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The effect of chronic morphine or methadone exposure and withdrawal on clock gene expression in the rat suprachiasmatic nucleus and AA-NAT activity in the pineal gland

Dominika Pačesová, Jiří Novotný and Zdeňka Bendová*

*Corresponding author:

Zdeňka Bendová

Department of Physiology, Faculty of Science, Charles University in Prague, Czech Republic

Viničná 7

Prague 2

128 43

Czech Republic

Tel. No. +4202 21951796

E-mail: zdenka.bendova@natur.cuni.cz

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Summary

The circadian rhythms of many behavioral and physiological functions are regulated by the major circadian pacemaker in the suprachiasmatic nucleus. Long-term opiate addiction and drug withdrawal may affect circadian rhythmicity of various hormones or the sleep/activity pattern of many experimental subjects; however, limited research has been done on the long-term effects of sustained opiate administration on the intrinsic rhythmicity in the suprachiasmatic nucleus and pineal gland. Here we compared the effects of repeated daily treatment of rats with morphine or methadone and subsequent naloxone-precipitated withdrawal on the expression of the *Per1*, *Per2*, and *Avp* mRNAs in the suprachiasmatic nucleus and on aralkylamine N-acetyltransferase activity in the pineal gland. We revealed that 10-day administration and withdrawal of both these drugs failed to affect clock genes and *Avp* expression in the SCN. Our results indicate that opioid-induced changes in behavioral and physiological rhythms originate in brain structures downstream of the suprachiasmatic nucleus regulatory output pathway. Furthermore, we observed that acute withdrawal from methadone markedly extended the period of high night AA-NAT activity in the pineal gland. This suggests that withdrawal from methadone, a widely used drug for the treatment of opioid dependence, may have stronger impact on melatonin synthesis than withdrawal from morphine.

Introduction

The circadian system, composed of master circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and the peripheral clocks, regulates daily variations in behavior including sleep/wake cycles, metabolism and neuroendocrine functions (reviewed in: Ramkisoensing and Meijer, 2015). The circadian rhythmicity is generated by interlocked molecular feedback loops of several clock genes and their protein products such as *Clock*, *Bmal1, Period 1 (Per1), Per2, Cryptochromes1 (Cry1)* and *Cry2, RevErbα* and *Rorα*. This molecular clockwork controls the temporal expression of many clock-controlled genes, such as the gene for *arginine vasopressin* (*Avp*), and this regulatory mechanism constitutes one of the clock´s output pathways (Reppert and Weaver, 2001). Expression and protein synthesis of these genes oscillates with a period that is close to 24 h (Zylka et al., 1998; Jin et al., 1999; Sumová et al., 2003). This endogenous period is adjusted by photic and non-photic entraining signals from the external environment to exactly align with a 24 h solar day. The major entraining signal is light that phase shifts the circadian rhythms when it impinges the SCN at night (Zylka et al., 1998; Ramkisoensing and Meijer, 2015). The SCN also developed sensitivity to a wide range of external non-photic cues such as stress stimuli or some drug intake that may also phase shift the endogenous period of molecular clockwork (Mistlberger et al., 2003; Jiang et al., 2011; Kosobud et al., 2007). The resulting timing signals from the SCN are neuronaly and humoraly transmitted to other parts of the brain and peripheral organs (reviewed in: Albrecht, 2012).

One of the most important SCN output signals relays to the pineal gland and drives the synthesis of the neurohormone melatonin. The essential enzyme in melatonin synthesis, arylkylamine N-acetyltransferase (AA-NAT), catalyzes the conversion of serotonin to N-acetylserotonin, which is the immediate precursor of melatonin (Hickman et al., 1999). The nocturnal induction of AA-NAT mRNA is mediated through an induction of adrenergic-cAMP mechanisms via multisynaptic signals from the SCN (Baler et al., 1997; Simonneaux et al., 2006). It has been shown that the increase in *Aanat* mRNA expression is followed by an increase in AA-NAT enzymatic activity, and this in turn is tightly correlated with the changes in melatonin synthesis (Zatz et al., 2000; Klein et al., 1997).

