Transplantation of the rat suprachiasmatic nucleus. Functional studies and ex vivo adenoviral vector-mediated gene transfer

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Vasopressin-deficient suprachiasmatic nucleus grafts re-instate circadian rhythmicity in suprachiasmatic nucleus-lesioned arhythmic rats

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It was investigated whether grafts of the suprachiasmatic nucleus could re-instate circadian rhythmicity in the absence of its endogenous vasopressin production and whether the restored rhythm would have the long period length of the donor. Grafts of 17-day-old vasopressin-deficient homozygous Brattleboro rat fetuses, homotopically placed in arhythmic suprachiasmatic nucleus-lesioned Wistar rats, re-instated circadian drinking rhythm within 20-50 days similar as seen for grafts of heterozygous control fetuses. Period length of the recovered rhythm revealed a similar difference (average 24.3 vs. 23.8 h) as reported for the rhythm between the adult Brattleboro genotypes. In all transplants, also those of the two-third non-recovery rats, a surviving suprachiasmatic nucleus was visible as a vasoactive intestinal polypeptide-positive neuronal cell cluster, whereas heterozygous transplants also revealed the complementary vasopressinergic cell part. Explanation of the absence of recovery failed since no undisputable correlation emerged between recovery of rhythm and vasoactive intestinal polypeptide, vasopressin and/or somatostatin immunocytochemical characteristics of the suprachiasmatic nucleus of the transplant. Special focus on the somatostatinergic neurons revealed their presence only occasionally near or in between the vasoactive intestinal polypeptidergic and (in the case of heterozygous grafts) vasopressinergic cell cluster. However, their aberrant cytoarchitectural position appeared not to have affected the possibility to restore drinking rhythm of the suprachiasmatic nucleus-lesioned arhythmic rat. It was concluded that grafted Brattleboro fetal suprachiasmatic nucleus develop their intrinsic rhythm conform their genotype.
and that vasopressin is not a crucial component in the maintenance nor in the transfer of circadian activity of the biological clock for drinking activity. Vasopressin of the suprachiasmatic nucleus may instead serve modulation within the circadian system.

Introduction

The suprachiasmatic nucleus (SCN) is the endogenous circadian pacemaker in mammals which controls diurnal cycles and undergoes photic and non-photic entrainment. Studies on the role of the vasopressinergic (VPergic) cells present in this 'biological clock' have made use of the hereditary VP-deficient Brattleboro rat. The absence of VP in the homozygous Brattleboro rat appeared not to alter the circadian aspect of behavioral and endocrine rhythms as compared to that of heterozygous VP-expressing Brattleboro rat or that of the ancestor Long-Evans rat [11,21,24,32,33,37]. Circadian behavioral rhythmicity of the homozygous Brattleboro rat remains intact under free-running conditions and is also present for instance in the cell firing rate of the SCN in \textit{in vitro} slices [22], and in the expression of (mutant) VP mRNA [46] and VP\textsubscript{1}a receptor mRNA of the \textit{in situ} SCN [53]. These findings seem to indicate that the intrinsic pacemaker driving the circadian rhythm is not dependent on VP.

In the VP-deficient homozygous Brattleboro rat, however, some parameters of the clock system are still different from controls, so that VP could have a modulatory role. The animals have a longer circadian period of the free-running activity rhythms [21], a reduced amplitude of circadian sleep [11] and pineal hormone rhythm [37], and an altered response on food entrainment [32], whereas also the \textit{in vitro} SCN cell firing rate shows a reduced amplitude [22]. A possible function of VP in the settings of the biological clock system seems also supported by many findings in normal VP-synthesizing rodents. VPergic neurons form intra-SCN synapses [14] and have extensive efferent pathways reaching many hypothalamic and thalamic areas involved in autonomic functions [12]. Alterations in the amplitude and diurnal pattern of motor activity rhythm relate to differences in VPergic cell content [13,18,52], chronic intraventricular infusions of VP in rat, masking the circadian rhythm in the CSF of VP derived from the SCN, alters the amplitude of wake/sleep cycles [3,28] and infusion of VP or its antagonists in hypothalamic target areas influence the diurnal rhythms of the pituitary-adrenal axis [26]. Whether the more subtle changes in the characteristics of the circadian system of the VP-deficient homozygous Brattleboro rat are caused by the intra-SCN absence of VP in adulthood or caused by developmental aspects is not known. Brain development of the VP-deficient homozygous Brattleboro rat is impaired [10] and, in normal rat, the SCN VPergic system matures (slowly) in the presence of hormonal VP generated by the early active magnocellular hypothalamus-neurohypophysial system [4]. Observations following transplantation of the VP-deficient fetal SCN in SCN-lesioned, but VP-expressing, rat, could shed some light on above aspects.

