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* Manuscript

Sexual Dimorphism in Environmental Epigenetic Programming

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Running title: Sexual dimorphism

ABSTRACT

The phenotype of an individual is the result of complex interactions between genotype and current, past and ancestral environment leading to a lifelong remodelling of our epigenomes. The vast majority of common diseases, including atherosclerosis, diabetes, osteoporosis, asthma, neuropsychological and autoimmune diseases, which often take root in early development, display some degree of sex bias, very marked in some cases. This bias could be explained by the role of sex chromosomes, the different regulatory pathways underlying sexual development of most organs and finally, lifelong fluctuating impact of sex hormones. A substantial proportion of dimorphic genes expression might be under the control of sex-specific epigenetic marks. Environmental factors such as social behaviour, nutrition or chemical compounds can influence, in a gender-related manner, these flexible epigenetic marks during particular spatiotemporal windows of life. Thus, finely tuned developmental program aspects, for each sex, may be more sensitive to specific environmental challenges, particularly during developmental programming and gametogenesis, but also throughout the individual's life under the influence of sex steroid hormones and/or sex chromosomes. An unfavourable programming could thus lead to various defects and different susceptibility to diseases between males and females. Recent studies suggest that this epigenetic programming could be sometimes transmitted to subsequent generations in a sex specific manner and lead to transgenerational effects (TGEs).

This review summarizes the current understanding in the field of epigenetic programming and highlights the importance of studying both sexes in epidemiological protocols or dietary interventions both in humans and in experimental animal models.

Keywords: Programming; Epigenetics; Sexual dimorphism; Nutrition; Environment; Transgenerational effects

ABBREVIATIONS

CYP, cytochrome P450; Cyp 2d-9, steroid 16 alpha-hydroxylase; DES, diethylstilbestrol; DMR/ICR, differentially methylated regions/ imprinting control region; DNMT1, DNA methyl transferase 1; fMRI, functional magnetic resonance imaging; GH, Growth hormone; GHRH, Growth Hormone Releasing Hormone; GR, glucocorticoid receptor; HC, high-carbohydrate; HDAC, histone deacetylase; HFD, high fat diet; HLG, high licking grooming; HNF3γ, HNF4α and HNF6, hepatocyte nuclear factors 3 gamma, 4 alpha and 6; IAP, intra-cisternal A particle; Igf2, insulin-like growth-factor 2; LG, licking and grooming ; LINE, long interspersed nucleotide element, LPD, low-protein diet; LTR, Long terminal repeat ;MeCP2, methyl CpG binding protein 2; PAR, pseudo autosomal region; PPARalpha, peroxisome proliferator- activated receptor-alpha; SINE, short interspersed nucleotide element; Slp, sex-limited protein, S/MARs, Scaffold/Matrix Attachment Regions; STAT5b, signal transducer and activator of transcription 5b; SRY, Sex-determining Region of the Y chromosome; TGE, transgenerational effects;

SEXUAL DIMORPHISM

The vast majority of common diseases, including atherosclerosis, diabetes, osteoporosis, asthma, neuropsychological and autoimmune diseases, display some degree of sex bias,. Moreover, quite often the risk of developing complex disease in offspring depends on the sex of the affected parent. The relevance of epigenetic mechanisms underlying the physiological differences between sexes, particularly in drug metabolism, fits well into the epigenetic theory of complex disease (reviewed in [1]).

Extent of global sexual dimorphism:

The regulatory pathways underlying sexual differentiation clearly result in extensive differences in gene expression in adults. The genetic and transcriptional mechanisms regulating differences between the sexes have intensively been investigated in the liver but dimorphic gene expression has also been reported in mouse kidney, blastocysts, lacrimal gland, and brain [2-5].

A recent microarray analysis of 23,574 transcripts by Yang *et al*. revealed the extent of sexual dimorphism in gene expression to be much greater than previously recognized. The degree of sexual dimorphism ranged from 14% (in the brain) to 70% (in the liver) of active genes. These genes displayed highly tissue-specific patterns of expression, correlated with high levels of activity of distinct pathways. Differences in expression level of a factor of less than 1.2 between tissues were observed for 70% of the sexually dimorphic genes. Most molecular studies of sexual dimorphism have focused on genes displaying large differences in expression between sexes. These genes are likely to be important for sex-specific physiological functions, but a large number of genes displaying small differences in expression between the sexes could well contribute to sexual biases in susceptibility to common diseases. Interestingly, these genes displayed evidence of clustering not only on the sex chromosomes, but also on several autosomes [6].

The sexual differentiation of the brain

The brain is bipotential but develops differently in males and females under the influence of sex steroid hormones during the perinatal period. In the brain, testosterone is metabolized to estradiol by aromatase or to dihydrotestosterone by 5a-reductase. Estradiol and dihydrotestosterone then act on estrogen and testosterone receptors, respectively, to sculpt the brain. In male rats, androgen secretion from the differentiated testis leads to two perinatal peaks in plasma testosterone concentration, the first of which occurs on day 18 of gestation, and the second, approximately 2h after birth [7,8].

While sex hormones undoubtedly play an important role in the sexual differentiation of the brain, other mechanisms may be involved in this phenomenon. Indeed, the identification of genes differentially expressed in male and female mouse brains even before the formation of the gonads (at E10.5) suggests that genetic factors may also influence the sexual differentiation of the brain [9,10].

Sex differences in nuclear volume or neuron number are often attributed to the hormonal control of cell death. The ratio of antiapoptotic proteins to proapoptotic proteins plays a key role in determining whether a cell survives or undergoes apoptosis [11-13]. In specific brain areas, testicular hormones decrease cell death during perinatal development. Males therefore have more neurons in these areas during adulthood. Conversely, more cells die during development and there are fewer neurons in adulthood in other areas of the hypothalamus of males than in that of females [13]. Recent advances in imaging technology i.e. functional magnetic resonance imaging (fMRI) have made it possible to show that numerous brain structures develop and/or function in a sexually dimorphic manner [14].

It's not all hormones. The roles of sex chromosomes

In order to accommodate recent findings, it has been proposed that sexual dimorphism precedes gonadal development. However this does not take into account the many important effects of perinatal secondary sexual differentiation and may only be true for a minority of sex-related traits.

Mammalian sexual differentiation was assumed to be initiated by the presence or absence of the testis-determining factor SRY, encoded on the Y chromosome, in a very narrow spatiotemporal window restricted to the Sertoli cells between 6 and 7 weeks of gestation. This maleness factor induces the production of testes, which secrete hormones responsible for male secondary sexual differentiation [15]. However, female development is not carried out by default since recent studies suggest that both Y and X sex-chromosomal primary mechanisms of sex determination probably exist [16]. In addition, sex-chromosomal sex-determining genes can influence not only the development of non-gonadal secondary sexual organs but also of organs outside of the reproductive system, such as brain [16].

