

# Correlative Association between *N*-Methyl-D-Aspartate Receptor-Mediated Expression of *Period* Genes in the Suprachiasmatic Nucleus and Phase Shifts in Behavior with Photic Entrainment of Clock in Hamsters

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## ABSTRACT

Because the rapid induction of *Period* (*Per*) genes is associated with the photic entrainment of the biological clock, we examined whether *N*-methyl-D-aspartate (NMDA) receptors were involved in the photic induction of *Per* genes in the hamster suprachiasmatic nucleus (SCN). In situ hybridization observation revealed that light during the early subjective night [circadian time (CT) 13.5] or the late subjective night (CT20) caused an induction of *Per1* and *Per2* but not *Per3* mRNA in the SCN. Photic induction of *Per* mRNA at CT13.5 was observed especially in the ventrolateral SCN, whereas that at CT20 was more widespread from the ventrolateral to the dorsal SCN. A non-competitive NMDA receptor antagonist, +MK801, dose-dependently (0.1–5.0 mg/kg) suppressed only the ventrolateral part of *Per1* and *Per2* mRNA induction by light at CT13.5 or CT20 in the

SCN. The suppressive effects of +MK801 on *Per* mRNA strongly correlated with the attenuating action of this compound on phase shifts by light at both CT13.5 and CT20. A competitive NMDA receptor antagonist, D-2-amino-5-phosphonovaleate (D-APV), also exhibited inhibitory actions on light (CT20)-induced *Per1* and *Per2* mRNA expression in the ventrolateral SCN. Furthermore, local injection of NMDA into the SCN resulted in the induction of *Per1* and *Per2* mRNA in the SCN. Among NMDA receptors, NR2B and NR2C mRNA were expressed in the ventrolateral and dorsal SCN, respectively. These results suggest that the activation of NMDA receptor is a critical step for photic induction of *Per1* and *Per2* transcripts in the SCN, which are linked to a photic behavioral entrainment.

Physiological rhythms with a period of 24 h, such as locomotor activity, feeding, sleep-wake, body temperature, and plasma adrenal corticosterone levels persist even in the absence of environmental time cues, suggesting the existence of an endogenous time-keeping system in animals. It is widely known that mammalian biological clocks are located in the suprachiasmatic nucleus (SCN) of the hypothalamus (for review, see Ralph et al., 1990), and daily light-dark cycles strongly entrain the self-oscillating circadian rhythms generated within the SCN (for review, see Inouye and Shibata, 1994). Light, a form of photic entrainment, conveys signals to the SCN mainly via glutamate release from the retinohypothalamic tract (RHT), a monosynaptic afferent from the retina to the SCN.

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Glutamate receptors are divided into three major subtypes; that is, *N*-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainic acid, and metabotropic receptors (Nakanishi, 1992). Although all three types of glutamate receptors are expressed in the SCN, NMDA receptors are reported to be principally involved in photic resetting of the biological clock in rodents. NMDA receptors are classified as NR1 and NR2 subtypes according to their sequence homologies, with the NR2 subtype further divided into four receptor subtypes (NR2A, NR2B, NR2C, NR2D). Furthermore, a heteromeric complex of NR1 with one or more NR2 subtypes is required for a functional NMDA receptor complex. In behavioral experiments, both competitive and noncompetitive NMDA receptor antagonists suppressed the photic resetting of behavioral rhythms (Colwell et al., 1991), and these NMDA receptor antagonists also reduced the photic induction of immediate-early genes such as *Fos* in the SCN (Abe et al., 1991; Ebbling et al., 1991, 1992; Mikkelsen et al., 1995; Edelstein and Amir, 1998;

**ABBREVIATIONS:** SCN, suprachiasmatic nucleus; NMDA, *N*-methyl-D-aspartate; CT, circadian time; RHT, retinohypothalamic tract; *Per*, *Period*; LD, light-dark; LV, lateral ventricle; DD, constant darkness; PB, phosphate buffer; PFA, paraformaldehyde; CRE, cAMP response element; CREB, CRE-binding protein; MAPK, mitogen-activated protein kinase; D-APV, D-2-amino-5-phosphonovaleate.

Guido et al., 1999). In addition, recent studies reported that NMDA application to the SCN elicited a phase shift in wheel-running rhythm in vivo (Mintz and Albers, 1997; Mintz et al., 1999), as well as in neuronal firing rhythm in SCN slices in vitro (Shibata et al., 1994). Because NMDA-induced phase shifts are very similar to those produced by light pulses, these lines of evidence suggest that NMDA receptor mediates photic entrainment of the biological clock.

On the other hand, current studies have described the molecular mechanisms underlying the generation of circadian rhythms in mammals. *Period* (*Per1*, *Per2*, *Per3*) genes were identified as mammalian putative clock genes that function in this process by forming a transcriptional/translational negative feedback loop in which their transcriptions are suppressed by their protein products (Dunlap, 1999). *Per1*, *Per2*, and *Per3* exhibited robust circadian rhythms with a peak in amplitude during daytime and a trough during the night in the SCN of mice (Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997; Takumi et al., 1998), rats (Yan et al., 1999), and hamsters (Maywood et al., 1999; Messenger et al., 1999), suggesting the essential role of *Per* genes in circadian rhythm generation among animal species. We and other groups have reported that photic stimulation elicits a transient increase in *Per1* and *Per2*, but not *Per3*, mRNA in the SCN only during subjective night when photic resetting also occurs (Shigeyoshi et al., 1997; Zylka et al., 1998). Furthermore, we have demonstrated that the photic induction of *Per1* mRNA in the SCN is strongly associated with photic resetting of the behavioral rhythm, because central administration of an antisense oligonucleotide targeting *Per1* mRNA inhibited the light pulse-induced phase shift of locomotor activity rhythm in vivo, as well as the glutamate-induced phase shift of neuronal firing rhythms in vitro (Akiyama et al., 1999). Although this study suggests that the

