Development of vasopressin neurons in the human suprachiasmatic nucleus in relation to birth

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The number of neurons expressing vasopressin (AVP) was determined in the suprachiasmatic nucleus (SCN) of the hypothalamus of 41 human subjects ranging in age from 27 weeks of gestation up to 30 years of age. At term, i.e. at 38–42 weeks of gestation, the fetal SCN is still immature, since it then contains only about 13% of the adult number of AVP-expressing neurons. The period of rapid increase in the number of AVP-staining neurons in the SCN during the first few months postnatally coincides with the appearance of overt diurnal rhythms in the child.

During late human gestation diurnal rhythms have been observed in fetal breathing¹⁸, heart rate^{20,34}, body movements^{6,34} and maternal plasma estriol levels¹⁹. Since diurnal metabolic changes have been observed in the fetal suprachiasmatic nucleus (SCN) in rat and squirrel monkey^{22–24}, it has been postulated that the fetal SCN could be responsible for the generation of these fetal rhythms. The observation that circadian oscillation in the myometrial activity of the rhesus monkey is abolished by fetal death³³ agrees with this possibility. In addition, the fetal SCN has been held responsible for the circadian gating of birth in the rat²⁶.

None of the human fetal rhythms, however, seem to persist immediately after birth^{12,16,21}. Diurnal rhythm in sleep–wakefulness starts to emerge only 2 months postnatally¹⁵, and plasma *N*-acetyltransferase (NAT) rhythm matures around 3 months of postnatal life³. These observations indicate that human fetal rhythms may, to a large extent, be driven by the mother, e.g., by maternal meals, glucocorticoids (for a review see ref. 11) or melatonin²⁷.

Vasopressin (AVP) is known to be a good marker for the localization of the human SCN³¹. The moment during development in which AVP is expressed in the rat SCN is closely related to the moment the first overt rhythms occur (see below). In order to assess the maturity of the human SCN with respect to the moment of birth, the number of AVP-expressing neurons has been determined in the present study from half way gestation up to adulthood.

For the present study the brains of 41 subjects (25 males and 16 females) from fetal week 27 up to 30 years of age were obtained by autopsy. General pathology and neuropathology were performed either at the Free University of Amsterdam (Dr. W. Kamphorst) or at the Academic Medical Center of the University of Amsterdam (Dr. D. Troost). Subjects had no neurological disease unless stated otherwise. Some neonates had periventricular hemorrhage. In addition, the SCN was measured in 1 case of pituitary aplasia in a 35-week-old female neonate, who had survived for 5 postnatal weeks. Brains were weighed, followed by generally 1 month of fixation in 4% formaldehyde at room temperature. The hypothalamic area was subsequently dissected, dehydrated and embedded in paraffin. Serial 6 μ m frontal sections were cut on a Leitz microtome, mounted on chrome-alum-coated slides, hydrated and brought to phosphate-buffered saline. Each 50th section was stained with thionine for orientation.

For immunocytochemistry the hydrated sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, for 10 min after which they were: (a) incubated with anti-AVP (Truus, 18/9/85) 1:800 in 0.5% Triton in PBS overnight at 4 °C; (b) washed in PBS (2 $\times$ 10 min); (c) incubated with goat anti-rabbit serum (Betsie) 1:50 in PBS for 30 min; (d) washed in PBS (2 $\times$ 10 min); (e) incubated with peroxidase-antiperoxidase (PAP) 1:500–1:1000 for 30 min; (f) washed in PBS (2 $\times$ 10 min); (g) rinsed in 0.05 M Tris-HCl (Merck), pH 7.6; (h) incubated in 0.05 mg/ml 3,3'-diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH

Fig. 1. The development of the human suprachiasmatic nucleus (SCN) in alternating thionine (A,C,E) and vasopressin (B,D,F) stained sections through the hypothalamus. A, B: 87.152.5, 42 weeks of gestation; C, D: 86.411.0, 5 months postnatally; E, F: 86.425.3, 3 years of age. The bars indicate 100 μ m. The arrows indicate a corresponding point in the thionine and vasopressin stained sections. Note the increase of the number of cells expressing vasopressin and the increase of staining intensity during development. The SCN cannot be delineated in the thionine sections.

7.6, 0.01% H_2O_2 (Merck) for 10 min; (i) washed in aqua dest; (j) dehydrated in ethanol and mounted in Entellan.

Specificity was checked by using non-immune serum (Ducky) 1:800 or anti-oxytocin (O-I-V) 1:800 in step (a), both of which failed to reveal any staining of SCN cells.

The morphometric procedures have been described extensively before³¹. In brief, area measurements of the vasopressinergic SCN and its cell nuclei profiles were performed unilaterally by means of a digitizer (Calcomp 2000) connected to a VAX 11/780 computer, using a Zeiss microscope equipped with 10 $\times$ and 40 $\times$ (PLAN) objectives, respectively, and with 12.5 $\times$ (PLAN) oculars. The volume of the SCN was determined by integrating all area measurements of the SCN sections that contained immunocytochemically stained cells. The number of AVP cells per unit SCN volume was estimated

using a discrete unfolding procedure. The total SCN cell number was estimated by counting the profile density per unit area in thionine-counterstained material using the same procedure. For this purpose, the AVP-stained section with the maximal SCN area was selected, counterstained and nuclear profiles of all cells were determined.

Comparison of alternating thionine- and AVP-stained sections (Fig. 1) revealed that the SCN cannot be reliably localized in conventionally stained sections although this is suggested in the literature^{7,14,27}. In fetuses younger than about 30 weeks of gestation, the possible site of localization of the SCN in thionine sections could not be confirmed by AVP staining and its exact site therefore remains obscure in this stage of development. But also when AVP was expressed during development, it was impossible to give a reliable delineation of the SCN in an alternating conventionally stained section. This means that, although the SCN cells are certainly born well before we are capable of staining them for AVP during late gestation, a good estimation of gestational age at which this nucleus is formed in human and its exact localization at that moment cannot be made at present.