Indeed at the level of the whole body, the sex chromosomes are crucial for establishment of sex-dimorphism of cellular functions. As illustrated in

figure 1, all male cells (Fig1A) possess a single X chromosome of maternal origin and a Y chromosome of paternal origin. Female cells consist of two populations, both of which possess two X chromosomes (Fig1B). In one population, the maternally inherited X is inactivated while in the second population the paternally inherited X is inactivated. Overall gene expression in a female tissue is the average of gene expression in these two populations.

Several classes of genes may be expressed in a sexually dimorphic manner, depending on their status and position on the X and Y chromosomes (Figure 1) (i) Y-specific genes are solely expressed in male, (ii) genes that escape X inactivation will be more highly expressed in female, (iii) maternally expressed X-linked imprinted genes subject to X inactivation are more highly expressed in male than in female and (iv) paternally expressed X-linked imprinted genes will be solely expressed in female. Other categories of genes might be equally expressed in male and female, This includes genes that are subject to X inactivation, maternally expressed X-linked imprinted genes which escape X inactivation and genes of the pseudoautosomal region (PAR), which is common to both X and Y chromosomes and escapes X inactivation. Gene expression in both male and female cells is likely to be influenced to some extent by external factors, including social influences and the hormonal milieu. As a consequence of this random female mosaicism, it is possible that certain traits, such as cognitive traits, show a greater degree of variability amongst females than amongst males.

EPIGENETIC PROGRAMMING

All our tissues contain the same 20,000 genes. However, only a few of these genes are expressed in a given tissue, at a given stage, and at a given time of day (or season), giving rise to the phenotype. To ensure proper gene expression, the epigenetic code comprises several levels of interconnected and interdependent codes: the DNA methylation code, the histone code (histone methylation, acetylation and phosphorylation) and the coregulator code that "orchestrate" the activity of the genome together with RNA interference. The epigenetic codes define a process involving the recruitment of a myriad of chromatin-remodeling complexes, insulator proteins, histone exchange chaperones, enzymes, coregulators and effectors, directing appropriate chromatin remodeling, i.e. tightly or loosely wound chromatin. DNA methylation and histone modification are mechanisms that participate to different but distinct processes. For instance, parental imprinting uses these mechanisms for monoallelic expression of certain genes, whereas perinatal environmental influences may use such mechanisms to modify the activity of certain promoters.

Moreover the nuclear compartimentalisation of ‘transcription factories’ and chromosome territories as well as the high-order level of chromatin conformation, enabling proper gene positioning, represent a new dimension of regulatory control that is related to epigenetic marks and organization [17,18].

The special case of genomic imprinting

Epigenetic mechanisms involved in the regulation of monoallelic expression of imprinted genes represent a special case. Their expression is determined according to their parent of origin. Imprinting affects between 90 (*bona fide*) and several hundreds (presumed) genes. A strong sexual dimorphism underlies major aspects of imprinted gene regulation [19]. Imprinting has been recognized as one of the epigenetic mechanisms, whose reprogramming occurs in the gametes. In these cells, the inherited imprints are erased and a new one, maternal or paternal specific is reestablished. Imprinted genes are particularly involved in embryonic development and metabolism, and imprinting dysregulation is linked to cancer, obesity, diabetes, and behavioral disorders such as autism and bipolar disease. Moreover there are several examples of long lasting impact of environmental factors on the epigenetic processes that modulate the expression of imprinted genes but the mechanisms involved may be different than those for « ordinary » biallelically expressed genes (for a review see [20]).

Dynamics of epigenetic programming

Upon fertilization, the gametes undergo a drastic reprogramming that includes erasure and changes in DNA methylation and histone modification. The paternal genome exchanges protamines for histones, undergoes DNA demethylation, and acquires histone modifications, whereas the maternal genome appears epigenetically more static [19]. During preimplantation development the erasure of DNA methylation is achieved and maintained to almost 10% overall [21,22]. How this residual methylation is distributed remains largely unknown. The removal of the epigenetic marks is essential to ensure the totipotency required for sustaining further development. Embryonic stem (ES) cells are characterized by the presence of bivalent domains of specific histone modifications that silence developmental genes while keeping them poised for activation [23].

After implantation, developmental stages proceed according to a temporally and spatially precise pattern of gene expression associated with changes in the chromatin structure. The epigenetic mechanisms in the early embryo not only involve

de novo DNA methylation and changes in histone modifications but may also include histone replacement [24]. Various replication-dependent and replication-independent epigenetic mechanisms and DNA repair are involved in developmental programming.

Mechanistic pathways for environmental factors involved in epigenetic reprogramming?

The flexibility of epigenetic marks may render possible for environmental, social and nutritional factors, or endocrine disruptors to alter whole genome- or gene-specific epigenetic landscapes, in a sex-specific manner. These marks include methylation or demethylation of specific CpGs, histone modifications, and transcription factor occupancy responsible for altered expression of a substantial proportion of genes.

Chemical and non chemical environmental factors: drugs, food, toxics, social cues, cultural factors, can have specific impacts, depending on their direct/indirect access to the epigenetic machinery and chromatin, on specific sets of target genes and/or at the whole genome level (Figure 2):

1) – Some environmental factors, ageing and gender may target chromatin modifying enzymes [25,26] or their substrate availability. Exogenous/endogenous substrates after passive or active entry through the cell membrane undergo cell specific metabolism. Folates and methionine are the precursors in the biosynthesis of S-adenosyl methionine (SAM), the principal methyl donor for the methylation of DNA and histones. Thus agents that modulate one carbon metabolism or directly affect levels of SAM might have an effect on epigenetic programming [27]. Moreover, metabolites such as resveratrol and sulphoraphane, or drugs such as valproate and trichostatine A (TSA) are specific inhibitors of different members of the large family of HDACs [28]. Some of these HDAC inhibitors were shown to achieve DNA demethylation in the presence of the DNA methylation inhibitor 5-azaC, thus emphasizing the links between DNA methylation and histone modifications mechanistic pathways [29]. Due to the complexity of the epigenetic machinery it is important to unravel the differential role of the different participants in a given physiopathological condition, at a given age and for the different sexes. Thus endogenous or exogenous compounds may lead to the alteration of a critical balance of ch romatin remodelling enzymes, not only for specific sets of dysregulated genes but also at the whole genome level.

2) – Some other compounds specifically bind to nuclear receptors (NR): Several mechanisms may be involved [30]: NRs, like steroid receptors, may be present in the cytoplasm, bind to their ligand, undergo several modifications and be subsequently translocated to the nucleus where they bind to their responsive elements (RE). Environmental compounds like endocrine disruptors may bind to estrogen and testosterone receptors and trigger the same (or slightly different) effect as natural ligands. Other NRs, like PPARs (peroxisome proliferator-activated receptor) and RXR (retinoid X receptor), are already dimerized in the nucleus on their RE within the promoter of target genes. Their binding to a complex of corepressors and HDAC prevents transcription of these genes in the absence of PPAR/RXR ligands. Upon binding with their natural polyunsaturated fatty acids ligands, or drugs like fibrates, allosteric rearrangements lead to the recruitement of coactivators and chromatin remodeling factors, forming a transcription-prone chromatin complex that activates or inhibits ch romatin modification enzymes. The appropriate modifications of the epigenetic marks at PPAR/RXR RE in target gene promoters modulate the expression of the set of genes, in a tissue-specific manner depending on the presence of appropriate cofactors [31].