Opioids are commonly used as analgesics to alleviate severe pain. Acute administration of opioids affects the circadian system and induces phase advance of the circadian clock when applied during the day, or interferes with photic entrainment when administered at night (Marchant and Mistlberger, 1995; Byku and Gannon, 2000; Meijer et al., 2000; Vansteensel et al., 2005). This effect might be mediated by changes in signaling cascades within the SCN neurons represented by time of day-dependent modifications of extracellular signal-regulated kinase 1/2 (ERK1/2) and glycogen synthase kinase-3β (GSK-3β) activity (Pačesová et al., 2015).

Long term use of opioids can in humans lead to physical dependence and addiction, which is associated with biochemical changes in the brain, sleep/wake cycle disruption and alterations in hormone levels (Shaw et al., 2005; Dimsdale et al., 2007; Wang et al., 2013; Licata and Renshaw, 2010). The sudden reduction or clearance of the opioids of abuse induces withdrawal symptoms that include disturbances in circadian rhythms such as restlessness and insomnia (Gossop et al., 1987; Pjrek et al., 2012). In animal models, the chronic morphine administration caused dampened circadian amplitude in locomotor activity rhythm and reduced total activity per 24 hour (Hood et al., 2011; Glaser et al., 2012). Abrupt morphine withdrawal in rats led to long-term disruption of the circadian rhythms in locomotor activity, in plasma corticosterone, ACTH, β-endorphin, melatonin, and orexin (Li et al., 2009, 2010; Glaser et al., 2012).

The period and phase of behavioral and humoral rhythms are regulated by the SCN, and acute morphine administration has been shown to change transiently clock gene expression within the SCN cells (Vansteensel et al., 2005; Pačesová et al., 2015). It is therefore legitimate to hypothesize that chronic morphine exposure and/or abrupt withdrawal may affect the clock gene expression in the SCN, which would result in subsequent modification of output rhythms in locomotion and hormone levels. This question has been addressed previously but with inconsistent conclusions. Real time-PCR of SCN extracts showed no difference in *rPer1* and *rPer2* in morphine-addicted rats, but significant difference after morphine withdrawal (Li et al., 2009, 2010). In this study, we used an *in situ* hybridization technique, which enabled the precise localization of gene expression within the brain sections and we explored the effect of repeated morphine exposure and naloxone-precipitated withdrawal on *Per1*, *Per2* and *Avp* mRNA rhythmicity in the rat SCN and AA-NAT activity rhythm in the pineal glands, all in one experiment. In addition, we investigated whether methadone, which is used for opioid substitution therapy, may differ from morphine in its impact on the rat circadian system.

Methods

Animals

Adult male Wistar rats (Velaz, Ltd; Koleč, Czech Republic) were maintained under the 12/12 h light–dark regime at a temperature of 23 ± 2°C with free access to the food and water at least 2 weeks before the experiment. Light was provided by overhead 40 W fluorescent tubes, and illumination was between 100 and 300 lux, depending on the cage position. All experiments were conducted under license no. A5228-01 of the U.S. National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (license no. MSMT-23852/2014-14).

Experimental design

Two experiments were performed with 81 adult rats in each. In 'Experiment 1', the group of 54 rats received an intraperitoneal injection of morphine for 10 consecutive days. Morphine (Zentiva Group, a.s., Prague, Czech Republic) was administered (i.p.) twice daily, at ZT0 and at ZT11.5, in gradually increasing doses (day 1 and 2: 5 mg/kg; day 3 and 4: 10 mg/kg; day 5 and 6: 20 mg/kg; day 7 and 8: 30 mg/kg; day 9 and 10: 50 mg/kg). Control group of 27 rats received saline. In ‘Experiment 2’, the group of 54 adult rats received an intraperitoneal injection of methadone for 10 consecutive days. Methadone (Zentiva Group, a.s., Prague, Czech Republic) was administered (i.p.) once daily at ZT0 in a dosage of 5 mg/kg of body weight because of a relatively long biological half-life of this drug. Control group of 27 rats received saline.

