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Block of c-Fos and JunB Expression by Antisense Oligonucleotides Inhibits Light-induced Phase Shifts of the Mammalian Circadian Clock

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Abstract

Light-induced phase shifts of circadian rhythmic locomotor activity are associated with the expression of c-Jun, JunB, c-Fos and FosB transcription factors in the rat suprachiasmatic nucleus, as shown in the present study. In order to explore the importance of c-Fos and JunB, the predominantly expressed AP-1 proteins for the phase-shifting effects of light, we blocked the expression of c-Fos and JunB in the suprachiasmatic nucleus of male rats, housed under constant darkness, by intracerebroventricular application of 2 μl of 1 mM antisense phosphorothioate oligodeoxynucleotides (ASO) specifically directed against c-*fos* and *junB* mRNA. A light pulse (300 lux for 1 h) at circadian time 15 induced a significant phase shift (by 125 ± 15 min) of the circadian locomotor activity rhythm, whereas application of ASO 6 h before the light pulse completely prevented this phase shift. Application of control nonsense oligodeoxynucleotides had no effect. ASO strongly reduced the light-induced expression of c-Fos and JunB proteins. In contrast, light pulses with or without the control nonsense oligodeoxynucleotides evoked strong nuclear c-Fos and JunB immunoreactivity in the rat suprachiasmatic nucleus. These results demonstrate for the first time that inducible transcription factors such as c-Fos and JunB are an essential part of fundamental biological processes in the adult mammalian nervous system, e.g. of light-induced phase shifts of the circadian pacemaker.

Introduction

The suprachiasmatic nucleus (SCN) is the predominant circadian pacemaker in mammals regulating a variety of basic physiological and behavioural rhythms (reviewed by Wollnik, 1992; Takahashi, 1993). The daily light–dark cycle synchronizes the endogenous circadian pacemaker within the SCN to the environmental 24 h rhythm. However, the molecular genetic mechanisms by which light entrains the circadian clock are poorly understood.

Previous studies have demonstrated that light-induced phase shifts of circadian rhythms induce immediate-early genes such as c-*jun*, *junB*, c-*fos* and *NGFI-A* (synonyms of *NGFI-A* are *Krox-24*, *Zif/268*, *Egr-1*) mRNAs in rodent SCN neurons (Rea, 1989; Aronin *et al.*, 1990; Earnest *et al.*, 1990; Kornhauser *et al.*, 1990, 1992; Rusak *et al.*, 1990; Ebling *et al.*, 1991; Sutin and Kilduff, 1992; Chambille *et al.*, 1993). These genes code for inducible transcription factors that bind to specific DNA elements regulating the transcription of their target genes (Ryseck and Bravo, 1991). Therefore, selective up-regulation of inducible transcription factors orchestrates the reactive alterations in the cellular programme according to the physiological or pathological context of events (Bravo, 1990; Morgan and Curran, 1991). For example, the selective inhibition of c-Jun, JunB and JunD proteins specifically affects cellular proliferation and neuron differentiation (Kovary and Bravo, 1992; Schlingensiepen and Brysch, 1992; Schlingensiepen *et al.*, 1993, 1994).

The following findings suggest the importance of inducible transcription factors for the function of the circadian pacemaker. (i) Inducible transcription factors are expressed in the SCN only during that circadian time when light is capable of shifting the circadian rhythm (reviewed by Takahashi, 1993). (ii) Expression of inducible transcription factors is mainly restricted to the ventrolateral part of the SCN, the terminal region of retinal afferents. (iii) The onset of

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circadian gating of inducible transcription factor induction is precisely correlated with the phase-shifting effects of the light (Kornhauser *et al.*, 1990; Mead *et al.*, 1992). Here we provide direct evidence that the light-induced shifts of circadian locomotor activity depend on the expression of the JunB and c-Fos proteins.