3) - Traditional membrane receptor–signalling cascades may be involved [32,33]. The basic idea proposed by Szyf and coworkers [34] is that behavioral exposures fire signalling pathways in the brain which in turn activate sequence specific factors that target HATs to specific targets facilitating DNA demethylation. Such a mechanism provides a conduit through which both social and behavioral experiences as well as chemical factors could affect our epigenome and thus gene expression and function. It is possible, depending on the type of ligand, or spatiotemporal conditions, that different pathways could be used. The maintenance of DNA methylation patterns is dependent on the preservation of the balance of factors such as DNA methyl transferase/demethylase, histone acetyl transferase/deacetylase, or histone methylase/demethylase. Extra or intracellular signalling pathways could trigger activation of one of these factors and result in loci-specific histone acetylation and tilt the balance toward DNA demethylation. Like the previous mechanism involving NR targeting, signalling pathways modulate the expression of specific sets of genes, in a tissue-specific manner depending on the presence of appropriate cofactors.

Target sequences

In developmental programming accumulating evidence suggest that specific environmental factors such as sex, diet, age, social, and generation can affect several types of sequences, associated with specific epigenetic chromatin alterations. These include unique copy genes [35-38], or multiple copies of genes such as rDNA[39] (within their promoters, CpG, CpG island) ; imprinted genes (promoters,

DMR/ICR) [40]; Scaffold/Matrix Attachment Regions (S/MARs) or AT islands, the mediators of nuclear compartmentalization and dynamics which, being targets for certain drugs, are also potential targets for nutrients[41]; Repeated sequences or transposons (LINE, LTR, DNA, SINE, Alu in man, Satα, Sat2, or IAP in the mouse) which can display altered epigenetic features due to abnormal growth, methyl donors supplementation or bacterial infection [42,43]. Altogether, in a given malprogramming situation, different types of sequences may be epigenetically altered implying the simultaneous involvement of different mechanistic pathways.

Implication of unique gene(s) is illustrated by increased pup licking and grooming (LG) by rat mothers which conditions the reactivity to stress of the offspring, in adulthood. This maternal behavior influences the offspring epigenome at the glucocorticoid receptor (GR) gene promoter in the hippocampus during the first week of life. Differences in DNA methylation and histone acetylation pattern between the offspring of female rats with high and low levels of LG behavior (HLG vs LLG) are associated with changes in transcription factor binding to the GR gene in the hippocampus [44].

Dietary protein restriction in pregnant rats induces gene-specific epigenetic modification of hepatic gene expression in the offspring. After weaning the hepatic level of GR and PPARalpha promoters methylation of the offspring was lower and the expression of the corresponding gene higher in restricted pups. Histone modifications are also induced at the GR promoter [36]. Uteroplacental insufficiency alters DNA methylation, with genome-wide DNA hypomethylation and large amounts of acetylated histone H3 after birth in the liver [45]. In the kidney, decreases in CpG methylation of a specific site within the promoter of the p53 gene and relative hypomethylation of the DNMT1 gene are observed [46]. Epigenetic determinants (DNMT1, MeCP2, HDAC and zinc levels) of chromatin structure are also affected in the brains of neonatal and juvenile rats born with IUGR [26]. Prenatal and suckling exposure to a diet rich in animal fat leads to whole body insulin resistance and pancreatic beta-cell dysfunction in adulthood, which is preceded by reduced tissue mtDNA content and altered mitochondrial gene expression [47]. The epigenetic alterations are both sex- and tissue-specific and the mechanisms involved are not mutually exclusive as different types of sequence may be simultaneously involved [26].

The epigenetic bases of sexual dimorphism:

Adult patterns of sexual dimorphism are set during the neonatal period by exposure to gonadal steroids, which programs the hypothalamo-pituitary axis and the regulation of growth hormone (GH) secretion at the onset of puberty and during adulthood. These effects are exerted at the level of the hypothalamus to modulate the number of hypothalamic neurons controlling GH secretion, their responsiveness to later steroids, and the establishment of synaptic connectivity and neuropeptide production. In the anterior pituitary, gonadal steroids modulate the numbers of somatotrophs and their responsiveness to inputs controlling GH synthesis and secretion. In the post-pubertal animal, androgens and oestrogens modulate hypothalamic somatostatin and GHRH synthesis respectively. These effects may be direct as somatostatin neurons express the androgen receptor and many GHRH neurons are oestrogen receptor positive [48] (Figure 3).

The difference in GH secretion in the plasma, which is pulsatile in males while continuous in females, appears to be crucial for sex-dependent effects on the liver in many species. Numerous hepatic genes, including those encoding cytochrome P450 (CYP) enzymes are indeed transcribed in a sex-dependent manner [49] (Figure 3). Sex differences in *CYP* expression are particularly striking in rats and mice (up to 500-fold differences between males and females), and such differences, although smaller, are also observed in humans. These differences are an important determinant of the sex dependence of hepatic drug and steroid metabolism. Mouse genes like the sex-limited protein (Slp), and the steroid 16-alpha-hydroxylase (Cyp 2d-9) display male-specific hepatic expression under the influence of male GH pulses at puberty and adulthood. Analyses of their promoter activity showed that these genes harbor a regulatory element in which a particular CpG is demethylated to a much higher degree in males than in females. The sex-specific expression patterns of these P450 genes highly correlate with DNA demethylation [50,51]. The sex-specific methylation profile of such specific CpGs or any other histone modification(s) underlying sex-specific expression might be altered by environmental nutritional factors, or pollutants (endocrine disruptors) in a sex-specific manner. In turn, this sex-specific epigenetic alteration may inhibit the binding of transcription factors, or have direct consequences for nucleosome positioning. Recent data showed that hypophysectomy abolished the sex specificity of approximately 90% of 1032 sex-dependent genes, consistent with the dominant role of pituitary GH in regulating liver sexual dimorphism [52]. It could be tempting to generalize these effects to other organs however it must be kept in mind that the examples cited above concern only sexual dimorphism of liver gene expression.

