ORGANOTYPIC SUPRACHIASMATIC NUCLEI CULTURES OF ADULT VOLES REFLECT LOCOMOTOR BEHAVIOR: DIFFERENCES IN NUMBER OF VASOPRESSIN CELLS

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ABSTRACT

This study is the first to demonstrate organotypic culturing of adult suprachiasmatic nuclei (SCN). This approach was used to obtain organotypic SCN cultures from adult vole brain with a previously determined state of behavioral circadian rhythmicity. We examined vasopressin (AVP) immunoreactivity in these organotypic slice cultures. AVP is one of the major neuropeptides produced by the SCN, the main mammalian circadian pacemaker. AVP immunoreactivity in the SCN of adult common voles in vivo has been shown to correlate with the variability in expression of circadian wheel-running behavior. Here, cultures prepared from circadian rhythmic and nonrhythmic voles were processed immunocytochemically for AVP. Whereas in all cultures AVP could be observed, AVP immunoreactivity differed considerably between vole SCN cultures. SCN cultures from rhythmic voles contained significantly lower numbers of AVP immunoreactive (AVPir) cells per surface area than cultures from nonrhythmic voles. The correlation between timing of behavior and AVP immunoreactivity in vitro is similar to the correlation found earlier in vivo. Apparently, such correlation depends on intrinsic AVP regulation mechanisms of SCN tissue, and not on neural or hormonal input from the environment, as present in intact brain. (Chronobiology International, 16(6), 745–750, 1999)

Key Words: Circadian wheel running—Common vole (Microtus arvalis)—Organotypic slice culture—Suprachiasmatic nucleus—Vasopressin.
INTRODUCTION

The hypothalamic suprachiasmatic nucleus (SCN) is the main circadian pacemaker in mammals. Vasopressin (AVP) is an important peptidergic product of the SCN and shows circadian fluctuations in content (Tominaga et al. 1992), release in vivo (Schwartz and Reppert 1985), and release in vitro (Earnest and Sladek 1987; Murakami et al. 1991; Shinohara et al. 1994; Gerkema et al. 1999). AVP-immunoreactive (AVPir) neurons in the SCN of circadian rhythmic common voles (Microtus arvalis) show time-dependent immunocytochemical changes (Jansen et al. 1998) that correspond with the release pattern of AVP in rats (Schwartz and Reppert 1985). Moreover, the number of AVPir neurons in the SCN correlates negatively with variability in expression of circadian wheel running in common voles in vivo (Gerkema et al. 1994). This raises the question whether this correlation depends on intrinsic properties of SCN tissue or on interactions with its environment (e.g., light input, humoral feedback). For the application of organotypic brain slice culturing, so far only SCN cultures from individuals up to 6 days have been used (Shinohara et al. 1994; Gerkema et al. 1999). In this study, circadian organization of wheel-running behavior was assessed in adult voles. Subsequently, SCN tissue of adult voles with or without expression of circadian wheel-running behavior was placed in an in vitro organotypic slice culture system for 2 weeks. Thereafter, cultures were subjected to AVP immunocytochemistry (ICC), and the number of AVPir neurons was established.

MATERIALS AND METHODS

Male common voles (1.5 months old, raised under light-dark [LD] conditions of 16h light, 8h dark) were obtained from our laboratory colony (Jansen et al. 1998). Voles were individually housed in a climate room (temperature 20°C, humidity 70%) in Lucite cages equipped with a running wheel. Food (Hope farm mouse pellets) and water were present ad libitum. Animals were subjected to a 12:12 LD cycle for 3 days. Subsequently, they were exposed to continuous low-light conditions (LL, 2 lux), while the number of running-wheel revolutions was counted via a microswitch and sampled every 2 minutes with an event recording system for activity data analysis. Slices were prepared from circadian rhythmic animals (n = 8) after 1 week and from nonrhythmic voles (n = 7) after 4 weeks.

The presence of circadian rhythmicity in individuals was assessed by visual inspection of double-plotted actograms of wheel-running activity and by chi-square (χ²) periodogram analysis of the last 10 days of the dim LL records (Sokolove and Bushell 1978). ΔQp values were obtained according to Gerkema and colleagues (1994).

Vole SCN explants were cultured according to the organotypic slice culture technique using the roller-drum method (Gähwiler 1984, 1997; Shinohara et al. 1994, 1995; Tominaga et al. 1994; Gerkema et al. 1999). In short, voles were deeply anesthetized. The brains were quickly removed, and a coronally cut 400-μm hypothalamic slice containing the two SCN was obtained using a tissue chopper. Slices containing the SCN were placed in Geys balanced salt solution and cooled for 2h at 5°C. The slice was embedded in a plasma clot (chicken plasma plus thrombin) on a coverslip and placed in a culture tube with 700 mL of culture medium (10% horse serum, 60% Eagle’s basal medium with 62 mM D-glucose and 4.16 mM NaHCO₃, and 30% Hanks’ balanced salt solution with 4.16 mM NaHCO₃). The SCN slice was cultured at 36°C with rotation (12...
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revolutions/h). In all cases, the medium was replaced 1–2 times a week, depending on the nutrient concentration. Finally, slices were fixed in 4% paraformaldehyde and AVP ICC was applied to all cultures as described elsewhere (Jansen et al. 1998).

AVPir neurons were counted in the dorsomedial area of the cultured SCN and expressed as the density of AVPir cells per 0.1 mm² (surface area ranged from 0.08 to 0.25 mm²). In this way, relative numbers of AVPir neurons could be compared instead of comparing absolute numbers, which are biased because of different surface areas.

