Sexual differentiation of the human and rodent forebrain
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2.0. SCOPE OF THESIS
1.1. RATIONALE

The involvement of gonadal steroid hormones in the regulation of behavior was shown early on, in experiments conducted by Berthold in 1849. His experiments showed that male-typical behaviors in roosters, such as crowing, aggression and male sexual behavior, disappeared after castration, whereas replacement of the missing gonads restored the male-typical behaviors. Similarly, female reproductive behavior in rats is controlled by the cyclic release of gonadotropins from the ovaries, a process which was thought by Pfeiffer et al., 1936 to be regulated by the pituitary. Harris and Jacobsohn in 1952 showed that the brain itself was targeted by gonadal steroid hormones to achieve the cyclic pattern in ovulatory gonadotropin release observed in female rats, since a male pituitary implanted under the hypothalamus of a female rat could support and maintain ovulation. However, it was not until Phoenix and his colleagues (1959) demonstrated that administration of testosterone in pregnant guinea pigs increased the chance that the female offspring displayed male sexual behavior as adults, that the idea that early gonadal steroid hormones organize sexual differentiation in behavior at the level of the central nervous system.

The first two studies confirming that the central nervous system contains specific regions that differ between males and females were published in 1971. Calaresu and Henry found a sex difference in the number of sympathetic neurons in the cat spinal cord, while Raisman and Field reported a sex difference in the number of synapses in the preoptic area of the rat forebrain, which was responsive to gonadal steroid hormones (Calaresu and Henry, 1971; Raisman and Field, 1971). Anatomical sex differences in the brain were soon directly related to behavioral sex differences, such as courtship behavior found in canaries and zebra finches, where song is produced only in males and not in females (Nottebohm and Arnold, 1976). These initial reports, as well as others that followed, solidified the idea that the phenotype of the brain is organized in a sex-dependent fashion, primarily regulated by gonadal steroid hormones in development (see reviews, Arnold and Gorski, 1984; Döhler, 1998; Breedlove, 1994; 1997; Cooke et al., 1998, Swaab et al., 2001; De Vries and Simerly, 2002).

Gonadal steroid hormones have been hypothesized to act on neurogenesis, migration, apoptosis, and/or differentiation of brain cell phenotype, all of which are important developmental processes required for normal brain organization. Here we utilized the far-reaching effects of gonadal steroid hormones to study how the brain is organized at an anatomical and neurochemical level. Central to these studies is the
gonadal steroid hormone-dependent regulation of apoptotic cell death during early brain development. For this purpose, we mainly focused, albeit not exclusively, on the fetal, neonatal and adult development of the bed nucleus of the stria terminalis (BST) in the human and rodent limbic system. This heterogeneous system contains a number of subdivisions that differ markedly between males and females in volume, cell number, and neurochemical content (Del Abril et al., 1987; Guillamon et al., 1988; Allen and Gorski, 1990; Zhou et al., 1995; Kruijver et al., 2000; De Vries and Simerly, 2002). Moreover, the BST is heavily interconnected with sexually dimorphic and non-sexually dimorphic nuclei found in other brain areas, such as the amygdala, preoptic area, hypothalamus and brainstem (Eiden et al., 1985; Moga et al., 1989; Alheid et al., 1995; Kozicz et al., 1998; Hutton et al., 1998; Ibanez et al., 2001). Both the BST and AM have been implicated in the regulation of a number of behaviors, such as reproduction, aggression, addiction, parental behavior and stress (Emery and Sachs, 1976; Dunn, 1987; Albert et al., 1989; Herman et al., 1994; Liu et al., 1997; Treit et al., 1998; Wang et al., 1994; Newman, 1999; Schulz and Canbelyli, 2000; Walker et al., 2000; De Vries and Simerly, 2002). In humans, the size of the BST has been correlated with a gender identity disorder called transsexuality, in which subjects express the strong feeling that they were born in the wrong body (Zhou et al., 1995; Kruijver et al., 2000). The following sections of this introduction are designed to illustrate the abundance of sex differences in the vertebrate brain, and also to give a concise description of the general hypotheses that have been formulated about the mechanisms that regulate sexual differentiation.

