Sexual differentiation of the human and rodent forebrain
Chung, W.C.J.

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7.1. MAIN RESULTS

The vertebrate brain is organized in a sex-dependent fashion under regulation of genetic and gonadal steroid hormone factors, which profoundly affect vertebrate physiology and behavior. Similar to other sexually dimorphic areas, the central subdivision of the bed nucleus of the stria terminalis (BSTc) in the human brain was thought to become sexually dimorphic during fetal, newborn and/or infant ages. To our surprise, the size of the BSTc was larger only in men than in women during adulthood suggesting that overt sex-dependent changes of the BSTc anatomy extended well into adulthood (chapter two). The mechanisms underlying sexual differentiation of the human BSTc, i.e., expression of estrogen receptor (ER) α, ER β, androgen receptor (AR) and progestin receptor (PR) were studied during fetal/neonatal, infant/pubertal and adult ages. The expression of these gonadal steroid receptors in the developing BSTc differed between males and females depending on developmental age (chapter three). Sex differences in “ligand-bound” gonadal steroid receptors may induce sex differences in the incidence of apoptosis during brain development in a sex-dependent fashion. This hypothesis was studied in the neonatal rat brain. The incidence of apoptosis during the first postnatal week in the rat BST and sexually dimorphic nucleus (SDN-POA) was higher in females than in males. Moreover, testosterone decreased the incidence of apoptosis, regardless of genetic sex (chapter four). However, these studies did not address whether sex differences in apoptosis alone were sufficient to cause marked anatomical differences between males and females. To study this, we compared the volume of the BST and amygdala in male and female Bax +/+ and Bax -/- mice. The BST and amygdala were larger in Bax -/- mice as compared to Bax +/+ mice suggesting that deletion of a single pro-apoptotic gene was sufficient to overtly change the morphological characteristics of the BST and amygdala. However, neither the BST nor the amygdala differed in size between males and females, regardless of genetic background. Consequently, these mice may not be well-suited for studying the involvement of apoptosis during sexual differentiation as found in the human and rat BST (chapter five). Evidence suggested that PRs may play a larger role in the regulation of sexual differentiation than envisaged earlier, specifically sex-dependent apoptosis. In a first study, we found that the incidence of apoptosis in the rat SDN-POA, which contains many more PR cells in males than in females did not differ between PR antagonist-treated males and females, while the incidence of apoptosis in the SDN-POA was
higher in vehicle-treated females than in vehicle-treated males (chapter six). These results are discussed in the following sections in relation to gonadal steroid hormone-dependent apoptosis during the sexual differentiation of the mammalian brain.

7.2. COMPARISON OF THE HUMAN AND RAT BST

Due to the existence of many disparate sets of nomenclatures used to describe BST subdivisions in human and rodent brain, it is difficult to directly envisage BST subdivision homologies between species. The lack of a coherent nomenclature hampers a clear and consistent analysis of the mechanisms that may be involved in the sexual differentiation of the human BST based on data already obtained from studies in other vertebrate species. The following section will discuss the comparative anatomy of the BST in human and rodent brain in more detail.

7.2.a. Nomenclature in Humans

Two major nomenclatures describe the medial-lateral BST subdivisions in the human brain. The first set of nomenclatures (Fig. 1A) advocates the presence of three major subdivisions: medial, central and lateral (Gaspar et al., 1985; Lesur et al., 1989; Walter et al., 1991), whereas the second set of nomenclatures (Fig. 1B) recognizes only two major BST divisions: medial and lateral (de Olmos, 1990; Martin et al., 1991; Heimer et al., 1999). The second set of nomenclatures embedded the central subdivision of the BST, as defined by the first nomenclature within the lateral BST (BSTL), which is called lateral dorsal central subdivision of the BST (BSTLDcn) (see Fig. 1). In order to limit confusion within this thesis, we referred to the central subdivision of the BST as BSTc, while recognizing that the BSTLDcn refers to the same. The BSTc core is surrounded by a cell sparse capsule, which contains thick tubular fibers and is called the lateral dorsal capsular subdivision of the BST (BSTLDc) (Heimer et al., 1999).

Neurochemically, the human BSTc is characterized by a dense network of fibers immunoreactive (IR) for vasoactive intestinal polypeptide (VIP) (Walter et al., 1991) and thick fibers and cell bodies IR for somatostatin (Gaspar et al., 1985; Lesur et al., 1989; Walter et al., 1991). The BSTc is also IR for glutamic decarboxylase, neurotensin, CCK, neuropeptide Y, enkephalin, synaptophysin, calbindin and acetylcholinesterase, whereas it is almost devoid of substance P and myelin binding protein, which are present in other subdivisions of the human BST (Gaspar et al.,
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7.2.b. Nomenclature in Rats

Parcellation of the BST in the rat brain is also disparate and depends on authors and their methodologies (see e.g., McDonald, 1983; de Olmos et al., 1985; Moga et al., 1989; Ju and Swanson, 1989; Ju et al., 1989). The medial division of the BST in the rat brain is comparable to the human BSTM, while the lateral division of the BST (BSTL) in the rat brain, which includes the dorsal division of the BSTL (BSTLD) is comparable to the BSTL in the human brain (Heimer et al., 1999)(Fig. 2). The BSTM is further parcellated, in an anterior BSTM (BSTMA) and a posterior BSTM (BSTMP) (Fig. 2). The rat BSTMP is at rostral levels located above the receding anterior commissure, while at more caudal levels the medial BSTMP (BSTMPM) and intermediate BSTPM (BSTPMI) are structures consisting of tightly packed neurons located diagonally along the fornix (Fig. 2B) (Paxinos and Watson, 1978; Del Abril et al., 1987; Guillamon et al., 1988; Moga et al., 1989). Recently, the BSTMA and BSTMP together have been referred to as the principal nucleus of the BST (BSTpr) (Ju and Swanson, 1989; Ju et al., 1989), a term adopted in our studies (Chung et al., 2000).

