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RESEARCH PAPER

Acute morphine affects the rat circadian clock via rhythms of phosphorylated ERK1/2 and GSK3β kinases and *Per1* expression in the rat suprachiasmatic nucleus

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BACKGROUND AND PURPOSE

Opioids affect the circadian clock and may change the timing of many physiological processes. This study was undertaken to investigate the daily changes in sensitivity of the circadian pacemaker to an analgesic dose of morphine, and to uncover a possible interplay between circadian and opioid signalling.

EXPERIMENTAL APPROACH

A time-dependent effect of morphine (1 mg·kg^{-1}, i.p.) applied either during the day or during the early night was followed, and the levels of phosphorylated ERK1/2, GSK3β, c-Fos and *Per* genes were assessed by immunohistochemistry and *in situ* hybridization. The effect of morphine pretreatment on light-induced pERK and c-Fos was examined, and day/night difference in activity of opioid receptors was evaluated by [^{35}S]-GTPγS binding assay.

KEY RESULTS

Morphine stimulated a rise in pERK1/2 and pGSK3β levels in the suprachiasmatic nucleus (SCN) when applied during the day but significantly reduced both kinases when applied during the night. Morphine at night transiently induced *Period1* but not *Period2* in the SCN and did not attenuate the light-induced level of pERK1/2 and c-Fos in the SCN. The activity of all three principal opioid receptors was high during the day but decreased significantly at night, except for the δ receptor. Finally, we demonstrated daily profiles of pERK1/2 and pGSK3β levels in the rat ventrolateral and dorsomedial SCN.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that the phase-shifting effect of opioids may be mediated via post-translational modification of clock proteins by means of activated ERK1/2 and GSK3β.

Abbreviations

CT, circadian time; dmSCN, dorsomedial suprachiasmatic nucleus; GSK3β, glycogen synthase kinase-3β; *Per1*, *Period1*; *Per2*, *Period2*; SCN, suprachiasmatic nucleus; vlSCN, ventrolateral suprachiasmatic nucleus; ZT, Zeitgeber time

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
δ receptor	Akt (PKB)
κ receptor	Clock
μ receptor	ERK1/2
Nuclear hormone receptors[b]	GSK3β
Rev-Erb-α	

LIGANDS	
Arginine vasopressin	GDP
cAMP	GTPγS
DADLE	Morphine
DAMGO	Neuropeptide Y
Enkephalin	Thiopental
GABA	U-50488
Gastrin	UTP

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Opioids are commonly used as effective analgesics to treat acute and chronic pain, but their use is complicated by their high potential for dose-dependent sleep/wake cycle disruption and alterations in hormone levels (Shaw *et al.*, 2005; Dimsdale *et al.*, 2007). Several studies in humans and rodents have shown diurnal variation in the analgesic effect of morphine, which is stronger during the day than during the night (Naber *et al.*, 1981; Yoshida *et al.*, 2003; Boom *et al.*, 2010). Similarly, the adverse effects of opioids may differ according to the time of their administration (Raymond *et al.*, 1992). In general, daily changes in susceptibility of an organism to opioids and their ability to alter biological rhythms point to the mutual interaction between opioids and the circadian system of the body.