TRANSGENERATIONAL EFFECTS

During critical periods of life (periconception, fetal and infantile development), exposure to deleterious environmental compounds, abnormal maternal behaviour or inadequate maternal feeding can induce, in the offspring, various lesions and susceptibility to diseases which can be sometimes transmitted to subsequent generations — leading to transgenerational effects (TGEs). Most early studies assumed that TGEs resulted from the malprogramming of epigenetic somatic processes. However paternal or maternal germline epigenetic inheritance may also account for these TGEs [53-55]. Moreover, both somatic and germline effects may be sexually dimorphic, and, through the maternal line, can affect both the mitochondrial and the nuclear DNA [47] (Figure 4). Although our understanding of the fundamental biological mechanisms underlying such sex-specific phenomena remains rudimentary, these effects could be due to cytoplasmic, hormonal or metabolic influences, selective effects on gametogenesis in one sex but not in the other, or the sex-specific reprogramming of imprinted genes (IG) expression (reviewed in [56]).

Vicious cycle of mother-to-offspring transmission through iterative somatic epigenetic malprogramming

Several recent studies have highlighted the influence of maternal physiological, behavioural or nutritional conditions on the establishment of a vicious cycle of mother-to-daughter transmission through modifications of the uterine environment triggering somatic developmental malprogramming.

Physiological changes in females associated with ageing, may affect the growth and reproductive traits not only in their direct offspring, but also in subsequent generations. Early-adolescent and middle-aged pregnant mice have less testosterone than young-adult pregnant mice. F2 pups with young-adult grandmothers are significantly heavier. A small increase in the levels of estradiol or other estrogens during the fetal development of female mice is also associated with earlier puberty [57].

In human, both the mother and father being small for gestational age significantly influence the risk of their offspring being small for gestational age. In addition, young women born small for gestational age tend to display hypergonadotrophinemia and a smaller uterus and ovaries than normal [58]. With the common shift toward very late pregnancies in human populations, the influence of age-related changes in levels of estradiol and testosterone requires further investigation. Moreover, vascular dysfunction in the programmed mother will also provide a deprived intrauterine environment to its offspring, thus perpetuating the cycle of fetal (mal)adaptation [59].

Recent studies in the rat suggest that the perpetuation of the disease risk through mother-to-offspring transmission may involve somatic epigenomic alterations. Early postnatal variations in maternal behavior (HLG vs LLG) are associated with differences in the cytosine methylation of the estrogen receptor ERα1b promoter and in ER expression in the medial preoptic area and are transmitted across generations. Mothers with HLG behavior will teach their female offspring to behave in a similar manner [60,61]. Thus mother-to-daughter epigenetic transmission may affect somatic tissues, not necessarily the germline, perpetuating the effect by affecting maternal metabolism or maternal behavior.

Sex-specific differences in germline transmission

In addition to these malprogramming epigenetic somatic processes there are now also clear examples of transmission through the germline for both sexes, with sex-specific effects [62-64].

The hypothesis that a female germline transmission can occur in addition or independently to the developmental somatic effects of the uterine milieu was recently demonstrated using cross-fostering experiments. Female F2 rats, procreated by F1 pre- and postnatally nutrient- and growth-restricted (IUGR) mothers but embryo transferred to gestate in control mothers were compared with similarly gestating age- and sex-matched control F2 progeny. It was shown that the transgenerational inheritance of aberrant glucose metabolism and skeletal muscle insulin signaling in the adult F2 IUGR female offspring was independent of the immediate intrauterine environment [35]. A male-mediated TGE on metabolism- and growth-related parameters has also been identified. Periconceptional paternal food deprivation induced, in both male and female offspring, a decrease in serum glucose concentration and changes in corticosterone and insulin-like growth factor-1 (IGF1) concentrations [64].

The proximity of transposable elements may render genes epigenetically labile, as demonstrated for two mutant mice harboring an insertion of an intra-cisternal A particle (IAP) retrotransposon: the Agouti viable yellow A^{vy} and Axin Fused $Axin^{Fu}$ mice. CpG methylation of the IAP varies considerably in A^{vy} mice and the intensity of expression of the gene depending on the methylation level, leads to a coat color ranging from yellow to pseudoagouti [54,65]. The proportion of pups with a phenotype corresponding to a methylated IAP depends on the mother's own phenotype, and therefore on the level of

methylation of the mother's own IAP sequence at the A^{vy} locus [66,67]. Thus the A^{vy} represents a sensitive epigenetic biosensor to assess the effects of dietary supplementation on locus-specific DNA methylation[68].

Epigenetic inheritance occurs at the A^{vy} locus. The A^{vy} epigenotype is partially transmitted when passed through the female line, but not through the male line. On the contrary, the $Axin^{Fu}$ locus displays epigenetic inheritance following both maternal and paternal transmission, but depending on the genetic background of the strain [54]. Maternal inheritance at the A^{vy} locus could be due to cytoplasmic, hormonal or metabolic influences, whereas paternal inheritance at the $Axin^{Fu}$ locus is not consistent with cytoplasmic influence. Indeed, in sharp contrast with the egg, the sperm does not contribute its cytoplasm to the zygote. Consistent with the transgenerational inheritance of epigenetic marks, Rakyan *et al.* showed that the methylation state of $Axin^{Fu}$ in mature sperm reflects the methylation state of the allele in the somatic tissue of the animal, suggesting that it does not undergo epigenetic reprogramming during gametogenesis [54].

It is widely accepted that the contribution of fathers to the next generation is limited to half their genome. However, this contribution appears to have been clearly underestimated, and the factors delivered by the sperm at fertilization are currently under research. Notably, a complex population of spermatozoan coding RNAs is delivered to the oocyte on fertilization and could have crucial developmental functions [63,69].

SEXUAL DIMORPHISM IN CONSEQUENCES ON OFFSPRING

In human and several species, sex-related differences in cardiovascular function, insulin sensitivity and subsequent susceptibility to diabetes and hypertension have been clearly demonstrated. Nevertheless, the precise mechanisms underlying these sex-related differences are still poorly understood. In human, small size at birth is associated with increased insulin resistance and hyperinsulinemia in young adult life but these relationships are restricted to the male gender in this age group [70]. Premenopausal women have lower arterial blood pressure than men matched for age and post-menopausal women, suggesting a role of ovarian hormones in blood pressure regulation [71]. In rats, feeding a diet rich in lard to pregnant females leads to gender-related cardiovascular dysfunction in normally fed offspring as blood pressure was found to be high in the female but not in the male offspring [72]. A maternal low-protein diet (LPD) during pregnancy and lactation modifies the growth and metabolism of the progeny (F2) of the female offspring (F1) [73,74]. Maternal undernutrition, restricted to the preimplantation period in rat development, causes blastocyst abnormalities and the programming of postnatal hypertension. Male blastocysts displayed a 30% decrease in H19 mRNA level, which was not observed in female blastocysts. Maternal undernutrition also led to significantly lower levels of H19 (9.4%) and Igf2 (10.9%) mRNA in male, but not in female, fetal liver. These differences may result from the sex-specific programming of imprinted gene expression within the preimplantation embryo itself [40,75,76]. Postnatal changes in cerebral chromatin conformation in rats born with IUGR are also sex-specific [26]. Prenatal exposure to dexamethasone of males mated with control females lowered birth weight in their progeny of either sex. However when dexamethasone-prenatally exposed female rats were mated with control males only the male part of the offspring was affected [62].

