The Suprachiasmatic Nucleus in Organotypic Slice Cultures of the Common Vole (Microtus arvalis): Comparison of Development with Rat and Hamster and the Effect of Age

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Abstract
The intrinsic properties of the suprachiasmatic nucleus (SCN), the site of the main circadian pacemaker in mammals, have recently been studied in vitro by means of organotypic slice culturing. So far, only neonatal rats and mice have been used for such developmental and functional analyses of the isolated pacemaker. Here, the authors present a comparative developmental study of the SCN of voles, rats, and hamsters in organotypic slice cultures. In contrast to strictly circadian organization of behavior in rats and hamsters, common voles (Microtus arvalis) are characterized by large variability in the strength of circadian organization of behavior. It is not known to what extent this variability is reflected in the intrinsic features of the SCN. Cultures were prepared from rat, hamster, and vole pups (6 to 9 days old) for the purpose of species comparison. In addition, the authors studied the relation between age and development in cultures from pup (7 to 10 days old), juvenile (15 to 16 days old), and young adult (1 to 2 months old) voles. In contrast to the situation in rat and hamster, the most striking feature in neonatal voles is the variability in shape of the final, fully developed culture and its poor resemblance with the in vivo SCN. The SCN of adult voles, however, could be cultured successfully while retaining its morphological organization seen in situ. Phase-contrast microscopy and immunocytochemical staining for vasopressin and glial fibrillary acidic protein revealed that cultures of pup and juvenile voles still have potential for neurogenesis and morphological reorganization. Young voles, therefore, can serve as a model to study the developmental establishment of a functional circadian pacemaker, while adult voles allow the study of intrinsic pacemaker properties in relation to previously recorded behavior of the donor and aging-related pacemaker dysfunction.

Key words: vasopressin, circadian pacemaker, glial cells, development, morphology, rat, Syrian hamster, common vole

The organotypic slice culture technique allows the assessment of intrinsic tissue properties because much of the in vivo tissue morphology is retained in organotypic slice cultures (Gåhlwier, 1984; Gåhlwier et al., 1997). This is of particular interest for the in vitro study of the suprachiasmatic nucleus (SCN), the major circadian pacemaker of mammals governing both behavioral and physiological circadian rhythmicity. Organotypic slice cultures of rat SCN have been widely used to study the morphological development of the isolated pacemaker and to determine circadian release patterns of neuropeptides and amino acids.
Central in this study is SCN tissue taken from common voles (*Microtus arvalis*) of different ages. Voles were used because of a clear individual variability in the strength of circadian organization of running-wheel activity (Gerkema et al., 1990, 1993), which correlates negatively with the number and staining intensity of arginine-vasopressin (AVP) positive cells in the SCN of young individuals (Gerkema et al., 1994). It is not yet known, however, to what extent the variation in circadian organization of behavior is related to intrinsic properties of the underlying pacemaker. Recent neuropeptide release data obtained from neonatal vole (6 to 7 days old) organotypic SCN tissue revealed the absence of vasoactive intestinal polypeptide (VIP) rhythmicity and considerable variability in AVP rhythmicity (Gerkema et al., 1999), which may suggest the absence of an organotypic nature of neonatal vole SCN in vitro in contrast to rat.

The main goal of the current article is to describe the developmental characteristics of the isolated vole circadian pacemaker in vitro. To our knowledge, only literature on rat SCN explants cultured in the roller tube is available. Therefore, one objective of this article is to study and compare the morphological development of SCN cultures of vole, rat, and hamster pups under identical culture conditions. The choice of rat and hamster is based on the difference of their circadian organization of rhythmicity, which allows comparison of the organotypic nature of pacemaker systems with different circadian strength. Hamsters have been used intensely in chronobiological research because of their strong expression of circadian rhythmicity. The rat, which has a tendency toward a bimodal distribution of circadian activity, is the main species in biomedical research, and rat SCN culturing has been thoroughly described in the literature (Tominaga et al., 1992; Tominaga et al., 1994a, 1994b, 1994c; Okamura et al., 1994a, 1994b; Wray et al., 1993; Shimohara et al., 1994; Belenky et al., 1996; House et al., 1998). The other objective of this article is to study effects of age on morphological development of vole SCN in vitro in order to be able to evaluate the feasibility of organotypic culturing of adult SCN tissue (and SCN tissue of old voles, which nearly lack AVP cells; Van der Zee et al., 1999) for future correlational studies with circadian behavioral characteristics of the donor and aging-related dysfunction at pacemaker level.

