A Putative Transcription Factor with Seven Zinc-Finger Motifs Identified in the Developing Suprachiasmatic Nucleus by the Differential Display PCR Method

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The suprachiasmatic nucleus (SCN) is a mammalian central circadian pacemaker. This nucleus develops in the last stage of fetal life and matures to make strong synaptic connections within 2 weeks of postnatal life to establish strong oscillation characteristics. To identify factors that initiate the circadian oscillation, we applied a differential display PCR method to developing SCN, and isolated a gene with seven zinc-finger motifs, *Lot1*, which encodes a gene that appeared at a very high level in the SCN during the early postnatal days. *Lot1* mRNA first appeared at postnatal day 1 (P1) at a very high level, and the signal in the SCN continued to be very high until P10 and thereafter rapidly decreased until P20 and was expressed

at a very faint level during adulthood. *Lot1* mRNA expression was observed only in neurons of the dorsomedial SCN throughout the course of development. During the developmental stage, *Lot1* mRNA expression shows a circadian rhythm with a peak in the day time and a trough at night time in both light-dark and constant dark conditions. These observations imply that *Lot1* is the first identified putative transcription factor expressed only in the period of active synaptogenesis in the SCN, where *Lot1* might play a role in establishing autonomous oscillation.

Key words: suprachiasmatic nucleus; *Lot1*; development; mRNA differential display; zinc finger; in situ hybridization

In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is a central circadian pacemaker that regulates numerous behavioral and physiological rhythms (Moore, 1973). Recent molecular studies have established a number of putative “clock genes” in mammals, including *mPer1*, *mPer2*, *mPer3*, *Clock*, *BMAL1*, and *mTim* (Dunlap, 1999). Unexpectedly, these genes are expressed not only in SCN but also in other brain areas and in peripheral organs, suggesting that the molecular machinery for the rhythmic oscillatory molecules exists in most cells. However, in mammals, it is already established that the oscillating activity of behavior and endocrine rhythms is governed solely by the SCN; among all the organs, the SCN is the only nucleus showing strong autonomous electrophysiological and metabolic activity (Inouye and Kawamura, 1979). Thus, there might be a mechanism unique to the SCN that produces high oscillatory activity.

The SCN is equipped with a pacemaker (composed of thousands of SCN cells) and efferent pathways. To the pacemaker, optic nerve innervates directly or indirectly as its entraining pathway. The pacemaking components are generated and equipped for a long developmental period. In rats, the neurogenesis of the SCN is completed by embryonic day 18 (E18) (Ifft, 1972; Altman and Bayer, 1978), and just after, SCN begin to oscillate (Reppert and Schwartz, 1984), the process of which is

entrained by the maternal circadian system (Weaver and Reppert, 1989). After birth, there is no light entrainment of circadian rhythm in the first week, and entrainment is established at 2–3 weeks (Moore, 1991). Neuronal activity is weak in the perinatal period and gradually increases to the adult level at 1–3 weeks (Shibata and Moore, 1988). Phenotypic change in neurotransmitters also occur during the first 2 weeks. We previously demonstrated phenotypic change in vasoactive intestinal peptide (VIP)-expressing neurons from postnatal day 10 (P10) to P20 in the SCN (Ban et al., 1997). These studies suggest that SCN acquires the characteristics of high oscillatory amplitude after the several sequential steps of development. Coupling of each oscillatory cell might be an important factor for the SCN to be the robust oscillator. To find molecules that are key to making the SCN oscillatory machinery robust, in the present study we applied a differential mRNA display method, which is based on RT-PCR (Liang and Pardee, 1992; Inokuchi et al., 1996a), to the developing SCN. From the SCN just after neurogenesis, we isolated cDNA encoding a putative transcription factor, *Lot1* [previously isolated in rat ovarian surface cell lines that are expressed only in the nontransforming state: lost on transformation 1 (Abdollahi et al., 1997)], which is a zinc-finger protein of the ZAC/LOT family important for apoptosis and cell cycle arrest (Spengler et al., 1997; Varrault et al., 1998; Pagotto et al., 1999). Furthermore, we characterized its expression characteristics at the developmental stage in relation to the maturation of the SCN.

