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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CHAPTER

SEX HORMONE RECEPTORS ARE PRESENT IN THE HUMAN SUPRACHIASMATIC NUCLEUS

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Abstract
The suprachiasmatic nucleus (SCN) is the clock of the brain that orchestrates circadian and circannual biological rhythms, such as the rhythms of 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 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, using immunocytochemistry in the present study, we investigated whether the human SCN expresses sex hormone receptors in 5 premenopausal women and 5 young men. SCN neurons appeared to contain estrogen receptor-α (ERα), estrogen receptor-β (ERβ) and progesterone receptors. Median ratings of ER immunoreactivity per individual and per gender group revealed a statistically significantly 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 progesterone receptors (p > 0.7). These data seem to support previously reported functional and structural SCN differences in relation to sex and sexual orientation and indicate for the first time that estrogen and progesterone may act directly on neurons of the human biological clock. In addition, the present findings provide a potential neuroendocrine mechanism by which SHRT can act to improve or restore SCN-related rhythm disturbances, such as body temperature, sleep and mood.

Introduction
The suprachiasmatic nucleus (SCN) is a hypothalamic nucleus which is located bilaterally on the rostro-basolateral side of the third ventricle, on top of the optic chiasm. It is the biological clock of the brain that orchestrates not only circadian day-night rhythms, but also shows seasonal fluctuations [1–3]. Environmental light serves as an important ‘Zeitgeber’ to entrain the clock by direct innervation via the retinohypothalamic tract [4].

In conjunction with animal experimental studies which show that the SCN communicates through synaptic pathways with various effector systems to orchestrate physiological, stress- and reproduction-related hormonal rhythms [2, 5–8], the human biological clock is thought to be involved in the regulation of body temperature [3, 9], sleep [9] and mood rhythm disturbances [10], in the mechanism of light therapy-induced alleviation of depressed mood in seasonal affective disorder [11], and in the activation of the hypothalamo-pituitary-adrenal (HPA) axis in depression [10, 12, 13].

The endogenous regulation of body temperature, sleep, HPA axis activity
and mood is frequently disturbed in menopause [14–17], while these disrupted rhythms can be improved or restored by sex hormone replacement therapy (SHRT) [18–23], with additional beneficial effects in the prevention of osteoporosis, cardiovascular disease and cognitive decline [24–29]. Also experimental studies [30–34] indicate that sex hormones can have a clear effect on the functioning of the clock. However, there is controversy in the literature as to whether sex hormones act directly or indirectly on the SCN [30, 34–36]. The present study was carried out in young males and females to investigate whether human SCN neurons can potentially be directly influenced by sex hormones via the expression of estrogen receptors (ERs, α and β) [37, 38] or progesterone receptors (PRs) [39].

Materials and Methods

Subjects

The brains of 10 subjects (5 men and 5 women) ranging from 20 to 39 years of age [40–42] were obtained at autopsy within the framework of The Netherlands Brain Bank. For clinicopathological data see table 1. Permission was obtained for brain autopsy and the use of the material and clinical records for research purposes. General pathology and neuropathology were performed either at the Free University of Amsterdam (Dr. W. Kamphorst, Prof. F.C. Stam or Prof. P. van der Valk) or at the Academic Medical Center of the University of Amsterdam (Prof. D. Troost). The subjects selected had no primary neuroendocrine, neurological or psychiatric disease. The menstrual cycle of
Table 2. Nuclear and cytoplasmic (−/−) sex hormone receptor expression in the SCN of young females and males

<table>
<thead>
<tr>
<th>NBB</th>
<th>Sex</th>
<th>ER</th>
<th>ERβ</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>86032</td>
<td>F</td>
<td>+++/-</td>
<td>+++/+</td>
<td>++/+</td>
</tr>
<tr>
<td>80008</td>
<td>F</td>
<td>+++/-</td>
<td>+/-</td>
<td>++/+</td>
</tr>
<tr>
<td>85027</td>
<td>F</td>
<td>+++/+</td>
<td>++/+</td>
<td>++/+</td>
</tr>
<tr>
<td>97055</td>
<td>F</td>
<td>+++/+</td>
<td>++/+</td>
<td>++/+</td>
</tr>
<tr>
<td>85041</td>
<td>F</td>
<td>+++/+</td>
<td>+/-</td>
<td>++/+</td>
</tr>
<tr>
<td>94040</td>
<td>M</td>
<td>++++</td>
<td>+/+</td>
<td>++/+</td>
</tr>
<tr>
<td>82020</td>
<td>M</td>
<td>+++/-</td>
<td>+/+</td>
<td>++/+</td>
</tr>
<tr>
<td>97075</td>
<td>M</td>
<td>+/+++</td>
<td>+/+++</td>
<td>++/+</td>
</tr>
<tr>
<td>84023</td>
<td>M</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>88035</td>
<td>M</td>
<td>+++/-</td>
<td>+++/+</td>
<td>+++/+</td>
</tr>
</tbody>
</table>

NBB = Netherlands Brain Bank; ERα = estrogen receptor α; ERβ = estrogen receptor β; PR = progesterone receptor; SCN = suprachiasmatic nucleus.

the patients was not known.