The developmental pattern of AVP-expressing neurons and total cell numbers are given in Fig. 2. The cells that stained for AVP during development did belong to the SCN because of their localization, their size and cytoarchitectonic characteristics (Fig. 1) and the fact that no oxytocin cells were found in this area, in contrast to the positive staining in the supraoptic and paraventricular nucleus. A clear SCN staining was only found from 31 weeks onwards. In 5 brains, no AVP was found in the SCN region: the youngest being a boy of 27 weeks of gestation and the oldest a 2-year-old girl (Fig. 2a). It is not clear why the latter case (urosepsis for 3 days) did not show any AVP staining in the SCN and only very weak staining in the otherwise strongly positive supraoptic and paraventricular nucleus. AVP and total cell numbers (Fig. 2a and b, respectively) rose rapidly in the neonatal period reaching a peak value around 1–1.5 years postnatally. The SCN cell number of a female neonate with a pituitary aplasia was fully within the range of its age. When the period around birth was studied in more detail, it became clear that most cells start to express AVP only after birth. At term, i.e. between 38 and 42 weeks of gestational age ($n = 7$), only about 13% of the adult numbers of AVP-expressing neurons was present. The developmental course was similar in boys and girls.

The moment AVP becomes clearly expressed in the SCN neurons in rat is closely related to the moment the first overt rhythms occur. The rat SCN neurons are formed between gestational days 15 and 17 (ref. 2). The retinohypothalamic tract, which is the primary pathway

Fig. 2. Development of the human suprachiasmatic nucleus (SCN) of the hypothalamus. Log-log scale. The period at term (38–42 weeks of gestation) is indicated by the vertical bar. Note that both vasopressin (AVP; 2a) and total cell number (2b) are low at the moment of birth (13% and 21%, respectively of the cell number found in adulthood). There is no difference in the developmental course of the SCN in boys and girls. In the SCN region of 5 subjects no AVP-expressing neurons were found; these subjects are indicated by symbols at the lower side of the time axis. Cell numbers around 1–1.5 years postnatally are more than twice the adult cell numbers. After these high levels a decrease to adult AVP cell number and total cell number is found.

through which the light stimulus reaches the SCN¹⁷, starts to penetrate the SCN on postnatal day 3–4 (ref. 29). From this period onwards, i.e. postnatal day 4–6, AVP positive neurons were visible in the SCN in all animals, whereas on the 7th postnatal day AVP-containing fibers were spread all over the SCN area⁵. The major increase in synaptic density in the SCN occurs in exactly the same period, i.e. between 4 days and 1 week of age¹³. On postnatal day 6, retina-mediated entrainment is functional and capable of overriding any remaining maternal influence⁸. The rapid maturation of the SCN around postnatal days 4–6, as judged from the increase in AVP-staining neurons⁵ is closely related to the appearance of the first overt rhythm, i.e. that of *N*-acetyltransferase (NAT)⁹. A similar relationship is present between the increasing number of human SCN neurons staining for AVP in the first postnatal months (Fig. 2a) and the occurrence in the first overt rhythms, i.e. temperature¹, NAT³ and sleep–wakefulness patterns¹⁵. This shows that, like in rats, also in humans AVP expression in the SCN coincides with the moment at which the first overt rhythms emerge.

On the basis of AVP staining, the human SCN at term seems to be comparable to the rat SCN around postnatal day 3, i.e. immature and not yet capable of producing overt rhythms. Our observations reinforce, consequently, the hypothesis that the human fetal diurnal rhythms are to a large extent driven by the mother. In addition, the diurnal variation in the timing of birth is probably largely determined by the mother as well (cf. ref. 11). SCN neurons seem to be able to express diurnal rhythmicity well before this nucleus is capable of coordinating overt circadian rhythms; in the rat the rhythm of SCN 2-deoxyglucose uptake appears on embryonic day 19 (ref. 23), a prominent day/night rhythm of AVP messenger RNA is evident in the SCN on day 21 of gestation²⁵, and the firing rate of SCN neurons shows a diurnal rhythm on

embryonic day 22 (ref. 28). Entrainment of the 2-deoxyglucose rhythm of the fetal SCN depends on the maternal SCN^{22,25}. This means that the SCN neurons express a metabolic rhythm long before they can communicate this rhythm to the rest of the brain. In order to coordinate overt rhythms fiber connections with other brain areas need to be developed and transmitters such as AVP have to be expressed.

Around the first year of postnatal life, both AVP and total cell numbers in the 3 cases investigated were more than twice the average numbers found in adult subjects^{10,31}. These high values may reflect a short lasting overshoot of SCN cell number. Redundance of cell number at some stage of development seems to be a general phenomenon in ontogeny⁴ and was also observed, for example, in the sexually dimorphic nucleus of women³⁰. The sexually dimorphic nucleus was localized and quantified using thionin-stained sections instead of by immunocytochemistry as used for the SCN. The high SCN cell numbers around 1 year postnatally correspond with those observed in one 30-year-old woman with Prader-Willi syndrome (an LH–RH deficiency) and 2 male-to-female transsexuals of 40 and 50 years of age³². This suggests that the decrease of SCN neurons observed in the reference group after the first year of postnatal life may not have occurred in these conditions. The finding of high SCN cell numbers in the Prader-Willi case points to the possibility that the reproductive axis may affect programmed postnatal cell death in the SCN.

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