1.2. SEX DIFFERENCES IN THE VERTEBRATE BRAIN: A BRIEF OVERVIEW

The concept that the brain differs in make-up between males and females is not new; it is well-established that anatomists in the nineteenth century already found sex differences in human brain weight (see Swaab and Hofman, 1984). This finding was confirmed by recent studies that accurately showed that the total volume and number of neurons in the human neocortex is about 10-15% larger in men than in women (Pakkenberg and Gundersen, 1997; Rabinowicz et al., 2001). Sex differences in the central nervous system have been found at every level of brain organization: brain area volume, cell number, cell cytoarchitecture, cell activity, synaptic connectivity and neurochemical content and in a large number of organisms, such as fish, lizards, songbirds, rodents and primates including humans (Swaab and Hofman, 1995; Cooke
et al., 1998). It was not until the early 1970's that anatomical sex differences were detected within specific regions of the central nervous system. The thoracolumbar intermediolateral nucleus (ILN) of the spinal cord in male cats was shown to contain more sympathetic motoneurons than in the female cat (Calaresu and Henry, 1971). In the same year, using electron microscopy, Raisman and Field (1971) reported that the preoptic area in rats contained more synapses from non-amygdaloid origin in females than in males in adulthood. This was of particular interest, because it was also shown that a single injection with testosterone in newborn female rats decreased these number of synaptic contacts, whereas neonatal castration increased the number of synaptic contacts (Raisman and Field, 1971; 1973). Similar sex differences in synaptic wiring were also found in the arcuate nucleus (Arc), ventromedial hypothalamus (VMH), and amygdala of the rodent brain (Greenough et al., 1977; Nishizuka and Arai, 1983; Matsumoto and Arai, 1986). Soon after the findings of Raisman and Field, much more dramatic gonadal steroid responsive sex differences were found in the vertebrate brain, in for instance, song-regulating brain areas, such as the hyperstriatum ventrale pars caudalis (HVc), nucleus robustus (RA), and magnocellular nucleus of the anterior neostriatum (MAN) in canaries and zebra finches, which are 6 times larger in males than in females (Nottebohm and Arnold, 1976).

1.2.1.2. a. Medial Preoptic Nucleus

The sexually dimorphic nucleus of the preoptic area (SDN-POA), which is located in the rat medial preoptic nucleus (MPN), is 4 to 6 times larger in males than in females (Gorski et al., 1978; Simerly et al., 1985). Similar sex differences in the preoptic area have been found in the ferret, gerbil, guinea pig and hamster brain (Bleier et al., 1982; Commins and Yahr, 1984; Hines et al., 1985; Tobet et al., 1986; Byne and Bleier, 1987). The first study showing a sex difference in the human preoptic area was published in 1985 by Swaab and Fliers, who showed that the human SDN-POA, also known as the interstitial nucleus of the anterior hypothalamus 1 (INAH-1) is about two times larger and contains more cells in young adult men than in young adult women (Swaab and Fliers, 1985; Swaab and Hofman, 1988; Hofman and Swaab, 1989). Since then, other reports showed the presence of additional sexually dimorphic nuclei in the human preoptic area. Notably, INAH-2 and INAH-3 were shown to be larger in men than in women (Allen et al., 1989;
LeVay, 1991; Byne et al., 2000), however, these studies did not confirm the sex difference in INAH-1. Studies in rodents showed that the SDN-POA is heavily interconnected with, among other brain regions, the lateral septum (LS), BST, VMH, posteromedial amygdaloid nucleus, ventral premamillary nucleus (PM) and periaqueductal gray (PAG) (Simerly and Swanson, 1988; De Vries, 1990; Pfaff et al., 1994; Micevych, 1998; Murphy and Hoffman, 2001).

The rat SDN-POA also shows sex differences in neuropeptide expression, such as in the number of galanin expressing cells in the SDN-POA, which is higher in males than in females (Bloch et al., 1993). The male rat MPN is also more heavily innervated with serotonin (5-HT) immunoreactive fibers than the female MPN (Simerly et al., 1985). The presence of galanin, thyrotropin releasing hormone, GABA and substance P-containing neurons has been found in both rat and human SDN-POA (Tatemoto et al., 1983; Simerly et al., 1986; Bonnefon et al., 1990; Gai et al., 1990; Gao and Moore, 1996; Chawla et al., 1997). Lesion studies in rats showed that the MPN, in which the SDN-POA resides, may be involved in the regulation of male sexual behavior (Arendash and Gorski, 1983; De Jonge et al., 1989; Meisel and Sachs, 1994). Because the rat MPN is also heavily connected with brain areas that are involved in gonadotropin release and female copulatory behavior, such as the anteroventral periventricular nucleus (AVPv), BST, AM and VMH it may also contribute significantly to the regulation of female sexual behavior (Gu and Simerly, 1997; Hutton et al., 1998; De Vries and Simerly, 2002). In humans, it is at present not known whether the SDN-POA is involved in similar behaviors.

1.2.b. Bed Nucleus of the Stria Terminalis and Amygdaloid Nucleus

The bed nucleus of the stria terminalis (BST) and the amygdaloid nucleus (AM) form a continuity through columns of sublenticular cell groups traversing the basal forebrain and cell groups that accompany the stria terminalis, which pass above and behind the thalamus (Fig. 1) (Johnston, 1923; Alheid et al., 1995; Heimer et al., 1999). The term extended amygdala, which describes this continuity consists of two major columnar subdivisions (Fig. 1), i.e., medial nucleus of the BST (BSTM)/medial amygdaloid nucleus (MeAM) and lateral BST (BSTL)/central amygdaloid nucleus (CeAM) (see review Alheid et al., 1995; Heimer et al., 1999). Beside the neuroanatomical columnar organization, the neurochemical phenotype is also mirrored in this respect. For example, the dense VIP innervation found in the BSTL is also de-
tected in the CeAM (Heimer et al., 1999).

Figure 1. Schematic representation of the extended amygdala (adapted from Heimer, 1999). Note two distinct columns connecting the BSTM with the MeAM and the BSTL with the CeAM, which traverse through the stria terminalis and sublenticular cell groups dorsal of the striatum. Abbreviations listed on page 140. For further details of BST parcellation see chapter seven, section 7.2.