**Figure 1.** Schematic representation of the human bed nucleus of the stria terminalis (BST) adapted from Walter et al., 1991 and Heimer et al., 1999. The medial-lateral subdivisions according to A) e.g., Lesur et al., 1989, Walter et al., 1991 and to B) e.g., de Olmos, 1990; Martin et al., 1991; Heimer et al., 1999. Note that the BSTc in A) refers to the same BST subdivision as the BSTLDcn in B). Abbreviations: ac = anterior commissure, BSTc = central subdivision of the stria terminalis, BSTL = lateral BST, BSTLDcn = lateral dorsal central subdivision of the BST, BSTM = medial BST, fx = fornix, ic = internal capsule, lv = lateral ventricle.
Although the rat BSTpr is larger and contains more cells in males than in females (Del Abril et al., 1987; Guillamon et al., 1988; Hines et al., 1990; Chung et al., 2000), it is probably not homologous to the human BSTc. The rostral-caudal extent of the rat BSTpr is mainly localized in close proximity of the fornix, whereas the rostral-caudal extent of the human BSTc is located in close proximity of the internal capsule (Fig. 1 and Fig. 2B). Moreover, the rat BSTpr does not contain dense VIP-IR terminal fields as found in the human BSTc.

The BSTLD in the rat brain is much more likely to be homologous to the human BSTc, because they share anatomical location (Fig. 1 and Fig. 2A) and extensive overlap in neuropeptide expression. The BSTLD in the rat brain, which is also referred to as oval nucleus by a number of cyto- and chemoarchitectural studies describing the rat BST (Moga et al., 1989; Ju and Swanson, 1989; Ju et al., 1989) is IR for VIP, somatostatin, neurotensin, CCK, enkephalin, substance P and corticotropin releasing hormone (CRH), whereas it is virtually devoid of galanin (Woodhams et al., 1983; Eiden et al., 1985; Ju et al., 1989; Gustafson and Greengard, 1990). However, there are discrepancies in, for example, substance P expression, which may be due to evolutionary adoptions in specific BST functions between humans and rats.

7.2.c. Origin of VIP-IR Fibers
VIP immunoreactivity has been used to delineate the human BSTc during different stages of life and several (non)-physiological conditions (Zhou et al., 1995; Chung et al., 2002). For instance, we showed that the BSTc, defined by its dense VIP-IR fibers was sexually dimorphic only in adulthood and not during earlier ages (Chung et al., 2002). Moreover, the VIP-IR BSTc in male-to-female transsexuals is similar in size as that found in control women (Zhou et al., 1995). To further and better understand the functions of the human BSTc, it is of utmost importance to establish the in- and outputs of the BSTc in the human brain. Recently, tracing studies have been developed using human post mortem brain tissue (e.g., Dai et al., 1998a; Dai et al., 1998b), however no such studies are available for the human BSTc. The efferents and afferents of the BST are well-known in the rat brain (See review Dong et al., 2001). Anterograde tracing studies showed that the rat BSTLD projected mainly to the central amygdala (CeAM), substantia inominata (SI), paraventricular nucleus (PVN), lateral hypothalamic area (LHA) and brainstem regions including the
periaqueductal gray (PAG), parabranchial nucleus (PB), dorsal (DR) and linear raphe nuclei (Cli), and nucleus solitary tract (NTS) (Dong et al., 2001). Retrogradely transported cholera-toxin B (CTB) injected in the rat BSTLD showed that it receives direct input from cell bodies located in the amygdala subdivisions: CeAM, CoAM, LBAM. Moreover, cells in the PVN, VMH, PAG, raphe nucleus, PB, NTS and dorsal vagal nucleus project directly to the BSTLD (Holstege et al., 1985; Eiden et al., 1985; Moga et al., 1989; Kozicz et al., 1998).

![Figure 2](image.png)

**Figure 2.** Schematic representation of the A) anterior and B) posterior portion of the rat BST (adapted from Paxinos and Watson, 1997; Ju et al., 1989; Moga et al., 1989). Abbreviations: ac = anterior commissure, BSTc = central subdivision of the stria terminalis, BSTL = lateral BST, BSTLD = dorsal lateral BST, BSTMA = anterior medial BST, BSTMP = medial posterior BST, BSTpr = principal nucleus of the BST, fx = fornix, ic = internal capsule, lv = lateral ventricle.

VIP containing fibers in the rat BSTLD originate from VIP-IR cell bodies located in the brainstem, because CTB and VIP double-labeled cell bodies were found only in the PAG, DR and Cli (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1998). Earlier studies showed that transsections of the amyidalofugal pathway, stria terminalis and medial forebrain bundle rostral to the DR resulted in dramatic decreases in VIP immunoreactivity in the BSTLD. These studies illustrate that the VIP-IR fibers in the human BSTc may not originate from the amygdala as proposed by Zhou et al., 1995, but ascend from the brainstem through the medial forebrain bundle, amyidalofugal pathway and stria terminalis (Fig. 3). Given that VIP-IR fibers cover a larger volume in the BSTc of adult men than in adult women, it is not unreasonably to hypothesize that the brainstem may contain sex differences in the number of VIP-IR cell bodies or VIP expression levels. This hypothesis can only be tested by directly

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examining in the human brainstem, specifically comparing the male and female PAG, DR and Cli. Circulating levels of gonadal steroid hormones during development can directly affect the organization of the human brainstem, since gonadal steroid receptors, such as ER $\alpha$ and PR may be present as suggested by reports in the rat brainstem (Alves et al., 1998). Moreover, sex differences have been found in the infant human median raphe nucleus, which contains more Golgi-Cox stained neurons in females than in males (Cordero et al., 2000).

