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

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CHAPTER

GENERAL INTRODUCTION
1.1. Rationale for studying sex differences in the human hypothalamus and adjacent areas

From the moment of conception until the moment we die we are living in a sex-differentiated world. Not only do men and women have different physiques, there are also sex differences in seeing, smelling, thinking, feeling and behaving (Savic et al., 2001; Rahman and Wilson, 2002; Collaer and Nelson, 2002; Gooren and Kruijver, 2002; Malcolm et al., 2002; Loring-Meier and Halpern, 2002; Karama et al., 2002; Canli et al., 2002; Fischer et al., 2004; Hamann et al., 2004). Also in terms of life expectancy and the risk to develop body and brain related diseases as well as in the way men and women respond to medical treatments, both sexes are known to differ from each other (MacLusky and Naftolin, 1981; Swaab and Hofman, 1995; Collaer and Hines, 1995; Swaab, 2002).

The brain orchestrates these sex differences by irreversible structural ("organizational") and reversible ("activational") functional sex differences. Sex differences are present in the brain at the macroscopic, microscopic, molecular and functional level. Examples of such differences are macroscopically sex differences in brain volume, weight and regional differences in size, shape or fiber connections (Swaab and Hofman, 1984; 1995; Kawata, 1995). Microscopically sex differences may exist in neuronal cell numbers, perikaryal size, nucleus and nucleolus, dendritic branching, as well as synaptic organization (density, type, axonal outgrowth), shape and quantitative differences in glia cells, while at the molecular level sex differences can exist at the level of neuropeptides, neurotransmitters, enzymes, proteins and mRNA (Kawata, 1995; Cooke et al., 1999; Swaab, 2002). Functional sex differences exist in various aspects of reproduction (e.g. in the sex dependent presence or absence of cyclic gonadotropin secretion required for the menstrual cycle), cognition (e.g. verbal fluency, visuospatial capacity, mechanical reasoning) and behavior (e.g. play- and sexual/social-behavior) (Collaer and Hines, 1995; Swaab et al., 2002; Gooren and Kruijver, 2002).

In analogy with the situation in animals, the interaction between sex hormones and their receptors is presumed to play an important role in the development and expression of organizational and activational sex differences in the human brain, thereby controlling neural development and neural communication (cf. Kawata, 1995; Simerly, 2002). Sex differences in a multitude of central systems are held responsible for sex differences in reproduction (e.g. in the presence of cyclicity in hypothalamo-pituitary-gonadal-[HPG]-axis), gender identity (i.e. the feeling to be male or female), sexual orientation (i.e. hetero-, bi-, or homosexuality), autonomic functions (differences in e.g. biological rhythms in body temperature, bloodpressure and sleep) as well as for sex differences in mood, cognition, behaviour and neuroprotection in health and disease (McEwen and Alves, 1999; Swaab et al., 2002; Swaab, 2003;2004; present thesis).
Animal studies have shown that the hypothalamus and its adjacent structures are important sexually dimorphic brain areas expressing androgen, estrogen and progesterone receptors (AR, ER, PRs) which are involved in the regulation of autonomic function, reproduction, sexual behavior, aggression and cognition (Kawata, 1995; McEwen and Alves, 1999; Simerly, 2002). Moreover, these brain areas have been implicated in functional sex differences in behavior, autonomic function, mood, cognition and reproduction (Swaab and Hofman, 1995; Collaer and Hines, 1995; McEwen and Alves, 1999; Simerly, 2002; Allen and Gorski, 2002). The present thesis was undertaken to investigate structural and functional differences in the human hypothalamus and adjacent areas in relation to sex, gender identity and sexual orientation by focussing on morphological sex differences, sex hormone receptors and their relation to endocrine status.

1.2. Sex determining genes

Sexual differentiation involves first the establishment of genetic sex which is followed by the establishment of gonadal and subsequently phenotypic sex (Jost, 1972). The process of sexual differentiation starts with the configuration of the sex chromosomes, which determine the undifferentiated gonads from the 7th week of gestation onwards to develop into testosterone producing testes in males (XY-configuration) or ovaries in females (XX-configuration) (Wilson, 2001).

A single gene determines the type of gonad, which in turn determines the hormone profiles of the developing fetus and infant. The gene for the testes determining factor (TDF) is located on the Y chromosome. This gene, called the sex-determining region of chromosome Y (SRY), induces eventually maleness of the genital tract. SRY switches the developmental program of the undifferentiated gonads from the pathway for follicle cell characteristics of the ovaries to that of Sertoli cells of the testes. The secretions from the fetal testes in turn determine subsequent events in the sexual differentiation of the male. The fetal testes secrete three hormones, testosterone, anti-Müllerian duct-inhibiting hormone (MIH/AMH) and insulin like growth factor 3 (InsI3). MIH is secreted by the Sertoli cells of the testes and actively induces the regression of the Müllerian ducts (paramesonephric ducts), thereby preventing the development of female reproductive organs. Simultaneously, testosterone is produced by the Leydig cells of the testes and induces the differentiation of (mesonephric) Wolffian ducts. The Wolffian ducts differentiate into the vas deferens, epididymis and seminal vesicles of the male reproductive system. Since females have no Y chromosome, the fetal ovaries do not produce MIH during development, creating thereby a permissive environment for differentiation of the Müllerian ducts, which differentiate into oviducts, uterus and upper part of the vagina of the female reproductive system. Furthermore, the absence of testosterone in females leads to passive regression of the Wolffian duct system (Kawata, 1995).
In contrast, the external genitalia and lower urogenital tracts of both sexes develop from common embryonic precursors: the genital tubercle, genital folds, and genital swellings. In the female the lower urogenital tract elongates and changes very little. In the absence of testosterone (and its derivate dihydrotestosterone that is formed from testosterone by reduction by the enzyme 5α-reductase) the genital tubercle becomes the clitoris, the genital folds become the labia minora and the genital swellings become the labia majora. In the male, conversion of testosterone into dihydrotestosterone by 5α-reductase is required to complete genital differentiation into the male direction. Dihydrotestosterone acts on the urogenital sinus to induce development of the male urethra and prostate and in the urogenital tubercle, swelling, and folds to cause the midline fusion, elongation and enlargement that eventuate in formation of the penis and scrotum (Wilson et al., 1993).

While hormones are assumed to determine the process of sexual differentiation beyond the formation of the gonads, also later genetic factors have been implemented as well. The SRY transcript encodes a DNA-binding protein that recognizes a response element the “high mobility group domain” or HMG box often in the promoter region of specific genes that are regulated by SRY. The downstream genes directly regulated by SRY may be involved in male sexual differentiation of the gonads and also of the brain (Arnold, 1996; McElreavey and Fellous, 1999). In addition to playing an important role in the sexual differentiation of the urogenital tract, SRY and related genes, such as ZFY, Ftzf1, SF-1, WT-1, SOX9, SOX18, are sexual dimorphically expressed in various organs, including the central nervous system (Mayer et al., 1998; Achermann et al., 1999; McElreavey and Fellous, 1999; Stanojcic and Stevanovic, 2000). Sexual differential expression of these genes in the brain may be a contributing genetic mechanism to establish sexual differentiation, in addition to the important epi-genetic effects of sex hormones on sex differences in the brain (MacLusky and Naftolin, 1981; McEwen, 1981; Kawata, 1995; Cooke et al., 1998; Mayer et al., 1998; Carruth et al., 2002).

1.3. Sex hormones

Steroid hormone metabolism

Sex hormones belong to the family of steroid hormones comprising many hormones and vitamins built on the teracyclic (cyclopentanoperhydrophenanthrene) nucleus. Steroid hormones are low in molecular mass (ranging from 240-370 Da) and sufficiently lipophilic to move across the blood-brain barrier by simple diffusion.