In SCN neurotransplantation studies, homotopic placement of fetal SCN in SCN-lesioned arhythmic rodents re-instates overt circadian rhythms such as for drinking, eating
and motor activity [1,2,17,19,29,34,36,40]. The presence of vasoactive intestinal polypeptide (VIP)-expressing SCN neurons has been mentioned as of more importance for rhythm restoration than that of VPergic neurons [29,40]. However the morphology of the grafted SCN develops many aspects of the in situ SCN, including the presence of clustered VPergic neurons [6,51]. VPergic neurite outgrowth is often extensive within the transplant but sparse towards the host brain, but these aspects could not be related to recovery of circadian rhythms. Grafting studies with the tau hamster mutant have indicated that the donor SCN determines the period of recovered circadian rhythm in SCN-lesioned hosts [34]. Since the circadian activity of the homozygous Brattleboro rat has a period different from that of its heterozygous Brattleboro genotype [21], one would expect, if the presence of VP plays no role during SCN development, similar differences to develop for the period of SCN graft-induced recovered rhythms.

To further investigate the role of VP or of the VPergic neurons of the SCN, and to see how the VP-deficient fetal SCN develops in the (hormonal) VP-containing environment of the host, in the present study SCN-lesioned arhythmic rats were subjected to neurotransplantation of VP-deficient SCN grafts dissected from homozygous Brattleboro fetuses with heterozygous Brattleboro SCN grafts as control. Daily drinking rhythm under continuous darkness was monitored as a marker for functionality of the SCN transplant. The morphological aspects of the SCN at 10-15 weeks post-transplantation were investigated as published before [8,19], in a renewed attempt to correlate structure and recovery of function under these conditions.

Experimental procedures
Animals and animal treatment
Male Wistar rats (Harlan, Zeist, The Netherlands), weighing approximately 300 g, were subjected to bilateral electrolytic lesion of the SCN and kept in rhythm cages under controlled conditions of continuous darkness (3-5 lux), temperature (22±1°C) and humidity (60±5%), ad libitum food and water supply and cage cleaning on an irregular basis [8,19]. The rhythm cages [35] were set up for automatic registration of drinking activity by recording the number of drinking nipple contacts per 30 min periods and these data were transmitted to a Digital VAX 4000 System for chi-square periodogram analysis of rhythm [41]. Rats without circadian drinking activity (P<0.01) between 3 and 7 weeks post-lesioning were defined as arhythmic, and were used for neurografting (n=27).

Homotopic grafting of fetal SCN tissue was performed in week 8 after the SCN lesion. Source tissue was obtained from embryonic day 17 fetuses of pregnant homozygous di/di Brattleboro females being either sired by homozygous (F1 homozygous) or Long-Evans (+/+) males (F1 heterozygous +/-di) [5,10]. The breeding stock was obtained from Harlan and the homozygous Brattleboro genotype of the dams was checked prior to mating by measurement of daily water consumption (>50% of body weight) [47]. Dissection of fetal SCN tissue and stereotactic neurografting were performed as described previously [7,19]. From the 27 available arhythmic rats, 9 animals received a heterozygous Brattleboro SCN graft (controls) and 18 animals a homozygous Brattleboro SCN graft (experimental
animals) both dissected from two source fetuses (about 2 µl) and directed at the SCN lesion site. After transplantation, drinking activity measurements were continued. Recovery of circadian drinking activity was judged by visual inspection of double-plotted actograms obtained up to 10 to 15 weeks post-transplantation. These data were subsequently confirmed by periodogram analysis [41] of the drinking frequency data of the 4 weeks prior to sacrifice.