MATERIALS AND METHODS

Animals

Pregnant female Wistar rats (Nihon Animal Care, Osaka, Japan) were obtained at 7–10 d of gestation (timed pregnancy). They were maintained in individual cages under standard laboratory conditions with diurnal lighting (LD; lights on at 6 A.M. and off at 6 P.M.), with free access to

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food and water. These rats typically give birth on the 22nd day after mating. The day after the mating was designated as E1. To study the fetuses, pregnant females were anesthetized deeply with ether, and the fetuses were removed and perfused. The fetuses were anesthetized by deep low-temperature anesthesia. The time of birth was carefully noted, and the day after the birth was designated as P1. All postnatal animals were anesthetized with ether before they were killed. We used fetuses of both sexes but only male postnatal rats.

For the developmental *in situ* hybridization histochemical study, we used E18 ($n = 4$) as well as E20 ($n = 4$), P1 ($n = 4$), P3 ($n = 4$), P5 ($n = 4$), P7 ($n = 4$), P10 ($n = 6$), P20 ($n = 4$), and P50 (adult; $n = 5$) animals.

The daily rhythm of the *Lot1* mRNA signals was analyzed in P10 rat SCN. Animals were adapted to LD conditions for 2 weeks, including the embryonic period. At P8, half of these rats ($n = 42$) were transferred to constant darkness (DD), and the other rats ($n = 42$) remained in LD conditions. In these experiments, circadian time (CT) 0 and CT12 are referred to as 6 A.M. and 6 P.M., respectively. At P10, rats in LD were killed at Zeitgeber time (ZT) 0 (0 was designed as the transition time from the dark to light phase), 4 (4 hr after the onset of the light phase), 8, 12, 16, and 20. The other rats were killed on the second day in DD, and the time point was at CT0, 4 (4 hr after the onset of the second subjective day), 8, 12, 16 (4 hr after the onset of the third subjective night), and 20. The dams were also associated in the same cage with their litters until the time of the experiment.

The experimental protocol of the current research was approved by the Committee for Animal Research at Kobe University School of Medicine.

Differential display

For the differential display study, P2, P10, P20, and P50 animals were used. These animals were killed at ZT4 or 16 under LD conditions. Brains were removed and soaked in ice-cold Tris buffer [Tris-HCl (50 mM), pH 7.5]. Coronal brain slices were cut to 500 μ m thickness on Brain-Matrix, and the SCN was punched out under a stereomicroscope with a microdissecting needle (inner diameter 500 μ m) and quickly homogenized in the Trizol Reagents (Life Technologies BRL, Gaithersburg, MD). As a control, we used a cerebral cortex at P50. Total RNA was prepared from samples using a single-step RNA isolation method (Chomczynski and Sacchi, 1987). mRNA differential display was performed as described previously (Liang and Pardee, 1992; Inokuchi et al., 1996a). Heat-denatured total RNA (0.5 μ g) was incubated with 300 U of Moloney murine leukemia virus reverse transcriptase (Toyobo) in a 20 μ l reaction volume for 60 min at 35°C in the presence of 20 μ M dNTP and 40 U of ribonuclease inhibitor (Toyobo), using the oligo (dT) primer T12MA, T12MC, T12MG, or T12MT (M, a mixture of A, C, and G) as anchor primer. An aliquot (0.5 μ l) of the samples was then added to 4.5 μ l of PCR solution containing 2 μ M dNTPs, 5 μ Ci [α -³²P]dATP (3000 Ci/mmol, New England Nuclear), 0.5 U of AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT), 1 μ M anchor primer (T12MN), and 0.5 μ M arbitrary primers. The arbitrary primer (10 mer, 40–60% GC content) was specifically designed for differential display. PCR parameters were 94°C for 3 min, 40°C for 5 min, 72°C for 5 min for the first cycle, 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec with 35 cycles, then 72°C for 5 min for elongation. Radio-labeled PCR amplification products were analyzed by electrophoresis in denaturing 6% polyacrylamide gels. Duplicate reactions from identical samples of each RNA preparation were performed and run side by side. Gels were run at 2000 V for 2.5 hr, dried, and exposed directly to XAR-5 film (Kodak) overnight at room temperature. The cDNA bands that showed high in P2 and P10 but low in P20 and P50 without positive signals in the cerebral cortex were cut from the gel and moved to microfuge tubes (0.5 ml). The obtained cDNA fragments were reamplified using the same primer set and PCR conditions used for the differential display reactions. The reamplified DNA fragments were subcloned to pGEM-T Easy vector using the TA-cloning system (Promega, Madison, WI). Using cRNA probes synthesized from PCR products, we performed *in situ* hybridization of the brain sections including the SCN (Shigeyoshi et al., 1997a). DNAs that expressed strong positive signals in the SCN at developmental stages were selected and sequenced. Six obtained DNA sequences were searched using the BLAST program and the EST and GenBank databases.

