Sex in the brain. Gender differences in the human hypothalamus and adjacent areas. Relationship to transsexualism, sexual orientation, sex hormone receptors and endocrine status
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CHAPTER

GENERAL DISCUSSION
General Discussion

Many aspects of life among vertebrates are affected by circulating sex hormones which have organizational and activational effects upon the functioning of the brain, body and behavior. Sex hormones are thus important epigenetic factors which act to differentiate and activate the brain according to sex. Not only do they bring brains together in order to mate, they are also presumed to exert an organizational effect on the developing human brain to establish two crucial aspects of the sexual spectrum, i.e. the feeling to be male or female (gender identity) and the erotosexual attraction towards an individual of the opposite or same sex (sexual orientation). In addition, sex hormones are implicated in the brain in many sexually dimorphic aspects of health and disease such as autonomic function, mood and cognition. The findings reported in the present thesis on the human hypothalamus and adjacent areas have provided evidence at the level of cells and cellular structures (proteins) which supports the paradigm that sex hormones interacting with the developing brain may contribute to the establishment of gender identity (Chapter 2, 3, 4, 5 and 6) and sexual orientation (Chapter 5, 6 and 8). In convergence with the available literature (cf. Österlund et al., 2000), there is virtually no adult brain area where sex hormones are not able to act in a sex dependent way (cf. Chapter 3, 4, 5, 6, 7 and 8).

In this chapter first some methodological considerations will be discussed regarding the brain material, staining procedures and antibodies used. Subsequently, the findings in relation to transsexuality and sexual orientation will be discussed. In addition, hypothalamus-brainstem axis interactions will be proposed to be involved in the regulation of sexual orientation, energy metabolism and various neuropsychiatric diseases. In this context, potential organizing and activating effects of circulating sex hormones are discussed and future research questions are put forward.

Methodological considerations

The data as obtained in the present thesis were obtained on paraffin embedded formalin fixed brain tissue.

A microwave tissue pre-treatment step at 700 watts for 10 minutes or a 90°C waterbath pre-treatment for 30 minutes appeared to be crucial to enable adequate antibody-antigen binding to the brain sections (cf. Kruijver et al., 2000; Ishunina et al., 2000; Pavao and Traish, 2001; Fodor et al., 2002). It has been proposed that microwave and waterbath antigen-retrieval techniques act by opening the formalin induced cross-links, which enables an adequate antibody penetration. For some antigens (AR; ERβ; PR) a tyramide-signal-amplification (TSA) step was introduced. TSA is based on biotin-labeled tyramide and peroxidase methodologies. In comparison with the standard streptavidin-bi-
otin (ABC-)method, TSA is about 50-100 times more sensitive because TSA introduces an extra second-antibody binding step (onto the second-antibody-primary-antibody-antigen-binding complex) with a larger molecule containing more biotinilated peroxidase sites which allow to detect very small quantities of the antigens (Kawai and Osamura, 2000).

A number of different antibodies were screened. In our hands the best ERα signal on paraffin embedded human brain tissue was obtained by MC-20. Other ERα antibodies, such as H222, 1D5, NCL-ER-bF11 (Novocastra) and other antibodies by Santa Cruz (Cat # 8002, F-10; sc-544,G-20; sc-7207,H-184), gave no to very weak nuclear and cytoplasmic ER-ir. The frequently used polyclonal-rabbit ERβ antibody Z8P, raised against the C-terminus of the mouse ERβ (Zymed Laboratories, South San Francisco, CA; cf. Shughru and Merchenthaler, 2001) revealed in human material much weaker ERβ nuclear and neuritic stainings and more prominent cytoplasmic staining than N-19. This observation was in contrast to Z8P’s very strong staining of only the nuclear compartment as was reported in rat (Shughru and Merchenthaler, 2001). ERβ-ir staining with the antibody N-19 was by far the most sensitive we could obtain in human material and had analogous to a ERα a low background as revealed by the staining protocols developed by F.P.M.K. et al.

Specificity tests on the antibodies as provided by the literature were extended by our tests i.e. by leaving out the primary antibody, adsorption tests, by verification of endocrine, pituitary and gonadal tissue staining patterns and by Western blots on human hypothalamic tissue to control for specificity of the AR-, ERα-, and ERβ-antibodies (respectively PG-21; MC-20 and N-19) (Chapter 3, 5 and 6). The antibody specificity tests as described in Chapter 2-7 were in good agreement with the literature. Moreover, we compared staining patterns of these antibodies with various other antibodies (that were raised against different epitopes of the AR, ERα, ERβ and PR). They showed similar receptor distribution patterns (Cf. Chapter 3, 5, 6 and 7). In addition, we have compared the receptor distribution patterns at the protein level with other studies at the messenger RNA in the human brain which also appeared to be in good agreement with each other (cf. Chapter 5 and 6).

Transsexuality

Transsexual individuals have the strong feeling, often from their earliest childhood memories onwards, of having been born the wrong sex. Gender identity disorder (GID) as defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994) consists of two components: (1) a strong and persistent cross-gender identification and (2) persistent discomfort with one’s biological sex or gender role behavior associated with one’s sex. The most extreme form, in which individuals need to adapt their phenotype with hormones and surgery to make it congruent with their gender identity, is called transsexualism (cf. ‘Definition and Synopsis of
the Etiology of Gender Identity Disorder and Transsexualism': www.GIRES.com). Transsexualism is thus the condition in which the transsexual person is convinced that he or she is actually a member of the opposite sex (reviewed by Gooren and Kruijver, 2002). Transsexuality is a rare condition. The annual incidence of transsexuality has been estimated in Sweden to be about 1/500,000 inhabitants. The sex ratio (genetic male:female) has been shown to vary from country to country between 1.4:1 and 3:1 (Landén et al., 1996; Garrels et al., 2000). In the Netherlands the prevalence of MTFs was found to be 1:11,900 and 1:30,400 for FTM (Bakker et al., 1993). The frequency of regret cases of sex re-assigned transsexual individuals varies from 3.8% in Sweden (Landén, 1999) to 0.4% in Germany and The Netherlands (Weitz and Osburg, 1996; van Kesteren et al., 1996). Transsexualism cannot be explained, in general, by variations in gonadal, genital or hormonal systems in adulthood (Gooren, 1990; Cohen-Kettenis and Gooren, 1999). In most cases, it cannot be clearly explained by variations in chromosomal patterns either (Gooren, 1990; Cohen-Kettenis and Gooren, 1999), although recent studies identified some sex chromosome anomalies. Six cases of male-to-female transsexuals with 47, XYY chromosome and one female-to-male transsexual with 47, XXX have been reported (Tayfun Turan et al., 2000). Also in men with Klinefelter syndrome (47, XXY) transsexualism has been reported (Wyler et al., 1979; Seifert and Windgassen, 1995).

A recent study on gender identity disorder (GID) in a child and adolescent mono- (n=96) and di-zygotic (N=61) pooled twin sample supports moreover the hypothesis that there is a heritable component to GID (Coolidge et al., 2002). This study also fits with other recent studies pointing to pairs of monozygotic female twins requesting for sex reassignment therapy and with familial cases of gender identity problems (Green, 2000; Sadeghi and Fakhrai, 2000). Together, these data do suggest a genetic basis in at least a subpopulation of transsexual people (reviewed by Swaab, 2002).

The paradigm of transsexuality as a neuro-developmental condition

"The brain is the sexiest hidden organ that we have"

Frank P.M. Kruijver, 2002

As pointed out in the introduction, sexual differentiation is a sequential process. At conception the configuration of the sex chromosomes determines the genetic sex, the genetic sex determines the gonadal sex and the gonadal sex influences the brain sex by gender specific secretion patterns of sex hormones: male by the presence of testicular androgens, female by the absence of testis and the lack of peaks in testicular androgen exposure, i.e. prenatally around 12-24 weeks of gestational age and postnatally around 4-24 weeks of neonatal age (reviewed by Hrabovszky and Hutson, 2002). The present thesis shows that
the human limbic brain expresses regional sex differences in gonadal hormone receptors (Chapter 3-8). Also during early development sex hormone receptors are present in the human brain in a stage-dependent sexually dimorphic way (cf. Chung, 2003). It thus appears conceivable that due to local hormone dependent changes during development at least some areas of the brain may follow a different course than the genitals during the process of sexual differentiation. A partial or even complete brain-body sex reversal may eventually be the result. This could lead to the development of female-like brain structures in a brain of a subject so far male differentiated or vice versa. If these brain areas are particularly involved in the establishment of e.g. an individual’s sexual orientation or gender identity a sex reversed partner preference or gender identity may be the result. The present thesis has provided new neurobiological evidence to support the view that transsexualism can be explained by a sex reversed brain status.

