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Sexual Differentiation of the Bed Nucleus of the Stria Terminalis in Humans May Extend into Adulthood

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Gonadal steroids have remarkable developmental effects on sex-dependent brain organization and behavior in animals. Presumably, fetal or neonatal gonadal steroids are also responsible for sexual differentiation of the human brain. A limbic structure of special interest in this regard is the sexually dimorphic central subdivision of the bed nucleus of the stria terminalis (BSTc), because its size has been related to the gender identity disorder transsexuality. To determine at what age the BSTc becomes sexually dimorphic, the BSTc volume in males and females was studied from midgestation into adulthood. Using vasoactive intestinal polypeptide and somatostatin immunocytochemical staining as markers, we found that the BSTc was larger and contains more neurons in men than in women. However, this difference became significant only in adulthood, showing that sexual differentiation of the human brain may extend into the adulthood. The unexpectedly late sexual differentiation of the BSTc is discussed in relation to sex differences in developmental, adolescent, and adult gonadal steroid levels.

Key words: bed nucleus of stria terminalis; sexual differentiation; plasticity; brain; adulthood; human

Several regions in the human brain differ in organization between men and women. For example, distinct cell groups in the preoptic and anterior hypothalamic area are larger, and the suprachiasmatic nucleus contains more vasoactive intestinal polypeptide-immunoreactive (VIP-IR) cells in young men than in young women (Swaab and Fliers, 1985; Allen et al., 1989; LeVay, 1991; Swaab et al., 1994; Byne et al., 2000). Clear anatomical sex differences have also been described in the human bed nucleus of the stria terminalis (BST). The darkly staining posteromedial component of the BST (BST-dspm) and the central subdivision of the BST (BSTc) are both larger in men than in women (Allen and Gorski, 1990; Zhou et al., 1995; Kruijver et al., 2000). Differences in the size of the human BSTc have been related to the gender identity disorder transsexuality, in which subjects voice the strong feeling of being born in the wrong sex. In male-to-female transsexuals, the BSTc was similar in size to that of control women, whereas in the only female-to-male transsexual studied so far, the BSTc was similar in size to that of control men (Swaab and Hofman, 1995; Zhou et al., 1995; Kruijver et al., 2000)

In general, perinatal sex differences in gonadal steroid levels are responsible for organizing the vertebrate brain in a sex-dependent manner (Döhler, 1991; Cooke et al., 1998). Studies showed that this was also the case for the sexual differentiation of the BST in the rat brain. For instance, perinatal sex differences in testosterone are required for the principal nucleus of the BST in the rat brain to become larger and contain more cells in males than in females (Del Abril et al., 1987; Guillamon et al., 1988; Chung et al., 2000). Moreover, these sex-dependent morphological changes occur within the first week of postnatal life (Chung et al., 2000). Therefore, gonadal steroids are presumed to play a role in the sexual differentiation of the human BSTc, which was predicted to be apparent early on during fetal or infant development. This idea is further supported by observations in humans, which indicate that dramatic changes in circulating gonadal steroid levels do not seem to alter the size of the BSTc in adult control subjects. For instance, high testosterone and androstenedione levels caused by an adrenal cortex tumor in a female control subject did not result in a larger BSTc, whereas gonadectomy in male control subjects with prostate cancer did not result in a smaller BSTc (Zhou et al., 1995; Kruijver et al., 2000).

In the present study, we used postmortem human brain tissue to determine at what stage of development the volume of the human BSTc diverges between men and women. Moreover, we assessed whether sex differences in total number of Nissl-stained BSTc neurons contributed to the sexual differentiation of the BSTc size.

MATERIALS AND METHODS

Human brain tissue. Brains of 50 control subjects (Table 1) were obtained through autopsies by the Netherlands Brain Bank following the required permissions for brain autopsy and use of tissue and medical information for research purposes. Brain tissue was fixed in formalin and embedded in paraffin. Serial coronal sections (6 μm) were made using a Leitz (Wetzlar, Germany) microtome and mounted on aminoalkylsilane-coated glass slides. Paraffin-embedded sections were processed for immunocytochemistry as described in previous studies (Zhou et al., 1995; Kruijver et al., 2000).