Other data suggest that environmental factors may have a direct influence on gametogenesis in one sex, but not in the other. The endocrine disruptor vinclozolin which acts as an anti-androgenic molecule, exerts transient effects at the time of embryonic sex determination, and leads to subfertility in F1 males associated with a spermatogenic cell defect [77]. This poor fertility resulted from modification of the methylation pattern of a series of genes and was inherited, through the male germline, by almost all the males in the next four generations [53]. This is also the case for paternal exposure to the anticancer drug, cyclophosphamide, which has been shown to modify germ cell quality, disrupt embryo development and dysregulate zygotic gene activation in the rat [78]. Sex- and tissue-specific methylation maintenance and *de novo* DNA methyltransferase synthesis following low-dose X-irradiation have also been observed in mice [79-81].

Epidemiological data and case studies suggesting or demonstrating the existence of TGEs with sexual dimorphism are also available for humans [82-86]. Undeniably, epigenetic processes provide the most plausible explanation for these observations, but the involvement of such processes in human developmental programming or in any epidemiological instance of transmission to subsequent generations has yet to be demonstrated.

Thus, sex-specific differences in the timing of and mechanisms involved in gametogenesis, post fertilization development, sexual differentiation of the gonads, gonad development and hormonal status may result in different effects of environmental challenges not only on the mother and father, but also on the female and male offspring with window-of-exposure-specific effects on the offspring.

CONTINUOUS/DISCRETE EXPOSURE TO THE INITIAL STIMULUS AND PERSISTENCE FOR SEVERAL GENERATIONS

In most animal models in which the existence of TGEs has been established, only the first-generation were subjected to the stimulus: endocrine disruptors, low-protein diets, betel-nut chewing, radiotherapy as used for cancer treatment, particular types of maternal behavior, folate-deficient diets, glucocorticoids etc., and still little is known about the cumulative effects of exposure over several generations. It has been proposed that for transgenerational inheritance, phenotypic changes should be maintained up to at least the F3 generation [87]. Accordingly it must be stressed that in some cases of maintenance of trait to F2, such cases can derive from direct exposure of F1 fetal gonadal cells via exposure of the F0 gestating female [88]. However, as previously described above, through a vicious cycle of mother-to-offspring transmission, iterative somatic alterations occurring in the womb and/or in the postnatal period under the influence of social, metabolic, nutritional or toxicological environmental factors do represent TGEs that do not necessarily affect the germline [34,89,90].

Effects present in the F1 persisting to the F2 and beyond

Endocrine disruptors have been shown to promote a transgenerational epigenetic phenotype involving a number of disease states (e.g. male infertility). Following exposure to vinclozolin of the F0 mother only, the phenotype was transferred through the male germline to all subsequent generations analyzed (F2 to F4). Small cell carcinoma of the ovary, a tumor generally rare in adolescence, was reported in a girl whose maternal grandmother had been taking diethylstilbestrol (DES) while pregnant with the patient's mother [88]. This example shows that TGEs may even skip generations. Therefore, since the insult and the deleterious effects may not be contemporaneous this has implications for assessments of the potential hazards of environmental toxins, mechanisms of disease etiology, and evolutionary biology [77].

Early malnutrition (LPD) impaired the development of the endocrine pancreas, decreasing beta-cell mass in the first generation of offspring and impairing subsequent beta-cell adaptation to pregnancy. This beta-cell alteration was also present in the next generation [91]. Glucose metabolism was shown to be altered in the adequately nourished offspring of the offspring of rats malnourished (LPD) during gestation and perinatal life, demonstrating the persistence of the effects in the third generation [89]. Undernourishment *in utero* produces striking insulin resistance in genetically normal, well-nourished second-generation rats. If these rats are fed a high fat diet (HFD), the effect is even more pronounced [92].

The male offspring of female rats exposed *in utero* to dexamethasone, but not exposed to this compound during their own pregnancy, also have a low birth weight, glucose intolerance and high levels of hepatic glucose production. However, these effects have been shown to resolve in the third generation [62].

In only one study, carried out two decades ago, Stewart *et al.* studied colonies of rats maintained for twelve generations on diets with adequate levels of protein or marginally deficient in protein. In the malnourished colony, the proportion of "small-for-gestational-age" offspring was ten times higher than that for the well-nourished colony [93]. Similarly, Pinto *et al.* recently reported the cumulative effects of exercise stress over successive generations [94].

Effects present in the F1 may not persist to the F2

It remains unclear whether these changes can always be transmitted to the next generation and whether there are cumulative effects of supplementation across successive generations. As shown by Waterland *et al*, maternal obesity during pregnancy can cause metabolic imprinting in the a/a offspring of A^{vy}/a obese mice, perpetuating obesity across generations. However it has been shown recently that diet-induced hypermethylation at the A^{vy} locus in mice is not inherited transgenerationally through the female [95]. These results suggest that, in the female germ line, diet-induced A^{vy} hypermethylation occurs in the absence of additional epigenetic modifications that normally confer transgenerational epigenetic inheritance at the locus [95]. Similarly, Armitage *et al* recently showed that programmed aortic dysfunction and reduced Na+K+-ATPase activity present in first generation offspring of lard-fed rats does not persist in the second generation [96]. Altogether these data strongly suggest that – due to probably subtle differences in genetic background, species, gender, age, diets, duration and trajectory, type of DNA target, and timing of epigenetic reprogramming – developmental misprogramming may or may not necessarily persist to the next generation(s).

ALLEVIATING MALPROGRAMMING BY DIET OR DRUG?

Epigenetic alterations during the course of developmental processes can lead to irreversibly damaged tissue/organ corresponding to a permanent and « no return » situation. Alternatively other

epigenetic alterations can be partially or completely reversible using appropriate epigenetic tools (nutrients, drugs, lifestyle?) [29,38].

There are now a few examples testing whether transfer of the malprogramming phenotype - due to high-fat, high-carbohydrate diets or to xenobiotic chemicals - to the progeny could be reversed or attenuated by maternal nutritional interventions.

In mice fed a HFD, a striking difference in sensitivity or resistance to the HFD between generations and sexes is observed. When HFD-induced obese mothers are fed a control diet during pregnancy and lactation, there is a shift in the stochastic resistance to a HFD in females. Even when the HFD was supplied *ad libitum*, a significantly increased proportion of F2 females were resistant and remained lean, with normal insulin sensitivity and normal glycemia, but mild hypercholesterolemia and glucose intolerance. These females but not males display a "satiety/resistance phenotype" [97].

Srinivasan *et al* have previously shown that artificial rearing of newborn female rat pups on a high-carbohydrate (HC) milk formula resulted in chronic hyperinsulinemia and adult-onset obesity (HC phenotype) and that the maternal HC phenotype was transmitted to their progeny. A mild dietary restriction reversed their HC phenotype and also prevented the development of the HC phenotype in their offspring [98].

