Sexual differentiation of the brain related to gender identity

- beyond hormones -

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Literature thesis
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Contents

1. Abstract

The sexual differentiation of the brain starts in the second semester of pregnancy, which is, after the development of the genitals which differentiate in the second month of pregnancy. Because these two processes have different timetables, it could be that these are initiated through different pathways. Male gonads synthesize testosterone, which can be converted into estrogen by aromatase in the brain. In humans, the exact mechanism of male and female brain development has still to be elucidated. Based on clinical evidence from genetic men (XY) suffering from a mutation in the androgen receptor gene (complete androgen-insensitivity syndrome) and who show a female phenotype of the external genitals as well as the brain, it can be proposed that direct action of testosterone is probably causing the brain to differentiate in the female direction. However, when the process of genital development and of brain sexual development does not match the same sex, females with a male brain and vice versa can arise. These transsexual people have problems with their gender identity and have the conviction of being born in the wrong body. Twin and family studies show that there are genetic factors influencing the chances of a gender identity problem. Genetic factors could play a large role in the sexual differentiation of the brain, as can be shown from studies where differential genetic expression is found before development of the gonads. These genes could also function in other tissues than gonads and influence the sexual differentiation of the brain. The DMRT gene family which encodes transcription factors or the amount of sex hormone binding globulin (SHBG) is possibly influencing the development of sex differences, just as sex-biased differential splicing. Epigenetic mechanisms such as X-inactivation and genomic imprinting are also good candidates for causing differences in the sexual differentiation of the brain. These observations indicate that probably many processes operate together in the sexual differentiation of the brain and that diverse mutations can lead to gender identity problems.

2. Introduction

During the intrauterine period, the fetal brain undergoes sexual differentiation under the influence of many factors, from which hormones are the best described so far (3; 4). Thus, sexual orientation and gender identity (the conviction of belonging to either the male or female gender, independent of the anatomical reality of the sex) are programmed into various structures of the brain before birth. A few decades ago, it was stated by Alfred Jost that in early ontogeny, the entire reproductive system exists in a basic, unspecialized state, identical in both sexes. A genetic factor produces testes from the undifferentiated gonads, which secrete hormones to induce male development. In the absence of the genetic factor, by default the gonads become ovaries which result in a female. Thus, the testes represent the induced state and the ovary the default state. The sex determining genes influence only the development of the gonads and by consequence the secretion of testicular hormones and are essential and sufficient to cause all secondary sexual developmental features (3). However, just the absence of testicular hormones induced by testicular development by the Y chromosome does not produce a female phenotype; ovarian genes and hormones are necessary. The default situation of the absence of Y does not produce normal ovaries in the absence of a second X (5). Thus, the female phenotype develops only in the presence of two functional copies of at least one X chromosomal gene and not by default. Differentiation of the sexual organs (first 2 months of pregnancy) takes place before the sexual differentiation of the brain (starting in the second semester of pregnancy). As these timetables are different, it might be possible that these two processes take different pathways under the influence of different factors (4). When these two processes do not match to the same sex, a transsexual individual can arise. Transsexuality is characterized by a strong conviction of belonging to the opposite sex. Prevalence is 1:10.000 in male to female transsexuals, and 1:30.000 in female to male transsexuals (6). Besides the feeling of being born in the wrong body, gender-related traits also resemble those of the opposite sex in transsexuals (7). The possible psychogenic or biological aetiology of transsexuality has been the subject of debate for many years. In history several ‘treatments’ against a different sexual orientation and sexual identity were performed: castration, administration of estrogens or testosterone, psychoanalysis, psychosurgery (hypothalamus lesions), electroshock treatment, chemical induction of epileptic seizures and imprisonment. It has never been proven that differences in gender identity are the effect of social learning (8). Therefore, increasing our understanding of how gender

identity develops is crucial to develop a policy of gender assignment for intersex infants. In this literature thesis, the sexual differentiation of the brain and alternative pathways, besides hormones, to induce sexual differentiation in relation to gender identity will be reviewed.

3. Gender development

Sexual differentiation causes permanent changes in the genitals and in brain structures and functions, by interactions of developing neurons with the environment. This environment consists of: surrounding cells, hormones of the mother and of the child, and substances circulating within the mother. When these environmental factors are not normal, differences in gender identity, sexual orientation, gender role and cognition (such as aggressive behaviour) can occur (4). For instance when a pregnant women is stressed, the chance to develop a homosexual son or a lesbian daughter is increased (9).

3.1 Genitals

The human sexual organs and genitals develop in the sixth week of pregnancy. This occurs under the influence of a cascade of genes, in males starting with the sex transcription factor gene on the Y-chromosome, sex determining region Y (SRY). In absence of this gene, the gonad will develop into an ovary. In the developing testes, the sex hormones are produced (fig. 1). Testosterone levels peak between week 6 and week 12, this is necessary for the development of the genitals. Testosterone is converted into dihydrotestosterone (DHT) by 5α-reductase and is essential for the development of the male sexual organs (10). In humans, the differentiation of the genitals has finished in the 13th week, a remarkably early event in a 40 week long pregnancy.

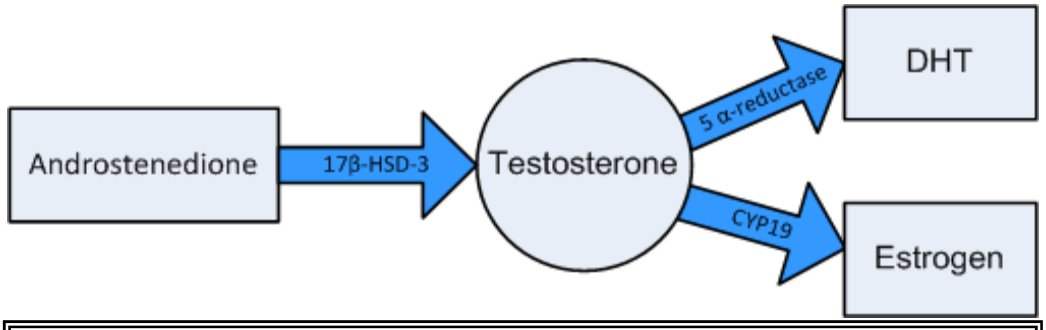

Figure 1 I Sex hormone production. Androstenedione is produced in the adrenal glands and the gonads and is converted by 17β-hydroxy-steroid dehydrogenase 3 (17β-HSD-3) into testosterone. Testosterone can be converted into estrogen by aromatase (CYP19) and by 5α-reductase into dihydrotestosterone, (DHT) which acts on the androgen and estrogen receptors.

3.1.1. Testicular development

SRY is the testes initiating transcription factor on the Y-chromosome, already expressed at the blastocyst stage, which is also known to be expressed in specific cells of the adult brain. When de testes are formed, testosterone is produced which induces masculinizing effects. Besides SRY, several downstream genes are involved (fig. 2). R-spondin 1 was found to induce XY sex reversal in the absence of SRY and can be differentially spliced (11). R-spondin 1 interacts with SRY-box 9 (SOX-9), the accepted candidate for the ultimate testes inducing gene. Evidence for SOX-9 as a testes inducing gene came from XX SOX-9 transgenic mice which develop as apparent males and human sex reversed XY mice with SOX-9 insufficiency. The WNT-4 gene appears to influence both testicular and ovarian development. Overexpression causes upregulation of orphan nuclear receptor DAX-1 and knockout induces masculinization of XX gonads in females. DAX-1 is an X-linked gene which is expressed in the developing ovary and is downregulated in the testis because it is thought to suppress SRY. Overexpression in transgenic animals delays testicular development and can result in sex reversal, which can be observed as ovaries in a genetic male (5). Mutations in human DAX-1 can lead to congenital adrenal hyperplasia (CAH), where females with abnormal masculinized external genitalia are raised as females but change their gender into male around puberty (12; 13).