Material and methods

Adult male rats (n = 12) were stereotaxically implanted under anaesthesia (100 mg/kg ketamine hydrochloride and 10 mg xylazine, i.m.) with a microinjection guide-cannula targeted to the bottom of the third ventricle at the level of the ventral SCN. After 2 weeks, the rats were placed in individual cages equipped with a running wheel and were housed under constant dark conditions for 16 weeks. Wheel-running activity was monitored on-line with a microcomputer system as described previously (Wollnik, 1991).

Every 4 weeks, the rats were subjected to one of the following treatments: (i) light pulse (300 lux, 1 h) at circadian time (CT) 15, when light pulses induce a maximal phase delay of circadian activity rhythms; (ii) intracerebroventricular (i.c.v.) application of antisense phosphorothioate oligodeoxynucleotides (ASO; 1:1 mixture of c-*fos* and *junB* ASO, each 1 mM in 2 µl physiological saline) at CT 9 alone or (iii) followed by a light pulse at CT 15; (iv) application of control nonsense oligonucleotides (NSO; 1:1 mixture of c-*fos* and *junB* ASO, each 1 mM in 2 µl physiological saline) at CT 9 followed by a light pulse at CT 15. Rats were reanaesthetized and killed 2–6 h after one of these treatments by transcardial perfusion with 4% paraformaldehyde. Following postfixation the brains were cryoprotected in 30% sucrose. Synthesis, stabilization protocol and proof of specificity of the phosphorothioate oligodeoxynucleotides complementary to the translation initiation site of *junB* and c-*fos* mRNA respectively and of the NSO were recently described in detail (Schlingensiepen *et al.*, 1993; Gillardon *et al.*, 1994). Briefly, the sequences of the c-*fos* ASO and the random c-*fos* NSO were CGA GAA CAT CAT GGT CGA AG and CCC TTA TTT ACT ACT TTC GC respectively; the NSO had some complementarity with the 5′ end of the c-*fos* mRNA. The sequences of the *junB* ASO and mismatch *junB* NSO were TTT CGT GCA CAT CC and GTC CCT ATA CGA AC respectively (Biognostik, Göttingen, Germany; batch S-2044). The mismatch control sequence of the *junB* NSO contains the same CG content as the anti-*junB* sequence. This is important since an identical CG content is required to achieve the same binding energy to a complementary sequence.

Phase shifts in the activity rhythm were determined by measuring the phase difference between eye-fitted lines connecting the onset of activity for a 14 day period before and after an experimental manipulation (according to Daan and Pittendrigh, 1976). Differences between experimental groups were evaluated by a univariate one-way analysis of variance (Statistica, StatSoft, Tulsa, USA). Sheffé's multiple t-test was used for comparison of individual means.

Serial cryostat cut sections (35 µm) were immunocytochemically processed as free-floating sections and staining was visualized by the conventional avidin–biotin complex protocol (Herdegen *et al.*, 1991). The primary polyclonal antisera against c-Jun (1:30 000), JunB (1:4000), c-Fos (1:25 000) and FosB (1:2000) proteins were incubated for 48 h. The specificity of the antisera has been described in detail previously (Kovary and Bravo, 1991).

Results

Rats exposed to the light pulse for 1 h at CT 15 showed a robust phase shift of the circadian rhythm of wheel-running activity by

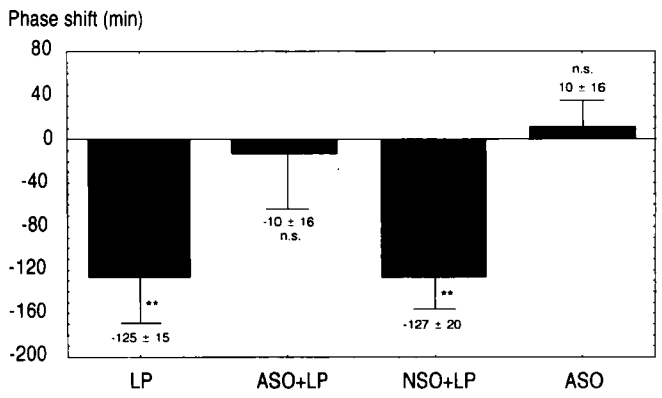

FIG. 1. Mean (±SD) of the phase shift of the circadian activity rhythm following application of a 1 h light pulse (LP, n = 9), LP preceded by ASO (n = 11) or NSO (n = 2), and ASO alone (n = 3). $^{**}P < 0.0001$; $n.s.$, non-significant phase shift compared to the free-running endogenous rhythm.

