# Hierarchical organization of the circadian timing system

Steensel, M.J. van

# Chapter 2

# Differential responses of circadian activity onset and offset following GABA-ergic and opioid receptor activation

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**SUMMARY**

The circadian pacemaker in the mammalian suprachiasmatic nuclei is responsive to photic and nonphotic stimuli. In the present study, the authors have investigated the response of activity onset and offset to application of nonphotic stimuli: the benzodiazepine midazolam and the opioid receptor agonist fentanyl. In correspondence with previous studies, both stimuli induced phase advances of the activity onset when given in the mid- to late subjective day. In contrast, activity offset did not phase advance following these injections. Injections during the early subjective day induced small phase delays of the activity onset, while large phase delays occurred in activity offset. Phase shifts, induced at both circadian time zones, were paralleled by an increase in the length of daily activity ($\alpha$). The increase in $\alpha$ remained present during several days after the injection. The different kinetics in phase shifting of the activity onset and offset indicate complexity in phase-shifting behavior of the circadian pacemaker in response to nonphotic stimuli. Moreover, the data show responsiveness of the circadian system to GABA-ergic and opioid receptor activation, not only during the mid- to late subjective day but also during the early subjective day. The data implicate that the early subjective day is an interesting phase for analysis of molecular and biochemical processes involved in nonphotic phase shifting.

## INTRODUCTION

A major mammalian pacemaker for circadian rhythmicity is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Takahashi et al., 2001). This pacemaker drives circadian rhythms in physiological and behavioral functions. Moreover, the circadian pacemaker plays an important role in photoperiodic functions, that is, the adjustment of physiological and behavioral functions to the change in day length in the course of the year (Goldman, 2001; Schwartz et al., 2001). The circadian pacemaker is responsive to light during the night. During the day, it is responsive to other types of stimuli, such as cage changes, novel-wheel running, and injections with, for example, triazolam, midazolam, and fentanyl (Turek and Losee-Olson, 1986; Mrosovsky et al., 1989; Wee and Turek, 1989; Meijer et al., 2000). These pulses have been referred to as “nonphotic” stimuli.

The responsiveness to both photic and nonphotic stimuli is commonly analyzed by using onset of behavioral activity as a phase marker. A major question is to what extent the phase shifts of the activity onset are representative for the behavior of the circadian pacemaker at other phases of the circadian cycle. Several studies have addressed this issue by using also activity offset as a phase marker. It was shown that in response to light pulses, the phase-shifting kinetics for onset and offset of activity differ. For example, a phase-advancing light pulse induces a gradual transient advance in the activity onset and an immediate and large phase advance in the activity offset (Honma et al., 1985; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995). These data have been explained by proposing a complex multioscillator system, with differentially responding components.

In this study, we used midazolam and fentanyl to investigate their phase-shifting effects in Syrian hamsters. Midazolam is a benzodiazepine, acting on GABA receptors, which are abundantly present in the SCN. GABA is a major neurotransmitter of the SCN and is also present in the projection from the intergeniculate leaflet (IGL) to the SCN (Moore and Speh, 1993; Morin and Blanchard, 2001). Several lines of evidence indicate that GABA is involved in synchronization and in phase shifting of SCN neurons (Liu and Reppert, 2000; Shirakawa et al., 2000). Fentanyl is a well-known opioid receptor agonist. Endogenous enkephalins, as well as opioid receptors of the $\delta$ subtype, have been found in the hamster IGL and SCN (Morin et al., 1992; Byku et al., 2000). Several opioids have been shown to induce phase shifts in hamster circadian activity rhythms (Byku and Gannon, 2000a; Byku and Gannon, 2000b; Meijer et al., 2000; Tierno et al., 2002).

In our present experiments, we found differences in phase-shifting responses of activity onset and offset to both midazolam and fentanyl injections in the early and the late subjective day. Importantly, the data indicate strong responsiveness of the circadian system to these stimuli during the early subjective day, which becomes apparent only when analyzing the animals’

wheel-running activity offset. The data are discussed in terms of the multioscillator model of the circadian pacemaker.

## MATERIALS AND METHODS

### Animals

Male Syrian hamsters (*Mesocricetus auratus*, Charlesriver, Maastricht, the Netherlands) were individually housed in a sound-attenuated and temperature-controlled room in cages that contained a running wheel. The occurrence of wheel-running activity was recorded every minute and stored by a computer. Food and water were available ad libitum. The experiments were performed under the approval of the Animal Experiments Committee of the Leiden University Medical Center.

### Experimental Protocol

All animals were entrained to a 14:10 light-dark schedule (LD). After entrainment, they were placed in constant darkness (DD) for 7 days. On the 7th day in DD, they received intraperitoneal injections with midazolam (5 mg, Roche, Basel, Switzerland, dissolved in 2 ml 0.9% NaCl with the aid of an equimolar quantity of hydrochloric acid), fentanyl (0.1 mg, 2 ml, fentanyl dihydrogen citrate, Genthon B.V., Nijmegen, the Netherlands), or saline (0.9 % NaCl, 2 ml) that were followed by 2 additional weeks of DD. Subsequently, the animals were re-entrained to the LD schedule for about 2 weeks and the experiment was repeated in such a way that the animals received an injection at a different time of the circadian cycle. In planning the times of the injections, effort was made to cover the whole circadian cycle, to be able to make phase response curves (PRCs) of the 3 substances. Individual animals did not contribute more than 3 data points to 1 PRC and did not contribute more than 6 data points to the whole dataset. Part of the fentanyl-induced phase shifts has been published previously (Meijer et al., 2000).

