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Participation of transcription factors from the Rel/NF-κB family in the circadian system in hamsters

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Abstract

We have studied the presence and activity of components of the nuclear factor-κB (NF-κB) transcription factor in the hamster circadian system analyzing wheel-running activity, protein expression and DNA binding activity by electrophoresis mobility shift assays (EMSA). Non-rhythmic specific immunoreactive bands corresponding to a NF-κB subunit (p65) were found in hamster suprachiasmatic nuclei (SCN) homogenates. The active form of NF-κB evidenced by EMSA was clear and specific in SCN nuclear extracts. The administration of the NF-κB inhibitor pyrrolidine-dithiocharbamate (PDTC) blocked the light-induced phase advance at circadian time 18 (vehicle + light pulse: 2.08 ± 0.46 h, PDTC + light: 0.36 ± 0.35 h). These results demonstrate the presence and activity of Rel/NF-κB family proteins in the hamster SCN and suggest that these proteins may be related to the entrainment and regulation of circadian rhythms.

Keywords: Circadian rhythms; Suprachiasmatic nucleus; Pyrrolidine-dithiocharbamate; Neuroimmune interactions; Photic entrainment

The mammalian circadian system is coordinated by a central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN). Photic entrainment of this system occurs through a retinohypothalamic pathway, resulting in a signal transduction cascade that ultimately regulates the activity of SCN 'clock genes'. Although several transcriptional activators have been proposed to be involved in phase resetting of the clock within the SCN, it is likely that additional factors in these nuclei fine-tune the phase of the central pacemaker (for review, see ref. [6]). Nuclear factor-kappa B (NF-κB), originally described as a transcription factor in B lymphocytes, has also been related to sleep, neuronal plasticity, and developmental responses [6,11,12,16]. NF-κB is a dimeric complex composed by members of the Rel protein family including p65 (Rel-A), p50/p105, p52/p100, Rel-B and c-Rel, most usually p65 and p50 [16,18]. NF-κB is latent in the cytoplasm and bound to an inhibitory κB protein (IκB). Upon activation IκB is degraded, allowing NF-κB to enter into the nucleus exerting its transcriptional activity, inducing the expression of nitric oxide synthase, neurotransmitters, cytokines, immunoglobulins, IκB, Rel family proteins, and several other genes [15,18]. Its activation is triggered by the rise of intracellular calcium, protein kinase activity, glutamate, phorbol esters, ultraviolet light, oxidative stress, and cytokines such as IL-1 and TNFα [1–3,13].

To address the possibility of NF-κB participation in the hamster SCN, we studied the presence and activity of NF-κB related proteins using running wheel activity analysis and molecular assays.

Animals used for all the experiments were adult male golden hamsters (*Mesocricetus auratus*) from our colony, housed under 14:10 light: dark photoperiods (LD) or constant dark (DD) conditions, with food and water ad libitum.

Western blot analysis of SCN whole cell homogenates from animals sacrificed at six different time points (i.e. at 4-h intervals) was performed in order to study p65 expression. Diurnal variations were studied in homogenates obtained from animals kept under a light-dark cycle (LD) (with

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zeitgeber time (ZT) 12 defined as the time of lights off), while animals kept in constant darkness (DD), 48 h prior to the experiment were used to analyze possible circadian variations. In both cases, four animals were sacrificed for each time point (a total of 48 animals). Twenty μg of protein per lane were loaded in 9% SDS-polyacrylamide gels and subsequently electrophoresed, transferred to nitrocellulose and immunoblotted with an antibody against p65 (1:1000 dilution) (Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). Light emission was assessed using an ECL kit (Amersham Pharmacia Biotech). A total of 2.5 μg of a p65 control protein were loaded in each assay as a positive control.