**METHODS**

**Experimental Design**

In experiment 1, pups of rats (*n* = 6 females and 6 males; age 6 to 7 days postnatal), hamsters (*n* = 7 females; age 7 to 9 days postnatal), and common voles (*n* = 6; 4 females and 2 males; age 6 to 7 days postnatal) were used. The voles (*M. arvalis*) were taken from a colony maintained in Haren that is based on individuals trapped in the Lauwersmeer, the Netherlands (53°20′N; 6°16′E). The hamsters (*Mesocricetus auratus*) and Wistar rats (*Rattus norvegicus*) were obtained from breeding colonies in Haren. Each neonatal animal, one SCN slice, containing the major part of the nuclei, was taken to culture. The morphological development of the cultured SCN was followed during 27 consecutive days. In experiment 2, the SCN of common voles of different ages (all males) were cultured. Voles were grouped in three age categories: group A (pups: age 7 to 10 days postnatal; *n* = 6), group B (juveniles: age 15 to 16 days postnatal; *n* = 10), and group C (young adults: age 1 to 2 months, *n* = 9). Each brain slice (cut coronally) containing SCN material was taken to culture. Hence, 45 cultures were prepared out of 25 voles (i.e., 20 “double cultures” were prepared, containing either the anterior or the posterior part of the SCN). In addition, one SCN culture of a vole at the age of 5 months (middle-aged; Van der Zee et al., 1999) was studied. The cultures were processed immunocytochemically for AVP or glial fibrillary acidic protein (GFAP) at the end of the experiments. Cultures in experiment 1 were immunoprocessed 27 days after explanting and, in experiment 2, 14 to 23 days after explanting. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and all experiments were approved by the Animal Experimentation Committee of the University of Groningen (Dec. No. 2091).

**Preparation of Suprachiasmatic Slice Cultures**

Organotypic slice culturing of rodent SCN was performed using the roller-tube technique as described by Shimohara et al. (1994, 1995). In short, the brains were quickly removed and a coronally cut hypotha-
lamic slice of 400 µm containing the SCN was obtained from different species at different ages (see experiment description) using a tissue chopper. Slices containing the SCN were placed in G ey’s balanced salt solution (G ibco Labs, G rand Island, N Y) and refrigerated for 1 h. Slices were embedded in a plasma clot (chicken plasma + thrombin Sigma) on a coverslip and placed in a culture tube (N unc) with 700 µl of culture medium (25% horse serum—heat inactivated at 68°C [G ibco], 50% Eagle’s basal medium [Sigma] with 62 mM D-glucose and 4.16 mM NaHCO$_3$ and 25% Hanks’ balanced salt solution [G ibco] with 4.16 mM NaHCO$_3$). SCN slices were cultured at 36°C with rotation (12 revolutions/h). In all cases, medium was replaced 1 to 2 times a week, using the shift in color of the medium from red (fresh medium) to yellowish (depleted medium) as an indicator for the quality of medium.