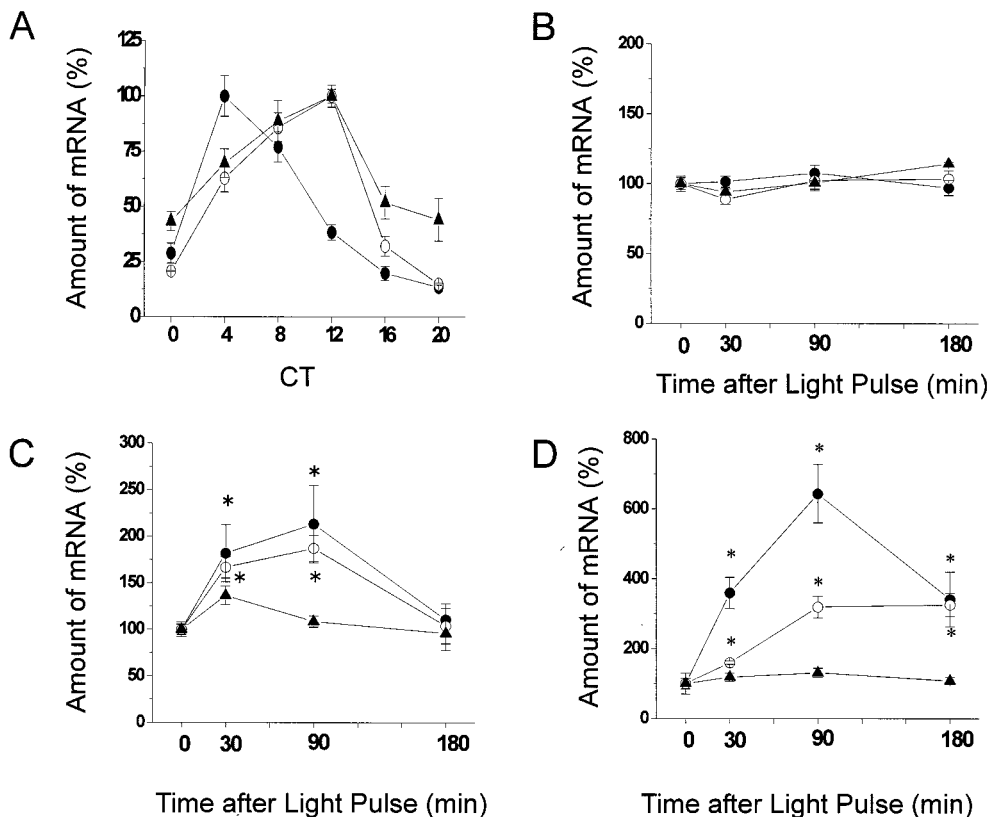
photic increase in *Per* mRNA via at least glutamate release from the RHT are critical steps for photic resetting, the types of neurotransmitter receptors involved in the photic induction of *Per* mRNA in the SCN remain to be clarified. To address this issue, we examined the effects of NMDA receptor antagonists on photic induction of *Per* mRNA and the effect of NMDA injection on *Per* mRNA in the SCN, using a quantitative in situ hybridization method. We further performed a correlation analysis between the inhibitory actions of NMDA receptor antagonists on the level of *Per* mRNA and the behavioral resetting produced by light pulses to clarify the physiological significance of *Per* induction. Finally, we examined the distribution of four NR2 subtypes of NMDA receptor mRNA in the hamster SCN because the expression of NMDA receptor subtypes in the SCN have been reported in rats (Mikkelsen et al., 1993; Gannon and Rea, 1994) and mice (Watanabe et al., 1993; O'Hara et al., 1995) but not in hamsters.

## Experimental Procedures

**Animals.** Male Syrian hamsters (*Mesocricetus auratus*) weighing 110 to 150 g were used in all experiments. Animals were housed in temperature-controlled animal quarters ( $23 \pm 2^\circ\text{C}$ ) under a 12:12 h light:dark (LD) cycle before use in the experiments. Food and water were given ad libitum. Animals were treated in accordance with the Law (no. 105) and Notification (no. 6) of the Japanese Government.

**Materials.** D-APV and +MK801 were obtained from Research Biochemicals, Inc. (Natick, MA). NMDA was supplied by Tocris Cookson, Inc. (Ballwin, MO).

**Intralateral Ventricle (LV) and IntraSCN Injection.** Hamsters were deeply anesthetized with sodium pentobarbital (75 mg/kg, i.p.), and a 22-gauge stainless steel cannula (total length: 11 mm for LV, 8 mm for SCN) was stereotactically implanted. Stereotaxic coordinates were as follows: LV: 0.4 mm anterior and 1.7 mm lateral to the bregma



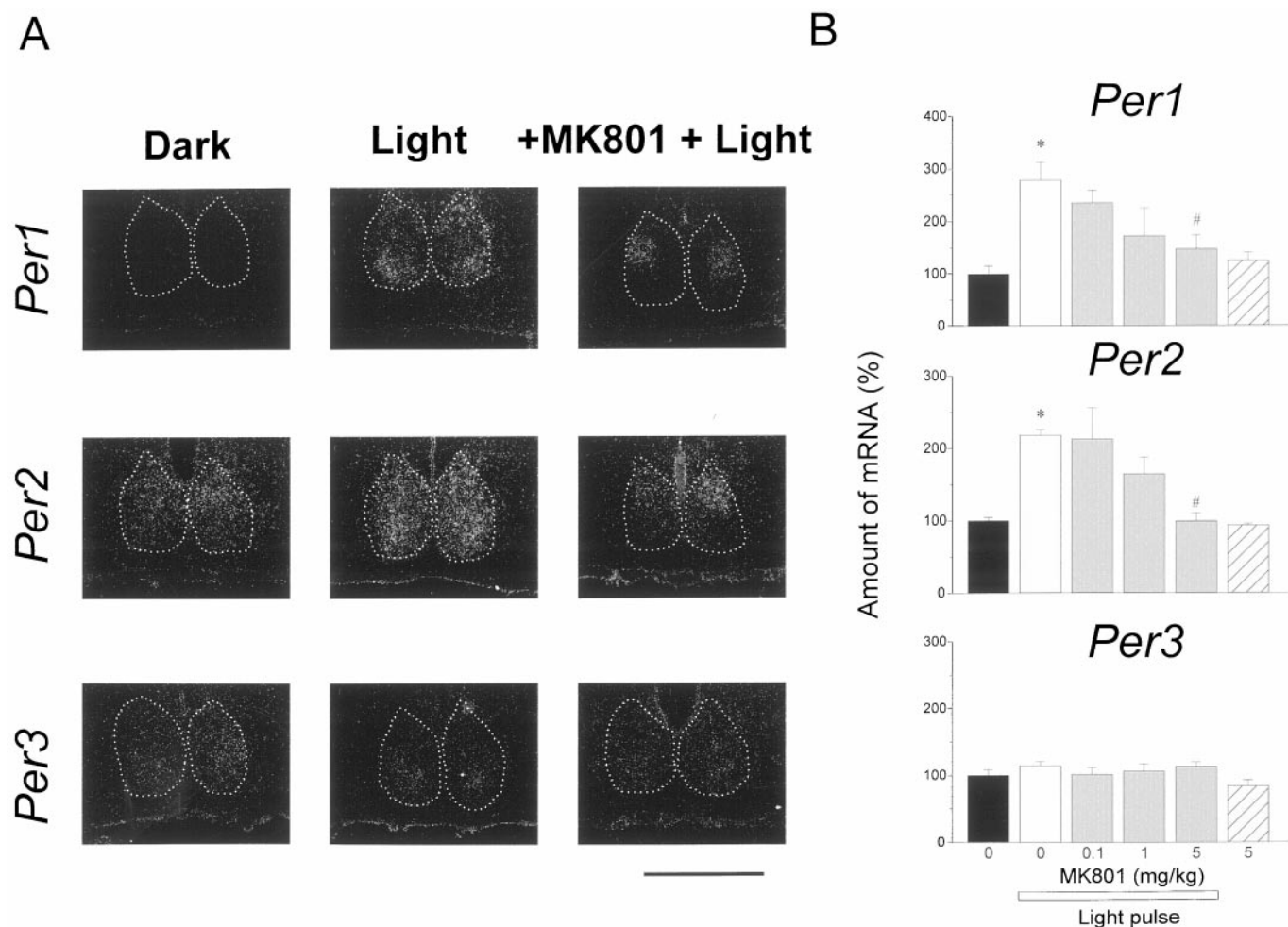
**Fig. 1.** Circadian expression and photic induction of *Per1*, *Per2*, and *Per3* mRNA in the SCN. A, circadian expression of *Per1*, *Per2*, and *Per3* mRNA under DD conditions 2 days after release from LD conditions. Photic induction of *Per1*, *Per2*, and *Per3* mRNA at CT6 (B), CT13.5 (C), or CT20 (D). After release into DD for 2 days, a light pulse (60 lux) was administered to hamsters for 15 min at CT6, CT13.5, or CT20 before they were returned to darkness. Then, 4%PFA was perfused intracardially in hamsters at the indicated time after light onset. The amount of *Per1* (closed circle), *Per2* (open circle), and *Per3* (closed triangle) mRNA in the SCN was quantitatively measured by the in situ hybridization method and is shown as a relative value. Asterisks indicate statistical significance ( $P < .05$ , one-way ANOVA followed by Dunnett's test) compared with the time 0 group (dark control group).  $n = 3$  to 4.

