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Photic and non-photic modulation of the mammalian circadian clock

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ABSTRACT

The suprachiasmatic nuclei (SCN) contain a major circadian pacemaker, which is regulated by photic and nonphotic stimuli. Although enkephalins are present in the SCN, their role in phase regulation of the pacemaker is largely unknown. The opioid agonist fentanyl, a homologue of morphine, is an addictive drug that induces phase shifts of circadian rhythms in hamsters. We observed that these phase shifts are blocked by naloxone, which is a critical test for true opioid receptor involvement, and conclude that opioid receptors are the sole mediators of the actions of fentanyl on the circadian timing system. A strong interaction between opioids and light input was shown by the ability of fentanyl and light to completely block each other's phase shifts of behavioural activity rhythms. Neuronal ensemble recordings *in vitro* provide first evidence that SCN cells show direct responses to fentanyl and react with a suppression of firing rate. Moreover, we show that fentanyl induces a strong attenuation of light-induced Syrian hamster *Period 1* (*shPer1*) gene expression during the night. During the subjective day, we found no evidence for a role of *shPer1* in mediation of fentanyl-induced phase shifts. Based on the present results, however, we cannot exclude the involvement of *shPer2*. Our data indicate that opioids can strongly modify the photic responsiveness of the circadian pacemaker and may do so via direct effects on SCN electrical activity and regulation of *Per* genes. This suggests that the pathways regulating addictive behaviour and the circadian clock intersect.

INTRODUCTION

The major circadian pacemaker of mammals is located in the suprachiasmatic nuclei (SCN) at the base of the anterior hypothalamus (Ralph et al., 1990; Takahashi et al., 2001). Generation of rhythmicity in the SCN is genetically determined and based on a transcriptional/translational feedback loop that involves several clock genes, such as *Period* (*Per*), *Cryptochrome*, *Clock* and *BMAL1* (Albrecht, 2002; Reppert & Weaver, 2002). Entrainment to the environmental light-dark cycle can be explained by the pacemaker's time-dependent responsiveness to light and is mediated by the retinohypothalamic tract (Morin, 1994; Meijer & Schwartz, 2003; Meijer & Takahashi, 2004). The clock genes *Per1* and *Per2* have appeared important for light-induced phase resetting during the night (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Moriya et al., 2000; Albrecht et al., 2001; Wakamatsu et al., 2001).

During the day, the SCN is responsive to pulses other than light; these are generally referred to as non-photic stimuli. Examples are behavioural activity, social interactions, benzodiazepines (e.g. midazolam), neuropeptide Y (NPY), opioids (e.g. fentanyl) and serotonin (5-HT) agonists (Albers & Ferris, 1984; Mrosovsky, 1988, 1996; Wee & Turek, 1989; Tominaga et al., 1992; Marchant & Mistlberger, 1995; Meijer et al., 2000; Vansteensel et al., 2003). Several nonphotic stimuli have been reported to result in suppression of *Per1* and *Per2* expression, which has led to the proposition that these genes play an important role in phase resetting also during the day (Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001; Maywood & Mrosovsky, 2001; Maywood et al., 2002).

The projection from the midbrain raphe nuclei towards the SCN contains 5-HT, whereas the afferent projection from the intergeniculate leaflet, the geniculohypothalamic tract, contains NPY, γ-aminobutyric acid (GABA), neurotensin and enkephalins in the hamster (Miller et al., 1996; Morin & Blanchard, 2001). The role of enkephalins in the circadian system has received increasing attention as δ-opioid receptors were identified in the hamster-SCN (Byku et al., 2000) and the δ- and μ-opioid agonists BW373U86, SNC-80, morphine and fentanyl were found to induce phase shifts in hamsters or mice when administered during the day. Whereas morphine-induced shifts were related to its direct effects on behavioural activity, phase shifts in response to BW373U86, SNC-80 and fentanyl were argued to occur independent of their direct behavioural effects (Marchant & Mistlberger, 1995; Byku

& Gannon, 2000a, b; Meijer et al., 2000). These findings open a new and promising avenue to study the interplay between the circadian clock and the mechanisms that regulate drug abuse-related behaviours. In the present experiments, we analysed the working mechanism of opioids in the circadian timing system by behavioural, electrophysiological and molecular investigations. Wheel running activity was recorded to study the phase shifting effects of fentanyl and light and the ability of naloxone to block these effects. The responsiveness of SCN neurons to fentanyl was analysed in acutely prepared SCN slices, and the expression levels of *shPer1* and *shPer2* were measured subsequent to fentanyl injections, both during subjective day and subjective night.

METHODS

Behavioural experiments (experiments 1-3)

Animals

All experiments were performed under the approval of the Animal Experiments Ethical Committee of the Leiden University Medical Centre. Male golden hamsters (*Mesocricetus auratus*, 80–100 g) were obtained from Charles River Laboratories (Kißlegg, Germany). The animals were housed individually in cages that were equipped with running wheels, in a sound attenuated and temperature controlled room. Food and water were available *ad libitum*. The presence of wheel running activity was recorded automatically by a computer system with a time resolution of 1 min. The animals were placed in a light-dark regime (LD = 14:10) for at least 10 days and in constant darkness (DD) for 7 days to assess their freerunning pattern. On the seventh day in DD, the animals were treated as described below (experiments 1, 2 and 3). The animals were kept in DD for another 14 days after the treatment day to establish steady-state phase shifts.