In rodents, the principal nucleus of the BST (BSTpr) is about two times larger and contains more cells in males than in females (Hines et al., 1985; 1992; Del Abril et al., 1987; Guillamon et al., 1988; Ju and Swanson, 1989), whereas the opposite trend occurs for the lateral anterior BST (BSTLA) and medial anterior BST (BSTMA) (Del Abril et al., 1987; Guillamon et al., 1988). The BST in the human brain also contains several subdivisions that differ in volume between men and women. Indeed, several studies reported that the darkly staining posteromedial component of the BST (BST-dspm) and the central nucleus of the BST (BSTc) are larger in men than in women in adulthood (Allen and Gorski, 1990; Zhou et al., 1995). The MeAM in the rodent brain is larger in males than in females (Mizukami et al., 1983; Hines et al., 1992) and this volumetric sex difference is maintained purely by the presence of circulating levels of testosterone in adulthood (Cooke et al., 1999). Although earlier studies suggested that the size of the human amygdala does not differ between men and women (Murphy, 1986), more recent studies using magnetic resonance imaging suggest that the human amygdala increases in size more rapidly in males than in
females during childhood and puberty (Giedd et al., 1996a). The BST and AM are heavily inter-connected with, among other brain regions, the AVPv, LS, MPN, paraventricular nucleus (PVN), VMH and Arc (Swanson, 1986; 1987; Alheid et al., 1995; Hutton et al., 1998; Newman, 1999).

The rodent BST also exhibits prominent sex differences in the expression of several neurochemicals. The number of vasopressin producing cells in the rat BST is larger in males than in females (Van Leeuwen et al., 1985; De Vries, 1990). More substance P and cholecystokinin (CCK) expressing cells in the BST and AM have been found in male rats than in female rats (Micevych et al., 1987; Malsbury and McKay, 1989). Recent studies showed that the human BSTc in men contains about 60% more neurons that are immunoreactive for somatostatin than in the BSTc in women (Kruijver et al., 2000). Functionally, the BST in animals is involved in the regulation of a number of behaviors, such as reproduction, aggression, addictions, parental behavior and stress (Emery and Sachs, 1976; Dunn, 1987; Albert et al., 1989; Herman et al., 1994; Liu et al., 1997; Treit et al., 1998; Wang et al., 1998; Schulz and Canbelyli, 2000; Walker et al., 2000). In humans the BSTc has been related to the gender identity disorder called transsexuality, in which subjects express the strong feeling of being born in the wrong body. These studies showed that the size of the BSTc in male-to-female transsexuals is similar to that found in control women, whereas in the only female-to-male transsexual studied so far the BSTc size was similar to that found in men (Zhou et al., 1995; Kruijver et al., 2000).

1.2.c. Anteroventral Periventricular Nucleus

Some brain regions are larger in females than in males. For example, the anteroventral periventricular nucleus (AVPv) in the rat, mouse, hamster and gerbil brain was shown to be larger in females than in males (Bleier et al., 1982; Sumida et al., 1993). The AVPv is heavily interconnected with other brain areas, such as the BST, organum vasculosum laminae terminalis (OVLT) and Arc (Hutton et al., 1998; Ibanez et al., 2001).

The rat AVPv also contains neurochemical sex differences that are biased in a female direction. For instance, more dopaminergic cells are found in the female AVPv than in the male AVPv (Gu and Simerly, 1997). Interestingly, the AVPv is more heavily innervated by the BST and MPN in males than in females (Hutton et al., 1998). The ascending AVPv projections terminate, in part, close to the OVLT where
gonadotropin-releasing hormone (GnRH) containing cells have been observed. Because descending AVPv fibers terminate in the periventricular nucleus and Arc, it has been suggested that the AVPv may function as a nodal point in the regulation of gonadotropin secretion. Indeed, lesioning of the AVPv eliminates surges in luteinizing hormone and prolactin, spontaneous ovulation and induces persistent vaginal estrus (Wiegand and Terawasa, 1982; Ronneklev and Kelly, 1986). At present, it is not known whether the human brain contains a similar analogous AVPv brain region.

1.2.1.2. Ventromedial Hypothalamic Nucleus
The ventromedial hypothalamus (VMH) of the rat brain is larger in males than in females (Matsumoto and Arai, 1983). Analysis of the number of synaptic contacts showed that this parameter is higher in males than in females (Matsumoto and Arai, 1986; Pozzo Miller and Aoki, 1991). Tracing experiments showed that the VMH projects to many sexually dimorphic and non-sexually dimorphic brain areas, such as the SDN-POA, LS, BST, AM and PVN (Canteras et al., 1994). Presently, it is unknown whether the VMH in the human brain is sexually dimorphic in volume. However, studies showed that metabolic activity in the VMH may correlate with sex. The size of the Golgi apparatus (GA) has been used as a marker for neuronal metabolic activity (Salehi et al., 1994; Lucassen et al., 1994). The neuronal metabolic activity in the human VMH seemed to be higher in young women than in young men. As the metabolic activity of the VMH appears to increase with age in men, it has been proposed that androgens may inhibit metabolic activity in the VMH (Ishunina et al., 2001). The VMH has been implicated in the regulation of feeding behavior, moreover, it also plays a central role in the regulation of male and female reproductive behavior (Pfaff and Sakuma, 1979; Blaustein and Olster, 1989; McGinnis et al., 1996). For instance, neurons residing in the lateral ventral portion of the VMH have been implicated in the regulation of lordosis after appropriate priming with estradiol and progesterone (Blaustein and Turcotte, 1989; Auger et al., 1995). The VMH in the human brain may be involved in the sexually dimorphic integration of pheromonal input. Positron emission tomography scan studies in humans showed that an androgen-like compound activated the female hypothalamus centering on the VMH, while in males the activation of the hypothalamus by an estrogen-like substance was centered on the PVN and dorsomedial hypothalamus (Savic et al., 2001).
1.2.e. Extra-hypothalamic Sex Differences