Cholesterol is the precursor of the five major classes of steroid hormones: progestins, estrogens, androgens, glucocorticoids and mineralocorticoids. Cholesterol contains 27 carbon groups, whereas steroid hormones contain 21 or fewer. Cholesterol is first converted into pregnanolone, which in turn is converted into progesterone. Various biochemical modification steps of the pro-
gesterone molecule (by oxidation and hydroxylation at various carbon groups) will directly yield the glucocorticoids cortisol and corticosterone, while the main mineralocorticoid aldosterone in turn is derived from corticosterone.

Synthesis of androgens and their derived estrogens starts with the hydroxylation of progesterone at C-17 into 17α-hydroxyprogesterone, which can be converted into androstendionc that can be either converted into the estrogen estrone or into the androgen testosterone. Testosterone can in turn be irreversibly converted (aromatized) at the A ring into estradiol by the enzyme aromatase of the cytochrome P450 family, while 5α-reduction of the C4-C5 double bond by the enzyme 5α-reductase, yields 5α-reduced testosterone or dihydrotestosterone (DHT)(McEwen et al., 1979; Wilson et al., 1993; Kawata, 1995). The conversion of testosterone to dihydrotestosterone is essential for many androgen actions. This reaction is irreversible and is mediated by two enzymes that are encoded by separate genes located on chromosome 5 and 2 (respectively 5α-reductase isoenzyme 1 and 5α-reductase isoenzyme 2; 5α-R1 and 5α-R2). 5α-R2 is the principal enzyme in the male urogenital tract and plays a critical role in the virilization of the external genitalia and urogenital sinus during embryogenesis. 5α-R1 is after puberty widely expressed in many tissues and may play a role in sebaceous glands. Although aromatase has clearly been implicated in sexual differentiation of the brain (cf Lephart, 1996; see below), the roles of 5α-R1 and 5α-R2 in the brain are less well understood. But both 5α-R1 and 5α-R2 are expressed in the brain, which indicates that they may be involved in sexual differentiated functions of the central nervous system (cf. Poletti et al., 1998; Wilson, 1999).

Progestins, estrogens and androgens are classically known to be involved in the process of phenotypic sexual differentiation, sexual maturation at the time of puberty and control of libido and potenti a in adults. The most important progestin is progesterone itself. In the normal non-pregnant female, progesterone is secreted by the corpus luteum cells in significant quantities only during the latter half of the ovarian cycle. In addition, large quantities of progesterone are secreted by the placenta during pregnancy, especially after the fourth month of gestation. The peripheral action of progesterone is to prepare the uterus for implantation of an ovum and the maintenance of pregnancy, and to prepare the mammary glands for lactation.

In the normal non-pregnant female, estrogens are mainly secreted by the ovaries, while during pregnancy large amounts of estrogens are secreted by the placenta. There are three estrogens: estradiol, estrone, and estriol. By far the most important of these estrogens is estradiol: the estrogenic potency of estradiol is 12 times that of estrone and 80 times that of estriol. The principal function of estrogens in the peripheral system is to cause cellular proliferation and growth of the tissues of the sex organs and of other tissues related to reproduction, while they are also responsible for development of the secondary sex characteristics in females. Before puberty, estrogens are secreted only in minute quantities,
but from puberty onwards the quantity of estrogens secreted under control of pituitary gonadotrophic hormones increases some 20-fold or more.

The testes secrete several male gonadal hormones, known collectively as androgens, which include testosterone, dihydrotestosterone (DHT) and androstendione. Testosterone is much more abundant than the other two steroids. Testosterone is synthesized in interstitial Leydig cells under the influence of the pituitary gonadotropic/luteinizing hormone. Leydig cells are almost absent in the testis during childhood, but are abundantly present from mid gestational to neonatal ages onwards and in the adult male after puberty (Kawata, 1995).

Recent emerging studies have shown that the largest organ producing sex hormones is, in fact, the vertebrate brain itself. The brain derived sex hormones are called neurosteroids and are locally de novo produced by neurons and/or glia cells (Baulieu et al., 2001; Mellon and Griffin, 2002). The potential functional significance of this newly discovered phenomenon for the human developing and adult brain in health and disease awaits, however, future investigations.

**Steroid receptors**

It is generally accepted that steroid hormones bind to their related steroid receptors in cells in order to act as transcription factors to regulate gene expression, which is followed by protein synthesis via translation of specifically transcribed mRNA's. Receptors for gonadal and adrenal steroids, thyroid hormone, vitamin D and retinoic acid comprise a superfamily of nuclear receptors that was classified more than a decade ago as a nuclear receptor superfamily (Parker, 1993). However, since these receptors may also exert extra nuclear cellular functions they may be best classified as a steroid receptor superfamily (McEwen et al., 2001). All the receptors in this superfamily consist of three main domains: a variable N-terminal (transactivating) domain, a well conserved central (DNA-binding) domain, and a relatively well conserved C-terminal (hormone-binding) domain. These three main domains are separated by small regions.

The classical concept on the action of steroid hormones is that they bind to their receptors in the cytoplasm followed by translocation of the resulting complexes to the nucleus, where they start to function as transcription factors altering gene expression (i.e. gene activation or repression; cf. Kawata, 1995). According to the classical concept the steroid receptor becomes only functional once inside the nuclear compartment of the cell. However, additional mechanisms of action have recently emerged indicating e.g. that cytoplasmic and/or membrane bound estrogen receptors can cross-talk with second messenger pathway systems, such as the cAMP- and MAPK-pathways which may result in non-genomic cellular metabolic effects of ERs (see e.g. Clarke et al., 2000; Beyer, 1999; Collins and Webb, 1999; Toran-Allerand et al., 1999; Kelly and Levin, 2001; McEwen et al., 2001; Vasudevan et al., 2001). Not only differences in nuclear steroid receptors but also differences in cytoplasmic steroid receptors may thus have a functional meaning (see also discussion Chapters 5 and 6).
Sex hormone metabolism is widespread throughout the brain

Sex hormones and their receptors are classically associated with the sexually dimorphic regulation of the HPG-axis and various specific brain areas known to be involved in the orchestration of reproduction and sexual behavior. However, only recently, it has become apparent that sex hormones and their receptors exert many actions beyond reproductive functions, including actions on brain areas that are important for learning and memory, emotions and affective state as well as motor coordination and pain sensitivity. This recognition is moreover in congruence with established sex differences in behavioral, mood and cognitive functioning (McEwen, 1999; see also discussion Chapter 6 and 9). The realization that sex hormones have widespread actions in the brain seems of clinical relevance for sex differences in the prevalence of various neuropsychiatric and neurodegenerative diseases which may to an important degree be due to sexual differentiation of the brain (Swaab, 2002; Swaab et al., 2003).

1.4. Sexual differentiation of the brain (structural and functional sex differences)

Already in the antique times, it was Hippocrates (460-377 B.C.) and Aristotle (384-322 B.C.) who claimed that there is a sex difference in the moment of human brain “animation”. The moment upon which the fetus receives its soul was in the male and female brain estimated by Hippocrates to take place at respectively 30 and 42 days of gestation and later by Aristotle at the 40th and 50th day of pregnancy (Swaab and Hofman, 1984). Only about a century ago scientists became interested in sex differences of the brain, initially by studying brain morphology and brain weight. Men were found to have about 15% more brain weight than women. Studies on the sexually dimorphic regulation of pituitary gonadotropin secretion followed later. Over more than the last 40 years, the role of androgens in sexual differentiation of the urogenital tract, behavior and subsequently on its central orchestrator, the brain have been demonstrated (reviewed by Swaab and Hofman, 1984; Arnold, 1996; Arai, 2000).

