SUMMARY

In the present thesis, sexual differentiation of limbic and anterior hypothalamic brain areas was examined in the human and rodent forebrain in relation to apoptosis. We focused, albeit not exclusively, on the human and rodent bed nucleus of the stria terminalis, which contains a number of neuroanatomical and neurochemical sex differences. For example, in adulthood the central subdivision of the bed nucleus of the stria terminalis (BSTc) in the human brain is larger in men than in women. The human BSTc is of further special interest, because the size of the BSTc has been related to a gender identity disorder called transsexuality, in which subjects convey the strong feeling of being born in the wrong sex. Indeed, the BSTc size in male-to-female transsexuals was similar to that found in control women, while in one female-to-male transsexual examined so far the BSTc size was comparable to that found in control men. In rodents, the BST has also been implicated in the regulation of a number of behaviors, such as reproduction, aggression, addiction, parental behavior and stress.

Fetal and/or neonatal sex differences in gonadal steroid hormone levels cause sexual differentiation of the rodent brain, and are presumed to be also responsible for the sexual differentiation of the human BSTc. To better understand the sexual differentiation of the human BSTc, we used postmortem human brain tissue to determine at which developmental age the volume of the BSTc diverged between males and females (*chapter two*). Using vasoactive intestinal polypeptide and somatostatin immunocytochemical staining as markers, we found that the BSTc is larger and contains more neurons in men than in women. However, this sex difference became significant only in adulthood, showing that sexual differentiation of specific areas in the human brain may extend into adulthood. The protracted sexual differentiation of the BSTc volume suggests that marked sex-dependent organizational changes in the human brain under influence of presumably gonadal steroid hormones are not limited to early development. However, the mechanism(s) underlying sexual differentiation of the human BSTc are presumed to occur during early fetal/neonatal and/or infant/pubertal ages, which may not be measurable as overt sex-dependent volumetric changes until adulthood.

Sexual differentiation of the mammalian brain is thought to be caused by sexually dimorphic circulating levels of testosterone acting on both estrogen as well as androgen receptors. Recent studies also suggest a role for progestin receptors. To study which of these receptors may play a role in the protracted sexual differentiation of the BSTc in the human brain, we investigated the development of estrogen receptor (ER) α, ER β, androgen receptor (AR), and progestin receptor (PR) expression in the

BSTc during fetal/neonatal, infant/pubertal and adult ages (*chapter 3*). Using immunocytochemistry, we found that ER α, ER β, AR and PR are expressed in the BSTc from fetal ages onwards. More nuclear stained ER β BSTc cells were observed in females than in males during fetal/neonatal ages, while there were no overt sex differences in ER α, AR and PR during the same developmental period. During infant/pubertal ages, more AR immunoreactive (IR) BSTc cells were observed in males than in females, whereas no overt sex differences in ER α, ER β and PR were detected. ER α and PR immunoreactivity in the BSTc were higher in men than in women only in adulthood, whereas no marked sex differences in ER β were observed in the adult BSTc. These results support the idea that sex differences in gonadal steroid hormone levels may have sex-dependent effects during early BSTc development as suggested by sex differences in ER β and AR during fetal/neonatal and infant/pubertal ages. Together with the sex differences in ER α and PR during adulthood, the subtle temporal-dependent sex differences in the expression of these four gonadal steroid receptors studied may ultimately result in the overt sex difference in the human BSTc size.

Much more research is required in order to obtain a more complete picture of the downstream mechanism(s) of gonadal steroid hormone/receptor-dependent sexual differentiation of the human brain. One strategy is to study the "temporal" expression of (multiple) gonadal steroid hormone-responsive mRNA species in brain areas, which develop in a sex-dependent fashion. In addition, expression of known *and* novel gonadal steroid hormone-dependent proteins underlying (late) sexual differentiation of the human brain can be studied using contemporary research tools, such as employed in proteomic studies. These types of studies, however, require availability of fetal and neonatal fresh-frozen postmortem human brain tissue of both sexes, which at this moment in time are not easily obtained. Studies using human brain tissue are invaluable, because they enable the development of hypotheses about the possible mechanism(s) involved in the sexual differentiation of the human brain.