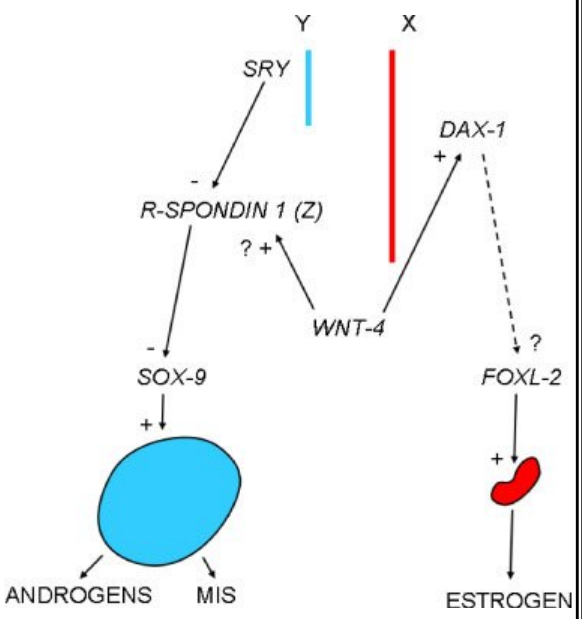

Figure 2 I Genes in sexual development. SRY is the male determining gene on the Y chromosome. It represses the function of a postulated gene, which in turn suppresses the testis-determining factor SOX-9. R-SPONDIN 1 is a recent candidate for this suppressor. The testes secrete androgens, which control secondary sexual differentiation in the male. The absence of this hormone is required for normal female development. DAX-1 is a likely candidate for an X-linked female determining gene, while recent research in goats suggests that FOXL-2 is the proximate ovary-determining gene. The pathway of interaction between the X-linked femaleness gene and the ovary-determining factor remains to be elucidated. Ovarian production of estrogen is essential for normal female secondary differentiation. The gene WNT-4 appears to influence both testicular and ovarian development (obtained from Blecher *et al.*, 2006)

3.1.2. Ovarian development

The classical concept of a default development for the female in the absence of male-determining genes is incorrect. This can be observed already before gonadal development, where in mice 51 genes are expressed differently between males and females before hormones come into play (14). Mutations in the gene forkhead box L2 (FOXL-2) induce a syndrome with ovarian failure and this gene was detected in human granulosa cells, which support normal maturation of the ova. FOXL-2 is therefore defined as the ovarian inducing gene. DAX-1 is a likely candidate for an X-linked gene which induces the female phenotype. However, it is unknown how this gene interacts with FOXL-2.

3.2 Brain sexual differentiation in rodents

The sexual differentiation of the brain is influenced by sex hormones produced by the recently differentiated gonads. This differentiation is a separate process from genital development and is conducted in mid to late gestation in primates and just before and after birth in rodents. The most important sex hormones include testosterone and estrogen. Estrogens are formed when testosterone is aromatized by the enzyme CYP19, better known as aromatase, in the brain (15). Estrogens and androgens (testosterone and DHT) bind to specific receptors, which then bind to specific DNA elements to result in the transcriptional activation of certain genes and thereby sculpting the brain. Estrogen levels in the hypothalamus, the major brain region undergoing sexual differentiation, are two to three times higher in newborn males than females (16), suggesting a sexual dimorphic action of this hormone in the male brain. Administration of testosterone and estrogen to fetal females induces masculinization and defeminization of the brain. In rodent species, the female fetal brain is protected against estrogens produced in high quantities by placenta and which would masculinize and defeminize the brain, by the presence of α-fetoprotein (17). This protein has strong estrogen binding capacities, but does not bind testosterone. At the end of pregnancy, α-fetoprotein levels decline rapidly and the fetus is more exposed to estrogens (4), which inhibit the hypothalamus-hypophysial-gonadal axis. Besides functioning as a transcription factor, estrogen receptors can also reside in the membrane, where they directly interact with various protein kinases to directly activate signal transduction pathways. To make it even more complex, estrogen shows region specific activity; it can

stimulate excitation and inhibit excitation, it is neuroprotective and neurodamaging, depending on the brain region and the conditions (fig. 3) (18).

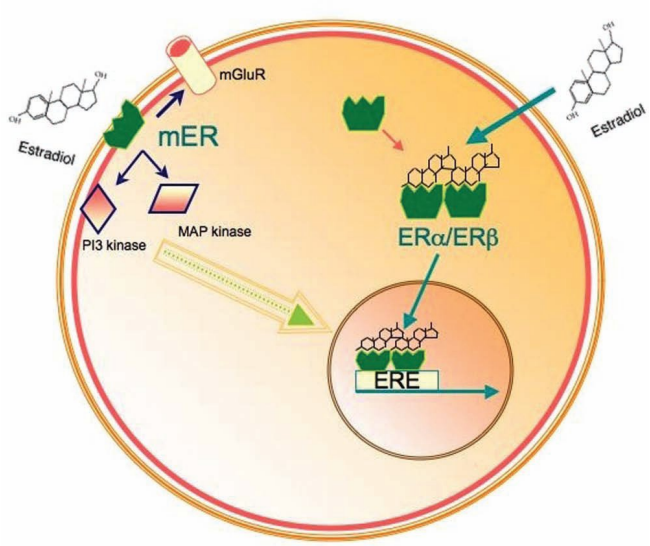

Figure 3 I Estrogen effects are slow, and also quick. Estrogen binds to the estrogen receptor (ER), either ERα or ERβ, which interact directly with the transcriptional complex and the genome, mostly at estrogen response elements (ERE) to alter gene expression. The resulting changes in protein synthesis are slow and enduring. Estrogen can also activate receptors located at the membrane (mER), which initiate signal transduction cascades that are relatively rapid and transient, but may lead to enduring effects through changes in gene expression (obtained from McCarthy *et al.*, 2009).

3.2.1. Masculinization and defeminization

Estrogen activates two independent processes in the brain: masculinization of neural circuits and networks that are essential for expression of male-typical adult behaviours by estrogen receptor alpha (ERα), and defeminization, the loss of the ability to display adult female-typical behaviours probably by estrogen receptor beta (ERβ). These observations were made with the use of knockout animals for each ER receptor (19). Without functional ERβ, females are more sensitive to estrogen, thus, ERβ normally decreases the effectiveness of ERα (20). ERα activation in the medial preoptic area (mPOA) during the sensitive period for sexual differentiation induces the formation of neuronal dendritic spines. Cyclooxygenase 1 and 2 (COX-1 and COX-2) are expressed by ER activation which lead to a 7-fold increase in prostaglandin E2 (PGE2) by cyclinization of arachidonic acid (21). An intracerebroventricular injection with PGE2 to newborn female rats is sufficient to induce full masculinization of dendritic spine density and adult sexual behaviour (22). By contrast, disrupting PGE2 neonatally blocks masculinization (21). PGE2 activates receptors EP2 and EP4, which increase protein kinase A (PKA) signalling and thereby enhance glutamate release, which activates AMPA receptors and helps to form new dendritic spines (fig. 4). Males show a larger density of dendritic spine synapses in the mPOA than females. The increase of dendritic spines in

the mPOA indicates an increase in afferent input, but its location is unknown. It could possibly be a different location between in males and females.