The BSTc

The human BSTc is sexually dimorphic in size and neuron number (Zhou et al., 1995a; Kruijver et al., 2000; Chung et al., 2002). Both measures are larger in males. No relationship between these BSTc measures and sexual orientation was found whereas a striking relationship with gender identity was observed (Zhou et al., 1995a; Kruijver et al., 2000). Male-to-female transsexuals had regardless of sexual orientation or adult endocrine status a BSTc with a female size and neuron number. Excitingly, the reversed pattern in the only available brain so far of a female-to-male transsexual was found (Kruijver et al., 2000). The functional implications of these sex dimorphic findings are still far from clear, but it is of note that in animals subdivisions of the BST have been implicated in the regulation of e.g. female reproductive (lordosis) and maternal behavior (Segovia and Guillamón, 1993; Sheehan and Numan, 2002). The BSTc expresses both ARs and ERs (Chapter 3,5,6) with more nuclear ERβ expression of low intensity in young adult men (present thesis; Chapter 6) that might be related to sexually dimorphic functions. The same holds true for the observation that men have more BSTc neuron numbers than women (Kruijver et al., 2000; Chung et al., 2002).

A conspicuous ERβ-ir of BSTc fibers was in addition observed both in men and in women (Fig 9.; Table 2; Chapter 6), which contrasted to the lack of such staining in the BSTm or BSTl (Fig.9; Table 2; Chapter 6). Together with literature of differences in neuropeptide content (Walter et al., 1991; Zhou et al., 1995a; Kruijver et al., 2000) these data clearly indicate that different subdivisions of the human BST may be functionally different. Furthermore, the presence of ARs, ERs and progesterone receptors (PRs) in the adult (Chapter 3,5,6) and developing BSTc (Chung, 2003) and the lack of effect of changed hormone levels in adulthood on the size and cell number of the BSTc (Zhou et al., 1995a; Kruijver et al., 2000) supports the idea that an altered interaction between sex hormones and the developing brain could have lead, at least in
part, to the BSTc’s sex reversal in size and neuron number and to the reversed
gender identity in transsexuals (Kruijver et al., 2003). Therefore it could be
argued that transsexualism is a sexual differentiation of the brain in disagreement
with the other characteristics of sexual differentiation. It should be emphasized
here that the BSTc size and neuron number is not related to sexual orientation.
Instead, the BSTc’s structural sex difference appears to be related to gender
identity per se rather than to a particular early onset, late onset or subtype form
of transsexuality, such as the recently intensely debated and claimed “homosexual”
and “autogynephilic” subtypes of male-to-female transsexualism (cf.
Blanchard, 1989ab; internet sites, literature and letters related to this topic by
JM Bailey; M. Italiano; K. Clarke & K. Gurney; C. Johnson).

The neurodevelopmental study by Chung et al., (2002) indicates that the
volume (neuron numbers were not counted during development) of the BSTc
area becomes, much to our surprise, only sexually dimorphic around young
adulthood. The volume of the BSTc therefore does not appear to be a good
early marker for GID. The developmental course of the BSTc could, however,
have been programmed already much earlier, i.e. by the pre- and/or postnatal
testosterone surges. A somatostatin and total neuron-count follow-up study on
the aforementioned investigations (Kruijver et al., 2000; Chung et al., 2002)
during the course of development might be of interest in order to determine
whether or not the higher circulating androgen levels in males at prenatal,
neonatal and/or pubertal ages (cf. Hrabovszky and Hutson, 2002) might be asso-
ciated with BSTc changes in neuronal cell division or apoptosis. The BSTc
may well be just the tip of the iceberg of a sex-reversed limbic brain circuit
which is involved in the establishment of the feeling to be male or female.
The search for potential additional brain areas, such as possibly the amygdala,
involved in the establishment of gender identity and the developmental age at
which the BSTc’s sex difference in terms of neuron numbers, synaptic branch-
ning, neuropeptide content, receptor-binding capacity and metabolic activity
becomes manifest still awaits future investigations. These investigations may
in the future hopefully profit from non-invasive in vivo visualisation techniques
(cf. Kok et al., 2001; Lamberts et al., 2002) by which e.g. somatostatin receptor
type 2(SR)-positive brain areas will be imaged in vivo by injection of a radioac-
tive-labeled non-peptidergic somatostatin analogues that can efficiently pass the
blood brain barrier (cf. Lamberts et al., 2002; Csaba et al., 2003). In contrast
to the human BSTm and BSTI but in parallel with its regional expression in
the rat BST (Csaba et al., 2003), the human BSTc expresses indeed robustly
somatostatin type 2 receptors at the protein level (Kruijver, Fodor, Epelbaum,
Dournoud and Swaab, unpublished observations). Interestingly, a recent clinical
study used the SR-binding technique in the cortex. The authors describe a
strong tendency in males for an increase in radioactively labeled somatostatin-
analogue binding. The reported relative enhanced 111-In-Pentetreotide-uptake in
men indicated a higher SR-density in the temporal and frontal cortex (Pichler

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et al., 2002). However, such a technique, even when refined, will not allow to reveal detailed microscopical information regarding e.g. neuron counts, synaptic branching, mRNA and neuropeptide expression levels, co-localization etc. Human postmortem brain studies will thus continue to be needed in order to further answer our detailed research questions.

**Genes**

Additional support for the paradigm that transsexualism is neuro-developmental condition, e.g. based upon a difference in interaction of sex hormones and the developing brain, comes from the findings by Landén et al., (1999). In a study from his thesis, Landén and colleagues found an association between sex steroid related genes and male-to-female (MTF) transsexualism. In a sample of 28 MFTs they found that MTFs display significantly more often than control subjects a long nucleotide sequence repeat polymorphism of the aromatase gene, AR gene or ERβ gene. It is known that the lengths of repeat nucleotide sequences may influence the transcription, translation and function of a gene (Comings, 1998; Landén et al., 1999). For example, a mutation, in terms of expansion of the CAG (40-72) repeats is known to be the cause of a rare X-linked inherited form of motor neuron disorder, termed Kennedy’s disease or spinal and muscular bulbar atrophy. The activity of the AR gene is inversely correlated to the length of a CAG trinucleotide repeat in exon 1 of the AR gene. This repeat region ranges from 10-30 in the normal population (MacLean et al., 1995). A relatively long CAG repeat length leads to a low transcriptional activity of the AR (cf. Chamberlain et al., 1994; Knoke et al., 1999). This is associated with a lower risk of prostate cancer and benign prostate hyperplasia, but with a higher risk of infertility. Thus, decreased aromatase and sex hormone receptor activities could be part of the functional consequences caused by the observed polymorphisms in MTF transsexuals (Landén et al., 1999).

Another potential direct genetic mechanism that might have contributed to the BSTC’s failure in MTFs to differentiate into the direction of their genetic sex is the possibility of an involvement of sex determining genes (Reisert and Pilgrim, 1991) such as the SRY gene, which is known to be transcribed in the male brain but not in the female brain of adult human subjects (Mayer et al., 1998). In addition, other potential sexually dimorphic genetic effects on the development of the brain cannot be ruled out (cf. Dewing et al., 2003; Galfalvy et al., 2003).

**Androgen exposure during early brain development**

Also differences in the level of intra-uterine testosterone (T) exposure and/or androgen receptor function of the developing brain could be a risk factor of GID.

Five α-reductase transforms testosterone into dihydroxytestosterone (DHT), which is necessary for the virilization of the male body and external genitals.
(cf. Wilson, 1999; introduction). Deficiency causes an autosomal recessive form of male pseudohermaphroditism in which the phenotype may resemble that in 17β-hydroxysteroid dehydrogenase-3 deficiency, which transforms androstendione into testosterone (Wilson, 1999; Stoffel-Wagner, 2001). In both situations virilization of the external genitalia is impaired and affected males are usually assigned a female gender at birth and raised as girls. Since the undescended testosterone producing testes are still present, these individuals virilize to a greater or lesser extent at the time of expected puberty (Wilson, 1999). Imperato-McGinley et al., (1979) reported that 18 of 19 affected individuals from one family with 5α-reductase deficiency in the Dominican Republic were initially raised as females but subsequently changed gender role behavior to male at the time of expected puberty. A similar phenomenon has been described in other parts of the world: about two-thirds of individuals raised as females change to male gender role after puberty (Wilson et al., 1993; Wilson, 1999).

Apparently, the influence of social factors in development is not sufficient to live as a female after puberty. Meyer-Bahlburg et al., (1996) reported a gender change from woman to man in four out of 31 46, XX individuals with classical congenital adrenal hyperplasia (CAH), which is characterized by high androgen levels during prenatal development (Wilson, 1999). Although it should be emphasized that the large majority of women with this disorder do not experience a marked gender identity conflict, the odds ratio that a genetic female with this disease would live, as an adult, as a male compared to genetic females in the general population was found to be 608:1 (Zucker et al., 1996). CAH thus seems to constitute a risk factor for the development of gender identity problems.