Immunocytochemistry

Animals were sacrificed by perfusion fixation with ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4. The brains were isolated and a 8-10 mm coronal slice containing the anterior hypothalamus was postfixed for 48 h. The slice was then embedded in gelatin under vacuum [20] in order to keep the site of lesion and transplantation intact upon subsequent 50 µm coronal vibratome sectioning. Alternating sections were immunostained for C-terminal propressophysin (CPP; identifies VP-containing structures), VIP or somatostatin using the three-step peroxidase-anti-peroxidase method with Ni/diaminobezidine in the final chromogen reaction. Primary antisera used were respectively C3-final 1:2000 (kindly supplied by J.P.H. Burbach, Rudolf Magnus Institute, University of Utrecht, Utrecht, The Netherlands), Viper 1:2000 and Somaar 1:2000 [19]. Stained sections were compared with maps from the rat brain atlas of Swanson [43] to locate the site of the transplant, and to facilitate comparison of consecutive sections of CPP, VIP and somatostatin staining. Evaluation was done by scoring the extent of the SCN lesion of the host, the presence of stained cells and fibers in the transplant and the occurrence of fibers crossing the transplant border. All microscopic observations were performed without knowledge of the donor genotype or of the occurrence of recovery of circadian drinking behavior. Transplants were considered SCN-positive if the staining visualized one or more clusters with VIP-positive cells.

Results

General microscopy

All grafted rats revealed the presence of a transplant, except for one heterozygous Brattleboro SCN-grafted rat which was excluded from the evaluations. Transplants were situated at the bottom of the third ventricle, i.e. the lesion site, occasionally with extensions in the host parenchyma (medial septum, periventricular area, thalamus or medial preoptic area). Only a single rat had its transplant (homozygous Brattleboro SCN graft) situated heterotopically, i.e., between hypothalamus and periventricular area of the thalamus. Although all acceptor rats were arrhythmic in their circadian drinking behavior prior grafting surgery, immunocytochemistry for CPP and VIP revealed small parts of the lesioned SCN to be present in several cases (2/8 and 10/18 in the heterozygous and homozygous Brattleboro graft group, respectively).

Immunocytochemical evaluation of the transplant

Irrespective of the heterozygous or homozygous Brattleboro genotype and the location, all
Vasopressin-deficient suprachiasmatic nucleus neurografting

transplants revealed positive staining for VIP. One or more clusters of VIPergic cell bodies were always found. Within these clusters VIP neurites had formed dense plexuses with extensions often throughout large parts of the transplants (Figs. 1C, D and 2B). VIP staining

Figure 1. Comparison of immunostaining in heterozygous Brattleboro and (hereditary VP-deficient) homozygous Brattleboro fetal SCN transplants at 10-15 weeks post-grafting in SCN-lesioned Wistar rats. Alternate sections of a control heterozygous Brattleboro (A, C, E) and a homozygous Brattleboro transplant (B, D, F) are immuno-stained for CPP (A, B), VIP (C, D) and somatostatin (E, F). Note the absence of CPP staining for mutant VPergic SCN cells in the homozygous Brattleboro transplant (B vs A), the comparable staining intensity of the transplanted SCN for VIP between heterozygous Brattleboro and homozygous Brattleboro transplants (C, D), the similar staining pattern for somatostatin and the presence of intensely stained somatostatinergic cells (arrowhead) (E, F), and the absence or low density of somatostatin staining (asterisk) in the area of VIPergic and/or VPergic SCN cells (A, C, E and D, F). The presented heterozygous Brattleboro transplant failed to restore circadian drinking behavior in the recipient SCN-lesioned Wistar rat, the homozygous Brattleboro transplant did. T, transplant; III, third ventricle (filled with gelatin of the embedding procedure); OC, optic chiasm. Dashed line indicate border of transplant. Bars indicate 200 μm.
Chapter 3

Figure 2. Complementary presence of VPergic and VIPergic SCN neurons in a heterozygous Brattleboro transplant and comparison of VP staining intensity with that of the VPergic neurons of the supraoptic nucleus of the recipient rat. This particular recipient Wistar rat revealed recovery of drinking behavior rhythm, the extent and staining intensity of the transplanted heterozygous Brattleboro SCN was not different from that in non-recovered rats (cf. Fig. 1A,C). T, transplant; III, third ventricle; OC, optic chiasm; SON supraoptic nucleus. Dashed line indicate border of transplant. Bar indicates 200 μm.

intensity between heterozygous Brattleboro and homozygous Brattleboro SCN transplants was comparable (Figs. 1C/2B vs. 2D/3C).

