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

Chapter 1

From the moment of conception until the moment we die we are living in a sex-differentiated world. Not only do men and women have different physiques, they also have sex differences in seeing, smelling, thinking, feeling, behaving, socializing and making love.

The brain orchestrates these sex differences by irreversible structural ("organizational") and reversible ("activational") sex differences. Examples of such differences are macroscopically sex differences in brain volume, weight and regional differences in size, shape or fiber connections. Microscopically sex differences may exist e.g. in neuronal cell numbers, perikaryal size, dendritic branching and synaptogenesis, while at the molecular level sex differences can exist e.g. at the level of neuropeptides, neurotransmitters, enzymes, proteins and mRNA. Functional sex differences exist in various aspects of reproduction (e.g. in the presence of the menstrual cycle in the hypothalamo-pituitary-gonadal-[HPG]- axis in women), gender identity (i.e. the feeling to be male or female), sexual orientation (i.e. hetero-, bi-, or homosexuality), autonomic functions (differences in e.g. biological rhythms in body temperature, stress hormones, bloodpressure and sleep) as well as in sex hormone dependent gender differences in the regulation of mood, cognition, behaviour and neuroprotection in health and disease.

The present thesis was undertaken to investigate structural and functional differences in the human hypothalamus and adjacent areas in relation to sex, gender identity and sexual orientation by focussing on morphological sex differences, sex hormone receptors (i.e. estrogen receptor- alpha [ERα], beta [ERβ], androgen receptors [ARs] and progesterone receptors [PRs]) and their relation to endocrine status. To this end potential structural sex differences were studied in human post mortem brain material by volume measurements and neuron counts (Chapter 2), while, as a basis for the detection of their site of action and the mechanisms involved in the functional sex differences, differences in the expression of gonadal hormone receptors were studied immunocytochemically (Chapter 3-8).

Chapter 2

First the central part of the human bed nucleus of the stria terminalis (BSTc) was studied in order to determine whether its previously reported sex difference in size and its striking sex reversed size in transsexual subjects were also reflected in neuronal numbers. Transsexuals experience themselves as being of the opposite sex, in spite of having all the biological characteristics of one sex. A crucial question resulting from a previous brain study in male-to-female transsexuals was whether the reported, gender identity related, female size of the central part of the bed nucleus of the stria terminalis (BSTc) was based upon
a neuronal difference in the BSTc itself or just a reflection of a difference in vasoactive intestinal polypeptide (VIP) innervation from the amygdala and other areas, which was used as a marker. Therefore, we determined in 42 subjects the number of somatostatin (SOM) expressing neurons in the BSTc in relation to sex, sexual orientation, gender identity and hormonal status. Regardless of sexual orientation men had almost twice as many somatostatin neurons in the BSTc as women (p<0.006). The number of neurons in the BSTc of male-to-female transsexuals was similar to that of the females (p=0.83). In contrast, the neuron number of a female-to-male transsexual was found to be in the male range. Hormone treatment or sex hormone level variations in adulthood did not seem to have influenced BSTc neuron numbers. The present findings of somatostatin neuronal sex differences in the BSTc and its sex reversal in the transsexual brain clearly support the paradigm that in transsexuals sexual differentiation of the brain and genitals may go into opposite directions and point to a neurobiological basis of gender identity disorder.