### Measuring Phase Shifts

Steady-state phase shifts in both activity onset and offset were determined by drawing straight lines through the activity onsets and offsets of the animals' wheel running activity rhythm, during at least 5 days before the injection day and at least 10 days after the injection day, after a steady-state free-running activity rhythm was regained. Phase advances ($+\Delta\varphi$) and phase delays ($-\Delta\varphi$) were measured on the 1st day after the injection and were plotted in PRCs.

### Alpha and Tau

The length of daily activity ($\alpha$) during 5 days before (day –5 to –1) and 10 days after (day 1 to 10) the injection was obtained by measuring the difference between the times of the activity onset and offset at these days. The start of activity on an individual day was defined as the activity onset. For the

termination of the activity, we used the straight line through all activity offsets. The values of $\alpha$ on the single days were expressed as a percentage of $\alpha$ on the last day before the injection day, which is termed $\alpha_{-1}$. Differences between $\alpha_{-1}$ and $\alpha$ on other days were tested for statistical significance with Student's *t* tests ($p < 0.05$).

The period ($\tau$) of the activity onset ($\tau_{onset}$) and offset ($\tau_{offset}$) before and after the injection day was obtained by calculating the slopes of the straight lines through the activity onsets and offsets. Subsequently, the changes of $\tau$ in response to the injection ($\tau$ after injection – $\tau$ before injection) were calculated for both activity onset and offset for each individual animal. These values were plotted in $\tau$ response curves ($\tau$RCs).

**Phase Response Curves and Tau Response Curves**

The time of every injection was expressed as a percentage of the length of daily activity ($\alpha$) on the day before the injection ($\alpha_{-1}$) or length of daily rest ($\rho$) on the day before the injection ($24 - \alpha_{-1} = \rho_{-1}$) of the individual animal. For example, when an injection was administered 2 h after activity onset of a particular animal and 8 h before activity offset, the time of injection was at 20% of $\alpha$. The average of all measured values of $\tau_{onset}$ and $\tau_{offset}$ was not significantly different from 24 h ($\tau = 24 \pm 0.005$, $p > 0.39$). For analysis, $\alpha_{-1}$ and $\rho_{-1}$ were divided in 4 quarters. The data of the injection times that belonged to the same quarters were averaged and plotted in a PRC or a $\tau$RC. The 1st quarter of $\rho$ and $\alpha$ will be referred to as "early," the second and third as "mid," and the fourth as "late" subjective day or night, respectively. For hamsters synchronized to a 14:10 LD schedule, $\alpha$ is close to 10 h and $\rho$ close to 14 h. For clarity, all PRCs and $\tau$RCs are double plotted.

PRCs and $\tau$RCs were investigated for significant effects of Condition or significant Time × Condition interactions with the use of ANOVAs. When a significant effect of Condition was found, or a significant Time × Condition interaction, post hoc Student's *t* tests were performed. Statistical significance was reached when $p < 0.05$.

## RESULTS

### Steady-State Phase Shifts

In total, 86 fentanyl injections, 61 midazolam injections, and 73 saline control injections were administered. When an animal displayed unclear circadian wheel-running patterns at 1 or more days during an experiment, the data were excluded from analysis. A total number of 68 fentanyl, 53 midazolam, and 59 saline injections could be used for analysis (Fig. 1).

Phase response curves were obtained for the activity onset and offset (Fig. 2). The responses of the activity onset to midazolam and fentanyl were significantly different from the responses to saline control injections (ANOVA: Time × Condition; Midazolam: $p < 0.05$, $F = 3.213$; Fentanyl: $p < 0.001$, $F = 5.866$).