Also a baby boy, who suffered accidentally from ablative penis during an operation for circumcision at 8 months of age and who was turned into a girl by plastic surgery of the genitalia and raised as a girl since then, strongly rejected, however, her female gender-role and became a man again at puberty (the John-Joan-John case; Diamond and Sigmondson, 1997). John never felt like a girl but like a boy/man like his male twin brother. Not even the estrogen treatment of this person at puberty could prevent him to return to his nature sex. Although just one case, it appears to illustrate that there is little, if any, support for the view that individuals are fully sexually neutral at birth. [At 38 years of age John’s life ended tragically by committing suicide during the spring of 2004, two years after his twin brother had committed suicide.]

In a second case of penile ablation in which the decision was made to reassign the patient as a girl and raise the baby as a girl, the remainder of the penis and testes were removed at a slightly earlier stage, at 7 months. Although her sexual orientation was bisexual and even though she was mainly attracted to women, her gender identity was female. The different outcome as compared with the former case is explained by the authors on the basis of the decision to reassign the sex at an earlier age (Bradley et al., 1998). These two cases illustrate that circulating testosterone abnormalities during development can be
considered as one of the risk factors and not the sole cause of gender identity disorder. However, a third case history appears to reinforce a testosterone-mediated/biological predisposition for gender identity. A child with true hermaphroditism, 45x(13%47XXY(87%) sex chromosome mosaic pattern in blood, uterus, fallopian tubes, phallus, testicular tissue and epididymis was assigned at birth to become a male. At 5 weeks the decision was made to reassign him to the female sex. At 13 months the testicle was removed and at 9 month and 5 years operations were carried out to make the genitalia female. She was raised as a girl, but had masculine interests and when she was around 8 years old she declared “that God had made a mistake” and that she “should have been a boy”. Apparently the presence of the male Y chromosome resulting in testicle derived sex hormones to which she had been exposed in utero had imprinted the male gender, although the authors also presumed postnatal psychosocial factors to have played a role (Zucker et al., 1987).

Individuals with a dysfunctional AR (or possibly AR-coactivator, see Adachi et al., 2000), i.e. known as complete androgen insensitivity syndrome (CAIS), result in XY heterosexual females with a female gender identity (Wisniewsky et al., 2000; Adachi et al., 2000). The idea that the direct interaction between androgens and the AR might play an important role in the establishment of a male gender identity is strengthened by the fact that in congenital deficiency of estrogens in men (by aromatase deficiency or estrogen resistance) gender identity is not affected (Carani et al., 1997;1999; Faustini-Fustini et al., 1999; Rochira et al., 2001; Smith et al., 1994; Morishima et al., 1995). The fact that throughout the world the prevalence of MTFs is on average three fold higher than that of FTM (Landén, 1999) could moreover be explained by the higher probability of androgen-AR brain interaction problems in men in contrast to women, where just the absence of elevated circulating androgens during early development seems to direct the establishment of a female gender (see also Hines et al., 2003; Sato et al., 2004).

Together, these observations support the view that the intrauterine and early postnatal exposure to normal androgen levels in males may contribute to the establishment of gender identity development into the male direction. In reverse, abnormal high androgen levels in females or androgen resistance in males may permanently affect brain development and consequently direct the establishment of gender identity into respectively male or female direction.

Possible factors overriding early androgenic effects

If indeed prenatal androgen exposure plays an essential role in the establishment of male gender identity it is conceivable that anything that may disturb the androgen-AR interaction, such as prenatal social stress of the pregnant mother, environmental neuroendocrine disruptors (e.g. plastics or pesticides), medicines taken during gestation or placenta dysfunction may result in abnormal hormone levels/action and so in gender identity problems. For example, prenatal (social)
stress was found to induce striking sex-reversed changes in brain and behavior in male and female rats and guinea pigs (Ward, 1972; Koehl et al., 1999; Kaiser et al., 2003ab). In the latter studies we found in respectively female and male guinea pigs a sex-reversed masculinization and demasculinization in social/play-behavior and ER/AR expression patterns in various limbic brain areas (such as the hypothalamic MPOA, ARC and hippocampal CA1 regions). The somatostatinergic BST area should in the future be studied. Also in the human the influence of prenatal stress on homosexuality might be inferred (see below), while in a recent prospective study on maternal self-reports on the effect of prenatal stress on gender behavior in children until 3 years of age no changes were found (Hines et al., 2002). However, we have to wait for longer follow-up studies to come to final conclusions.

Another exciting observation comes from a study by Dessens et al., (1999). They reported that 3 children born of a group of 243 women exposed to the anticonvulsants phenobarbital or diphtantoin were found to be transsexuals, while, in addition, there were a few other subjects with gender dysphoria/cross-gender behavior. Gender problems thus occurred remarkably often in view of the rarity of this disorder. Phenobarbital has widely been used as prophylactic treatment in neonatal jaundice, and greatly elevated the postnatal rise in testosterone (Forest et al., 1981). This important observation on the effect of medicines that are known to alter steroid levels leads to the question whether intrauterine sex hormone levels are changed by alcohol, cannabis, nicotine, cocaine or ecstasy use during pregnancy and if so whether these compounds are potentially related to the occurrence of transsexualism. Interestingly, it was found by Hines et al., (2002) that prenatal nicotine exposure induces more masculine/sex-reversed gender behavior in young girls of 3 years of age. There is now also growing evidence of potential interactions between sex hormones and the above mentioned compounds (cf. Purohit et al., 1980; Raum et al., 1990; Parrott and Lasky, 1998; Tchernitchin et al., 1999; Ward et al., 1999; Eagon et al., 2001; Mani et al., 2001; Mendelson et al., 2002). For example, elevated circulating estrogens, decreased circulating androgens and AR/ER cellular activity as well as inhibited androgen binding at the receptor level have been reported for alcohol and canabis respectively (Purohit et al., 1980; Eagon et al., 2001). One may also have concerns about many other compounds. Melatonin, for example, is currently popular and widely used for sleep and jet-lag problems and freely available in the USA (Rohr and Herold, 2002). However, recent in vitro experimental studies showed that melatonin is able at nanomolar (nM) levels to diminish nuclear androgen receptor concentrations of prostate cancer (PC3) cells by nuclear exclusion (Rimler et al., 2002) and to destabilize the binding of the estradiol-estrogen receptor complex to DNA estrogen responsive element in MCF7 breast cancer cells (Rato et al., 1999). Whether or not the use of melatonin during pregnancy may interfere with the process of sexual differentiation of the brain remains to be studied.
**Sexual orientation**

Throughout the animal kingdom the spectrum of sexual orientation (i.e. from hetero-, bi- to homo-sexuality) is known to exist from Drosophila and birds to lions, rams and primates including Homo Sapiens (Bagemill, 1999; Kitamoto, 2002; Roselli et al., 2003). It is estimated that 2-5% of men are exclusively homosexual and 1-2% of the women (for references see Rahman and Wilson, 2002). Biological variations may underly shifts from the species-typical pattern of heterosexuality. There are two consistent correlates of sexual orientation that point to an early developmental genesis. The first is the relationship between childhood gender nonconformity and adult sexual orientation (Bailey and Zucker, 1995). The second is the fraternal birth order in males. In diverse samples and independent replications, homosexual men are found to have a greater number of older brothers than heterosexual men (Blanchard et al., 1995; Blanchard, 1997; Bailey et al., 1999; Bogaert, 1998; Purcell et al., 2000; Ellis and Blanchard, 2001). It has been estimated that each older brother increases the relative risk of being a homosexual man by 33- 48%, although these odds translate into population probability estimates of only a few percent (Blanchard, 2001; Rahman and Wilson, 2003). The hypothesis advanced is that the late birth order, with more male siblings born earlier, could lead to a progressive immune response of the mother to androgens and/or Y-linked minor histocompatibility (H-Y) antigens which, by maternal transfer of these immune antibodies to the fetus, could impair brain masculinization of the fetus (Blanchard, 2001). However, why this mechanism would selectively impair only certain presumed androgen dependent processes, such as the brain programming, and not others, like formation of the genitalia, is not explained by this hypothesis (Gooren and Kruijver, 2002).

**Family studies and genetics**

Genetic and epigenetic factors, in particular hormonal actions have also been implicated (for recent review cf. Swaab et al., 2003).

Sexual orientation is influenced by a number of genetic factors as appears from studies in families and twins, and from molecular genetics (Kallman, 1952; Pillard and Weinrich, 1986; Bailey and Pillard, 1991; Bailey and Bell, 1993; Hamer et al., 1993; Turner, 1995; Hu et al., 1995; Pillard and Bailey, 1998; Bailey et al., 1999). Homosexual men have more homosexual brothers and homosexual women have more homosexual sisters as compared to respectively heterosexual men or women. Twin studies also suggest that this familality is at least partly genetic. Monozygotic twins show around 30-60% greater concordance for homosexuality than dizygotic twins (Rahman and Wilson, 2003). As reviewed by Swaab et al., (2003), Hamer and colleagues found linkage between DNA markers on the X-chromosome and male sexual orientation. Genetic linkage between the microsatellite markers on the X chromosome, i.e. Xq28, was detected for the families of gay males but not for the families of lesbians.
(Hamer et al., 1993; Hu et al., 1995). However, Rice et al., (1999), found an absence of linkage to microsatellite markers at Xq28 and this controversy will probably continue for some time.