As already mentioned, unbalanced prenatal nutrition (LPD) induces persistent, gene-specific epigenetic changes that alter mRNA production levels, and folic acid supplementation prevents these changes [99,100].

Genistein, a phytoestrogen from soybean, induces gene hypermethylation. In the Agouti A^{vy} offspring maternal supplementation with genistein protected from obesity through modification of the fetal epigenome. This marked phenotypic change was significantly associated with higher levels of methylation of six CpG sites in the IAP retrotransposon at the *Agouti* A^{vy} locus [68]. Maternal nutrient supplementation, with either methyl donors like folic acid or the phytoestrogen genistein, counteracts the bisphenol A-induced DNA hypomethylation of the IAP retrotransposon at the A^{vy} and $Cabp^{IAP}$ loci in mice early development [43].

Central infusion of the HDAC inhibitor (trichostatin A), in adult LLG offspring normalized the hippocampal GR gene expression by enhancing histone acetylation and DNA demethylation that allow increased NGFI-A transcription factor binding. This leads to reversion eliminated the maternal effect on hippocampal GR expression and HPA responses to stress.

In contrast, L-methionine treatment reverses the benefic effect of high maternal LG by inducing active remethylation of the NGFI-A binding site on the GR promoter. Thus despite the inherent stability of the epigenomic marks established early in life through behavioral programming, they are potentially reversible in the adult. Increase in one amino acid (methionine) in the brain could alter DNA methylation and alter behavior in adult [32,101]. Thus dietary changes in methyl contents could affect DNA and or histone methylation and gene expression programming [102].

In contrast, as recently shown by Benyshek et al, postnatal diet determines insulin resistance in fetally malnourished (LPD), low birthweight rats (F1). Insulin sensitivity was significantly reduced in all F2 animals versus control animal. However insulin resistance was not dependent on offspring birthweight and persisted regardless of dietary treatment [89].

CONCLUSION

There is a clear need for us to understand the programming of gene expression in response to the environment for both genders, in early life, throughout life, and beyond. However, we still have too little information to evaluate the actual impact of environmentally triggered TGEs. Are we dealing with an all-or-nothing process? Does it depend on the type of sequence altered? Is a given type of sequence equally affected in every individual? Are there genetic backgrounds conferring susceptibility/resistance to environmentally induced epigenetic alterations and epigenetic inheritance?

Unlike genetic changes, epigenetic marks may be reversible. If the epigenetic marks acquired during developmental programming and through germline inheritance do indeed prove to be reversible, then we will need to determine when, how, and whether to use preventive methods or treatments, such as specific diets, drugs or lifestyle changes. Are there specific epigenetic signatures associated with replication-dependent, replication-independent and repair processes, specific histone variants or posttranslational modifications that might respond differently to specific interventions? Optimal sex-specific "epigenetic diets" should be investigated as part of the prevention and treatment of all these conditions.

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LEGEND TO THE FIGURES

Fig. 1 – Role of the sex chromosome dosage and imprinting in sexual dimorphism

Sex chromosomes consist in a non-automosal pair in which one is inherited from the mother (red rectangle) and the other from the father (blue rectangle). In male (A), the pair is composed of an X and a Y. In female (B), there is two X, one of which is randomly inactivated (black rectangle), leading to two distinct cell populations. A small region is homologous between X and Y: the pseudoautosomal region (white rectangle).

Different classes of genes (arrows) may be expressed in a sex-dimorphic manner: Y specific genes (blue), genes that escapes X inactivation and have a functionnaly different homologue on the Y (red and pink), maternally expressed imprinted genes subject to X-inactivation (white) and paternally expressed imprinted genes subject to inactivation or escaping (orange and purple respectively).

Other genes may be expressed equally in male and female: PAR genes (black), genes subject to X-inactivation (green) and maternally expressed imprinted genes that escape inactivation (yellow).

With permission of [103]).

Fig 2 - Mechanistic pathways for environmental factors involved in epigenetic reprogramming

There is three ways to link environmantal factors such as nutrients or drugs from the cell membrane to the chromatin structure: 1) activation or inhibition of chromatin machinery by metabolites of these substrates, 2) Activation of nuclear receptor by ligands and 3) Traditionnal membrane receptor signalling cascade. Adapted from Sharma et al, with permission of [31]).

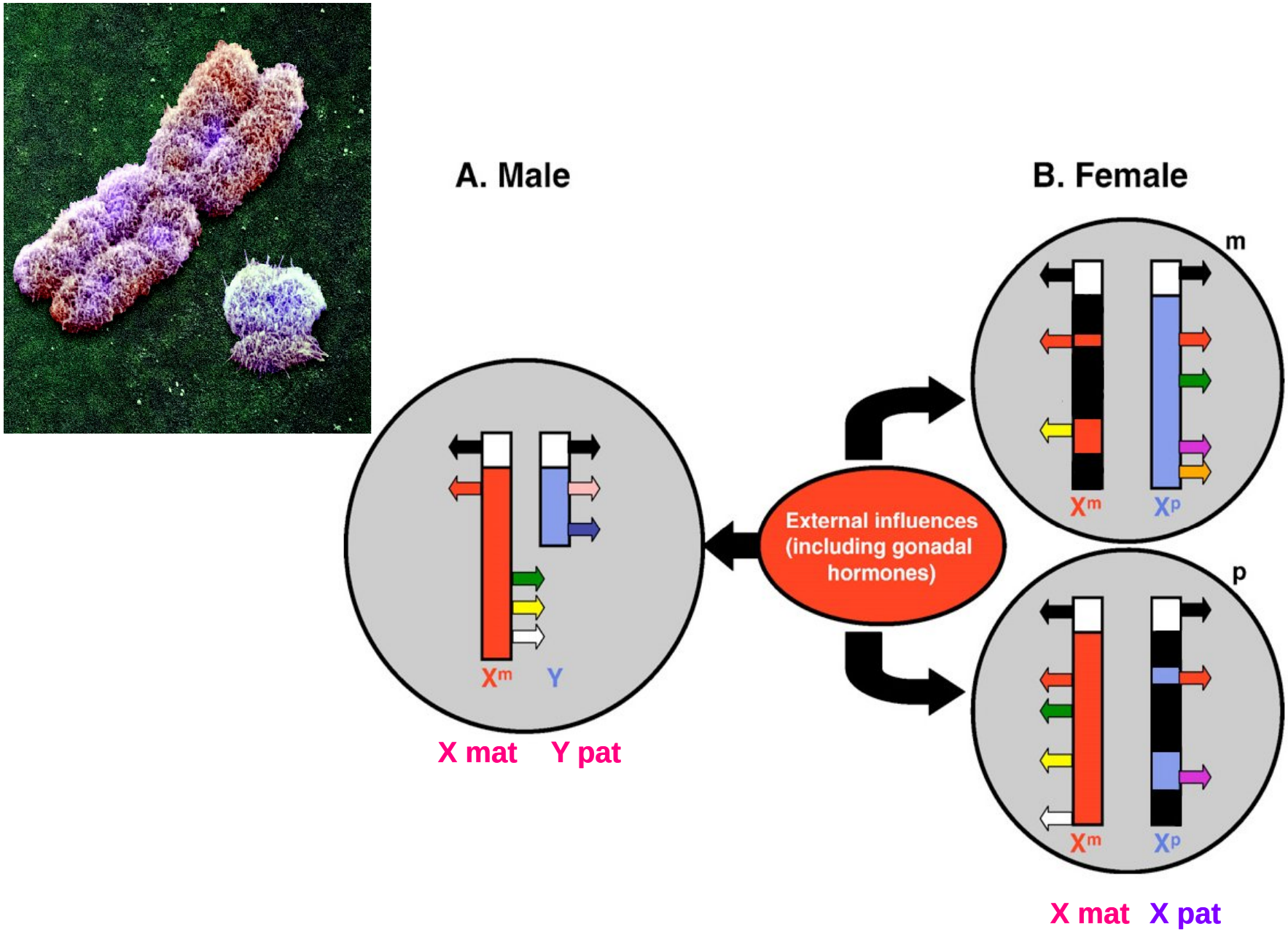
Fig. 3 - The "pulsatile male-specific" versus the "continuous female-specific GH plasma on pathways leading to differential methylation on target genes.