Immunocytochemistry. After deparaffinization and rehydration using xylene and decreasing grades of ethanol, sections were placed in 0.05 M citrate buffer, pH 4.0, microwave treated (for antigen retrieval) at 90°C for 10 min, cooled at room temperature (RT) for 30 min, rinsed three times for 5 min each in 0.05 M Tris-buffered saline (TBS), pH 7.6, and placed into 0.3% Triton X-100 (Sigma, St. Louis, MO) and 5% milk powder (Elk; Campina Melkunie, Eindhoven, The Netherlands) diluted with TBS (TBS-XM) for 30 min to reduce nonspecific staining. Sections were then incubated overnight at 4°C with rabbit anti-VIP polyclonal (VIPER; 1:600) or rabbit anti-somatostatin polyclonal (SOMAAR;

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Table 1. Control human brain material

	NBB number	Sex	Age	Brain weight (gm)	Postmortem delay (hr)	Fixation (d)	VIP (mm^3)	SOM (mm^3)	Clinicopathological diagnosis
Males (n = 25)	Fetal/neonatal period								
	81024	m	26 4/7 weeks	110	1:52	51	0.216	0.376	Pulmonary failure
	84032	m	27 weeks	142	21:45	45	0.241	0.462	Cardiac failure
	97154	m	33 6/7 weeks	186	7:55	32	0.729	1.136	Respiratory insufficiency
	82002	m	35 1/7 weeks	322	<41:00	32	0.766	1.088	Pericardial rupture
	94054	m	36 2/7 weeks	357	<24:00	118	0.703	1.238	Pulmonalis atresia, cardiac insufficiency
	90095	m	38 1/7 weeks	320	14:05	26	0.624	1.018	Respiratory insufficiency
	88120	m	40 weeks	440	<17:00	31	1.284	2.125	Asphyxia
	Infant/pubertal period								
	88053	m	3 months	485	<41:00	35	1.445	2.520	Pulmonal insufficiency, Fallot's tetralogy
	85036	m	3 months	635	<17:00	73	1.583	2.582	Cardiac failure, aortic stenosis, cerebral ischemia
	84019	m	3 months	710	<11:00	792	1.034	2.318	Sudden infant death syndrome
	86041	m	6 months	800	<6:30	14	1.515	2.663	Sudden infant death syndrome
	88092	m	1 year	920	<41:00	31	2.587	4.536	Penthotal intoxication, hypoxia
	88058	m	1 year	1070	<35:35	28	2.597	3.843	Bacterial meningitis, sepsis
	84016	m	5 years	1565	23:55	100	2.083	3.074	Sepsis
	87057	m	6 years	1550	3:30	41	3.836	5.637	Peritonitis
	98116	m	8 years	nd	<17:10	103	2.826	5.595	Cardiomyopathy
	87036	m	14 years	1640	<41:00	32	4.968	8.059	Lymphadenopathy
	Adult period								
	97083	m	22 years	1334	<16:29	26	4.067	5.897	Cardiomyopathy
	97173	m	24 years	1364	<33:30	31	3.969	6.708	Accidental death
	86042	m	28 years	1450	<17:00	46	3.618	5.254	Guillain-Barré syndrome
	96406	m	35 years	1430	13:30	1214	3.288	6.598	Pulmonary aspergillosis
	99071	m	39 years	1400	<16:30	130	3.362	6.919	Cardiac failure
	88011	m	41 years	1500	20:30	33	4.464	6.745	Suicide
	92011	m	47 years	1500	<89:00	77	4.258	4.915	Sepsis
	97159	m	48 years	1500	5:30	42	3.757	5.948	Diabetes mellitus type I, euthanasia
Females (n = 25)	Fetal/neonatal period								
	89056	f	25 6/7 weeks	100	<65:00	31	0.362	0.579	Idiopathic (infant) respiratory distress syndrome
	96401	f	27 2/7 weeks	77	<41:00	1301	0.217	0.460	Congenital infection
	98193	f	30 6/7 weeks	116	3:00	82	0.579	1.023	Sepsis
	86030	f	31 2/7 weeks	200	<41:00	33	1.011	1.545	Hypoxia
	87024	f	34 5/7 weeks	180	<3:15	38	0.368	0.770	Idiopathic (infant) respiratory distress syndrome
	96403	f	34 4/7 weeks	197	<57:19	1288	0.385	0.951	Developmental syndrome