Experiment 1

On the seventh day in DD, 15 hamsters received three injections during the mid-subjective day as indicated in protocol 1 (Table 1): a naloxone injection (100 µg, 0.25 mL, i.p., naloxone hydrochloride, AZL, Leiden, the Netherlands); a fentanyl injection (100 µg, 2 mL, i.p., fentanyl dihydrogencitrate, Genthon B.V., Nijmegen, the Netherlands); and a second naloxone injection. This injection scheme was used because of the short half-life of naloxone compared with fentanyl in rats (Berkowitz

et al., 1975; Misra et al., 1976; Cox et al., 1998). A similar injection scheme was used for control experiments (saline control injection, 2 mL, i.p., 0.9% NaCl; protocols 2 and 3; **Table 1**). The experiments were performed as specified in Table 1 with intervals of at least 1 month between the protocols.

Protocol	CT 5.75	CT6	CT6.75
1	Naloxone	Fentanyl	Naloxone
2	Naloxone	NaCl	Naloxone
3	NaCl	Fentanyl	NaCl

Table 1. Injection protocols of experiment 1. The animals in experiment 1 were subsequently exposed to protocol 1, 2 and 3. In each injection protocol, the animals received three injections at the indicated circadian times. Naloxone-fentanyl-naloxone is referred to as 'fentanyl plus naloxone' in the text, naloxone-NaCl-naloxone as 'naloxone', and NaCl-fentanyl-NaCl as 'fentanyl'.

Experiment 2
A total of 21 hamsters received a fentanyl injection (100 µg, 2 mL, i.p.) at circadian time (CT) 6 on the seventh day in DD. The injections were followed by a 2.5-h light pulse of 100 lux. In a second series, the animals received a fentanyl injection at CT 6 that was not followed by a light pulse of 2.5 h (control experiment).

Experiment 3
On the seventh day in DD, 23 hamsters received a fentanyl (100 µg, 2 mL, i.p.) injection at CT 18.25, 45 min before a 15-min light pulse of 2–10 lux that was aimed at CT 19. In the control experiment, the animals received a saline injection (2 mL, i.p.) before the light pulse. Application of fentanyl alone at this circadian time did not induce significant phase shifts of the hamsters' activity rhythm (Meijer et al., 2000).

Note that in experiments 2 and 3, the duration and intensity of the light pulses differ. The respective duration and intensities were used to render maximal detectability of effects. In experiment 2, saturating light intensity was used and the duration of the light pulse was based on the half-life of fentanyl after intravenous administration in rats (these data are not available for hamsters). Fentanyl reaches the brain within

several minutes and has a half-life of 73 min (Cox et al., 1998). We assumed therefore that a 2.5-h light pulse would cover the principal component of the presence of fentanyl. In experiment 3, a light pulse of half-maximal light intensity and standard duration was used, based on the intensity response curve described for the same strain of hamsters (Meijer et al., 1992), as a saturating light pulse may prevent potential effects of inhibiting agents such as fentanyl to be detected.

Data analysis of the behavioural experiments

The magnitudes of the steady state phase shifts were measured by fitting straight lines through the activity onsets of the days before and after the injection day. The fitted lines were extrapolated to the day subsequent to the injection day to measure phase advances or phase delays. All values are given as mean ± SEM. The differences within the animals between the experimental- and control-induced phase shifts were analysed with paired Students t-tests. Statistical significance was reached when $P < 0.05$.

***In vitro* electrophysiology (experiment 4)**

Animals and tissue preparation Male golden hamsters of at least 4–5 weeks old were housed individually for 10 days or more under a light)dark cycle of LD = 14:10 in plastic cages equipped with a running wheel, and food and water available *ad libitum*. Wheel running activity was recorded in 1-min bins by a computer. Two hours after lights on, hamsters were killed by decapitation, and their brains were rapidly dissected from the skulls and placed in ice-cold artificial cerebrospinal fluid (ACSF). A block containing the hypothalamus, optic chiasm and adjacent tissue was dissected and transferred to a cooled McIllwain–type tissue chopper. Coronal hypothalamic slices (~400–500-µm-thick), containing the SCN, were prepared and placed on a platinum grid in a laminar flow chamber. Slices were mechanically stabilized and kept under a thin fluid layer by using a tungsten fork. In the chamber the slices were perfused at a rate of 2.5 mL⁄min with oxygenated (95% O_2 : 5% CO_2) ACSF that was prewarmed to 35 °C. The humidified and prewarmed O_2⁄CO_2 mixture was blown over the slices. The chamber was maintained at 36.0 ± 0.1 °C with a temperature controller. The composition of the ACSF was (in mm): NaCl, 116.3; KCl, 5.3; $CaCl_2$, 1.8; NaH_2PO_4, 1.0; $MgSO_4$, 0.8; $NaHCO_3$, 26.2; glucose 10; and 5 mg⁄L gentamycin sulphate. The slices were equilibrated in the chamber for 1 h before the start of the recording. Recording procedure In order to record multiunit discharge activity, single metal electrodes coated with Enamel (90% platinum - 10% iridium; resistance ~100 kΩ; tip diameter 75 µm) were lowered 25–100 µm in the left and⁄or right SCN using micromanipulators under direct visual control. The electrical activity was amplified

with a low noise amplifier, filtered (bandpass 300 Hz-3 kHz) and displayed on an oscilloscope to visualize spike activity. After further amplification, the electrical events were converted to pulses by window discriminators that were set to measure multiunit activity. The signal-to-noise ratio was at least 2:1. The pulses were counted by a computer in 10-s bins, using custom made software, and were stored for offline analysis. During the recording of the neuronal activity, fentanyl, solved in the perfusion fluid at a concentration of 2 µM, was applied to the slices for 30 min. Fentanyl was applied during the subjective day, defined by the previous light-dark regime, by switching from the normal perfusion fluid to the fentanyl-containing perfusion fluid. After fentanyl application, the recording was continued for at least 30 min to determine washout characteristics.