![Figure 3. Schematic representation of VIP-IR fibers in the human BSTc thought to originate from the brainstem as inferred from rodent studies indicated by black arrows. Select BSTc afferents from the amygdala indicated by light gray broken arrows. Abbreviations: BSTc = central subdivision of the BST, CeAL = lateral central amygdala, CeAM = medial central amygdala, Cli = linear raphe nucleus, DR = dorsal raphe nucleus, PAG = periaqueductal gray, SI = substantia inominata, st = stria terminalis.]

VIP fibers terminating in the human BSTc may play a role in the regulation of stress responses. Indeed, VIP-IR fibers in the rat BSTLD synapse onto CRH cell bodies, which in turn project back to the dorsal vagal nucleus, PB and midbrain central gray, modulate the cardiovascular system during stress (Kozicz et al., 1997; Dong et al., 2001a). In addition, the rat BSTLD projects to the CRH component of the PVN, which is a major component of the hypothalamic-pituitary-adrenal (HPA) axis. The lack of data on whether VIP-IR fibers terminate on CRH containing cell bodies in the human BSTc, can be easily addressed in future studies using double-
immunocytochemistry for VIP and CRH and comparing males and females.

**7.3. PROTRACTED SEXUAL DIFFERENTIATION OF THE HUMAN BST**

The adult human BSTc is larger and contains more Nissl-stained neurons in men than in women (Zhou et al., 1995; Kruijver et al., 2000; Chung et al., 2002a). Moreover, the number of somatostatin-IR BSTc neurons is higher in men than in women (Kruijver et al., 2000). Studies in the rat brain showed that sexual differentiation of the BST is signaled and regulated by sex differences in circulating levels of testosterone during fetal and perinatal development (Del Abriu et al., 1987; Guillamon et al., 1988; Chung et al., 2000). Therefore, we expected the BSTc size to diverge in a sex-dependent fashion during a similar early period of life. Surprisingly, we found that the size of the BSTc, as defined by its VIP or somatostatin immunoreactivity, did not differ between males and females from the 25th week of pregnancy until 16 years of age, while the BSTc was larger in men than in women during adulthood (Chung et al., 2002).

Late divergence of BSTc volume between males and females may be a general characteristic of the human BST. The human darkly-staining posteromedial BST (BST-dspm) seems to become sexually dimorphic around puberty as suggested by the developmental time points in the study by Allen and Gorski (1990). Indeed, the BST-dspm appeared to be smaller in females than in males from around 14 years of age (Allen and Gorski, 1990). The number of cells in the sexually dimorphic vasopressin and oxytocin-containing nucleus in the pig hypothalamus even increases in (post)-adolescent females but not in males (Van Eerdenburg and Swaab, 1994). Recent studies also showed that several regions in the adult human and primate brain continuously produce new neurons and change in gray - and white matter volume (Eriksson et al., 1998; Gur et al., 1999; Sowell et al., 1999; Gould et al., 1999). Therefore, marked morphological changes in the human brain including sexual differentiation may not be limited to childhood but may extend into adulthood.

There are several possible explanations for the lack of a sex difference in the BSTc volume shortly after fetal or neonatal sex differences in testosterone levels emerge. Organizational effects of testosterone on sexual differentiation may become clear much later in life. An example of long delayed gonadal steroid hormones effects on brain organization is the development of the sexually dimorphic anteroventral periventricular nucleus (AVPv) in the rat brain, which is larger in females.
than in males. Although, perinatal sex differences in testosterone cause the adult sex difference in AVPv size, its volume becomes only significantly different around puberty (Davis et al., 1996). Alternatively, it is possible that sex differences in peripubertal or adult gonadal steroid levels establish sexual differentiation of the BSTc volume in adulthood. Indeed, androgens and estrogens in puberty cause the development of secondary sexual characteristics in peripheral body structures.

Further details about possible mechanisms involved in the development of sex differences in the human BSTc size were obtained by studying the distribution of steroid receptors for estrogens, androgens and progestins. As we found that gonadal steroid receptors are expressed from fetal development onwards. Sexual differentiation of the BSTc may be a resultant of combined effects of estrogens, androgens and progestin throughout life as suggested by the temporal presence of sex differences in gonadal steroid receptors in the fetal/neonatal, infant/pubertal and adult BSTc (see below).

7.3.7.3. a. Effects of Estrogens

Sex-dependent actions of testosterone or its estrogenic metabolite are mediated by their specific "gonadal" steroid receptors, which are part of a large family of nuclear steroid receptors (Evans, 1988; Robyr et al., 2000). Studies in the adult human brain including the BSTc clearly showed the presence of ER α, ER β, AR and PR-IR cells (Puy et al., 1995; Beyenburg et al., 2000; Österlund et al., 2000a, b; Fernandez-Guasti et al., 2000; Ishunina et al., 2000; Ishunina and Swaab, 2001; Kruijver and Swaab, 2002; Kruijver et al., 2002 accepted). We showed the presence of ER α and ER β-IR cells in the BSTc from 25 weeks of gestation onwards suggesting that gonadal steroid hormones can potentially exert direct effects on the sexual differentiation of the BSTc (chapter 3). Testosterone-derived estrogen effects may furthermore be sexually dimorphic, because more nuclear ER β containing cells were found in females than in males during fetal/neonatal ages (i.e., between 25 and 41 weeks of gestation).