Chapter 3

The next step was to find out whether the BSTc and other hypothalamic areas are sex hormone sensitive as judged by the presence of gonadal hormone receptors. For this purpose immunohistochemical protocols for paraffin embedded formalin fixed hypothalamic human brain material were developed. First androgen receptor (AR) distribution was described throughout the rostro-caudal hypothalamus and adjacent areas. In this chapter we report for the first time the distribution of androgen receptor immunoreactivity (AR-ir) in the human hypothalamus of 10 human subjects (5 men and 5 women) ranging between 20 and 39 years of age using the antibody PG21. Prolonged post mortem delay (72:00 hours) or fixation time (100 days) did not influence the AR-ir. In men, intense nuclear AR-ir was found in neurons of the horizontal limb of the diagonal band of Broca, of the lateromamillary nucleus (LMN) and in the medial mamillary nucleus (MMN). An intermediate nuclear staining was found in the diagonal band of Broca, sexually dimorphic nucleus of the preoptic area, paraventricular nucleus, suprachiasmatic nucleus, ventromedial nucleus and infundibular nucleus, while weaker labeling was found in the bed nucleus of the stria terminalis, medial preoptic area, dorsal and ventral zone of the periventricular nucleus, supraoptic nucleus and nucleus basalis of Meynert. In most brain areas women revealed less staining than men. In the LMN and the MMN a strong sex difference was found. Cytoplasmic labeling was observed in neurons of both sexes, while women showed a higher variability in the intensity of such staining. No sex differences in AR-ir were, however, observed in the bed nucleus of the stria terminalis, the nucleus basalis of Meynert (NBM) and islands of Calleja. Species differences and similarities of the AR-ir distribution were discussed. The present results suggest the participation of androgens in the regulation of various hypothalamic processes that are sexually dimorphic.
Chapter 4
In the previous study we found androgen receptor (AR) sex differences in several regions throughout the human hypothalamus. Generally men had a stronger nuclear androgen receptor immunoreactivity (AR-ir) than women. The strongest nuclear labeling was found in the caudal hypothalamus in the mamillary body complex (MBC), which is known to be involved in aspects of sexual behaviour. The study in this chapter was carried out to investigate whether the sex difference in AR-ir of the MBC is related to sexual orientation or gender identity (i.e. the feeling to be male or female) or rather to circulating levels of androgens, since nuclear AR-ir is known to be upregulated by androgens from animal experiments. Therefore, we studied the MBC in the following groups: young-heterosexual men, young-homosexual men, aged-heterosexual castrated and non-castrated men, castrated and non-castrated transsexuals, young-heterosexual women and a young virilized woman. Nuclear AR-ir did not differ significantly between heterosexual and homosexual men but was significantly stronger in men than in women. A female-like pattern of AR-ir (i.e. no to weak nuclear staining) was observed in 26 to 53 year old castrated male-to-female transsexuals and in old castrated and non-castrated males of 67 to 87 years of age. In analogy with animal studies showing strong activational effects of androgens on nuclear AR-ir, the present data suggest that nuclear AR-ir in the human MBC is dependent on the presence or absence of circulating levels of androgens. The group data were, moreover, supported by the fact that a male-like AR-ir (i.e. intense nuclear AR-ir), was found in a 36 year old bisexual non-castrated male-to-female transsexual and in a heterosexual virilized woman of 46 years of age with high levels of circulating testosterone. In conclusion, the sexually dimorphic AR-ir in the MBC seemed to be related to circulating levels of androgens and not to sexual orientation or gender identity. The functional implications of these alterations are discussed in relation to reproduction, cognition and neuroprotection.

Chapter 5
In 1996 a novel second genomic ER subtype of ERs was cloned in rodents and humans and designed ERβ. Subsequently it has been demonstrated that the original ‘classical’ ERα and the second ERβ subtype may play different often opposite (e.g. activating [ERα] versus inhibiting [ERβ]) roles in gene regulation. In order to determine the putative sites of action of estrogens, mediated by ERα and ERβ in the human hypothalamus and adjacent areas immunocytochemical protocols were developed for systematic rostro-caudal mapping studies in relation to sex and endocrine status in the same young adults studied for AR-ir in chapter 3. Hypothalamic material taken from 10 subjects (5 men and 5 women), ranging between 20 and 39 years of age, was investigated.
Since it is known from various animal and human studies that ERs can be down- or upregulated by circulating levels of estrogens in a region dependent
way, hypothalami of a few rare cases with well documented abnormal estrogen levels were also studied: a castrated, estrogen treated 50 year old male-to-female transsexual (T1), a 31 year old man with an estrogen producing tumor (S2) and an ovariec tomized 46 year old woman (S8).