The mammalian circadian system comprises a master circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and a peripheral clock in other brain parts and body organs. It regulates daily rhythmicity of behaviour and bodily homeostasis, including the sleep/wake cycle and neuroendocrine functions (reviewed in Buhr and Takahashi, 2013). The circadian rhythmicity is generated by interlocked molecular feedback loops composed of several clock genes and their protein products such as *Clock*, *Bmal1*, *Period 1* (*Per1*), *Period2* (*Per2*), *Cryptochromes1* (*Cry1*) and *Cry2*, *Rev-Erb-α* and *Rora*. The loops spontaneously oscillate in a period close to 24 h and produce timing signals that are neuronally and humourally transmitted to other parts of the brain and peripheral organs (reviewed in Ko and Takahashi, 2006). The stability of the circadian period is supported by post-translational modifications of clock components by several kinases, including casein kinase Iε and δ, glycogen synthase kinase-3β (GSK3β) and ERK1/2 (Reischl and Kramer, 2011). GSK3β promotes the nuclear translocation of PER2, CRY2, Rev-Erb-α and Bmal1; its overexpression advances the phase of clock gene expression in serum-shocked NIH3T3 cells and haplo-insufficiency in GSK3β causes lengthening of the intrinsic period in mice (Harada *et al.*, 2005; Ko *et al.*, 2010; Sahar *et al.*, 2010; Lavoie *et al.*, 2013). Moreover, lithium, a direct inhibitor of GSK3β that is widely used as a mood stabilizer, affects clock gene expression *in vitro* and also *in vivo* (Osland *et al.*, 2011; McCarthy *et al.*, 2012). ERK1/2 is an important modulator of the negative loop in the molecular clockwork. It phosphorylates Bmal1 and directly interacts with CRY1 and CRY2, facilitating the repression of CLOCK/Bmal1 transactivation *in vitro* (Sanada *et al.*, 2002; 2004).

The endogenous period of the circadian pacemaker is adjusted by photic and non-photic entraining signals from the external environment to a 24 h solar day. The major entraining signal is light, which phase shifts the circadian rhythms when impinging the SCN at night and induces the expression of clock genes *Per1*, *Per2* and immediate early gene *c-fos* (Trávníčková *et al.*, 1996; Zylka *et al.*, 1998). These effects are mediated via activation of the cAMP-responsive element by phosphorylated CRE-binding protein and require phosphorylation of ERK1/2 (Obrietan *et al.*, 1998; Dziema *et al.*, 2003). Phosphorylated ERK1/2 (pERK1/2) is thus an integral part of the endogenous circadian clockwork as well as photic input into the SCN.

The wide range of non-photic external stimuli that affect the SCN includes alcohol and drugs. Opioids induce phase advance of the circadian system when applied during the day or interfere with photic entrainment when administered at night (Marchant and Mistlberger, 1995; Byku and Gannon, 2000a,b; Meijer *et al.*, 2000; Vansteensel *et al.*, 2005).

The SCN is a complex structure that consists of two main parts. The ventrolateral part (vlSCN) is characterized by the rhythmic expression of vasoactive intestinal peptide; its cells are weakly intrinsically rhythmic and receive direct retinal input via the glutamatergic retinohypothalamic tract. They receive other input from the intergeniculate leaflet that contains neuropeptide Y, GABA and enkephalins, and 5-hydroxytryptaminergic projections from the midbrain raphe nuclei. The dorsomedial part (dmSCN) contains intrinsically rhythmic cells and integrates the self-sustained circadian oscillations with information from vlSCN. It is characterized by the rhythmic expression of arginine vasopressin that constitutes one of the major outputs from the SCN (reviewed in Morin, 2007; Kalsbeek *et al.*, 2010). Besides these two main parts, there is a specific cluster of light-sensitive cells in the central SCN that express either a high level of calbindin in hamsters or a gastrin-releasing peptide in mice and rats (Karatsoreos *et al.*, 2004; Antle *et al.*, 2005a,b).

At the molecular level, both the analgesic or reward actions of opioids require initial interaction with opioid receptors. There are three main groups of GPCR subtypes: μ, δ and κ receptors. These have been shown to modulate several signalling pathways such as the cAMP pathway, Akt or MAPK–ERK pathways (Li and Chang, 1996; Borgkvist *et al.*, 2007; Merighi *et al.*, 2013). In the brain, both acute and chronic morphine results in either up-regulation or down-regulation of ERK1/2 activity depending on the brain region, and induces *c-fos* and *junB* in striatum and nucleus accumbens (Liu *et al.*, 1994; Ortiz *et al.*, 1995; Berhow *et al.*, 1996; Eitan *et al.*, 2003). Activation of GSK3β is associated with the development of morphine tolerance, and inhibition GSK3β has been shown to attenuate morphine-induced antinociception (Dehpour *et al.*, 1994; Raffa *et al.*, 1995).