Estrogens are envisaged to organize sexual differentiation of BSTc at multiple cellular levels during fetal/neonatal development. Studies using HeLa cells containing an AP-1 luciferase reporter gene showed that estrogens and anti-estrogens stimulate partial-to-full transcriptional activity with ER α, while estrogens repressed transcriptional activity with ER β (Paech et al., 1997; Weatherman and Scanlan,
As a consequence, the sex difference in the number of nuclear ER $\beta$-IR BSTc cells may indicate lower ER-related transcriptional activity in the female BSTc during fetal/neonatal ages.

A large proportion of ER $\beta$ immunoreactivity in the BSTc was localized in punctate terminal varicosities from fetal/neonatal ages onward, which during infant/pubertal (i.e., between 3 month and 16 years) and adult (i.e., between 22 years and 49 years) ages concentrated around BSTc cells bodies in a basket-like manner. Similar basket-like ER $\beta$ immunoreactivity was also detected in the adult male and female DBB and NBM of the human brain (unpublished observations W.C.J.C.).

Many studies clearly showed that estrogens induce rapid non-genomic changes, such as in membrane potential and synaptic spine density in rodents indicating direct actions of estrogens at the membrane site (Woolley and McEwen, 1993; Murphy and Segal, 1996; Pozzo Miller et al., 1999; Toran-Allerand et al., 1999; Kelly and Levin, 2001). These actions may be mediated by ER $\alpha$ and/or ER $\beta$ that are, in part, targeted to the cell surface as found in Chinese hamster ovary (CHO) - and pituitary cells (Razandi et al., 1999; Watson et al., 1999). Evidence showed that both membrane ER $\alpha$ and ER $\beta$ in CHO cells directly influence G-protein-induced intracellular signaling (Razandi et al., 1999). Membrane-targeted ER $\alpha$ and ER $\beta$ were also shown to have opposite effects on activation of intracellular signaling pathways. For instance, c-Jun kinase (i.e., a mitogen-activated kinase) activity in CHO cells was increased by ER $\beta$ activation, while ER $\alpha$ activation inhibited c-Jun kinase activity (Razandi et al., 1999). Similarly, estrogens in developing human BSTc cells interacting with ER $\alpha$ may also have opposite effects to estrogens interacting with ER $\beta$ on second messenger systems.

$\gamma$-aminobutyric acid (GABA), which during development influences neuronal survival, neurite outgrowth, synapse formation and membrane potential (Barbin et al., 1993; Ikeda et al., 1997; Obata, 1997) has been viewed as a possible candidate for the modulation of sex-dependent actions of gonadal steroid hormones during development (McCarthy et al., 2002). ER $\beta$ may directly regulate glutamate decarboxylase (GAD), the rate limiting enzyme of GABA synthesis, which is also localized in similar terminal varicosities arranged in a peribasket-like fashion around BST cell bodies (Sun and Cassell, 1993; Heimer et al., 2000). In rats, GABA concentration and GAD mRNA in the hypothalamus are responsive to estrogens and are higher expressed in neonatal males than in females (McCarthy et al., 1995;
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Sagrillo and Selmanoff, 1997; Davis et al., 1999). Activation of the GABA_A receptor during early rat hypothalamic development resulted in sex-dependent responses in the phosphorylation of cAMP response element binding protein (CREB) (Auger et al., 2001). Modulation of these proteins affect critical developmental processes involved in sexual differentiation of the vertebrate brain, such as neuronal survival and synaptic plasticity (Shaywitz and Greenberg, 1999). Sexual differentiation of the BST in rats is dependent on a sex difference in apoptotic cell death during early postnatal development, which is regulated by circulating levels of testosterone presumably mediated by its estrogenic metabolite (Arai et al., 1996; Chung et al., 2000; see also section 7.4). Together, our results suggest that fetal/neonatal sex-dependent organization of the BSTc may be an interplay between sex differences in ER α/ER β mediated transcriptional activity at the level on the cell nucleus and ERs mediated “rapid” effects at extra-nuclear sites, such as cell cytoplasm and/or cell membrane.

7.3.b. Effects of Androgens

Testosterone may also influence sexual differentiation of the BSTc by acting on ARs. In humans, sexual differentiation seems to depend more on androgens than on estrogens (Robbins et al., 1996). For example, men with complete androgen insensitivity syndrome (AIS) have a female body - and psychosexual phenotype, while men with a non-functional ER α or defective aromatase enzyme remain phenotypically and behaviorally male (Smith et al., 1994; Morishima et al., 1995; Quigley et al., 1995; Bilezikian et al., 1998; Carani et al., 1999; Rochira et al., 2001). Studies in rodents also show direct involvement of androgens during sexual differentiation, in for example the rat spinal nucleus of the bulbocavernosus (SNB) (Forger et al., 1992). Both the male and female BSTc during fetal/neonatal and infant/pubertal development contained many AR-IR cells. Our results do not support an androgenic role during fetal/neonatal ages in the BSTc, since there were no overt sex differences in AR expression during this developmental period. Androgenic actions on the developing BSTc during fetal/neonatal ages, however, can not be ruled out, because testosterone levels are inherently higher in males than in females (Abramovich and Rowe, 1973; Griffin and Wilson, 1980).