Sexual differentiation is a sequential process which starts from the moment of conception when the configuration of the sex chromosomes determines the genetic sex, which subsequently determines the gonadal sex, which in turn determines the developing brain sex: male in the presence of testicular androgens, female in the absence of testis and the lack of gestational and neonatal testicular androgen exposure. It were Phoenix, Goy, Gerall and Young in 1959 who reasoned that if testosterone was critical to the masculine differentiation of genitalia, it might also be important in the differentiation of the brain. To test this idea, they exposed female guinea pig fetuses to testosterone, and found that as adults the females showed permanently more masculine copulatory behaviors and less feminine lordosis behavior. Phoenix et al., inferred that testosterone had organized neural circuits in the brain responsible for masculine copula-
tory behaviors and had prevented the organization of circuits responsible for feminine behaviors. These studies placed testicular secretion of testosterone at the heart of sexual differentiation, and were the first to strongly suggest that testosterone has a differentiating action on the brain (cf. Whalen and Edwards, 1967; reviewed by Arnold, 1996).

Since then, many studies in various mammalian species, including primates and rodents, have shown that circulating androgens are in the brain converted into estrogens by the enzyme aromatase. Local conversion of testosterone (T) into estradiol (E) by aromatase plays a crucial role in the sexual differentiation of the rodent brain, so it is in fact the estrogenic component of testosterone that mediates to an important extend the masculinizing and defeminizing effects of testosterone on rodent brain function (McEwen et al., 1977; Baum, 1979; MacLusky and Naftolin, 1981; Cooke et al., 1998).

In rats and humans pre- and early postnatal circulating testosterone levels are higher in males than in females. In rats, there is a testosterone surge on days 18 and 19 prenatally, while postnatally there is a rise in serum testosterone in male pups directly after birth followed by a sharp decline approximately 6 hours after birth. Interestingly, in male rats hypothalamic estradiol levels increase dramatically between 0 h in utero and 1 h after delivery and decrease between 2 and 24 hour after birth. The striking presence in male rats (and its absence in female rats) of the postnatal estradiol surge seems to be due to neuronal aromatization of testosterone to estradiol, a process that is presumably also responsible for the very low circulating estradiol levels produced by the testes (Rhoda et al., 1984). Testosterone levels remain also higher in the postnatal ‘critical-period’ in males than in females until day 10, when they drop and remain low until puberty (Corbier et al., 1978; Baum, 1979; Weisz and Ward, 1980).

In humans, testosterone levels start to surge in males between 10 and 20 weeks of gestation, followed by a surge around birth and 3 month postnatally, after which circulating testosterone levels cease until puberty (cf. Wilson, 1999; Zup and Forger, 2002). In human neonates testosterone peaks occur at midgestation, around birth and neonatally (cf. Wilson, 1999; Zup and Forger, 2002). At 34-41 weeks of gestation, testosterone levels are ten-fold higher in boys than in girls (De Zegheer et al., 1992). Circulating estrogen levels presumably do not play a crucial role in the process of sexual differentiation of the mammalian brain because they will be bound to high concentrations of the plasma glycoprotein-alpha-fetoprotein- which prevents passage to the brain (Toran-Allerand, 1984). Aromatase is expressed in the human brain (cf. Lephart, 1996; Sasano et al., 1998), however, to date, no information is available on hypothalamic aromatase activity and estradiol levels in the human brain during development (cf. Chapter 9; Gooren and Kruijver, 2002; for the lack of an effect of aromatase or ER deficiency on the development of sexual orientation).
**Aromatase**

Aromatase is the product of the Cyp 19 gene (located at the long arm of chromosome 15), and is a member of the P-450 cytochrome superfamily. The physiological and organizational implication of aromatase activity is relatively well known, at least in rodents, since estrogens derived from androgens are responsible for the major effects of brain masculinization (cf. Negri-Cesi et al., 2000). Masculinization of the brain results in rats in the display of male typical sexual behaviors in adulthood, such as mounting, intromissions and ejaculations. Defeminization results in rats in a loss of the cyclic release of gonadotropins necessary for ovulation and the display of female typical sexual behaviors in adulthood (lordosis, presenting, ear wiggle and hopping; Bakker, 1996).

The central masculinizing/defeminizing effects by aromatized androgens have lead to "the aromatization hypothesis".

**The aromatization hypothesis**

The aromatization hypothesis proposes that testosterone secreted by the testes in male fetusses and newborns acts to masculinize and defeminize the developing brain in a permanent way through intracellular conversion of testosterone to estradiol by the enzyme aromatase. Data supporting the aromatization hypothesis are: (1) aromatizable androgens (e.g. testosterone propionate (TP)), but not non-aromatizable androgens, such as dihydrotestosterone propionate(DHTP) mimic the masculinizing effects of testosterone in some of the nervous centres of neonatally treated female rats (Lutgge and Whalen, 1970), and are also able to prevent the demasculinizing effect of neonatal orchidectomy in the male rat(Arai Y, 1972); (2) 19-hydroxytestosterone, an intermediate in the aromatization process, is a more potent masculinizing agent than testosterone when administered to neonatal female rats (McDonald and Doughty, 1974) (3) Estradiol and other steroidal estrogens, given to neonatal females, have a masculinizing effect at doses much lower than those of testosterone, especially if administered into the hypothalamus (Doughty et al., 1975; Docke et al., 1975); in particular estrogens appear to masculinise the SDN-POA, an effect which may be antagonized by anti-estrogens (Döhler et al., 1984; see also below); (4)pretreatment of newborn females with anti-estrogens, with antisense oligonucleotides to the ER mRNA, or with inhibitors of the aromatisation process counteract many aspects of testosterone-induced masculinization (McDonald and Doughty, 1973; Morali et al., 1977; McCarthy et al., 1993); (5) estrogens and aromatizable androgens stimulate neurite outgrowth and differentiation in cultured slices of the hypothalamic/ preoptic area of the newborn mouse and rat, whereas nonaromatizable androgens are ineffective (cf. Negri-Cesi et al., 2000); (6) neonatal administration of of an aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), via silastic capsules to male rats, strikingly increases males’ ability to display female sexual behaviors, not only after castration and treatment with ovarian hormones in adulthood, but also if they
remain intact. Neonatal ATD treatment of male rats induces bisexual partner-preference behavior and enhances the number of vasopressin neurons in the suprachiasmatic nucleus or biological clock (SCN) (cf. Swaab et al., 1995; Bakker, 1996; Negri-Cesi et al., 2000).

However, in contrast to rats, perinatal exposure to testosterone and/or estradiol in females does not defeminize sexual behavior in some other species such as hamsters, ferrets and rhesus monkeys (Baum, 1979; Bakker, 1996). Moreover, in analogy with females of the primate species, early exposure to supraphysiological androgen levels does not masculinize the human female brain either by blocking the capacity of the hypothalamo-pituitary-gonadal (HPG-) axis to respond with an estrogen positive feedback of LH, as was found in girls with congenital adrenal hyperplasia (Reiter et al., 1975; reviewed by Gooren and Kruijver, 2002).

In addition, it should be kept in mind that there are also direct, aromatase independent, actions known of androgens that can establish sex differences in the vertebrate brain. It was e.g. reported in sheep that prenatal dihydrotestosterone (DHT) masculinizes the hypothalamo-pituitary tonic control of the gonadotropin releasing hormone (GnRH) system in order to permit high GnRH secretion, while conversion of testosterone to estrogen is required for sexual differentiation of the LH surge (Masek et al., 1999). In addition, in the African clawed frog (Xenopus laevis), a DHT effect is responsible for the enlarged laryngeal motor nucleus in males which is related to the sexually differentiated vocalization system (Kay et al., 1999). DHT has also been shown to reduce postnatal apoptotic cell-death in the developing sexually dimorphic rat visual cortex (Nunez et al., 2000). The latter group showed that postnatal androgen receptor activation by DHT in female rats can establish the male-like reduced expression pattern of neuronal cell death in the rat visual cortex, explaining its sex difference with more neuron numbers in males (Nunez et al., 2000;2001;2002). Another sexually dimorphic nucleus in rat is the spinal nucleus of the musculus bulbocavernosus (SNB), in which direct postnatal organizational effects of DHT has been shown to increase in female rats its neuron number and morphology (connectivity, dendritic length, and soma size) to male characteristics (Goldstein and Sengelaub, 1992). Interestingly, demasculinizing and feminizing effects of DHT have also been reported on some rat brain nuclei e.g. the accessory olfactory bulb (AOB) and the bed nucleus of the accessory olfactory tract (BAOT) (see below) where postnatal (day 6 to 20) DHT treatment in male rats induces a drastic reduction in overall volumetric sizes to female levels (Valencia et al., 1992; Collado et al., 1992). These two nuclei belong to the sexually dimorphic vomeronasal system (VNS).
1.5 The sexually dimorphic vomeronasal system (VNS): organizational and activational effects of circulating steroids