A strong sex difference with more nuclear ERα-ir in women was observed rostrally in the diagonal band of Broca (DBB) and caudally in the medial mamillary nucleus (MMN). Less robust sex differences were observed in other brain areas with more intense nuclear ERα-ir in men, e.g., in the sexually dimorphic nucleus of the medial preoptic area (SDN-POA), paraventricular nucleus (PVN) and lateral hypothalamic area (LHA), while women had more nuclear ERα-ir in the suprachiasmatic nucleus (SCN) and ventromedial nucleus (VMN). No nuclear sex differences in ERα were found e.g. in the central part of the BST (BSTc). In addition to nuclear staining, ERα-ir appeared to also be sex-dependently present in the cytoplasm of neurons and was observed in astrocytes, plexus choroi deus and vascular cells.

The differences in ERα-ir in subjects T1,S2 and S8 indicated the presence of some activating effects of estrogens on hypothalamic ERα-ir. The female expression pattern of ERα-ir in the VMN and MMN of the genetic male subjects (T1) and (S2) (see e.g. Fig. 14C) were related to higher circulating estrogen levels. On the other hand, no clear changes occurred in the BSTc, SDN or DBB, and a strikingly low ERα-ir was found in the NBM (cf Fig. 7E; Fig. 8A; Fig. 11A.. These data seem to suggest that in addition to differential activational effects of estrogen on ERα-ir, also other regulatory mechanisms (that are independent on circulating estrogen levels occur, such as effects on an organizational level) might be involved in the regional control of some ERα-ir sex differences. However, ERα-ir in T1,S2 and S8 suggested that the majority of the observed sex differences in ERα-ir are “activational” (e.g., VMN/MMN) rather than “organizational” in nature. Species similarities and differences in ERα-ir distribution and possible functional implications for the human brain are discussed.

Chapter 6

Subsequently a systematic rostro-caudal distribution of ERβ-ir was studied in the human hypothalamus and adjacent areas in 5 males and 5 females between 20-39 years of age and compared to the ERα distribution (Chapter 5) in the same patients. ERβ-ir was generally observed more frequently in the cytoplasm than in the nucleus and appeared to be stronger in women. In addition, basket-like fiber stainings, suggestive for ERβ-ir in synaptic-terminals, were observed in various areas.

Men showed more robust nuclear ERβ-ir than women in the medial part of the bed nucleus of the stria terminalis (BSTm), paraventricular and paratenial nucleus of the thalamus (PV and PT), while less intense, but more nuclear, ERβ-ir appeared to be present in e.g. the BSTc,SDN-POA, DBB and VMN.
Women revealed more nuclear ERβ-ir than men of a low to intermediate level e.g. in the SCN, supraoptic (SON), PVN, infundibular (INF) and MMN.

ERβ-ir expression patterns in subjects with abnormal hormone levels, i.e., a 50 year old castrated estrogen treated male-to-female transsexual, a 31 year old man with an estrogen producing tumor and a 46 year old ovarietomized woman, suggest that the majority of the observed sex differences in ERβ-ir are “activational” rather than “organizational” in nature. Similarities, differences, potential functional and clinical implications of the observed sex and hormone dependent ERα and ERβ distributions are discussed in relation to reproduction, autonomic-function, mood, cognition and neuroprotection in health and disease.