The main goal of this study was to determine the day/night difference in the SCN sensitivity to a low dose of morphine. Our data show a bidirectional effect of morphine on pERK1/2 and the pGSK3β level in the SCN and suggest the role of central SCN in morphine-induced effects at night. We also demonstrate the diurnal variation in the activity of κ and μ receptors (but not δ receptors) in the SCN and characterize the rhythmic profile of pGSK3β in the rat SCN.

Methods

Animals

Male Wistar rats (Velaz, Ltd, Koleč, Czech Republic) were maintained under a 12/12 h light–dark regime at a temperature of 23 ± 2°C with free access to 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 animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 292 rats was used in this study.

Experimental design

Daily profiles of pERK1/2 and GSK3β in the SCN. Four adult male rats were deeply anaesthetized by i.p. injection of thiopental (50 mg·kg^{-1}) at 2 h intervals, perfused through the ascending aorta with heparin-treated saline followed by PBS (0.01 M sodium phosphate/0.15 M NaCl, pH 7.2) and then with 4% paraformaldehyde in PBS. Brains were removed, postfixed for 12 h at 4°C, cryoprotected in 20% sucrose in PBS overnight at 4°C, frozen on dry ice and stored at –80°C. Time is expressed as Zeitgeber time (ZT), where ZT0 corresponds to the time of lights-on and ZT12 corresponds to the time of lights-off.

The effect of morphine on pERK1/2, pGSK3β and c-Fos level in the SCN. Adult rats received an i.p. injection of morphine (1 mg·kg^{-1}; i.p.) at ZT7 (7 h after lights on) or ZT15 (3 h after lights off). Control animals received saline. Four experimental and control animals were anaesthetized with thiopental at 15–30 min; 1, 2 and 4 h later; and perfused as described earlier. This experiment was repeated twice.

The effect of morphine on light-induced elevation of pERK1/2 and c-Fos levels in the SCN. Adult rats were released into constant darkness at the time of dark to light transition (designated as circadian time zero; CT0). At CT14 (during the first part of subjective night) animals in experimental groups received morphine (1 mg·kg^{-1}; i.p.) and the control group received saline; 1 h later (CT15), both groups were exposed to a 30 min light pulse (400 lux). The second control group received saline and was left in darkness. Four experimental and control animals were anaesthetized with thiopental at 15 and 30 min; 1, 2 and 4 h after the start of each light pulse; and perfused as described previously.

The effect of morphine on Per1 *and* Per2 *expression in the SCN.* Animals were released into constant darkness and at CT3 (during the early subjective day) and CT15, animals in experimental groups received morphine (1 mg·kg^{-1}; i.p.). Control animals received saline. Four experimental and control animals were anaesthetized with thiopental 1, 3 and 5 h later. Animals were killed by rapid decapitation; their brains were frozen on dry ice and stored at –80°C.

The activity of κ, μ and δ receptors in the SCN. For the *in situ* [^{35}S]-GTPγS binding assay, four animals were killed either at ZT7 or at ZT15 by rapid decapitation under thiopental anaesthesia. Their brains were frozen on dry ice and stored at –80°C.

Immunohistochemistry

Brains were sectioned into a series of 30 μm thick free-floating coronal slices throughout the rostral-caudal extent of the SCN. Levels of phospho-p44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}), phospho-GSK3β (Ser^{9}) (antibody purchased from Cell Signalling Technology, Inc., Danvers, MA, USA) and c-Fos (Merck Group, Darmstadt, Germany) were assessed by immunohistochemistry using the avidin/biotin method with diaminobenzidine as the chromogen (Vectastain ABC Kit, Vector, Burlingame, CA, USA). All brain sections were processed simultaneously under identical conditions. Immunopositive cells in the SCN mid-caudal region were manually tagged and counted using an image analysis system (ImageJ, US National Institutes of Health, Bethesda, MD, USA). To delineate the position of the ventrolateral and dorsomedial SCN, the boundaries of pERK1/2 signal at ZT15 and ZT7, respectively, were saved as regions of interest and applied to all immunohistochemical images. The data are expressed as the means of values from the left and right SCN. For binary black and white images, photomicrographs were converted to 16 bit images and adjusted by auto-threshold using the triangle method.