On the 10th day, the experimental groups were divided into two subgroups. One group of 27 animals of morphine or methadone-exposed animals received naloxone (Sigma-Aldrich, Inc.; 1 mg/kg, i.p.) instead of the usual dose of the drugs just before lights-on, i.e. between ZT23-ZT24, and the other group of 27 animals received the drugs as usual. Control groups received saline as usual. All animals were released into the constant darkness. Time was expressed as circadian time (CT), where CT0 corresponds to the time of lights-on and CT12 corresponds to the time of lights-off. Starting from CT1, three adult male rats from each group were deeply anaesthetized by intraperitoneal injection of thiopental (50 mg⁄kg) in 3 h intervals and sacrificed by rapid decapitation. Each brain was sectioned into series of 12 μm-thick serial coronal sections through the mid-caudal SCN using a cryostat and stored at -80° C until use.

In situ **hybridization**

The cDNA fragments of rat *Per1*, *Per2* and *Avp* were used as templates for *in vitro* transcription of complementary RNA probes (SP6/T7 MAXIscript kit, Applied Biosystems, Austin, TX, USA). Probes were labelled by [α-^{35}S]-UTP (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) and purified using Chroma-Spin 100-DEPC H_2O columns (Clontech Laboratories Inc., Mountain View, USA). *In situ* hybridization was performed as described previously (Sládek et al., 2004). Briefly, sections were hybridized for 21 h at 60 °C. Following a post-hybridization wash, the sections were dehydrated in ethanol, dried and exposed to BIOMAX MR film for 10 days. For each gene, brain sections from control and experimental rats were processed simultaneously under identical conditions.

Autoradiographs were analyzed using NIH Image J software to detect relative optical density (OD) of the specific hybridization signal. In each animal, the signal was quantified bilaterally at the mid-caudal SCN section. Each measurement was corrected for nonspecific background by subtracting OD values from the adjacent area in the hypothalamus with consistently low OD. The ODs for each animal were calculated as a mean of values for the left and right SCN.

AA-NAT enzymatic activity assay

AA-NAT activity was determined as described previously (Trávníčková and Illnerová, 1997). Briefly, pineal glands were stored frozen in dry ice until homogenization in a reaction mixture of 0.1 M phosphate buffer (pH 6.8) containing 0.25 mmol [1-^{14}C] acetyl coenzyme A (specific activity, 37 Mbq/ mmol) and 10 mmol tryptamine hydrochloride in a final volume of 100 µl. The reaction mixture was incubated at 37° C for 20 min. At the end of the incubation period, the reaction was stopped by the addition of 1 ml of chloroform. After 1 min vortexing, the aqueous phase was removed, and the organic phase was washed three times with 0.2 ml phosphate buffer (pH 6.8). The organic phase was transferred to a scintillation vial and dried by evaporation. Radioactive acetylated product was determined by scintillation counting. AA-NAT activity was expressed as nanomoles of N-acetyltryptamine formed per hour per 1 mg of pineal tissue.

Data analysis and statistical procedures

Data are reported as the mean ± SEM of at least three animals. The data were analyzed by two-way ANOVA followed by Sidak – Bonferroni post hoc pair-wise comparisons with P values less than 0.05 for significance.

Results

Circadian profiles of *Per1*, *Per2* and *Avp* mRNA levels were investigated for control, morphine- or methadone-exposed and naloxone-treated rats. After 10 days of drug treatment, all experimental animals expressed the somatic signs of opioid addiction and immediate opioid withdrawal (teeth chattering, sniffing, piloerection, and body tremor) as characterized before (Lu et al., 2000; Laorden et al., 2012). As shown in Fig. 1, the mRNA level of the genes changed over the 24 h cycle. A two-way ANOVA showed the significant effect of time ($F (11, 100) = 11.42$; $P < 0.0001$) but no difference between the groups, although interaction was significant for *Per1* mRNA level for morphine ($F (16, 54) = 2.997$; $P = 0.0013$) and methadone-treated groups ($F (16, 54) = 1.840$; $P = 0.0491$) (Fig. 1A, B). Pairwise comparisons using the Sidak-Bonferroni method showed no difference between the values of control, drug-exposed and naloxone-treated animals.