and 3.5 mm ventral to the skull surface; SCN: 0.3 mm anterior and 1.6 mm lateral to the bregma and 4.6 mm ventral to the skull surface at a 10° angle toward the midline, with the incisor bar 2 mm below the interaural line. After recovering from surgery for at least 7 days under LD conditions, animals were anesthetized during the appropriate circadian time (CT) phase with ether for 40 s, and a 27-gauge injection cannula (total length: 8.5 mm for LV, 14.6 mm for SCN) was inserted. Drugs or vehicle (total volume: 2  $\mu$ l for LV, 0.2  $\mu$ l for SCN) was administered for 2 min under dim red illumination (<1 lux) by a 1- $\mu$ l (for SCN injection) or 5- $\mu$ l (for LV injection) Hamilton syringe to hamsters gently restrained by hand. After injection, the injection cannula was left in position for 15 s to facilitate drug diffusion.

**Recording of Wheel-Running Rhythm.** Hamsters were housed individually in transparent plastic cages (35  $\times$  20  $\times$  20 cm), each equipped with a running wheel 15 cm in diameter, which turned a microswitch with each revolution. Wheel-running activity was continuously recorded in 6-min epochs by a PC-9801 computer. At least 10 days after releasing the animals into constant darkness (DD) conditions, hamsters received intraperitoneal injections of vehicle or drugs 60 min before light pulse (60 lux) for 15 min at CT13.5 or CT20 (CT12 is defined as activity onset time) and were then returned to their cages. Hamsters received three injections at most, and the drug and vehicle groups were crossed-over so that they were given the

opposite drug treatment. Eye-fitted lines were drawn for consecutive activity onset by two observers without knowledge of the treatment conditions, and the averaged difference between these two lines was designated as the phase shift.

**In Situ Hybridization using Radioisotope-Labeled cRNA Probe.** In situ hybridization was executed to determine the quantity of *Per* and NMDA receptor subtype mRNA expression in the SCN using hamster *Per1*, *Per2*, and *Per3* cRNA probes and rat NMDA receptor subtype (NR2A, NR2B, NR2C, NR2D) cRNA probes. Hamsters were deeply anesthetized with ether, and 0.1 M phosphate buffer (PB) (pH 7.4) containing 4% paraformaldehyde (PFA) was intracardially perfused. Brains were removed, postfixed in 0.1 M PB containing 4% PFA for 24 h at 4°C, and transferred into 20% sucrose in PB for 72 h at 4°C. Slices (30  $\mu$ m thick), including the SCN, were made using a cryostat (model HM505E, Microm, Walldorf, Germany) and divided equally from rostral to caudal parts into three groups for the measurement of *Per1*, *Per2*, and *Per3* mRNA. These slices were then placed in 2 $\times$  standard saline citrate until processing for hybridization. Slices were treated with 1  $\mu$ g/ml proteinase K in 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA for 10 min at 37°C, followed by 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 10 min. The slices were then incubated in hybridization buffer [60% formamide, 10% dextran sulfate, 10 mM Tris-HCl,



**Fig. 2.** Inhibitory effects of +MK801 on light pulse-induced (CT13.5) *Per* mRNA induction in the SCN. After release into DD for 2 days, saline (2 ml/kg) or +MK801 (0.1–5 mg/kg) was injected intraperitoneally into hamsters 60 min before light onset. Animals were then exposed to a light pulse (60 lux) for 15 min at CT13.5, and 4%PFA was perfused intracardially 90 min after light onset. A, representative emulsion autoradiograms showing that +MK801 suppressed the photic induction of *Per1* and *Per2* mRNA in the ventrolateral part of the SCN. Dotted circles indicate the border of the SCN. Scale bar, 0.5 mm. B, quantitative analysis of *Per* mRNA in the SCN. The amount of *Per* mRNA in the SCN was measured as described in the legend for Fig. 1. Asterisks, Statistical significance: \*,  $P < .05$  (Student's  $t$  test) compared with the dark group, in which saline was injected; #,  $P < .05$  (one-way ANOVA followed by Dunnett's test) compared with the light group with saline.  $n = 3$  to 7.



pH 7.4, 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.2 mg/ml transfer RNA, 0.25% sodium dodecyl sulfate] containing  $^{33}\text{P}$ -labeled cRNA probes for 16 h at 60°C. Radioisotope-labeled [ $\alpha$ - $^{33}\text{P}$ ]UTP (New England Nuclear, Boston, MA) antisense cRNA probes were made from restriction enzyme-linearized cDNA templates kindly donated by Dr. H. Okamura (Kobe University, Japan). After high-stringency posthybridization washes with 2× standard saline citrate/50% formamide, slices were treated with RNase A (10  $\mu\text{g}/\text{ml}$ ) for 30 min at 37°C. The radioactivity of each SCN on BioMax MR film (Kodak) was analyzed using a microcomputer interface to an image analysis system (Imaging Research, Inc., St. Catherine's, Ontario, Canada) after conversion into absorbance by  $^{14}\text{C}$  autoradiographic microscopes (Amersham, Buckinghamshire, UK). For data analysis, we subtracted the intensities of the absorbance of the corpus callosum from those of the SCN in each section and regarded it as the net intensity in the SCN. The intensity values of the sections from the rostral-most to the caudal-most part of the SCN (five sections per hamster brain) were then summed; the sum was considered to be a measure of the amount of *Per1*, *Per2*, or *Per3* mRNA in this region. We used relative mRNA abundance, which means that the intensity values of the peak point (Fig. 1A) of the dark control group (Fig. 1, B–D; Fig. 2B; Fig. 3B) or saline group (Fig. 4B and 6B) were adjusted to 100. For emulsion autoradiography, all mounted slices were dipped into emulsion (NTB2, Kodak, Rochester, NY; diluted 1:1 with distilled water) after exposure to X-ray film, air dried for 3 h, and stored in light-tight slide boxes at 4°C for 2 weeks. The slides were developed with a D19 developer (Kodak), then fixed with Fujifix

