ELSEVIER

Developmental Brain Research 85 (1995) 273–279

DEVELOPMENTAL BRAIN RESEARCH

Research report

Increased number of vasopressin neurons in the suprachiasmatic nucleus (SCN) of 'bisexual' adult male rats following perinatal treatment with the aromatase blocker ATD

D.F. Swaab [a,*], A.K. Slob [b], E.J. Houtsmuller [b,d], T. Brand [b], J.N. Zhou [a,c]

[a] *Graduate School Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands*
[b] *Department of Endocrinology and Reproduction, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands*
[c] *Ningxia Medical College, Yinchuan, Ningxia, PR China*
[d] *Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA*

Accepted 13 December 1994

Abstract

In an earlier article an enlarged subpopulation of vasopressin containing neurons was found in the suprachiasmatic nucleus (SCN) of homosexual men as compared to heterosexuals. The present study investigates the possibility that the number of vasopressin neurons in the SCN and sexual partner preference behavior in male rats are both influenced by sex hormones during brain development. For this purpose, we studied groups of adult male rats that had been treated either prenatally or pre- and postnatally with the aromatase inhibitor ATD (1,4,6-androstatriene-3,17-dione) which blocks the aromatization of testosterone to estradiol. Rats treated with ATD in both pre- and postnatal periods showed 'bisexual' partner preference behavior and appeared to have 59% more vasopressin-expressing neurons in the SCN than the controls. The prenatally treated rats did not differ from the controls. This observation supports the hypothesis that the increased number of vasopressin neurons found earlier in the SCN of adult homosexual men might reflect differences that took place in the interaction between sex hormones and the brain early in development.

Keywords: Vasopressin; Suprachiasmatic nucleus; Sexual behavior; Sexual partner preference; Hypothalamus; Aromatase blocker; Male rat

1. Introduction

In the past few years several structural differences have been described in the human hypothalamus in relation to sexual orientation. We found that the subpopulation of vasopressin-containing neurons of the suprachiasmatic nucleus (SCN), the clock of the brain, was twice as large in homosexual men as in heterosexuals [28]. This observation was followed by LeVay's [21] report that an interstitial nucleus of the anterior hypothalamus (INAH-3) was twice as large in heterosexual men as in homosexual men, and by Allen and Gorski's [1] paper showing that the commissura anterior is larger in homosexual men than in heterosexual men and women. However, neither the functional meaning of these differences in the context of sexual orientation, nor the mechanism that causes them, are as yet known for any of these three structural differences.

A prominent theory of sexual orientation, gender and sexual differentiation of the brain is that they develop as a result of the interaction between the developing brain and sex hormones [11,18,29]. This theory is mainly based on experiments in rats. Whether the mechanism described also works in primates remains to be determined [6,13]. Sexual partner preference for female rats and sexual differentiation of the male rat brain are both largely determined by the presence of testosterone during the perinatal phase of development. However, before testosterone acts on brain differentiation it must generally be converted into estrogens by means of aromatase [7,10,18]. Perinatal treatment of male rats with the aromatase inhibitor

* Corresponding author. Fax: (31) (20) 696-1006.

Elsevier Science B.V.
SSDI 0165-3806(94)00218-5

1,4,6-androstatriene-3,17-dione (ATD) blocks the aromatization of testosterone to estradiol and induces 'bisexuality'. Such males, when tested in a three compartment box interact sexually both with tethered male and female conspecifics. With the female partner in heat they mount and intromit, with the male they present, hop and dart and they readily display lordosis behavior when mounted [7]. A remarkable recent finding was that in these ATD male rats partner preference behavior shows a nocturnal rhythmicity, i.e., it switched depending on the hour of testing. When tested late in the dark phase ATD males show a clear preference for the female partner, and when tested early in the dark phase they showed a lesser preference for the female, or no preference at all [4]. The time dependency of this phenomenon points to the possible involvement of the SCN.

The present study was carried out to determine the number of vasopressin-containing neurons in the SCN in the ATD-treated male rats.