The double-blind observations on the presence of CPP immunostaining confirmed the VP deficiency of the homozygous Brattleboro SCN transplants as well as the presence of VPergic cells in the heterozygous Brattleboro SCN transplants. CPP-containing cell clusters of the heterozygous Brattleboro transplants were always situated in complementary fields of the area in which in the alternate sections VIP-containing cells were present (Figs. 1A,C and 2), thereby resembling the cytoarchitecture of an intact SCN. CPP-immunopositive fiber plexuses were also present in the heterozygous Brattleboro SCN cell cluster areas and fiber outgrowth had occurred within the transplant as observed for VIP fibers.

Perikaryal and fiber somatostatin staining was found throughout the transplant without differences between heterozygous and homozygous Brattleboro genotyped tissue (Fig.
Figure 3. High magnification comparison of VIP and somatostatin staining pattern in the transplanted SCN.

The area of the VIPergic cells is often hardly stained for somatostatin (heterozygous Brattleboro transplant, A,B), however in 34% of the cases, independent of the heterozygous or homozygous genotype of the transplant, a group of weakly stained somatostatinergic cells (asterisk) is visible, characterized by a halo of absence of staining (homozygous Brattleboro transplant C,D). See also Fig. 1. Note the presence of the intensely stained somatostatinergic cells in the transplant that resemble cells (arrowheads) lining the host ventricular ependyma (B). T, transplant; III, third ventricle. Dashed line indicate border of transplant. Bar indicates 50 μm.
Chapter 3

1E,F). The fiber staining was intense and numerous plexuses were visible. Strongly stained cells were present as single cells or in groups, the morphology of which resembles the somatostatinergic neurons lining the ependyma of the posterior part of the third ventricle of the host (Fig. 3B). Weakly stained cell groups were present as well. In most transplants, these areas did not match the location of the SCN VIP/VPerigic neuronal complex in the alternate sections (Fig. 1). Some of these somatostatinergic cell clusters however did match the location of the VIP/VP-positive cell cluster, the cluster was then characteristically surrounded by an halo of tissue devoid of somatostatin fiber staining (Figs. 1 and 3).

Recovery of circadian rhythm
From the 8 rats that had a heterozygous Brattleboro SCN transplant, 3 rats revealed significant recovery of circadian drinking behavior with an onset around 20 days post-grafting and an average period length ~ of 23.8 h (SEM 0.09) as determined between week 7-15 post-grafting (Table 1). From the 18 homozygous Brattleboro SCN-grafted rats, 6 rats showed rhythm recovery, resulting a similar percentage of recovery as in the homozygous Brattleboro graft group (33 vs. 38%, $\chi^2 0.22$, P 0.64). Onset of re-instatement of drinking rhythm occurred between 20 and 50 days, i.e. often later than seen for the heterozygous Brattleboro-grafted group (Fig. 4), whereas the average of 24.3 h was significantly longer...

| Table 1. Survey of the results for SCN-lesioned arhythmic Wistar rats revealing re-instatement of their circadian drinking rhythm following placement of homotopic SCN-containing grafts dissected from either control heterozygous or VP-deficient homozygous E17 Brattleboro rat fetuses. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Rat number     | Latency to      | Period length   | Transplant      | Apposition of   | Residue lesioned |
|                | recovery (days) | (h)             | location        | grafted SCN to  | host SCN        |
| Heterozygous   |                 |                 |                 | host brain      |                 |
| Brattleboro-grafted (control) |
| 704            | 20              | 23.8            | 3V+MS           | 3V/ventral      | -               |
| 713            | 25              | 24.0            | 3V              | hypothalamos    | -               |
| 723            | 20              | 23.7            | 3V              | intra-transplant| -               |
| Homozygous     |                 |                 |                 |                 |                 |
| Brattleboro-grafted (VP-deficient) |
| 304            | 30              | 24.5            | d3V+PTV         | intra-transplant(1) | -               |
| 309            | 50              | 24.2            | 3V              | ventral hypothalamos(1) | -               |
| 310            | 30              | 24.2            | 3V+PTV          | intra-transplant(2) | -               |
| 330            | 20              | 24.0            | 3V              | ventral hypothalamos(1) | -               |
| 710            | 20              | 24.3            | 3V              | intra-transplant(2) | -               |
| 741            | 25              | 24.7            | 3V              | ventral hypothalamos(2) | -               |