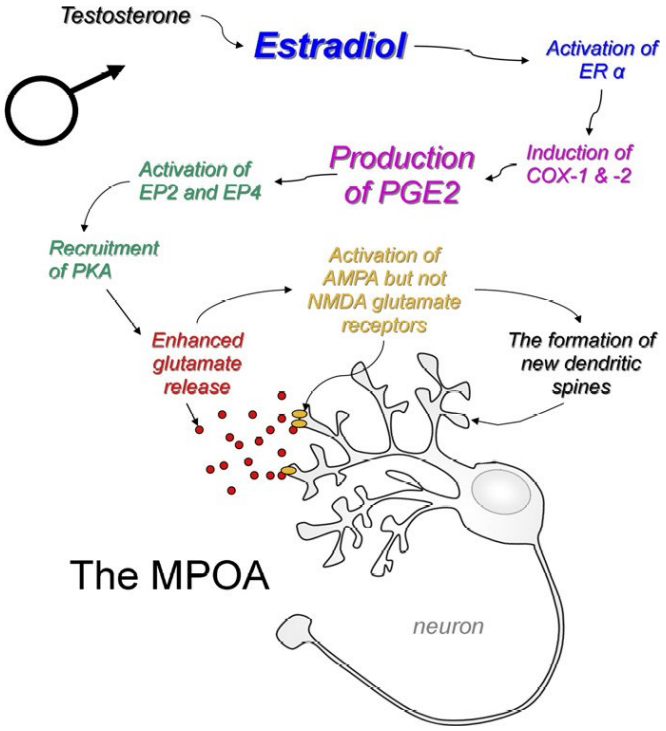

Figure 4 I Estradiol induces PGE2 synthesis to promote dendritic spine formation and sexual differentiation in the mPOA. Estradiol aromatized from testicular androgens binds to ERα and induces transcription of COX-1 and COX-2, the rate limiting enzymes in PGE2 synthesis. PGE2 activates EP2 and EP4 receptors, both of which are linked to activation of PKA. Through mechanisms that remain poorly understood, PKA enhances the actions of glutamate at AMPA, but not NMDA receptors, and the formation of new dendritic spines and the organization of a higher density of spine synapses on male POA neurons compared to female (obtained from McCarthy *et al.*, 2009)

Perhaps projections from the amygdala relevant to olfactory stimuli or reciprocal connections to the ventromedial nucleus (VMN) of the hypothalamus, a critical brain region in the control of female sexual behaviour, could be the input source. Because of the particular function of this region in female sexual behaviour, it could be a substrate for defeminization, which is the loss of the capacity to express female sexual behaviour in adulthood. Defeminization is induced by ERβ signalling. This can be shown from studies where ERβ antagonist was applied to female mice, these mice showed impaired lordosis, a characteristic female receptive behaviour. Male ERβ knockout mice show normal masculinization but incomplete defeminization (19). Similar to the mPOA, male VMN neurons have more dendritic spine synapses than females, but this is not the result of an increased density but of a more complex dendritic tree. This increase in spines is the result of an increase of glutamate release from the presynaptic terminal, which activates AMPA and NMDA receptors, leading to calcium influx and activation of MAP kinase. The enhanced glutamate release requires estrogen-induced activation of PI3 kinase. This mechanism is confirmed by applying glutamate to induce defeminization and blocking glutamate disrupts estrogen-induced defeminization (23).

3.2.2. Steroid receptor coactivators

Estrogen and androgen receptors activate the transcription of genes by binding to specific DNA sequences in humans, as well as in primates and rodents (24; 25). The specific binding of steroid hormones leads to a conformational change and hyperphosphorylation of the receptor which induces dimerization and binding to a specific DNA sequence, the Hormone Response Element. After this dimerization, several proteins of the transcription machinery are recruited. It is unknown when the coactivators come into play, but they bind to the sex hormone receptors (fig. 5) and are rate-limiting in steroid receptor-mediated gene transcription (26). These coactivators can be involved in a large array of enzymatic processes, including acetylation and methylation of histones H3 and H4, ATPase-dependent chromatin remodelling, mRNA splicing and localization of receptors (27). To fully understand the actions of sex hormones on physiology, behaviour and development, it must be taken into account that there are differences in the responsiveness of various tissues and between individuals. For instance, steroid receptor coactivator-1 (SRC-1) from the rat

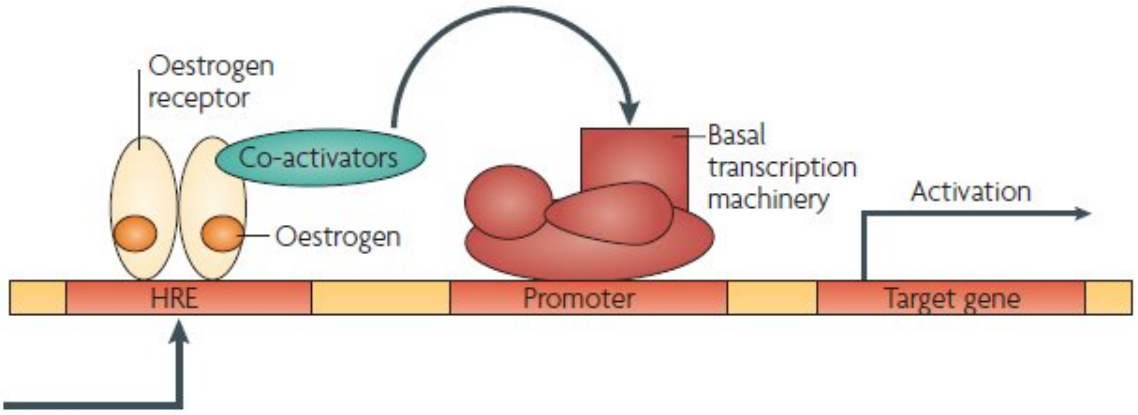

Figure 5 I Regulation of gene expression by hormone receptors. Oestrogen molecules form a complex with oestrogen receptors (ERs). This complex dimerizes and then binds to a hormone-responsive element (HRE) (in this case an oestrogen-responsive element (ERE)), usually located upstream of the target gene. The ERE–ER complex interacts through co-activators with the basal transcription machinery to increase the transcription of target genes in a hormone-dependent manner (obtained from Jazin & Cahill, 2010).

hippocampus interacts equally with ERα and ERβ, while SRC-1 from the rat hypothalamus interacts more with ERα than with ERβ (26). This suggests that these different brain areas could have distinct post-translational modification of SRC-1. Studies in rodents showed that SRC-1 expression in several brain regions is sex-

dependent, which could lead to sex-specific phenotypes in reaction to the same sex hormones (28). Injection of antisense SRC-1 RNA in the Japanse quail brain showed a decrease of male sexual behaviour in response to testosterone, indicating the importance of the SCR-1 coactivator in testosterone signalling. Furthermore, the amount of aromatase neurons (which convert testosterone into estrogen) in the medial preoptic nucleus was decreased, as compared to animals injected with scrambled oligonucleotides (29). Female rats which are treated with testosterone shortly after birth usually show high levels of male-typical sexual behaviour. However, when these testosterone-treated females and males were treated with antisense SRC-1 one day before- or after testosterone treatment, they exhibited adult female sexual behaviour, suggesting that SRC-1 is critical in mediating the defeminizing actions of testosterone (30).

3.2.3. ARs in masculinization and de novo estrogen synthesis

Because of the various mechanisms through which ERs are functioning, it is not very likely that only one of these mechanisms is involved in masculinizing and defeminizing the brain. Besides ER activated mechanisms, there is increasing evidence of AR induced masculinization. Several sexual dimorphic brain regions appear to show deficits when ARs are not functional (31). Although the amount of androgens in the female is much lower than in the male, equally high amounts of estrogen have been found in the neonatal hippocampus and the cerebellum. This could be the effect of de novo synthesis of estrogen from cholesterol within select brain regions. All of the enzymes that are required for this conversion have been identified in the brain (32). By synthesizing estrogen locally, females can maximize the beneficial effects in certain areas, while avoiding the masculinizing and defeminizing effects in other areas. As can be noted, there are many mechanisms involved in the sexual differentiation of the brain in rodents, much more research has to be performed to fully elucidate the exact pathways.