The mammalian VNS expresses sex hormone receptors, such as androgen receptors, estrogen receptors, and progesterone receptors (ARs, ERs, and PRs) and consists of a multisynaptic sexually dimorphic circuit which contains the vomeronasal organ (VNO), the accessory olfactory bulb or (AOB), the bed nucleus of the accessory tract or (BAOT), the medial amygdala or (MeA), the bed nucleus of the stria terminalis (BST), the medial preoptic area (mPOA), the anteroventral periventricular nucleus AVPV and the ventromedial nucleus (VMN) (Segovia and Guillamon, 1993). The VNS is a limbic circuit, crucially involved in the regulation of reproduction and sexual behavior. With the exception of the bed nucleus of the stria terminalis (BST) of which some subdivisions are larger in females, the pattern of sexual dimorphism in the whole VNS pathway seems to be homogeneous: males show greater values for volume and neuron number than females (Panzica et al., 1995). The input enters a sense organ (the VNO) and the output involves endocrine, motivational, and motor centers such as the amygdala-BST complex, hypothalamus, basal ganglia, brain stem and spinal cord in order to orchestrate its neuroendocrine and behavioral effects (Panzica et al., 1995; Cooke et al., 1998).

The human vomeronasal organ (VNO, i.e. also called Jacobson’s organ) measures up to 2 mm and is filled with fluid (Johnson et al., 1985; cf. Swaab, 2003). It originates from the embryonic medial olfactory placode and is enclosed in a cartilaginous capsule that is separated from the main olfactory epithelium and is located in the most anterior part at the inferior margin of the nasal septum. It is sensitive to certain airborne chemical signals defined as pheromones (vomeropherins) that are secreted into the environment in sweat or urine by one individual of a species which can exert a behavioral or physiological response in another individual of the same species (Berliner, 1996). Thus pheromones dissolved in the fluid of the VNO can reach the receptor cells of the cavernous tissue (Tirindelli et al., 1998; Keverne, 1999; reviewed by Swaab, 2003). It is generally claimed that adult humans do not have a vomeronasal organ and an accessory olfactory bulb, but that they would be present only in the human fetus where they disappear before birth (Price, 1990; Trotier et al., 2000). However, recent studies and autonomic electrical and psychological responses provide evidence for a selective and sensitive response from the human vomeronasal organ (Meredith, 2001). Since the accessory olfactory bulb is absent in humans it is uncertain via which pathway exactly pheromone signals derived from the VNO can reach the human brain (Keverne, 1999; Savic et al., 2001). Interestingly, the latter group has demonstrated by PET studies a substrate for a sexually dimorphic reaction to pheromones. They showed that smelling an androgen-like compound activates the hypothalamus of women with the center of gravity in the preoptic and ventromedial nucleus. In contrast, in men the paraventricular and dorsomedial nucleus is activated when smelling an estrogen-like substance.
These data are fully in agreement with the concept of the presence of a VNO-mediated sexually dimorphic human limbic brain circuit that is involved in monitoring olfactory stimuli related to reproduction.

*Examples of organizational actions of androgens on the size of some VNS brain areas (SDN; AVPV; BST; SNB)*

The hypothalamus plays a critical role in coordinating expression of reproductive behaviors and physiological responses in relation to environmental cues. Its close anatomical and physiological relationship with the pituitary gland provides an effective means for coordinating diverse homeostatic processes through neuroendocrine regulation. The hypothalamus also shares strong connections with other limbic regions of the brain (e.g. septal area, DBB, BST, amygdala, and hippocampus) so that it can effectively coordinate neuroendocrine processes.
to removal of T may extend even until postnatal day 29 in the rat (Woodson and Gorski, 2000).

The functions of the SDN-POA are still largely unknown, although there is some evidence which points to its regulatory role in autonomic function (e.g. temperature regulation), reproduction (i.e. HPG-axis regulation by LHRH release and sexual behavior (cf. Silva and Boulant, 1986; Van der Beek et al., 1997; Swaab et al., 2003). Lesion experiments in rats have indicated that the SDN-POA may be involved in aspects of male sexual behavior, i.e. mounting, intromission and ejaculation (Turkenburg et al., 1988; De Jonge et al., 1989). However lesions confined to the SDN-POA had only relatively modest inhibitory effects on these behaviors. Penile erection following medial preoptic area stimulation in the monkey and the rat (cf. Giuliano et al., 1996) could be one of the sexual functions this area is mediating, although, following medial preoptic area lesions, rats do not lose the ability to achieve an erection (McKenna, 1998).

Interestingly, where lesions of the SCN failed to find an effect on potential changes in partner preference behavior/sexual orientation (Kruijver et al., 1993), such lesions in the area of the SDN-POA in the ferret and rat caused a significant switch in the males’ preference shifting from estrus females to stud males, i.e. from a male-typical pattern of sexual behavior to a more female-typical pattern, that was accompanied by reduced copulatory behavior in male (Paredes and Baum, 1995; Kindon et al., 1996; Paredes et al., 1998; Swaab, 2002).

Regarding the structural sexual differentiation of the SDN-POA, it is important to note that exposure to steroid signals during its critical period affects neuron fate in the SDN-POA differently from other sexually dimorphic brain areas. Another such area that is likewise sensitive to hormone signalling perinatally, but, unlike the SDN-POA, develops its volumetric sexual dimorphism only peripubertally, is the hypothalamic anteroventricular nucleus (AVPV). The AVPV is sensitive to castration on postnatal Day 1 (PN1) but does not develop its structural sex difference until about PN30- PN40 (reviewed by Woodson and Gorski, 2000). Interestingly, while it has been shown that estrogen regulates the developing SDN-POA’s neuronal number by inhibiting apoptosis (Chung et al., 2000), Arai et al (1996) found that estrogen facilitates apoptotic cell death in the developing AVPV, yielding a sexually dimorphic dopaminergic nucleus that is larger in females than in males and was found to be involved in the (cyclic) regulation of GnRH release.

Thus as previously described for DHT, it appears that also with estrogens there are paradoxal organizational actions known of these steroid hormones while acting on the brain: in one region estrogens form larger nuclei in males (e.g. the SDN-POA) and in another nearby region estrogens cause a nucleus to become smaller (e.g. in the AVPV). Similar sex- reversed organizational effects by steroid hormones have been described in subnuclei of the rat BST. The medial posterior portion of the BST (BSTMP or principal nucleus, BSTpr) is sexually dimorphic with males having larger volumes and neuron numbers
than females. Neonatal castration decreases its volume to female levels, while treatment with TP on PND 1 in female rats prevents apoptotic cell death in the BSTMP/BSTpr and increases its volume to male levels (Del Abri et al., 1987; Segovia and Guillamon, 1993; Chung et al., 2000). In contrast, the lateral anterior BST (BSTLA) and medial anterior BST (BSTMA), have both more neurons in females than in males, and neonatal orchidectomy in males results in a significant increase in the adult number of neurons over control males. Interestingly, treatment of newborn females with androgens reduces the number of cells in the adult BSTLA to values equal to those of control males, whereas TP treatment in females further increases the cell number in the BSTMA beyond that of control females. The cellular mechanisms by which these sex differences in neuronal number arise in the BSTLA and BSTMA are likely to be related to apoptosis (Chung et al., 2000), but have not yet been entirely clarified (for review see Segovia and Guillamon, 1993).