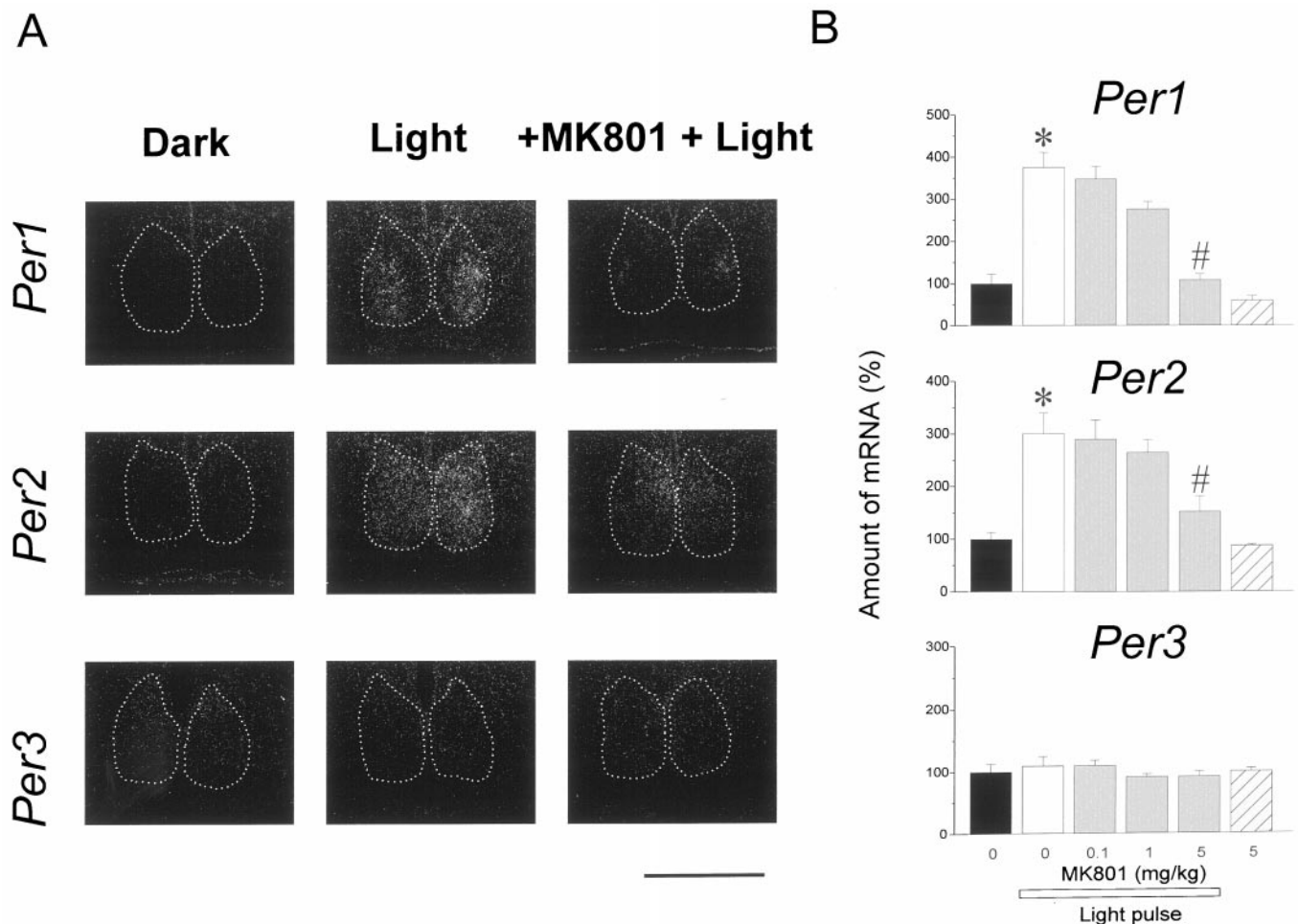
(Fuji Film, Tokyo, Japan), and counterstained with cresyl violet. Subnuclear silver grain distribution in the SCN of all slices was examined using optical microscope. We did not adopt the quantitative analysis of emulsion autoradiogram because thickness of coating could not be controlled with the present emulsion-dipping method.

**Statistical Analysis.** The values are expressed as means  $\pm$  S.E.M. For statistical analysis, one-way ANOVA, followed by Dunnett's test or the Student's *t* test, was applied.

## Results

### Photic Induction of *Per* mRNA in the Hamster SCN.

The level of *Per1*, *Per2*, and *Per3* mRNA in the SCN showed a robust circadian rhythm under DD conditions 2 days after release from LD conditions (Fig. 1A). Peaks in *Per1*, *Per2*, and *Per3* mRNA rhythm under DD conditions were observed at CT4, CT12, and CT12, respectively. As shown in Fig. 1, C and D, brief light exposure at CT13.5 or CT20 elicited an increase in *Per1* and *Per2* mRNA in the SCN with peaks at 90 or 180 min, respectively, after light onset. Light exposure both at CT13.5 and CT20 failed to affect the amount of *Per3* mRNA in the SCN (Fig. 1, C and D). During CT6, at which the amount of *Per* mRNA still remained high, light exposure had little effect on the level of *Per1*, *Per2*, or *Per3* mRNA in the SCN (Fig. 1B). Emulsion autoradiograms revealed that