Table 1. Continued

NBB number	Sex	Age	Brain weight (gm)	Postmortem delay (hr)	Fixation (d)	VIP (mm^3)	SOM (mm^3)	Clinicopathological diagnosis
88123	f	37 6/7 weeks	350	<41:00	30	1.067	2.544	Cardiac failure
89092	f	40 1/7 weeks	380	30:45	28	0.696	1.066	Asphyxia
88077	f	40 2/7 weeks	350	<65:00	56	0.782	1.378	Asphyxia
87028	f	41 4/7 weeks	350	<41:00	33	0.880	2.444	Aspiration of maternal blood
Infant/pubertal period								
86027	f	5 months	735	10:00	40	1.028	1.890	Sudden infant death syndrome
89027	f	6 months	780	<17:00	28	1.033	2.890	Cardiomyopathy
89036	f	1 year	820	nd	31	2.366	3.956	Hypoglycaemia
85031	f	2 years	nd	<65:00	48	1.028	4.699	Kidney dysplasia, sepsis
87077	f	7 years	1320	<9:45	33	1.033	5.287	Astrocytoma
87035	f	13 years	1250	<13:00	48	2.366	7.243	Histiocystic lymphoma, cardiac failure
99060	f	16 years	1364	<43:00	238	3.251	6.662	Diabetes mellitus, acidosis
Adult period								
85041	f	28 years	nd	5:25	44	3.218	6.384	Cardiac failure
85027	f	29 years	1150	13:10	60	4.318	3.131	Coma, liver cirrhosis
92037	f	32 years	1280	30:00	45	3.518	3.334	Bronchitis, pneumonia
86032	f	33 years	1035	<41:00	20	2.411	4.179	Adenocarcinoma
84002	f	36 years	1420	85:40	51	1.893	3.688	Suicide, multiple fractures, aortic rupture
97131	f	43 years	1345	<92:00	63	2.293	3.647	Cardiac failure, liver cirrhosis
89104	f	49 years	1260	<41:00	32	3.877	6.512	Lung carcinoma, septic shock
96423	f	49 years	1253	<17:00	806	3.183	5.316	Adenocarcinoma, thromboembolism

M, Male; F, female; nd, not determined; NBB, Netherlands Brain Bank; SOM, somatostatin.

1:800) diluted in TBS-XM. Afterward, sections were rinsed one time for 5 min in TBS-XM and two times for 5 min each in TBS and incubated with biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) diluted in TBS-XM for 60 min at RT. Sections were rinsed three times for 5 min each in TBS and incubated with ABC Elite kit (1:600; Vector Laboratories) diluted in TBS for 60 min and, after an additional three rinses for 5 min each in TBS, reacted with 0.25% nickel–ammonium sulfate-enhanced 3,3′ diaminobenzidine tetrahydrochloride (0.5 mg/ml) and 0.01% H_2O_2 in TBS. The reaction was stopped after 10–20 min, dehydrated with increasing grades of ethanol, cleared with xylene, and coverslipped using entellan (Merck, Darmstadt, Germany).

Volume measurements. The volume of the human BSTc in control subjects (males, $n = 25$; females, $n = 25$) was assessed both by using VIP and somatostatin immunocytochemical staining as markers (Walter et al., 1991; Zhou et al., 1995; Kruijver et al., 2000). Both markers have been shown previously to delineate clearly the borders of the BSTc in adult males and females (Zhou et al., 1995; Kruijver et al., 2000). The present study showed that the same markers delineate the BSTc from 25 weeks of pregnancy onward (Table 1). The volume of the BSTc was estimated by measuring the cross-sectional area delineated by VIP or somatostatin immunoreactivity in approximately every 25th section (fetal/neonatal and infant/pubertal subjects) or every 50th section (adult subjects) using a 2.5× objective (Plan-Neofluar) on a Zeiss (Oberkochen, Germany) Axioskop microscope mounted with a Sony (Tokyo, Japan) B/W CCD camera (model XC77CE) connected to an IBAS imaging analysis system (Kontron Elektronik, Eching, Germany). The total volume of the BSTc was calculated according to the Cavalieri principle (Gundersen et al., 1988).