Non-genetic factors related to homosexuality

Although not without controversy, the prenatal social environment, in particular maternal stress during the first trimester, has been implicated in increased occurrence of homosexuality in boys (Dorner et al., 1983; Ellis et al., 1988; Schmidt and Clement, 1995; Ellis and Cole-Harding, 2001). Interestingly, recent studies have indicated that prenatal nicotine exposure has masculinizing/defeminizing effects on sexual orientation of the female offspring and increases the probability of lesbianism, especially if the nicotine exposure occurred in the first trimester along with prenatal stress in the second trimester (Ellis and Cole-Harding, 2001).

Sex hormones during early brain development (i.e. prenatal, but also peri- and neonatal; cf. Chapter 1) are very likely to have an important epigenetic influence on the establishment of sexual orientation. In addition to experimental studies (Adkins-Regan, 1993; Swaab et al., 1995), this possibility is underlined by studies showing an increased proportion of bi- and homosexuality in girls with congenital adrenal hyperplasia (CAH) (Money et al., 1984; Dittmann et al., 1992; Meyer-Bahlberg et al., 1996) and in girls whose mothers received a synthetic estrogen-like compound diethylstilbestrol (DES) during pregnancy to prevent miscarriage (which it does not do) (Ehrhardt et al., 1985; Meyer-Bahlburg et al., 1995). Also, complete androgen insensitivity syndrome, which results in the development of 46, XY females who are both in phenotype and gender identity heterosexual (i.e. male oriented), provide additional support for this concept (Wnisiewski et al., 2000; Adachi et al., 2000).

Although postnatal social factors are generally presumed to be involved in the development of sexual orientation (Byrne and Pearson, 1993; Zucker et al., 1996), solid evidence in support of such an effect has so far not been reported. On the contrary, the observation that children raised by lesbian couples or by transsexuals generally have a heterosexual orientation (Green, 1978; Kirkpatrick et al., 1981; Golombok et al., 1983) does not support the possibility that the social environment, in which the child is raised, is an important factor for determining sexual orientation, nor is there scientific support for the idea that homosexuality has psychoanalytical or other psychological or social learning explanations, or that it would be a “life-style choice”(Ellis, 1996; cf. Swaab, 2002; Swaab et al., 2003). Furthermore, the findings of the present thesis showing sex hormone sensitivity of the SCN, INAH-3 and the anterior commissure, brain areas from which the size has been related to sexual orientation (Swaab and Hofman, 1990; LeVay, 1991; Allen and Gorski, 1992) and the presence of sex hormone receptors during brain development (Chung, 2003) support the idea that the interaction between sex hormones and the developing brain may play an organizing role.
on these brain structures (Chapter 4, 5, 6 and 8). Further support for the idea that early developmental processes are involved comes from the ratio of the index finger (2D) to the fourth digit (4D), which is sexually dimorphic and shows in males a reduced ratio and in females a higher ratio. This difference, most likely, reflects a difference in prenatal actions of androgens on males (Manning et al. 1998; 2000). Recently, Williams et al., (2000) reported that the right hand 2D:4D ratio in homosexual women was significantly more masculine (i.e. smaller) as compared to that in heterosexual women and not different from that in heterosexual men. This group reported no differences between homosexual and heterosexual men. However, segregating men by birth order showed that only homosexual men with a later birth order (i.e. two or more older brothers) had a more masculine right hand 2D:4D ratio than homosexual men with one or no older brothers. On the basis of this birth-order effect the authors concluded that men with older brothers, including those who may become homosexual, are exposed to higher prenatal testosterone levels (reviewed by Rahman and Wilson, 2003). Rahman and Wilson, (2003) also confirmed that, particular on the right hand, both homosexual males and females have lower 2D:4D ratios than heterosexual controls. Altogether, these data suggest that homosexual men and women may have been exposed to alterations in sex hormone levels during early development.

Potential hypothalamus-brainstem axis involvement in sexual orientation

“We must recollect that all of our provisional ideas in psychology will presumably one day be based on an organic substructure.”

Sigmund Freud, 1914

A fascinating question is which sexually dimorphic brain system can be held responsible for the establishment of sexual orientation. To date there is some literature pointing to two neuropeptide systems that appear to be involved in pair-bonding/partner-preference behavior, i.e. the vasopressin (AVP) system in male and the oxytocin (OXT) system in female voles (cf. Chapter 1). Partner preference (i.e. heterosexual orientation) has been shown to be dependent on AVP/V1a-receptor interactions in the brain of male voles (Winslow et al., 1993; Cho et al., 1999; Insel and Young, 2001;), whereas central oxytocin/OT-receptor interactions are required for heterosexual preference in females (Williams et al., 1994; Insel and Hulihan, 1995; Cho et al., 1999).

AVP and OXT are centrally produced by the hypothalamic supraoptic nucleus (SON), paraventricular nucleus (PVN) and suprachiasmatic nucleus (SCN). In addition, neuronal pathways immunoreactive for AVP and OXT were demonstrated in a large number of areas in the rat brain (Buijs et al., 1978; Buijs, 1990) and human brain (Swaab, 1998). In this respect, the area of the brainstem optic tectum or colliculus superior seems to be of particular inter-
est. This evolutionary very old neuroanatomical structure is also an important vasopressinergic and oxytocinergic relais station (Loup et al., 1989; 1991) that receives (cortically mediated) auditory, tactile, olfactory and visual information (Kahle et al., 1986; Huffman et al., 1990; Stein et al., 2002). It might be inferred that the SC could play a role in the integration of sensory information needed for all kinds of automatical behavioral strategies, including sexual orientation. In this respect it seems of interest to note that within the salamander Taricha granulosa the optic tectum has AVP-precursor arginine vasotocin concentrations which are sexually dimorphic (higher in males than in females) and reach peak levels in males at the time point of high partner preference behavior, i.e. during the breeding season (Zoeller et al., 1986; Moore, 1992).

The superior colliculus may also be a logical site for the integration of multiple sensory information to another sexual orientation related brain area i.e. the SCN.

The presumed existence of a SCN- superior colliculus connection, which may collaborate to orchestrate various physiological aspects of behavior (Morin et al., 1994), was just recently demonstrated (Krout et al., 2002). In this respect it could be postulated that a retina-SC-SCN-connection may be involved in respectively SCN-mediated timing and SC-mediated perception and orienting performance of sexual orientation/partner preference behavior (cf. Swaab and Hofman, 1990; Kruijver et al., 1993; Bakker et al., 1993; Rahman and Silber, 2000; Swaab, 2002). Future studies will be needed to determine whether the human SC is structurally and/or functionally sexually dimorphic.

"A behavioral trait such as sexual orientation almost certainly is not caused by a single gene, a single alteration in a hormone or in brain structure, or a single life experience. The continuing progress in studies of sexually dimorphic characteristics will no doubt help psychoanalysts to better understand gender identity and sexual orientation."

Eric Kandel, 1999

Reproduction, autonomic function, mood and cognition

Potential functional implications

It seems clear by now that there is hardly any human brain area that is completely devoid of sex hormone receptors. Except for the external globus pallidus ARs, ERα and ERβ are ubiquitously expressed, albeit in a distinct and gender dependent way (see also Table 2, Table 3 and Table 4 in Chapter 3 and 6 respectively). In addition PR-ir is observed in many different brain areas (Kruijver and Swaab, 2002; Chung, 2003). It is thus no surprise that human neuronal ERs are colocalized with many different neuropeptides such as somatostatin (SRIF) and prepro-enkephalins (e.g. in the BSTc), galanin (e.g. in the SDN-
MPOA, DBB, NBM, SON, PVN and INF), vasopressin and oxytocin (e.g. in the PVN and SON), CRH (e.g. in the hypothalamic PVN and thalamic PV,PT and AM), vasopressin, vasoactive intestinal polypeptide and neurotensin (e.g. in the SCN), acetylcholine (e.g. in the DBB, NBM), histamine (e.g. in the TM), GABA and glutamate (e.g. in the INF, NTL and presumably BSTc).

In our AR, ERα and ERβ distribution studies (Chapter 3, 5, 6) we discussed various brain areas such as the BSTc, Ac, SDN-POA/INAH-1-4, BM, PV, PT, SCN, VMN, INF, ZI and MBC in relation to sex, sex hormone sensitivity, gender identity, sexual orientation, sexual behavior, autonomic function and reproduction (Chapter 2-6).

Here we will focus on the way sex hormones may be involved in human reproduction, energy metabolism, stress, mood and cognition.