2. Materials and methods

2.1. Animals

The experimental animals used for this study were Wistar albino rats. The stimulus animals were F1 hybrids of two inbred Wistar strains (R × U). They were housed 2–4 to a cage with food and water available ad libitum and kept on a reversed light–dark cycle of 14-h light/10 h dark (lights on 5.30 p.m. to 7.30 a.m). Temperature in the animal room ranged from 20–22°C. Female rats were timed mated (day of mating = day 0 of pregnancy) and parturition occurred 22 days later. Prenatal treatment consisted of daily injections of the mothers ($n = 8$) with ATD (5 mg/day in 0.1 ml propylene glycol) or solvent ($n = 4$; propylene glycol; 0.1 ml/day) from days 10–22 of pregnancy. Neonatal treatment consisted of implantation (between 3 and 9 h after birth s.c. in the back under ice anaesthesia) of a Silastic capsule (inner diameter 1.5 mm, outer diameter 2.1 mm, length 5 mm) filled with either ATD or cholesterol. The implants were removed when the pups were 21 days of age. Pups were weaned at 21 days of age and housed 2–4 to a cage of the same sex and treatment. The animals were left undisturbed until the onset of behavioral testing at 11 weeks of age.

2.2. Behavior

Behavior tests were carried out with intact males, i.e. the testes in situ. Detailed behavioral procedures and behavioral data for a larger group have been presented elsewhere [7,19]. In the present paper only the behavioral data of the 5 rats per group that were histologically examined are given. Stimulus animals were sexually active intact males and ovariectomized female rats. The latter were brought into estrus with 30 μg estradiol benzoate (EB) 24–48 h prior to testing and 2.5 mg progesterone 3–4 h before testing. These hormones were dissolved in oil and injected subcutaneously.

2.3. Tissue treatment

At 48 weeks of age 5 males of each group were randomly chosen for histological examination of the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the suprachiasmatic nucleus (SCN). The results on the SDN-POA have been reported previously [19]. All males were injected with pentobarbital (Nembutal 0.5 ml/rat i.p.) and perfused with 500 ml fixative (4% paraformaldehyde, pH = 7.2) between 10 a.m. and 4 p.m.

The brains were removed and stored in fixative at 4°C for one day. Subsequently the brain was dehydrated and embedded in paraffin. Serial 6 μm frontal sections were cut according to the coronal plane of the atlas of the rat brain by Paxinos and Watson (1986), mounted on chrome-aluminium coated slides and stained 1:10 with thionine.

2.4. Immunocytochemistry

For immunocytochemistry every 10th paraffin section was hydrated and rinsed in phosphate-buffered saline (PBS 0.01 M), pH 7.4 for 10 min, after which they were (1) incubated with anti-vasopressin (Truus, 18/9/85) 1:800 in 0.5% Triton in PBS overnight at 4°C; (2) washed in PBS (2 × 10 min); (3) incubated with goat-anti-rabbit serum (Betsie) (1:50 in PBS for 30 min; (4) washed in PBS (2 × 10 min); (5) incubated with peroxidase-antiperoxidase (PAP) (1:500 to 1:1000 for 30 min); (6) washed in PBS (2 × 10 min); (7) rinsed in 0.05 m Tris-HCl (Merck), pH 7.6; (8) incubated in 0.05 mg/ml 3,3-diaminobenzidine (Sigma) in 0.05 m Tris-HCl, pH 7.6, 0.01% H_2O_2 (Merck) for 10 min; (9) washed in aquadest; (10) dehydrated in ethanol and mounted in Entellan [28].