Another well known example of an androgen dependent sexually dimorphic nucleus is coming from a group of motor neurons, located in the lower lumbar spinal cord, which innervate the striated bulbocavernosus (BC) and levator ani (LA) muscles which are attached to the penis. The motoneurons form the spinal nucleus of the musculus bulbocavernosus (SNB) and are located in the dorsomedial portion of the ventral horn in lumbar segments 5 and 6 in rats. Male rats have at least three times as many motoneurons as females and the neurons themselves are almost twice as large in males as in females. Both male and female rats have bulbocavernosus muscles before birth and have SNB cells which synaps upon those muscles. However, due to the lack of postnatal androgen exposure, first the muscles and subsequently the motoneurons degenerate shortly after birth in females. This results in the adult neuronal sex differences of the SNB and their target muscle fibers (Breedlove and Arnold, 1980; 1983; Nordeen et al., 1985). Furthermore, androgen treatment, but not estrogen treatment, of newborn female rats results in more SNB motoneurons and more and larger BC muscle fibers, while androgen deprivation of perinatal males results in a feminine SNB system in adulthood. Moreover, it has been shown that androgen-androgen receptor interactions play a crucial role in these organizational effects. The importance of the androgen receptor in the masculinization of the SNB system is clearly illustrated by the fact that male rats with a defective structural gene for the androgen receptor, i.e. the testicular feminization mutation-Tfm- rat, develop a feminine phenotype, including the absence of BC muscles and fewer and smaller SNB neurons than in normal males (reviewed by Cooke et al., 1998). Neonatal estrogens may further masculinize the size of SNB neurons in female rats, whereas neonatal estrogen with androgen increases the size of adult SNB motor neurons even more (Breedlove, 1997; Cooke et al., 1998).
Examples of activational actions of androgens on the size of some brain nuclei (MePD; POA; VMN; POM)

In addition to organizational effects of androgens on the development of structural and functional sex differences in the brain, also activational effects by androgens on some volumetric sex differences in the vertebrate brain have been described. Recently, such an effect was reported in the rat posterodorsal nucleus of the medial amygdala (MePD), which has a greater volume in male rats than in females. However, adult castration of males causes the volume to shrink to female values within four weeks, whereas androgen treatment of adult females for that period enlarges the MePD to levels equivalent to normal males. It was shown that this sex difference and androgen responsiveness was purely due to activational effects of androgens upon the soma size of MePD neurons (Cooke et al., 1999). In another species, the reptile whiptail lizard (Cnemidophorus inoratus) the POA is larger in males whereas the VMN is larger in females. Crews and colleagues have shown that the volume and cell size of these structures are also controlled by gonadal hormones in adulthood (Panzica et al., 1995). Another example is the sexually dimorphic medial preoptic nucleus (POM) of the Japanese quail. Its volume is approximately 30% larger in adult males than females. Increases in POM volume are found in T-treated ovariectomized adult females, and the volume of the nucleus decreases in castrated adult males or intact males kept in short day conditions (Panzica et al., 1995).

Examples of combined organizational and activational actions of androgens on the size of some mammalian brain nuclei

Species specific combined organizational and activational effects have also been observed in e.g. the mammalian gerbil and ferret. The sexually dimorphic area pars compacta (SDApc) of the gerbil medial preoptic area-anterior hypothalamus (MPOA-AH) is larger in males than in females and steroid sensitive in the adult given its disappearance in castrated males. It re-appears after T treatment of castrated males in contrast to females (Commins and Yahr, 1984; cf. Panzica et al., 1995).

In the ferret POA-anterior hypothalamus, Tobet and colleagues described a dorsal nucleus that was apparent only in males, irrespective of hormonal status (castrated, or supplied with T or progesterone). There was, however, a significant effect of hormonal treatment on the cell area with an increase of its soma size in T-treated adult males (Tobet et al., 1986). This is another example of a sexually dimorphic nucleus due to both organizational as well as activational effects of androgens (reviewed by Panzica et al., 1995).

Examples of organizational actions of sex hormones on neuronal networks

In addition to regulating the volume and number of neurons in sexually dimorphic nuclei, sex steroids appear to regulate the development of sexually
dimorphic patterns of connectivity in telencephalic, and diencephalic brain regions. These regions are thought to play a role in mediating reproductive functions. Arai and his colleagues found sex differences in synaptic morphology in many of these limbic regions and were one of the first to recognize that sexually dimorphic nuclei and their sexually dimorphic connections together form the backbone of a dimorphic forebrain circuit involved in sexually differentiated behaviors and physiological responses (Simerly, 2000).

It has for example been found in rat that there is a sexually dimorphic projection from the SCN to the AVPV and from the AVPV to the ARC. Females have a more robust SCN projection pattern to GnRH neurons in the AVPV and from the AVPV to the ARC as opposed to males. In turn, males have a stronger projection from their BSTpr to the AVPV (Simerly, 2000;2002). It is conceivable that these sex differences in neuronal pathways may play a role in the sexually dimorphic regulation of e.g. the HPG-axis. Simerly and colleagues established that the projection from the BSTpr to the AVPV occurs between postnatal day (PN) 9 and PN10 in male rats and appears to be maintained during the juvenile period. Although labeled fibers extended from the BSTpr toward the preoptic region in both male and female neonates, a strong connection with the AVPV was not apparent in female rats at any of the ages studied and by PN10 the density of labeled axons in the AVPV of males was approximately 20-fold greater than that of females (Gu and Simerly, 1997). These results are also consistent with the greater synaptic density in the AVPV of males relative to that of females (reviewed by Simerly, 2000). Interestingly, postnatal treatment of female rats with testosterone caused a dramatic sex reversal in the density of BSTpr inputs (axon terminals) to the AVPV, but had a less pronounced effect on other terminal fields, such as that in the medial preoptic nucleus (MPN), which suggests that sex steroid hormones exert a target-specific influence on the development of BSTpr projections (Simerly, 2000).

Axon terminals, i.e. synapses that contain synaptic vesicles, can make contact with the shaft or spines of dendrites or somata. Dominique Toran-Allerand was the first to show that estradiol causes a dramatic proliferation of neurites extending from explant cultures of mouse preoptic tissue (Toran-Allerand, 1976). Additional studies have indicated that estrogens can act as a neurotrophic factor on neonatal brain tissue by stimulating axonal and dendritic growth and synapse formation. For example, estrogens markedly enhance axodendritic synapse formation in the ARC during the neonatal period, while the synaptic density in the ARC is progressively increased during the course of development. The number of axodendritic and axosomatic synapses in the ARC is very small at the neonatal period and reaches a plateau around the onset of puberty (Matsumoto et al., 2000).

Sexual dimorphism in synaptic patterns has been found in several brain regions. In the ARC, the number of spine and somatic synapses is approximately twice as great in female rats as in males, whereas there is no sex difference in
the number of shaft synapses. A similar sexually dimorphic distribution pattern is found in the dorsal part of the POA. In contrast, in the ventrolateral part of the VMN, the number of shaft and spine synapses is greater in males than in females. In the suprachiasmatic nucleus (SCN), the incidence of spine synapsis is also higher in males than in females (Guldner, 1982; LeBlond et al., 1982). All these nuclei contain sex hormone sensitive neurons. Together these findings suggest that synaptic organization may vary according to nucleus specific responses to organizational actions of sex steroids (reviewed by Matsumoto et al., 2000).

**Examples of activational actions of sex hormones on neuronal networks (dendrites, synapsis, projection pathways)**

Estrogen actions in the ventromedial nucleus of the adult hypothalamus of the female rat induce new synapses and these effects are absent in adult castrated males. According to McEwen, (1999), this may point to an overriding effect of developmental actions of sex hormones, i.e. that the ability to show adult plasticity in response to hormone is programmed by early developmental actions of testosterone. Work from McEwen’s lab has shown that estrogen induction of synapse formation is not restricted to the hypothalamus, but also occurs in e.g. the hippocampus, where it shows a dependency on circulating estrogens by expressing synaptic fluctuations according to the estrous cycle of the female rat (Woolly and McEwen, 1994; Woolly et al., 1997). In contrast, the adult male is refractory to the synapse-inducing effects of testosterone unless testosterone actions on sexual differentiation have been prevented at birth (Lewis et al., 1995).