One of these genes completely matched the 3' untranslated region of *Lot1* (GenBank accession no. U72620). Because this gene was originally isolated from a rat ovarian surface epithelium cell line, we tried to confirm that the *Lot1* gene expressed in the SCN of the brain was the same as that of the cell line. The gene sequence at the translated region was obtained by the hypothalamic cDNA by PCR method. Primers were

5'-GTGGAACAGTGGTTCATCTC-3' (483–502) and 5'-GTCAGATA-TGACTGACAACC-3' (3113–3132) for the first PCR reaction, and 5'-GAAAGTGCAGAGAAGCAGAGG-3' (526–545) and 5'-GAAAACG-AAGACACCGACAG-3' (3003–3022) for the second reaction.

Northern blot analysis

Total RNA was prepared from rat peripheral and brain tissue of P50 animals using a single-step RNA isolation method. Poly(A⁺) RNA was separated from total RNA using Oligotex-dT30 (Takara). For Northern blot analysis, Poly(A⁺) RNA (2 μ g) was electrophoresed in a 1.2% agarose gel containing 5.4% formaldehyde and 1 $\times$ MOPS buffer (1 $\times$ MOPS buffer = 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA). Transfer of RNA to Hybond-N + nylon transfer membrane (Amersham, Arlington Heights, IL) was performed by capillary blotting with 10 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl and 0.015 M sodium citrate). After transfer, the nucleic acids were cross-linked to the membrane in a UV cross-linker. For analysis of RNAs, cDNA probes (*Lot1*; 4697–4955) were labeled with [³²P]dCTP (New England Nuclear) by random priming and hybridized in 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution (50 $\times$ Denhardt's solution = 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), 50 mM phosphate buffer, pH 7.5, 0.5% SDS, and 100 μ g/ml denatured salmon sperm DNA at 42°C for 16 hr. After hybridization, filters were washed in 2 $\times$ SSC and 0.1% SDS for 10 min at room temperature, twice in the same buffer for 30 min at 60°C, and in 0.1 $\times$ SSC and 0.1% SDS for 1 hr at 50°C. The washed filters were exposed to XAR-5 film (Kodak) for 12 hr at –80°C.

In situ hybridization using radiolabeled probes

Probes. Vectors containing the PCR product (*Lot1*; 4697–4955) were linearized with restriction enzymes and used as templates for sense or antisense cRNA probes. Radiolabeled probes for *Lot1* were made using ³⁵S-dCTP (New England Nuclear) with standard protocols for cRNA synthesis. To check the specificity of hybridization, we synthesized antisense probes of two *Lot1* cDNA fragments (954–1599 and 2387–3022) and sense cRNA probe of original PCR products. All three antisense probes produced the same pattern of positive signals (data not shown), and the sense probe produced no positive signals. In all experiments, we used cRNA probe from the original PCR product (*Lot1*; 4697–4955) for *in situ* hybridization.