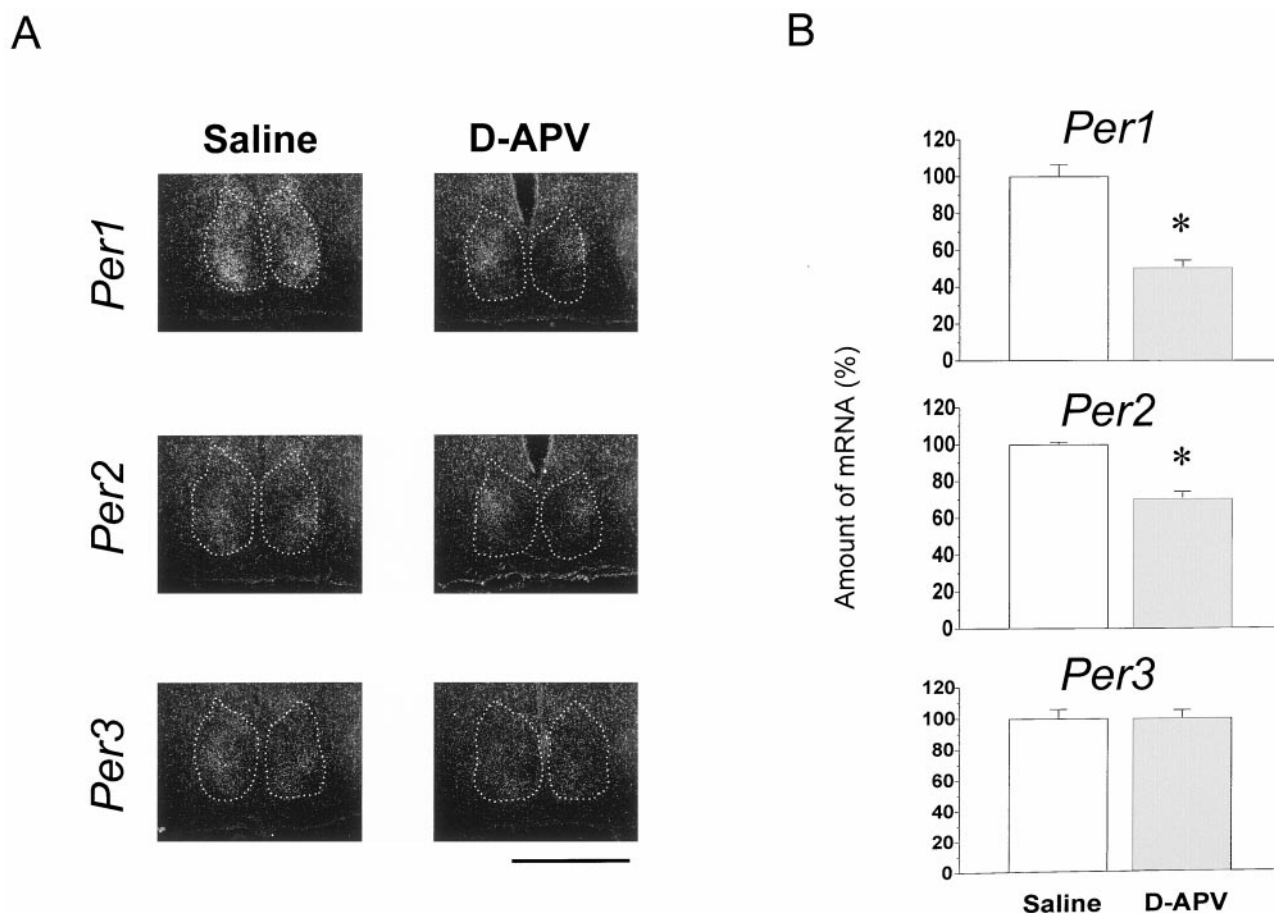
**Fig. 3.** Inhibitory effects of +MK801 on light pulse-induced (CT20) *Per* mRNA induction in the SCN. Details are identical to the legend for Fig. 2. *n* = 3.

photic induction of *Per1* and *Per2* mRNA during both CT13.5 and CT20 was observed especially in the ventrolateral portion of the SCN, and a weak induction was also seen in the dorsal part of the SCN (Figs. 2A and 3A). The photic induction of *Per1* and *Per2* mRNA was more widely distributed and more intensively observed in the SCN of hamsters in response to light at CT20 compared with that at CT13.5 (Figs. 2 and 3).

**Inhibitory Actions of NMDA Receptor Antagonists on Photic Induction of *Per* mRNA in the SCN.** Systemic injections of +MK801, a noncompetitive NMDA receptor antagonist, suppressed an increase in *Per1* and *Per2* mRNA normally elicited by light exposure at both CT13.5 and CT20 in a dose-dependent manner (0.1–5 mg/kg) (Figs. 2 and 3). These injections did not, however, affect the basal level of *Per1* and *Per2* mRNA in the absence of light exposure (Figs. 2 and 3). From emulsion autoradiograms of all examined slices, it appears that +MK801 suppressed the photic induction of *Per1* and *Per2* mRNA mainly in the ventrolateral part of the SCN at CT13.5 and CT20 (Figs. 2A and 3A). On the other hand, the cells in the dorsal part of the SCN still exhibited a photic induction of *Per1* and *Per2* mRNA even after +MK801 treatment. At CT13.5, +MK801 did not reduce, but rather slightly augmented, photic induction of *Per1*

and *Per2* mRNA in the dorsal part of the SCN. The amount of *Per3* mRNA in the SCN was not affected by light exposure and/or +MK801 treatment at CT13.5 and CT20. The induction of *Per1* and *Per2* mRNA in the SCN resulting from a light pulse at CT20 was also inhibited by an intralateral ventricular injection of D-APV, a competitive NMDA receptor antagonist (Fig. 4). Topological features related to the inhibitory action of D-APV were similar to that of +MK801. D-APV failed to affect the level of *Per3* mRNA in the SCN.

**Correlative Inhibitory Action of +MK801 between Photic Induction of *Per* mRNA and Photic Resetting of Behavioral Rhythm.** Under DD conditions, pretreatment with +MK801 dose dependently attenuated the phase delay or phase advance of wheel-running rhythm induced by a light pulse at CT13.5 or CT20, respectively, without affecting the phase of behavioral rhythm in hamsters receiving no light stimulation (Fig. 5A). Correlative analysis demonstrated that the attenuating effects of +MK801 on photic resetting of the behavioral rhythm correlated well with the inhibitory action of this compound on photic induction of *Per1* and *Per2* mRNA but not with that of *Per3* mRNA in the SCN at both CT13.5 and CT20 (Table 1 and Fig. 5B). In addition, for both *Per1* and *Per2* mRNA expression, the correlation values were higher at CT20 than at CT13.5.



**Fig. 4.** Inhibitory effects of D-APV on light pulse-induced (CT20) *Per* mRNA induction in the SCN. After release into DD for 2 days, saline or D-APV (40 nmol/2  $\mu$ l) was injected intralaterally into hamsters 10 min before light onset. Animals were then exposed to a light pulse (60 lux) for 15 min at CT20 and 4% PFA was perfused intracardially 90 min after light onset. A, representative emulsion autoradiograms showing that D-APV suppressed the photic induction of *Per1* and *Per2* mRNA in the ventrolateral part of the SCN. Dotted circles indicate the border of the SCN. Scale bar, 0.5 mm. B, quantitative analysis of *Per* mRNA in the SCN. Statistical significance: \*,  $P < .05$  (Student's  $t$  test) compared with the saline group.  $n = 4$ .