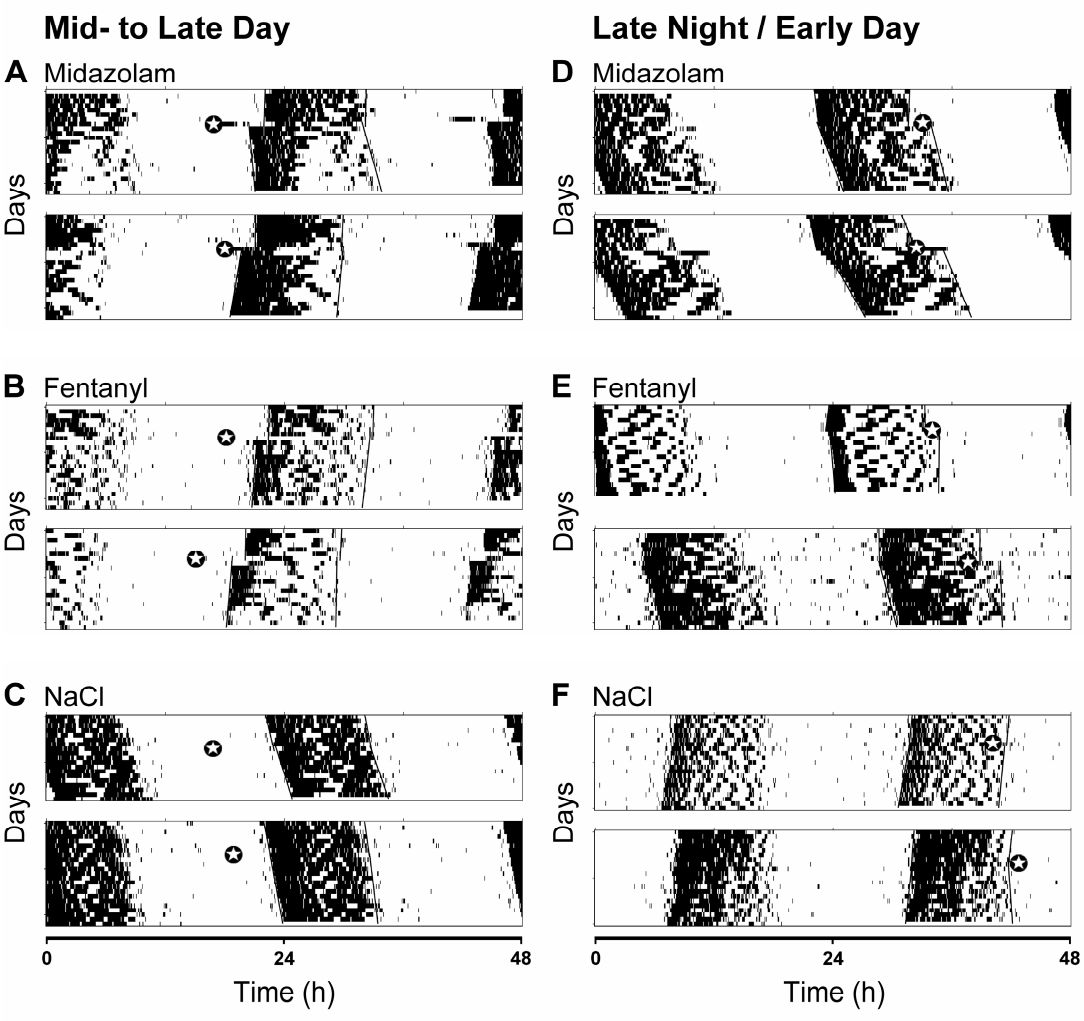


**Figure 1.** Responses of hamster wheel-running activity rhythms to midazolam, fentanyl, and saline injections

Examples of hamster wheel-running activity records and their responses to all 3 treatments at 2 different time points. Activity bouts are indicated in black, per minute. The activity records are double plotted to enable visualization of the activity rhythms. Consecutive days are plotted underneath each other. The times of the injections are indicated by asterisks. A-C. Midazolam (A) and fentanyl (B) injections during the mid- to late subjective day induce phase advances in the activity onset, but not in the activity offset. Saline injections (C) at this time of day do not phase-shift the activity onset or offset. D-F. Midazolam (D) and fentanyl (E) injections during the late subjective night or early subjective day induce large phase delays in the activity offset, while the activity onset delays only slightly. Saline injections (F) at this time of day have no phase-shifting effect.

Moreover, the midazolam- and fentanyl-induced phase shifts in the activity offset were significantly different from the responses to saline (ANOVA: Midazolam: Condition, $p < 0.05$, $F = 12.613$; Fentanyl: Time × Condition, $p < 0.001$, $F = 6.281$). Post hoc Student's $t$ tests revealed significant phase

advances of the activity onset induced by midazolam and fentanyl in the mid-subjective day, compared to saline injections (Fig. 2 a,b). In the early subjective day, both midazolam and fentanyl induced small but significant phase delays of the activity onset (Fig. 2 a,b). Surprisingly, midazolam and fentanyl also induced large, significant phase delays of the activity offset when administered in the early subjective day (Fig. 2c,d).

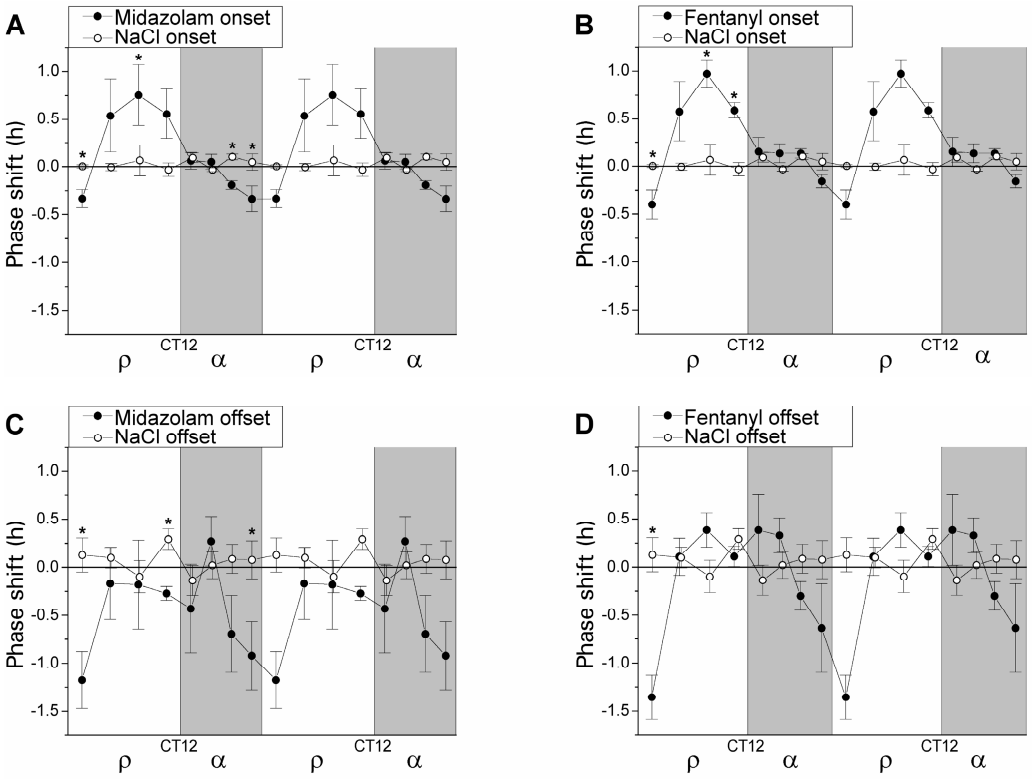


**Figure 2.** Phase response curves of the activity onsets (A and B) and offsets (C and D) of wheel-running activity rhythms following midazolam and fentanyl versus saline injections