In order to characterize NF-κB activity in the SCN we performed electrophoretic mobility shift assays (EMSAs) from whole brain, hypothalamus and SCN nuclear extracts obtained according to the method of Korner et al. [10], slightly modified [5]. Briefly, samples were incubated for 30 min at 0 °C and 1 ng (γ^{32}-P) ATP labeled oligonucleotide probe was added, incubating for another 30 min at 0 °C. In each competition assay, homologous unlabeled κB oligonucleotides were added before the first incubation. All samples were subsequently electrophoresed in a 6% polyacrilamide gel, dried and autoradiographed for 24 h at −70 °C with intensifying screens. For the SCN EMSAs, the nuclear extracts of tissue from ten animals/lane killed at ZT 4 (midday) or ZT 16 (midnight) were used (a total of 40 animals), and EMSAs were repeated three times. Due to the high number of animals required to obtain the SCN nuclear extracts, the characterization of the bands (competition assay) was done with whole brain extracts. For the supershift assays the same protocol was followed except for the use of 4% polyacrilamide gel and an exposition time of 72 h. In this case a p65 antibody (Santa Cruz Biotechnology) diluted 1:200 was added to the samples prior to the first incubation.

Behavioral analysis was performed recording the wheel-running activity of hamsters with a Dataquest III acquisition system (Mini-mitter, Sunriver, OR). The activity onset defined as CT 12 was used as a phase reference point to calculate phase shifts. Twenty-six ga. cannulae were implanted in the third ventricle (coordinates: AP = +0.6, DV = −8.2 from bregma) under deep anesthesia (90 mg/kg sodium pentobarbiturate). After surgery animals were kept in DD and their running wheel activity was recorded for 30 days. Animals were treated at CT 18, when light pulses should produce phase advances of the free running rhythm. Animals (6/group, 18 for the whole experiment) received 0.5 μl i.c.v. injections of vehicle or the NF-κB inhibitor PDTC (7 μM) [17] followed 15 min later by a 700-lux, 15-min light pulse.

The aim of the first experiment was to analyze the expression levels of p65 in the hamster SCN at different time points in LD and DD conditions (Fig. 1). Strong expression of a single band was observed, with a molecular

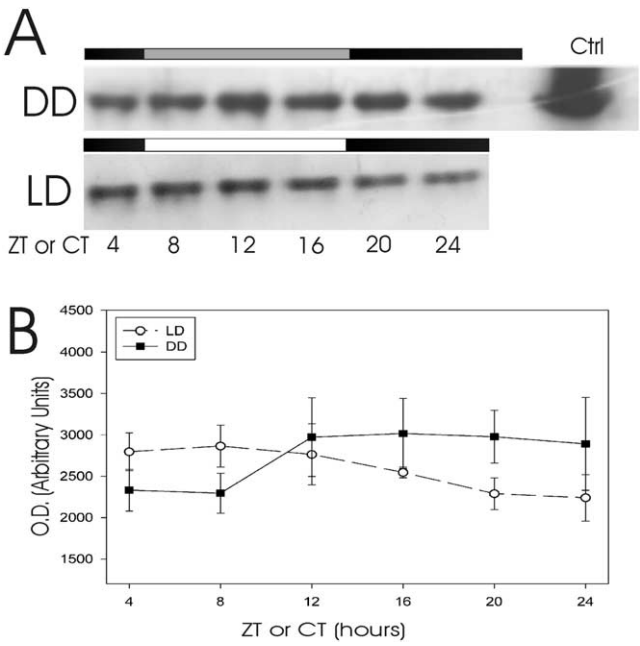

Fig. 1. p65 expression in the hamster SCN. (A) Representative western blots of SCN whole cell extracts from animals sacrificed at different times of the day in DD (CT 4; 8; 12; 16; 20 and 24) or LD conditions (ZT 4; 8; 12; 16; 20 and 24). (B) Quantitative analysis (mean ± SEM) of western blots of SCN whole cell extracts from animals sacrificed at different times of the day in LD or DD conditions (ZT or CT 4; 8; 12; 16; 20 and 24). No diurnal or circadian variations were found in p65 expression ($n = 4$ for each time point, one-way ANOVA, $F = 0.6757$ for LD and $F = 0.7271$ for DD, $P > 0.5$). No differences in variance were found with a Bartlett test. The lane labeled as ctrl, was loaded with a p65 purified protein as a positive control.

weight of approximately 65 kD. Quantitative analysis showed neither diurnal nor circadian variations in intensity or mobility in SCN p65 expression (one-way ANOVA, $F = 0.6757$ for LD and $F = 0.7271$ for DD, $P > 0.5$). Similar results, namely an absence of daily and circadian variations of protein expression, were found for the p50 protein and its precursor p105 (data not shown).