Estimation of neuronal density and total neuronal number. Somatostatin-stained sections of the BSTc from adult males and females (between 22 and 49 years) (Table 2) were counterstained with Nissl staining to reveal all cells and to estimate total neuronal number in the adult BSTc. Cross-sectional digital images (every 50th to 100th section) were made using a 2.5× objective (Plan-Neofluar) on a Zeiss Axioskop microscope, mounted with a Sony B/W CCD camera (model XC77CE), that was connected to an IBAS imaging analysis system (Kontron Elektronik). The somatostatin-stained BSTc was outlined at 2.5× magnification; subsequently, the imaging analysis system overlaid a grid of rectangular fields within the outlined cross-sectional area. Each field was equal in size to the area displayed by the camera at 63× objective (Plan-Apochromat). For analysis, 25% of the rectangular fields (each field covering at least 10% of the outlined area) was selected by a random systematic sampling procedure. To prevent double counting, only neurons containing a nucleolus (~2 μm diameter) were counted. This counting procedure is first based on the assumption that the cell nucleus only contains one nucleolus. No multi-nucleolated nuclei were observed in our sections, confirming the observation of Kruijver et al. (2000) for the BSTc. Second, nucleoli are considered to be hard particles that will not be sectioned by a microtome knife but instead are pushed either in or out the paraffin when hit by a microtome knife (Jones, 1937; Cammermeyer, 1967; Koningsmark, 1970; Braendgaard and Gundersen, 1986). All visible neurons with a nucleus containing a clear nucleolus, within the exclusion lines, were counted using a 63× objective. The neuronal density was calculated by multiplying the total number of nucleoli counted by the sampled volume. The total number of neurons was then estimated for the adult BSTc by multiplying neuronal density with the total BSTc volume. The measurements were made without knowledge of age and sex.

Statistical analysis. The data were categorized in a fetal/neonatal period (between the 25th and 41th weeks of gestation), an infant/pubertal period (between 3 months and 16 years), and an adult period (between 22 and 49 years) and were tested for significant differences using one-way ANOVA and *t* tests (see Fig. 2). A $p < 0.05$ was considered as significant.

RESULTS

BSTc volume defined by VIP immunostaining was ~60.7 ± 3.1% (SEM) smaller than BSTc volume defined by somatostatin immunostaining across all age groups (Figs. 1, 2) because of the

Table 2. Total number of neurons in the BSTc

	NBB	Sex	Age (years)	Brain weight (gm)	Postmortem delay (hr)	Fixation (d)	Neuronal density (mm^3)	Total number of BSTc neurons
Males ($n = 8$)	97083	m	22	1334	<16:29	26	12930	76249
	97173	m	24	1364	<33:30	31	17012	114120
	86042	m	28	1450	<17:00	46	18240	96200
	96406	m	35	1430	13:30	1214	18095	119383
	99071	m	39	1400	<16:30	130	16346	113095
	88011	m	41	1500	20:30	33	15158	102247
	92011	m	47	1500	<89:00	77	18267	89782
	97159	m	48	1500	5:30	42	15070	89630
Females ($n = 7$)	85041	f	28	nd	5:25	44	16255	103774
	85027	f	29	1150	13:10	60	19597	61353
	86032	f	33	1035	<41:00	20	19541	81669
	84002	f	36	1420	85:40	51	20204	74509
	97131	f	43	1345	<92:00	63	20639	75268
	89104	f	49	1260	<41:00	32	13552	88245
	96423	f	49	1253	<17:00	806	17443	92717

NBB, Netherlands Brain Bank; F, female; M, male; SOM, somatostatin.