**Histology and Immunohistochemistry**

After autopsy the hypothalamus was fixed in 4% formaldehyde at room temperature, dehydrated and embedded in paraffin. Serial 6-μm frontal sections were cut on a Leitz microtome. Paraffin-embedded sections of human hypothalamus were mounted onto Super-Frost/Plus (Menzel, USA) slides and dried overnight on a hot plate at 58°C followed by 24–36 h in an oven at 37°C. The sections were deparaffinized and rehydrated by a series of decreasing ethanol concentrations followed by rinsing in distilled water. The staining and antibody specificity procedures for ERα and ERβ were previously described by Ishunina et al. [43]. Staining for the PR was performed with the mouse monoclonal antibody 1A6 (NCL-PGR; code 2272 MPGR, Eurodiagnostica [39, 44], which recognizes both the human PRα and β isoforms according to Bevitt et al. [39], and Euro-Diagnostica, Arnhem, The Netherlands [Rachel Osborne, personal commun.]). The PR-staining procedures were performed according to the ERβ protocol [43] with adjustments of the following steps: rinsing occurred in TBS instead of (TBS)-high salt. Incubation with the primary human PR antibody was performed in the recommended 1:10 dilution, while the incubation with the secondary antibody was performed with biotinylated antimouse IgG (Vector Laboratories, Inc., Burlingame, Calif.) in the standard 1:200 dilution.

**Analysis of the SCN Area**

The sections were systematically stained and analyzed throughout the rostrocaudal axis of the hypothalamus that included the SCN area, as previously
Table 3. Sexually dimorphic median intensity of nuclear and cytoplasmic (−/−) labeling of ERα, ERβ and the PR in human SCN neurons

<table>
<thead>
<tr>
<th></th>
<th>Female SCN</th>
<th>Male SCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+++/-</td>
<td>+/+*</td>
</tr>
<tr>
<td>ERβ</td>
<td>++/+</td>
<td>+/-</td>
</tr>
<tr>
<td>PR</td>
<td>++/+</td>
<td>++/+</td>
</tr>
<tr>
<td>AR</td>
<td>+/-</td>
<td>++/+</td>
</tr>
</tbody>
</table>

The androgen receptor (AR) data were previously published by our group. Note that no median sex difference is present in nuclear PR labeling as opposed to the median gender differences in ERα/ERβ and AR labeling. The strongest median nuclear labeling was observed for ERα which was in addition statistically significant for gender (*p < 0.05; Mann-Whitney U test; two-tailed correction for ties; see Results section). ERα = Estrogen receptor α; ERβ = estrogen receptor β; PR = progesterone receptor; SCN = suprachiasmatic nucleus.

described by Fernandez-Guasti et al. [40]. The SCN was identified based on its neuroanatomical location according to Mai et al. [45] and if necessary with the help of vasoactive intestinal polypeptide (VIP), vasopressin (AVP) or neurotensin stainings [10]. The sections were rated for receptor staining intensity by 3 independent investigators. The few differences in rating were agreed by settlement. The category assigned to the SCN area corresponds to the predominant cell type according to the following scale: − = no staining; + = diffuse and transparent staining; ++ = non-transparent staining but individual granules of the reaction product still distinguishable, and +++ = intense opaque staining. The staining range was established systematically in both the nucleus and cytoplasm. The estimates were made at three different microscopic magnifications using x2.5, x10, and x40 objectives. Median SCN ratings were taken per individual (table 2) and subsequently per gender group (table 3) for the nuclear and cytoplasmic staining intensity for all sex hormone receptor subtypes analyzed.

Statistical Analysis

The assigned median categories of ERα, ERβ and PR immunoreactivity in the SCN were compared using the non-parametric Mann-Whitney U test at the two-tailed level (corrected for ties). Group comparisons for postmortem delay (PMD), fixation time (FT) or age were also made with the Mann-Whitney U test. Differences were considered significant at a p level of <5%.