2.5. Morphometry

The morphometric procedures, performed blind for the group to which the animals belonged, have been described extensively before [31]. In brief, area measurements through the SCN were performed unilaterally by means of a digitizer (Calcomp 2000) using a Zeiss microscope with 2.5×, 10× and 40× (PLAN) objectives and 12.5× (PLAN) oculars. Area measurements of the SCN were performed in thionine-stained material by measuring every tenth section in the SCN. At the rostral and caudal ends of the SCN every fifth section was analyzed. In order to describe the shape of the SCN, the rostrocaudal axis and the maximum cross-sectional area were determined as separate parameters. The SCN volume was determined by integrating the surface measurements [33]. Total cell density was estimated by counting the profile density per unit area in thionine-stained sections. By using an unfolding procedure, correcting for section thickness (6 μm) and including the modification proposed by Cruz-Orive (9), cell density and mean nucleus diameter were calculated. The total cell number (neurons and glial cells) was estimated by multiplying cell density with SCN volume in each subject. In addition, the number of vasopressin-expressing neurons – i.e. staining clearly more intensely than the low background – and the volume of vasopressin cell population in the SCN was measured per SCN in the adjacent sections within the borders of the thionin stained SCN by means of the same procedure. Profiles of all immunocytochemically stained cells were measured in 8.6 ± 1.1 (mean ± S.E.M.) sections per animal.

2.6. Statistics

The Kruskal–Wallis and Mann–Whitney U-test (two-tailed) were used to test the differences, using a 5% level of significance.

3. Results

3.1. Behavior

The behavioral results for the animals used in the present studies were as follows: male rats pre- and

Table 1
Partner preference and sexual behavior during partner preference testing of male rats perinatally treated with ATD and controls

Group	(n)	partner preference † (seconds)	% time near male #	mount + introm + ejac	ejaculation per test	number of rats ejaculating
Control	5	−597 ± 45 ***	2.6 ± 0.7 ***	25.7 ± 1.2 **	2.4 ± 0.2 ***	5
Pre	5	−643 ± 70 ***	2.1 ± 1.0 ***	23.1 ± 4.1	1.6 ± 0.4 *	4
Pre + post	5	−121 ± 30	33.8 ± 2.9	15.9 ± 3.1	0.1 ± 0.05	1

Data are given as mean ± S.E.M.
Abbreviations used: *n*: number of rats; mount + introm + ejac: mean number of mounts plus intromissions plus ejaculations.
* $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$ compared to pre + post treatment group.
† A preference score calculated by subtraction the time spent with female partner from time spent with male partner.
# of total time spent with stimulus animals.

postnatally treated with ATD had significantly lower preference scores in a three-compartment partner preference test for the estrous females than prenatally ATD-treated or control males. They spent about one third of their time with the male partner (Table 1). The latter 2 groups did not differ during partner preference testing. One out of 5 pre- and neonatally ATD-treated males ejaculated, whereas 4 out of 5 prenatally ATD-treated males and all controls did (Table 1). In pair tests (early in the dark phase of the light-dark (LD) cycle) with an estrous female none of the 5 pre- and neonatally ATD-treated males ejaculated, whereas 4 out of 5 controls and prenatally ATD treated males did [19]. In a pair test with a sexually active male all 5 pre- and neonatally-ATD treated males were readily mounted (mean lordosis quotient 78 ± 9%), whereas only 2 out of 5 control males were mounted, of whom none displayed lordosis.

3.2. Staining

Vasopressin staining was slightly more intense in the pre- and postnatally ATD-treated males and slightly less intense in the prenatally ATD-treated males as compared to the controls (Fig. 1), indicating activity changes of the vasopressin neurons in the treated groups. The number of vasopressin neurons was significantly increased in the pre- and postnatally ATD-treated males as compared to both the control group and the animals that had only undergone prenatal treatment. The data are given in Table 2. There were no significant differences between the total SCN cell number of the three groups, although they showed a similar trend as the vasopressin cell countings.