The rhythmic profiles of AA-NAT for morphine-treated groups are shown in Fig. 2A. A two-way ANOVA showed the significant effect of time ($F (8, 81) = 81.34$; $P < 0.0001$), the significant difference between the groups ($F (2, 81) = 6.775$; $P = 0.0019$), and significant interaction ($F (16, 81) = 6.638$; $P < 0.0001$). Pairwise comparisons using the Sidak-Bonferroni method did not, however, showed any difference between the values of the control, morphine and naloxone treated groups.

The rhythmic profiles of AA-NAT for methadone-treated groups are shown in Fig. 2B. A two-way ANOVA showed the significant effect of time ($F (8, 81) = 126.1$; $P < 0.0001$), the significant difference between the groups ($F (2, 81) = 10.15$; $P < 0.0001$), and significant interaction ($F (16, 81) = 10.82$; $P < 0.0001$). Pairwise comparisons using the Sidak-Bonferroni method showed significant difference between control and naloxone-treated group at CT15 ($P < 0.0001$), and between methadone and naloxone-treated groups at CT15 ($P = 0.0028$) and CT 21 ($P = 0.0043$).

Discussion

We have previously shown that acute morphine applied at night transiently induced *Per1* but not *Per2* and modified the level of pERK1/2 and pGSK3β in the rat SCN (Pačesová et al., 2015). In the present study, we found that 10 days of morphine exposure and subsequent abrupt withdrawal precipitated by naloxone do not change the circadian rhythmicity of *Per1*, *Per2* and *Avp* mRNA in the rat SCN. A few studies have addressed this question before; Li et al., (2009) showed no difference in *Per1* and *Per2* mRNA rhythms in the SCN under chronic morphine abuse, and marked difference in both rhythms under protracted morphine withdrawal (Li et al., 2010). In contrast with this study, we found that acute morphine withdrawal precipitated by naloxone did not affect the phase and amplitude of clock gene expression in the SCN. The mRNA rhythms showed a maximum during the subjective day and a minimum during the subjective night, as typical for endogenous rhythms in the rat SCN (Shearman et al., 1997; Zylka et al., 1998; Jáč et al., 2000; Sládek at al., 2004; Sumová et al., 2003). Nevertheless, our data are in agreement with the general conclusions of Li et al., (2009) and indicate that repeated morphine does not affect the rhythms of clock genes in the SCN. In support, no significant difference between control and morphine addicted rats has been demonstrated also for PER2 protein level measured at ZT1 and ZT13 in the rat SCN (Hood et al., 2011). In supraoptic nucleus, acute morphine injection elevated AVP level while repeated administration of morphine led to the decrease of AVP expression as well as to the decrease of plasma AVP levels (Milanés et al., 1997, Yousefpour et al., 2014). Specific morphine-induced inhibition of magnocellular neurons in the supraoptic nucleus (Yousefpour et al., 2014) may explain the differences with our data showing no effect of repeated morphine or methadone on the AVP expression in the SCN.

In contrast to morphine, no study has focused so far on the effect of methadone on the circadian pacemaker in the SCN. In humans, actigraphic assessment of patients under methadone treatment revealed significant changes in the phase of rest-activity cycle, sleep disturbances and overall reduction in the relative amplitude of their behavioral circadian rhythms (Pjrek et al., 2012). This suggests that the SCN driving these rhythms might be the subject of methadone action. Several studies have shown the difference in morphine and methadone-induced desensitization and internalization of μ-opioid receptors (μ-OR) and superactivation of adenylyl cyclase (AC) (Finn and Whistler, 2001; Bohn et al., 2000, 2002, 2004; Borgland et al., 2003). In the SCN, there are all three types of G-protein coupled opioid receptors and μ-ORs show a subtle day/night difference in activity (Pačesová et al., 2015). The AC activity in the SCN induces signaling events enabling intercellular synchronization within the SCN network. This synchronization is underlied by the cAMP/CREB-dependent transcription initiation of *Per* genes (Hastings et al., 2014). Moreover, methadone but not morphine inhibited the L-calcium currents in neuroblastoma cells independently of μ-OR (Yang et al., 2000). Ca^{2+} channels in the SCN contribute to spontaneous activity in neurons and the genes coding for T-type and L-type channel subunits are rhythmically expressed in the SCN (Panda et al., 2002; Nahm et al., 2005). These data suggest that morphine and methadone could exhibit distinct effects on the intercellular coupling within the SCN and thus affect differently the molecular clockwork generating the endogenous circadian oscillations in the SCN. Despite all these indices, our data show that repeated treatment with neither morphine nor methadone significantly affects the clock gene expression in the SCN and expression of clock-controlled *Avp* gene. Furthermore, expression of these genes was not changed even by naloxone-precipitated withdrawal from both these drugs.