Analysis of the *in vitro* electrophysiology

The data were analysed offline. To determine the effect of fentanyl application, the trend in the data was removed by linear regression. For every application, up to 60 min of baseline preceding the pulse was used for the regression and the resulting line was subtracted from the data. Subsequently, the data were smoothed (low-pass box filter) and the maximum response during the fentanyl application was determined. A response was considered significant if it was larger than two-times the standard deviation of the baseline before smoothing. The response magnitudes were normalized by expression of the maximum responses during fentanyl application as a percentage of the average discharge rate over the last 5 min before fentanyl was applied. To determine regional differences in responsiveness within the SCN, the effects of fentanyl on the multiunit activity in the dorsal and ventral SCN were analysed. The magnitudes of the effects were compared using an independent Students t-test. All values are expressed as means ± SEM. Statistical significance was reached when $P < 0.05$.

In situ hybridization experiments (experiments 5 and 6)

Animals

Male golden hamsters (40–60 g) were treated as described in experiments 1, 2 and 3 until the 7th day in DD.

Experiment 5

The effects of light and fentanyl on *shPer1* and *shPer2* expression during the subjective night were examined in 16 hamsters that were randomly assigned to one of four experimental groups. On the seventh day in DD, the first group was left untreated; the other three groups received a 15-min light pulse of 2–10 lux starting at

CT 19. One of these groups received a fentanyl injection (100 µg, 2 mL, i.p.) at CT 18.25 and one of these groups received a saline control injection (2 mL, i.p.) at CT 18.25. All animals were decapitated at CT 20.25 for in situ hybridization.

Experiment 6

In the first series of experiments, 16 hamsters were released into DD after they were stably entrained to a light-dark cycle. On the seventh day in DD, the animals were randomly assigned to one of four experimental groups. One of the groups was left untreated; the second group received a fentanyl injection at CT 5.75 (100 µg, 2 mL, i.p.); the third group received a saline (2 mL, i.p) injection at CT 5.75 and the fourth group received a fentanyl injection at CT 5.75 followed by a 1.5-h light pulse of 100 lux. At CT 7.25, all animals were decapitated for in situ hybridization analysis. In the second series, 16 hamsters were randomly assigned to one of four experimental groups. These groups were treated as described above, apart from the fourth group, which received a 2.5-h light pulse of 100 lux subsequent to the fentanyl injection. At CT 8.25, all animals were decapitated for in situ hybridization analysis. The expression levels of *shPer1* and *shPer2* in the SCN were quantified 1.5 h and 2.5 h after treatment, as similar intervals are known to result in substantial *Per1* and *Per2* suppression (Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001).

In situ *hybridization*

After decapitation, the hamster brains were quickly removed from the skulls and fixed overnight in ice-cold 4% paraformaldehyde. The tissue was dehydrated and embedded in paraffin and in situ hybridization was carried out according to Albrecht et al. (1998) using probes for *Per1* and *Per2* as described in Albrecht et al. (1997) and Sun et al. (1997). Coronal sections (7 µm) of hamster brain were cut and alternate sections were hybridized with ^{35}S-uridine triphosphate (New England Nuclear, NEG-039H)-labelled cRNA. In most cases, three sections for each hamster SCN were analysed. Sections were exposed to Hyperfilm (Amersham) and the signal was analysed with a microcomputer using NIH-image. The difference in signal intensity between the SCN and an equal area of the lateral hypothalamus was used for normalization. The data were normalized with the no treatment group being 100% in all experiments. Values are expressed as means ± SEM (n = 3 or n = 4 animals). Differences were tested for statistical significance with analyses of variance (ANOVA) and Bonferroni's *post hoc* test. Statistical significance was reached when $P < 0.05$.

RESULTS

Behavioural experiments

Experiment 1

Fifteen animals received fentanyl, naloxone and NaCl injections as described above (**Fig. 1**). The average phase advance induced by fentanyl was 0.9 ± 0.1 h (**Fig. 1A**), whereas fentanyl in the presence of naloxone resulted in shifts of 0.02 ± 0.1 h (**Fig. 1B**). Naloxone by itself induced mean shifts of 0.03 ± 0.1 h (**Fig. 1C**). In all but one animal the phase shifts induced by fentanyl were larger than those induced by naloxone or fentanyl plus naloxone application. The fentanyl-induced phase shifts were significantly different from the phase shifts induced by naloxone and by fentanyl plus naloxone ($P < 0.001$ in both cases). The phase shifts induced by naloxone and fentanyl plus naloxone were not significantly different ($P > 0.8$).