Immunocytochemical Procedure

SCN cultures attached to coverslips were fixated in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) in culture tubes for 2 to 4 h. Thereafter, cultures were rinsed in phosphate-buffered saline (PBS; pH 7.4), preincubated with normal goat serum (5%), and incubated with primary polyclonal IgG antibody rabbit anti-AVP (Tuus ’86; 1:1000; G erkema et al., 1994; Jansen et al., 1998) or rabbit anti-G FA P (1:500; D akopatts) overnight at 36°C in culture tubes in the roller drum. Then, cultures were thoroughly rinsed with PBS and preincubated again with normal goat serum (5%) and incubated with biotinylated goat anti-rabbit IgG (1:500; Z ymed) for 2 h at 36°C in culture tubes in the roller drum. After rinsing in PBS, the sections were exposed to HRP-conjugated streptavidin (1:500; Z ymed) for 2 h at 36°C in the roller drum. Triton-X-100 (0.5%) was added during all incubation steps. Finally, the sections were processed by diaminobenzidine (D AB)-H$_2$O$_2$ reaction (30 mg D AB and 0.01% H$_2$O / 100ml PBS) at room temperature, guided by visual inspection.

Light Microscopic Analysis and Quantification

The morphological development of the SCN cultures during experiment 1 was followed daily by light microscopic inspection. Drawings as well as photomicrographs were made by phase-contrast microscopy. At the end of experiments 1 and 2, cultures were immunoprocessed. The number of A VP- and G FA P-positive cells were counted in the cultured SCN and expressed per 0.1 mm$^2$. The longest axis of AVP-positive cell bodies was measured and taken as the diameter of the cell. Quantification of the density of G FA P processes in the neuronal zone of vole, rat, and hamster pup SCN and young adult vole SCN was estimated by way of counting line crossings. For this purpose, an ocular grid was used and the density of processes was randomly determined in the neuronal zone at 16 positions. The used lines had a length of 65 µm. A nalysis of variance followed by a post hoc S tu dent’ s t-test was used with a probability level of $p<0.05$ used as an index of statistical significance.

RESULTS

Experiment 1: Comparison of Vole SCN Cultures with Rat and Hamster

Morphological Analysis

The SCN was easily recognized morphologically under the stereomicroscope in hypothalamic brain slices at postnatal days 6 to 9 in all three species. After explanting the SCN, the morphology of the cultures was studied by means of phase-contrast microscopy on a daily basis for 27 days. All 19 cultures survived during the course of the experiment. On day 2, the slices became somewhat swollen and clouded, an indication for degeneration of cells damaged at the cut surfaces of the slice. This phenomenon was seen in all cultures of the three species. Thereafter, on days 3 to 5, the slices began to spread out. After 8 to 10 days, rat and hamster cultures developed with only minor morphological changes with time, whereas it took 20 to 24 days for vole cultures to fully develop and to become morphologically stable (i.e., the final stage in which the occurrence of gross morphological changes is terminated) (Figs. 1 and 2).

In rat and hamster, a clear morphological organization in ependymal zone, neuronal zone, and dispersed cell zone was seen. The development of the vole cultures, however, deviated from that of rat and hamster. In the vole, the final shape and size of the cultured SCN were highly variable. The surrounding non-SCN tissue started to degenerate 1 week after explanting. This was reflected in the appearance of lacunae in the tissue, which were traversed by long processes arising from G FA P-positive cells (see immunocytochemical
After 2 to 3 weeks, nearly all surrounding tissue had disappeared, and predominantly the SCN cells (the neuronal zone) survived in an isolated, clusterlike, and multilayered fashion. The distended extracellular space between the neuronal zone and the dispersed cell zone could be several hundred micrometers wide. Different patches of neuronal zones could be encountered in four of the six cultures, which varied in shape from circular to elongated, multipolar structures. At the edge of the cluster, a layer of large cells oriented perpendicularly to the cluster (the so-called radial arrays) was always present (Figs. 2 and 5a). Judged under phase contrast microscopic inspection, these radial arrays appeared to consist of living cells and a mixture of thin and relatively broad neural processes. The width of this layer varied from 0.1 to 0.5 times the size of the neuronal zone.