The phase response curves are double plotted. The gray areas indicate the length of daily activity (α), the white areas the length of daily rest (ρ), as determined by the average values of activity onset and activity offset. For clarity, the time of the activity onset (CT 12 by convention) is indicated. Open dots indicate saline injections. In the figures, means (± SE) of the following group sizes were used: midazolam: *n* = 10, 9, 7, 6, 5, 4, 4, and 8; fentanyl: *n* = 10, 9, 14, 11, 4, 6, 9, and 5; saline: *n* = 7, 7, 9, 6, 11, 6, 6, and 7. Asterisks indicate significant differences with the responses to the corresponding saline injections (Student's *t* tests, $p < 0.05$ after significant ANOVA). A and B. Phase response curves of the activity onset in response to midazolam or fentanyl injections and saline injections. Saline, injected at any of the time zones, did not induce large phase shifts in activity onset. Midazolam and fentanyl injections, given during the mid-subjective day, induced large phase advances. When administered during the early subjective day, midazolam and fentanyl induced small phase delays. C and D. Phase response curves of the activity offset in response to midazolam or fentanyl injections and saline injections. Saline injections did not induce large phase shifts in activity offset. Midazolam and fentanyl injections during the early subjective day induced large phase delays in the activity offset.

ANOVAs were used to investigate the differences in responses of activity onset and offset. Significant differences were found between the responses of the activity onset and offset to both midazolam and fentanyl (Midazolam: $p < 0.001$, $F = 12.113$; Fentanyl: $p < 0.001$, $F = 12.980$), but not saline. Post hoc Student's *t* tests indicated significant differences between the responses of the activity onset and offset to midazolam injections in the early and the late subjective day ($p < 0.05$). The phase shifts of the activity onset and offset following injections in the early subjective day were $\Delta\varphi = -0.33 \pm 0.09$ h, $n = 10$, and $\Delta\varphi = -1.18 \pm 0.30$ h, $n = 10$, respectively. In response to injections in the late subjective day, the phase shifts were $\Delta\varphi = 0.56 \pm 0.26$ h, $n = 6$, and $\Delta\varphi = -0.27 \pm 0.07$ h, $n = 6$. Also in response to fentanyl, significant differences between the activity onset and offset were found following injections in the early and late subjective day ($p < 0.05$). The phase shifts induced by fentanyl injections in the early subjective day were $\Delta\varphi = -0.40 \pm 0.15$ h, $n = 10$, and $\Delta\varphi = -1.36 \pm 0.23$ h, $n = 10$, in the activity onset and offset, respectively. Fentanyl injections in the late subjective day induced phase shifts of $\Delta\varphi = 0.59 \pm 0.08$ h, $n = 11$, and $\Delta\varphi = 0.11 \pm 0.11$ h, $n = 11$, respectively.

The phase-shifting responses of the activity onset as well as the activity offset to midazolam and fentanyl were similar (Fig. 3). For the PRCs of the activity onset, ANOVAs revealed no effect. For the PRCs of the activity offset, an effect of Condition was found ($p < 0.05$, $F = 4.535$). Student's *t* tests indicated a significant difference between the response of the activity offset to midazolam and fentanyl in the late subjective day (Fig. 3b).

**Alpha**

The data of the early subjective day and the late subjective day were used for analysis of the effects on $\alpha$, as these 2 time points showed significant differences in the responses of the activity onset and offset to both midazolam and fentanyl. The values of $\alpha$ were calculated for 5 days before and for 10 days after the injection (Fig. 4). Before the injections, $\alpha$ increased slightly, but not significantly ($p > 0.1$), except for the group that received fentanyl injections in the early subjective day ($p < 0.05$). After midazolam and fentanyl injections in both the early and late subjective day, $\alpha$ showed an immediate and significant increase ($p < 0.05$, Fig. 4). During the days that followed, $\alpha$ remained larger than baseline values. Saline injections did not induce a sudden increase in $\alpha$ ($p > 0.7$). The small increase of $\alpha$ that was observed in the days before the injections proceeded in the days after the saline injections.

**Tau**

The differences between the values of $\tau_{onset}$ or $\tau_{offset}$ before and after the injection day were plotted in $\tau$RCs (Fig. 5). The $\tau$RCs of the activity onsets indicate a circadian rhythm in the response of $\tau_{onset}$ with lengthening of $\tau_{onset}$ in response to midazolam and fentanyl injections in the late night and early day.

Significant differences between the effects on $\tau_{onset}$ of midazolam and fentanyl on the one hand and saline on the other were found (ANOVA; Midazolam: Condition, $p < 0.001$, $F = 21.133$; Fentanyl: Condition, $p < 0.001$, $F = 33.545$ and Time × Condition, $p < 0.05$, $F = 2.437$). The post hoc Student's *t* tests indicated significant differences between the responses of $\tau_{onset}$ to midazolam and saline injections during the late subjective night and early to mid-subjective day and to fentanyl and saline injections during the subjective night and early subjective day ($p < 0.05$). With the use of ANOVAs, no significant differences were observed between the $\tau RCs_{onset}$ of midazolam and fentanyl (Fig. 5).