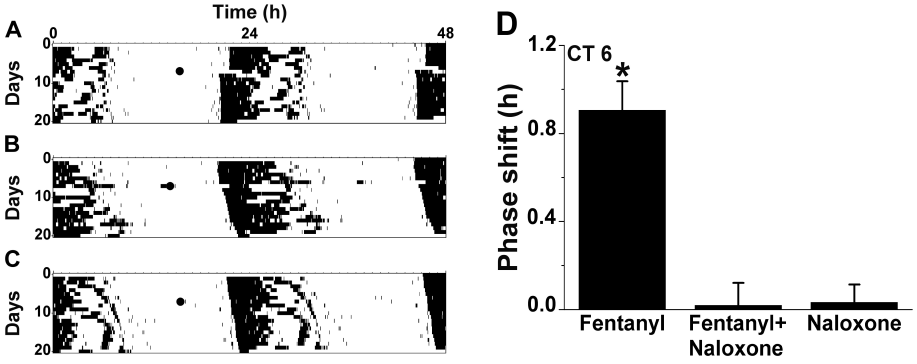

Fig. 1. Naloxone blocks phase advances induced by fentanyl at CT 6. (A)C) Double plotted actograms of hamster wheel running activity rhythms in constant darkness (DD). Days are plotted underneath each other and black vertical marks indicate the presence of wheel running activity. CT 6 is indicated by a black dot. (A) Injection of fentanyl at CT 6 induces a phase advance. (B) Injection of fentanyl plus naloxone as well as (C) injection of naloxone fail to induce phase shifts. (D) Phase shifts (mean ± SEM) of the circadian wheel running activity rhythms of hamsters. The animals received fentanyl, fentanyl plus naloxone and naloxone injections at about CT 6. The phase shifts induced by fentanyl were significantly different (*) from the phase shifts induced by fentanyl plus naloxone and by naloxone.

Experiment 2

Twenty-one animals received a fentanyl injection at CT 6 followed by a light pulse of 2.5 h (**Fig. 2**). In the control experiments, the fentanyl injections were not followed by

a light pulse. The average phase advance induced by fentanyl alone was 1.2 ± 0.2 h (**Fig. 2A**). The average phase shift induced by fentanyl with a light pulse was 0.3 ± 0.1 h (**Fig. 2B**). In all but two animals, the phase shift induced by fentanyl alone was larger than the phase shift induced by fentanyl with light. The fentanyl-induced phase shifts were significantly different from those induced by fentanyl and light ($P < 0.05$).

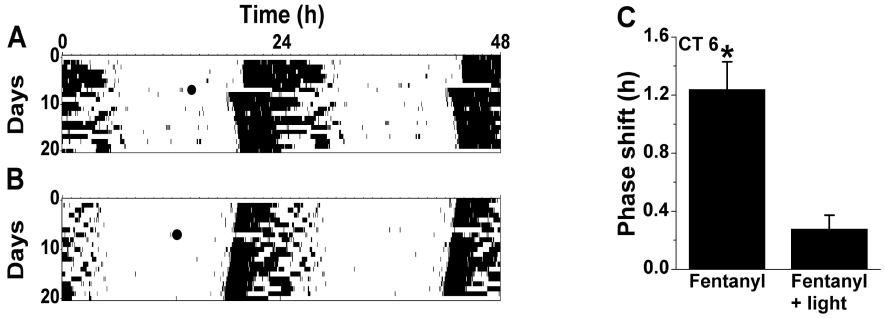

Fig. 2. Light blocks phase advances induced by fentanyl at CT 6. (A and B) Double plotted actograms of hamster wheel running activity rhythms in constant darkness (DD). Days are plotted underneath each other and vertical black marks indicate the presence of wheel running activity. CT 6 is indicated by a black dot. (A) Injection of fentanyl at CT 6 without a subsequent light pulse induces a phase advance whereas (B) injection of fentanyl at CT 6, followed by a 2.5-h light pulse fails to change the phase of the wheel running activity rhythm. (C) Phase shifts (mean ± SEM) of the circadian wheel running activity rhythms of hamsters. Hamsters received fentanyl alone at CT 6, and fentanyl followed by a 2.5-h light pulse. The phase shifts induced by fentanyl plus light were significantly different (*) from the phase shifts induced by fentanyl alone.

Experiment 3

Twenty-three hamsters received a saline control or a fentanyl injection at CT 18.25 that was followed by a 15-min light pulse at CT 19 (**Fig. 3**). A light pulse preceded by a saline injection induced an average phase shift of 0.9 ± 0.1 h (**Fig. 3A**) and a light pulse preceded by a fentanyl injection induced an average phase shift of 0.1 ± 0.1 h (**Fig. 3B**). For all animals, the phase advance induced by light with fentanyl was smaller than the phase advance induced by light with saline. The differences in light-induced phase shifts, with application of saline or fentanyl, were highly significant ($P < 0.001$).

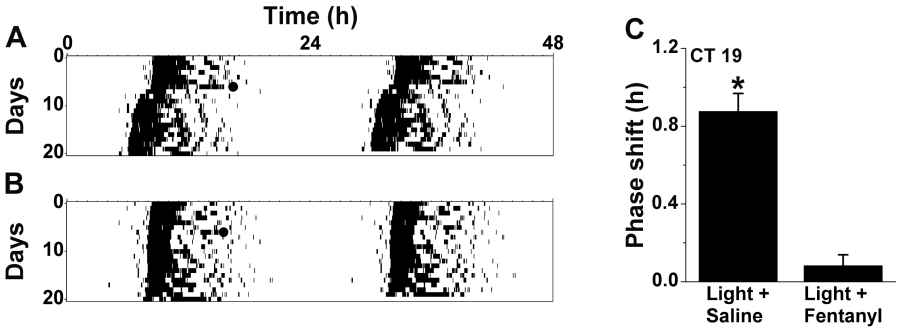

Fig. 3. Phase advances induced by light at CT 19 are blocked by fentanyl. (A and B) Double plotted actograms of hamster wheel running activity rhythms in constant darkness (DD). Days are plotted underneath each other and vertical black marks indicate the presence of wheel running activity. CT 19 is indicated by a black dot. (A) A 15-min light pulse at CT 19, preceded by a saline control injection at CT 18.25 results in a phase advance of the wheel running rhythm. (B) If the light pulse is preceded by a fentanyl injection at CT 18.25, no phase shift is induced. (C) Phase shifts (mean ± SEM) of the circadian wheel running activity rhythms of hamsters. Hamsters received a saline or a fentanyl injection at CT 18.25 and a light pulse at CT 19. The phase shift induced by fentanyl plus light was significantly different (*) from the phase shifts induced by saline plus light.