Patterns common for each species are presented in Fig. 2. It should be noted, however, that 2 of the 12 rat cultures and 2 of the 7 hamster cultures showed a neuronal zone with a clusterlike organization, which less clearly resembled the SCN in vivo. Small distended extracellular spaces with radial arrays of cells were seen in parts of these rat cultures and in one of these hamster cultures. Hence, 83% of the rat cultures and 71% of the hamster cultures (but none of the vole cultures) showed a clear topographic organization of the SCN like in vivo.
In all cultures, independent of the species, the density of the cells within the SCN was higher than that of the cells adjacent to the SCN, which originally belonged to co-cultured tissue of the surrounding hypothalamus. The multilayer organization of the neuronal zone was most apparent in the cultures of the common vole and least in rat cultures. Cultures from hamster had intermediate values in SCN cell density. At the end of the experiment, 27 days after explanting, the thickness of the SCN was highest in the vole, intermediate in the hamster, and lowest in the rat (Table 1). Only in the rat, almost transparent, monolayer cultures were found. The optic chiasm, present at the time of dissection in all species, was absent at the end of the experiment in all vole cultures, but remnants of the optic fibers could be found in most of rat and hamster cultures.

In the first days of culturing, the ependymal cells of the third ventricle could be recognized in all cultures. These cells were rearranged or proliferated outward from the wall of the third ventricle and characteristically formed aggregates or tiers encircling a small lumen. A circulation of fluid, probably due to pulsating activity of ependymal cells, was visible within such aggregates in most cultures. In the vole, however, only occasionally a clear ependymal zone remained visible. This zone usually became incorporated in one of the separated clusters after 8 to 12 days of culturing (Fig. 2).

Immunocytochemical Analysis

AVP-immunoreactive (AVP-ir) cells were seen in all cultures (Fig. 4). In general, AVP immunoreactivity was predominantly present in the neuronal zone. Some scattered AVP-ir cells were found in the dispersed cell zone, but the ependymal zone was always devoid of AVP-ir cells. In the vole, the several patches or clusters of neurons always contained AVP-ir cells (Figs. 4a and 5b), which suggests that these clusters are separated parts of the original SCN. Fine AVP-positive fibers lacking obvious varicosities were occasionally seen in the neuronal zone in rat and hamster SCN cultures. In vole SCN cultures, some darkly stained AVP-ir cells gave rise to fibers with varicosities, which could be followed in the neuronal zone over short distances (20 to 40 µm) only.

Immunocytochemistry revealed that the cells in the radial arrays were predominantly AVP-positive. GFAP-positive cells were intermingled and gave rise to radially oriented processes (Fig. 5). The broad processes represent dendrites, whereas the thin processes are of radial glial origin (Fig. 5c, d).

To estimate the numerical density, the number of AVP-immunopositive cells was counted in a square of 0.1 mm² projected on the neuronal zone. This measure was taken instead of the total number of AVP-ir cells since this number depends largely on the (variable) size of the cultured SCN. The numerical density of AVP-ir cells was significantly higher in vole than in rat and hamster (p < 0.05, Student’s t-test, Table 1; vole AVP cell number was 5.2 and 3.5 times higher than that of rat and hamster, respectively). Taking into account the thickness of the neuronal zone for the three species, the number of AVP cells was still significantly higher in voles than in rat and hamster (p < 0.05, Student’s t-test, Table 1; corrected vole AVP cell number per cell layer was 1.6 and 2.1 times the numbers of, respectively, rat and hamster). Diameters of AVP-ir cells of rat, hamster, and vole did not differ significantly (Table 1).

In all species, large GFAP-ir cells of the protoplasmic type (Figs. 5d and 6d) were found in the dispersed cell zone and smaller GFAP-ir cells of the fibrous type in the neuronal zone (Fig. 6f). GFAP-ir cells were also detected inside the plasma clot surrounding the dispersed cell zone or lacunae in all species. In the neuronal zone, a dense plexus of GFAP-positive fibers was present with scattered GFAP-positive cell bodies (Fig. 6e, f). Bundles of GFAP-positive fibers bridging

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Table 1. Morphological and immunocytochemical characteristics of SCN cultures obtained from rat, hamster, and vole pups.