4. Discussion

From earlier work it appeared that neonatally ATD-treated male rats can readily display 'bisexual' behaviors in partner preference testing (see also Introduction). They respond with mounts and intromissions when confronted with the estrous female partner, and with proceptive and lordosis behavior when mounted by a male. In addition, neonatally ATD-treated males showed significantly fewer ejaculations than controls. They are less masculinized in partner preference and ejaculatory behavior, and less defeminized in lordosis behavior in adulthood. These behavioral experiments show that estradiol derived from testosterone plays a

Table 2
Morphometrical SCN parameters (mean ± S.E.M.) of male rats pre- or perinatally treated with ATD, and controls

Group	(n)	Cell number (×1000)	Volume (mm^3)	Cell nuclear diameter (μm)	Density ($\times 10^3/mm^3$)	RCA (mm)	MCA (mm^2)
Total							
Control	(5)	13.15 ± 1.78	0.021 ± 0.002	5.80 ± 0.130	628.11 ± 41.654	0.43 ± 0.06	0.071 ± 0.004
Pre	(5)	12.61 ± 0.81	0.020 ± 0.001	5.51 ± 0.234	634.51 ± 22.160	0.44 ± 0.02	0.069 ± 0.005
Pre + post	(5)	14.54 ± 0.72	0.025 ± 0.003	5.63 ± 0.148	617.15 ± 62.284	0.46 ± 0.02	0.075 ± 0.008
AVP							
Control	(5)	1.58 ± 0.22 *	0.005 ± 0.001	5.22 ± 0.146	314.91 ± 52.735	0.36 ± 0.03	0.023 ± 0.004
Pre	(5)	1.42 ± 0.23 **	0.006 ± 0.001	5.30 ± 0.174	254.75 ± 18.869	0.40 ± 0.03	0.024 ± 0.004
Pre + Post	(5)	2.51 ± 0.35	0.007 ± 0.001	5.46 ± 0.287	374.00 ± 52.457	0.45 ± 0.03	0.026 ± 0.004

Data are given as mean ± S.E.M.
Abbreviations used: *n*: number of rats; RCA: rostro-caudal axis; MCA: maximal cross-sectional area
* $P < 0.05$; ** $P < 0.02$ compared to pre + post treatment group. SCN: suprachiasmatic nucleus
Total: total cell number of the SCN as determined in thionine-stained sections.
AVP: number of vasopressin expressing neurons in the SCN.