Testosterone or its androgenic metabolite may differentiate the BSTc in a sex-dependent fashion during infant/pubertal development, because more AR-IR BSTc
cells were detected in males than in females. Studies in rodents also showed that AR expression in the perinatal - and adult BST is higher in males than in females (Wersinger et al., 1998; McAbee and DonCarlos, 1998; Wood and Newman, 1999). The sex difference in AR expression in the human BSTc is likely controlled by the estrogenic and androgenic metabolites of testosterone. Both estradiol and DHT treatment increased AR expression in the adult and neonatal rodent preoptic and hypothalamic areas (Wood and Newman, 1999; McAbee and DonCarlos, 1999). In adult humans, AR-IR in the mamillary bodies in the hypothalamus also depends on endocrine status (Kruijver et al., 2001). Although the consequences of an AR sex difference during infant/pubertal ages are unclear, given that earlier studies showed that the BSTc size does not differ between males and females during infant/pubertal ages (Chung et al., 2002), we hypothesize that increases in androgens at puberty in males may be required for the maintenance and/or further differentiation of the BSTc in a sex-dependent fashion in this period of life. This idea seems to be supported by data showing that the male BSTc size stabilizes, while the female BSTc size decreases around late puberty and early adulthood (Chung et al., 2002).

7.3.c. Effects of Progestins

In many cases sex differences in testosterone or its estrogenic metabolite levels during fetal and newborn development are prerequisite for sex-dependent anatomical and neurochemical organization of the vertebrate brain (McEwen et al., 1977; MacLusky and Naftolin, 1981; MacLusky et al., 1985; Sasano et al., 1998; Holloway and Clayton, 2001). Additional layers of complexity have been identified, because recent research showed that sex-dependent actions of testosterone or its metabolites may also be modulated by circulating levels of progestins during perinatal ages (Wagner et al., 1998; Quadros et al., 2002).

Progestins may modulate sexual differentiation of vertebrate brain (Peppe et al., 1972; Sanyal et al., 1978; Weisz and Ward, 1980; Wagner et al., 1998). Maternal and fetal progestin levels increase during pregnancy followed by a dramatic decrease during the end-stages of pregnancy. Progestin treatment during pregnancy may affect a number of sexually dimorphic behaviors, such as physical aggression in the offspring (Ehrhardt et al., 1977; Reinisch, 1981), while elevated maternal progesterone levels, to which the fetal brain is exposed to during pregnancy have been correlated to changes in male and female sexual behavior in rats (Hull, 1981; Wagner et al.,
1986; Quadros et al., 2002). Studies in rats during development showed the presence of PR containing cells in several preoptic and hypothalamic regions, such as the BST indicating that progestins may directly affect sexual differentiation of the vertebrate brain (Wagner et al., 1998). Progestins may also influence sexual differentiation of the human brain as suggested by the presence of PR containing cells during fetal/neonatal, infant/pubertal and adult ages in the BSTc. Moreover, the number of PR-IR cells in the adult BSTc is higher in men than in women. Therefore, it is can be speculated that progestins may potentially exert sex-dependent effects on the adult human BSTc.

7.3.d. Further Layers of Complexity

The understanding of molecular and cellular pathways by which estrogens act on sexual differentiation is further complicated by the existence of ER α and ER β splice variants, which are expressed in a region-specific and age-dependent fashion in vertebrates (e.g., Österlund et al., 2000c; Price et al., 2001). Studies showed the existence of a smaller AR-A (about 87 kDa), in addition to the full length human AR-B (about 110 kDa) (Wilson and MacPhaul, 1994). However, in contrast to the PR-A and PR-B isoforms, in which the truncated PR-A acts as a dominant inhibitor of the full length PR-B, no functional relationships have been described for the AR isoforms (Vegeto et al., 1993; Hiort and Holterhus, 2000). The AR gene furthermore contains a variable stretch of CAGₙ(CAA) triplets in exon 1, which encode for a variable number of glutamine residues in the transactivation domain of the N-terminal portion of the AR protein (Lubahn et al., 1988; La Spada et al., 1991; Simental et al., 1992). The number of CAGₙ(CAA) repeats in humans ranges normally between 6-to-33 repeats (Brinkmann, 2001). Interestingly, the number of CAGₙ(CAA) repeats is highly expanded (38-to-75) in Kennedy’s disease, which is a X-linked neurodegeneration disorder characterized by a selective loss of motoneurons in the spinal cord and atrophy of the bulbar muscles (La Spada et al., 1991; Kennedy et al., 1968). Similar TA and CA repeat polymorphisms have been detected in the promotor region of the ER α (Del Senno et al., 1992) and non-coding 3' portion of the ER β gene (Tsukamoto et al., 1998), respectively. Recent studies further showed that the levels of circulating androgens are correlated with the number of CAGₙ(CAA) repeats in AR and CA repeats in ER β (Krithivas et al., 1999; Westberg et al., 2001). However, the implications of gonadal steroid receptor splice variants and poly-
morphism on the sexual differentiation of the vertebrate brain remain unclear.

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). 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 uterine 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., 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).