In situ *hybridization*

The cDNA fragments of rat *Per1* and rat *Per2* were used as templates for *in vitro* transcription of complementary RNA probes (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 Laborato-

ries, 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.

In situ *[^{35}S]-GTPγS binding assay*

Agonist-stimulated [^{35}S]-GTPγS autoradiography was performed as described in earlier studies (Wang *et al.*, 2011). Briefly, sections were incubated with [^{35}S]-GTPγS (0.04 nM) and 10 μM of the μ receptor agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) or the δ receptor agonist [D-Ala2, D-Leu5]-enkephalin (DADLE), or the κ receptor agonist U-50488 (Sigma, St. Louis, MO, USA) and 2 mM GDP at 25°C for 2 h. Basal activity was assessed with GDP in the absence of an agonist, and non-specific binding was assessed in the presence of 10 μM unlabelled GTPγS. After being washed, slides were dried and exposed to Biomax MR film for 5 days. Brain sections from day and night groups were processed simultaneously under identical conditions.

Autoradiograph analysis

Autoradiographs were analysed using NIH ImageJ software to detect relative optical density (OD) of the specific hybridization or [^{35}S]-GTPγS binding signal. In each animal, the signal was quantified bilaterally at the mid-caudal SCN section. Each measurement was corrected for non-specific 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.

Data analysis and statistical procedures

Data are reported as the mean ± SEM of at least four animals. The data were analysed by one-way ANOVA followed by Tukey's *post hoc* test with *P*-values less than 0.05 for significance. A two-way ANOVA was also used to reveal whether the profiles of pERK1/2 and pGSK3β differed between dorsomedial and ventrolateral SCN and *post hoc* pair-wise comparisons were performed by the Sidak–Bonferroni method.

Results

Daily profiles of pERK1/2 and pGSK3β within the SCN of adult rats

Daily profiles of phosphorylated forms of both kinases were investigated separately for the vlSCN and dmSCN. As shown in Figure 1A, the protein level of the pERK1/2 changed over the 24 h cycle in antiphase between the vlSCN and dmSCN. In the vlSCN, the number of pERK1/2 immunoreactive cells (–ir cells) increased significantly between ZT13 and ZT15 [$F(7) = 202.7$; $P < 0.01$], and decreased between ZT23 and ZT01 [$F(7) = 31.3$; $P < 0.01$]. In the dmSCN, the first significant increase occurred between ZT13 and ZT17 [$F(7) = 6.1$; $P < 0.05$] and further between ZT15 and ZT23 [$F(7) = 8.7$; $P < 0.05$]. The first significant decrease occurred between ZT9 and ZT11 [$F(7) = 21.4$; $P < 0.01$]. A two-way ANOVA showed the significant effect of time [$F(11, 100) = 11.42$; $P < 0.0001$] but no difference between vlSCN and dmSCN ($P = 0.8776$); however, interaction was significant [$F(11, 100) = 24.62$; $P < 0.0001$]. Pair-wise comparisons using Sidak–Bonferroni method showed that the values of the dmSCN were significantly different from the values of the vlSCN ($P < 0.01$) (Figure 1A).