−125 ± 15 min that was almost identical to the phase shift induced by the i.c.v. application of NSO at CT 9 followed by light at CT 15 (phase shift = −127 ± 20 min) (Fig. 1). In contrast, i.c.v. application of anti-c-*fos* and anti-*junB* ASO 6 h prior to the light pulse at CT 15 completely blocked the phase shift (−10±16 min). Injection of ASO without a subsequent light pulse neither changed the free-running circadian rhythm nor shifted the locomotor activity (phase shift = +10 ± 16 min). Figure 2A shows representative activity records. In all animals, the injections of ASO or NSO did not alter the endogenous free-running circadian rhythms.

A light pulse at CT 15 alone or following application of NSO at CT 9 induced distinct nuclear immunoreactivity for c-Jun, JunB, c-Fos and FosB in the ventral SCN that persisted at least for 6 h (Figs 2B–D and 3). The numbers of c-Fos- and JunB- immunoreactive nuclei were strongly reduced by ~40–65% and the intensity of labelling was substantially lowered when application of ASO preceded the light pulse (Fig. 2B, C) whereas expression of c-Jun and FosB was not affected (Fig. 2D). Application of ASO alone did not induce inducible transcription factors.

Discussion

Here we present findings about physiological effects of the inducible transcription factors c-Fos and JunB in the adult mammalian nervous system *in vivo*. Block of expression of c-Fos and JunB by ASO prevented the light-induced phase shift of the circadian activity rhythms driven by molecular genetic changes in the SCN and strongly reduced the immunoreactivities of c-Fos and JunB. This block in phase shift was due to selective inhibition of c-Fos and JunB expression, because ASO alone did not interfere with the free-running rhythms, and NSO altered neither the effect of the light pulse nor the immunoreactivities. Thus, inducible transcription factors exert an important role in the adaptive synchronization of the endogenous clock to the environmental *zeitgeber* formed by the dark–light cycle. In addition, we have shown that light-induced phase shifts also induce, but to a lower degree, the expression of c-Jun and FosB proteins.

Nuclease-resistant phosphothioate oligodeoxynucleotides allow the selective inhibition of the synthesis of individual proteins (Neckers *et al.*, 1992), and the ensuing loss of function permits conclusions on the physiological function of the missing protein (Nishikura and Murray, 1987; Gerdes *et al.*, 1992; Kovary and Bravo, 1992). Recently,