How the human SCN may regulate the sexually dimorphic hypothalamo-pituitary-gonadal (HPG)-axis

The suprachiasmatic nucleus (SCN) is the hypothalamic biological clock (Rusak and Zucker, 1979; Swaab, 1999; Buijs and Kalsbeek, 2001). Environmental light serves as the main “Zeitgeber” to entrain the clock, which receives its input via the retinohypothalamic tract (Dai et al., 1998a). In turn, the SCN and its projections communicate through synaptic pathways with various effector systems. These areas are known to be involved in the autonomic regulation of body temperature, blood pressure, sleep, arousal, energy metabolism, stress (HPA-axis), thyroid hormone (HPT-axis) and growth hormone regulation and in the regulation of the reproductive gonadal (HPG-)axis, via the cyclic release of gonadotropin-releasing- or luteinizing-hormone-releasing hormone (GnRH or LHRH) (Van der Beek et al., 1997; Dai et al., 1998b; Swaab, 1999; Saeb-Parsy et al., 2000; Aston-Jones et al., 2001; Kalsbeek et al., 2000a; Kalsbeek and Buijs, 2002).

During early embryonic brain development LHRH neurons originate from the olfactory placode and from here differentiate and migrate into the mammalian brain along the terminal nerve into the ventromedial rostro-basal forebrain as well as preoptic and medio basal hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; 1990; Schwanzel-Fukuda et al., 1996). As a result there is a prominent medio-ventral zone of LHRH expressing neurons, rather than a single LHRH expressing nucleus, which seems to be responsible for the regulation of the SCN-mediated cyclic release of LHRH by the HPG-axis (Pape and Herbison, 2001; Buijs and Kalsbeek, 2001). The rat SCN has been shown to be responsible for the ovarian reproductive cycle, through a direct monosynaptic innervation of LHRH neurons by VIP fibers (Van der Beek et al., 1993; 1997) and indirectly by SCN-derived AVP fibers projecting to the MPOA from which the signal is further transmitted to LHRH expressing neurons (Palm et al., 1999; Funabashi et al., 2000). Lesioning of the SCN area results indeed in failure of ovulation in the female rat (Brown-Grant and Raisman, 1977). One may wonder which
neurons participate in the estrogen dependent cyclic preovulatory LHRH surges (positive estrogen feedback) (Pape and Herbison, 2001). Rodent and primate literature suggest that, in addition to the periventricular Pe/PVN-zone and mediobasal INF, also the SON and DBB may be involved in the regulation of LHRH release. The DBB, Pe-zone, SON and INF have 4 important features in common: 1) they have reported reciprocal anatomical and/or functional connections with the SCN (Stephan et al., 1981; Dai et al., 1998b; Saeb-Parsy et al., 2000; Krout et al., 2002; 2) all four areas have been reported to express LHRH, at least in the human and non-human developing and adult primate brain (Thind et al., 1991; Rance et al., 1994; Quanbeck et al., 1997; Dudas et al., 1998) 3) all four areas have been reported to project, at least partly, to the ME (Wiegand and Price, 1980; Silverman et al., 1987; Thind et al., 1993; Saeb-Parsy et al., 2000; Simmerly, 2002) 4) all four areas have been reported to express sex hormone receptors in vertebrates, including non-human and human primates (Cf. Chapter 5 and 6).

Observations in the human hypothalamus revealed LHRH neurons to be present in the DBB and ventromedial SON (Rance et al., 1994; Dudas et al., 2000; Dudas and Merchenthaler, 2001/2002). A subpopulation of DBB and SON neurons project in turn to the ME in addition to classical projection areas such as, respectively, DBB-hippocampus or SON-neurohypophysial stalk projections (Mikami et al., 1978; Wiegand and Price, 1980; Mesulam et al., 1983; Silverman et al., 1987; Thind et al., 1993; Saeb-Parsy et al., 2000). Also the neurons of the periventricular zone (involving to some extend PVN neurons; cf. Rance et al., 1994; Dudas et al., 2000; Dudas and Merchenthaler, 2001/2002) and INF are known to be under direct control of the SCN (Marani et al., 1987; Saeb-Parsy et al., 2000; Buijs and Kalseek, 2001; Simmerly, 2002) and to project to the ME (Wiegand and Price, 1980), where cyclic LHRH can be released into the portal capillaries. Release of LHRH into portal vessels is of importance in order to regulate the menstrual cycle by LHRH signalling to FSH and LH producing cells of the anterior pituitary (Simonian et al., 1999; Saeb-Parsy et al., 2000). The sex hormone sensitive SCN may thus, in principle, orchestrate cyclic LHRH release either by direct (vasopressinergic/VIPergic) projections onto LHRH producing neurons and/or by direct projections onto INF/ME LHRH terminal endings on the portal vessel system (cf. Marani et al., 1987). Whether or not the observed sex differences in nuclear ERα and ERβ in DBB neurons with respectively higher and lower expression patterns in young women and the higher expression patterns of nuclear ERβ in young women in the VPe, SON and INF reflect potential functional sex differences of the HPG-axis awaits further investigations. So far at least two points have been resolved concerning the LHRH system. The first is the fact that LHRH neurons indeed do express ERs, since LHRH neurons in rat coexpress at least ERβ (Pape and Herbison, 2001). The second is the fact that the SCN itself does indeed express sex hormone receptors in both the developing and adult rodent,
primate and human brain, thereby enabling the SCN to monitor circulating levels of sex hormones (cf. Chapter 3,5,6, and 8 and the literature referred to within chapter 8). However, the idea that the DBB (cf. Chapter 5) and SON, in principle, may play a SCN mediated role in the sexually dimorphic regulation of the HPG-axis puts both the DBB and SON in a new context. Since in most rodent and primate species (cf. Silverman et al., 1977; Kawano and Daikoku, 1981; Witkin et al., 1982; King et al., 1982; Silverman et al., 1982; Glass, 1986) the most prominent neuronal LHRH population does not reside in the caudal ARC/INF area (including possibly the human INF; Hestiantoro et al., unpublished observations) but rather in the rostral septal/DBB/preoptic/SON neurons, the sexually dimorphic DBB and SON may thus constitute a neuronal basis in this region for SCN mediated cyclic LHRH release. In connection to the sexually dimorphic regulation of the HPG-axis, it is presumed by some researchers (Gladue et al., 1984; Dorner, 1998) that transsexuals and homosexuals alike are neuroendocrinologically different from heterosexuals with regard to the feedback response of LH to an estrogenic stimulus, inferring that this feedback response is of the female-type in male homosexuals and MTF transsexuals, and, vice versa. However, better designed research shows that transsexuals and homosexuals are neuroendocrinologically indistinguishable from heterosexuals with regard to the dynamics of the HPG-axis (Gooren et al., 1990; Gooren, 1990).

ERs in relation to autonomic function and energy metabolism

Autonomic function

In contrast to premenopausal women, ER expression patterns in postmenopausal women shifts towards a greater nuclear ERα/ERβ ratio in vasopressin-ergic SON neurons, indicating an enhanced ERα-mediated vasopressinergic activity in SON neurons of older women as a result of a decrease in circulating estrogen levels (Chapter 7). A functional consequence of the hyperactivity of the SON may, at least in part, be the presence of increased blood pressure in postmenopausal women. A similar nuclear ERα/ERβ shift appears to occur in postmenopausal PVN neurons, which may, in principal, stimulate enhanced CRH-release (cf. Chapter 6; Goncharuk et al., 2002) and elevate blood pressure levels (vide infra).

Another hypothalamic nucleus connected to blood pressure regulation is the sex hormone sensitive SCN (Goncharuk et al., 2001; Kruijver and Swaab, 2002). The SCN innervates the ventromedial SON reciprocally (Dai et al., 1997; 1998; Saeb-Parsy et al., 2000) and shows a decreased number of AVP and VIP neurons in hypertensive patients (Goncharuk et al., 2001). Since the human AVP gene contains an estrogen responsive element (cf. Ishunina and Swaab, 2002) and since vasopressin neurons from the rat SCN were shown to inhibit CRH neurons in the PVN (Kalsbeek et al., 1992; Gomez et al., 1997),
it could be reasoned that the decrease in postmenopausal circulating estrogen levels may contribute to a decrease in SCN derived AVP release in aged people (Hofman and Swaab, 1994;1995; cf. Chapter 8). Analogous to its effects in rat, reduced SCN derived AVP may in turn induce enhanced corticotropin releasing hormone (CRH) release in PVN neurons innervated by AVP fibers from the SCN (cf. Swaab et al., 2000; Zhou et al., 2001). An activated hypothalamus-pituitary (HPA)-axis results in higher plasma cortisol levels which may even further inhibit SCN activity. The adult rat SCN expresses glucocorticoid receptors (GRs) (Morimoto et al., 1996) and the human SCN appears to be sensitive too to glucocorticoids, e.g. by the fact that glucocorticoid-treated patients have reduced AVP mRNA levels in their SCN (Liu et al., 2001), but also restores the enhanced diurnal bloodpressure values back to premenopausal levels (Mercuro et al., 1998). In addition, SHRT in postmenopausal women restores changes in the circadian fluctuation of body temperature, impaired sleep and mood (Sherwin and Gelfand, 1985; Polo-Kantola et al., 1998; Gudmundsson et al., 1999). Together, these data suggest that sex hormone withdrawal effects on the SCN may mediate autonomic functional changes in postmenopausal women and that such effects may be reversed by “activation” effects of SHRT (cf. Chapter 8).