Total number of SCN-grafted arhythmic rats was 8 and 18 animals for the heterozygous and homozygous Brattleboro group respectively. Latency to recovery is based on visual inspection of the drinking rhythm actograms. Average values for the period length $\tau$ for the heterozygous and homozygous Brattleboro groups are respectively (± SEM) 23.8 ± 0.09 and 24.3 ± 0.10 h. Apposition data are given for each SCN VIPergic cell cluster of the transplant (number between brackets in case of more than one), intra-transplant means no apposition to host hypothalamos. The presence of a host SCN lesion residue concerns small groups of cells primarily in the ventral aspect of the original nucleus near the optic chiasm. 3V, ventral aspect of third ventricle; d3V, dorsal aspect of third ventricle; MS, medial septum; PVT, periventricular area of the thalamus.
Vasopressin-deficient suprachiasmatic nucleus neurografting

(SEM 0.10, P<0.01; Student t-test, Table 1). Circadian drinking behavior was weak as compared to that observed in intact rat under the same conditions (data not shown).

Both recovery and non-recovery heterozygous or homozygous Brattleboro SCN-grafted animals had a transplant in which VIPergic SCN neurons were present. The number of SCN cell clusters within the transplant as well as the apposition of the SCN areas to the host brain was variable (Table 1). VIP-positive fibers had grown from transplant to host parenchyma in 6 out of the total of 9 rhythm-recovered rats. From the three heterozygous Brattleboro SCN-grafted recovery rats, one had no discernible transplant-to-host VIP fiber connection, but all revealed CPP fiber outgrowth to the host brain. The density of fiber outgrowth was generally very sparse, and only occasionally clearly recognizable as sprouting bundles (Fig. 5). Fibers were directed dorsally to the periventricular area of the hypothalamus.

VIP fibers passing the transplant/host brain interphase were also found in 13 out of 17 cases without recovery of rhythm. Thus, this finding was not particularly correlated with recovery of circadian drinking rhythm ($\chi^2 0.43$, P 0.51). Also other matches failed, such as with (i) the extent of SCN neuron mass in the transplant, (ii) the site of integration of the transplant with the host hypothalamus, (iii) the location of the SCN within the transplant (ventral, dorsal or lateral apposition towards host brain, or central presence, cf. Table 1), (iv) the presence or absence of the weakly stained somatostatinergic cell group within the hypothalamus.

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Figure 4. Examples of recovery of circadian drinking behavior in SCN-lesioned arhythmic Wistar rat after homotopic grafting of SCN tissue dissected from heterozygous or homozygous Brattleboro E17 fetuses.

Figures are double-plotted actograms of the frequency per 30 min of drinking nipple contacts monitored under DD conditions, before and after grafting (arrow). Recovery of circadian drinking activity by the fetal heterozygous Brattleboro SCN tissue occurs around 20 days after the grafting surgery, by fetal homozygous Brattleboro SCN tissue onset of recovery varied between 20 (homozygous Brattleboro no. 2) and 50 days (homozygous Brattleboro no. 1).
area of intense VIP or CPP cell staining, (v) the presence of other characteristics of the somatostatin staining pattern, or (vi) the presence of some residual SCN cells in the recipient rat (cf. Table 1).