Tissue preparation. Under deep anesthesia by ether, postnatal animals were perfused via the left cardiac ventricle with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. Brains were removed and post-

Figure 1. Representative autoradiogram of mRNA differential display with an anchor primer, T12MC, and an arbitrary primer, 5'-TGTAACGAAAT-3'. Arrow shows cDNA band of *Lot1*. Samples were collected from SCN at P2, P10, P20, and P50 and from the cerebral cortex at P50. SCN, Suprachiasmatic nucleus; CCx, cerebral cortex.

Figure 2. *A*, Amino acid sequence of LOT1. Zinc-finger motifs are highlighted by bold underlining. Boxes indicate the Cys and His of zinc finger. *B*, Northern blot analysis of *Lot1* in rat tissue RNA. Two micrograms of mRNA in P50 rat tissues were loaded on each lane. β -actin served as a loading control. *C*, *In situ* hybridization using antisense probe and sense probe in P50 rat brain. Photomicrographs were taken using Bio-max film (Kodak). Arc, Arcuate hypothalamic nucleus; LS, lateral septal nucleus; Me, medial amygdaloid nucleus; OVLT, organum vasculosum lamina terminalis; PirCx, piriform cortex; Pur, Purkinje cell layer. Scale bars, 2 mm.

fixed in the same fixative for 12 hr at 4°C. For the preparation of fetal animals, pregnant dams were deeply anesthetized with ether, and fetuses were removed and immersed in the same fixative for 24 hr at 4°C. Fixed brains were then transferred into 20% sucrose in PB for 72 hr. Frontal sections (40 μ m in thickness for anatomical localization study and 50 μ m in thickness for quantitative hybridization study) were made using a cryostat and processed for the free-floating *in situ* hybridization method as described previously (Ban et al., 1997; Shigeyoshi et al., 1997b). For the quantitative analysis, we collected all sections from the rostral end of the SCN to the reticular area. We also thaw-mounted frontal and sagittal sections (40 μ m in thickness) on silane-coated microscope slides (Okamura et al., 1990) and processed them for *in situ* hybridization.

Hybridization. Free-floating and thaw-mounted sections were processed following the same protocols of prehybridization, hybridization, and posthybridization washes. The thaw-mounted and free-floating sections were treated with 0.1 mg/ml proteinase K (Sigma, St. Louis, MO), 10 mM Tris buffer, pH 7.4, and 10 mM EDTA for 5 min at 37°C, then with 4% paraformaldehyde in 0.1 M PB for 5 min. Because free-floating sections from developing rats are fragile, the proteinase K treatment was omitted in free-floating sections obtained from rats younger than P10. Then sections from rats of all ages were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in hybridization buffer (60% formamide, 10% dextran sulfate,

1 $\times$ Denhardt's solution, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.3 M NaCl, 10 mM NaPB, pH 8.0, 0.2% *N*-laurylsarcosine, and 500 mg/ml yeast tRNA) containing the 35 S-dCTP-labeled cRNA probe for 12 hr at 60°C. After hybridization, these sections were rinsed in 20 $\times$ SSC/50% formamide for 15 min twice at 60°C, and the sections were treated with a solution containing 20 mg/ml RNase A, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5 M NaCl for 30 min at 37°C. The sections were rinsed further in 2 $\times$ SSC/50% formamide for 15 min twice at 60°C and in 0.5 $\times$ SSC for 30 min at 60°C.

Sections for free-floating *in situ* hybridization were mounted onto gelatin-coated microscope slides. All sections were air-dried and dehydrated through a graded alcohol series. The slides were exposed to BioMax film (Kodak) at 4°C for 3 d. The films were developed using a Kodak D19 developer for 5 min at 20°C. The slides were dipped in Kodak NTB2 nuclear track emulsion (dilution 1:1 distilled water), developed after 2 weeks, and counterstained by Nissl staining (cresyl violet).