In vitro electrophysiology

Experiment 4

Multiunit recordings were performed on hypothalamic slices containing the SCN. In nine slices, successful recordings were obtained from two electrodes and in four slices from one electrode. Two recordings from one slice will be treated as independent recordings in the analysis as there was no significant correlation between the responses to fentanyl in separate recordings from one slice ($R = 0.47$, $P > 0.15$). Fentanyl was applied for 30 min in the mid-subjective day, starting between Zeitgeber Time (ZT) 4 and ZT 6 (ZT 12 is defined as the projected time of lights off; **Fig. 4**). Fentanyl application induced a significant decrease of SCN multiunit activity in all 22 recordings. The average magnitude of the decrease was 17 ± 2%. After termination of fentanyl application, the electrical activity showed a slow increase in most recordings. We analysed whether subdivisions of the SCN reacted differently to the application of fentanyl. To this purpose, results obtained from dorsal and ventral parts of the SCN were analysed separately. The average magnitude of fentanyl-induced decrease of electrical activity in the ventral and dorsal SCN was 20 ± 3% and 13 ± 2%, respectively. These values were not significantly different from one another ($P > 0.05$).

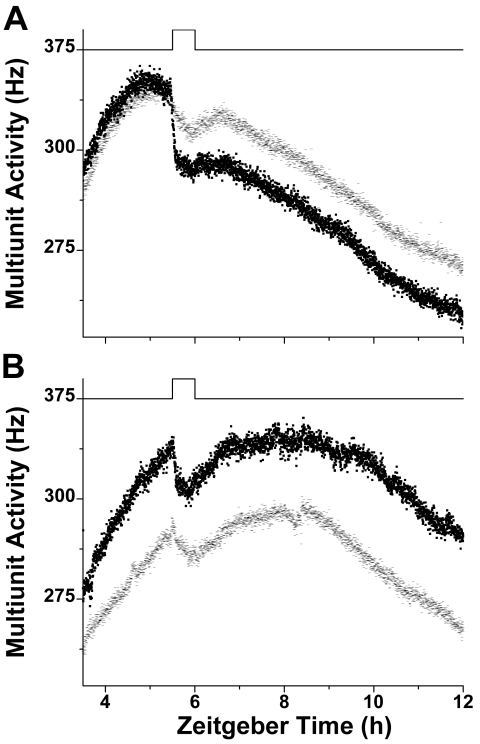

Fig. 4. Fentanyl induces a decrease of SCN neuronal firing rate. (A and B) The effect of 30 min fentanyl application on the neuronal discharge rate in the SCN *in vitro* was measured in 22 recordings from 13 slices. Two representative graphs show the responsiveness to fentanyl when applied during the midsubjective day. Zeitgeber time (based on earlier photoperiod) is plotted on the x-axis and the multiunit neuronal activity from the SCN neurons on the y-axis. In the examples shown, two electrodes were placed in the slice. The results from the first electrode are indicated by black squares, from the second by black horizontal dashes. The time interval of fentanyl application is indicated by black lines on top of the graphs. The application of fentanyl during the subjective day caused a decrease in the discharge rate of SCN neurons. When fentanyl was removed from the perfusion fluid, the discharge rate recovered in most cases to baseline levels (see A with recovery in one trace and no recovery in the other trace).

In situ hybridization experiments

Experiment 5

Four groups of four hamsters were treated as indicated. A 15-min light pulse at CT 19 increased the *shPer1* levels in the SCN dramatically, compared to the control group that did not receive a light pulse ($P < 0.001$). This effect of light was strongly inhibited by fentanyl ($P < 0.001$), but not by saline ($P > 0.05$), administered at CT 18.25 (**Fig. 5**). In the same animals, *shPer2* expression could not be detected in any of the experimental groups (data not shown).

Experiment 6

Two series of experiments were carried out to quantify *shPer1* and *shPer2* expression levels at two time points after fentanyl injection at CT 5.75 (i.e. CT 7.25 and CT 8.25). Due to damaged brain tissue in one case, three hamsters per group were used for *in situ* hybridization analysis at CT 7.25. Four hamsters per group were used in the

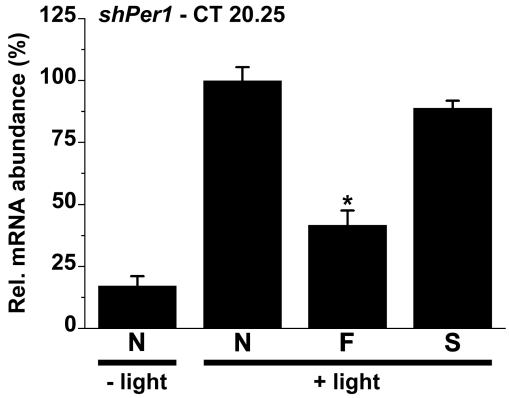

Fig. 5. Fentanyl blocks light-induced *shPer1* expression. Effects of a 15-min light pulse with or without a fentanyl injection on *shPer1* expression during the subjective night. Injections were given at CT 18.25 and light pulses started at CT 19. A light pulse significantly increased *shPer1* expression. This effect was significantly attenuated by fentanyl, but not by saline. Treatments are indicated on the x-axis (N, no treat; F, fentanyl; S, saline) and the relative mRNA abundance of *shPer1* is presented on the y-axis.