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Number of Cell Layers in the Neuronal Zone</th>
<th>Number of AVP Cells per 0.1 mm² in the Neuronal Zone</th>
<th>Number of AVP Cells per Cell Layer</th>
<th>AVP Cell Diameter (in µm)</th>
<th>Number of Crossings of GFAP Processes</th>
<th>Number of Protoplasmic GFAP Cells per 0.1 mm² in the Dispersed Cell Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (12)</td>
<td>1-3</td>
<td>13 ± 2</td>
<td>6.5 ± 1.0</td>
<td>13.20 ± 0.35</td>
<td>14.3 ± 0.8</td>
<td>3.4 ± 1.3</td>
</tr>
<tr>
<td>Hamster (7)</td>
<td>3-5</td>
<td>19 ± 5</td>
<td>4.8 ± 1.3</td>
<td>14.63 ± 0.58</td>
<td>17.5 ± 1.4</td>
<td>6.2 ± 1.8</td>
</tr>
<tr>
<td>Vole (6)</td>
<td>3-10</td>
<td>67 ± 7*</td>
<td>10.3 ± 1.1*</td>
<td>12.88 ± 0.28</td>
<td>31.5 ± 3.5*</td>
<td>18.7 ± 2.6*</td>
</tr>
</tbody>
</table>

**NOTE:** Mean ± SEM. *p < 0.05.
parts of the neuronal zone and the dispersed cell zone were seen in cultures of all three species, but most frequently in rat (Fig. 6e). In general, GFAP immunoreactivity in the SCN was dense in vole, moderate in hamster, and low in rat. Quantification of the density by counting the crossings of GFAP-ir processes revealed a significantly ($p < 0.05$; Student’s $t$-test) higher density in vole cultures compared with rat and hamster (Table 1). In the vole, the divergence in terms of cell morphology and staining intensity of GFAP-positive elements was large. The four most frequently encountered types of GFAP-positive cells are depicted in Fig. 6a, d. In some cases, the large protoplasmic type of GFAP-ir cell was seen with nuclei in close proximity, which suggests mitotic activity (Fig. 6d). This was most often observed in vole and occasionally in hamster, but only rarely in rat. In line with this observation, the number of protoplasmic GFAP-ir cells per 0.1 mm$^2$ area in the dispersed cell zone was highest in the vole, intermediate in the hamster, and lowest in the rat (Table 1).

Experiment 2 Effect of Age on Vole SCN Culturing

Culture development time needed to arrive at a stable condition of the cultures decreased with increasing age. In group A (7- to 10-day-old pups), B (15- to 16-day-old juvenile animals), and C (1- to 2-month-old young adult animals), it took 20 to 24, 12 to 16, and 8 to 10 days, respectively, before this stage was realized (Fig. 3). Of all 45 cultures used in experiment 2, 43 (95.6%) survived until the end of the experiment. The development of the SCN cultures of voles of group A was similar to those of the voles of 6 to 7 days old described in experiment 1. The most striking feature in cultures of those vole pups is the variability in shape of the final, stable culture and its poor resemblance with the in vivo SCN. Examples of the three age groups are shown in Fig. 4a through c. Patterns common for each age group are depicted in a schematic representation in Fig. 3, although it should be noted that 7 out of 18 cultures of group B partly developed like those of group A (i.e., radial arrays of cells at the edge of the neuronal zone). The development and final shape of the SCN cultures of 7- to 16-day-old voles (groups A and B), therefore, are highly variable and morphologically unpredictable at the time of explanting. Only in cultures of group C, the topographic organization of the neuronal zone looked like that of the SCN in vivo (Figs. 4c through d, 6g). The thickness of the neuronal zone varied from 1 to 4 cell layers. A main difference with the cultures of groups A and B is the absence of cells radiating from the edge of the neuronal zone. As such, radiating cells are only seen when the neuronal zone is isolated from the dispersed cell zone. Moreover, also the organization of the surrounding hypothalamic tissue, usually reduced to a monolayer, resembles that in situ.