3.3 Brain development in humans

The human brain shows structural and functional resemblance with the brains of rodents. However, with regard to the sexual differentiation of the brain, there appear to be large differences in hormone action. Thus, in rodents male brain development is largely dependent on ER signalling, whereas it seems that in humans direct action of testosterone is crucial for the development of the male brain. For instance, men with

aromatase deficiency and as a result are deficient in estrogens, do not show any gender identity problems (33; 34). The sexual differentiation of the human brain is thought to be determined in the two first periods during which sexually dimorphic peaks in testosterone levels are found – during gestation and the perinatal period. From puberty onwards, sex hormones induce adult activation of specific networks and alter the function of previously organized neuronal systems, and behavioural patterns that are originated much earlier in development are expressed (35).

3.3.1. Direct testosterone action

In contrast to the sexual differentiation of the rodent brain, testosterone seems to have a direct action on the developing human brain. This theory can be supported by investigating androgen related disorders. Various mutations in androgen receptor (AR) on X chromosome at Xq11-12 can lead to insensitivity for androgens (androgen-insensitivity syndrome) (36). Although this male person (XY) has normal testis and androgen biosynthesis, the external and behavioural phenotype is female. Phenotypic women (XX) with androgen-insensitivity syndrome perceive themselves as highly feminine and do not have any gender problems (37). When a boy fetus (XY) has a 5α-reductase deficiency which prevents peripheral testosterone from being transformed into DHT, the male external organs as well as the prostate cannot develop properly; the boy is looking like a girl and has a large clitoris. This is also true for fetuses with a 17β-HSD-3 deficiency, the isoenzyme that is required for testosterone production during the fetal life, which leads to a lower testosterone production (there is still some production by other isoenzymes). These children are raised as girls and when testosterone increases during puberty, the clitoris grows into a penis and the testicles descend. The children also masculinize and become muscular. Despite their rearing, a large part of these children change into males (60%), apparently due to the effect of testosterone on their brains during early development (38).

4. Sex differences in the human brain

Due to the process of sexual differentiation, it is likely that the brains of the two sexes are different during development as well as in adult stages. This is also expected from differences in cognitive domains between the sexes (39) and differences in susceptibility to neurological and psychiatric disorders (40). These differences could involve structural

differences in sizes of brain regions and connections between those. However, numerous studies have found sexual differences in neural activity when there were no differences in behaviour. De Vries argues that these sex differences can arise to not create differences in behaviour, but to prevent them through compensation (41). Functional differences, for instance, differences in aspects of neurotransmitter function or in genetic or metabolic response to experience can also be the result. For instance serotonin, γ-Aminobutyric acid (GABA), acetylcholine opioids and monoamines are expressed in a sexually dimorphic manner (40). Sex differences in nuclear volume or neuron number are often attributed to the hormonal control of cell death. The ratio between antiapoptotic proteins to proapoptotic proteins plays a key role in determining whether a neuron survives or undergoes apoptosis. In specific brain areas, testicular hormones decrease cell death during perinatal development. Males therefore have more neurons in these areas during adulthood (42).

4.1 Brain regions

With the use of *in vivo* magnetic resonance imaging (MRI), the presence of sexual dimorphisms in different brain regions was examined (tab. 1). Greater sexual dimorphism was found in brain areas which are homologous to those identified in animals showing large differences in steroid receptor density during critical periods of brain development (1). A large sex difference was found in the thickness of many areas of the cortex (1; 43; 2). The hippocampus, a region associated with learning and memory, is larger in women than in men. Animal research reveals additional hippocampal differences, for instance differences in neurotransmitter systems (e.g. adrenergic, corticosterone and serotonergic systems), long-term potentiation (LTP) and the enzymatic reaction related to memory consolidation (40).

Larger in women	Larger in men
Gray matter	White matter
Hippocampus	Lateral ventricles
Precentral gyrus	Third ventricle
Frontoorbital cortex	Amygdala
Superior frontal gyrus	Hypothalamus
Lingual gyrus	Frontomedial cortex
	Angular gyrus

Table 1 I Structural dimorphism in different adult human brain areas. The sizes of structures are measured using MRI and are adjusted for total brain volume and cerebrum size (1; 2).

Sex hormones can alter the excitability of hippocampal cells, influence the dendritic structure and augment N-methyl-D-aspartic acid (NMDA) receptor binding (44). It has not yet been investigated how sex hormones influence hippocampal function in humans, but the animal results indicate that there is a possibility that this will be similar in humans. The amygdala is a structure in the temporal lobe which plays a key role in emotional responses and emotional memory. Most, if not all, nuclei of the amygdala have been shown to be sexually dimorphic (45). There are functional differences in how the amygdala is connected with the rest of the brain as well; the left amygdala of women covaried with the rest of the brain significantly more than in men, while for the right amygdala the opposite was found (46). The anterior commissure was 12% larger in females (10). The interthalamic adhesion, a grey structure that crosses the third ventricle between the two thalami, was present more in females (78%) than in males (68%). From the subjects having a massa intermedia, the structure was 53% larger in females (4). These observations suggest a larger connectivity between the female hemispheres than those of males.

4.1.1. Hypothalamus

The hypothalamus is an important brain region which consists of many nuclei with various functions, linking the nervous system to the endocrine system through the pituitary gland. Various nuclei and adjacent structures are found to be sexually dimorphic (fig. 6). The sexually dimorphic nucleus of the preoptic area (SDN-POA) is 2.5 times larger in men than in women and contains 2.2 times as many cells. The size of this nucleus varies over age, but it is always larger in men (10). This sexual dimorphism was not found by other research groups, possibly because this nucleus varies strongly in size over age. Because four hypothalamic nuclei in the literature were not described yet, they were named ‘Interstitial nuclei of the anterior hypothalamus’ (INAH), in numerical order from lateral to medial (47). The SDN-POA is now defined as INAH-1. INAH-2 and INAH-3 were found to be larger in the male brain as well (10). Another sex