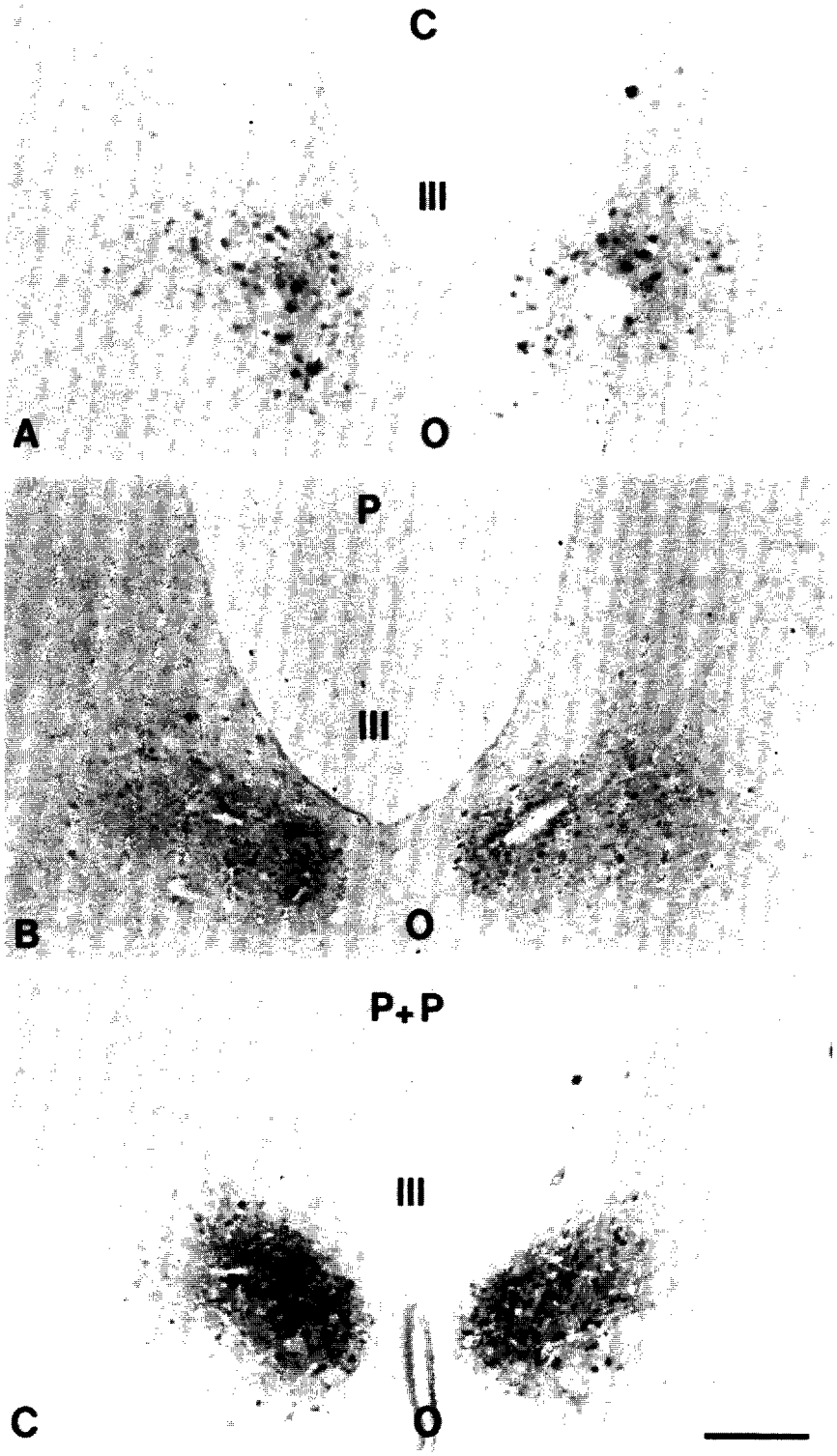

Fig. 1. Vasopressin immunocytochemistry in the suprachiasmatic nuclei of (a) a representative control rat (C), (b) a rat treated prenatally with ATD (P) and c) a rat treated pre- and postnatally with ATD (P + P). III, third ventricle; O, optic chiasm. Bar = 100 μm. Note the increased staining in the P + P animal and the decreased staining in the P animal as compared to the control (C).

major role in programming male rat sexual partner preference [4,5,7,34].

The medial POA is considered to be involved in both sexual arousal and performance in male rats [25], although not without debate [12]. The decreased size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in ATD rats [19] is in line with the suggestion that the brain is also less masculinized in these

animals. The smallest size was found in pre- and neonatally ATD treated males. However, since the SDN-POA was also smaller in rats that were treated only prenatally with ATD, while their sexual partner preference behavior was not affected, the size of the SDN-POA does not seem to be causally related to partner preference. A similar conclusion was reached for the human SDN-POA, which is of similar size in homosexual and heterosexual men [28]. The homology between the rat and human SDN-POA is, however, still controversial [29].

In contrast to these observations the number of vasopressin-expressing neurons in the SCN seems to be related to partner preference behavior in rat and sexual orientation in man. In the present study we have shown that there is an increased number of vasopressin-containing neurons in the pre- and neonatally ATD-treated 'bisexual' rats. Earlier, we found twice as many vasopressin-expressing neurons in the SCN of homosexual men [28] and in two out of three male-to-female transsexuals [29]. The present study cannot distinguish between three possible interpretations for the increased number of vasopressin neurons, i.e. (1) that pre- and neonatal ATD treatment induces the formation of extra neurons, (2) that ATD prevents programmed cell death of some of the vasopressin neurons during brain development, or (3) that more neurons start to express vasopressin beyond the detection limit. Although the pre- and neonatally ATD treated males had the largest total cell number, they did not differ statistically from the other groups. To answer the question posed above, future research is needed, e.g., with [^{3}H]thymidine as a marker for cell division. Our results are also in agreement with earlier studies showing that in the rat neonatal androgens decrease diurnal rhythmicity [16,24,26,27].