Comparison of the development of double cultures from one animal (containing either the anterior or the posterior half of the SCN) of groups A ($n = 3$) and B ($n = 8$) revealed no developmental similarities between the two SCN cultures. In other words, also different SCN explants taken from one individual vole do not develop in a predictable way. In contrast, little vari-
ability in development was found in double cultures of group C (n = 9).

The presence of AVP-ir fibers and varicosities in the SCN cultures increased with increasing age of the donor. In cultures of group A, very few AVP-ir fibers were observed, whereas in group C clear AVP-positive fibers with large and numerous (punctate) varicosities were found in the neuronal zone (Fig. 4C through E). Such fibers were also frequently seen in the surrounding hypothalamic tissue and in the dispersed cell zone (Fig. 4C). The main branch of some fibers could be followed up to 500 µm running through the culture. In the SCN of young adult voles, AVP-negative cells were found embraced by AVP-ir boutons such as those seen in vivo. This was not observed in group A and only occasionally in group B.

GFAP immunoreactivity revealed some differences in the neuronal zone between groups A and B compared with C. In groups A and B, few, relatively small (6-8 µm) GFAP-positive cells were seen in this area (Fig. 6F), which was covered by a dense plexus of GFAP-positive processes. In group C, numerous, relatively large (10-12 µm) GFAP-ir cells with a round appearance were present in the neuronal zone, but much less GFAP-positive processes could be detected here (Fig. 6G). The variation in types of GFAP-positive cells seen in the dispersed cell zone, however, was similar in all age groups. GFAP-positive cells of the protoplasmic type were predominant in the dispersed cell zone in all groups (Figs. 5d and 6d). They were frequently seen with nuclei in close apposition in cultures of all ages. Quantification of GFAP immunoreactivity in SCN cultures revealed a significantly lower number of GFAP-ir processes in the neuronal zone as well as the number of large GFAP-positive protoplasmic cells in young adults (12.1 ± 1.2 and 5.6 ± 2.4 per 0.1 mm², respectively) versus pups (31.5 ± 3.5 and 18.7 ± 2.6 per 0.1 mm², respectively; p < 0.05, Student’s t-test, Table 1).

One additional animal was 5 months of age at the time of explanting. In this culture, of which only AVP data are available, less non-SCN tissue survived than in the 1- to 2-month-old voles. The AVP cell distribu-
tion and appearance, however, did not differ from that of 1- to 2-month-old voles. The varicosities of the AVP-positive fibers were larger (up to 6 µm versus 3 µm in younger animals) and usually characterized by multiple empty spots or vacuoles, a feature rarely seen in SCN cultures of younger voles. Moreover, the varicosities had an aberrant appearance with more irregularly shaped axonal swellings (Fig. 4f).

DISCUSSION

Developmental Aspects of Vole, Rat, and Hamster SCN Cultures

The most striking findings of the present study are the remarkable age-dependent differences in morphological development of vole SCN in vitro and the successful culturing of the pacemaker of young adult (and even middle-aged) voles. These findings are not due to unusual culture conditions since the development and morphological organization of rat SCN cultures in this study resembled findings reported in the literature: the overall shape and thickness (one to three cell layers) of the rat SCN cultures and the morphological appearance and size of AVP and GFAP cells in this study are comparable with that described by others (Tominaga et al., 1994b, 1994c; O Kamura et al., 1994a, 1994b; W Ray et al., 1993). Moreover, a comparable percentage of rat cultures in our study and in the study of Tominaga and coworkers (1994c) (83% vs. 71%, respectively) showed a clear topographic organization like in vivo. AVP cell numbers were significantly higher in vole compared with those in rat and hamster, even after correction for the average differences in cell layer thickness of the neuronal zone for the three species. Such a difference in AVP cell density cannot be deduced from the literature on rat and vole SCN in vivo (Jansen et al., 1998; Madeira et al., 1997; Wollnik and Bihler, 1996). Neurogenesis of SCN cells expressing AVP is currently the most likely explanation for this difference (see discussion below).