Once NF-κB members were identified in the SCN, the κB binding activity was assessed. Four clear bands were obtained in the EMSAs when whole brain extracts were used (Fig. 2A), while only three bands were visualized with SCN or hypothalamic extracts (Figs. 2B,C). In all cases the bands observed in the SCN and hypothalamic extracts were the same as the first, second and fourth bands observed in the whole brain extracts (counting from the top). The missing band was the third one and might correspond to an NF-κB complex without constitutive activity in the hypothalamus. Overall, no gross variations in the active form of NF-κB were found in nuclear extracts from animals sacrificed at different times of day (Fig. 2C). All bands were competed by the addition of cold κB oligonucleotide to the incubation mixture but they were not competed by the addition of cold oligonucleotides containing unrelated consensus sequences (Fig. 2A). The presence of p65 in the observed bands of SCN extracts was evidenced by super-

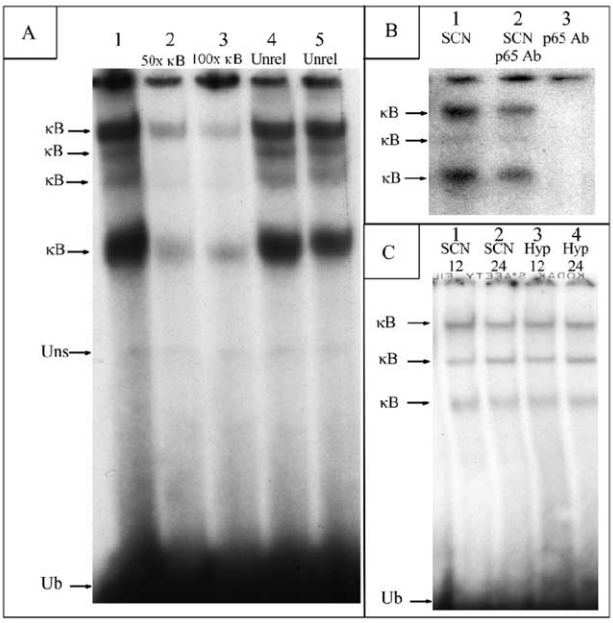

Fig. 2. NF-κB activity in hamster whole brain, hypothalamus and SCN. (A) Brain nuclear extracts: lane 1, incubation with κB probe; lanes 2 and 3, incubation with κB probe and preincubation with 50 × and 100 × excess of unlabeled κB oligonucleotide, respectively; lanes 4 and 5, incubation with κB probe and preincubation with 200 × excess of different unlabeled unrelated oligonucleotides. (B) SCN nuclear extracts: lane 1, incubation with κB probe; lane 2, incubation with κB probe and preincubation with p65 antibody; lane 3, incubation with κB probe and preincubation with p65 antibody without SCN nuclear extract. (C) SCN and hypothalamic nuclear extracts: lanes 1 and 2, SCN extracts obtained from animals sacrificed at midday or midnight, respectively; lanes 3 and 4, hypothalamic extracts obtained from animals sacrificed at midday or midnight, respectively, incubated with the κB probe. Bands corresponding to specific binding are marked as κB, unspecific bands are marked as Uns and the free probe band is marked as Ub.

shift assays performed by adding anti-p65 antibody to the binding mixture (Fig. 2B, lane 2). A decreased κB-binding activity was found for the three characteristic bands, suggesting an impaired DNA binding activity due to the antibody-p65 interaction. Therefore p65 is probably a component of all κB binding complexes found within the SCN.

The role of NF-κB in photic entrainment was addressed in behavioral experiments by administrating the NF-κB inhibitor pyrrolidine-dithiocharbamate (PDTC) to animals housed in DD prior to a light pulse at CT18. As shown in the actograms and the bar plot of Fig. 3, light induced a significant phase advance (2.38 ± 0.36 h) that was blocked by the previous administration of PDTC (0.38 ± 0.32 h) ($P < 0.002$, Student's t-test). No alterations of circadian rhythms were observed when PDTC was administered without a light pulse (data not shown).

Altogether this study shows for the first time that Rel/NF-κB-related factors are expressed in the hamster SCN in correlation with κB binding activity and that NF-κB activation might be implicated in photic entrainment, since the i.c.v. administration of the NF-κB inhibitor PDTC blocks the phase advance in response to light pulses at CT 18.