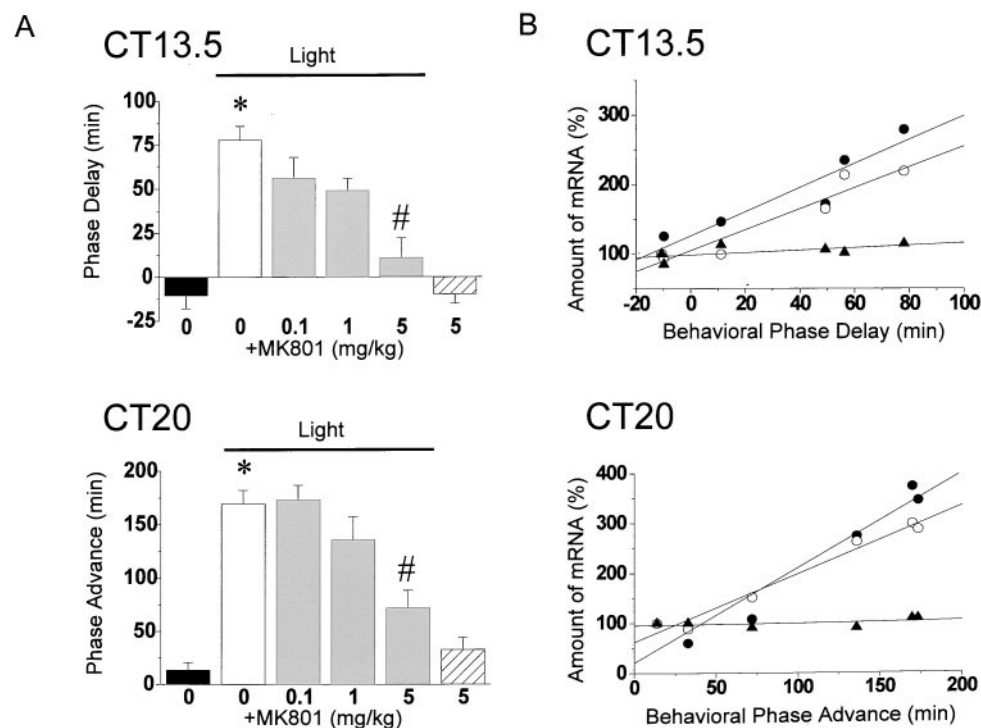
**Effect of Local Injection of NMDA on *Per* mRNA in the SCN.** We next investigated whether or not NMDA injected directly into the SCN caused *Per* mRNA induction in the SCN in a manner similar to light exposure. At first, we confirmed that the tip of the injection cannula was successfully inserted just above the SCN (data not shown). Fast green stain, which was infused through the injection cannula, was observed throughout the entire SCN bilaterally, as well as the optic chiasm, but not in the supraoptic or hypothalamic paraventricular nuclei. As shown in Fig. 6, NMDA injection into the SCN at CT20 significantly elicited a response from *Per1* and *Per2* but not *Per3* mRNA in the SCN, whereas vehicle injection failed to elicit a response. The distribution of NMDA-induced *Per1* and *Per2* mRNA expression varied among animals because of the differences in cannula position, i.e., two of five animals treated with NMDA exhibited the robust induction in ventrolateral part in the SCN, whereas in the remaining three hamsters, *Per1* and *Per2* mRNA induction was observed throughout the entire SCN. The levels of *Per1* and *Per2* mRNA after NMDA injection were 85 and 76% of that seen in light-activated (60 lux for 15 min at CT20) SCN, respectively. NMDA injection into the SCN did not affect *Per1*, *Per2*, or *Per3* mRNA expression in the hypothalamic paraventricular nucleus (data not shown).

**Subtype Distribution of NMDA Receptor mRNA in the SCN.** Similar to previous reports (Watanabe et al.,

1993), NR2A and NR2B, but not NR2C and NR2D, mRNA were abundantly expressed in the forebrain, including the cortex and the hippocampus, in hamsters (data not shown). In the hamster cerebellum, NR2C (high) and NR2A (moderate) were expressed, whereas neither NR2B nor NR2D signals were observed (data not shown). Emulsion autoradiograms in Fig. 7 show the distribution of mRNA for NMDA receptor subtypes NR2A, NR2B, NR2C, and NR2D in the hamster SCN. Among four subtypes of NMDA receptors, the expression of NR2C mRNA was most abundant in the SCN, and its signal was restricted to the SCN in the hypothalamus. Furthermore, NR2C mRNA was expressed especially in the dorsal part of the SCN. NR2B mRNA was also detected in the hamster SCN, and the expression was observed from the ventrolateral to the lateral part of the SCN, at which NMDA receptor antagonists exhibited the inhibitory action on *Per* gene expression. On the other hand, neither NR2A nor NR2D mRNA was expressed in the hamster SCN.

## Discussion

In the present study, we demonstrated that photic induction of *Per1* and *Per2* mRNA is suppressed by NMDA receptor antagonists +MK801 and D-APV in the ventrolateral part of the SCN, at which NR2B subtypes of NMDA receptors were expressed. Furthermore, NMDA injection into the SCN elicited



**Fig. 5.** Correlation between the inhibition of photic induction of *Per* mRNA and the inhibition of photic resetting of behavioral rhythm by +MK801. A, effect of +MK801 on phase shift of wheel-running rhythm induced by light pulse at CT13.5 (top) or CT20 (bottom). Under DD conditions, hamsters received an intraperitoneal injection of saline or +MK801 followed by light exposure (60 lux) for 15 min at CT13.5 or CT20. The vertical axis indicates the phase delay (top) or advance (bottom) in behavioral rhythm calculated from two regression lines drawn by two observers unaware of test conditions. Statistical significance: \*,  $P < .05$  (Student's *t* test) compared with the dark group, injected; #,  $P < .05$  (one-way ANOVA followed by Dunnett's test) compared with light group, in which saline was injected.  $n = 5$  to 10. B, correlation analysis between photic induction of *Per1* (closed circle), *Per2* (open circle), and *Per3* (closed triangle) mRNA and photic resetting of behavioral rhythm, based on data from Figs. 2 and 3.

TABLE 1

Correlative analysis between the inhibition of photic induction of *Per* mRNA and the inhibition of photic resetting of behavioral rhythms by +MK801

		CT13.5 <sup>a</sup>			CT20 <sup>b</sup>		
<i>Per</i> mRNA		<i>Per1</i>	<i>Per2</i>	<i>Per3</i>	<i>Per1</i>	<i>Per2</i>	<i>Per3</i>
<i>R</i> value		0.9486	0.9583	0.6089	0.9642	0.9818	0.5536
<i>F</i> (1,4) value		35.95	45.00	2.35	52.85	107.02	1.77
Statistical significance		$P < .01$	$P < .01$	$P > .05$	$P < .01$	$P < .001$	$P > .05$

<sup>a</sup> Correlation between behavioral phase delay and *Per* mRNA induction at CT13.5.

<sup>b</sup> Correlation between behavioral phase advance and *Per* mRNA induction at CT20.