**Examples of organizational and activational actions of sex hormones on some neuropeptide and neurotransmitter systems**

Adjacent structurally different brain areas and even subdivisions of a particular brain region may show different or even opposite sexually dimorphic expression patterns of a specific neuropeptide or neurotransmitter system. Such sex differences can be organizationally determined during a critical period of brain development or rather be due to activational events, such as age and endocrine status. Thus, in addition to structural sex differences in the wiring of the brain, sex differences at the neurotransmitter and neuropeptide level in vertebrates may also contribute to sex differences in the regulation of e.g. gonadotropin secretion (HPG-axis), somatostatin (SOM) and growth hormone (GH) secretion and be involved in the sexual dimorphic response of the hypothalmo-adrenal axis to stressors. At the behavioral level they may contribute to sex differences in social, sexual and aggressive behaviors. A sexual dimorphism has for example been reported for several neurotransmitters, such as adrenaline, noradrenaline, dopamine and serotonin, whereas various neuropeptide systems that may act as neurotransmitters or neuromodulators, such as substance P, cholecystokinin
(CCK), vasoactive intestinal polypeptide (VIP), vasopressin (AVP) and oxytocin (OXT) have also been shown to be sexually dimorphic (for refs cf. Cooke et al., 1998; Negri-Cesi et al., 2000; Swaab, 2002). Another example is the rat AVPV sex difference which has more more Nissl stained cells in females than in males, while females also possess greater numbers of tyrosine hydroxylase immunoreactive (TH)-ir neurons in the AVPV (Simerly et al., 1985). Simerly and colleagues found that TH immunoreactivity was feminized in male estrogen receptor knockout mice but normal in affected Tfmr males. These data do suggest that AVPV dopaminergic neurons are organized to its masculine pattern/masculinized by estrogen receptors alone, independently of androgen receptors (Simerly et al., 1997). Interestingly, other neuropeptides were shown to display reversed sex differences in this nucleus. Male rats have e.g. more AVP-ir cells and enkephalin-ir cells in the AVPV than do females (reviewed by Cooke et al., 1998).

Other nuclei that are known to display activational rather than organizational sex differences in the pattern of immunoreactivity of several neuropeptides in rodents are e.g. the interconnected medial amygdala (MeA) and BST. Substance P-immunoreactive perikarya reside in the MePD and their terminals are found in the BST. Substance P staining in the MePD is more than two times stronger in males than in females, and this difference depends on androgen levels. Eight weeks postsurgery, castrated adult males have approximately 42% smaller substance P-ir areas whithin the MePD than their TP-treated counterparts. A similar adult castration effect has also been observed in the BST (Cooke et al., 1998).

Two other neuropeptides, CCK and vasopressin, are also present whithin the MePD and are accordingly known to have sexually dimorphic distributions. Males have more CCK-ir cells in the MePD than females (Micevych et al., 1987). This sex difference also appears to depend on circulating levels of adult androgen concentrations, since castration of adult males dramatically reduced CCK-ir, and this could be reversed with testosterone replacement (Simerly and Swanson, 1987; Cooke et al., 1998).

Sex differences in arginine vasopressin (AVP) immunoreactivity have previously been characterized by De Vries and colleagues. A neonatal-testosterone dependent sexual dimorphism in AVP fiber density was demonstrated in the rat brain, males having a denser AVP innervation of several brain structures than females (De Vries et al., 1981; 1983). In the adult rat this sexual dimorphism is maintained by circulating sex steroids, since castration of adult male rats was shown to cause a decrease in AVP fiber density in a number of brain structures, which could be restored by testosterone (De Vries et al., 1984; 1985). Dependency on sex steroids was evident in projections of the BST and medial amygdala (MeA), whereas projections of the PVN and the SCN were not affected by castration or administration of gonadal hormones (De Vries et al., 1985). We may thus conclude from these data that, in adulthood, part of the extrahypothalamic
vasopressinergic innervation of the rat brain is more androgen dependent. This is in particular the case for the lateral septum to which vasopressinergic fibers, originating from the bed nucleus of the stria terminalis, project. In males the density of vasopressinergic nerve fibers in the lateral septum is higher than in female rats (De Vries et al., 1981). It is reduced by castration and restored to normal rates following testosterone replacement therapy (De Vries et al., 1984). These authors subsequently showed that the 90% decrement of the lateral septum vasopressin content by castration could be restored by either testosterone or estradiol (E) plus dihydrotestosterone (DHT) treatment. E alone, however, was only half as effective as E plus DHT. Whereas DHT enhanced the response to E it had little effect on its own. Neither castration nor the hormonal substitution treatments had an effect on the oxytocin (OXT) content of the septum or on the vasopressin or OXT content of the dorsal vagal complex (De Vries et al., 1986).

Interestingly, the results of Johnson et al., (1991) indicate an analogous hormonal mechanism regarding OXT-receptor binding in the ventromedial hypothalamic nucleus (VMN). Castration decreased endogenous OXT-receptor binding levels, whereas treatment with testosterone propionate (TP) or estradiol benzoate (EB) plus dihydrotestosterone benzoate (DHTB) restored the expression patterns back to control levels. Again, treatment with EB alone only partially reinstated binding to the levels in intact males, while DHTB treatment was without effect. These findings are of particular interest as structures of the vasopressinergic and oxytocinergic neural circuit (like e.g. the SCN, BST, LS, MeA, SDN-POA and VMN) have been associated not only with morphological sex differences, but also with functional sex differences related to male and female copulatory behaviors, social memory and most interestingly with pair bonding and partner-preference behavior (Blunthé et al., 1990; Carter et al., 1992; Insel, 1992; Winslow et al., 1993; Caldwell et al., 1994; Lévy et al., 1995; Swaab et al., 1995).

Partner preference behavior

A striking example of gender dependent sexual and concomitant social behaviors is provided by studies of pair-bond formation in the monogamous prairie vole (Microtus orogaster). Partner preference (i.e. heterosexual orientation) is dependent on AVP in males (Winslow et al., 1993), whereas oxytocin, and not AVP, is required for heterosexual preference formation in females (Williams et al., 1994; Insel and Hulihan, 1995). Although these studies indicate sex differences in the regulation of sexual orientation by AVP in males and OXT in females, a recent study has demonstrated that either peptide may nonetheless facilitate heterosexual preference in both male and female voles (Cho et al., 1999; cf. Goodson and Bass, 2001). Both neuropeptide systems act, at least in part, via their receptors that are known to be organized by perinatal sex hormone levels and show distinct differences in expression patterns in social
monogamous voles versus non-social polygamous voles (Carter, 1998; Insel and Winslow, 1998). It has moreover been shown that these social differences are due to differences in the promoter regions of the AVP/OXT receptors (reviewed by Insel and Young, 2001). Interestingly, perinatal ATD-treatment in rats was shown to induce a diurnal rhythm in partner preference behavior in rats with more homosexual partner preference behavior towards at the beginning of the dark period and more heterosexual partner preference behavior at the end of the dark period pointing to an involvement of the SCN (Bakker et al., 1993). A subsequent study in such pre-, and neonatally ATD-treated male 'bisexual' rats indicated an organizational change in its vasopressinergic subnucleus with 59% more AVP neurons as control rats (Swaab et al., 1995). These findings have a striking parallel with the enlarged vasopressinergic subnucleus in the SCN of homosexual men which was the first discovered human brain structure showing a relationship with sexual orientation (Swaab and Hofman, 1990).

