraty et al. 1998) as well as stimulating the proceptive and receptive behaviours that accompany it (Blache et al. 1991). Track tracing studies have concluded that a trans-synaptic pathway links this
region of brain with the preoptic area, where the majority of ovine GnRH neurones reside (Goubillon et al. 2002), providing a potential conduit for information about an animals’ steroidal status to be relayed to the reproductive neuroendocrine axis. Within the ovine VMN, initial studies were focused on a population of neurones that synthesise somatostatin (Fig. 3a), largely because these comprise about 70% of the oestrogen receptive cells in this region (Herbison 1995). Further, somatostatin fibres are found in close apposition to GnRH neurones (Fig. 3b). Although the percentage of oestrogen-responsive somatostatin neurones is not sexually differentiated, we have recently determined that these neurones are ‘activated’ in the ewe in a manner that is dependent on the prenatal androgen environment of the fetus. Specifically, neurones in the VMN that were activated by oestrogen were identified using immunocytochemistry for the protein product of the immediate early gene c-fos (Fig. 3a). Ovariectomised ewes were exposed to late follicular phase concentrations of oestrogen that triggered an LH surge in the control, but not the androgenised ewes. On examination of their hypothalami, we noted that the percentage of cells in the VMN of the control ewes that were immunoreactive for fos following oestrogen administration was approximately double that of androgenised animals (Fig. 3c; Robinson et al. 2003). Further, the androgenised ewes had a similar number of fos-positive neurones in the VMN to a control group that had not been exposed to exogenous oestrogen. On further examination, we found that approximately 20% of somatostatin neurones were ‘activated’ by oestrogen in the controls but significantly fewer in the androgenised animals and controls not given exogenous steroids. These data support the suggestion that the activation of steroid responsive somatostatin neurones in the VMN is one of the early events in a chain by which oestrogen triggers the preovulatory surge of GnRH. This conjecture is also supported by a study by Pillon et al. (2004) in the normal ewe. Further, our data suggest that this mechanism is compromised in animals that have been treated with testosterone in utero, which do not exhibit an LH surge. Our future studies include those in which somatostatin will be infused directly into the brain in an attempt to alter GnRH surge dynamics. Similar studies have been carried out in the rat, where centrally administered somatostatin has been shown to inhibit the oestrogen-induced LH surge by preventing activation of the GnRH neurones (Van Vugt et al. 2004). Furthermore, it is clear that other neural phenotypes in the region of the ovine VMN are differentially activated by oestrogen and these will be the focus of future studies.

Figure 3 (a) Cells in the ventrolateral aspect of the ventromedial nucleus (vlVMN) of the ewe stained for somatostatin (brown cytoplasmic stain) or fos (black nuclear staining). (b) A cell in the preoptic area that is immunoreactive for GnRH (brown cytoplasmic stain). An axon immunoreactive for somatostatin is in close apposition to the GnRH cell body. (c) Control ewes given high follicular phase concentrations of oestrogen (+E) have significantly more fos-positive cells in the vlVMN than similarly treated androgenised ewes or control animals not treated with oestrogen (−E). (d) About 20% of the fos-positive cells in the oestrogen-treated control animals are somatostatin. The oestrogen-treated androgenised animals and the non-steroid-treated controls have significantly fewer fos-positive somatostatin neurones in the vlVMN. See text for further details. *P<0.05, **P<0.01, ***P<0.001.
Conclusion

Exposure of the developing female to male steroid hormones has disruptive actions on the organisation of all the levels of reproductive axis from the GnRH neuronal network to the gonad. These organisational actions take place during specific windows of time during an individuals’ development and these so-called ‘critical periods’ are species-specific. The precise mechanism by which early exposure to male hormones programmes the reproductive neuroendocrine axis of the female to malfunction in postnatal life is unclear. However, we do know that the gonadal steroid feedback mechanisms that modulate GnRH release are central to this process. The challenge is to identify those steroid-responsive inputs. This will facilitate a better understanding of the neural mechanisms by which the activity of the GnRH neurone is regulated in normal conditions as well as which specific circuits are disrupted by the programming influences of neonatal steroids.

Acknowledgements

The author wishes to thank the BBSRC and Wellbeing and the Royal College of Obstetricians and Gynaecologists for their financial support. Several collaborators contributed substantially to the work described in this manuscript: Neil Evans, Rachel Forndike, Douglas Foster, Jo Grindrod, Vasantha Padmanabhan, James Taylor and Will Unsworth. Andrew Dady, Jonah Jones, Malcolm McColl and Martin White who took care of the animals made these studies possible. The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 13 June 2006
First decision 20 July 2006
Accepted 14 August 2006

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