The rhythmic melatonin synthesis in the pineal gland and its release to blood in rats has been shown responsive to both acute and repeated morphine administration (Esposti et al., 1988). Chronic morphine also increased AA-NAT activity and its mRNA level, and enhanced melatonin secretion in the pineal gland in culture (Govitrapong et al., 1998; Chchuen et al., 2004; Chetsawang and Govitrapong, 2005). These effects seem to be mediated by enhanced cAMP production that has also been recorded in morphine-treated pinealocytes (Chchuen et al., 2004). In this study, the significant difference in peak points at CT18 and CT21 between control and morphine stimulated animals has not been confirmed by pairwise comparisons. Yet, the significant difference of both profiles supported by two-way ANOVA analysis might ensure that our data do not challenge the previous conclusions and we do not contest that chronic morphine treatment increases AA-NAT activity and melatonin secretion.

No analogic study exists that compares the effect of repeated morphine and methadone exposure on melatonin or AA-NAT activity in the pineal gland. Here, we did not detect any change in AA-NAT activity in methadone-treated rats compared to control animals. Kreek et al. (1983) reported that the normalization of circadian rhythm in levels of cortisol, ACTH, β-endorphin in former heroin addicts has been achieved during long-term methadone maintenance treatment. Supposing that AA-NAT activity corresponds to melatonin synthesis, it is possible to infer that the stabilization and normalization of these hormones and melatonin rhythmicity may be one of ameliorating effects of methadone substitution in opioid addicts.

In sharp contrast with no effect of methadone treatment on AA-NAT activity is the strong impact of acute naloxone-precipitated withdrawal of this drug. While the effect of acute morphine withdrawal was manifested rather by immediate reduction of excessive AA-NAT activity in morphine-treated animals, the acute withdrawal from methadone abuse caused significant widening of AA-NAT peak activity. Our data are supported by the observation that the methadone withdrawal in patients results in an increase of sulfatoxymelatonin in the urine, a non-invasive marker of elevated melatonin secretion in humans (Bearn et al., 2002). The negative physiological states of opioid withdrawal involve induction of the hypothalamo-pituitary-adrenocortical axis and elevation of noradrenergic activity that has been followed mainly in the paraventricular hypothalamic nucleus and locus coeruleus (Aghajanian, 1978; Maldonado and Koob, 1993; Nestler, 2004). The withdrawal-induced hyperexcitability of noradrenergic neurons has been associated with the upregulation of the cAMP/CREB pathway that leads to transcription activation of target genes, which besides the *Per* genes include also the *Aanat* gene (Nestler and Aghajanian, 1997; Parlato et al., 2010; Baler et al., 1997). Interestingly, this upregulation of CREB-dependent genes in the locus coeruleus by opioid withdrawal has been established predominantly on morphine-induced abuse. However, in the pineal gland, the observed naloxone-precipitated morphine withdrawal was much less effective compared to methadone withdrawal. One possible explanation could be related to the distinct pharmacokinetic profiles of both drugs. Methadone has a slower onset and longer duration of action than morphine (Kreek, 1992; 2000). Thus, the administration of naloxone 12 hours after last morphine injection may precipitate very little of residual morphine, but naloxone injected 24 hours after last methadone administration may affect a much higher level of active drug. Whether these differences may account for the distinct effect on NA release in the pineal gland and affect the activity of AA-NAT should be explored.