Results

ERα and β and PRs were present throughout the rostrocaudal axis of the human biological clock with a more robust expression pattern for ERα compared with ERβ throughout the entire SCN area (i.e. the NT-staining area that covers both the VIP- and AVP-staining area [10] (fig. 1-3). More intense median nuclear ERα and ERβ labeling was found in the SCN neurons of women than men (fig. 1, 2; table 2, 3) of which nuclear ERα was found to be statistically significant (p < 0.05; nuclear ERβ p > 0.1). PR labeling showed no median sex
Fig. 1. Illustration of a human female SCN (A, C, E) and male SCN (B, D, F) identified by NT (A) and AVP (B), respectively, with adjacent sections immunoreactive for ERα (C, D). A, C, E The SCN was obtained from female subject No. 80008. B, D, F SCN was obtained from male subject No. 94040. Note the sex difference in C vs. D and in E vs. F, showing a stronger intensity of nuclear ERα in female SCN neurons as compared to male SCN neurons. E is a higher magnification of C, and F is a higher magnification of D. The insertions in A and B represent cytoplasmic NT and AVP staining in SCN neurons as opposed to nuclear staining for ERα in C and D. The asterisks point to blood vessels as a reference point (A–D). Scale bar in D represents 200 μm (and represents 100 μm when extrapolated to the small insertions in A–D), and the scale bar in F represents 25 μm.

differences (table 2, 3; fig. 2, 3; p > 0.7). No statistically significant median differences were present for cytoplasmic ERα, ERß or PR staining. No statistical differences were present between males and females for PMD, FT or age (p = 0.70, p = 0.35 and p = 0.20, respectively), so that the tendency for sex differ-
Fig. 2. Illustration of the sex difference in nuclear intensity for ERβ immunoreactivity (A vs. B) and the lack of such difference for PR immunoreactivity (C vs. D) in SCN neurons as represented by female subject No. 97055 (A, C) and male subject No. 82020 (B, D). More intense nuclear staining for ERβ in females is represented by A and the clearly visible neuronal fiber stained for the PR is indicated in D (arrows). No median sex difference was found for nuclear PR staining (table 3). The asterisk (B) points to strong nuclear ERβ immunoreactivity in small smooth muscles and endothelial cells of a capillary. Nuclear and cytoplasmic ERα signal is illustrated in E. The smaller arrow points to cytoplasmic ERα immunoreactivity and the larger arrow points to nuclear ERα immunoreactivity in an SCN neuron. Scale bar represents 25 μm.

Discussion

Here, for the first time we provide immunocytochemical evidence that the human adult SCN expresses ERs and PRs, allowing a direct influence of sex hormones on SCN functions. In addition, nuclear expression patterns revealed a statistically significant sexually dimorphic pattern for ERα but not for ERβ or PR presence.

The strong reduction in postmenopausal sex hormone levels coincides with a disruption of the circadian regulation of body temperature, sleep and mood that can be improved or restored by SHRT [18–23]. These observations point to a
causal relationship between circulating levels of sex hormones and functioning of the biological clock but do not indicate whether such effects on the SCN can be direct or indirect in nature. The results of the present study, in which we demonstrate the presence of ERs and PRs in human SCN neurons, show that sex hormones may, in principle, act directly on the SCN. Interestingly, more intense nuclear ERα labeling was found in the SCN neurons of women as compared with the SCN neurons of male subjects, which is an opposite sexually dimorphic tendency to that found previously for nuclear androgen receptor immunoreactivity in SCN neurons (table 3) [40]. The fact that men in general do not suffer that much from ‘andropausal’ disturbances is generally explained by the fact that testosterone levels in men do not decrease as strongly as estrogen levels in menopausal women, although hot flushes, insomnia and mood disturbances (irritability, anxiety, depression) can be manifestations of the male climacteric as well [for reviews see, 46–48]. There are indeed human data which indicate that sex hormones have effects on mood [18, 22, 48, 49], sleep [19, 20] and heat balance [21, 50]. The temperature effects are believed to be mediated by the temperature- and sex hormone-sensitive hypothalamic neurons of the anterior preoptic area [51], an area which is known to be under direct control of the SCN [2, 6, 52]. In turn, the human SCN has recently been shown to be a crucial brain site in the regulation of body temperature [3, 9] and sleep [9], and has been implicated in the regulation of mood [4, 11, 12, 53]. Body temperature, sleep and well-being, including the psychosexual aspects of mood, are also known to fluctuate during the menstrual cycle [54–56]. The presence of sex hormone receptors in the neurons of the human SCN may, therefore, not only be of importance for the sexually dimorphic regulation of the hypothalamopituitary-gonadal axis [3, 52], but also for the sexually dimorphic regulation of the circadian rhythms of temperature and sleep [57–59].

Gender differences in the elderly exist, e.g., in the circadian course of body core temperature, with a phase advance of 1.25 h in its acrophase and with a larger amplitude of its temperature curve in women versus men. Women are also reported to wake up earlier and sleep for shorter durations than men [57]. The sexual dimorphisms in the human SCN regarding its vasopressinergic shape, VIP content [60–62] and its sex differences in ERα (present study) and androgen receptors [40] are thus consistent with the idea of the SCN having sexually dimorphic functions.