For the $\tau RCs_{offset}$, the ANOVAs did not reveal significant differences between the effects of midazolam or fentanyl and saline. A trend of an effect of Condition was present, however, in the $\tau RC_{offset}$ of fentanyl ($p = 0.056$, $F = 3.738$). Despite this trend, the Student's *t* tests revealed no significant differences between the responses of $\tau_{offset}$ to fentanyl and saline ($p > 0.05$).

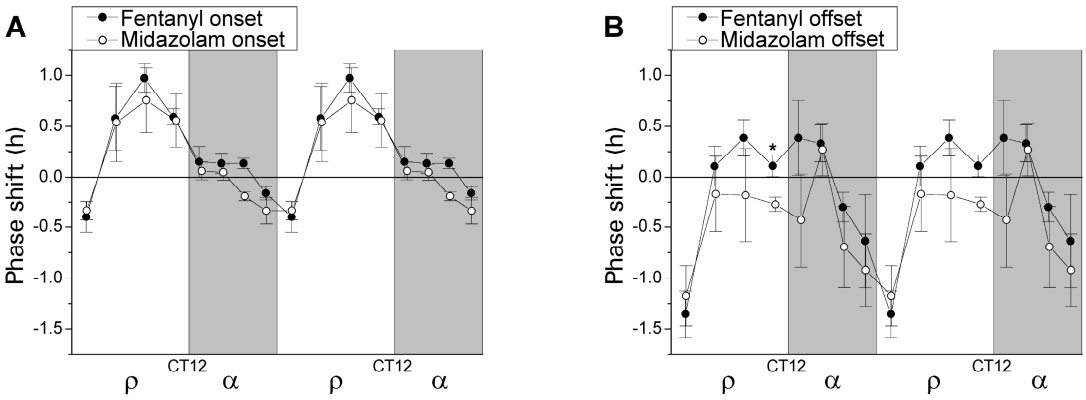


**Figure 3.** Comparison of midazolam versus fentanyl phase shifting effects

Double-plotted phase response curves of the responses of activity onset (A) and activity offset (B) after midazolam and fentanyl injections. For group sizes, see legend of Fig. 2. Gray areas indicate the length of daily activity ($\alpha$); white areas indicate the length of daily rest ($\rho$). The asterisk indicates a significant difference between responses to fentanyl and midazolam injections (Student's *t* tests, $p < 0.05$ after significant ANOVA). Note the similarities between the phase response curves of both activity onset and offset in response to midazolam and fentanyl injections.

## DISCUSSION

In the present experiments, we measured the phase shifts of the onset and offset of behavioral activity rhythms in response to midazolam and fentanyl injections. As reported previously, midazolam and fentanyl induced phase advances of the activity onset when injected during the mid- to late subjective day (Wee and Turek, 1989; Meijer et al., 2000). Despite considerable phase advances in the activity onset, the offset of activity did not phase advance.