In summary, our data provide a strong support for the previous conclusion that repeated morphine treatment does not affect the intrinsic rhythmicity of the SCN. While the acute morphine insult affects the SCN significantly (Pačesová et al., 2015), its repeated application leads to the development of tolerance and the SCN readjusts the clock gene expression to normal phase. In contrast, such a tolerance is not developed in the pineal glands and, in accord with previous reports, repeated morphine treatment leads to elevation of AA-NAT activity in this region. Acute naloxone-precipitated morphine withdrawal does not affect the rhythmic clock gene expression in the SCN and normalizes the AA-NAT activity in the pineal gland to the control level. Furthermore, repeated administration of methadone and its withdrawal also failed to affect clock gene and *Avp* expression in the SCN. We propose that the changes in the sleep/wake cycle, locomotor activity or humoral rhythms observed in morphine or methadone-treated subjects originate in structures downstream from the SCN regulation. Naloxone-precipitated methadone withdrawal leads to substantial acceleration of AA-NAT nighttime activity, which suggests that withdrawal from this substitution drug widely used in opioid addiction pharmacotherapy may have a stronger impact on melatonin synthesis than withdrawal from morphine addiction.

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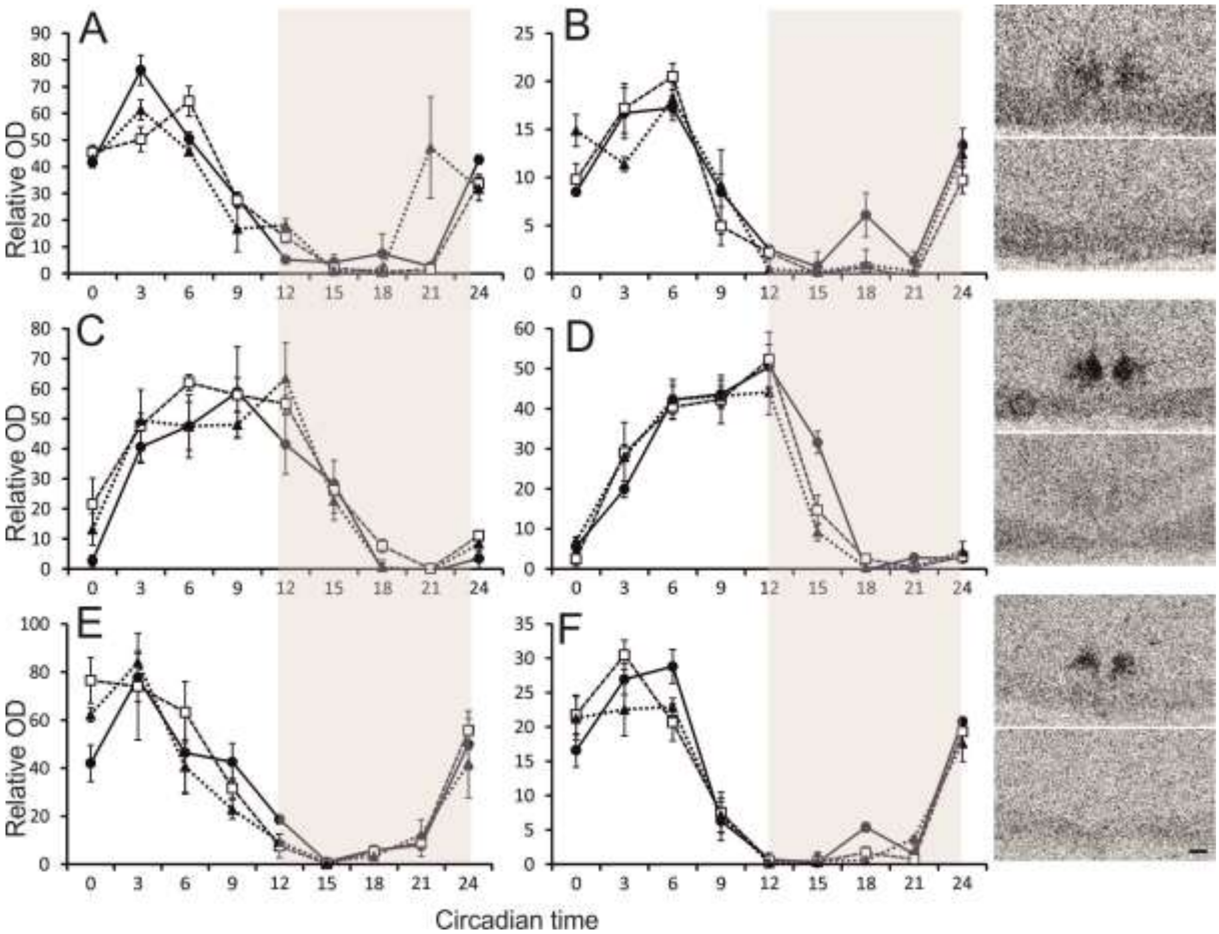