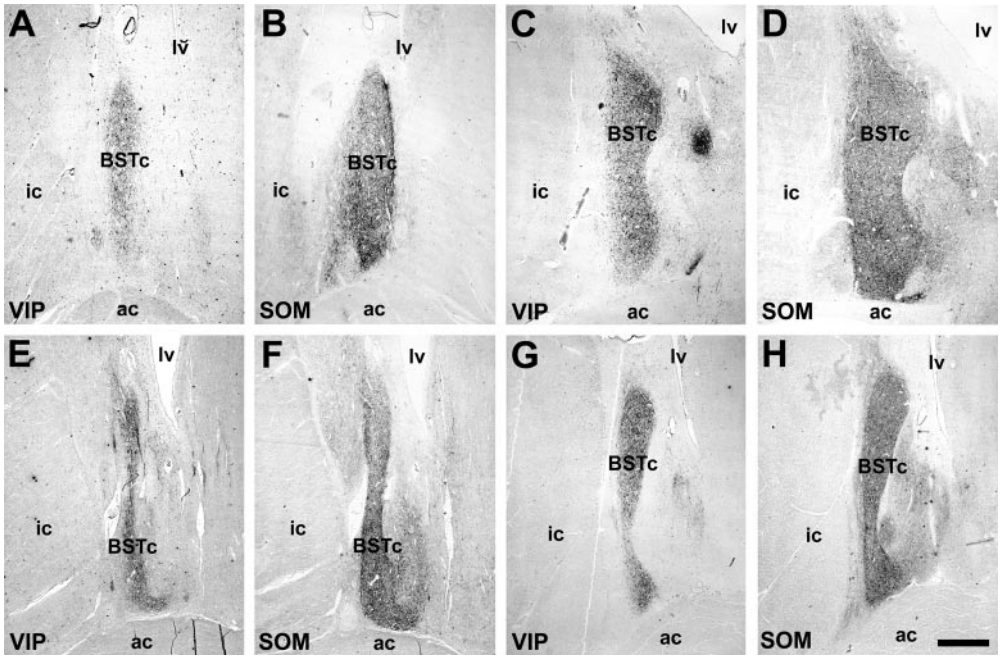

Figure 1. Representative photomicrographs depicting the BSTc in males (*top row*) and females (*bottom row*) in sections stained immunocytochemically for VIP (*A*, *C*, *E*, *G*) and somatostatin (*SOM*) (*B*, *D*, *F*, *G*) during development. *A*, *B*, #87036, 14 years old; *C*, *D*, #99071, 39 years old; *E*, *F*, #99060, 16 years old; *G*, *H*, #92037, 32 years old. Note that the BSTc in males is larger than in females only in adulthood (#99071 vs #92037). Scale bar, 1 mm. *ac*, Anterior commissure; *ic*, internal capsule; *lv*, lateral ventricle.

presence of somatostatin-IR fibers in the cell-sparse shell that surrounds the VIP-IR fibers in the BSTc core. No postmortem delay or fixation time effects on the immunocytochemical staining were observed. Regression analysis showed that BSTc volume in both males and females defined by VIP immunocytochemistry is correlated with BSTc volume defined by somatostatin immunocytochemistry (in males, $r = 0.96$, $p < 0.0001$; in females $r = 0.73$, $p < 0.0001$) (Fig. 2).

One-way ANOVA showed that the male BSTc volume significantly increased with age as based on its VIP ($F_{(2,24)} = 27.4$; $p < 0.0001$) or somatostatin ($F_{(2,23)} = 28.2$; $p < 0.0001$) immunocytochemical staining. *Post hoc* analysis showed that the fetal/neonatal, infant/pubertal, and adult age groups significantly differed from each other ($p < 0.05$). The female BSTc volume significantly increased with age as based on its VIP staining ($F_{(2,24)} = 22.2$; $p < 0.0001$) or somatostatin staining ($F_{(2,23)} = 18.1$; $p < 0.0001$). *Post hoc* analysis showed that only the fetal/neonatal–infant/pubertal age groups and the fetal/neonatal–adult age groups ($p < 0.05$), but not the infant/pubertal–adult age group, significantly differed from each other (Fig. 2*A*,*B*). Consequently, adult BSTc volume was on average 39% larger in males than in females ($t = 2.14$, $p < 0.001$ for VIP; $t = 2.14$, $p < 0.01$ for somatostatin; t test), thereby confirming previous studies, which showed that the adult BSTc size is larger in males than in females (Zhou et al., 1995; Kruijver et al., 2000) (Fig. 2*C*,*D*). Moreover, the total number of BSTc neurons in adulthood (i.e., between 22 and 49 years) was significantly ($t = 2.16$; $p < 0.05$) larger in males (100,088 ± 5247) than in females (82,505 ± 5242) (Fig. 2*E*), which is consistent with the larger number of somatostatin-IR neurons found in males compared with females in adulthood (Kruijver et al., 2000).