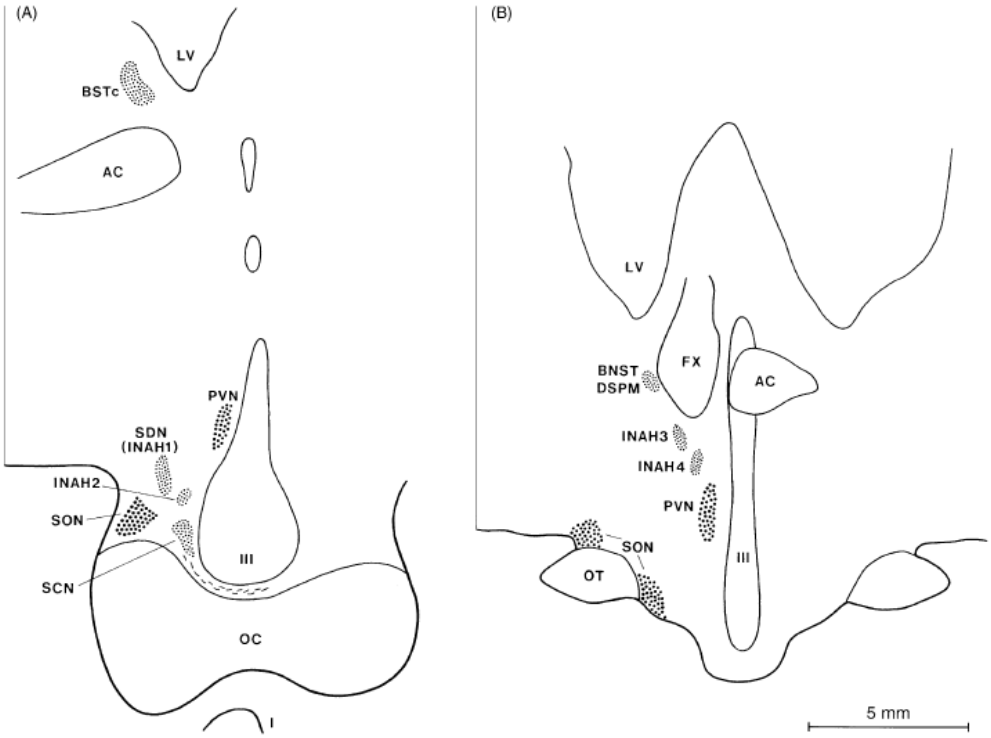

Figure 6 I Sexual differences in the human hypothalamus and adjacent regions. (A) is more rostral than (B). Abbreviations: III, third ventricle; AC, anterior commissure; BNST-DSPM, darkly staining posteriomedial component of the bed nucleus of the stria terminalis; FX, fornix; I, infundibulum; INAH1-4, interstitial nucleus of the anterior hypothalamus 1-4; LV, lateral ventricle; OC, optic chiasm; OT, optic tract; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus of the preoptic area = INAH-1; SON, supraoptic nucleus. Scale bar = 5 mm. AC, BSTc, BNST-DSPM, INAH2-4, SCN and SDN were reported to vay according to sex (obtained from Swaab *et al.*, 2003).

difference was found in the 'darkly staining posteriomedial component of the bed nucleus of the stria terminalis' (BNST-dspm), which was again larger in males than in females (48). The central part of the bed nucleus of the stria terminalis (BSTc) is 40% larger in men and contains almost twice as many somatostatin neurons as in women. A sex difference in shape of the suprachiasmatic nucleus (SCN) suggests sex differences in circadian patterns and sexual orientation (10).

4.2 Sex hormones and receptors

Sexual differentiation of the brain is regulated by the presence of sex hormones and receptors, which are very different between males and females. Proposed mechanisms of action of sex hormones in the brain include influences on neurogenesis, cell

migration, cell differentiation, cell death, axon guidance and synaptogenesis (25). Within the hypothalamus much less AR staining is observed in women as compared to men, in almost all sexually dimorphic areas, especially within the lateral and medial mammillary nucleus (49). However, a female-like AR pattern was found in two castrated men, indicating that the amount of AR staining is dependent on the circulating levels of androgens. INAH-1 was more intensely stained for ARs as well, just as the horizontal diagonal band of Broca, SDN-POA, paraventricular nucleus (PVN) and supraoptic nucleus (SON). In young adults, higher levels of ERα staining have been observed in the male SON and the PVN, but also the SDN-POA. In females, a higher ERα staining was observed in the SCN and the mammillary bodies (50). The BSTc does not show any sex differences in ERα staining, but does show a higher ERβ staining in males. The male SDN-POA shows a higher ERβ staining as well. Women showed more ERβ staining in the SCN, the SON and the PVN. Observations in subjects with abnormal estrogen levels revealed that ERβ immunoreactivity patterns were consistent with the level of circulating estrogens (4), just as was found with AR staining. These findings suggest that the majority of sex differences in AR and ERβ staining were activated by circulating sex hormone levels rather than organized beforehand.

4.3 Transsexuality

Transsexuality or gender identity disorder is characterized by a conviction of having been born in the wrong body, the body of the opposite sex that the person feels he or she belongs to. These feelings probably have a biological cause and are not the result of the social environment, as was shown by the famous John/Joan/John story, where the genitals of a boy were made into those of a girl. This 'girl' was having problems with her gender identity at puberty and switched into a man again (8). There are three times as many male-to-female (MtF) transsexuals than female-to-male (FtM) (35). Feelings of transsexuality are often expressed early in neonatal development, a few years after birth, but only in 23% of the cases does a childhood gender problem lead to transsexuality in adulthood (6). Transsexuals do not only have the feeling that they belong in the body of the opposite sex, they also show the same traits, such as hobby preferences and expressiveness (7). Gender identity is a unique feature and this can thus not be investigated in animal models.

4.3.1. Causes

Because the brains of males and females are functionally and structurally different, it is unknown in MtF and FtM transsexuality has the same cause in both sexes. There is little information known about factors that may increase the risk of developing a gender identity disorder (table 2). Twin and family studies show that genetic factors play a large part (51; 52). A few cases of MtF transsexuals with 47,XYY, a FtM case with 47,XXX and a MtF transsexual with Kleinfelter (47,XXY) have been reported (10). No differences in SRY have been identified in transsexuals (53). Polymorphisms of ERα, ERβ, AR repeat length and polymorphisms of the aromatase gene CYP19 increased the risk of a gender identity problem (54; 55; 52). In addition, various hormonal disorders increase the risk of transsexuality. For instance, cloacal exstrophy is a disorder where genetic males (XY) are born with exstrophy of the bladder and absence of a penis. These males were traditionally changed into females after birth, but a large part (53%) did not continue to live as a female but changed their gender into male (56). CAH is a female disorder where there are high androgen levels during prenatal development in 90% of the patients due to a defect in 21-hydroxylase (57) which can lead to male-like genitals. Nevertheless, these girls are raised as females, but some of them change their gender during puberty into male. These examples show that for the development of gender identity, direct androgen action on the developing male brain and a lack of androgens in the female brain is a crucial factor. Furthermore, studies on cloacal exstrophy suggest that the

Genetic factors	rare chromosomal disorders: 47,XYY (MtF), 47,XXX (FtM)
	Twin studies
	Kleinfelter XXY (MtF)
Epigenetic factor	Genomic imprinting
Phenobarbital/diphantoin	taken by pregnant mother
Hormones	cloacal extrophy
	5-α reductase 2 or 17β-hydroxy-steroid-dehydrogenase 3 deficiency
	CAH girls with gender problems
	Complete androgen insensitivity syndrome → XY females
	DES sons: 35.5% gender problem
	MtF sex reassignment
Social factors	postnatally no evidence

Table 2 I Factors influencing gender identity (obtained from Swaab, (2004).

postnatal testosterone peak is not crucial for the development of gender identity, because the testicles of these girls are removed shortly after birth (4). Drugs can alter gender identity as well, as was found in a group of epileptic women received phenobarbital or diphantoin during pregnancy, which change the sex hormone metabolism. An increased number of transsexuals was found as well as a few others with less radical gender problems (58). A controversial finding is that an increased number of transsexual sons are observed in women who received diethylstilbestrol, an estrogen-like substance during pregnancy. Since estrogen is causing a male phenotype, it is hard to explain how this can cause feminization and therefore this should be investigated further.

4.3.2. Brain differences in transsexuals

The theory of the origin of transsexuality is based on the fact that the differentiation of the genitals takes place during the first couple of months of pregnancy, before the sexual differentiation of the brain (35). Because these are separate processes, it is possible to find people with female genitals and a male brain and vice versa. The hypothalamic nucleus BSTc has been found to be larger in men than in women. This region is involved in many aspects of sexual behaviour in rats, for instance it plays an essential role in masculine sexual behaviour and in the regulation of gonadotrophin (LH, FSH) release. It contains estrogen and androgen receptors and it is a major aromatization centre in the developing rat brain. Furthermore, the BSTc receives projections from the amygdala and is a strong input for the preoptic-hypothalamic region. In genetically male transsexuals, a female size BSTc was found and in one female-to-male transsexual a male size BSTc was found (59; 60). In homosexual males, this was not the case, indicating that the reduced size in transsexuals was not related to sexual orientation. However, the difference in size between men and women is only apparent in early adulthood (4) and therefore it cannot play a part in the early diagnosis. Besides this difference in BSTc size, a female INAH-3 was found in MfF transsexuals and a male INAH-3 was found in a FtM transsexual (4). The difference in BSTc size cannot be explained by differences in adult sex hormone levels (59) but is probably established during development. Besides these structural differences, functional differences that match the desired sex in transsexuals have been shown as well. Using fMRI, it was shown that non-homosexual MtF (erotically attracted to women) showed that a number of brain areas in the hypothalamus were activated by estrogen-derived pheromones in a

female way. MtF transsexuals also showed a female cerebral activation pattern when viewing erotic stimuli (61). A recent study has found a decrease in cerebral blood flow in the left anterior cingulate cortex and an increase in the right insula in FtM transsexuals as compared to controls (62), pointing to new and still not investigated areas of the brain in relation to gender identity disorders.