second series. No significant differences in *shPer1* mRNA expression between the four groups were observed in either series of experiments (ANOVA, $P > 0.1$; $P > 0.1$, respectively; **Fig. 6**). This indicates that both at CT 7.25 and 8.25, neither fentanyl nor saline changed the *shPer1* expression in the SCN compared with their control values.
shPer1 expression in the SCN was also unaltered when fentanyl injections were followed by a 1.5-h or a 2.5-h light pulse. Statistical analysis of *shPer2* expression, measured at CT 7.25 and at CT 8.25, revealed no significant differences between the four treatments (ANOVA, $P > 0.05$; $P > 0.1$, respectively, **Fig. 6**). In response to fentanyl injection, a substantial, but not significant, *shPer2* decrease of 28% and 38% (as compared with no treatment and saline-injected groups, respectively) was observed at CT 7.25. At CT 8.25 these values were 24% and 30%, respectively. No decrease was observed when fentanyl injections were combined with a 1.5- or 2.5-h light pulse. Despite the absence of significant effects on either time point, it is remarkable that *shPer2* expression level profiles were consistent between the two time points (cf. **Fig. 6C and D**). Interestingly, when the *shPer2* data from CT 7.25 and CT 8.25 were pooled, the fentanyl-induced suppressions were significant (ANOVA, $P < 0.05$; fentanyl vs. saline $P < 0.01$ by *post hoc* LSD test, $P > 0.05$ by *post hoc* Bonferroni test; fentanyl vs. fentanyl plus light $P < 0.05$ by *post hoc* LSD test, $P > 0.1$ by *post hoc* Bonferroni test).

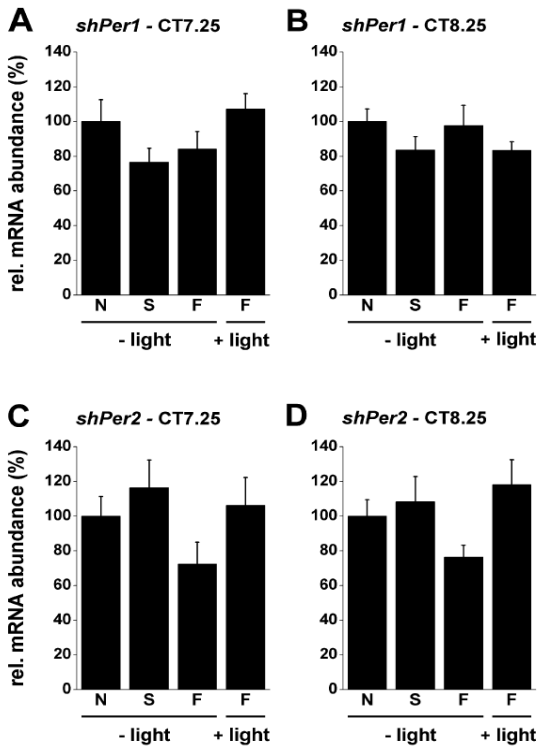

Fig. 6. The effects of fentanyl on the *shPer1* and *shPer2* expression in the SCN. (A and B) Effects of a fentanyl injection (CT 5.75) during the subjective day on *shPer1* expression at CT 7.25 (A) or CT 8.25 (B) with and without accompanying light pulses that lasted for 1.5 h or 2.5 h, respectively. None of the treatments changed the *shPer1* expression significantly. (C and D) Effects of a fentanyl injection during the subjective day (CT 5.75) on *shPer2* expression at CT 7.25 (C) or CT 8.25 (D) with and without accompanying light pulses that lasted for 1.5 h or 2.5 h, respectively. Although fentanyl seemed to suppress *shPer2* by about 30%, none of the treatments changed the *shPer2* expression significantly. Treatments are indicated on the x-axis (N, no treat; F, fentanyl; S, saline) and the relative mRNA abundance of *shPer1* or *shPer2* is presented on the y-axis.

DISCUSSION

Opioid receptor activation

We used the opioid receptor agonist fentanyl and the antagonist naloxone to investigate opioid responsiveness of the circadian system. The difference in the half-life of fentanyl and naloxone made it necessary to apply naloxone with an interval of 1 h. This protocol appeared effective, as naloxone completely blocked the phase advances induced by fentanyl in the mid-subjective day. As fentanyl is a selective and potent opioid receptor agonist (Chen et al., 1993), our data implicate the opioid receptor system as a mediator of the phase shifts observed in response to fentanyl injection. The blockade after naloxone administration further strengthens our argument. Naloxone blocks μ-, δ, and κ-opioid receptors (Lord et al., 1977; Minami & Satoh, 1995) and effectively renders the preparation into a pharmacological opioid receptor knockout animal (Dahan et al., 2001). We therefore argue that the observed

fentanyl-induced phase advances were solely mediated by opioid receptors, indicating their role in the circadian timing system.