Notwithstanding the importance of studies using postmortem human brain tissue, experiments in animals are equally required to elucidate further novel mechanism(s) of sexual differentiation. One possible mechanism resulting from sex differences in early levels of gonadal steroid hormones acting on gonadal steroid receptor during development may be a sex difference in the incidence of apoptosis. This idea was tested by comparing the incidence of apoptosis in the early postnatal male and female BST in the rat brain (*chapter four*). More apoptotic nuclei were found in the rat principal nucleus of the BST (BSTpr) in females than in males, whereas the reverse was true for the lateral division of the BST (BSTL). Moreover, the volume of the BSTpr was larger in males than in females, whereas there was no sex

difference in the volume of the BSTL. Our results also confirmed earlier reports indicating that the incidence of apoptosis in the central part of the medial preoptic nucleus (MPNc) is higher in females than in males. In the BSTpr of gonadal steroid hormone-treated animals, the incidence of apoptosis was lower when compared to animals treated with oil-vehicle, which was also true for the MPNc. These results are consistent with the hypothesis that gonadal steroid hormones acting on gonadal steroid receptors contribute to the sexually dimorphic differentiation of the BST by controlling the incidence of apoptosis.

Although the rat BSTpr is larger and contains more cells in males than in females, it is probably not homologous to the human BSTc. The rostral-caudal extend 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. The lateral dorsal BST (BSTLD) in the rodent brain is much more likely to be homologous to the human BSTc, because they share anatomical location and extensive overlap in neuropeptide expression. Consequently, we compared the volume of the VIP-IR component in the BSTL and central lateral amygdaloid nucleus (CeL) in *Bax* wildtype (+/+) mice with *Bax* knockout (-/-) mice (*chapter five*). *Bax* is a proapoptotic member of the *Bcl-2* gene family, that activates pro-apoptotic caspases. We also investigated whether the BSTL and CeL size in *Bax* mice is sexually dimorphic. Our results showed the BSTL and CeL were larger in *Bax* -/- mice than in *Bax* +/+ mice. However, there was no difference between males and females as far as the BSTL and CeL volume in *Bax* +/+ and *Bax* -/- were concerned. These results indicate that attenuation of apoptosis is sufficient to overtly change the BST and amygdala volume. However, these mice did not seem to be as suited for studying sex differences in the BST and amygdala in humans and rats.

The sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rat brain is much larger in males than in females and is one of the best examples of involvement of testosterone-dependent apoptosis during sexual differentiation, in addition to the BSTpr. As described earlier, the incidence of apoptosis in the developing rat SDN-POA is significantly attenuated by testosterone or its estrogenic metabolite estradiol. The mechanism(s) that underlie this protective effect are not known. Recent studies suggest a role for PRs during sexual differentiation of the SDN-POA, because ligand-bound PRs prevent apoptosis in endometrial cells derived from the uterus, while PR antagonists: ZK 98,299 or RU 486 negate this protective effect. Indeed, the number of PR containing SDN-POA cells is much higher in males than in females. Therefore, we hypothesized that testosterone-derived estradiol protects male SDN-POA cells by increasing PR expression during early development (*chapter six*). This was tested in an initial study comparing the incidence of apoptosis

and the volume of the SDN-POA between male and female rat pups on postnatal day (PN) 8, which were injected daily until PN 7 with vehicle, ZK 98,299 or RU 486. In vehicle-treated animals, the incidence of apoptosis was higher in females than in males and the SDN-POA volume was larger in males than in females. Treatment with PR antagonist did not significantly affect the incidence of apoptosis or SDN-POA volume. However, *post hoc* tests showed that neither the incidence of apoptosis nor the SDN-POA volume significantly differed between males and females. The absence of sex difference in the incidence of apoptosis or SDN-POA volume between PR antagonist-treated males and females indicate that postnatal treatment with ZK 98,299 or RU 486 affects sexual differentiation of the SDN-POA in an unexpected fashion. A possible explanation for these results may be found in data from recent studies, showing that progestins elicited phosphorylation of Akt to protect cortical explants against cell death, which could not be inhibited by PR antagonists, such as RU 486. This suggests that progestins in brain cells may not act directly through PRs to facilitate/modulate cell survival in the developing vertebrate brain.

Together, our results, described in the present thesis, suggest that sexual differentiation of the brain, in particular the sexually dimorphic development of BSTc in the human brain is not limited to early perinatal development, but can extend well into adulthood. Moreover, we found evidence supporting the hypothesis that sex differences in gonadal steroid hormone levels during human fetal/neonatal development, possibly acting on their specific gonadal steroid receptor, may organize the sex-dependent development of the human brain, which may regulate the incidence of apoptosis as found in the rodent BST during perinatal development. The latter inference requires additional research in order to be confirmed both in rodent and human brain. Results from preliminary studies showed the presence of apoptotic cells in the perinatal human BST only, but since apoptosis is a rapid process it will be difficult to obtain reliable quantitative data on the incidence of apoptotic cell death.