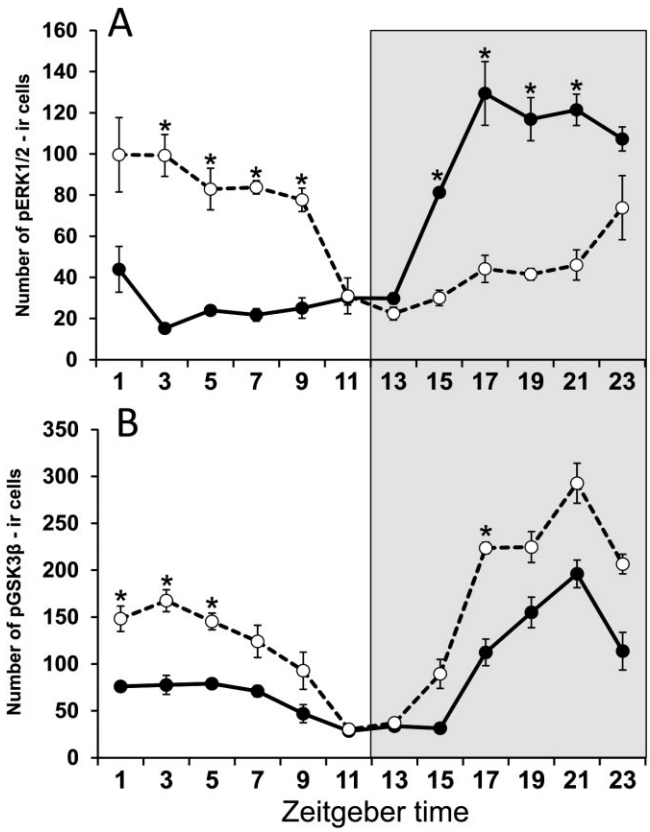

Figure 1

Daily profile of pERK1/2 (A) and pGSK3β (B) in the rat SCN. Adult rats were sampled at 2 h intervals within a 24 h period, and numbers of pERK1/2-ir and pGSK3β-ir cells were assessed separately for the ventrolateral (closed circles) and dorsomedial SCN (open circles). Each point represents the mean of four values ± SEM. Time is expressed as ZT, where ZT12 corresponds to the time of lights off. A grey rectangle delineates the dark phase of the daily cycle. *$P < 0.001$ for Sidak–Bonferroni pair-wise comparison between vlSCN and dmSCN.

The rhythmic profile of pGSK3β proceeded in parallel in both vlSCN and dmSCN, although the duration of every basal level was much shorter in the dmSCN (Figure 1B). In the vlSCN, the number of pGSK3β-ir cells increased significantly between ZT15 and ZT17 [$F(7) = 35.9$; $P < 0.01$], and decreased between ZT21 and ZT23 [$F(7) = 11.4$; $P < 0.01$]. In the dmSCN, the significant increase occurred between ZT13 and ZT15 [$F(7) = 11.2$; $P < 0.01$] and the first significant decrease occurred between ZT21 and ZT23 [$F(7) = 11.7$; $P < 0.01$]. A two-way ANOVA showed the significant effect of time [$F(11, 89)$