Discussion

Homotopic grafting of the fetal VP-deficient homozygous Brattleboro SCN can re-instate circadian drinking rhythm of SCN-lesioned arhythmic Wistar rats. The percentage of animals that showed recovery was similar to that in the group of control heterozygous Brattleboro SCN-grafted animals (33 vs. 38%), and quite comparable to the recovery percentage of approximately 40% reported in earlier similarly performed Wistar-to-Wistar studies [8,19]. The biological clock mechanism of the homozygous Brattleboro SCN, at least in the cases of rhythm recovery, appeared therefore to ‘tick’ circadianly, and is able to transfer its rhythm to the host brain. Thus, as expected, the lack of VP in the SCN did not hamper the graft to mediate functional recovery. The current findings confirms that VP is not a signal of crucial importance either for the intrinsic circadian pacemaker activity of the SCN nor for transfer of rhythm to host target areas. The difference in period length $\tau$ of the circadian activity between the heterozygous and homozygous Brattleboro SCN graft-mediated rhythm-recovered Wistar rats (24.3 vs. 23.8), in the same magnitude of that described for intact adult heterozygous and homozygous Brattleboro rats (25.4 vs. 24.8 h) [21], could indicate that the fetal SCN develops its intrinsic rhythm mechanisms without the influence of endogene VP hormonal signals and/or of intercellular VP neurotransmission.
Period length
The longer period length $\tau$ of the intact homozygous Brattleboro rat is reported not to be shortened by supplementation of the circulating VP [21], so that the value of $\tau$ seems a particular characteristic of the mutant not related to the absence of hormonal VP in adulthood. Grafting studies with $\tau$au mutant hamsters and across rodent species indicated $\tau$ to be genetically determined [34,42]. The longer $\tau$ of the circadian rhythm of recovered homozygous Brattleboro-grafted compared to that of heterozygous Brattleboro-grafted rats may therefore be another example of the finding that the donor animal determines the period. However, the $\tau$ of recovered circadian rhythm also depends on the SCN volume of the transplant [16] and can still have been influenced by the abnormal maturational conditions for the fetal SCN in the adult host or the allograft situation [6,8,9,42]. Presently, VIP expression in the heterozygous and homozygous Brattleboro transplants is not conspicuously different (as for the in situ SCN of both genotypes [27]), so that differences in cell survival and maturation of the grafted SCN have therefore unlikely contributed to the differences in $\tau$. Although the present outcome seems to indicate that the homozygous Brattleboro E17 SCN develops its intrinsic rhythm independent of the presence or absence of VP, one cannot yet completely exclude that the absence of VP expression and release within the SCN during the development of the homozygous Brattleboro rat would not have altered aspects of its clock system [9]. Circulating VP of the Wistar host cannot replace intra-SCN synaptic VP neurotransmission, and presently only circadian drinking rhythm parameters have been studied. It is for instance known that neonatal VP antagonist treatment, blocking the SCN V1a receptors, permanently increases the number and VP expression of the VPergic SCN neurons up to adulthood [30]. However, so far no studies have been reported to investigate whether this treatment affects $\tau$ or any other circadian rhythm parameter. In a study on the level of expression of VP in the SCN among rat species, the $\tau$ of wheel-running activity rhythm appeared to correlate positively with VPergic cell content [52].

Possible role of vasopressin
The re-instatement of circadian drinking rhythm by VP-deficient SCN transplants cannot be interpreted as VP playing no role in the circadian system of normal rat. VP-containing synapses [14] and VP receptors [53] are normally present in the SCN, and VP is released in a circadian fashion within the in situ SCN [25], where it can act excitatory on SCN cells [22]. However, it is not the ruling factor in the pacemaker system of the SCN. Intranuclearly released VP may serve to modify and/or to synchronize the intrinsic pacemaker activity. The observed dampened circadian rhythm of spontaneous bioelectrical activity in the in vitro homozygous Brattleboro SCN, i.e. in the absence of VP, may support this [22]. Also the relatively poor rhythm of the recovered heterozygous Brattleboro and homozygous Brattleboro SCN-grafted rats in the present study (partial and no VP expression respectively) as compared to those of previous studies with Wistar-to-Wistar grafts [8,9], and observations that low expression of VP in the SCN results in less stringent behavioral rhythm [13,18,52], could be indications for such an intra-SCN role of VP. However, it could also relate to
differences in the output strength at the SCN target areas, since the SCN has an extensive VPergic afferent system. These pathways to e.g. the subzone of the paraventricular nucleus in the hypothalamus, the dorsomedial hypothalamus and the periventricular area of the thalamus are thought to be involved in autonomic nervous system control, hormone secretion, and sleep/wake and motor activity [12]. Thus, VP may serve to modulate yet another output signal in these SCN afferent pathways. Nearly all peptidergic cells of the SCN are GABAergic [44], whereas GABA amplifies the oscillations in the firing rate of the SCN [50]. An interplay with VP may be postulated.