There are indeed a number of indications that the SCN is involved in reproduction and sexual behavior. Postcoital ultrastructural signs of activation have been described in the female rabbit SCN, i.e. a well developed Golgi apparatus and rough endoplasmic reticulum and an increase in nuclear and neuronal size [8], while increased neuronal activity was also found in the rat SCN around puberty as indicated by increased nucleolar and nuclear size [3,22,23]. In addition, the ovarian reproductive cycle in the female rat is controlled by the SCN, possibly by direct innervation of LHRH neurons by vasoactive intestinal polypeptide (VIP) neurons [32]. Moreover, several morphological sex differences have been reported in the human SCN [31,36]. The two times larger vasopressin subdivision of the SCN in homosexual men [28] points to a possible role of the SCN in sexual orientation. Of course, neither this observation in man, nor the increased number of vasopressin neurons in the SCN of ATD treated male rats necessarily means that the number of vasopressin neurons in the SCN bears a causal relation to sexual partner preference. Lesion of the SCN in adult male rats did not affect sexual partner preference [20], although, in the light of Bakker et al.'s findings on nocturnal rhythmicity of sexual partner preference [4,5], such animals should be studied in more detail over different periods of the night.

An alternative possibility is that both sexual partner preference and the number of vasopressin neurons in the SCN are determined by estrogen levels during development, whereas the SCN is not directly involved in sexual partner preference. In this respect the number of vasopressin-expressing neurons in the adult SCN can be considered as reflecting the interaction between sex hormones and the brain in a certain period of development. This sensitive period for the estrogen effects on the developing vasopressin neurons in the rat SCN seems to be situated between birth and 21 days postnatally since prenatal ATD treatment did not affect the vasopressin cell number in the SCN (see Table 2). Alternatively, for a positive effect of the lack of estrogens on vasopressin neurons ATD might be required during both the pre- and postnatal period. This possibility should be explored in future studies by giving ATD during the postnatal period alone. Concerning the effect of sex hormones on SCN development it is of interest that several sex differences in the fine morphology of the SCN have been described both in rat [14,15] and human [30,31,36]. One functional consequence of the effect of sex hormones on the developing SCN is that during normal development in male rats, the estradiol metabolite of testosterone may affect the organization of circadian rhythms [4,5,26]. The observation that neonatal castration of male gerbils resulted in a reduction of some 70% of the SCN volume in adulthood [17] might mean that the SCN can be influenced by sex hormones during development in a different way in different species, although vasopressin cell counts are not available in that study.

Sexual differentiation of the brain is not only epigenetically influenced by sex hormones but also by other factors such as fetal drug exposure [35] and maternal stress [2]. It would be of considerable interest, therefore, to study the effect of such factors on the developing SCN as well.

In conclusion, we might say that the rat data presented in this paper support the hypothesis that the increased number of vasopressin neurons in the SCN of homosexual men and two out of three male-to-female transsexuals as observed earlier [28,29] may reflect an altered interaction between sex hormones and brain development. It seems worthwhile, therefore, to study steroid receptors and aromatase in the human hypothalamus in relation to gender and sexual orientation in order to reveal the possible mechanisms behind the structural differences in this brain structure.

Acknowledgements

We are grateful to R.N.J.M.A. Joosten and B.F. Fisser for their technical assistance and to Ms. W.T.P. Verweij for her secretarial help, to M.A. Hofman for his advice on the statistics, to J.B. Hutchison and M.J. Baum for their valuable comments on and suggestions for an earlier version, and to Psychon (560-258-038), BIOMED I (PL 931536) and NWO (900-552-134) for financial support.

References

[1] Allen, L.S. and Gorski, R.A., Sexual orientation and the size of the anterior commissure in the human brain, *Proc. Natl. Acad. Sci.*, USA 89 (1992) 7199–7202.

[2] Anderson, R.H., Fleming, D.E., Rhees, R.W. and Kinghorn, E., Relationships between sexual activity, plasma testosterone, and the volume of the sexually dimorphic nucleus of the preoptic area in prenatally stressed and non-stressed rats, *Brain Res.*, 370 (1986) 1–10.