7.3.e. Transsexuality and Late Sexual Differentiation

The BSTc size has been related to a gender disorder called transsexuality, in which subjects feel that they were born in the wrong sex (Zhou et al., 1995; Kruijver et al., 2000). Late sexual differentiation of the human BSTc affects our perception of the relationship between BSTc volume and transsexuality. Although, transsexuals receive their first consultation between the ages of 20 and 45 years, which coincided with the period of sex-dependent divergence of BSTc volume found in our studies (Chapter 2; Chung et al., 2002), epidemiological studies show that the awareness of gender problems is generally present much earlier. Indeed, about 67% to 78% of transsexuals in adulthood report to have strong feelings of being born in the wrong body from childhood onwards (Van Kesteren et al., 1996) during which the BSTc size did not differ between males and females (Chung et al., 2002). Phenobarbital or diphantoin usage during pregnancy, which affect gonadal steroid hormone levels, increased prevalence of transsexuality in the offspring (Dessens et al., 1999). Also
girls who had been exposed to high androgen levels as infants caused by congenital adrenal hyperplasia show an increased incidence of gender problems, which supports early developmental programming of this disorder (Meyer-Mahlburg et al., 1996; Zucker et al., 1996). These results suggest that disturbances in circulating levels of gonadal steroid hormones during fetal/neonatal and/or infant development may underlie the development of transsexuality.

This idea is supported by the presence of gonadal steroid receptor containing cells in the human BSTc during fetal/neonatal and infant/pubertal development (chapter 3). Consequently, disturbances in gonadal steroid hormones levels may have resulted in a female-sized BSTc in male-to-female transsexuals or in a male-sized BSTc in one female-to-male BSTc studied so far during adulthood (Zhou et al., 1995; Kruijver et al., 2000). Although the size of the human BSTc was correlated with transsexuality, inherent effects of gonadal steroid hormones during early development may not be measurable in terms of volumetric changes. For instance, gonadal steroid hormones may affect transcriptional activity, resulting in alterations in mRNA and protein expression. Alternatively, occupied gonadal steroid receptors located in the cytoplasm or cell membrane may also act on second messengers systems. Both of which may not be reflected directly in overt BSTc size changes.

To better understand how gonadal steroid hormones and their respective steroid receptors dynamically affect the anatomical organization and functions of the BST in the human brain on a cellular and molecular level, additional research techniques should be used. In particular, we must use our ability to culture postmortem human brain tissue to our full advantage (Verwer et al., 2002). For example, microarray techniques could be used to examine gene expression changes in postmortem human brain tissue from the BST cultured in the presence or absence of gonadal steroid hormones. Similar approaches have already yielded several interesting sex-dependent and gonadal steroid hormone-responsive genes, such as in the neonatal rat hypothalamus (Yonehara et al., 2002), hypothalamus of food-deprived rats (Li et al., 2002) and human carcinoma breast tissue (Bouras et al., 2002). These studies should be following-up by functional proteomic studies, which examine changes in protein levels.
7.4. APOPTOSIS AND SEXUAL DIFFERENTIATION

Gonadal steroid hormones acting through their specific receptors regulate sexual differentiation of the mammalian brain by affecting one or more of four major developmental processes: neurogenesis, neuronal migration, apoptosis and/or differentiation of cell phenotype. To date no studies have found evidence for the direct involvement of gonadal steroid hormone-dependent neurogenesis or neuronal migration in the sexually dimorphic organization of the BST anatomy (Henderson et al., 1999). Earlier studies investigating sexual differentiation of the rat SDN-POA also found no evidence indicating that neurogenesis or neuronal migration differed between males and females during perinatal development (Jacobson and Gorski, 1981; Jacobson et al., 1985). Far more evidence confirms that gonadal steroid hormones regulate sexual differentiation through apoptosis and/or differentiation of cell phenotype. For instance, the incidence of apoptosis in the rat SNB, SDN-POA, bed nucleus of the olfactory tract was higher in females than in males (e.g., Nordeen et al., 1985; Davis et al., 1996; Collado et al., 1998), while the incidence of the apoptosis in the AVPv was higher in males than in females (Arai et al., 1994). The ferret male nucleus of the preoptic area and the rat medial amygdaloid nucleus are examples of differentiation of cell phenotype (Cherry et al., 1992; Cooke et al., 1999).

7.4.a. Apoptosis Sex Difference in the BST

Apoptosis is an important developmental mechanism during fetal/newborn human brain development (e.g., Chan and Yew, 1998; Simonati et al., 1999; Rakic and Zecevic, 2000; Itoh et al., 2001). In agreement with these studies, apoptotic cells as visualized by Nissl-staining and an antibody against the activated form of the pro-apoptotic factor, caspase-3 were detected in the human BSTc during perinatal ages (unpublished observations W.C.J.C.; Srinivasan et al., 1998; Stadelman et al., 2000). These initial studies and results from the rodent brain suggest that apoptosis may be an important developmental mechanism involved in the organization of the human BST. However, more extensive and quantitative studies are required to test the idea whether gonadal steroid hormones differentiate the human BST in a sex-dependent fashion through apoptosis in the human brain.

The importance of apoptosis during sexual differentiation of the BST was recognized and supported by results from earlier studies. The most prominent cytoarchitectural sex difference in the rat BST is the principal nucleus of the BST (BSTpr),
which is larger and contains more cells in males than in females (Del Abril et al., 1987; Guillamon et al., 1988; Hines et al., 1992; Chung et al., 2000). Sexual differentiation of the BSTpr is controlled by early circulating levels of testosterone (Del Abril et al., 1987; Guillamon et al., 1988; Chung et al., 2000). Our studies clearly showed that the incidence of apoptosis in the BSTpr during the first two postnatal (PN) weeks of development was higher in females than in males. Moreover, the size of the BSTpr became larger in males than in females only after the sex difference in apoptosis (Chung et al., 2000). In addition, the incidence of apoptosis was higher in animals devoid of testosterone (i.e., castrated male pups and intact female pups) than in animals with testosterone (i.e., castrated male pups and intact female pups injected with testosterone) (Chung et al., 2000). These results strongly suggest that sex differences in developmental apoptosis are prerequisite for the gonadal steroid hormone-dependent sexual differentiation of the BSTpr in the rat brain.