Another brain area that has been associated with various autonomic functions such as the regulation of body temperature, sleep, sexual development of the reproductive axis as well as with circadian rhythm disturbances during ageing and in Alzheimers disease is the pineal gland (Cagnacci and vole, 1996; Liu et al., 1999; Satoh and Mishima, 2001; Van Someren et al., 2002) At night it produces the sleeping hormone melatonin under control of the SCN (Teclemariam-Mesbah et al., 1999; Kalsbeek et al., 2000b; Perreau-Lenz et al., 2003), while melatonin, in turn, is considered to be a “fine-tuner” of rhythms that are generated by the SCN (Cagnacci and vole, 1996; Liu et al., 1999). Recently sex hormone receptors have also been shown in the human pineal gland, which provides an additional entrance for sex hormones on circadian rhythm regulation (Martin et al., 1996; Luboshitzky et al., 1997; 1998; Brzezinski, 1997). Interestingly, it has been shown in the non-primate brain that testosterone and estrogen indeed stimulate the nocturnal release of melatonin from the pineal gland (Martin et al., 1996). Not only ageing is associated with decreased levels of sex hormones and melatonin and with sleep rhythm disturbances, but an even more dramatic disruption is found in Alzheimer patients (Swaab et al., 1996; Manly et al., 2000; Schönknecht et al., 2001). The circadian rhythm disturbances in Alzheimer patients are often so severe that they are even thought to contribute to mental decline (Moe et al., 1995). Therefore, it would be of interest to investigate whether sex hormone replacement therapy may not only improve cognition, regional cerebral blood flow and EEG activity (Ohkura et

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al., 1994; Resnick et al., 1998), but also circadian functions such as sleep quality and temperature regulation by restoring the decreased nighttime melatonin levels that were observed in elderly people and in Alzheimer patients (Liu et al., 1999; Wu et al., 2003).

**Energy metabolism**

Two other sex hormone sensitive areas that are known to be reciprocally connected with the SCN and that are functionally implicated in energy metabolism are the zona incerta (ZI) and the infundibular nucleus (INF or ARC) (Marani et al., 1987; Akabayashi et al., 1994; Horvath, 1997; Jamali and Tramu, 1999; Saeb-Parsy et al., 2000; Leak and Moore, 2001; Krout et al., 2002; Mungarndee et al., 2002).

**ZI**

The rostral zona incerta (ZI) is continuous with the lateral hypothalamic area and has been implicated in nociceptive and somatosensory perception, locomotion, sexual behavior, feeding and drinking, arousal and attention (Brown and Grossman, 1980; Edwards and Isaacs, 1991; Heeb and Yahr, 1996; Greco et al., 1996; Ongur and Price, 1998; Iqbal et al., 2001; Mungarndee et al., 2002; Nandi et al., 2002). ERβ is strikingly present in both neurons and (basket-like) fibers of the human zona incerta (Chapter 6). Therefore, it seems of interest to investigate (e.g. by conditional knock-out studies of ERβ signal in the ZI) to what extent ERβ is involved in one or more of these processes.

**INF**

The INF expresses appetite-driving peptides such as neuropeptide Y (NPY), opioids and galanin (GAL) as well as appetite-inhibiting (anorexigenic) neuropeptides such as alpha-melanostimulating hormone (α-MSH) and cocaine and amphetamine-regulated transcript (CART) (Kalra et al., 1999). In addition to previously observed anatomical connections between the SCN and the INF (Marani et al., 1987), a recent study provided functional evidence for reciproque connections between the INF and SCN (Saeb-Parsy et al., 2000), suggesting not only that the INF may be regulated by the SCN (cf. Akabayashi et al., 1994; Jamali and Tramu, 1999), but also that the INF may feed-back “energy-balans” information to the SCN (cf. Rivest, 2002). In turn, the SCN balances sympathetic and parasympathetic tonus by innervating respectively the intermediolateral columns (IML) and the sensoric nucleus tractus solitarius (NTS) as well as dorsal motor nucleus of the vagus(DMV) in the brainstem via multisynaptic pathways (cf. Buijs and Kalsbeek, 2001; Kreier et al., 2002).

Our studies show that the human INF expresses nuclear and cytoplasmic ERα, ERβ and ARs (Chapter 3, 5 and 6). In young subjects a sex difference was observed with males expressing a stronger nuclear AR-ir than females (Fernandez-Guasti et al., 2000; Chapter 3), while the reversed pattern was observed
with women expressing stronger nuclear ERβ (Chapter 6). The most prominent nuclear ER expression observed, without a sex difference, was that of ERα. Interestingly, in the INF, nuclear expression patterns of ERα and AR shift towards predominant cytoplasmic expression patterns in elderly (Hestiantoro and Swaab, submitted; unpublished observations). This shift appears to result from decreased circulating androgen and estrogen levels as also seen in the SON, MBC and PVN (cf. Ishunina et al., 2000; Kruijver et al., 2001; Chapter 6). The cellular redistribution of sex hormone receptors by shifting to the cytoplasm might functionally be related to the trend during aging to accumulate body fat, especially within the visceral depots (Svendsen et al., 1995; Sternbach, 1998; Jankowska et al., 2000; Tchernof et al., 2000). Thus, during normal aging it seems that a decrease in circulating sex hormones shifts the sympathetic/parasympathetic equilibrium towards the parasympathetic-lipogenetic tonus of fat tissue (cf. Kreier et al., 2002). During Alzheimer’s disease (AD) it seems as if just the opposite is happening with a shift towards the sympathetic-lipolytic tonus of fat tissue, which results in enhanced energy expenditure and finally a cachectic state. An INF state that triggers a pathophysiological domino stone effect may start with decreased circulating sex hormone levels which stimulate the translocating shift of nuclear sex hormone receptors to the cytoplasm. Body weight gain might subsequently result from a reduction of energy expenditure and sympathetic activity induced by stimulation of NPY- and Gal-production (cf. Palkovits et al., 1987; Leibowitz et al., 1998).

Reduced sex hormone levels appear to stimulate enhancement of HPA-axis activity and thereby cortisol levels (Gudmundsson et al., 1999; Prinz et al., 2001). Enhanced HPA-axis activity occurs during aging and even more so in AD, where hypercortisolism is correlated with severity of disease (Swaab et al., 1994; Giubilei et al., 2001). Both the reduction of the HPG-axis activity and the increase in HPA-axis activity may affect in the INF the production and release of NPY and GAL in AD and/or stimulate energy expenditure by enhancing (INF-SCN mediated) sympathetic activity. In this respect it might be of interest to note that resistin was recently found to be produced by fat and brain tissue (Steppan et al., 2001; Morash et al., 2002). In the hypothalamus it has been shown to be mainly present in the INF while in the periphery it decreases insulin stimulated glucose uptake in adipocytes (Steppan et al., 2001). Future research in the INF could reveal whether or not changes in NPY and Gal, at mRNA and protein levels, can be related to cachexia in AD and whether enhanced expression of resistin is involved in hypercortisolism induced insulin insensitivity in the brain and body.

The SCN and sex hormones in relation to mood

This thesis shows that the human biological clock is sex hormone sensitive. The SCN has been proposed to be involved not only in the gender and sex hormone dependent regulation of the hypothalamo-pituitary-gonadal (HPG)-
axis, but also in the sex dependent regulation of body-temperature, sleep and mood-rhythm disturbances (Dijk and Czeisler, 1995; Zhou et al., 2001; Kruijver and Swaab, 2002). The SCN is involved in mood since its decreased activity causes an activation of the hypothalamo-pituitary-adrenal (HPA)-axis in depression (Swaab et al., 2001; Zhou et al., 2001; Overeem et al., 2002). The mechanism of light-therapy induced alleviation of depressed mood may also be mediated by the SCN (Eastman et al., 1998; Benedetti et al., 2001; Oren et al., 2002). The mechanism of the beneficial “activational” effect of light-therapy on mood in humans may involve a stimulatory effect of light on e.g. the production of serotonin in the raphe nucleus (RN) either by a direct retina-raphé nucleus pathway (Foote et al., 1978; Shen et al., 1994; Fite et al., 2001) or by an indirect light-retinohypothalamic-SCN-raphé nucleus-pathway (Stephan et al., 1981; Dai et al., 1998a). Brain serotonin levels in mammals are higher during wakefulness (arousal) than during sleep (Portas et al., 2000; Carlsson et al., 1980). This day-night rhythm is paralleled by a higher electrical activity during wakefulness in the rat raphe nucleus (Sakai and Crochet, 2001), which projects to various brain areas, including the SCN (Morin, 1999). Via the latter pathway the raphe nucleus is able to influence circadian rhythms and modulate the SCN’s sensitivity to light (Morin, 1999). Enhanced light induced serotonin activity onto SCN neurons may lead to enhanced vasopressin (AVP) activity of SCN neurons. In rodents, the CRH producing PVN cells are inhibited by AVP neurons from the SCN (Buijs et al., 1993ab; Kalsbeek et al., 1996). Light therapy could stimulate SCN-derived AVP activity, which may subsequently result in reduced PVN-derived CRH-release or hypothalamo-pituitary-Adrenal (HPA-) axis activity and thus improve or even cure the status of depressed mood. Light therapy in rat has been shown to prevent the age related decrease in SCN AVP neurons (Lucassen et al., 1995). It seems thus rather likely that the light-therapy induced improvement of the circadian rest-activity rhythm in Alzheimer patients is based, at least in part, upon re-activation of AVP neurons in the SCN (van Someren et al., 1997; Swaab et al., 1998).