Quantitative *in situ* hybridization. Serial frontal sections (50 μ m in thickness) of rats were made from the rostral end to the caudal end of the SCN using a cryostat. In this experiment, we used P10 rats to obtain a signal intensity sufficient for analyzing the diurnal rhythm of *Lot1* mRNA signals in the SCN. Quantification of mRNA has been described (Okamura et al., 1995; Ban et al., 1997). The radioactivity of each section of the BioMax film (Kodak) was analyzed using a microcomputer inter-

Figure 3. Expression of *Lot1* mRNA in fetal rat (E20) detected by *in situ* hybridization using antisense probe. Sense controls performed in the adjacent sections showed no signals. Scale bar, 2 mm.

faced to an image analyzing system (MCID, Imaging Research) after conversion into the relative optical density using ^{14}C -acrylic standards (Amersham). The rostralmost to the caudalmost of the SCN (13 sections per rat) were then summed; the sum was considered a measure of the amount of *Lot1* mRNA in this region. Statistical analyses of the data were made using one-way ANOVA followed by Scheffé's multiple comparisons.

Developmental change of the relative amount of *Lot1* mRNA in the SCN was also examined by film autoradiographic image at E20, P1, P3, P5, P7, P10, P20, and P50 rats. The value at P3 is adjusted to 100.

RESULTS

cDNA cloning using mRNA differential display in rat SCN

We used mRNA differential display (Liang and Pardee, 1992; Inokuchi et al., 1996b) to isolate cDNA clones that are differentially expressed in the developing SCN of the rat brain. Eighty combinations of primer sets made of four anchor primers and a group of 20 arbitrary 10-mers were used for screening. By this method, seven independent cDNAs were isolated in which mRNA levels were markedly modulated in the SCN at ZT4 during early neonatal periods. When a primer pair of T12MC and an arbitrary primer 5'-TGTACGAAAT-3' was used, we detected PCR products of 259 bp. Figure 1 shows that signals of the PCR product at P2 and P10 were strong, but they were weak at P20 and very weak at P50. DNA sequence analysis of the PCR product using the BLAST program and GenBank databases revealed that the sequence of obtained PCR products matched 259 bp (4697–4955) of the 3' untranslated region of *Lot1* cDNA (Abdollahi et al., 1997), which has seven zinc-finger motifs of the C_2H_2 type, as well as proline-rich, glutamine-rich, and glutamic acid-rich areas (GenBank accession no. U72620). We cloned the translated region of *Lot1* from the hypothalamic cDNA using PCR. Among the coding sequences (548–2299), the following DNA sequences from hypothalamic cDNA were different from the reported sequence: C $\rightarrow$ T (916, 1836), T $\rightarrow$ C (1816, 2263), A $\rightarrow$ G (2053, 2071, 2089, 2095, 2117), and A $\rightarrow$ T (2188). However, the translated amino acid sequence of *Lot1* from the hypothalamic cDNA was identical to the reported one (Fig. 2A).

Tissue distribution of *Lot1* mRNA in adult rats: Northern blot analysis and *in situ* hybridization

First, we examined *Lot1* expression in the rat body and brain before examining its expression in the developing SCN. To de-

Figure 4. Expression of *Lot1* mRNA in the developing SCN. *A*, Film autoradiographic image of *in situ* hybridization of *Lot1* mRNA in the SCN of E20, P1, P3, P5, P7, P10, P20, and P50 rats. Scale bars, 2 mm. *B*, Developmental change of the relative amount of *Lot1* mRNA in the SCN. The value at P3 is adjusted to 100.

termine the tissue distribution of *Lot1*, we performed Northern blot analysis using obtained PCR products (4697–4955) of the 3' untranslated region of *Lot1* cDNA as a probe. A transcript of ~5.5 kb was detected in all rat tissue, including brain, skeletal muscle, heart, lung, kidney, and testis (Fig. 2B). The amount of *Lot1* mRNA was highest in the kidney, high in the lung, moderate in the brain, testis, and skeletal muscle, and low in the heart.