NF-κB was first known as an immune transcription factor, but in the last few years its activity has been reported in different physiological processes including sleep regulation [11], neuronal plasticity [12] and memory [4,5]. It is currently considered as a transcription factor involved in rapid and precise responses to complex stimuli such as stress, infection or training and is capable of responding to neurotrophic factors, neurotransmitters, proinflammatory cytokines, nitric oxide, bacterial endotoxin (LPS) and phorbol esters. Among neurotransmitters, NF-κB may be induced by glutamate [2,8,13,15], which is the main messenger of the retino-hypothalamic tract [7]. It is interesting that LPS, another positive modulator of NF-κB, increases the production of arginine-vasopressin in SCN slice cultures [14], since it is known that arginine-vasopressin is an output of the molecular machinery of the circadian clock [9]. This is in accordance with our preliminary results indicating that LPS induces a photic-

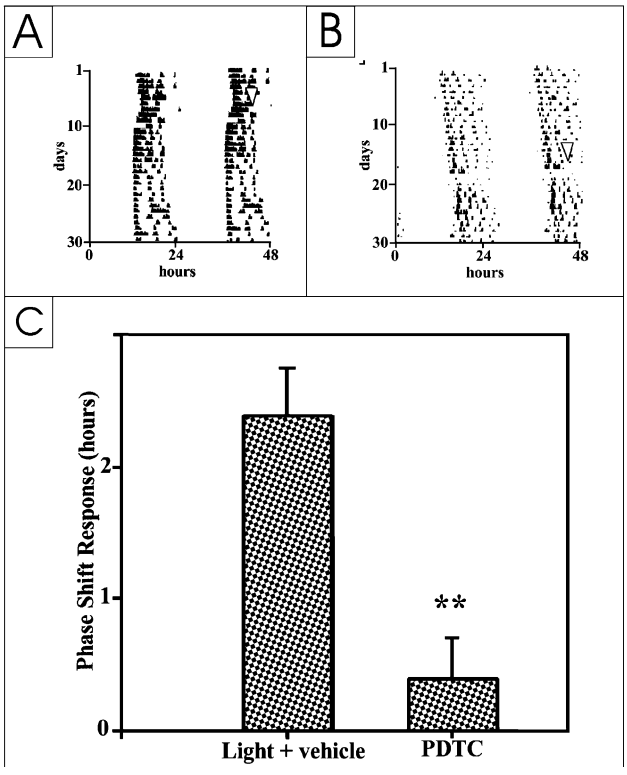

Fig. 3. Effect of PDTC on light-induced phase advances. (A, B) Representative actograms of animals treated with vehicle (A); or PDTC (7 μM) (B) prior to a light pulse. Drugs were administered at CT 18 (white triangle), with the activity onset defined as CT 12. (B) Summary of light-induced phase advances with or without previous administration of PDTC. This NF-κB inhibitor blocked the phase advances induced by light (*** = $P < 0.002$, Student's t-test). Shapiro–Wilk test showed both samples were normally distributed ($w = 0.9139$, $P = 0.4236$ for light pulse + PDTC, and $w = 0.9305$, $P = 0.5554$ for light pulse + vehicle) while no differences in variance were found with a Bartlett test ($F = 1.592$, $P = 0.2932$). No evident effects on circadian rhythms were observed when PDTC or vehicle were administered without a light pulse after the injection.

like phase response curve in mice (Bekinschtein et al., unpublished). Moreover, the expression of some other potent activators of this transcription factor such as IL-1 and TNF-α was clearly demonstrated in the hypothalamus [19]. We hypothesize that SCN NF-κB may be activated by the release of glutamate from the retino-hypothalamic tract and/or by hypothalamic or circulating cytokines. Indeed, we have recently found that TNF-α activates NF-κB in transfected SCN primary cell cultures (Marpegan et al., unpublished).

This putative interaction between cytokines and the biological clock might represent a cross-talk between the immune and the circadian system, where the SCN modulates the circadian variation of immune circulating factors which feedback into the clock to fine-tune phase output. This is supported by the fact that immunosuppressants affect the phase of SCN-controlled rhythms [7]. This interaction may be relevant both in physiological and pathological conditions, when synchronization of immune activities may be crucial for survival. The activity of this immune-circadian interaction in proinflammatory or immunodeficient situations deserves to be investigated.

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