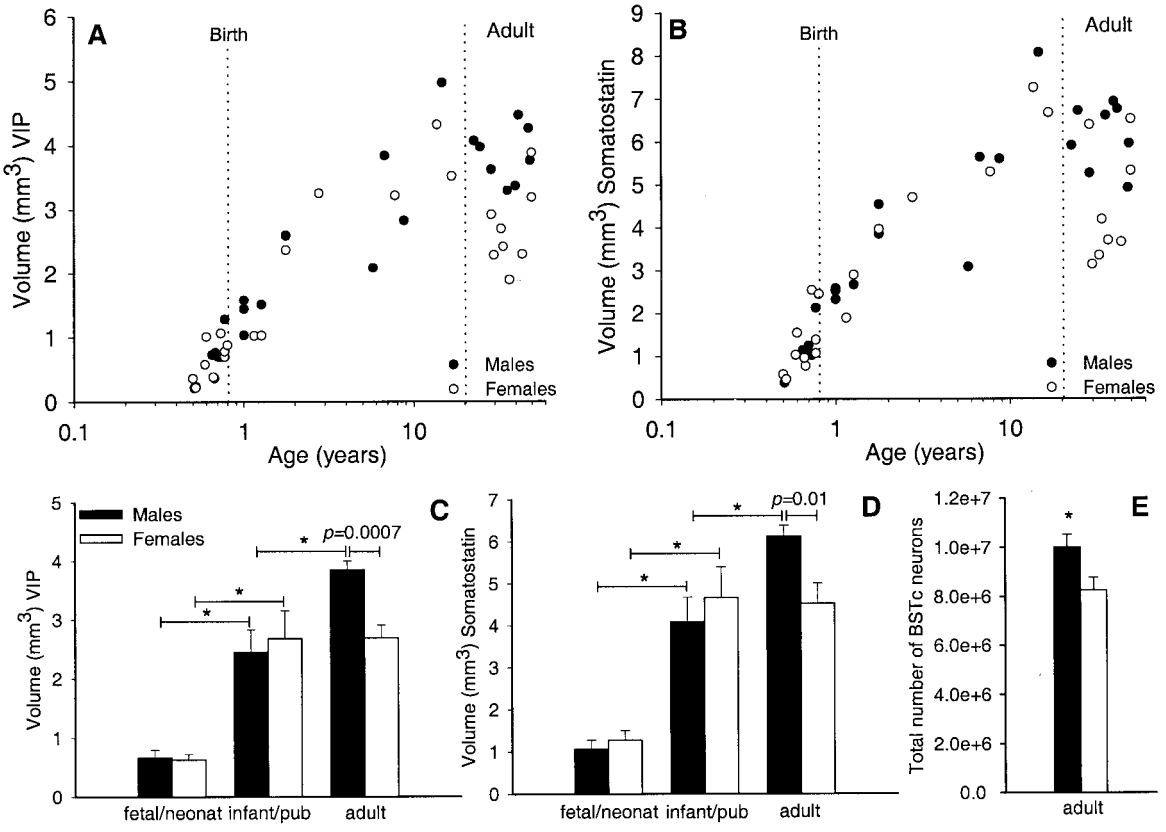

Figure 2. BSTc development in males and females. *A*, *C*, BSTc volume as delineated by its VIP innervation. *B*, *D*, BSTc volume as delineated by its somatostatin innervation. *E*, Total number of BSTc neurons in males and females in adulthood.

DISCUSSION

The sex difference in BSTc volume, which reached significance only in adulthood, developed much later than we expected. Sexual differentiation of the rat BST occurs in the first weeks after birth and requires perinatal sex differences in testosterone levels (Del Abril et al., 1987; Chung et al., 2000). In humans, testosterone levels during fetal and neonatal development are higher in males than in females (Abramovich and Rowe, 1973; Winter, 1978). In addition, dramatic alterations in adult testosterone levels have no obvious effects on the volume of the BSTc in either males or females (Zhou et al., 1995; Kruijver et al., 2000). Therefore, the BSTc was presumed to diverge between males and females early on in development. Moreover, sexual differentiation of the sexually dimorphic nucleus of the preoptic area and other areas in the human anterior hypothalamus occurs between 4 and 10 years of age (Swaab and Hofman, 1988; Swaab et al., 1994).