Expression of *Lot1* mRNA in the adult rat brain was examined by *in situ* hybridization. We found high levels of signals in the hypothalamic arcuate nucleus and moderate levels in the piriform cortex, lateral septum, medial amygdaloid nucleus, and the Purkinje cells of the cerebellar cortex (Fig. 2C). No signals were detected using the sense probe.

Figure 5. Dark-field emulsion image of *Lot1* mRNA in P5 (*A*) and P10 (*B*, *C*) rat SCN. SCN is outlined by broken lines in *A* and *B*. *C*, Bright-field high-power photomicrograph of the dorsomedial SCN in *B*. *oc*, Optic chiasma; *v*, third ventricle. Scale bars: *A*, *B*, 100 μ m; *C*, 10 μ m.

The expression of *Lot1* mRNA in developing rats

The expression of *Lot1* mRNA was also examined during the perinatal age (E18 and E20) by *in situ* hybridization using the antisense cRNA probe (*Lot1*; 4697–4955). At both E18 and E20, we found clear positive signals in most organs in the rat; high in the lung, tongue, intestine, and vertebral bone but weak in the heart and liver (Fig. 3). Using the sense probe, we found no signals in any of these structures by our present hybridization condition (data not shown).

In the brain, moderate levels of positive signals were detected in the external germinal layer of the cerebellar cortex, the tegmental neuroepithelium, the hypothalamus, and the cortical plate of the cerebral cortex (Fig. 3). The positive signals detected in the developing cerebral cortex disappeared at the adult stage.

The expression of *Lot1* mRNA in the developing and adult SCN

To observe the developmental modification of the expression of *Lot1* mRNA in the SCN, we performed *in situ* hybridization using *Lot1* cRNA probes with coronal sections of the brain at E20, P1, P3, P5, P7, P10, P20, and P50 (Fig. 4*A*). *Lot1* mRNA signals of the SCN were very weakly expressed at E20. At P1, when the animals were just born, very intense signals appeared in the SCN. This high level of signals increased to the highest level at P3, and then gradually decreased but was still high until P10 (Fig. 4*B*). However, the level of signals at P10 was much higher than that at P20 and P50 (adult rats).

To delineate the localization of positive signals in the SCN, we performed emulsion autoradiogram. At both P5 and P10, *Lot1* mRNA signals were found in the dorsomedial part of the suprachiasmatic nucleus (DMSCN) but not in the ventrolateral part of the suprachiasmatic nucleus (VLSCN) (Fig. 5). DMSCN neurons expressed abundant *Lot1* mRNA signals (Fig. 5*C*).

Rhythm of *Lot1* mRNA expression in the SCN at P10

To explore the daily expression of *Lot1* mRNA at the developmental stage, we compared the *Lot1* signals at 4 hr intervals in LD and DD conditions at P10. In both LD and DD conditions, sections at ZT/CT4 (4 hr after dawn or subjective dawn) showed the highest signals. We quantified *Lot1* mRNA in the SCN by the quantitative *in situ* hybridization method (Fig. 6). The peak–trough rhythm profiles were similar in both LD and DD conditions. In the LD condition, *Lot1* mRNA showed a clear diurnal rhythm, forming a peak at ZT4 and a trough at ZT0. The increase from ZT0 to ZT4 was very sharp, but the decline was very gradual. The level of the peak was ~ 1.7 -fold higher than that of the trough. In the DD condition, *Lot1* mRNA showed a clear circadian rhythm, forming a peak at CT4 and a trough at CT20, with the same peak/trough ratio as in LD.

DISCUSSION

In this study, we identified a putative transcription factor, *Lot1*, expressed in the SCN only at specific developmental stages. The results of the first screening by gel-loading of amplified genes were consistent with those of *in situ* hybridization, which suggests that our screening procedure is effective in cloning genes differentially expressed in the SCN. Many researchers have applied the differential display method to rat SCN, retina, and pineal body to identify genes expressed in a circadian manner, but only some preliminary reports are available (Gauer et al., 1995; Chong et al., 1996). The only success was the trial of Green and Besharse (1996b), who identified a specifically night-expressed photoreceptor mRNA nocturnin in *Xenopus* retina (Green and Besharse, 1996a).