1.6. Sexual dimorphisms in the human hypothalamus and adjacent areas and their relationship with sexual orientation and gender identity

Clear sex differences exist in reproduction related functions, such as the presence of a menstrual cycle in women. Moreover, statistical sex differences are present in cognitive performance with e.g. women performing generally better on language related tasks and verbal fluency, while men perform better than women on visuo-spatial related abilities and mechanical reasoning (Collaer and Hines, 1995). Childhood play behavior also differs clearly between boys and girls. More rough-and-tumble play (RTP) is seen in boys than in girls, which seems to be influenced by pre- and neonatal hormone levels. Male rats treated with antiandrogen during neonatal life show reduced RTP. Likewise female monkeys and girls with congenital adrenal hyperplasia (CAH)exposed in utero to androgens exhibit more male-like/RTP play behavior, including more outdoor play activity and expenditure of physical energy. CAH is a genetic defect in the adrenal steroid hormone P-450c21 hydroxylase enzyme system, resulting in lower adrenal cortisol and/or aldosterone levels with higher endogenous adrenal androgen levels due to a lack of negative feedback which results in hyperstimulation of the hypothalamo-pituitary-adrenal [HPA]-axis by enhanced ACTH-secretion.

Homosexual and transsexual men and women report a higher incidence of gender atypical behavior during childhood than do heterosexual and non-transsexual people, which supports the concept that the hormonal milieu of the developing brain may not only be a major determinant of structural sex differences in the brain, but also of gender identity and sexual orientation (cf. Kruijver et al., 2001; Berenbaum, 1999; Berenbaum and Bailey, 2003; Allen and Gorski, 2002; Rahman and Wilson, 2003; Swaab, 2002; Cohen, 2002; Gooren and Kruijver, 2002). Sexual orientation is an individual's erotosexual attraction towards a person of the opposite sex, same sex or of both sexes, respectively
hetero-, homo-, or bi-sexuality. Gender identity is the feeling to be male or to be female. Gender identity disorder (GID) is defined as an incongruence between self-identification as male or female and the physical phenotype. The experience of this incongruence is termed gender dysphoria. The most extreme form, in which individuals feel the need to adapt their phenotype with hormones and surgery to make it congruent with their gender identity is called transsexualism. Those individuals experiencing this condition are referred to as transsexual individuals or trans people, that is male-to-female (MTF) or female-to-male (FTM) transsexuals. (GID definitions are derived from the International Synopsis Colloquy Meeting, UK, 2002). Seventy five percent of transsexual individuals are MTF transsexuals (Landen, 1999).

A major question is which structural differences in the brain may be the basis for sex differences in behavior, sexual orientation and gender identity. Several structural sex differences have been reported throughout the human brain in cortical-, hypothalamic and adjacent limbic-areas and spinal cord. For example in the neocortex, in limbic nuclei (SDN-POA or INAH-1; INAH-2 and 3; SCN and the BST-subdivisions-BSTdmp/BSTc) and in the spinal cord Onuf’s nucleus[i.e. the human analogue of the rat SNB that innervates muscles involved in penile erection] more neurons were found to be present in men than in women (Swaab and Fliers, 1985; Forger and Breedlove, 1986; Allen et al., 1989; Allen and Gorski, 1990; Zhou et al., 1995; Swaab and Hofman, 1995; Pakkenberg and Gundersen, 1997). In contrast language related areas (planum temporale, dorsolateral prefrontal cortex and superior temporal gyrus) and structures connecting the left and right brain (corpus callosum, anterior commissure and massa intermedia of the thalamus) are proportionally larger in women than in men (Morel, 1947; Wada et al., 1975; De Lacoste-Utamsing and Holloway, 1982; Allen and Gorski, 1991; Witelson et al., 1995; Schlaepfer et al., 1995; de Courten-Myers et al., 1999; Gur et al., 1999).

The anterior part of the human hypothalamic area contains various nuclei or subnuclei which are sexually dimorphic (see Fig 1A,B.). In 1985 Swaab and Fliers discovered a sexually dimorphic nucleus in the anterior hypothalamic preoptic area (SDN-POA). The volume of the human SDN-POA was found to be twice as large in young adult men as in women and to contain twice as many cells in young men, a sex difference that was found to occur during development between the fifth year of age and adulthood (Swaab and Fliers, 1985; Hofman and Swaab, 1989). Since Allen et al., (1989) failed to confirm this SDN-POA area to be sexually dimorphic, they called the SDN-POA “interstitial nucleus of the anterior hypothalamus number 1 (INAH-1; vide infra). In addition, Allen and Gorski described three other “interstitial nuclei of the anterior hypothalamus” (INAH-2-4 (Allen et al, 1989; LeVay, 1991; Byne et al., 2000; 2001). Interestingly statistical re-analysis of the INAH-1 volumes published by Allen et al., (1989) showed a strong trend for a sex difference by the use of the non-parametric Mann-Witney U test (n=22; p=0.053). Moreover,
exclusion of the 2 brains of a 4 and 5 year old boy and girl from their otherwise (n=20) adult brain sample, since at that age no sex difference is yet present in the SDN-POA (cf. Swaab and Hofman, 1988; Swaab et al., 1992), indicates now INAH-1 to be sexually dimorphic in a statistically significant way (n=20; p<0.02 or p=0.019). With the parametric student T test, as used by Allen and Gorski, (1989), this sex difference remains present p=0.042; Kruijver et al., present thesis/in prep). The findings by the Allen and Gorski, 1989 study can therefore be considered as a confirmation of the SDN-POA by Swaab and Fliers, (1985). The re-definition of the SDN-POA into INAH-1 is solely due to the inclusion of 2 “confounding” non-adult brains of a 4 and 5 years old boy and girl to the otherwise adult brain sample (n=20).

From the four INAHs Allen et al., (1989) reported only INAH-2 and INAH-3 to be sexually dimorphic. In subsequent studies, neither LeVay, 1991 nor Byne, et al., 2000 could “also” confirm INAH-1 or INAH-2 to be sexually dimorphic but they did confirm INAH-3 to vary with sex. These authors also proposed that that INAH-3 and not INAH-1 would be homologous to the SDN-POA of the rat. However, the human INAH-1/SDN-POA is more likely to be the rat’s SDN-POA homologue as judged from the sex difference in young adults in size and cell number, as found by our group by the cytoarchitecture and neurotransmitter /neuromodulator content. Immunocytochemical studies support such a homology between the SDN-POA in rat and human on the basis of the presence of e.g. thyrotropin-releasing hormone, cholecystokinin, glutamic acid decarboxylase and galanin (Gal)(Swaab, 1997). A recent study on the development of the human hypothalamus confirmed, moreover the homology between the rat SDN-POA and the human SDN-POA/INAH-1 and explained the fact that in the human this area is localized more laterally than in the rat (Koutcherov et al., 2002). They showed that during gestational development, the human sexually dimorphic nucleus of the medial preoptic area migrates from a dorso-medial into a ventro-medio-lateral position due to the development of the lateral preoptic area. In the human adult, the more laterally located SDN-POA should, in our opinion, thus be regarded to be homologous to the SDN-MPOA in rat. Recently we found an age dependent sex difference in the SDN-POA that support this idea. Between 10-40 years of age men had about twice the SDN-POA volume and number of Gal containing neurons as women. Interestingly, such a sex difference was not found any longer between 40 and 70 years of age. The disappearance of Gal neurons in the SDN-POA may be explained by decreased Gal expression and/or apoptosis in the periphery of this nucleus (Kruijver, Ligtenberg, Zhou, Unmehopa, Pool, Herikhuize, Hofman, Swaab, unpublished observations). This preliminary finding, based upon 30 age and sex matched subjects, appears to confirm and extend previous work from our group (Swaab and Fliers, 1985; Hofman and Swaab, 1989) on sex difference of the SDN-POA. The remarkable early disappearance of the galanin sex difference may be related to age dependent endocrine changes influencing
the SDN-POA in men (cf. Chapter 3, 4, 5 and 6) and seems to underline the importance of age as a variable when studying sex differences.