Rhythm recovery and structure of the suprachiasmatic nucleus of the transplant

Nearly all SCN transplantation studies have tried to find a correlate between the parameters of the morphology and host connectivity of the grafted SCN and the presence or absence of circadian rhythm recovery and, if recovery was observed, the strength and entrainability of the rhythm. This proved to be a topic of continuous debate, since results are not yet conclusive. Homotopic graft placement seems not of importance for restoration of circadian activity in SCN-lesioned hamster [1,2], but heterotopic placement in lesioned rat never revealed an effect [7,8,19]. The presence of a VIP-positive SCN appeared to be a necessary but not a sufficient condition for recovery of circadian activity in SCN-lesioned rodents [17,29,40], especially in rat [19]. The same is reported for the VIPergic and VPergic fibers crossing the transplant/host brain interface [8,19,42]. Both statements are presently confirmed. The importance of efferent connections has been challenged by the observation that a yet undefined diffusible signal from the SCN transplant appeared sufficient for recovery of rhythms in hamster [39]. Hypothetically it may be possible that at the denervated SCN projection areas of the rhythm-recovered hosts, circadian humoral signals arrive that are released from SCN neuronal processes within the transplant and mimic a synaptic input. The diffusible humoral replacement is obviously not fully matching the normal circadian input. In fact, restoration of the circadian clock system by SCN neurografting has sofar been found limited to rhythms in wheel-running activity and eating and drinking behavior and is not reported for other measures.

Somatostatinergic neurons of the SCN appeared another important cell group for the circadian time-keeping system. Temporal profiles of somatostatin expression resemble that of VP, but with a peak time of somatostatin mRNA preceeding that of VP mRNA, and both show endogenous circadian rhythmicity independent of photic information, which is not observed for VIP expression [23]. Somatostatinergic cells form a distinct group [45] and are located in the ventral part of the 'core' of the SCN, the area supposed to include the pacemaker cells [31]. They send their axons to the VPergic and VIPergic neurons of the SCN [15]. Somatostatinergic neurons may therefore even be good candidates as circadian oscillator cells. Previously we reported that within the VPergic/VIPergic cell complex of the transplanted SCN, somatostatin immunostaining is virtually absent and that somatostatinergic SCN-like cell clusters often show up in the transplant at some distance [8,19]. The heterozygous and homozygous Brattleboro SCN transplants confirmed this observation, but, possibly by improvement of our staining methods, sometimes weakly
stained small cluster of somatostatinergic cells were identified in the VIP/VP-stained SCN area. These cells showed very limited neuritic outgrowth. In fact, a halo of absence of somatostatin staining seem to indicate their local and isolated function. This halo contained the VIPergic and (in case of the homozygous Brattleboro transplant possibly the mutant) VPergic SCN neurons, so that communications with the somatostatinergic cells can take place. Presence or absence of somatostatinergic cell staining in the VIP/VPergic cell area could however not be correlated with recovery of drinking rhythm of the animal. Thus, no further clue on the possible primary oscillator function of these cells could be given. Since above observations could be made both in heterozygous and homozygous Brattleboro transplants, the immunocytochemical parameters of the homozygous Brattleboro SCN appeared not differently affected by the growth conditions of the host.

Conclusions

In conclusion, the recovery of circadian drinking behavior of the SCN-lesioned rat by the application of VP-deficient SCN grafts re-establishes that VP is not the crucial factor maintaining the intrinsic circadian pacemaker activity in the SCN biological clock system, nor that it is a crucial component in transfer of circadian activity. As in several previous transplantation studies, no undisputable correlation emerges between particular VIP, VP (in the heterozygous Brattleboro controls) and somatostatin immunocytochemical characteristics of the transplanted SCN and the occurrence of recovery of rhythm in the SCN-lesioned/grafted arhythmic animal. The recent evidence that somatostatin may be a key substance for the circadian time-keeping system in rat, could neither be supported or rejected on the basis of the present experiments. The observed genotypic difference in period length $\tau$ of the circadian activity between the heterozygous and homozygous Brattleboro SCN graft-mediated rhythm-recovered Wistar rats as described for adult heterozygous and homozygous Brattleboro rats [21], seem to indicate that the fetal SCN develops its intrinsic rhythm mechanisms independently, that is without the influence of endogene VP hormonal signals. However the requirement of intercellular VP neurotransmission for the fine-tuning of the rhythms can not completely be excluded.

Yet, the present findings do not exclude the VPergic neurons, rather than their VP signal to be of pacemaking importance. Indeed, specific ablation of VPergic neurons in normal rat SCN has recently shown to abolish circadian rhythm of drinking behavior [38]. In the homozygous Brattleboro mutant rat, the cells that would normally produce VP are still present [48] and may thus act in the neuronal timing system using another messenger like GABA [44,50]. The function of the VP signal in the circadian system will then most probably be modulatory.

References


Chapter 4