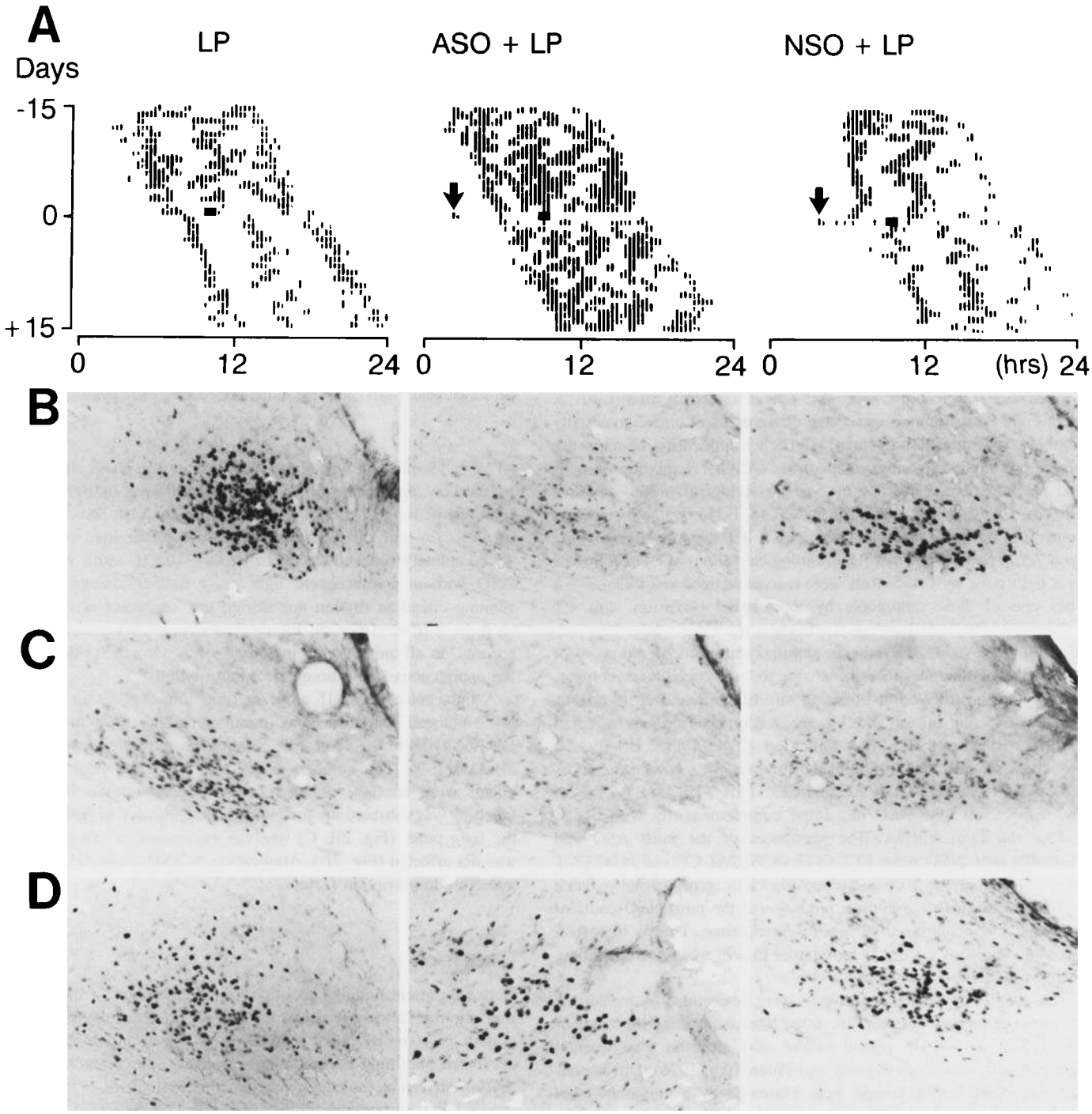

FIG. 2. (A) Representative recordings of wheel-running activity illustrating the phase shifts following a 1 h light pulse at CT 15 (LP, black rectangles), LP preceded by i.c.v. application of ASO at CT 9 (arrow) and LP preceded by i.c.v. application of NSO (arrow). Each line depicts a single 24 h period, and successive days are plotted from top to bottom. The recordings give the running activity between 15 days before (+15) and 15 days after (–15) the experimental treatment. Corresponding expression of (B) c-Fos, (C) JunB and (D) c-Jun in the SCN 4 h after LP (left column), ASO + LP (middle column) and NSO + LP (right column). Magnification (B–D), 400×.

it was demonstrated that the ASO used in the present study selectively prevents translation of JunB in hippocampal neurons *in vitro* (Schlingensiepen *et al.*, 1993) and of c-Fos in spinal cord neurons *in vivo* (Gillardon *et al.*, 1994). Function assays have conclusively demonstrated that reduced levels of c-Jun or JunB proteins induced by specific ASO selectively alter the differentiation or growth of hippocampal neurons (Schlingensiepen and Brysch, 1992; Schlingensiepen *et al.*, 1993, 1994).