The specific type of opioid receptor involved in these effects of fentanyl on the circadian timing system is presently unknown. Fentanyl is a potent μ-opioid receptor agonist but is also able to bind to (albeit to a lesser extent) δ- and κ-opioid receptors (Chen et al., 1993). No general agreement exists about the presence of the several types of opioid receptors in the rat SCN (Desjardins et al., 1990; George et al., 1994; Mansour et al., 1994; Arvidsson et al., 1995; Ding et al., 1996). Interestingly, recent studies suggested that the d-opioid receptor is involved in phase shifting mechanisms (Byku & Gannon, 2000a,b), and is amply present in the hamster SCN (Byku et al., 2000). Despite its presence in the SCN (Allen et al., 1999), the orphanin-FQ/nociceptin receptor, a structural homologue to the classical opioid receptors, is unlikely to mediate the effects of fentanyl on the circadian timing system as its affinity for this receptor is extremely low (Zaveri et al., 2001). Future experiments should elucidate the involvement of μ-, δ, or κ-opioid receptors or an interaction between them in mediating fentanyl-induced shifts (Palazzi et al., 1996; O'Neill et al., 1997; Matthes et al., 1998).

Opioid–light interaction

Our experiments indicated that a light pulse blocks phase advances of wheel running activity rhythms induced by fentanyl at CT 6, whereas application of light by itself is ineffective during the day. Conversely, fentanyl blocks phase advances induced by light at CT 19, but does not induce phase shifts during the night (Meijer et al., 2000). The latter finding is consistent with findings of Tierno et al. (2002), who found that δ-opioid agonists, but not antagonists, block phase shifts induced by light during the late night. We conclude that fentanyl and light interact at the level of phase shifting mechanisms during the day and the night, indicating convergence of photic and opioid-activated pathways.

An interaction between light-induced phase advances and other non-photic stimuli has been described before. Phase advances induced during the subjective day by NPY, the 5-HT agonist 8-OH-DPAT or wheel running, are blocked by subsequent light exposure (Biello & Mrosovsky, 1995; Ehlen et al., 2001). Phase advances induced during the night by light are amongst others blocked by NPY, the 5-HT agonists TFMPP, CGS 12066 A and 8-OH-DPAT and by wheel running (Rea et al., 1994; Pickard et al., 1996; Weber & Rea, 1997; Mistlberger & Antle, 1998). The suppression of phase shifting effects at both phases of the circadian cycle is often substantial. In

our study a nearly 100% suppression of phase shifting effects was observed at the two circadian time zones that were investigated. The data suggest that a large number of SCN neurons involved in phase shifting is under the control of the opioid system. As the projections from the geniculo-hypothalamic and retino-hypothalamic tracts overlap in the SCN, a neuroanatomical basis exists for convergence of opioid and photic influences on SCN neurons (Miller et al., 1996; Moga & Moore, 1997).

***In vitro* electrophysiological response to fentanyl**

In vitro electrophysiological experiments showed that application of fentanyl to SCN neurons induces consistent decreases in the multiunit activity. This decrease is in agreement with the inhibitory effect of opioid agonists in most brain areas (Duggan & North, 1984). Our results are somewhat different from data by Cutler et al. (1999), who found a rebound excitatory response in the SCN upon removal of morphine or enkephalins, despite the absence of direct effects of enkephalins or morphine on the basal or NMDA-evoked firing rate of single neurons. This rebound excitatory response is consistent with the general inhibitory effects of opioids, but was not observed in our experiments with fentanyl. The differences between the results may be caused by the difference in substance used or by the difference in recording technique (single unit recording vs. multiunit recording). Moreover, in the experiments by Cutler et al. (1999), a criterion was set of 20% change from baseline firing rate, which is common for single unit recordings. The responses that were observed in our experiments were somewhat smaller (mean: 17%), but significant due to the low variability in the multiunit recording traces.

Slice preparations were made from hamsters housed in LD 14:10, whereas in the behavioural experiments animals were kept on DD for 7 days before injection of fentanyl. These protocols are routinely used for *in vitro* and behavioural studies, respectively. It could be possible that the sensitivity to fentanyl changes after several days in constant darkness. However, in the present study we aimed to establish in a qualitative way whether or not the SCN neurons are responsive to fentanyl. From the present data we conclude that fentanyl is able to change directly the electrical activity of SCN neurons and we propose that, *in vivo*, fentanyl might use this mechanism for the induction of phase shifts.

Opioid-stimulated pathways during the night

A light pulse during the late subjective night strongly induced *shPer1* expression, which is in correspondence with previous studies (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Horikawa et al., 2000; Moriya et al., 2000; Yokota et

al., 2000). A fentanyl injection that preceded the light pulse by 45 min attenuated this induction, indicating that the observed blockade of light-induced phase shifts by fentanyl could have been mediated by suppression of *shPer1*. In contrast, no *shPer2* induction was detected 1.25 h after the start of a light pulse during the late subjective night. This might be explained by the relatively slow responses that have been observed for *Per2* in the hamster (Fukuhara et al., 2001). A previous study has suggested that the attenuation of light-induced phase shifts during the late subjective night by injection of the benzodiazepine brotizolam is accompanied by a suppression of *Per1* (Yokota et al., 2000). Novelty-induced wheel running, however, blocks photic shifts via mechanisms other than *Per1* (Edelstein et al., 2003). We conclude that nonphotic stimuli inhibit light-induced phase shifts via diverse intracellular pathways.