Chapter 7

The hypothalamic supraoptic nucleus (SON) is the main production site of plasma arginine vasopressin (AVP), a neurohormone that is involved in water-balance, electrolyte, HPA-axis and bloodpressure regulation, while it is also known to exert various central effects. Plasma AVP levels are elevated during stress. In humans plasma AVP levels are higher in males than in females. Sex hormones may be involved in this sex difference, as plasma AVP levels change e.g. during the menstrual cycle and after administration of oral contraceptives. Therefore, we investigated whether previously observed age related sex differences in neuronal activity in the SON might be related to postmenopausal hormonal changes. As estrogens are presumed to inhibit AVP production in a receptor-mediated way, we studied ERα and ERβ immunoreactivity in the dorsolateral (dl)-SON in young and old men and women. To this end hypothalami of 34 controls were subdivided into 4 groups within a 50-yr boundary (young men, young women, elderly men and elderly women). The AVP part of the dorsolateral dl-SON of young women contained 50 times more neurons with ERβ nuclear staining than in young men and 250 times more than in elderly women. In addition, young women also showed more ERβ cytoplasmic staining than young men and elderly women. The proportion of nuclear ERα-positive neurons was higher in young men than in young women (P=0.018) and higher in young men than in elderly men (p=0.015). A significant positive correlation between age and nuclear ERα-positive SON neurons was found in women only. These data show a strong decrease of nuclear ERβ-ir and increase of nuclear ERα-ir in AVP neurons of the dl-SON in postmenopausal women. Both ER changes are proposed to participate in the activation of the AVP neurons in postmenopausal women.

Chapter 8

The suprachiasmatic nucleus (SCN) is the central clock of the brain that orchestrates circadian and circannual biological rhythms, such as the rhythms of -stress- hormones, body-temperature, sleep and mood. These rhythms are
frequently disturbed in menopause and even more so in dementia and can be restored in postmenopausal women by sex hormone replacement therapy (SHRT). Although it seems clear, both from clinical and from experimental studies, that sex hormones influence circadian rhythms, it is not known whether this is by a direct or an indirect effect on the SCN. Therefore, we investigated in the present study by immunocytochemistry whether the human SCN expresses sex hormone receptors in 5 premenopausal women and 5 young men. SCN neurons appeared to contain ERα, ERβ and progesterone receptors (PRs). Median ratings of ER- immunoreactivity per individual and per gender group revealed a statistically significant stronger nuclear ERα expression pattern in female SCN neurons (p<0.05). No significant sexual dimorphic tendency was observed for nuclear ERβ (P>0.1) and PRs (p>0.7). These data extend the sexually dimorphic presence of ARs in SCN neurons (Chapter 3) and appear to support previously reported functional and structural SCN differences in relation to sex and sexual orientation by indicating for the first time that not only testosterone but also estrogen and progesterone may, in principal, act directly on neurons of the human biological clock. From the findings reported in this chapter it may be inferred that the SCN is capable of monitoring the circadian, menstrual and circannual rhythms of circulating levels of sex hormones in humans. Since body-temperature, sleep and well-being, including psychosexual aspects of mood, are also known to fluctuate along the menstrual cycle, the presence of sex hormone receptors in neurons of the human SCN may not only be of importance for the sexually dimorphic regulation of the hypothalamo-pituitary-gonadal (HPG)-axis, but also for the sexually dimorphic regulation of circadian rhythms, such as temperature and sleep. The sexual dimorphisms in the human SCN regarding its sex differences in e.g. ERα and ARs are consistent with the idea of the SCN having sexually dimorphic functions. Furthermore the presence of sex hormone receptors in the SCN provide a potential neuroendocrine mechanism by which SHRT can act to improve or restore directly SCN-related rhythm disturbances, such as body-temperature, sleep, HPA-axis activity and mood.

Chapter 9

The findings (Chapter 2-8), are subsequently summarized and discussed in relation to sex, gender identity, sexual orientation, autonomic function, mood, cognition and circulating hormones. First some methodological considerations are pointed out. Hereafter some background regarding transsexualism is given. The paradigm of transsexuality as a neuro-developmental condition is discussed in relation to the findings of the present thesis. To the same end the findings in relation to sexual orientation and its possible genetic-, epigenetic- determinants and brain areas involved are discussed. Subsequently the potential role of various SCN-target areas related to the HPG-, HPA-axis, autonomic system, energy metabolism and regulation of mood and neuropsychiatric diseases are discussed.

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