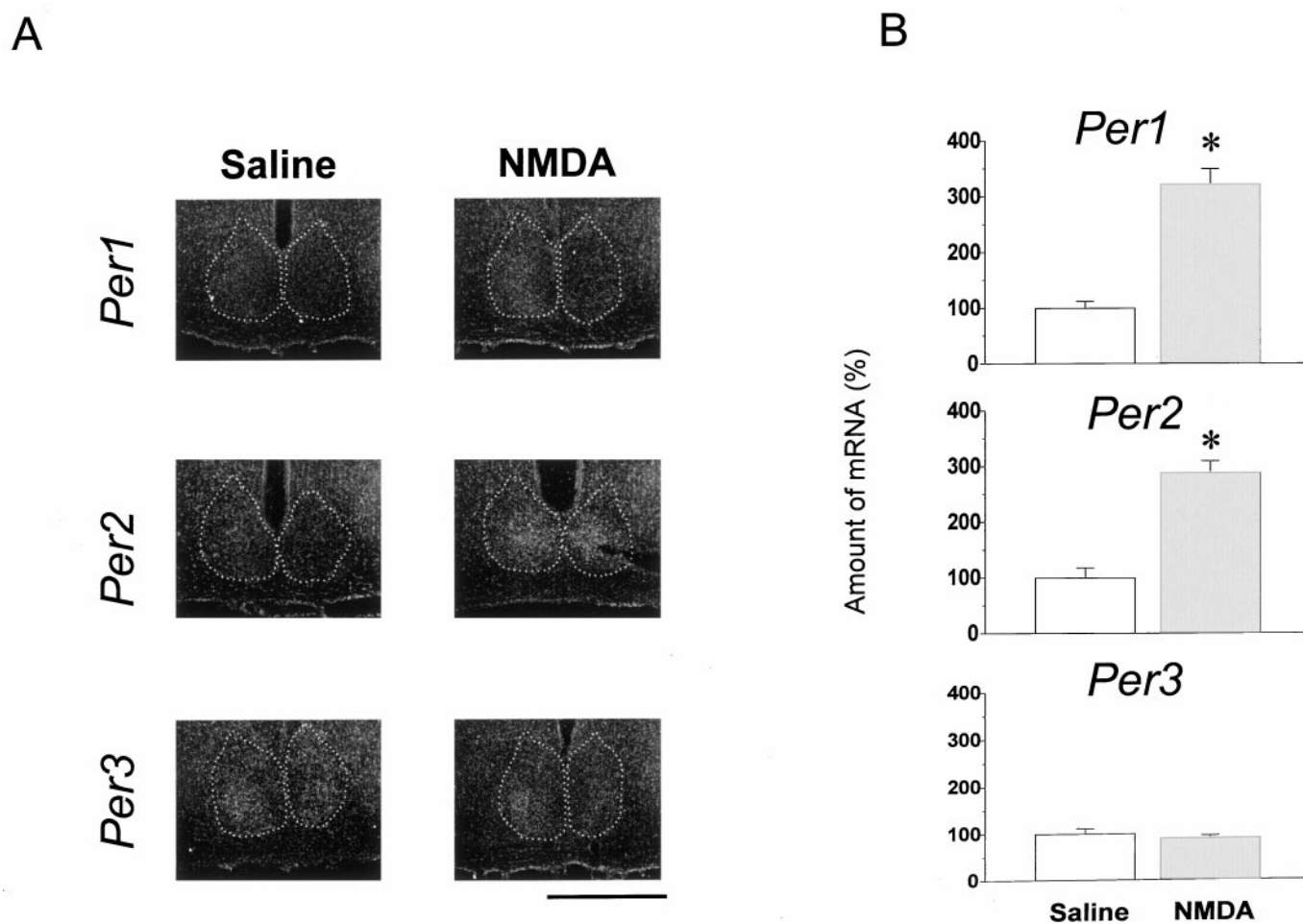


*Per1* and *Per2* mRNA expression, suggesting that transient, but not tonic, activation of NMDA receptor is necessary for photic induction of *Per1* and *Per2* genes. We also showed that the photic induction of the *Per* gene via the activation of NMDA receptor was strongly associated with photic resetting of the behavioral rhythm. In one of our previous studies, both the light-induced phase shift in behavioral rhythm in vivo and the glutamate-induced phase shift of SCN neuronal firing rhythm in vitro were attenuated by pretreatment with an antisense oligonucleotide targeting *Per1* mRNA (Akiyama et al., 1999). Our previous and present findings together suggest that *Per1* and *Per2* mRNA induction via glutamate/NMDA receptor activation is a critical pathway for photic resetting of the biological clock in mammals.

The activation of NMDA receptor increases intracellular  $\text{Ca}^{2+}$  concentration, thereby eliciting a varying gene expression, resulting in the appearance of synaptic plasticity that underlies learning and memory. Similarity in the topological distribution of the *Per* gene and Fos induction in the hamster SCN (Rea, 1992) led to the supposition that the transcripts of both genes may be regulated by a common signal pathway in cells. Of the transcription factors that trigger *Per1* and *Per2*, as well as *c-fos* gene expression, one candidate is cAMP response element (CRE)-binding protein (CREB), which up-regulates transcriptional activity upon

phosphorylation at the Ser<sup>133</sup> residue. It was reported that light stimulation caused CREB activation and CRE-mediated gene expression in the SCN only during the subjective night, when light has the capacity to reset the biological clock (Obrietan et al., 1999). Furthermore, the phosphorylation of CREB was elicited by glutamate application in the cultured SCN (McNulty et al., 1998). Multiple protein kinases including protein kinase A,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II, and mitogen-activated protein kinase (MAPK) have been shown to have the ability to activate the CRE/CREB pathway. Recently, Obrietan et al. (1998) demonstrated that both light stimulation and glutamate application during the subjective night activated MAPK in the SCN. Therefore, we consider MAPK to be the likely kinase linking NMDA receptor activation to *Per* gene expression in the SCN, although more experiments clarifying the involvement of other kinases like  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II and/or protein kinase A will be required.