A new focus of potential confusion is the Gal containing ventrolateral preoptic nucleus (VLPO), which is a sleep regulation associated Gal cell cluster located ventro-lateral to the SDN-POA in the rat. The VLPO has recently been declared to be homologous to the SDN-POA (=INAH-1) in human (Gaus et al., 2002). However, albeit that both nuclei express Gal, the VLPO is located more ventro-laterally adjacent to NBMC neurons in the rat, while the human galaninergic SDN-POA neurons are located more dorso-medially in between Gal expressing PVN and SON and other neurons (cf. Swaab and Fliers, 1985; Gaus et al., 2002). We have to wait for specific markers for the VLPO neurons to solve this new homology problem.

A sex difference in shape has been reported in the human biological clock or SCN, which was found to be more elongated in women and more spherical in men (Swaab et al., 1985). Interestingly, an age dependent sex difference was also found to be present for the SCN’s vasoactive intestinal polypeptide (VIP) containing subnucleus which was found to be twice as large in volume and neuron number in young men as compared to young women between 10-40 years of age. This sex difference was found to be sex reversed in the 41-65 years of age group, after which the VIP sex difference disappeared (Swaab et al., 1994; Zhou et al., 1995).

Another sex difference, close to the hypothalamus, was found in the anterior limbic BST area, which the authors (Allen and Gorski, 1990) called the “darkly staining posteriomedial component of the bed nucleus of the stria terminalis (BNST-dspm). In their study, the volume of the BNST-dspm was found to be 2.5 times larger in males than in females. Five years later the central subdivision of the bed nucleus of the stria terminalis (BSTc) was found to be 40% larger in men than in women (Zhou et al., 1995).

In some of the above mentioned areas volumetric differences according to sexual orientation (SCN; INAH-3; anterior commissure) and gender identity (BSTc) have been observed. Swaab and Hofman, (1990) reported for the first time a neurobiological correlate with sexual orientation. A two times enlarged vaspressinergic SCN was observed in homosexual men. A year later LeVay (1991), observed a smaller female-like INAH-3 in the hypothalamus of homosexual men as compared to heterosexual men. Interestingly, Allen and Gorski found that the anterior commissure is larger in homosexual men than in heterosexual men and women (Allen and Gorski, 1992). The anterior commissure mediates interhemispheric transfer of visual information, including the visual recall of dreams, auditory and olfactory information (Swaab, 2002). The observation of Allen and Gorski, (1992) may thus point to a greater connectivity between the cerebral hemispheres of homosexual men and was the third neurobiological correlate to homosexuality. It should be noted, however, that in addition to INAH-1’s reported lack of a sexual dimorphism (Allen et al.,
1989 > however see previous section; LeVay, 1991; Byne et al., 2000) Byne and colleagues again failed to replicate the INAH-3’s and anterior commissure (AC)’s association with male homosexuality (Byne et al., 2001; Lasco et al., 2002). Unfortunately, in the Byne studies no detailed information regarding variability in fixation time, postmortem delay, neuroanatomical dissection and delineation procedures are given. Furthermore the heterosexual control group was more than twice as large as opposed to the homosexual group suggesting a lack of power to detect potential differences in that group. In addition, it is not clear from the Lasco et al., (2002) study whether all the AC-measurements were performed by only one or various investigators.

In 1995, Zhou et al., (1995) described for the first time a neurobiological correlate with gender identity in the central component of the bed nucleus of the stria terminalis or BSTc. Based on vasoactive intestinal polypeptide (VIP) fiber staining, the BSTc was found to be larger in men than in women with a female-like volume in male-to-female transsexuals (Zhou et al., 1995). The human BST area and hypothalamic medial preoptic area, where the brain differences related to gender identity and sexual orientation were reported, are also known from animal studies to play a role in sexually dimorphic aspects of sexual behavior, social/parental behavior, partner preference behavior and reproduction (Insel and Winslow, 1998; Insel and Young, 2001; Swaab, 2002). However, an experimental model for transsexuality is not available.

1.7. Sex dependent neuropsychiatric diseases

Sex differences in the brain may be the basis not only for sex differences in gender identity, sexual orientation, reproduction and behaviour, but also for sex differences in the prevalence of psychiatric and neurological diseases and in age-related neurodegeneration. The proportions of cases range up to 100% prevalence in women in Rett syndrome, 96% in anorexia and 75% in bulimia nervosa, 90% in central precocious puberty, 74% in senile dementia of the Alzheimertype, 67% in multiple sclerosis, 67% in anxiety disorder, 66% in posttraumatic stress disorder and 63% in unipolar depression/ dysthymia to more than 60% prevalence in men in amyotrophic lateral sclerosis, 66% in severe learning disability, 100% substance abuse, 71% in stuttering, 73% in schizophrenia, 76% in REM sleep behavioral disorder, 72% in transsexualism, 77% in dyslexia, 80% in ADHD, 80% in autism, 82% in sleep apnoea, 83% in Kallman syndrome, 87% in rabies,90% in Gilles de la Tourette and 100% in Kleine-Levin syndrome (Swaab and Hofman, 1995; Swaab et al., 2003; Zup and Forger, 2002; Swaab, 2002).

1.8. Scope of the thesis

A major question is which structural differences in the brain may be the basis for sex differences in behavior related to gender identity, sexual orientation,
reproduction, autonomic function, mood and cognition. The present thesis was aimed at exploring differences in the human hypothalamus and adjacent areas in relation to sex, gender identity, sexual orientation and endocrine status, which may underly the sex differences in these functions. Potential structural sex differences were studied by volume measurements and neuron counts (Chapter 2), while, as a basis for the mechanism behind the functional sex differences, differences in the expression of gonadal hormone receptors were studied immunocytochemically in human post mortem brain material (Chapter 3-8).

First the central part of the human bed nucleus of the stria terminalis (BSTc) was studied in order to determine whether its previously reported sex difference in size and its striking sex reversed size in transsexual subjects were also reflected in neuronal numbers. This appeared to be the case while no relationship with sexual orientation or actual endocrine status was detected (Chapter 2).

The next step was to find out which hypothalamic areas are sex hormone sensitive as judged by the presence of gonadal hormone receptors. For this purpose immunohistochemical protocols for paraffin embedded formalin fixed hypothalamic human brain material were developed. First androgen receptor (AR) distribution was described throughout the rostro-caudal hypothalamus and adjacent areas (Chapter 3). A robust sex difference was observed in the caudal hypothalamic mamillary body complex (MBC), with males having a much stronger nuclear AR immunoreactivity (AR-ir) expression pattern. We therefore investigated whether this sex difference in nuclear AR-ir was related to gender identity or sexual orientation or rather to sex differences in actually circulating androgen levels. The latter appeared to be the case (Chapter 4).

Estrogen receptor α and β (ERα and ERβ) protocols were subsequently developed for systematic rostro-caudal mapping studies of the human hypothalamus and adjacent areas in relation to sex and endocrine status (Chapter 5 and Chapter 6). The similarities, differences and potential functional and clinical implications of the sexually dimorphic distribution patterns in both receptor subtypes are described in Chapter 6.

Subsequently, we investigated whether the previously observed hypothalamic functional sex difference in neurons of the vasopressin (AVP) producing supraoptic nucleus (SON), which are more active in young males who also have higher serum AVP levels than females, might be accompanied by a difference in ERs in women compared to men. Therefore, we studied in Chapter 7 the expression of ERα and ERβ in the SON in relation to sex and pre- and post-menopausal age.

The suprachiasmatic nucleus (SCN) is the central clock of the brain that is known to display sex hormone dependent functional differences. However, it has been a controversial issue whether the SCN is directly or indirectly influenced by sex hormones. Therefore we investigated the sex dependent presence or absence of ERα, ERβ, AR and PR in SCN neurons (Chapter 8).
The obtained neuroendocrine data (Chapter 2-8), in relation to sex, gender identity, sexual orientation, autonomic function, mood, cognition and circulating hormones are subsequently summarized and discussed in Chapter 9.