Although sex differences in the preoptic and hypothalamic brain areas have received most attention, sex-dependent organization is not restricted to these brain areas. For instance, in humans the volume and number of neurons of the total neocortex is approximately 10-15% larger in males than females (Pakkenberg and Gundersen, 1997; Rabinowicz et al., 2001). More regional cortical sex differences have been found in the visual cortex by Reid and Juraska, 1992, who showed that the male visual cortex in rodents contains more neurons than the female visual cortex.

Notably, sex-dependent structures have also been found in the hippocampus, which is the limbic part of the isocortex. The volume of the hippocampal Ammon's horn CA1 region is larger and contains more neurons in males than females (Madeira et al., 1992). There may also be sex differences in the organization of the corpus callosum and anterior commissure (De Lacoste-Utamsing and Holloway, 1982; Clarke et al., 1989; Allen et al., 1990; Cowell et al., 1992). However, many studies have disputed the validity of these findings (e.g., Bell and Variend, 1985; Byne et al., 1988; Giedd et al., 1996b; Byne et al., 2002).

1.2.f. Sex differences in Cognitive Function and Neurological Diseases

Sex differences are not only found in the organization of the brain, but also in cognitive functions, such as verbal skills, mathematical skills and visiospatial tasks (see review Swaab and Hofman, 1995). However, at present these sex differences have not been correlated to neuroanatomical differences. The incidence of neurological and psychiatric diseases is also highly dependent on sex (Swaab and Hofman, 1995). For instance, the incidence of anorexia nervosa and bulimia is much higher in women than in men, whereas the opposite is true for dyslexia, sleep apnea and Gilles de la Tourette (Block et al., 1979; Caine et al., 1988; Whitaker et al., 1989; Castle and Murray, 1991). The incidence of gender identity disorders depends on sex as well. In the Netherlands, there are about three times fewer male-to-female transsexuals than female-to-male transsexuals (Van Kesteren et al., 1996).

1.3. FORMATION OF THE GONADS

Numerous studies showed that sexual differentiation begins with the sex-dependent development of the fetal gonads under influence of genetic sex. In early fetal development, the gonads (i.e., primary source of plasma gonadal steroid hormones) do not
differ between males and females, and have therefore been called indifferent or bipotential gonads. Differentiation of the male fetal gonads into testes is caused by a number of sex specific genetic factors, such as the testis determining factor (TDF), which is encoded by the sex determining region-Y chromosome (Sry) gene located on the short arm of the Y chromosome (Palmer et al., 1989; Sinclair et al., 1990; Jager et al., 1990; Berta et al., 1990; Koopman et al., 1991; McElreavey and Fellous, 1999; Koopman, 2001). In the absence of TDF, as is the case in the female fetus, the bipotential gonads differentiate into ovaries. The formation of the testes induces the masculinization of the internal Wolffian ducts. Leydig cells in the fetal testes produce testosterone, which stimulates the formation of the ductus vas deferens, seminal vesicles, and epididymis from the Wolffian ducts, while Sertoli cells in the fetal testes produce Müllerian inhibiting hormone (MIH), which facilitates the regression of the Müllerian ducts, thereby preventing the formation of female reproductive organs, such as the uterus. Moreover, testosterone is converted into dihydrotestosterone (DHT) by 5α-reductase, which in turn facilitates the development of the penis and scrotum. Similar to the differentiation of the bipotential gonads into ovaries, no hormonal signaling seems to be required for the feminization of the internal ducts in fetal females.

1.4. SEX DIFFERENCES IN CIRCULATING TESTOSTERONE LEVELS

Inherent to testes formation, overall testosterone levels (Fig. 2) are higher in males than in females during fetal and perinatal development (Corbier et al., 1978; Döhler and Wuttke, 1975; Weisz and Ward, 1980). Interestingly, circulating levels of testosterone are markedly increased in males at specific time points in development. In rats, circulating testosterone levels peak around embryonic day 18 and 19, which is followed by a lower peak in testosterone levels just hours after birth (i.e., within 1 to 2 hours) (Weisz and Ward, 1980; Corbier et al., 1978; 1990). Not surprisingly, testosterone plasma levels are also higher in males than in females during human fetal and newborn development. Testosterone levels in the male human fetus begin to rise in the second month of the first trimester and reach their highest levels in the second trimester, which are maintained until late gestation (i.e., third trimester) when testosterone levels are only slightly higher in males than in females at the time of birth. In the first neonatal year, a second surge in testosterone plasma levels has been observed, which subsides to levels that are again slightly higher in males than in
females until the onset of puberty (Abramovich and Rowe, 1973; Winter, 1978; Corbier et al., 1990; Griffin and Wilson, 1998).