Indications for mitotic activity of the large, protoplasmic type of GFAP-positive astrocytes were seen in voles of all studied ages. Mitotic activity in morphologically identical GFAP cells was proven with the marker 5-bromodeoxyuridine in organotypic slice cultures of the hippocampus (del Rio et al., 1991). The various shapes of GFAP cells is most likely related to different stages of migration and postmigratory differentiation in glial cells. Apparently, in vitro glial cell activity in the pup vole SCN is considerably higher than that in the SCN of rat and hamster pups or in juvenile and young adult voles.

It has been suggested that glial-neuronal interaction may play an important role in the generation and/or entrainment of circadian rhythms in the SCN (Lavialle and Serviere, 1995; Tamada et al., 1998; Prosser et al., 1994), although the number of glial cells in dispersed cell cultures proved not to be essential for the generation, expression, and synchronization of...
SCN functioning reflected in AVP release rhythms (Honma et al., 1998). Not much is known about a possible role of glia in SCN functioning. GFAP immunoreactivity is indicative of reactive astrocytes (but also radial glia; Rickmann et al., 1987) abundantly expressing this intermediate filament (Malhotra et al., 1990; Ridet et al., 1997). Our data suggest that the shape of vole SCN cultures is related to the density of GFAP processes and number of GFAP cells. Higher numbers of GFAP cells in the dispersed cell zone and a higher density of GFAP processes in the neuronal zone correlate positively with the degree of developmental reshaping of the SCN culture. GFAP cells are reactive astrocytes, which produce and release numerous neurotrophic factors (such as growth factors) and cell adhesion molecules (Ridet et al., 1997). Large quantities of these substances may be responsible for the formation of isolated SCN clusters, whereas lower quantities allow a more organotypic development of the SCN culture. Further studies are necessary to evaluate this possibility.

Vole SCN Cultures and the Effect of Age

Gähwiler (1984) showed that slices from rat brain did not preserve the organotypic structure when prepared at a developmental stage with neurogenesis and neuronal migration, whereas cultivation of brain tissue derived from rats older than 7 days needed oxygenation continuously. Tominaga et al. (1994b) reported that the choice of the developmental stage at which rat SCN slices are explanted is crucial for obtaining proper cultures (i.e., a time window of several days following the stage of neurogenesis and neuronal migration; rat pups of 8 days or older could not be cultured successfully). As a rule, therefore, only animals of 7 days old are being used for the preparation of SCN cultures. Here, we report that SCN explants of pup and juvenile voles (in contrast to those of adult voles) did not develop organotypically. The radial array of neurons extending into the periphery of these cultures could best be thought constituted by cells migrating away from the neuronal zone during the period of morphological reorganization, guided by radial glial-like cells. Radial glial cells and migrating neurons are typical features seen during ontogeny. Apparently, SCN explants of young voles are prone to extensive morphological development with characteristics that may suggest a return to an ontogenetic state. Also, the remarkable high number of AVP cells in cultures of young voles, notably at the periphery of the neuronal zone, indicates neurogenesis (e.g., of AVP neurons). Neurogenesis can occur in already developed brain tissue, as has been reported for the granule cells in adult rat hippocampus in vivo (Kuhn et al., 1996). Aiso in this region, GFAP-positive radial glia have been found (Rickmann et al., 1987), which probably subserve the same function as in ontogeny: guiding migrating neurons to their final destination.