5. Alternative explanations for sexual differentiation and gender identity disorders

Based on elaborate scientific research in the past, we now know that sex hormones play an important role in the sexual differentiation of the brain. However, in practice it is more likely that hormonal pathways and genetic pathways are intertwined and dependent upon each other (41). For example, the AR gene is X-linked, and therefore it is expressed in a sexually dimorphic way. This receptor binds androgens that are available in at different concentrations between males and females. Consequently, the development of the brain will be dependent on the combined function of receptor expression level and androgen concentrations (63). However, there are also alternative explanations to induce sexual differentiation, which come into play even before gonadal development. Hormonal disorders such as the androgen-insensitivity disorder and CAH indicate a role for hormones in causing gender identity disorders together with physical abnormalities. However, a large group of transsexuals do not suffer from gender dysphoria. This indicates that there have to be other explanations than hormones for the existence of the phenomenon transsexuality. One important explanation may involve the genetic background, several twin- and family studies show higher concordance rates for transsexuality in monozygotic than in dizygotic twins (51; 64). Genes related to steroid hormone function are the most likely to be involved in transsexuality. However, a recent association study of gender identity disorder and sex-hormone related genes did not show any genetic variants between transsexuals and controls in AR, ERα, ERβ and aromatase (65). Furthermore, there is still a large part of the population that shows gene patterns of disorders which can lead to gender identity problems and yet are not transsexuals, as well as there is a large proportion of transsexuals who do not have these gene patterns. For this reason, attention has to be drawn away from the genetic determinants of hormones, into the direction of genetics and epigenetics.

5.1 Genetics

Genetic control of sex-specific brain development in mammals may result in the formation of sex-specific neural networks, which can be determined very early in development. Some fetal brain cells undergo sexual differentiation, even in tissue culture, without the involvement of sex hormones (63). Already in 1977, it was found that male rat fetuses at postnatal day 12.5 were larger than females, had higher protein content and a faster rate of cell division, which is confirmed in humans (5). 51 genes are expressed differently in the brains of male and female mouse fetuses before hormones come into play and before the formation of the gonads (at E10.5), suggesting that genetic factors may influence sexual differentiation (14). Studies in adult mice have shown that a large part of the genes is expressed in a sexually dimorphic manner, in the human brain this is approximately 14% (650 genes) (66). When the formation of the gonads and the adrenal glands is prevented by knocking out steroidogenic factor 1 (SF-1), sex differences are still found in the adult VMN and the POA. This suggest sex hormone-independent sexual differentiation of the brain (67). In the human brain, sexual dimorphic gene expression has been shown, especially in genes involved in translation (68). Sex linked genes could contribute fundamentally to neural sexual dimorphism prior to the onset of gonadal hormone secretion (and thereafter), and these genes may in turn elicit sex-specific expression of autosomal genes (14; 69). It is important for new studies to take sex differences into account, especially when knocking out sex-specific genes. A genetic manipulation may have an effect in only one sex or may have the opposite effect between the sexes (25).

5.1.1. Doublesex and mab-3 related transcription factor (DMRT) family

Studies in *C. Elegans* showed that neural sexual differentiation was ultimately controlled by *tra-1*, a transcriptional repressor which blocks the expression of male-specific genes in hermaphrodites (70). The terminal factor of the TRA-1 regulated pathway is MAB-3. This class of genes are homologous to the *doublesex* in *Drosophila* and to the DMRT family in vertebrates, which encode putative transcription factors (25). Although genes of the DMRT family are located on chromosome 9 and not on sex chromosomes, combining of DMRT1, DMRT2 and DMRT3 mutations have been associated with sex reversal of the XY embryonic gonad into ovarian tissue (71), which could be interesting in relation to gender identity problems.

5.1.2. Differential splicing

Sexual dimorphic differential mRNA splicing between males and females, especially many transcription factors and splicing factors has been observed in multiple species. In humans, Ca^{2+} channels are extensively spliced in a age- and gender-biased manner (72). These channels have key roles in neurotransmitter release and synaptic strength and could influence the differences in number of synapses and spines in sexually dimorphic brain areas. Sexual dimorphic splicing is different between specific tissues, suggesting that the same genes have different functions and control mechanisms depending on the tissue (25).

5.2 Epigenetic control mechanisms

Changes in phenotype caused by mechanisms other than changes in the underlying DNA sequence are called epigenetic mechanisms. These mechanisms include DNA methylation, histone modifications, nucleosome repositioning, higher-order chromatin remodeling to loosen or tighten wound chromatin, mechanisms involving non-coding RNA, and RNA and DNA editing (73). Methylation of histones is generally associated with repression of gene transcription and acetylation with stimulating gene expression. Histone demethylases are encoded on X and Y chromosomes could contribute to epigenetic mechanisms that encode sex differences.

5.2.1. X-inactivation

Genes important in brain function are expressed threefold higher on the X chromosome than on autosomes (74). Because males have only one copy of the X chromosome, whereas females have two, genes on the X chromosome can be expressed in higher doses in females. The significance of these compensation mechanisms is shown by individuals expressing more or fewer copies of sex chromosomes. These individuals exhibit multiple neurodevelopmental and behavioural abnormalities, such as people with X-monosomy (Turner syndrome) (75). These people show volume changes in various brain areas, as compared to XX individuals. A reason for these differences can be that certain genes are not expressed in a sufficient level. Due to X-inactivation, dosage differences on X chromosomal genes between XX and XY individuals can be alleviated and X chromosome genes will be expressed equally in both sexes. In this process, which is imprinted in the blastocyst stage, one X chromosome is active and the other is partially inactive by heterochromatin.