**Figure 2.** Sex differences in testosterone levels during human male A) and rat development B). Note the peaks in testosterone plasma levels during fetal and neonatal human and rat development, which are thought to be important for the sex-dependent anatomical and neurochemical development of the central nervous system (adapted from Döhler and Wuttke, 1975; Winter, 1978; Griffin and Wilson, 1980; Weisz and Ward, 1980).

### 1.5. AROMATIZATION HYPOTHESIS

The gonadal steroid hormone testosterone is crucial for the sex-dependent organization of the brain. The *aromatization hypothesis* states that effects of testosterone on sexual differentiation of the brain are mediated by its metabolite estradiol (Fig. 3), a conversion process facilitated by aromatase which occurs locally in brain cells (McEwen et al., 1977; MacLusky and Naftolin, 1981; MacLusky et al., 1985; Sasano et al., 1998; Simpson et al., 2000; Holloway and Clayton, 2001). Evidence from studies investigating the development of sex differences in animals support the aromatization hypothesis. Indeed, numerous studies showed that perinatal estradiol treatment reproduced the masculinizing effects of testosterone on brain organization in gonadectomized animals, whereas perinatal treatment with a non-aromatizable androgenic metabolite of testosterone, DHT, was unable to affect brain organization in a testosterone-like manner (see review McEwen, 1983; Balthazart and Ball, 1998; Cooke et al., 1998). For example, estradiol treatment in female and castrated male rat pups increased the size of the SDN-POA, whereas perinatal DHT treatment did not
Moreover, aromatase is highly expressed in newborn rodent and human brain areas that contain anatomical and neurochemical sex differences in adulthood, such as the preoptic area, hypothalamus and limbic system (Naftolin et al., 1975; 1996; Sasano et al., 1998).

The conversion of testosterone into estradiol by aromatase may be responsible for the dramatic surge in estradiol levels in the male rodent hypothalamus around birth, which is absent in the female rodent hypothalamus (Rhoda et al., 1984). Masculinizing effects of testosterone were also effectively blocked by estrogen antagonists and aromatase inhibitors (McDonald and Doughty, 1972; 1974; McEwen et al., 1977; Vreeburg et al., 1977; Brandt et al., 1991; Bakker et al., 1993; 1995). These studies strongly support that testosterone-derived estrogens facilitate sexual differentiation of the rodent, and possibly the human brain.

![Diagram of testosterone conversion by aromatase or 5α-reductase into estradiol or dihydrotestosterone (DHT) respectively. Sexual differentiation is thought to be mainly mediated by estradiol derived from testosterone, however there are evidence suggesting that testosterone and or DHT may also directly affect sexual differentiation of the central nervous system.](image)

**Figure 3.** Schematic representation of testosterone conversion by aromatase or 5α-reductase into estradiol or dihydrotestosterone (DHT) respectively. Sexual differentiation is thought to be mainly mediated by estradiol derived from testosterone, however there are evidence suggesting that testosterone and or DHT may also directly affect sexual differentiation of the central nervous system.

### 1.6. ORGANIZATIONAL VERSUS ACTIVATIONAL EFFECTS

A pivotal concept for understanding the actions of gonadal steroid hormones on vertebrate brain sexual differentiation was put forward by Phoenix and colleagues in 1959, who proposed that gonadal steroid hormone effects in vertebrates can be categorized as organizational or activational. Organizational effects of gonadal steroid hormones are thought to be permanent and mainly occur during perinatal development, whereas activational effects are thought to be transient and mainly
restricted to adulthood. An example of an organizational effect is the inability of testosterone treatment to markedly affect the anatomical organization of the female SDN-POA in adulthood, while testosterone treatment on postnatal day 1 markedly increases the SDN-POA size in female rats (Gorski et al., 1978; Jacobson et al., 1981). A good example of a transient activational effect of gonadal steroid hormones is the induction of female sexual behavior in rats during the estrous cycle, which depends on an increase in circulating estradiol followed by an increase in progesterone (Fig. 4). Although, these changes in serum levels increase the expression of the immediate early gene c-fos and progestin receptors in the VMH, these changes do not markedly affect VMH anatomy and more importantly are transient (Auger et al., 1996). These hormonal changes prime the female brain for behavioral estrus, in which female rats are receptive for males.

**Figure 4.** The four day estrous cycle in female rats is characterized by an increase of estrogen plasma levels, which is followed by an increase of progesterone plasma levels (adapted from Carter, 1992). These hormonal changes prime the female rat brain for behavioral estrus, in which female rats are receptive to males.