However, these mechanisms may not necessary underlie to the sexual differentiation of the human BSTc, given that the rat BSTLD is the best homology candidate for the human BSTc and not the BSTpr (section 7.2.). A small overall sex difference in the incidence of apoptosis favoring females was found in the neonatal rat BSTL, in which the BSTLD is embedded. However, this small sex difference may not have been sufficient to cause a sex-dependent organization of the BSTL (Chung et al., 2000). Further studies in the rat BSTLD are required in order to be able make any statements about the role of apoptosis in the human BSTc. For example, it is unknown whether the size of the BSTLD in rat brain differs between males and females as delineated by VIP-IR fibers. In addition, the temporal incidence of apoptosis should be studied in the rat BSTLD as delineated by VIP-IR fibers.

VIP-IR fibers were used to delineate the BSTLD and CeAL in mice, in which the proapoptotic Bax gene was deleted. We showed that the deletion of Bax in mice resulted in an increase of the BSTLD and CeAL size, when compared to wild type mice. Bax knockout mice may not be an appropriate model, however, as neither BSTLD nor CeAL size differed between males and females. These mice are therefore unsuitable for studying the development of sex differences in the BST, that have been found humans and rats. The absence of a sex difference as detected in the human brain may be due to strain - and species differences. For instance, the SDN-POA was not be detected in the mouse preoptic area (Brown et al., 1999). However, the fact remains that attenuation of developmental apoptosis in Bax knock-out mice,
may underlie overt sex-dependent changes in BST and amygdala size.

7.4.b. Apoptosis Sex Difference in the SDN-POA

Apoptosis is an important underlying the differential effects of gonadal steroid hormones on the vertebrate brain. In addition to the BSTpr, the incidence of apoptosis in the rat SNB, SDN-POA, bed nucleus of the olfactory tract was higher in females than in males (e.g., Nordeen et al., 1985; Davis et al., 1996; Collado et al., 1998), while the incidence of the apoptosis in the AVPV was higher in males than in females (Arai et al., 1994). In our studies, we confirmed that the incidence of apoptosis in the early postnatal rat SDN-POA was higher in females than in males on PN 8 (Arai et al., 1996; Davis et al., 1996; Chung et al., 2000). Although, testosterone treatment in male and female pups eliminated the sex difference in the incidence of apoptosis on PN 8, a small peak of apoptosis was observed around PN 10. These SDN-POA results seem to be inconsistent with earlier findings that showed that testosterone completely inhibited developmental apoptosis in the SDN-POA (Davis et al., 1996). However, this disparity may well be explained by differences in the timing of testosterone treatment. In earlier studies, pups received testosterone several days after birth (Davis et al., 1996), whereas in our studies testosterone was given on the day of birth, which may have resulted in differences in testosterone levels during the SDN-POA cell death period (Chung et al., 2000). Differences in the pattern of ontogenesis between BSTpr and SDN-POA cells may be responsible for the difference in effectiveness of testosterone in curtailing the incidence of apoptosis in these two areas. Counting the day of conception as embryonic day 1, BSTpr cells are born around embryonic day 16 and 17, whereas SDN-POA cells are born one to two days later (Jacobson et al., 1981b; Bayer, 1987; Bayer and Altman, 1987). Hence, perinatal BSTpr cells may well be more advanced in their development than MPNc cells during the same perinatal period. An indication for difference in developmental maturity may be the level of androgen receptor (AR) mRNA expression, which appears to develop more rapidly in the BSTpr than in the MPN (McAbee and DonCarlos, 1998), supporting the idea that neonatal testosterone administration may protect the more mature BSTpr cells better against postnatal apoptosis than the more immature SDN-POA cells.
7.4.c. Gonadal Steroid Hormone Regulation of Apoptosis

Although testosterone was shown to be effective in protecting BST and MPN cells against apoptosis; this effect is facilitated by its estrogenic metabolite acting on ERs (Arai et al., 1996). Estrogen-bound ERs form homodimers or heterodimers and translocate to the cell nucleus to associate with specific estrogen response elements (EREs) on DNA (Pettersson et al., 1997; Donaghue et al., 1999), which are palindromic enhancer sequences located in promoter regions to modulate the transcriptional activity of genes involved during apoptosis. For example, members of the Bcl-2 gene family, including Bcl-2 and Bcl-X<sub>L</sub> have the putative EREs in their promoter regions supporting the idea that estrogens may modulate directly the transcriptional activity of genes involved apoptosis (Dong et al., 1999; Pike, 1999). Indeed, estrogen increased Bcl-2 and Bcl-X<sub>L</sub> expression in neuronal cell lines (Gollalpudi and Oblinger, 1999; Pike, 1999) and in the arcuate nucleus of the adult rat brain (Garcia-Segura et al., 1998), while decreasing the expression of Bad mRNA, which is a proapoptotic Bcl-2 family member (Pike, 1999). Estrogens also decrease the expression of cellular factors, such as Bnip-2 mRNA which in turn down-regulate Bcl-2 expression (Belcreditto et al., 2001). Conversely, estrogen removal increased mRNA expression of two proteolytic so-called initiator Caspases (i.e., 1 and 2) in chick oviduct studies. While at the same time activating the executioner proenzymes, caspase-3 and caspase-6 (Monroe et al., 2002). Together these studies suggest that estrogen-bound ERs, in part, oppose apoptosis through genomic actions.