[3] Anderson, C.H., Nucleolus changes at puberty in neurons of the suprachiasmatic nucleus and the preoptic area, *Exp. Neurol.*, 74 (1981) 780–786.

[4] Bakker, J., Van Ophemert, J. and Slob, A.K., Organization of partner preference and sexual behavior and its nocturnal rhythmicity in male rats, *Behav. Neurosci.*, 107 (1993) 1049–1058.

[5] Bakker, J., Brand, T., Van Ophemert, J. and Slob, A.K., Hormonal regulation of adult partner preference behavior in neonatally ATD-treated male rats, *Behav. Neurosci.*, 107 (1993) 480–487.

[6] Baum, M.J., Carroll, R.S., Cherry, J.A. and Tobet, S.A., Steroidal control of behavioural, neuroendocrine and brain sexual differentiation: studies in a carnivore, the ferret, *J. Neuroendocrinol.*, 2 (1990) 401–418.

[7] Brand, T., Kroonen, J., Mos, J. and Slob, A.K., Adult partner preference and sexual behavior of male rats affected by perinatal endocrine manipulations, *Horm. Behav.*, 25 (1991) 323–341.

[8] Clattenburg, R.E., Singh, R.P. and Montemurro, D.G., Postcoital ultrastructural changes in neurons of the suprachiasmatic nucleus of the rabbit, *Z. Zellforsch.*, 125 (1972) 448–459.

[9] Cruz-Orive, L.M., Particle shape-size distributions: the general spheroid problem. II. Stochastic model and practical guide, *J. Micr.*, 112 (1978) 153–167.

[10] Döhler, K., The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus, *Int. Rev. Cytol.*, 131 (1991) 1–57.

[11] Dörner, G., Neuroendocrine response to estrogen and brain differentiation in heterosexuals, homosexuals, and transsexuals, *Arch. Sexual Behav.*, 17 (1988) 57–75.

[12] Everitt, B.J. and Stacey, P. Studies of instrumental behaviour with sexual reinforcement in male rats (Rattus norvegicus): II. Effects of preoptic area lesions, castration and testosterone, *J. Comp. Psychol.*, 101 (1987) 407–419.

[13] Gooren, L., Fliers, E. and Courtney, K., Biological determinants of sexual orientation, *Annu. Rev. Sex. Res.*, 1 (1990) 175–196.

[14] Güldner, F.-H., Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats, *Exp. Brain Res.*, 50 (1983) 373–376.

[15] Güldner, F.-H., Sexual dimorphisms of axo-spine synapses and postsynaptic density material in the suprachiasmatic nucleus of the rat, *Neurosci. Lett.*, 28 (1982) 145–150.

[16] Hansen, S., Södersten, P., Eneroth, P., Srebro, B. and Hole, K., A sexually dimorphic rhythm in oestradiol-activated lordosis behaviour in the rat, *J. Endocrinol.*, 77 (1979) 381–388.

[17] Holman, S.D. and Hutchison, J.B., Differential effects of neonatal castration on the development of sexually dimorphic brain areas in the gerbil, *Dev. Brain Res.*, 61 (1991) 147–150.

[18] Hutchison, J.B., Hormonal control of behaviour: steroid action in the brain, *Curr. Opin. Neurobiol.*, 1 (1991) 562–570.

[19] Houtsmuller, E.J., Brand, T., De Jonge, F.H., Joosten, R.J.N.M.A., Van de Poll, N.E. and Slob, A.K., SDN-POA volume, sexual behavior and partner preference of male rats affected by perinatal treatment with ATD, *Physiol. Behav.*, 56 (1994) 535–541.

[20] Kruijver, F.P.M., De Jonge, F.H., Van den Broek, W.T., Van der Woude, T., Endert, E. and Swaab, D.F., Lesions of the suprachiasmatic nucleus do not disturb sexual orientation of the adult male rat, *Brain Res.*, 624 (1993) 342–346.