### 1.7. GONADAL STEROID RECEPTORS

Actions of gonadal steroid hormones are classically described as being mediated through their specific receptors, which are part of a large family of nuclear steroid hormone receptors (Evans, 1988; Robyr et al., 2000). Despite the large diversity in steroid hormone receptors, they are highly conserved transcription factors, which are
variations of a general basic modular organization. They contain a ligand binding domain, DNA binding domain and transactivation domain (Fig. 5). The ligand - and DNA binding domain of the steroid receptor recognize specific ligand and hormone response elements on DNA, thereby conveying specificity in “gonadal” steroid hormone action. Functional hybrids between one functional domain from a steroid receptor and a functional domain from another steroid receptor, nicely illustrate the modular organization of steroid receptors. For instance, swapping the DNA binding domain of an estrogen steroid receptor with a similar domain from a glucocorticoid receptor will render normally glucocorticoid responsive genes to be estrogen-sensitive.

Classical activation of steroid hormone receptors requires binding of their specific ligand, which causes the translocation of the ligand-receptor complex to the nucleus, where it dimerizes with another ligand-receptor complex, which in turn will interact through the DNA binding domain with its specific hormone response element on DNA in order to regulate gene transcription (Fig. 6). This process requires interaction with activation function 1 (AF-1) and recruitment of co-regulatory proteins (see below), which bind to AF-2 on the ligand binding part of the steroid receptor. DNA binding capability is conveyed by the cysteine-rich motif called “zinc finger” present in the DNA binding domain. This zinc finger motif is based on a zinc ion with a surrounding tetrahedral protein structure containing either four cysteine residues or two cysteine and two histidine residues.

![Figure 5](image.png)

**Figure 5.** Basic modular organization of steroid receptors (Adapted from Lemke, 1992; Auger, 2001). Ligand-dependent activation of steroid receptors is conferred by the ligand binding domain (LDB). The ligand binding domain moreover contains an activation function 2 (AF-2) site which associates with specific co-regulatory proteins. Ligand-independent activation of steroid receptors are thought to be mediated through the activation function 1 (AF-1) site. Abbreviations: COOH = C-terminal domain, NH$_2$ = N-terminal domain.

Estrogen receptors (ER) were the first nuclear receptors to be discovered (Toft and Gorski, 1966), after which specific nuclear receptors were found for androgens (AR), progestins (PR), glucocorticoids (GR), mineralocorticoids (MR), thyroid hormone (TR), all-trans retinoic acid (RAR), 9-cis retinoic (RXR), and vitamin D$_3$.
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(VDR). The nuclear receptor superfamily also includes a new class of receptors called orphan receptors (McKenna et al., 1999; 2000; Mueller and Korach, 2001).

1.7.a. Estrogen Receptors

Until recently, it was believed that the mammalian brain only expressed one type of estrogen receptor (ER). However, several laboratories found a second type of ER in the mouse, rat, and human brain (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997; Kuiper et al., 1998). The classical ER was referred as ER α, and the newly discovered ER was termed ER β. The two receptors are encoded by separate genes that are located on different chromosomes (Kuiper et al., 1996). ER α and ER β expression in the rat brain shows considerable overlap in brain areas, such as the MPN, BST, MeAM, cortical AM, lateral habenula (LH), PAG, parabranchial nucleus (PB), locus coerules (LC), and nucleus of the solitary tract (NTS) (Simerly et al., 1990; Kuiper et al., 1996; Shughue et al., 1997; 1998; 2001). On the other hand, the VMH and subfornical organ contain almost exclusively ER α, while neurons of the olfactory bulb, SON, PVN, suprachiasmatic nucleus (SCN), zona incerta, ventral tegmental area, cerebellum (Purkinje cells), and pineal gland among other areas are exclusively ER β positive (Shrughue et al., 1997; 1998; 2001). ER α and ER β distribution in the human brain is largely consistent with studies in experimental animals. Examples of human brain areas that contain ER α expressing cells are the diagonal band of Broca (DBB), nucleus basalis of Meynert (NBM), SDN-POA, BST, AM, PVN, SON, Arc, hippocampus, and cerebral cortex (Goldsmith et al., 1997; Blurton-Jones et al., 1999; Donahue et al., 2000; Österlund et al., 2000a, b; Ishunina et al., 2000; see review Österlund and Hurd, 2001). ER β expression in the human brain can be found in similar brain areas and shows considerable overlap with the expression pattern of ER α (Ishunina et al., 2000; Österlund and Hurd, 2001).

Recent data from our laboratory indicate that both ER α and ER β may differ in expression in the SDN-POA and BST between men and women in adulthood (Kruijver et al., 2002 accepted; Kruijver unpublished observations). Expression of ERs in rodents and primates is also regulated by circulating levels of gonadal steroid hormones. Unlike ARs, removal of circulating levels of testosterone or its metabolite estradiol increases expression of ER α in the rat AVPv, MPN, BST, VMH and Arc
(Simerly and Young, 1991; Lisciotto and Morrell, 1993; DonCarlos et al., 1995; Simerly et al., 1996; Bethea et al., 1996; Funabashi et al., 2000). Gonadal steroid hormone regulation of ER β expression is presently not well-studied; however, gonadotropins seem to down-regulate ER β expression in the rat ovary (Byers et al., 1997). Similarly, ER β expression in the PVN of the rat brain was shown to be decreased by estradiol, while estradiol had no effect on ER β expression in the MPN or BST (Patisaul et al., 1999). On the other hand, estradiol seems to up-regulate ER β expression in the Arc of the rat brain (Österlund et al., 1998). In humans, ER β expression may also be affected by circulating levels of gonadal steroids. For example, ER β expression was higher in the SON of young women as compared to postmenopausal women, while ER α expression was lower in young women than in postmenopausal women (Ishunina et al., 2000).