Figure 1. Circadian profiles of *Per1* (A, B) *Per2* (C, D) and *Avp* (E, F) in the rat SCN. Adult rats were treated either with morphine (A, C, E) or methadone (B, D, F) and sampled in 3 h intervals within a 24 h period. Relative OD was assessed for the control groups (closed circles) morphine/methadone-treated groups (open squares) and naloxone-treated groups (closed triangles). Each point represents the mean of 3 values ± SEM. Time is expressed as circadian time, where CT0 corresponds to the time of lights-on. Grey rectangle delineates the dark phase of the daily cycle. Representative autoradiographs of coronal sections of the SCN probed for *Per1* mRNA (left to A, B), *Per2* mRNA (left to C, D) and *Avp* mRNA (left to E, F) illustrate the maximum level at CT6 (upper images) and minimum level at CT18 (bottom images) in the morphine experiment. Scale bar = 100 μm.

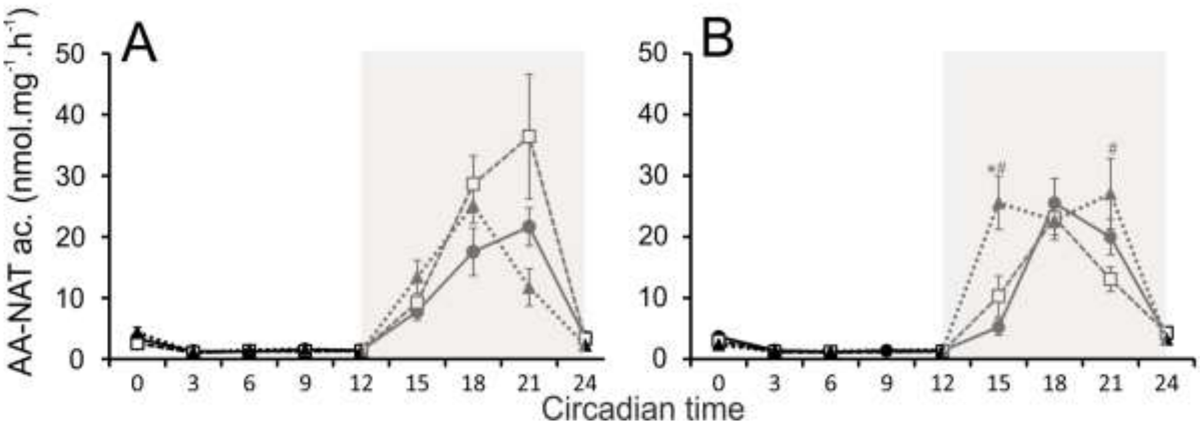

Figure 2. Circadian rhythm of AA-NAT activity in the rat pineal gland. Adult rats were treated either with morphine (A) or methadone (B) and sampled in 3 h intervals within a 24 h period. AA-NAT activity was assessed for the control groups (closed circles) morphine/methadone-treated groups (open squares) and naloxone-treated groups (closed triangles). Each point represents the mean of 3 values ± SEM. Time is expressed as circadian time, where CT0 corresponds to the time of lights-on. Grey rectangle delineates the dark phase of the daily cycle. * $P < 0.0001$ for Sidak-Bonferroni pairwise comparison between control and naloxone-treated groups, # $P< 0.05$ for Sidak-Bonferroni pairwise comparison between methadone and naloxone-treated groups.