In addition to light, circulating estrogen levels in humans may also have direct activating effects on clock neurons (Chapter 8) by modulating the production serotonin in the raphe nucleus (cf. McEwen and Alves, 1999) and AVP in the SCN. In the human SCN the circadian and circannual fluctuations of AVP diminish with aging, while the HPA-axis activity becomes even more activated in aged women than in men (Swaab et al., 1994; Hofman and Swaab, 1994; Hofman and Swaab, 1995; Seeman et al., 2001). Sex hormone replacement therapy inhibits the enhanced circadian HPA-axis activity in postmenopausal women (Gudmundson et al., 1999; Prinz et al., 2001), indicating a direct or indirect effect on estrogen sensitive PVN and vasopressinergic SCN neurons (Chapter 6 and 8; see below).

The SON and PVN produce AVP and oxytocin (OXT) that regulate water balance, bloodpressure, reproduction, labour and the HPA-axis (see Raadsheer
et al., 1994; Swaab, 1997; Ishunina et al., 2000; Keck and Holsboer, 2001; Keck et al., 2002; Goncharuk et al., 2002). A characteristic hallmark of depression is elevated hypothalamic CRH-production. CRH activity is potentiated by AVP (Raadsheer et al., 1994; Keck and Holsboer, 2001; Keck et al., 2002; Van London et al., 1997; 1998; 2001). An inhibiting effect of estrogens on PVN CRH neurons may be mediated via nuclear ERβ, while a stimulating effect of estrogens on CRH expression in PVN neurons may be mediated via nuclear ERα. This possibility is supported by the fact that nuclear ERα increases and nuclear ERβ decreases in PVN neurons of postmenopausal women (Kruijver and Swaab, unpublished observations), while the reversed pattern is observed in young women (cf. Chapter 6; Table 3). The observation that higher estrogen levels are paralleled by stronger nuclear ERβ-ir in PVN neurons, may explain the inhibiting effect of estrogens on CRH expression in premenopausal women (cf. Gudmundsson et al., 1999; Prinz et al., 2001) and would also fit with the reported anti-depressant effects by SHRT in postmenopausal women (Sherwin and Gelfand, 1985; Halbreich, 1997). It could also be inferred that a decrease in circulating sex hormone levels during aging may contribute to the enhanced CRH activity in the PVN and the reduced AVP activity in the SCN in elderly with major depression (Raadsheer et al., 1994; Zhou et al., 2001). In this respect it is of note that our group observed more nuclear ERα co-localization in CRH neurons in the PVN of depressed patients than in control patients (Bao et al., submitted). This observation reinforces the idea of an ERα mediated stimulatory role on CRH neurons. The enhanced nuclear ERα-ir activity and reduced nuclear ERβ-ir in vasopressinergic SON neurons of postmenopausal women (Chapter 7) with low circulating estrogen levels may, at least to some extend, contribute to elevated postmenopausal CRH-ACTH-cortisol production and so to mood disturbances. The modulatory role of sex hormones on mood, not only via the PVN, but also via the raphe nucleus, SCN or SON could be of interest for further studies.

Relation of sex hormones with schizophrenia and bipolar disorder

Schizophrenia

In the brain and CSF of schizophrenic patients abnormal levels have been found, not only for dopamine, but also for histamine, noradrenaline, acetylcholine, glutamate and GABA (Prell et al., 1995; Gluck et al., 2002; Kapur, 2003; reviewed by Swaab, 2003). A potential dysbalans of SCN-catacholaminergic connections may be present in schizophrenia. This idea seems to be supported by the fact that the mean level of 24 hour serum dopamine level (mesor) is higher in schizophrenic patients than in healthy subjects (Rao et al., 1994;1995). In addition, other SCN-related rhythms can either be abnormally enhanced (e.g. cortisol) or be lowered in this disorder. The mesor of prolactin and thyroid stimulating hormone [TSH] is lower in drug-free schizophrenic patients than in
healthy ones (Rao et al., 1994; 1995; reviewed by Swaab, 2003). Elevated levels of histamine metabolites in the CSF of schizophrenic patients (Prell et al., 1995) suggest e.g. an increased activity of the histamine producing tuberomamillary neurons. The present thesis shows that these histaminergic TM neurons are sensitive to estrogens (Chapter 5 and 6). Interestingly, estrogens are presumed to be a protective factor for schizophrenia and are associated with its related clinical sex differences. Women suffering from schizophrenia have significantly lower estradiol levels and experience the first onset or recurrence of a psychotic episode more often in a low estrogen phase of the cycle (Huber et al., 2001). In contrast, testosterone levels have been reported to be higher in schizophrenic patients (Mason et al., 1988), and androgen-like anabolic steroids are known to have psychosis inducing “psychotogenic” properties (Malone et al., 1995; Markowitz et al., 1999; Pope et al., 2000). Since the SCN and its connected catecholaminergic neurons express ARs and ERs (McEwen and Alves, 1999; Fernandez-Guasti et al., 2000; Leranth et al., 2000; Kruijver and Swaab, 2002; Ravizza et al., 2002; Stevens, 2002; Kruijver et al., 2002) it is proposed here that disturbed sex hormone receptor mediated SCN-catecholaminergic interactions might play a role in the sexually dimorphic aspects of schizophrenia.

Bipolar disorder

Bipolar women who were not using SHRT were significantly more likely than those who were using SHRT to report worsening of symptoms during perimenopause/menopause (Freeman et al., 2002). Interestingly, both depression and mania are associated with disrupted sleep and activity levels, which provide further arguments for a probable involvement of the human SCN in unipolar (major) and bipolar depression (BPD). Sleep deprivation is a symptom of mania and can cause mood cycling by precipitating switches from depression into mania (Leibenluft, 1995). The fact that total sleep deprivation combined with sleep phase advance is followed by improvement in mood in bipolar depressed patients (Benedetti et al., 2001), hints at the potential involvement of the SCN in this disorder. Moreover, lithium, the most frequently prescribed medicine to treat BPD, is a potent modulator of circadian function, acts directly on SCN-neurons and lengthens the free-running period of individual SCN neurons (Abe et al., 2000). Clinically, lithium is also known to lengthen the period of the sleep-wake cycle in humans (Johnsson et al., 1983; Wirz-Justice, 1983) and to induce a phase delay of SCN-mediated circadian rhythms, such as melatonin, temperature and REM-sleep rhythms, in addition to its improvement of mood in bipolar patients (cf. Campbell et al., 1989; Abe et al., 2000).

The human SCN expresses sex hormone receptors (cf. Chapter 3 and 8) which suggests that sex hormones can act directly upon SCN neurons and affect sleep as one of its effector systems (Dijk et al., 1995; Kruijver and Swaab, 2002). Although it is tempting to relate the difference in sex hormones (i.e. higher androgen levels in men versus women) to the greater prevalence
of manic episodes in men than in bipolar women (cf. Leibenluft, 1995), one may wonder, however, whether the in the literature reported mania-inducing properties of androgens are purely activational. Since sex hormone receptors are present in the human developing SCN, at least from 27 weeks of gestation onwards (Kruijver and Swaab, unpublished observations), it seems of interest to note that men who were prenatally exposed to diethylstilbestrol (DES), were more likely to develop major depressive disorder with recurrent depressive episodes, than their nonexposed brothers (Pillard et al., 1993). An abnormal programming of the fetal SCN, by abnormal steroid hormone exposure (such as DES) and/or severe maternal stress, could result in a more vulnerable circadian system in adulthood with various clinical implications, such as the development of bipolar disorder or depression (cf. Pillard et al., 1993; Hoek et al., 1996; Kochl et al., 1999; Brown AS et al., 2000; Hulshoff et al., 2000; Zhou et al., 2001; Kennaway, 2002; Kaiser et al., 2003ab).

Age related changes in ER α/β-ratios and brain disease

Not only may alterations in local neurosteroidogenesis [see e.g. Schumacher et al., 2000]) change a neuron’s activity, also age related changes in ER α/β-ratios and ERs are likely to mediate functional neuronal changes. An example of an ER α/β-ratio change is the shift from predominant nuclear ERβ in premenopausal women to predominant nuclear ERα and enhanced activity in SON neurons of postmenopausal women (Chapter 7). However, age and brain area dependent ER α/β-ratio changes may affect gene transcription and neuronal activity, not only in health and but also in disease (cf. Ishunina et al., 2000; Ishunina and Swaab, 2001; Hu et al., 2003; Swaab et al., 2004).