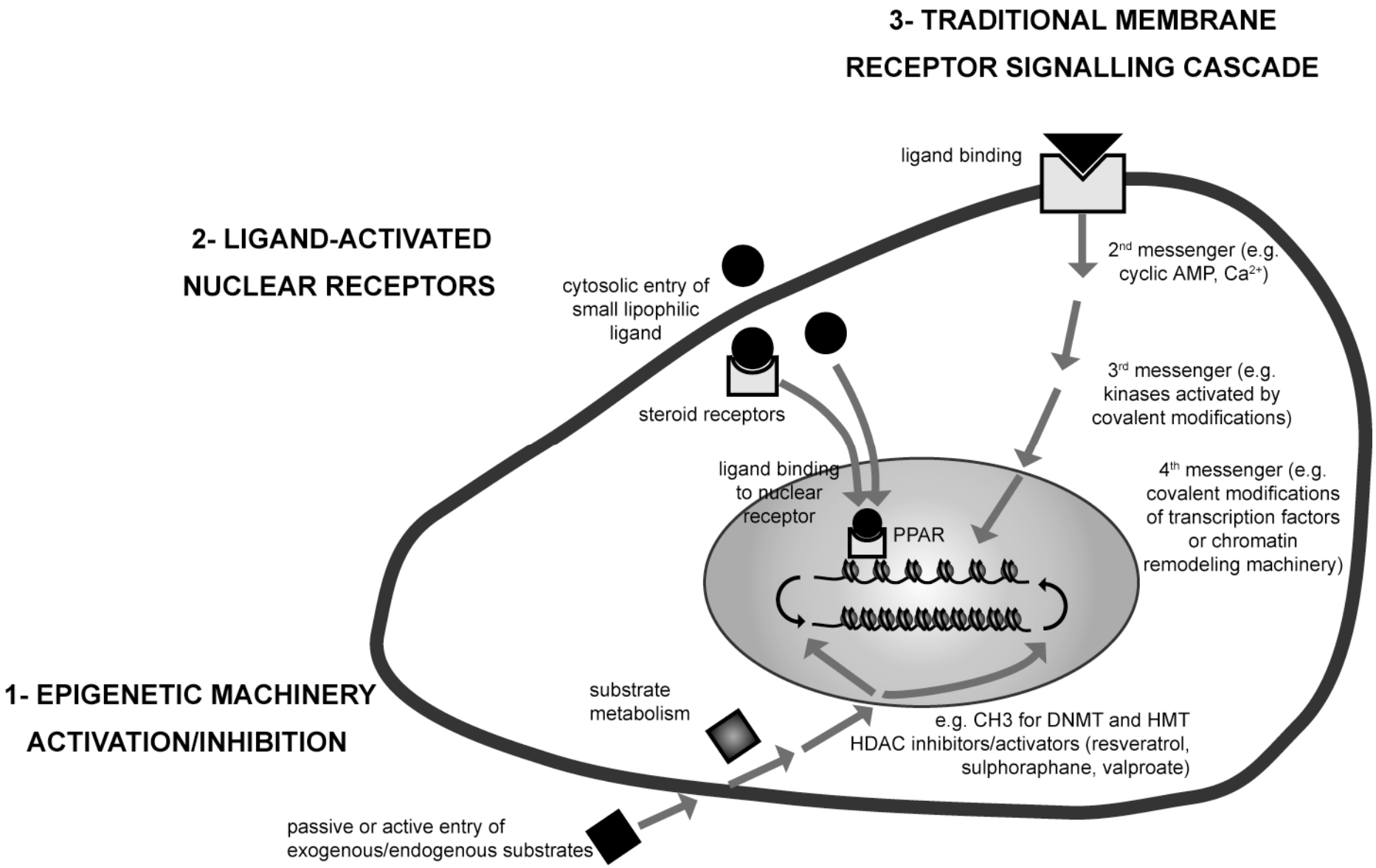
Adult patterns of sexual dimorphism are set during the neonatal period by exposure to gonadal steroids, which programs the hypothalamus and its regulation of pituitary GH secretion at the onset of puberty and during adulthood. Recent findings have implicated the GH-regulated transcription factor STAT5b, hepatocyte nuclear factors 3 gamma (HNF3γ), 4 alpha (HNF4α) and 6 (HNF6), and sex differences in DNA methylation and chromatin structure in the sex-dependent actions of GH [49,104]. The "pulsatile male-specific" GH exposure activates liver STAT5b (signal transducer and activator of transcription) 5b) by tyrosine phosphorylation, leading to dimerization, nuclear translocation, and transcriptional activation of the STAT, which is thought to regulate the sexual dimorphism of liver gene expression induced by pulsatile plasma GH. No such activation occurs with the "continuous female-specific" GH exposure. STAT5b gene disruption has shown that STAT5b is required for the maintenance of sexual dimorphism in body growth rates and liver gene expression, suggesting that STAT5b may be the major, and perhaps only STAT protein mediating the sexually dimorphic effects of GH pulses in the liver and possibly other target tissues [105]. (With permission from [104]).

Fig. 4 - Sexual dimorphism in the modes of transmission and in the effects on the offspring on successive generations:

The sex-specificity of these effects operates at different levels: 1) the maternal transmission during pregnancy and postnatal periods; 2) the sex of the parent transmitting the consequences of stimulus exposure via the germline; 3) the sex of the offspring displaying the maternal effect or paternal and/or maternal germline TGE.