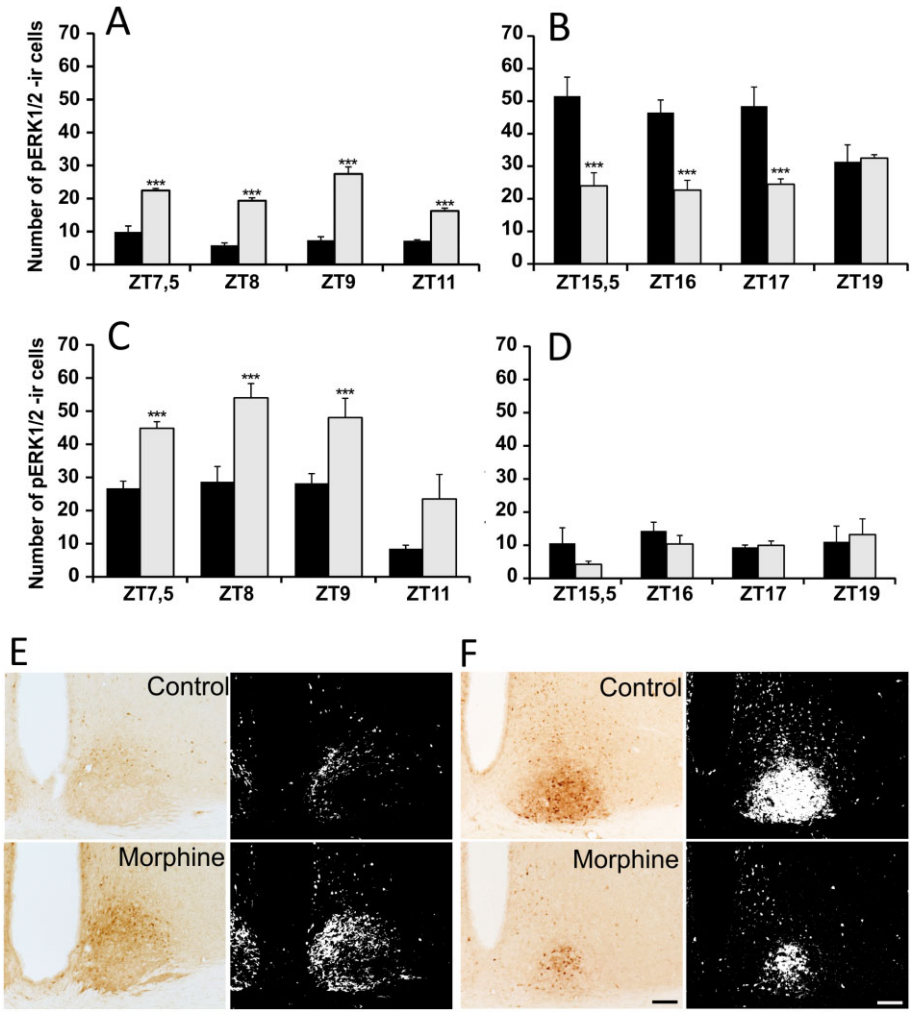

Figure 2

Effect of acute morphine on ERK1/2 phosphorylation within the rat SCN. Adult rats were injected with morphine (1 mg·kg^{-1}) either during the day at ZT7 (A, C) or during the night at ZT15 (B, D) and sampled 30 min and 1, 2 and 4 h later (grey columns) (controls; black columns). Levels of pERK1/2 were assessed separately for the ventrolateral (A, B) and dorsomedial SCN (C, D). Each column represents the mean of six to eight values ± SEM. ***$P < 0.01$. Representative photomicrographs of coronal sections of the SCN demonstrate the intensity and distribution of pERK1/2 in control and morphine-treated animals 2 h after morphine at ZT7 (E) or at ZT15 (F). Black and white photomicrographs are used to highlight the spatial distribution of immunopositive signals. Scale bar = 200 μm.

= 48.76; $P < 0.0001$], the significant difference between vlSCN and dmSCN [$F(1, 89) = 127.2$; $P < 0.0001$] and interaction [$F(11, 89) = 3.047$; $P = 0.0017$]. Pair-wise comparisons using Sidak–Bonferroni method showed that the values of the dmSCN were significantly different from the values of the vlSCN ($P < 0.01$) (Figure 1B).

Dual effect of acute morphine on pERK1/2 and pGSK3β level in the rat SCN, and no effect on endogenous c-Fos

Acute application of morphine at a dose of 1 mg kg^{-1} showed opposite effects on the level of both kinases when applied during the day or night. Morphine applied at ZT7 significantly induced pERK1/2 in the vlSCN within 30 min [$F(11) = 43.5$; $P < 0.01$], 1 h [$F(11) = 89.7$; $P < 0.01$], 2 h [$F(11) = 88.5$; $P < 0.01$] and 4 h [$F(11) = 68.2$; $P < 0.01$] (Figure 2A), and in the dmSCN within 30 min [$F(11) = 47.1$; $P < 0.01$], 1 h [$F(11) = 16.8$; $P < 0.01$] and 2 h [$F(11) = 11.5$; $P < 0.01$] (Figure 2C). The pERK1/2 elevation after 4 h from the morphine injection in the dmSCN was not statistically significant. Although morphine-induced pERK1/2-ir cells thickened the dmSCN uniformly, in the vlSCN, this occurred at the ventral border of the SCN (Figure 2E). Morphine applied at ZT15 significantly reduced the number of pERK1/2 immunopositive cells in the vlSCN within 30 min [$F(11) = 17.2$; $P < 0.01$], 1 h [$F(11) = 21.7$; $P < 0.01$] and 2 h [$F(11) = 12.1$; $P < 0.01$] (Figure 2B). This reduction occurred within the entire vlSCN, except for its central part (Figure 2F). Interestingly, the intensity of the immunopositive signal within individual cells was not changed (Supporting Information Fig. S1). There was no significant change in pERK1/2 level in dmSCN at night (Figure 2D).