In addition, functional ER differences can originate at the DNA- or RNA-level. ER-gene polymorphisms have been associated with central nervous system related diseases such as hypertension, anxiety and Alzheimer’s disease (Lehrer et al., 1993; Comings et al., 1999; Ji et al., 2000). However, according to a recent study the association between ER-gene polymorphisms and Alzheimer’s disease was absent for each ER subtype separately and appeared to be present only when both ERα and ERβ polymorphisms were present (Lambert et al., 2001).

Another possibility by which ERs can be implicated in activity changes is by changes in the proportion of splice variants. ERα and ERβ splice variants are known to have varying estrogen dependent transcriptional activities and expression patterns in normal or malignant brain and peripheral tissue (Chaidarun et al., 1998; Friend et al., 1997; Shupnik et al., 1998; Lu et al., 1998; Jazaeri et al., 1999; Speirs et al., 2000; Price et al., 2000/2001; Österlund and Hurd., 2001).
Neuroprotection by SHRT

Postmenopausal women are more vulnerable to develop Alzheimer’s disease than men, which is presumed to be related to the lack of premenopausal circulating estrogen levels (reviewed by Smith and Zubieta, 2001; Swaab et al., 2002). Postmenopausal estrogen replacement therapy was indeed found to delay the onset of AD, lower the risk of disease and improve mood and cognition in a number of studies (Tang et al., 1996; Yaffe et al., 1998; Asthana et al., 1999; Lerner, 1999; Dubai et al., 1999; Halbreich, 1997; Sherwin, 1997). The effects of SHRT on AD are certainly not without debate (see also discussion Chapter 5 and 6), but a recent study underlines the potential preventive effects of endogenous bioavailable free estrogens (Yaffe et al., 2000).

Restored levels of bioavailable free forms of circulating sex hormones by SHRT in elderly people may protect specific cognitive domains (cf. Yaffe et al., 2000; Manly et al., 2000; Cunningham et al., 2001; Chapter 6). Neuroprotection by SHRT could be mediated, at least in part, by increasing cerebral blood flow, reducing corticosteroid mediated oxidative injury, reducing homocysteine levels, preventing the formation of neurotoxic free radicals, β-amyloid and aberrant proteins as well as by preventing neuronal decreases in the endogenous production of specific peptides, such as neurotrophins, serotonin, acetylcholine and histamine (Toran-Allerand et al., 1992/1999; Salehi et al., 1995; Ohkura et al., 1995; Goodman et al., 1996; Tang et al., 1996; Gonzalez-gonzalez et al., 1996; Xu et al., 1998; Gibbs and Aggerwal., 1998; Mufson et al., 1999; Swaab et al., 1998; van Leeuwen et al., 1998/2000; Behl et al., 1999; Whitmer et al., 2003). However, SHRT may also have some unwanted deleterious effects on the brain. An example of this possibility is the enhanced activation of INF neurons in postmenopausal women having low circulating estrogen levels which is accompanied by neuroprotection effects against Alzheimer changes in these activated neurons, a principle that was paraphrased as “use it or lose it” (Swaab, 1991; Swaab et al., 1998; 2001). One may thus presume that SHRT may inhibit the activity of INF neurons and therefore allow AD changes to occur. It should be investigated whether such side effects of SHRT occur indeed in the brain.

SHRT has recently been claimed also to have potential beneficial effects in the treatment of Parkinson’s disease, major depression and schizophrenia (Blanchet et al., 1999; Tsang et al., 2000; Schneider et al., 1997; Amsterdam et al., 1999; Kornstein et al., 2000; Seeman and Lang, 1990; Kulkarni et al., 2001; Lindamer et al., 2001; Garcia-Segura et al., 2001; Stevens, 2002). Such effects may be mediated by ERα and ERβ that are expressed in a multitude of brain areas such as the nucleus accumbens (Ac) and corpus striatum, the basal forebrain, amygdala, entorhinal- and temporal-cortex, SCN, PVN, SON, TM and MBC (Chapter 5,6,7,8). The recently developed in vitro technique by Verwer et al., (2002;2003), to keep metabolically still active human postmortem brain tissue alive for some weeks in culture, might be a valid approach to further study the stimulatory and inhibitory effects and mechanisms of action of e.g. sex hormones on the human brain.
Androgens

The studies as described in Chapter 3, 4, 5 and 6 of the present thesis have revealed that brain areas that are associated with cognition, such as the cholinergic nucleus basalis of Meynert complex (NBMC) and mamillary body complex (MBC), are androgen and estrogen sensitive. These brain areas express ARs and ERs in a gender dependent way, dependent on circulating levels of sex hormones rather than being related to sexual orientation or gender identity (cf. Kruijver et al., 2001; Chapter 4 and 5). These findings underline the potential effects of circulating sex hormones on specific brain areas that are involved in cognition (Chapter 4, 5 and 6). This notion is supported by the strong nuclear to cytoplasmic shift and decrease of AR-ir in the MBC of 5 old male gonadally intact male subjects (Kruijver et al., 2001; Chapter 4) which appeared to be due to decreased circulating levels of androgens during ageing (Sternbach, 1998; Seidman and Walsch, 1999). The MBC is involved in memory function (Tanaka et al., 1997). Mamillary bodies atrophy in vivo with age (Raz et al., 1992; but see Begega et al., 1999) and are affected in Alzheimer’s disease (McDuff and Sumi, 1985) and in alcohol-associated Korsakoff’s disease (Koppelman, 1995). The decline with age of nuclear AR-ir in the male MBC as reported in Chapter 4 may be reflected in functional changes. The MBC is involved in various aspects of reproduction, such as sexual motivation and penile erection (see Chapter 4). Whether the observed changes of a decrease in nuclear AR-ir in the MBC in elderly men (Chapter 4) play a role in the impairment in sexual and cognitive functioning (Sternbach, 1998), or in the increased prevalence of sporadic Alzheimer’s disease in the elderly (Swaab et al., 2002) should be further investigated. Protective actions of androgens on neurons (Ahlblom et al., 1999; Hammond et al., 2001; Pike, 1999) and memory loss (Adinoff et al., 1993; Kalmijn et al., 1998; Carlson et al., 1999) have been described. It may thus be of interest to investigate the possible neuroprotective effects of androgens in age-related diseases in men, in a similar way as has been and is done for estrogen replacement therapy in postmenopausal women. Obviously, more studies will be necessary to further complete our neuroendocrine understanding of the sex-dimorphic brain and its relation to human sexuality, neurophysiology and neuropsychiatric diseases.
Fig. 1  Topography of the sexually dimorphic structures in the human hypothalamus and adjacent areas. A is a more rostral view 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; and SON, supraoptic nucleus. Scale bar, 5mm.

Fig. 2  SCN regulated network of various hormone and transmitter systems in the human brain that can, in principal, be modulated by sex hormones at the organizational and activational level. All these systems are implicated in effects of sex hormones on (sexually dimorphic) brain functions related to autonomic function (e.g., the arousal/alertness/sleep rhythm of the day/night cycle), cognition and mood. In addition to its involvement in major depression, a dysbalans of the SCN itself and SCN-(catecholaminergic) connections is proposed to play a pathophysiological role in respectively bipolar disorder and schizophrenia. In bipolar and schizophrenic men elevated circulating androgen levels appear to have “psychotogenic” rather than “psychotolytic” effects, in contrast to the neuro-protective effects that estrogens seem to play in bipolar and schizophrenic women (cf. Leibenluft, 1995; Kulkarni et al., 2001; Freeman et al., 2002).
**Future studies**

Future studies will be necessary to further study:

1. the potential pathophysiological role of ER splice variants in various brain related diseases.

2. ER α/β-ratio mediated functional changes in neuronal systems that are known to be affected in brain diseases, such as e.g. the serotonergic-, CRH- and TRH-system in depression, the dopaminergic system in Parkinson’s disease as well as the cholinergic, noradrenergic, GABA-ergic, glutaminergic and histaminergic system in Schizophrenia and Alzheimer’s disease.

3. the potential role of (splice variant induced) functional AR and GR changes in relation to neuropsychiatric and neurodegenerative diseases.

4. the potential pathophysiological role of hormone co-activators in neuroendocrinology (cf. Adachi et al., 2000).

5. the potential existence of neurosteroidogenesis in the human brain and its possible relation to sex dependent neurodevelopmental conditions regulating human sexuality, autonomic function, mood and cognition.

In addition, studies of aromatase, 5α-reductase and progesterone receptor distributions and functions in the human brain will be necessary to further complete our understanding of the nervous system and its relation to sexual orientation, gender identity, autonomic, neuroendocrine, neuropsychiatric and neurodegenerative diseases.

"Life is a lot about hormones, brain and behaviour"

Frank. P.M. Kruijver, 2002

"The brain is our biggest sexual organ. A pity it is hidden in the skull"

Dick F. Swaab., 2003