Figure


A. Male
B. Female
m
p
External influences (including gonadal hormones)
Xm
Y
Xm
Xp
Xm
Xp
X mat
Y pat
X mat
X pat


3- TRADITIONAL MEMBRANE RECEPTOR SIGNALLING CASCADE
ligand binding
2nd messenger (e.g. cyclic AMP, Ca2+)
3rd messenger (e.g. kinases activated by covalent modifications)
4th messenger (e.g. covalent modifications of transcription factors or chromatin remodeling machinery)
2- LIGAND-ACTIVATED NUCLEAR RECEPTORS
cytosolic entry of small lipophilic ligand
steroid receptors
ligand binding to nuclear receptor
PPAR
1- EPIGENETIC MACHINERY ACTIVATION/INHIBITION
substrate metabolism
e.g. CH3 for DNMT and HMT
HDAC inhibitors/activators (resveratrol, sulphoraphane, valproate)
passive or active entry of exogenous/endogenous substrates

Neonatal period

Puberty

Sex hormones

↓

Hypothalamus

↓

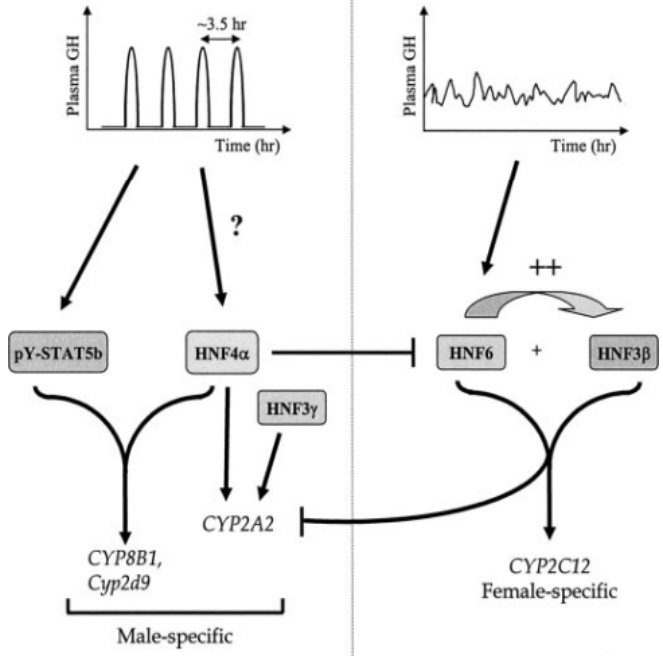
Anterior pituitary

GH

Inputs controlling GH synthesis and secretion

Male

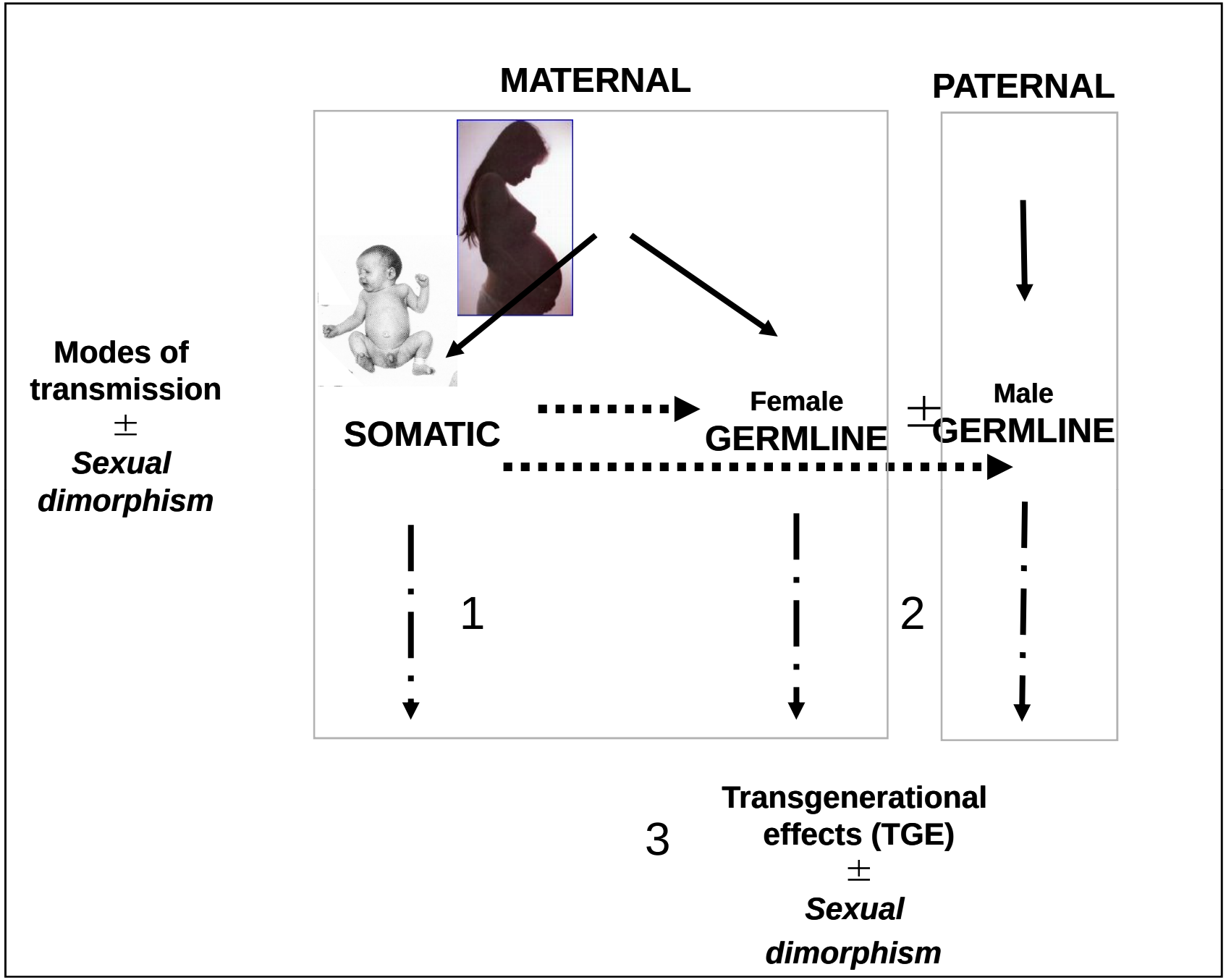
Female



Adult patterns of sexual dimorphism

Sexually dimorphic epigenetic marks

TTCCGGGC **TTC^{m5}CGGGC** **(TF binding site)**


MATERNAL
PATERNAL
Modes of transmission ± Sexual dimorphism
SOMATIC
Female GERMLINE
±
Male GERMLINE
1
2
3
Transgenerational effects (TGE) ± Sexual dimorphism