RESULTS

Actograms of running-wheel activity of nonrhythmic (Fig. 1A) and rhythmic voles (Fig. 1B) illustrate the difference in the expression of daily wheel-running behavior between animals of both behavioral categories. Mean $\Delta Q_p$ values of $\chi^2$ periodogram analysis were 309 (SEM 167) and 1709 (SEM 213) for the nonrhythmic and rhythmic groups, respectively, indicating a significant difference in expression of circadian rhythmicity between the two groups of voles ($P < .001$; $t$ test, two tailed). No significant difference was found after comparing the mean $\Delta Q_p$ value (309, SEM 167) of the nonrhythmic group with $\Delta Q_p$ equal to 0 ($P > .05$; Student $t$ distribution, $t = 1.85$, $df = 5$).

FIGURE 1. Representative actograms of (A) nonrhythmic ($\Delta Q_p = 0$) and (B) circadian rhythmic ($\Delta Q_p = 1702$) voles; photomicrographs showing AVP immunoreactivity in the SCN and surrounding hypothalamic tissue in organotypic slice cultures of (C) nonrhythmic and (D) rhythmic voles 2 weeks after explanting. The insets show AVP immunopositive cells located in the rectangular boxes at a higher magnification. AVP immunoreactivity is higher in SCN cultures of nonrhythmic voles than in cultures of rhythmic voles. Arrows point to AVP immunopositive fibers, characterized by varicosities. hyp = hypothalamus; scn = suprachiasmatic nucleus. Scale bars = 40 μm.
The SCN of adult voles retained their in vivo cytoarchitectural organization in culture. Daily examinations with a phase contrast microscope revealed no visible morphological differences in cytoarchitectural organization between SCNs derived from circadian rhythmic and nonrhythmic voles during the 14 days of culturing. The morphological reorganization of the SCN explants stabilized within 8–10 days in all cases, as described elsewhere (Van der Zee, Jansen, and Gerkema, unpublished data). AVP immunoreactivity was predominantly present in the dorsomedial aspect of the SCN in all cultures, which is similar to the in vivo morphological localization of AVP. The SCN of nonrhythmic voles, however, revealed a much higher AVP staining intensity than the SCN of rhythmic voles (Figs. 1C, 1D). This was seen in the AVP cell bodies, the boutons, and punctate varicosities. These punctate varicosities frequently were observed around AVP cells (Fig. 1C, inset), as well as around AVP immunonegative cells. Moreover, clear AVP immunopositive fibers were frequent in the hypothalamic tissue of nonrhythmic voles, but rare in that of the rhythmic voles. Quantification of the number of AVPir cells expressed per 0.1 mm² in the SCN revealed a significantly higher numerical density of AVPir SCN cells in nonrhythmic voles (mean 62.3, SEM 18.03) compared with rhythmic voles (mean 16.6, SEM 6.46; \( P = .022 \), Mann-Whitney \( U \) test, two tailed). Proportionally, the difference in the number of AVPir cells in rhythmic and nonrhythmic vole slice cultures (nonrhythmic/rhythmic AVP ratio = 3.75) is similar to that found in vivo by Gerkema and colleagues (1994).

**DISCUSSION**

Our findings are consistent with the hypothesis that AVP is retained within SCN neurons of nonrhythmic voles, but not in circadian rhythmic voles (Gerkema et al. 1994). This idea, originally based on findings in vivo that voles without circadian rhythmicity of wheel-running behavior showed an increase in AVP immunoreactivity, were confirmed in slice cultures, even after morphological reorganization of the tissue. Moreover, from this study, it has become clear that, independent of input from extra SCN origin, AVPir cells in the isolated vole SCN show a negative correlation with previous expression of circadian wheel-running behavior. Neither a complete SCN nor the original neuronal network within and around the SCN is required to result in such correlation, which apparently depends on AVP regulation mechanisms of vole SCN tissue itself.

In contrast to anatomical observations on unstained cultures by phase contrast microscopy, detailed analysis of AVP ICC revealed that not only AVPir neurons, but also the immunocytochemical expression of AVPir boutons, punctate varicosities, and axons is higher in the cultures of nonrhythmic voles. When AVP is not released, or is released slowly, this may result in accumulation of AVP and in more intense AVP immunoreactivity. Interestingly, both circadian rhythmic and nonrhythmic patterns of AVP release are observed in SCN slices obtained from vole pups. In these pups, however, behavioral rhythmicity could not be assessed (Gerkema et al. 1999).

In view of all the results, the role of AVP in the vole circadian system still remains to be clarified. We observed that almost no AVPir neurons were present in the SCN of aging voles, although some of them still showed circadian rhythmicity of wheel-running behavior (Van der Zee et al. 1999). These older voles, however, show a clear reduction in stability and precision of the circadian rhythmicity. Perhaps other neurochemical sys-
tems are involved more directly in the generation of an SCN output signal, whereas AVP is likely involved in the strength of the pacemaker output (Van der Zee et al. 1999).

This study reveals the potentials of SCN cultures obtained from adult brain tissue, in addition to earlier studies using very young animals in which individual behavioral activity could not yet be recorded (Shinohara et al. 1994; Gerkema et al. 1999). The analysis of pacemaker characteristics, including peptidergic activity, in complete neural and humoral isolation can thus be related to behavioral characteristics of the circadian system. The association between behavioral rhythmicity and AVP in the SCN in vitro shown here suggests that interindividual variation in circadian organization of behavior is traceable at the pacemaker level.

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REFERENCES