What could set in motion the return to an ontogenetic state of (vole) SCN cultures? Horse serum, which makes up 25% of the medium in our experiments, contains (partly unknown) trophic factors (Kawahara et al., 1994). Trophic factors may stimulate mitotic activity of glial cells, which in turn produce neurotrophic agents as discussed earlier. The SCN of pup and juvenile voles may be more sensitive to such agents than rat and hamster SCN, while this sensitivity may be lost in adult vole SCN tissue. Interestingly, SCN cultures of juvenile vole SCN maintained in culture medium with 10% horse serum revealed minimal cell death and almost no mitotic activity of glial cells or neurogenesis and hence barely changed morphologically (E. A. Van der Zee, unpublished observations).

The results obtained with the juvenile, young-adult, and middle-aged voles indicate that at least for this species, organotypic SCN culturing is not restricted to an age of about 7 days only as originally suggested by Gähwiler (1984). The SCN of the young-adult vole retains its cytoarchitectural organization as in the brain, notwithstanding the loss of afferent neuronal (and hormonal) input and the processes of flattening and spreading that take place in vitro. Besides the reduction in AVP staining in the varicosities in the middle-aged vole, which may be related to the severe reduction in AVP staining in old voles in vivo (Van der Zee et al., 1999), the culture was as viable as that of young adult voles. Axonal aberrations, as seen in cultures of the middle-aged vole, are reported in other aging species in vivo as well (Van der Zee et al., 1997).

Important issues in chronobiology are the study of the morphological development and structural organization of the SCN and the transfer of SCN output to the rest of the brain. Cultures of vole pups, therefore, seem to be ideal for testing the effect of various trophic factors on morphological development and functional maturation of the SCN. Culturing adult vole SCN offers the possibility to correlate the strength of circadian organization of behavior with SCN features that can be studied best in vitro. Recently, AVP tempo-
ral release patterns in organotypic SCN cultures of pup and young adult voles have been established (Gerkema et al., 1999; K. Jansen, unpublished observations), indicating viability of cultured SCN slices of voles of different ages. The survival of SCN cultures of adult animals opens the avenue to study parameters such as peptide release patterns and electrical activity of behaviorally characterized subjects, as well as to study aging-related alterations in these parameters. A as a first step, we recently studied organotypic SCN cultures of circadian rhythmic and nonrhythmic young adult voles. A remarkable difference was found in their AVP content (Jansen et al., 2000), which corroborates earlier findings of the vole SCN in vivo (Gerkema et al., 1994).

Functional Considerations

As in embryonic/ neonatal SCN of rats and hamsters in vitro, morphological and functional maturation occurs in embryonic/ neonatal SCN grafts of both species, either in the presence or absence of an adult host SCN (Romero et al., 1993; A. Guilar-Roblero et al., 1992; Boer and G riffioen, 1990; Ralp et al., 1990; Silver et al., 1990). These observations led to the suggestion that the embryonic/ neonatal SCN is programmed to develop to maturity, even in isolation. The age-dependent development of vole SCN cultures suggests that the capacity for morphological reorganization of the SCN of pups and juveniles is not yet lost. Only at a higher age does the vole SCN in vitro retain an "organotypic" nature as seen in vivo. In voles of younger age, the SCN does not develop in an organotypic fashion, but rather in a "nucleo-typical" fashion or even in a "neuronal-typical" fashion in case of separated clusters of cells. In other words, the vole SCN is more "plastic" or "reactive" than that of rat and hamster. It is conceivable that this plasticity relates to the difference in the organization of timing of behavior between the species. In contrast to rats and hamsters, voles are characterized by strong ultradian organization of drinking, feeding, and locomotor behavior (Gerkema et al., 1990, 1993). A as a consequence, vole pups have to organize their behavior in an ultradian, rather than circadian, manner. This would leave a less important role for the (neonatal) SCN in voles compared with rat and hamster, which might be reflected in delayed loss of morphological plasticity of vole SCN tissue. It leaves us with the intriguing question whether flexibility in morphological development of the SCN is determined by the functional necessity of circadian organization of SCN tissue in a species-specific way.

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