**Figure 6.** Classical activation of gonadal steroid receptors by their ligands, which readily pass the cell membrane. Upon binding their specific receptors in the cytoplasm or the nucleus, the ligand-receptor complex in the cytoplasm translocate to the nucleus and dimerizes with another ligand-receptor complex. This dimer complex binds to the hormone response element (HRE) and interacts with co-regulatory protein and general transcriptional proteins to alter gene expression, and thereby modifying cell function (adapted from Auger, 2001).

1.7.b. Androgen Receptors
High levels of AR mRNA and protein have been detected in many of the sexually dimorphic and non-sexually dimorphic nuclei of the rat preoptic and hypothalamic
brain area. Examples are the LS, AVPv, MPN, BST, AM, and VMH (Simerly et al., 1990; Zhou et al., 1994; Kerr et al., 1995; Handa et al., 1996). ARs are also present in the main and accessory olfactory bulbs, hippocampus, neocortex, and in several nuclei in the brain stem and spinal cord (Murphy and Hoffman, 2001). The expression of ARs during early postnatal development of the rat AVPv, MPN, and BSTpr is higher in males than in females, while no sex difference in AR expression was found in the VMH (Simerly et al., 1990; Herbison et al., 1995; McAbee and DonCarlos, 1998). The expression of AR in the human brain has recently been studied using in situ hybridization and immunocytochemistry (Puy et al., 1995; Tohgi et al., 1995; Fernandez-Guasti et al., 2000; Beyenburg et al., 2000). The expression of AR in the adult human brain also shows a sexually dimorphic pattern in, for example, the SDN-POA among other areas in the human preoptic and hypothalamic area (Fernandez-Guasti et al., 2000; Kruijver et al., 2001).

The expression of AR in the rodent brain is regulated by circulating levels of testosterone (Kerr et al., 1995; Handa et al., 1996; McAbee and DonCarlos, 1999). Recent studies suggest that aromatization of testosterone into its metabolite estradiol facilitates the induction of AR in the rat brain (McAbee and Don Carlos, 1999). The regulation of AR in the human brain is also thought to be regulated by gonadal hormone status as suggested by recent studies, in which the expression of AR in the mamillary bodies is increased following castration and aging (Kruijver et al., 2001).

1.7.c. Progestin Receptors

Progestin receptors (PR) protein and mRNA, of which there are two major isoforms: PR-A and PR-B are like AR and ER widely distributed in the central nervous system (Olster and Blaustein, 1991; Kato et al., 1993; Turcotte and Blaustein, 1993; Wagner et al., 1998). PR-B is the full length receptor, whereas PR-A lacks 40 amino acids at the N-terminal part of the receptor. Functional studies suggest that PR-A may act as a repressor of PR-B (Tung et al., 1993; Vezeto et al., 1993; Mulac-Jericevic et al., 2000). In the rodent brain, high expression of PR mRNA and protein has been detected in AVPv, MPN, BST, AM, VMH, and Arc (Romano et al., 1989; Simerly et al., 1996; Wagner et al., 1998). Interestingly, a dramatic sex difference in the number of PR immunoreactive cell nuclei has been found in the perinatal rat MPN, indeed many more PR containing rat MPN cells can be found in males than in females during perinatal development (Wagner et al., 1998). The expression of PR
is differentially regulated by estradiol, depending on brain area. For instance, estradiol increases PR expression in the AVPv, MPN, and VMH, whereas no changes in PR expression were detected in the MeAM (Romano et al., 1989; Simerly et al., 1996; Wagner et al., 1998).

1.8. STEROID RECEPTOR CO-REGULATORS
Ligand-bound steroid hormone receptor dimers are stable and bind with high efficiency to hormone response elements on DNA. The steroid receptor complex bound to DNA can interact with several combinations of so-called co-regulatory proteins, which influence genomic transcription (McKenna et al., 1999). Two main classes of co-regulatory proteins have been found; those that increase steroid receptor action are called co-activators, those that decrease steroid receptor action are called co-repressors. These co-regulatory proteins were biochemically identified through their ability to interact with the hormone binding domain of gonadal steroid receptors in a ligand-dependent fashion and contain ATPase, acetyltransferase, methyltransferase and ubiquitin ligase activity (McKenna et al., 1999; 2000). For example, ligand-bound gonadal steroid receptors can recruit steroid receptor coactivator 1 (Src-1), a histone acetyltransferase (HAT), which is involved in the disruption of the nucleosome causing the unwinding of chromatin (McKenna et al., 1999), a process which is thought to be required for the recruitment of general transcription factors and RNA polymerase to enhance or repress gene transcription. For instance, transcriptional activity of ligand bound ER can be modulated by coregulator RIP140 (Brinton, 2001).