[21] LeVay, S., A difference in hypothalamic structure between heterosexual and homosexual men, *Science*, 253 (1991) 1034–1037.

[22] Morishita, H., Kawamoto, M., Masuda, Y., Higuchi, K., Tomioka, M., Nagamachi, N., Mitani, H., Ozasa, T. and Adachi, H., Quantitative histological changes in the hypothalamc nuclei in the prepuberal and postpuberal female rat, *Brain Res.*, 76 (1974) 41–47.

[23] Morishita, H., Nagamachi, N., Kawamoto, M., Tomioka, M., Higuchi, K., Hashimoto, T., Tanaka, T., Kuroiwa, S., Nakago, K., Mitani, H., Miyauchi, Y., Ozasa, T. and Adachi, H., The effect of prepuberal castration on the development of the nuclear sizes of the neurons in the hypothalamic nuclei of female rats, *Brain Res.*, 146 (1978) 388–391.

[24] Rusak, B. and Zucker, I., Neural regulation of circadian rhythms, *Physiol. Rev.*, 59 (1979) 449–526.

[25] Shimura, T., Yamamoto, T. and Shimokochi, M., The medial preoptic area is involved in both sexual arousal and performance in male rats: re-evaluation of neuron activity in freely moving animals, *Brain Res.*, 640 (1994) 215–222.

[26] Södersten, P. and Eneroth, P., Neonatal treatment with antioestrogen increases the diurnal rhythmicity in the sexual behaviour of adult male rats, *J. Endocrinol.*, 76 (1980) 331–339.

[27] Södersten, P., Hansen, S. and Srebro, B., Suprachiasmatic lesions disrupt the daily rhythmicity in the sexual behaviour of normal male rats and of male rats treated neonatally with antioestrogen, *J. Endocrinol.*, 88 (1981) 125–130.

[28] Swaab, D.F. and Hofman, M.A., An enlarged suprachiasmatic nucleus in homosexual men, *Brain Res.*, 537 (1990) 141–148.

[29] Swaab, D.F., Gooren, L.J.G. and Hofman, M.A., The human hypothalamus in relation to gender and sexual orientation, *Prog. Brain Res.*, 93 (1992) 205–219.

[30] Swaab, D.F., Zhou, J.N., Ehlhart, T. and Hofman, M.A., Development of vasoactive intestinal polypeptide neurons in the human suprachiasmatic nucleus in relation to birth and sex, *Dev. Brain Res.*, in press.

[31] Swaab, D.F., Fliers, E. and Partiman, T., The suprachiasmatic nucleus of the human brain in relation to sex, age and dementia, *Brain Res.*, 342 (1985) 37–44.

[32] Van der Beek, E.M., Wiegant, V.M., Van der Donk, H.A., Van den Hurk, R. and Buijs, R.M., Lesions of the suprachiasmatic nucleus indicate the presence of a direct VIP containing projection to gonadotropin releasing hormone neurons in the female rat, *J. Neuroendocrinol.*, 5 (1993) 137–144.

[33] Van Eden, C.G., Uylings, H.B.M. and Van Pelt, J., Sex difference and left–right asymmetries in the prefrontal cortex during postnatal development in the rat, *Dev. Brain Res.*, 12 (1984) 146–153.

[34] Vreeburg, J.T.M., Van der Vaart, P.D.M. and van der Schoot, P., Prevention of central defeminization but not masculinization in male rats by inhibition neonatally of estrogen biosynthesis, *J. Endocrinol.*, 74 (1977) 375–382.

[35] Ward, O.B., Fetal drug exposure and sexual differentiation of males. In A.A. Gerall, H. Moltz and I.L. Ward (Eds.), *Handbook of Behavioral Neurobiology, Vol. 11: Sexual Differentiation*, Plenum Press, New York, 1992, pp. 181–219.

[36] Zhou, J.N., Hofman, M.A. and Swaab D.F., Vasoactive intestinal polypeptide (VIP) neurons in the human suprachiasmatic nucleus (SCN) in relation to sex, age and Alzheimer's disease, *Neurobiol. Aging*, in press.