The specific structural features of LOT1, the existence of zinc-finger motifs in their putative protein sequence, indicate that LOT1 is a DNA-binding protein that may play a role as a transcriptional regulator. Originally, *Lot1* was isolated from rat ovarian surface epithelial cell lines by the differential display method (Abdollahi et al., 1997). The deduced amino acid sequence from the open reading frame contains seven zinc-finger motifs of the C_2H_2 type, which belongs to the C_2H_2 class of zinc-finger proteins, typified by the *Xenopus* leaves TIIIF (Pelham and Brown, 1980; Miller et al., 1985) and mouse Zif268 (Christy et al., 1988). Some zinc-finger proteins, such as *Zic1*, contribute to development of the brain (Aruga et al., 1998). Similarly, abundant *Lot1* mRNA in the developmental brain and poor expression in adults suggest that *Lot1* may contribute to the process of CNS development. During the prenatal period, many regions of the brain express a large amount of *Lot1* mRNA, and this strong expression continues until P10, but from then to the adult stage, brain signals are markedly suppressed. In the SCN, transient but strong expression of *Lot1* was observed in the early postnatal days (P1–P10), and later, the signal intensity rapidly decreased to a very faint level during the adult stage.

Early postnatal days with strong expression of *Lot1* in the SCN correspond to the period when each SCN neuron attains unity of each oscillating neuron by mutual innervation. Moore and Bernstein (1989) reported that synaptogenesis of SCN occurs after birth, and in particular it occurs predominantly during the early postnatal days until P10, when *Lot1* is highly expressed. These synapses may be derived from the mutual connection of intrinsic neurons of the SCN, because innervation from external origins is reached later (Takatsuji et al., 1995). Retinal terminals reach to the SCN during the late gestational period (Bunt et al., 1983), but Güldner (1978) reported that optic nerve terminals containing

Figure 6. Diurnal and circadian profiles of *Lot1* mRNA levels in the SCN of P10 rats. Quantitative *in situ* hybridization analysis of *Lot1* mRNA in the P10 rat brain under 12 hr LD and total darkness (DD) is shown. Values are means $\pm$ SEM ($n = 7$). * $p < 0.05$, compared with the value at ZT4 or CT4 (ANOVA). Representative sections are shown in top panels; SCN is indicated by arrows. Scale bars, 2 mm.

characteristic tubular mitochondria make synapses first at P9, and those synapses increase and mature from P17 to P27. Raphe serotonergic and geniculohypothalamic neuropeptide Y innervations most vigorously increase between P10 and P20 (Takatsuji et al., 1995). Thus the strong timing correlation of mutual intrinsic synaptic connection and the expression of *Lot1* suggest that the essential function of *Lot1* is in coupling among SCN neurons. During the period of *Lot1* expression, the amplitude of circadian rhythm of firing rate of SCN neurons greatly increases (Shibata and Moore, 1987), although only a weak day–night difference in electrical activity was observed at the late gestational period.

Because *Lot1* mRNA expression is confined to the DM part of the SCN from the beginning to the adult stage, *Lot1* may contribute to the development and differentiation of DMSCN neurons. In the rat, cytoarchitectonic subdivision of DM and VL regions is evident at least from P6 (Moore, 1991). In adult, neurons in the DMSCN are small, with scant cytoplasm, and well packed compared with VLSCN, with more widely spaced neurons and more cytoplasm. In addition to the cytoarchitectonic difference, the chemical and physiological characteristics of neurons are different between DMSCN and VLSCN. Most neurons of the DMSCN express arginine–vasopressin (AVP) and aromatic L-amino acid decarboxylase with endogenous oscillation, whereas neurons in the VLSCN produce VIP and gastrin-releasing peptide with light-dependent expression. Ontogeneti-

cally, AVP appears first in the DMSCN at P2 and gradually increases to the adult level at P10 (De Vries et al., 1981), and this maturation period mimics the period of expression of *Lot1* in the SCN. Together with the finding that endogenous *c-fos* expression is enhanced in the dorsomedial part during the first 10 postnatal days (Joyce and Barr, 1995), *Lot1* expression might help maturation of DMSCN neurons as a putative transcription factor.