Gonadal steroid hormones may regulate cell survival by acting on the transcription level of neurotrophic factors. Indeed, the gene encoding for brain-derived neurotrophic factor (BDNF) contains a putative ERE. Moreover, estrogen increased mRNA levels of BDNF in the rat cerebral cortex and olfactory bulb (Sohrabji et al., 1995). Similarly, androgens rescue motoneurons in the spinal nucleus of the bulbocavernous androgens is facilitated by ciliary neurotrophic factor (CNTF) expressed in the perineal muscles, which act on CNTF α receptors located on the motoneurons (Forger et al., 1998; Xu et al., 2001). These studies indicate that gonadal steroid hormone regulate cell survival not only directly, by targeting the expression of specific components of the apoptotic cell death mechanisms, but also indirectly by modulating the expression of neurotrophic factors.

Recent in vitro studies strongly suggest that gonadal steroid hormones can also regulate apoptosis through non-genomic pathways. In particular, estrogen-bound
ERs interact directly with phosphatidylinositol-3 kinase (PI-3K) through protein-protein binding, which in turn phosphorylates the downstream effector Akt to rescue cortical neurons (Tommaso et al., 2000; Honda et al., 2000; 2001). Estrogen-dependent rescue through the PI-3K/Akt pathway was prevented by ICI 182,780, a selective ER antagonist or LY 294002, a selective PI-3K inhibitor (Honda et al., 2001). Estrogen activation of PI-3K/Akt pathway rapidly (i.e., within 15 minutes) resulted in the accumulation of serine\(^{133}\) phosphorylated CREB, which promoted the observed increase in the expression of the anti-apoptotic factor Bcl-2 (Du et al., 1998; Pugazhenthhi et al., 2000; Honda et al., 2001; Campbell et al., 2001) as shown in cultured sympathetic neurons (Riccio et al., 1999).

*In vitro* also showed that PI-3K/Akt phosphorylation of the AR in similar prostate cancer cells may inhibit apoptosis (Sharma et al., 2002). In addition, PI-3K/Akt was shown to increase AR mRNA expression (Manin et al., 2002). Although, there are no similar data available for the vertebrate brain, these studies suggest that apoptosis-dependent sexual differentiation of the vertebrate brain possibly mediated by PI-3K/Akt may also involve ARs.

Recent studies showed that the number of PR-IR cells in the perinatal rat SDN-POA is much higher in males than in females (Wagner et al., 1998). *In vitro* studies showed that ligand-activated PRs protected endometrial cells derived from the uterus against apoptosis, which was negated by treatment with PR antagonists: ZK 98,299 (onapristone) or RU 486 (mifepristone) (Pecci et al., 1997). Furthermore, PRs have been shown to induce overexpression of Bcl-xL, which is an apoptosis inhibiting factor (Pecci et al., 1997; Tsujimoto and Shimizu, 2000). Therefore, we hypothesized that estrogens may protect male SDN-POA cells by increasing PR expression during early development. This was tested by blocking PRs in male and female rat pups with PR antagonists: ZK 98,299 or RU 486 from postnatal day (PN) 1 (is day of birth) until PN 7 (*chapter 6*). In agreement with earlier studies, the incidence of apoptosis is higher in vehicle-treated females than in vehicle-treated males, which was also reflected by the smaller SDN-POA volume found in females as compared to males (Arai et al., 1996; Davis et al., 1996; Chung et al., 2000). However, statistical analysis showed no significant effects of PR antagonist on the incidence of apoptosis in the SDN-POA (*chapter six*). These results seem to be in agreement with a recent study investigating neuroprotection pathways in cortical explants showing that progestins elicited phosphorylation of Akt, which could not be inhi-
bited by RU 486 (Singh, 2001). Therefore, indicating that progestins in brain cells may not act directly through PRs to facilitate/modulate cell survival in the developing vertebrate brain.

Gonadal steroid hormone effects on apoptosis, i.e., cell survival during sexual differentiation are as described above many fold. A good strategy to capture and elucidate which gonadal steroid hormone-dependent apoptosis regulating mechanisms are important for the development of the various sexually dimorphic brain regions is to apply microarrays, which are specifically focused on genes involved in apoptosis. For instance, in a first study this approach can be used to compare gonadal steroid hormone effects on the incidence of apoptosis during the sexual differentiation of the BST (i.e., larger in males than in females) with that of the AVPv (i.e., larger in females than in males). Coincidently, changes in apoptosis-related protein expression should be examined using proteomic techniques in order to visualize “network-like” changes in protein interactions upon the presence or absence of gonadal steroid hormones.

7.4.d. Sex Chromosomal Influences

Although gonadal steroid hormones have been shown to be able to affect the sexual differentiation of the vertebrate brain in dramatic fashion, recent studies showed that the inherent sex difference in sex chromosome configuration may also affect the sexual differentiation process in the developing vertebrate brain. For instance, in vitro studies showed that sex differences of cultured rodent dopaminergic neurons in soma size and dopamine uptake were dependent only on genetic sex (Reisert et al., 1989; Kolbinger et al., 1991; Sibu et al., 1996). Several sex chromosomal genes in rodents are expressed in a sex-dependent fashion (Xu et al., 2001). Similarly, in human brain the sex chromosomal genes, ZRY and SRY are continuously expressed during life in men only (Mayer et al., 2000). Although, these fundamental differences gene expression exist between males and females it remains to be seen, whether they have overt and far-reaching effects on the sexual dimorphic organization of the rodent and human brain.