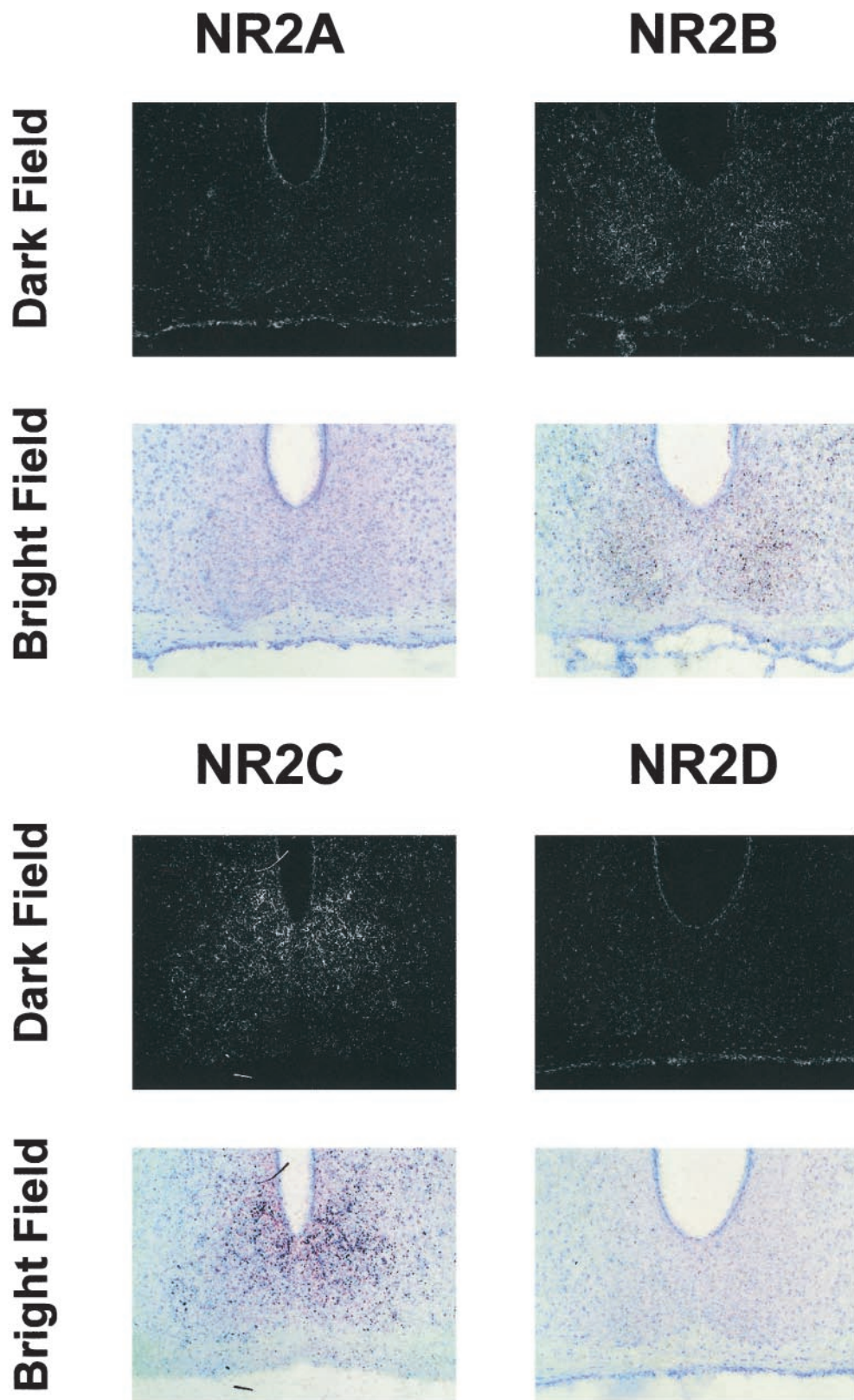
The peak level in the photic induction of *Per1* and *Per2* genes was found to be higher at CT20 (6.5-fold for *Per1* and 3.2-fold for *Per2* higher than basal level, based on data from Fig. 1) than at CT13.5 (2.2-fold for *Per1* and 1.7-fold for *Per2* higher than basal level, based on data from Fig. 1). These differences in degree of photic induction of *Per* gene corre-



**Fig. 6.** NMDA injection into the SCN elicited induction of *Per1* and *Per2* but not *Per3* mRNA. A, representative emulsion autoradiograms showing that NMDA injection (2 nmol/0.2  $\mu$ l) into the SCN induced *Per1* and *Per2*, but not *Per3*, mRNA in the SCN. Dotted circles indicate the border of the SCN. Scale bar, 0.5 mm. B, quantitative analysis of *Per* mRNA in the SCN. The amount of *Per* mRNA in the SCN was measured as described in the legend for Fig. 1. Statistical significance: \*,  $P < .05$  (Student's *t* test) compared with the saline group.  $n = 4$  to 5.

lated well with the behavioral phase shift; i.e., the large phase advance at CT20 (170 min) and relative small phase delay (78 min) at CT13.5 in our hamsters agreed with other

reports (Rea, 1992). Different pathways of signal transduction for light-evoked phase delays during early subjective night and phase advances during late subjective night (Ding



**Fig. 7.** Distribution of NMDA receptor subtype mRNA in the SCN. Representative dark-field (top) and bright-field (bottom) emulsion autoradiograms for NR2A, NR2B, NR2C, and NR2D mRNA are shown. Note that abundant NR2C and NR2B mRNA are expressed in the SCN, whereas NR2A and NR2D mRNA could not be observed. Scale bar, 0.5 mm.



et al., 1998) may account for these differences in photic induction of *Per* genes in the SCN. Alternatively, there may be a difference in the degree of gating for *Per1* and *Per2* gene expression with light exposure. The roles of brain-derived neurotrophic factor in generating circadian gating to photic signal were recently reported for the SCN (Liang et al., 2000).

It should be noted that light exposure at both CT13.5 and CT20 elicited *Per1* and *Per2* gene induction exclusively in the ventrolateral, and modestly in the dorsal, part of the SCN, but NMDA receptor antagonists suppressed *Per1* and *Per2* mRNA induction only in the ventrolateral part of the SCN. These topological features elicited by light stimulation and NMDA receptor antagonists were also reported in the induction of immediate-early genes, such as Fos proteins in the hamster SCN (Abe et al., 1991; Vuilleuz et al., 1998). In hamsters, distribution of the RHT terminal within the SCN expands more to the dorsal part compared with that in other species, such as rats (Johnson et al., 1988). As shown in our present study, NMDA receptor subtype NR2B was expressed dominantly in the ventrolateral and lateral parts of the SCN, where inhibitory actions of NMDA receptor antagonists were also observed. In contrast, subtype NR2C was observed in the dorsal part of the hamster SCN. Earlier papers demonstrated the presence of NR2C mRNA in the dorsomedial and ventrolateral areas of the SCN in rats (Mikkelsen et al., 1993, 1995) and mice (Watanabe et al., 1993) and abundant expression of NR2B mRNA in the mouse SCN (O'Hara et al., 1995). Interestingly, we recently reported that gene deletion of NR2C or NR2A failed to affect photic resetting of the behavioral rhythm in mice (Moriya et al., 2000). Therefore, NR2B itself with or without forming complexes with other NMDA receptor subtypes could mediate the photic induction of the *Per* gene in the rodent SCN. Thus, different neuronal mechanisms for the photic induction of *Per1* and *Per2* mRNA might underlie the multiple cell populations in the SCN, i.e., NMDA receptor-dependent induction in the ventrolateral and NMDA receptor-independent induction in the dorsal part of the SCN.

In summary, our present study suggests that activation of the NMDA receptor, including NR2B subtype, mediates the photic induction of *Per1* and *Per2* genes in cell populations of the ventrolateral SCN, which is then followed by resetting of the biological clock in hamsters.

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