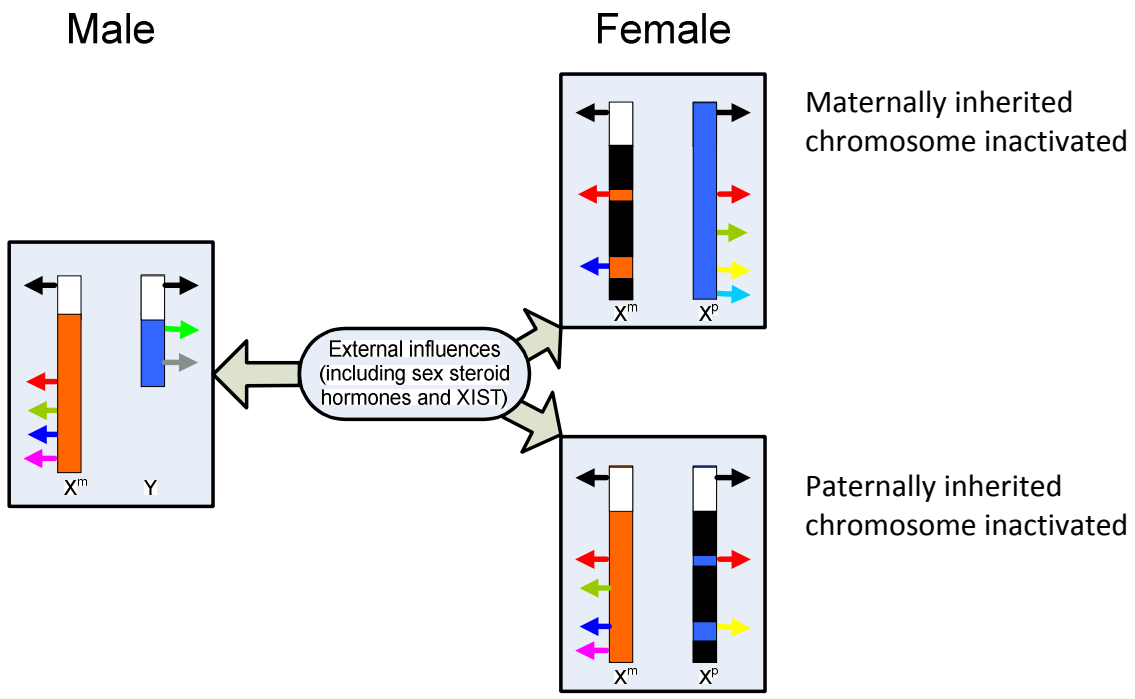

Figure 7 I X-inactivation. Male brain cells have an X chromosome of maternal origin (Xm, orange rectangle) and a Y chromosome of paternal origin (blue rectangle). Female brain cells consist of two populations, both of which possess a single Xm and a single Xp. In one population Xm is inactivated (shown by black shading) and in the second population Xp is inactivated. Overall gene expression is the average of gene expression in these two populations. The pseudoautosomal (PAR) region (white rectangle) is common to both chromosomes, and escapes X inactivation. Several classes of genes may be expressed in a sexually dimorphic manner: Y-specific genes (grey) which are solely expressed in male brain, non-imprinted X-linked genes that escape X inactivation (red) and that have a functionally different Y homologue (light green) will be higher expressed in female brain, Xm genes subject to X inactivation (pink) which are higher expressed in male than in female brain, and Xp genes (X inactivation or escaping) which will be solely expressed in female brain (light blue and yellow respectively). Several other categories of genes will be equally expressed in male and female brains including PAR genes (black), non-imprinted X-linked genes that are subject to X inactivation (green) and Xm which escape X inactivation (blue). Gene expression in both male and female brain cells is likely to be influenced to some extent by external factors, including the environment in which the cell finds itself and the initiating gene XIST. (Adapted from Davies *et al*., 2006)

All the descendants of the inactivated cells show the same X inactivation mosaic (63). Effects of X-inactivation are dependent on which of the chromosomes is inactivated, either the paternally or the maternally inherited X chromosome and external influences, including sex hormones (fig. 7). The process of X-inactivation is initiated by X (inactive)-specific transcript (XIST) which does not encode a protein, but remains in the nucleus where it coats the inactive X chromosome to prevent transcription. The XIST RNA only coats the chromosome from which it is expressed (76). 15% of genes on X chromosome are thought to escape X inactivation (77) and will be expressed at a higher level in

females than in males. Therefore the contribution of these dosage effects to neurobiological sexual dimorphism could be quite large. The contribution of paternally versus maternally inherited X-linked genes has to be investigated further, because when maternally expressed X-linked genes are subject to random X-inactivation, their dosage in females will be reduced relative to that in males (63). Xu *et al.* have found sex differences in mRNA levels of the X-inactivation escaping gene eukaryotic translation initiation factor 2 (Eif2s3x), but not in protein levels (78). Although mRNA levels of X-inactivation escaped genes may be higher in the brains of females; this does not necessarily mean that protein levels will be higher as well. For complete understanding of the genetically based control of sex differences, it is important to conduct future studies of genes that escape X-inactivation and the corresponding protein levels. As a result of X-linked gene dosage effects, mutations or polymorphisms will be more overt in males as a result of hemizygosity, which could explain the higher prevalence of transsexuality in males.

5.1.2. Genomic imprinting

Genomic imprinting is a process occurring in about 1% of the genes, involving methylation and histone modification in order to influence gene expression without altering the genome. In this way, only one of the two alleles for a gene is expressed, depending on the parental origin. Some of the genes are preferentially expressed from the paternally allele, while other genes are preferentially expressed from the maternally allele. Because only females inherit a paternally X chromosome, the expression of paternally imprinted genes will be limited to this sex. Imprinted genes highly expressed in the brain and can affect neurodevelopment, ongoing brain function and behaviour (79). Imprinting mechanisms could lead to the disparate maternal aunt-uncle ration that was found in MfF transsexuals (fig. 8). This could be due to a mutation in XIST, such that the ancestral imprinted epigenotype is retained, meaning that the X^p chromosome in the daughter remains its imprinted pattern when it is passed on to her sons. Because the X^p is normally silenced in early stages of development, higher lethality is expected in sons that inherit the X^p from the mother (80). Studies on patients with X-monosomy revealed that females who inherited their single chromosome maternally (X^mO) showed higher numbers of impairments in IQ, social cognition and behavioural inhibition, but also boy-ish assertiveness, than females who inherited their single chromosome paternally (X^pO), indicating the importance of X-linked parent-of-origin effects on brain

development (63). These results also lead to the expectation that genomic imprinting is causing these differences. In the generation where sons inherit X^p from their X^pX^p mother (X^pY sons), probably more social female-like behaviour and MtF transsexuality will be the result (80).

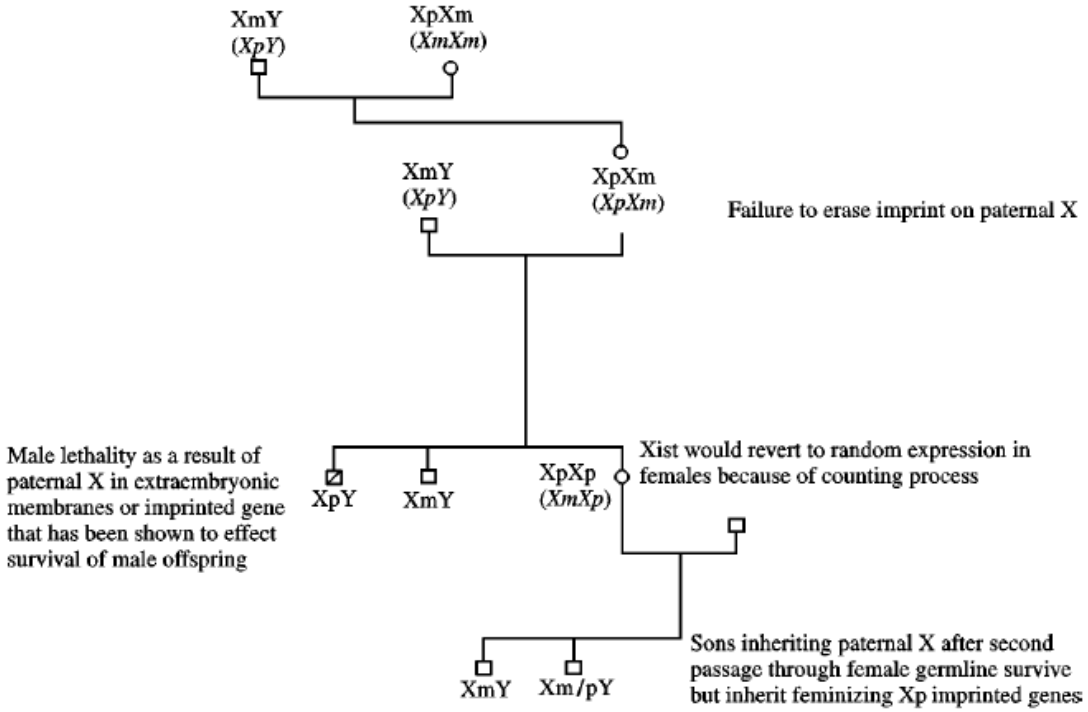

Figure 8 I Effects of genomic imprinting on offspring. XpXm somatic cell; (XmXm) gametes; -XIST silenced; Xm/pY imprinted genes expressed as if in the female (obtained from Green *et al.*, 2000).