The late divergence of BSTc volume in males and females may be a general characteristic of the human BST. The human BST-dspm seems to become sexually dimorphic at approximately puberty, as suggested by the developmental time points that were included in the study by Allen and Gorski (1990). Indeed, the BST-dspm appeared to be smaller in females than in males from ~14 years of age (Allen and Gorski, 1990). Relatively late sexual differentiation has also been observed in the pig hypothalamus. The number of cells in the sexually dimorphic vasopressin and oxytocin-containing nucleus in the pig hypothalamus 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; Gould et al., 1999; Gur et al., 1999; Sowell 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 a long delay in organizational effects of gonadal steroids 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 this sex difference in AVPv size, its volume becomes only significantly different at approximately puberty (Davis et al., 1996). Alternatively, it is possible that sex differences in peripubertal or adult gonadal steroid levels establish the sex difference in BSTc volume in adulthood. Although androgens and estrogens in puberty cause the development of secondary sexual characteristics in peripheral body structures, as far as we know, no data exist on similar effects on human brain structures. However, data from six cases reported in previous studies suggest that the BSTc volume, as delineated by VIP or somatostatin immunocytochemical staining, is not affected by marked increases or decreases in gonadal steroid levels in adulthood. A normal female-sized BSTc was found in one control female with increased androgen levels and in two postmenopausal control females with low gonadal steroid levels. Furthermore, a normal male-sized BSTc was found in a control male with high

estrogen levels caused by a feminizing adrenal tumor and in two control males who were orchidectomized as a result of prostate cancer. The possibility that gonadal steroid-dependent changes in VIP or somatostatin neuropeptide expression underlie the changes BSTc volume, such as, in quail preoptic area, rat medial amygdala and human amygdala (Panzica et al., 1987; Giedd et al., 1996; Cooke et al., 1999), is also not supported by these six cases who had marked changes in gonadal steroid levels, although their the BSTc volume was normal for their gender (Zhou et al., 1995; Kruijver et al. 2000).

In addition to direct actions of gonadal steroids on the BSTc, the late emergence of sex differences in BSTc volume may reflect relatively late sex-dependent changes in brain areas that supply the BST with its VIP-IR innervation, such as the amygdala (Eiden et al., 1985), which increases in size at a higher rate in males than in females between 4 and 18 years of age (Giedd et al., 1996). Although sex differences in gonadal steroids are the most likely factor to cause sexual differentiation of the BSTc and the areas that innervate the BSTc, we cannot exclude gonadal steroid-independent mechanisms on brain sexual differentiation, such as local expression of sex chromosomal genes (Reisert and Pilgrim, 1991). A candidate gene for such an effect is the *SRY* gene, which was shown to be transcribed in the adult human hypothalamus and cortex of males but not in females (Mayer et al., 1998).

Late sexual differentiation of the human BSTc volume also affects our perception about the relationship between BSTs volume and transsexuality. Interestingly, transsexuals receive their first consultation between the ages of 20 and 45 years, which coincides with the period of sex-dependent divergence of BSTc volume found in the present study (Van Kesteren et al., 1996). However, epidemiological studies show that the awareness of gender problems is generally present much earlier. Indeed, ~67–78% of transsexuals in adulthood report having strong feelings of being born in the wrong body from childhood onward (Van Kesteren et al., 1996), supporting the idea that disturbances in fetal or neonatal gonadal steroid levels underlie the development of transsexuality. Moreover, observations that phenobarbital or diphantoin usage during pregnancy, which affect gonadal steroid levels, increases the prevalence of transsexuality in the offspring support this idea (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-Bahlburg et al., 1996; Zucker et al., 1996). The lack of marked sexual differentiation of the BSTc volume in our study before birth and in childhood certainly does not rule out early gonadal steroid effects on BSTc functions. As suggested by animal experiments, fetal or neonatal testosterone levels in humans may first affect synaptic density, neuronal activity, or neurochemical content during early BSTc development (Döhler, 1991; Park et al., 1997). Changes in these parameters could affect the development of gender identity but not immediately result in overt changes in the volume or neuronal number of the BSTc. Alternatively, it must also be taken into consideration that changes in BSTc volume in male-to-female transsexuals may be the result of a failure to develop a male-like gender identity. In summary, our finding of a sex difference in BSTc volume only in adulthood suggests that marked sex-dependent organizational changes in brain structure are not limited to early development but may extend into adulthood.

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