We applied ASO analogues complementary to *junB* and c-*fos* mRNA because previous data (Rusak *et al.*, 1990, 1992; reviewed by Takahashi, 1993) and our results have shown that their protein products are the predominantly expressed proteins of the AP-1 family.

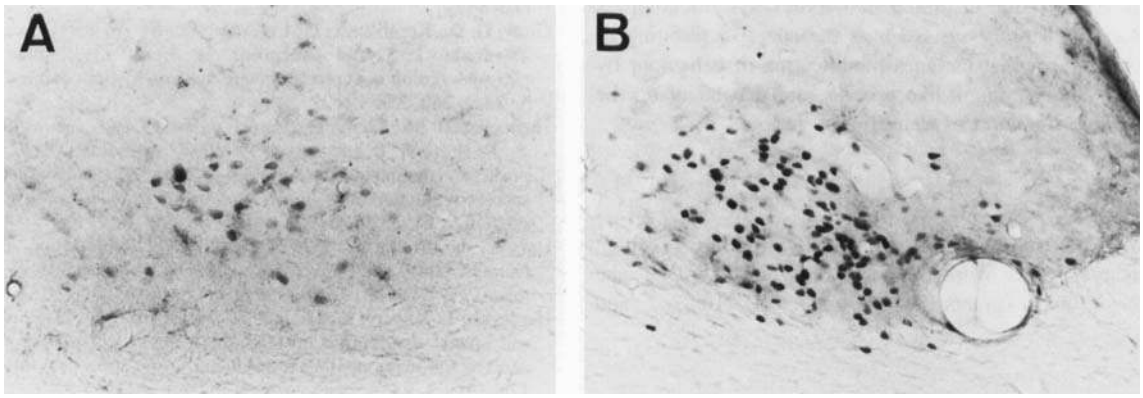

FIG. 3. (A) Basal expression of FosB in the SCN at CT 17. The rats were kept for 4 weeks in constant darkness. (B) Application of a 1 h light pulse at CT 15 increases the expression of FosB. Magnification, 400×.

Furthermore, since light pulses also induce other AP-1 binding proteins such as c-Jun and FosB, these might compensate for the absence of only one transcription factor. Knock-out mice carrying a null-mutation of c-*fos* or c-*jun* show apparently no alterations in the embryonic and neonatal phenotype of the nervous system (Hilberg *et al.*, 1993; Wang *et al.*, 1992). Therefore, we decided to block the expression of both c-Fos and JunB, because these proteins are supposed to exert the main inducible transcription activity of AP-1 proteins *in vitro* (Kovary and Bravo, 1991) and *in vivo* following light pulse (Kornhauser *et al.*, 1992) and other trans-synaptic excitation paradigms such as noxious cutaneous stimulation, epileptic seizures and cortical spreading depression (Herdegen *et al.*, 1991, 1993b, 1994; Demmer *et al.*, 1993; Gass *et al.*, 1993). Therefore, cocktails containing two or more ASO against inducible transcription factors offer a good strategy to determine significant effects of the presumably dominant dimers and/or combinations of transcription factors.