5.1.3 Regulation of X and Y chromosome paralogues

Many of the genes that escape X-inactivation have Y-linked homologues, alleviating the dosage difference and providing a possible compensation for X-inactivation escaped X-linked genes. The degree of this alleviation will depend upon whether the X-linked gene and its Y-linked version exhibit similar levels, patterns of expression and the extent of functional homology between the proteins (25). One example is protocadherins (PCDHs), which are involved in cell-cell interactions during the development of the central nervous system. The human-specific X-linked (PCDHX) and the Y-linked (PDCHY) genes share 98.3% amino acid identity, which may result in different functions. Furthermore, PCDHX escapes X-inactivation and the genes show different region specific expression (81). Another example is the histone demethylase UTX with paralogue UTY,

which show different expression patterns in the brain, particularly in the hypothalamus and the amygdala (82), two regions which are sexually dimorphic (4). In mice, UTX escapes X-inactivation and differences in expression may result in altered demethylation of histone 3 and therefore differences in epigenetic regulation of gene expression between the sexes.

5.2. Sex hormone binding globulin

In the rodent brain, but especially in the human brain, the glycoprotein sex hormone binding globulin (SHBG) has a high binding affinity for androgens and a lower affinity for estrogens. SHBG is produced by the liver influences the bioavailability of these hormones by inhibiting the function through binding those in the bloodstream. Only 'free' hormones can bind to steroid receptors. Because more than 50% of the circulating hormones is bound to SHBG, the release of hormones can be targeted to specific tissues (26). The concentration of SHBG is tightly regulated by a balance of enhancing and inhibiting factors, these include the hormones themselves. Androgen decreases SHBG levels, while estrogen is increasing the amount of SHBG (83). Therefore, SHBG levels in women tend to be higher than in men. Besides buffering hormones, SHBG is associated to diabetes, obesity and cardiovascular diseases (84), but also to masculine features of FtM transsexuality .

7. Conclusion and discussion

This thesis shows that genetic and epigenetic factors are very important in sexual differentiation of the brain, and a failure in expression of several of these factors can lead to disturbances in sexual differentiation and gender identity. Hormones seem very important in the sexual differentiation of the brain, but there are many genes expressed sexually dimorphic before hormones come into play. Sex chromosomes are good candidates for being the first step of the controlling molecular pathways. Genes on the X chromosome which escape X-inactivation as well as Y linked genes that are expressed in early development are likely candidates for mediating the early phase of brain sex differences. Before the formation of the gonads, several genes are expressed upstream and downstream of the Y linked gene SRY and the possible female inducing X linked gene DAX-1. Mutations in a few of these genes in the same pathway, could possibly lead to similar effects influencing the development of the gonads. Disturbances in gonadal

development can have large effects on the sex of an individual and sometimes even lead to sex reversal, which means that the genetic sex differs from the gonadal sex. Some of the genes expressed in early development have also been identified in the brain. A recent study showed that SRY not only influences genes to induce the development of the testes, but also regulates the X-linked gene monoamine oxidase A (MOA), which catalyzes the oxidative deamination of neurotransmitters such as serotonin and dopamine (85). These neurotransmitter systems are sexually dimorphic and have a large impact on brain functioning (2). The MOA gene is influenced by X-inactivation and methylation systems which are regulated differently between the sexes. Due to an escape in X-inactivation, MOA levels could be higher in females, leading to a decrease in neurotransmitters in the synaptic cleft. Differences in the amount of neurotransmitter can have large effects on synaptic plasticity, which can influence connections between different brain areas. This can be a factor influencing gender identity, since structural and functional differences between transsexuals and controls have been identified in the BSTc and the INAH3. These differences are independent of hormone levels (59), which further supports the importance of alternative explanations for the sexual differentiation. The dopamine system is besides the influencing deamination of dopamine also dependent on tyrosine hydroxylase, a precursor for DOPA, which is converted into dopamine. Tyrosine hydroxylase expression is upon the direct influence of sex-linked genes in the ventral striatum (63), which was found to be functionally different in transsexuals in a fMRI study (62).

Although a certain amount of transsexuals shows sex steroid receptor mutations and mutations in for instance the aromatase gene, the most recent association study could not identify any differences between transsexuals and controls (65). Furthermore, many people with the same steroid receptor mutations as were found in a few transsexuals do not show any gender identity problems. A very important finding is that there is still sexual differentiation of the brain without gonadal development, indicating that other genes, outside the hormonal system, have to play a role as well. Because there are many genes and pathways that could be involved, it is very difficult to make predictions on the genes implicated in gender identity disturbances, other than performing association studies on transsexual subjects. Probably there is interplay between the direct effects of genes and the indirect effect of hormones. This can either be in concert to exaggerate certain sex-specific phenotypes, or in the opposite way (41). This latter theory is supported by a recent finding where a male sex chromosome complement

stimulates immune response, whereas this is attenuated by hormones (86). Similar situations can occur in the brain as well.

Unlike genetic mutations, epigenetic modifications such as X-inactivation, are dynamic and may be reversible. If these epigenetic modifications are triggering or inhibiting gene expression which induces developmental disturbances and leads to gender identity problems, perhaps it can be identified when, how and whether to use methods or treatments, such as specific drugs or lifestyle changes in the pregnant mother or the newborn child to prevent these gender problems leading to transsexuality. For example, it is known that unbalanced prenatal nutrition in rats can lead to persistent, gene-specific epigenetic changes that alter mRNA production levels, which can be prevented by folic acid (87).

A large problem in studying gender identity is that it cannot be studied in animals, because behaviour has to be observed and one cannot simply measure gender identity in an animal. There are sex differences found in the BSTc of rats and mice, but there is no evidence of BSTc functions in rodents which are in any way analogous to human gender identity (88). Therefore, we are forced to keep using human 'experiments of nature' to investigate gender identity.

In recent years, there has been much evidence for genetically and epigenetically based sex influences on brain function and structure. Different findings indicate the importance of this course of research and more knowledge will increase our understanding on sexual differentiation and related gender identity problems and perhaps it will lead to new treatment and prevention targets.

8. List of abbreviations

17β-HSD-3	17β-hydroxy-steroid dehydrogenase 3
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	androgen receptor
BSTc	central part of the bed nucleus of the stria terminalis
CAH	congenital adrenal hyperplasia
COX-1	cyclooxygenase 1
COX-2	cyclooxygenase 2
DHT	dihydrotestosterone
DMRT	doublesex and mab-3 related transcription factor
DNA	deoxyribonucleic acid
Eif2s3x	eukaryotic transcription initiation factor 2
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response elements
fMRI	functional magnetic resonance imaging
FOXL-2	forkhead box L2
FtM	female-to-male
GABA	γ-Aminobutyric acid
INAH	interstitial nucleus of the anterior hypothalamus
LTP	long term potentiation
MAP	mitogen-activated protein
mER	membrane estrogen receptors
MOA	monoamine oxidase A
mPOA	medial preoptic area
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MtF	male-to-female
NMDA	n-methyl-D-aspartic acid
PGE2	prostaglandin E2
PI3	phosphoinositide 3
PKA	protein kinase A
PVN	paraventricular nucleus
RNA	ribonucleic acid
SCR-1	steroid receptor coactivator 1
SDN-POA	sexual dimorphic nucleus of the preoptic area
SF-1	steroidogenic factor 1
SHBG	sex hormone binding globulin
SON	supraoptic nucleus
SOX-9	SRY-box 9
SRY	sex determining region Y
VMN	ventromedial nucleus of the hypothalamus
XIST	X (inactive)-specific transcript
X^mO	single maternally inherited X chromosome
X^pO	single paternally inherited X chromosome

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