To understand the effects of opioids on photic entrainment, it is important to identify their working mechanism. The major transmitter of the retinohypothalamic tract involved in phase shifting by light is the excitatory amino acid glutamate (De Vries et al., 1993, 1994). The first site of action of fentanyl may have been pre- or postsynaptically on SCN neurons or afferents. Our data agree with opioids affecting the SCN via presynaptic modulation of the retinal input, as was proposed by Cutler et al. (1999). Based on our findings that fentanyl directly decreases SCN neuronal firing rate, it is also possible that fentanyl affects the SCN neurons postsynaptically by hyperpolarization of the membrane, thereby counteracting the glutamate-induced depolarization. Opposing effects of light- and behaviourally induced changes in electrical discharge have been shown *in vivo*, indicating that such mechanisms may exist in the SCN (Schaap & Meijer, 2001). Finally, fentanyl may affect the light-induced intracellular signalling pathway. The binding of glutamate to *N*-methyl-D-aspartate (NMDA) receptors results in membrane depolarization, which is followed by calcium influx during the night. From there, the light-induced signalling pathway has been proposed to contain, e.g. nitric oxide synthase, nitric oxide, the ryanodine receptor, guanylyl cyclase, cyclic GMP, protein kinase G, MAP kinase, Ca^{2+}/cAMP response element binding protein and the clock genes *Per1* and *Per2* (Ding et al., 1997; Shearman et al., 1997; Obrietan et al., 1998; Gillette & Mitchell, 2002). Opioid receptors belong to the superfamily of seven-transmembrane domain receptors coupled to G-proteins. Opioid-induced activation of G-proteins may lead to calcium channel blockade, an important step in the photic signalling pathway (Minami & Satoh, 1995). It is possible therefore that opioids have suppressed light-induced *hPer1* expression and phase shifts in behavioural activity by interfering with known components of the photic input pathway, either pre- or postsynaptically.

Opioid-stimulated pathways during the day

Previous studies have indicated a causal relationship between suppression of the clock gene *Per1* and the occurrence of nonphotically induced phase shifts in behavioural activity rhythms (Hamada et al., 2004). Several studies described a decrease in *Per1* expression of up to 60% in response to: (i) confinement to a novel running wheel; (ii) intraperitoneal injections with brotizolam or 8-OHDPAT; (iii) injections adjacent to the SCN with NPY; (iv) NPY application to a brain slice containing the SCN; or (v), dark exposure (Maywood et al., 1999; Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001; Maywood & Mrosovsky, 2001; Maywood et al., 2002; Mendoza et al., 2004; but see Yannielli et al., 2002; Poirel et al., 2003). Interestingly, a fentanyl injection at CT 5.75 did not change the levels of *shPer1* expression significantly. Compared to the no treatment group, fentanyl induced a 3–16% decrease and compared to the saline group a 9-17% increase in *shPer1*, which is small relative to the *Per1* suppression levels found in aforementioned studies. Despite the lack of *shPer1* suppression in our study, the fentanyl-induced phase shifts of the behavioural activity rhythm were in the same order of magnitude as those induced by other nonphotic stimuli (Horikawa et al., 2000; Yokota et al., 2000). Our results suggest that fentanyl-induced phase shifts are not correlated with changes in *shPer1* expression, indicating that opioids exploit routes other than *Per1* to phase shift the clock during the subjective day.

Additional analyses were performed to study whether fentanyl application affects *Per2* expression, because of the putative role of *Per2* in non-photic phase shifting (Maywood et al., 1999, 2002; Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001; Yannielli et al., 2002; Mendoza et al., 2004; but see Poirel et al., 2003). Generally, in these studies *Per2* levels are suppressed by 10–40%, depending on the stimulus applied. In our study, the average *Per2* expression levels were decreased by about 30% as compared to no treatment and saline injected groups at CT 7.25 and also at CT 8.25. Despite the consistency of the effects at both time intervals, none of these suppressions was statistically significant. This is probably due to relatively large standard errors as pooling the data from the two time points revealed an effect of fentanyl on *shPer2*. On the basis of the present results we hypothesize a direct effect of fentanyl on the molecular clock during the subjective day. Future studies should provide more information on the involvement of *shPer2* in the mediation of fentanyl-induced phase shifts.

The present study shows that opioids have phase-shifting effects on the circadian timing system and strong interactions with photic input pathways. This is in line with

other reports showing that flies and mice mutant in clock components exhibit changes in cocaine sensitization (Andretic et al., 1999; Abarca et al., 2002) and alcohol intake (Spanagel et al., 2005) suggesting that the circadian clock and modulator mechanisms of drug abuse-related behaviours intersect (Yuferov et al., 2003). It is noteworthy that methamphetamine injection causes an increase of *Per* gene expression in the caudate putamen of the mouse (Masubuchi et al., 2000; Nikaido et al., 2001). Our finding that opioids affect the circadian timing system is of importance insofar that endogenous enkephalins and their receptors are present in the SCN. The question arises under what circumstances these endogenous enkephalins are released in the SCN and have functional significance for photic and nonphotic entrainment. Apart from their role under physiological conditions, opioids might affect the circadian clock in patients when administered exogenously in a clinical setting. This is especially important after ambulatory surgery and in the treatment of chronic pain.

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Abbreviations

5-HT, serotonin; ACSF, artificial cerebrospinal fluid; CT, circadian time; DD, constant darkness; LD, light-dark; NMDA, *N*-methyl-D-aspartate; NPY, neuropeptide Y; *Per1* (2), Period 1 (2); SCN, suprachiasmatic nuclei; *shPer1* (2), Syrian hamster Period 1 (2); ZT, Zeitgeber time.

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