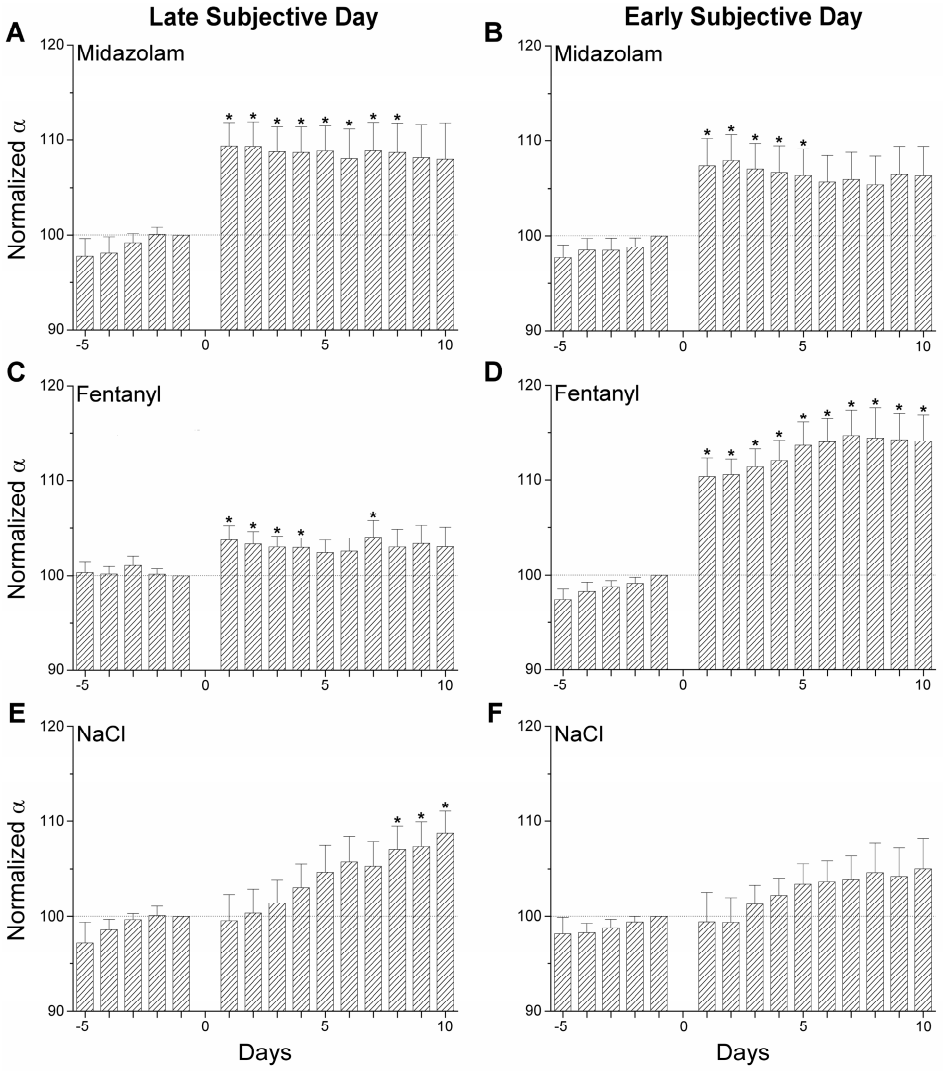


**Figure 4.** Lengthening of $\alpha$ by midazolam and fentanyl

The average length of daily activity ($\alpha$) ± SE, expressed as a percentage of $\alpha$ on the last day before the injections ($\alpha_{-1}$). Values were calculated for 5 days before and 10 days after the injection. The panels on the left indicate changes in $\alpha$ following injections during the late subjective day. The panels on the right indicate changes in $\alpha$ following injections during the early subjective day. Asterisks indicate significant differences with $\alpha_{-1}$ (Student's *t* tests, $p < 0.05$). A and B. During the days before the injection, $\alpha$ lengthens gradually, but not significantly. After a midazolam injection during the late ($n = 6$) or early ($n = 10$) subjective day, $\alpha$ increases immediately and remains larger than baseline values for several days or more. C and D. During the days before the injection, $\alpha$ lengthens gradually. After a fentanyl injection during the late ($n = 11$) or early ($n = 10$) subjective day, $\alpha$ increases immediately and remains increased. E and F. During the days before the injection, $\alpha$ lengthens gradually. After a saline control injection in the late ($n = 6$) and the early ($n = 7$) subjective day, $\alpha$ does not change immediately, but increases gradually in a manner similar to the preinjection days.

When midazolam and fentanyl were injected in the late subjective night and early subjective day, we observed small phase delays in the activity onset. Surprisingly, the activity offset delayed strongly after injections at this circadian phase. The phase delays in the activity offset were largest following injections in the early subjective day and were significantly different from the phase delays in the activity onset. The phase shifts induced by midazolam and fentanyl injections in both activity onset and offset were consistent. Shifts in activity onset were not significantly different at any circadian time; shifts in activity offset were especially similar at those phases where the effects were large.

This is the 1st study that compares the responses of activity onset and offset to nonphotic stimuli. Different responses of the evening rise and morning decline of N-acetyltransferase in response to melatonin administration have been shown previously (Humlova and Illnerova, 1990), as well as different responses of the activity onset and offset after the administration of light pulses. Light pulses in the late subjective night induce rapid, large phase advances in the activity offset and transient, smaller phase advances in the activity onset (Honma et al., 1985; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995). In the early night, light pulses induced somewhat different effects in different studies (Honma et al., 1985; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995). We conclude that photic as well as nonphotic stimuli induce differential phase shifts in activity onset and offset that depend on the circadian phase of application.

The differential phase shifts of activity onset and offset resulted in changes in $\alpha$. Midazolam and fentanyl injections in both early and late subjective day resulted in an immediate expansion of $\alpha$ on the 1st day postinjection. The increase in $\alpha$ lasted for about 1 week after the injection. Saline injections did not induce an immediate expansion of $\alpha$ in the present study. Instead, $\alpha$ increased gradually upon LD to DD transition, which is a known phenomenon, also described in other studies (Elliott and Tamarkin, 1994; Boulos et al., 1996). We conclude that photically induced shifts are generally accompanied by a decrease in $\alpha$ (Honma et al., 1985; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995), while phase shifts induced by the nonphotic stimuli midazolam and fentanyl are accomplished by a temporal increase of $\alpha$. Our finding corresponds with observations by Humlova and Illnerova (1990), who found an expansion of the duration of elevated N-acetyltransferase activity in response to melatonin application.

Our 3rd observation was that midazolam and fentanyl induced changes in free-running period. Lengthening of $\tau_{onset}$ occurred when midazolam was administered during the late subjective night and early to mid-subjective day and when fentanyl was administered during the subjective night and early subjective day. These changes in $\tau_{onset}$ in response to midazolam and fentanyl injections are in general correspondence with those for triazolam injections and novelty-induced wheel running (Joy et al., 1989; Mrosovsky, 1993). The magnitude and direction of changes in $\tau_{offset}$ also seemed to depend on

circadian time, but this was not significant due to rather large standard errors. Visual inspection of Figure 5 indicates that the $\tau RC_{offset}$ of midazolam is shifted by about 3 h compared to the $\tau RC_{offset}$ of fentanyl. Note that this difference was not present in the PRCs.