Interestingly, mouse *Lot1* is isolated in the expression cloning study independently from the study of rat *Lot1* cloning, and from its ability to induce apoptosis and cell cycle arrest, the mouse *Lot1* gene is called *Zac1* (a zinc-finger protein that regulates apoptosis and cell cycle arrest) (Spengler et al., 1997). In the developing SCN, neuronal cell death was observed in the first postnatal days (Moore, 1991). Recently Müller and Torrealba (1998) performed quantitative analysis of cell death in the Syrian hamster and found that 40% cell loss occurred in the SCN between P2 and P6, just before the arrival of retinal fibers. The timing of expression of the apoptotic-inducing factor *Lot1* and cell death seemed similar, but we must be cautious about this conclusion because there is a difference of several days in development among the two species. However, the apoptotic role of *Lot1* gene expression at the very strict time point of the developing SCN may be important because the reorganization of neurons by apoptosis is an inevitable step for the differentiation of neurons in many nervous systems.

Although the circadian clock in adults is entrained by the light–dark cycle, in the fetal and neonatal animals environmental light–dark cycles cannot be monitored directly; thus the nonphotic entrainment system by maternal cues is very important in these animals (Takahashi et al., 1989; Hastings et al., 1998). Cross-fostering studies have shown that maternal cues exert a powerful effect on the neonatal clock, with newborn litters adopting the circadian phase of their foster mother if fostered within the first weeks of life (Takahashi et al., 1989). Because the underlying mechanisms of maternal entrainment are not fully understood, the postnatal expression of *Lot1* in dorsomedial SCN is possible to associate with the postnatal entrainment of the SCN by maternal-derived cues and the development of nonphotic entrainment of the SCN (Weaver and Reppert, 1995; Hastings et al., 1998; Duffield et al., 1999).

The *Lot1* mRNA transcription in the SCN might be controlled by a circadian clock. The SCN exhibits circadian oscillation of *Lot1* mRNA levels, forming a peak during the (subjective) day and a trough during the (subjective) night in both LD and DD conditions at P10. The circadian profile of this expression pattern mimics that of chemical substances expressed in DMSCN neurons: AVP formed a peak during the subjective day and a trough during the subjective night at both mRNA and peptide levels (Tominaga et al., 1992; Cagampang and Inouye, 1994). The daytime peak and nighttime trough patterns are similar to the expression pattern of *rPer1* and *rPer2* (Yan et al., 1999) and suggest that all of these bioactive substances expressed in the DMSCN are under the control of mammalian putative clock period genes. For AVP, Jin et al. (1999) recently demonstrated that CLOCK-BMAL1 heterodimers bind to the E-box of the 3' flanking sequence of AVP promoter and accelerate transcription, and mPER1 negatively regulates its gene transcription. The similarities between the profiles of circadian mRNA fluctuation in AVP and *Lot1* suggest that *Lot1* is also under a similar regulatory mechanism and directly controlled by circadian clock genes.

In conclusion, we detected a putative transcription factor, *Lot1*, expressed in neurons of developing DMSCN by the differential mRNA display method. *Lot1* mRNA is expressed very strongly in the limited phase of development between P1 and P10, which is identical to the period of massive synaptogenesis that occurs among intrinsic neurons. This putative transcription factor is expressed under the control of circadian rhythm and may contribute to the maturation of autonomous oscillation of oscillator cells with little direct influence of light stimulation (Shigeyoshi et al., 1997b; Yan et al., 1999).

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