Gonadal steroid receptor co-regulatory proteins are required for the anatomical, neurochemical and behavioral sexual differentiation of the vertebrate brain. Mice in which Src-1 was deleted showed partial resistance to gonadal steroid hormone dependent responses in the periphery. For example, testis, prostate and urethral weight in Src-1 knockout male mice is lower as compared to wildtype mice (Xu et al., 1998). Moreover, the increase in uterus weight caused by estradiol is much smaller in Src-1 knockout mice as compared to Src-1 wildtype mice (Xu et al., 1998). Centrally, reduction of Src-1 protein by means of Src-1 antisense oligodeoxynucleotide infusions interfered with the masculinizing effects of testosterone on the developing female SDN-POA. Testosterone-treatment on day of birth in female rats increases the SDN-POA volume and moreover decreases female sexual behavior (Wilson et al.,
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1940; Feder et al., 1966; Gorski et al., 1978). However, the size of the SDN-POA in testosterone-treated female rats was approximately 50% smaller in Src-1 antisense treated animals versus control animals. Scr-1 antisense infusion also attenuated the defeminization of female sexual behavior caused by early testosterone treatment (Auger et al., 2000).

1.9. GONADAL STEROID HORMONES AND APOPTOSIS

One of the proposed resultant gonadal steroid hormone effects during sexual differentiation in the vertebrate brain has been hypothesized to be discrete sex-dependent elimination of brain cells through “apoptotic cell” death (See reviews Arnold and Gorski, 1984; Breedlove, 1994; 1997). Apoptosis is a highly regulated distinct form of cell death that histologically is characterized by shrinkage of cell cytoplasm, condensation of chromatin, blebbing of cell membrane and formation of membrane-bound apoptotic bodies containing intact organelles and condensed chromatin (Fig. 8; Kerr et al., 1972). Biochemical analysis indicated that fragmentation of DNA during apoptosis occurs in multiples of 180-200 base pairs (Arends et al., 1990; Schwartz and Osborne, 1993). Many studies showed that apoptosis is a widespread phenomenon during early brain development, which is required to remove brain cells that do not migrate, differentiate and/or form appropriate neuronal circuits in a given developmental time period (Davies, 1994; Oppenheim, 1991). Examples of apoptotic cell death during brain development have been described early on, for instance in the rodent striatum, hippocampus, amygdala and cerebellum (Mensah, 1982; Janowski and Finlay, 1983). The presence of apoptotic cell death has also been documented in a number of studies investigating fetal human brain (e.g., Chan and Yew, 1998; Simonati et al., 1999; Rakic and Zecevic, 2000; Itoh et al., 2001).

Apoptosis has been observed during sexual differentiation of a number of regions in the preoptic area in the rat brain. For example, the incidence of apoptosis in the perinatal AVPv was higher in males than in females, while the incidence of apoptosis in the postnatal SDN-POA was higher in females than in males (Arai et al., 1994; Arai et al., 1996; Davis et al., 1996). Moreover, these studies showed that testosterone or its metabolite estradiol increased the incidence of apoptosis in the perinatal rat AVPv, whereas testosterone or its metabolite estradiol decreased the incidence of apoptosis in the developing rat SDN-POA. These studies suggest that
gonadal steroid hormones may indeed influence the sexual differentiation of the vertebrate brain by inducing or preventing apoptotic cell death.

Figure 7. Morphological characteristics during apoptosis (adapted from Kerr et al., 1972). A) Normal healthy cell, B-C) Shrinkage of cell nucleus and cytoplasm, D-E) condensation of cell nucleus followed by blebbing of cell membrane, F) disintegration of cell into apoptotic bodies.

2.0. SCOPE OF THESIS

In the present thesis, sexual differentiation of limbic and anterior hypothalamic brain areas in the human and rodent forebrain in relation to apoptosis was examined. We focused, albeit not exclusively, on the human and rodent bed nucleus of the stria terminalis.

In humans, the BST is larger in men than in women, however, it was not clear at what moment during development the BST became sexually dimorphic. This question was studied in chapter two, where the BST size was measured from fetal development onwards. Several seminal studies in animal experiments showed that developmental and adult gonadal steroid hormone actions are conveyed by their specific steroids receptors. In chapter three, we examined the distribution of gonadal steroid hormone receptors in the human brain from fetal ages onward.

Gonadal steroid hormone actions presumably working through their specific steroid receptors may differentiate the vertebrate BST in a sex-dependent fashion by regulating apoptosis during brain development. In chapter four, we examined whether apoptosis indeed plays a role in the sexual differentiation of, amongst other brain regions the neonatal rat BST. Moreover, we investigated whether testosterone
whether sex differences in apoptosis only are sufficient to differentiate the vertebrate brain in a sex-dependent fashion. In chapter five, we examined the effects of the proapoptotic Bax gene deletion on the organization of the BST and amygdala in the mouse brain. Recent studies showed that progestins may also be involved in the modulation of sexual differentiation in the vertebrate brain. In chapter six, we investigated in an initial study whether antiprogestins affect the incidence of apoptosis in the neonatal rat SDN-POA.
Chapter One