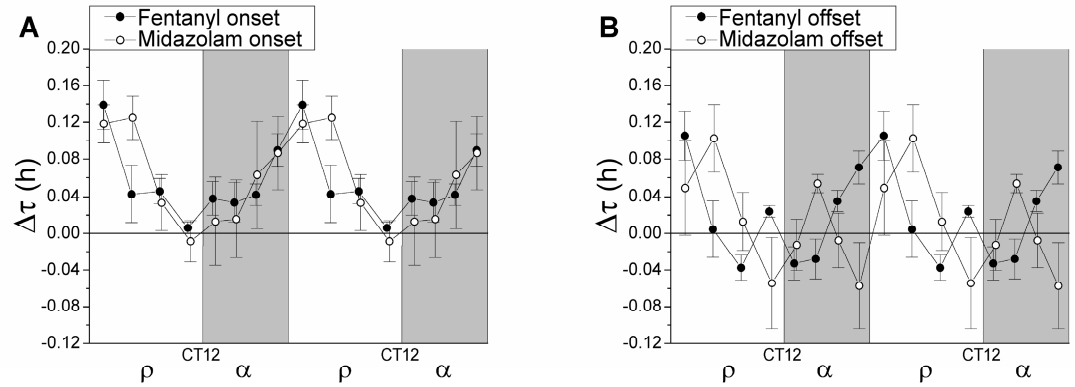


**Figure 5.** $\tau$ response curves of the activity onsets (A) and offsets (B) of hamster wheel-running activity rhythms after midazolam and fentanyl injections

The $\tau$ response curves are double plotted with gray areas indicating the length of daily activity ($\alpha$) and white areas indicating the length of daily rest ($\rho$). The time of the activity onset (CT 12) is indicated on the time axis. Closed dots indicate $\tau$ responses to fentanyl, and open dots indicate $\tau$ responses to midazolam. In the figures, the means (± SE) from the same groups as in Fig. 2 were used. A. In response to midazolam injections during the late night and early to mid-subjective day, $\tau_{onset}$ lengthens. Fentanyl injections during the entire night and the early subjective day induce a lengthening of $\tau_{onset}$. B. Fentanyl injections during the late night and early day seem to lengthen $\tau_{offset}$, but this effect is not significant. In response to midazolam injections, $\tau_{offset}$ shows a similar response that is slightly shifted along the time axis.

A valid question is whether differences in the phase shifts of the activity onset and offset reflect complex oscillator properties, or whether alternatively they reflect a complex response of structures downstream to the SCN. Differential onset and offset shifts have often been discussed in the literature in terms of a 2-oscillator model (Pittendrigh and Daan, 1976; Daan et al., 2001; Hastings, 2001; Watanabe et al., 2001). The 2-oscillator model proposes that a “morning oscillator” (M) couples to dawn and an “evening oscillator” (E) to dusk and that their phase relationship carries information on day length (Pittendrigh and Daan, 1976).

It has been shown that changes in $\alpha$ are paralleled by changes in electrical activity patterns in the SCN in vitro, suggesting that day length, and thus phase relation between activity onset and offset, is encoded in the SCN (Mrugala et al., 2000). Indication for the presence of 2 distinct components inside the SCN was obtained in slices that were cut in a horizontal plane. Two peaks in electrical activity were observed that occurred at the projected onset of dawn

and dusk, respectively (Jagota et al., 2000). Interestingly, the 2 peaks showed differential responsiveness to glutamate application that was consistent with behavioral data on onset and offset shifts. Moreover, differential phase shifting of the evening rise and morning decline of light-induced c-Fos production in the SCN has been observed (Sumova and Illnerova, 1998).

Our present study indicates that the activity onset advances immediately and offset delays immediately in response to nonphotic stimuli, depending on the time of application. Previous studies indicated that activity offset advances immediately and onset delays immediately in response to photic stimuli. While the neurobiological basis of transient cycles remains to be determined (Watanabe et al., 2001), the immediate and large phase shifts are interpreted as responses of the circadian clock itself. If activity onset and offset are representatives of 2 functional groups of oscillators, the data indicate that these oscillators are able to phase shift in both directions. The results also indicate that phase shifts obtained with 1 particular phase marker are not representative for the behavior of the circadian clock at other phases of the cycle. In other words, the circadian clock does not shift by a linear transition along the time axis.

Our data indicate that the circadian system is responsive to midazolam and fentanyl in the early subjective day to a rather large extent. The latter became especially apparent when the activity offset of the animals was analyzed. In future molecular or biochemical experiments, it would be most interesting to include this circadian time zone. It has previously been suggested that different clock genes are responsible for phase advances and phase delays (Albrecht et al., 2001). The present study raises the possibility that different mechanisms are responsible for the control of activity onset and offset.

Several studies have addressed the effects of nonphotic stimuli on the *Per* clock genes in the hamster in the early day. Horikawa et al. (2000) did not see changes in *Per1*, *Per2*, and *Per3* expression after injection with the serotonin agonist 8-OH-DPAT at circadian time (CT) 1, while a significant decrease in *Per1* and *Per2* expression after injection at CT 6 was observed. Yokota et al. (2000) observed significant effects of the benzodiazepine brotizolam on hamster *Per1* and *Per2* expression at CT 6, but not at CT 1. At CT 20, a brotizolam-induced decrease in *Per1* and *Per2* expression was observed, but the effect was not significant. The question remains whether specific clock genes change their expression in response to GABA-ergic and opioid stimulation during early day, resulting in phase delays of the activity offset.

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## REFERENCES


Albrecht U, Zheng B, Larkin D, Sun ZS, and Lee CC (2001) *mPer1* and *mPer2* are essential for normal resetting of the circadian clock. J Biol Rhythms 16:100-104.

Boulos Z, Macchi M, Houpt TA, and Terman M (1996) Photic entrainment in hamsters: effects of simulated twilights and nest box availability. J Biol Rhythms 11:216-233.

Byku M and Gannon RL (2000a) Opioid induced non-photic phase shifts of hamster circadian activity rhythms. Brain Res 873:189-196.