Furthermore, the fact that sex hormone receptors are expressed in human SCN neurons corroborates the possibility that sex hormone/SCN neuron interactions during a ‘critical sensitive period’ of early brain development might ‘organize’ the size and function of its adult vasopressinergic subnucleus, which has been related to sexual orientation [63, 64]. In order to further corroborate this possibility sex hormone receptors should also be demonstrated in the SCN during early human development.

Experimental studies point both to ‘organizing’ sex hormone effects dur-
ing early development on the SCN as well as to immediate 'activational' effects of sex hormones on the SCN function during adulthood. An example of an organizational effect is the finding of a female type of synaptogenesis in male rat SCN neurons that can be induced by neonatal castration of males [32]. Examples of activational effects are, e.g., the findings that estrogens and testosterone increase SCN-mediated wheel running activity in adult male and female rodents, while castration attenuates this immediate effect in both sexes [for review see, 34]. In addition to inducing an increase in the amplitude of activity, estrogen shortens the circadian period and advances its phase [30, 31]. Estrogens have also been shown to induce the formation of gap junctions in adult rat SCN neurons [33] and were recently reported to increase mRNA levels of the clock gene Cry2 [65].

It is intriguing that our studies on the human hypothalamus [40] reveal the presence of different types of sex hormone receptors in the SCN, whereas studies in the rat revealed conflicting data regarding the presence or absence of ERα in the SCN. With the ERα antibody H222, no ERα was reported to be present in the adult rat SCN [66]. However, an in situ hybridization (ISH) study in the adult rat [36] revealed a robust expression of ERα mRNA in the SCN, which corresponds well with our data in adult humans. In other ISH studies in the adult rat no such hybridization signal was detected in this area [35, 67]. However, Shughrue et al. [35] found low ISH expression levels of ERβ in the rat SCN, which is in agreement with our data. Receptor staining was also observed in the adult rat and guinea pig SCN [Kaiser et al., personal commun.] with the same ERα antibody as used in the present study. Interestingly, a very recent paper shows ERα and ERβ immunoreactivity in neonatal rat SCN neurons [68], which is completely in line with our observations of the presence of ERα and ERβ immunoreactivity in the human SCN. Differences in the detection threshold may, at least partly, account for the differences in the literature on sex hormone receptors in the SCN. The presence of PR in the human SCN is in agreement with data in the nonhuman primate SCN [69] and with the progesterone sensitivity of the rat SCN, which is necessary for the progesterone-induced luteinizing hormone surge [52].

From the present findings 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 from early puberty onwards until late adulthood [70-75].

The presence of ERs and PRs in human SCN neurons support the possibility that immediate 'activational' effects of sex hormones on the SCN are responsible for the sex hormone withdrawal-induced desynchronization of the SCN in menopause in which sex hormone receptors are still present [Kruijver and Swaab, unpublished observations]. The previously reported decreased amplitude of circadian and seasonal peaks in the activity of human vasopressinergic SCN neurons after the age of 50 [76, 77] may functionally be associated with
the decreased amplitude of circadian temperature regulation [78] as well as the disappearance of the menstrual cycle around this age, and be due to the diminishment or disappearance of circadian testosterone and estrogen rhythms [70, 74, 79]. The reduced plasma concentrations of sex hormones during aging [47, 70, 73, 74] and the reduced AVP neurons in the SCN in elderly are thus presumed to contribute to the desynchronization of the clock in these subjects [80], and even more so in Alzheimer patients [3, 81–85], resulting in a disturbed regulation of temperature, sleep and mood. On the basis of these data it seems of interest to investigate whether there is a relationship between the degree of circulating levels of sex hormones and the severity of circadian rhythm disturbances in elderly subjects and Alzheimer’s patients and, if so, whether SHRT is especially effective in improving or restoring disturbed circadian rhythms in individuals that have very low sex hormone levels. In this respect, it seems of particular interest to note that SHRT has recently been shown to restore: changes in the circadian fluctuation of temperature; 24-hour cortisol levels; impaired sleep and mood [23, 86, 87], and blood pressure rhythms in postmenopausal women [88]. The latter finding also fits with the close relationship between the SCN and blood pressure [89].

In conclusion, we have demonstrated that the human SCN is a sex hormone-sensitive brain area. The present findings provide a direct mechanism by which SHRT can act to improve or restore SCN-related rhythm disturbances.

References
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63 Swaab DF, Slob AK, Houtsmlller EJ, Brand T, Zhou JN: Increased number of vasopressin neurons in the suprachiasmatic nucleus (SCN) of 'bisexual' adult male rats following perinatal treatment with the aromatase blocker ATD. Dev Brain Res 1995;85:273–279.