Synthetic oligodeoxynucleotides can interact with biological systems, via non-specific associations with DNA, RNA and proteins. Oligodeoxynucleotides also exhibit the potency to block or even to strip membrane receptors. In general, these compounds can interfere with signalling pathways on various levels of synaptic–nuclear information transfer. The present study contains two internal controls that argue against non-specific actions of the applied synthesized oligodeoxynucleotides: (i) the investigation of several related proteins, and (ii) the computer-assisted monitoring of behaviour, i.e. circadian locomotor activity. Also, (i) ASO selectively reduce the expression of c-Fos and JunB but not that of c-Jun and FosB. Such an expression pattern of AP-1 proteins has not been observed so far. Numerous experimental paradigms for trans-synaptic stimulation of neurons evoke parallel induction of *fos* and *jun* mRNAs and proteins (Saffen *et al.*, 1988; Sonnenberg *et al.*, 1989; Cole *et al.*, 1990; Wisden *et al.*, 1990; Herdegen *et al.*, 1991, 1993b; Gass *et al.*, 1993), and this is also true for Fos and Jun expression in the SCN following light pulses (Rusak *et al.*, 1990, 1992; Kornhauser *et al.*, 1992). In contrast, selective up-regulation of c-Jun and JunD *without* Fos proteins is a general feature of the cell body response following axotomy (Herdegen *et al.*, 1993a). (ii) Neither application of ASO or NSO alone nor the preceding application of light pulses interfered with the rhythm of locomotor activity. Circadian locomotor activity also depends on *de novo* protein synthesis (Takahashi and Turek, 1987; Wollnik *et al.*, 1989), and the non-specific activation or inhibition of intraneuron signalling pathways affecting protein synthesis should result in dissociation of the monitored rhythmic activity. Our results clearly demonstrate robust circadian behaviour (i) that is shifted but does not disintegrate following the common application of both control NSO with subsequent light pulses, and (ii) that maintains its stability following application ASO or NSO alone.

The latency between application of ASO and light pulses also offers the possibility that other immediate-early genes are rapidly induced or that pre-existing transcription factors such as Sp-1 are post-translationally activated in a sequence-independent manner (Perez *et al.*, 1994). However, it is rather unlikely that these mechanisms mimic the specific alterations in inducible transcription factor expression and in locomotor activity.

The reduction of c-Fos and JunB levels affects several transcriptionally operating systems since Jun/Fos proteins bind to AP-1, CRE/CaRE consensus sequences that are present in the promoters of many genes (Rauscher *et al.*, 1988; Ivaskiv *et al.*, 1990; Ryseck and Bravo, 1991). Moreover, these proteins dimerize with members of different transcription factor families, i.e. CRE-BP and ATF proteins (Hai and Curran, 1991; Masquilier and Sassone-Corsi, 1992; Hagmeyer *et al.*, 1993), ligand-dependent transcription factors (Zhang *et al.*, 1991; König *et al.*, 1992) or liver-regenerating factor 1 (Hsu *et al.*, 1992).

The expression of inducible transcription factors in the SCN by light are in the centre of a complex sequence of neurogenetic processes that starts with the excitation of SCN neurons by excitatory amino acid input via the retinohypothalamic tract (Kim and Dudek, 1991; Vindlacheruvu *et al.*, 1992), activation of second messengers with subsequent phosphorylation of the CREB transcription factor (Ginty *et al.*, 1993), and terminal expression of inducible transcription factors (Rusak *et al.*, 1990, 1992; Kornhauser *et al.*, 1992; Sutin and Kilduff, 1992). The target genes that are finally responsible for the shifted locomotor activity rhythm have still to be elucidated. While inducible transcription factors are involved in the adaptive synchronization of environmental stimuli, the genetic constituents of the mammalian circadian clock that drives the truly endogenous and self-sustained pacemaker are not yet known.

During the past decade, the importance of inducible transcription factors such as c-Fos and c-Jun in basic biological processes such as cell growth, mitosis and differentiation has been shown at the single-cell level and has founded a new area in biology (Curran *et al.*, 1984; Ryseck *et al.*, 1987; Almendral *et al.*, 1988). Recently it was shown by the application of antisense against c-Fos *in vivo* that inducible transcription factors such as c-Fos play a functional role in behaviour, i.e. anxiety and pharmacologically modulated locomotion (Chiasson *et al.*, 1992; Dragunow *et al.*, 1993; Möller *et al.*, 1994). Here

we show that in the adult mammalian nervous system complex physiological and vital processes, such as the adaptive resetting of the circadian pacemaker that includes modification of behaviour by formation of a memory engram-like process, are dependent on the activity of inducible Jun and Fos transcription factors.

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Abbreviations

ASO	antisense oligodeoxynucleotide
CT	circadian time
i.c.v.	intracerebroventricular
NSO	nonsense oligodeoxynucleotide
SCN	suprachiasmatic nucleus

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