Byku M and Gannon RL (2000b) SNC 80, a delta-opioid agonist, elicits phase advances in hamster circadian activity rhythms. Neuroreport 11:1449-1452.

Byku M, Legutko R, and Gannon RL (2000) Distribution of $\delta$ opioid receptor immunoreactivity in the hamster suprachiasmatic nucleus and intergeniculate leaflet. Brain Res 857:1-7.

Daan S, Albrecht U, van der Horst, GTJ, Illnerova H, Roenneberg T, Wehr TA, and Schwartz WJ (2001) Assembling a clock for all seasons: are there M and E oscillators in the genes? J Biol Rhythms 16:105-116.

Elliott JA and Tamarkin L (1994) Complex circadian regulation of pineal melatonin and wheel-running in Syrian hamsters. J Comp Physiol A 174:469-484.

Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283-301.

Hastings M (2001) Modeling the molecular calendar. J Biol Rhythms 16:117-123.

Honma K, Honma S, and Hiroshige T (1985) Response curve, free-running period, and activity time in circadian locomotor rhythm of rats. Jpn J Physiol 35:643-658.

Horikawa K, Yokota S, Fuji K, Akiyama M, Moriya T, Okamura H, and Shibata S (2000) Nonphotic entrainment by 5-$HT_{1A/7}$ receptor agonists accompanied by reduced *Per1* and *Per2* mRNA levels in the suprachiasmatic nuclei. J Neurosci 20:5867-5873.

Humlova M and Illnerova H (1990) Melatonin entrains the circadian rhythm in the rat pineal N-acetyltransferase activity. Neuroendocrinology 52:196-199.

Jagota A, de la Iglesia HO, and Schwartz WJ (2000) Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro*. Nat Neurosci 3:372-376.

Joy JE, Losee-Olson S, and Turek FW (1989) Single injections of triazolam, a short-acting benzodiazepine, lengthen the period of the circadian activity rhythm in golden hamsters. Experientia 45:152-154.

Liu C and Reppert SM (2000) GABA synchronizes clock cells within the suprachiasmatic circadian clock. Neuron 25:123-128.

Meijer JH and De Vries MJ (1995) Light-induced phase shifts in onset and offset of running-wheel activity in the Syrian hamster. J Biol Rhythms 10:4-16.

Meijer JH, Ruijs ACJ, Albus H, van de Geest B, Duindam H, Zwinderman AH, and Dahan A (2000) Fentanyl, a $\mu$-opioid receptor agonist, phase shifts the hamster circadian pacemaker. Brain Res 868:135-140.

Moore RY and Speh JC (1993) GABA is the principal neurotransmitter of the circadian system. Neurosci Lett 150:112-116.

Morin LP, Blanchard J, and Moore RY (1992) Intergeniculate leaflet and suprachiasmatic nucleus organization and connections in the golden hamster. Vis Neurosci 8:219-230.

Morin LP and Blanchard JH (2001) Neuromodulator content of hamster intergeniculate leaflet neurons and their projection to the suprachiasmatic nucleus or visual midbrain. J Comp Neurol 437:79-90.

Mrosovsky N (1993) $\tau$ changes after single nonphotic events. Chronobiol Int 10:271-276.

Mrosovsky N, Reebs SG, Honrado GI, and Salmon PA (1989) Behavioural entrainment of circadian rhythms. Experientia 45:696-702.

Mrugala M, Zlomanczuk P, Jagota A, and Schwartz WJ (2000) Rhythmic multiunit neural activity in slices of hamster suprachiasmatic nucleus reflect prior photoperiod. Am J Physiol 278:R987-R994.

Pittendrigh CS and Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents: V. Pacemaker structure: a clock for all seasons. J Comp Physiol 106:333-355.

Schwartz WJ, de la Iglesia HO, Zlomanczuk P, and Illnerova H (2001) Encoding *Le Quattro Stagioni* within the mammalian brain: photoperiodic orchestration through the suprachiasmatic nucleus. J Biol Rhythms 16:302-311.

Shirakawa T, Honma S, Katsuno Y, Oguchi H, and Honma K (2000) Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons. Eur J Neurosci 12:2833-2838.

Sumova A and Illnerova H (1998) Photic resetting of intrinsic rhythmicity of the rat suprachiasmatic nucleus under various photoperiods. Am J Physiol 274:R857-R863.

Takahashi JS, Turek FW, and Moore RY (2001) *Handbook of Behavioral Neurobiology. Vol 12: Circadian Clocks*, Kluwer Academic/Plenum, New York.

Tierno A, Fiore P, and Gannon RL (2002) Delta opioid inhibition of light-induced phase advances in hamster circadian activity rhythms. Brain Res 937:66-73.

Turek FW and Losee-Olson S (1986) A benzodiazepine used in the treatment of insomnia phase-shifts the mammalian circadian clock. Nature 321:167-168.

Watanabe K, Deboer T, and Meijer JH (2001) Light-induced resetting of the circadian pacemaker: quantitative analysis of transient versus steady-state phase shifts. J Biol Rhythms 16:564-573.

Wee BEF and Turek FW (1989) Midazolam, a short-acting benzodiazepine, resets the circadian clock of the hamster. Pharmacol Biochem Behav 32:901-906.

Yokota S, Horikawa K, Akiyama M, Moriya T, Ebihara S, Komuro G, Ohta T, and Shibata S (2000) Inhibitory action of brotizolam on circadian and light-induced *Per1* and *Per2* expression in the hamster suprachiasmatic nucleus. Br J Pharmacol 131:1739-1747.