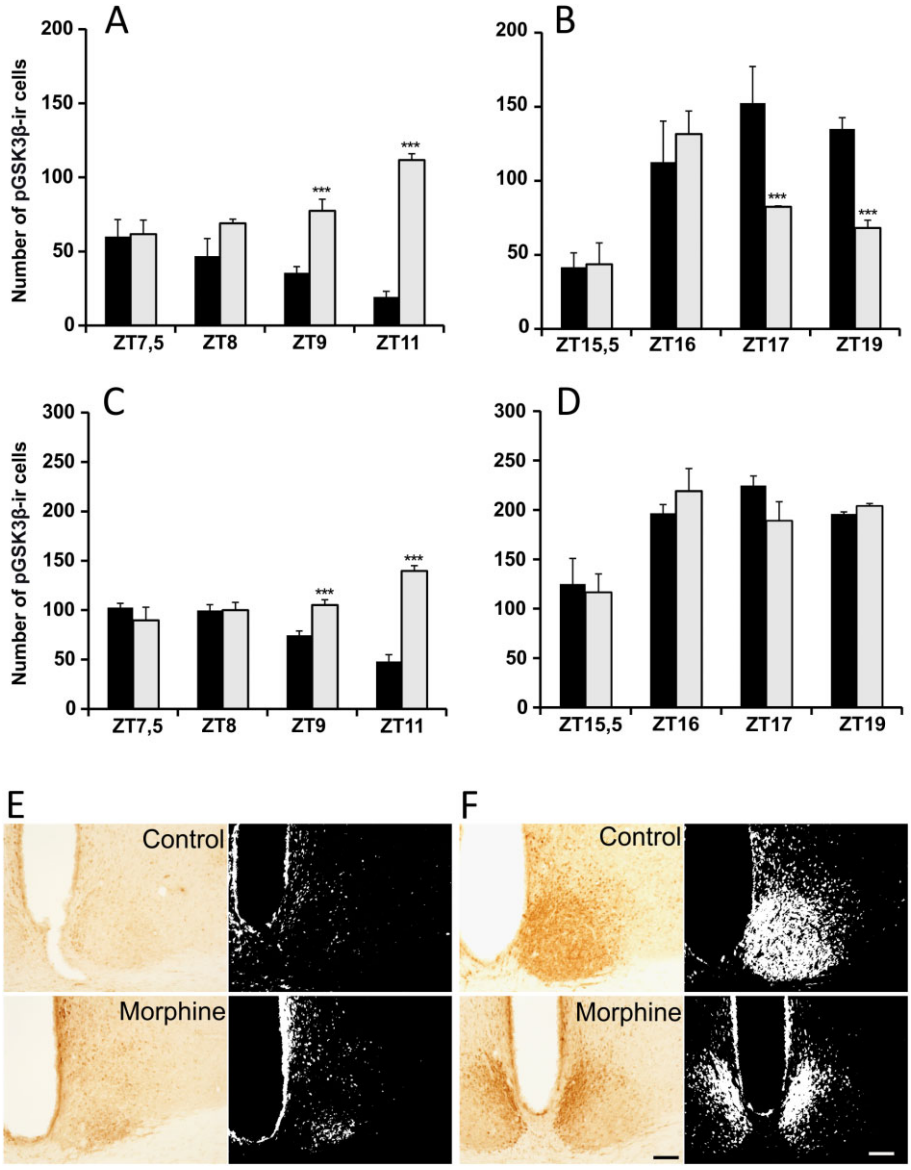

Figure 3

Effect of acute morphine on GSK3β phosphorylation in rat SCN. Adult rats were injected with morphine (1 mg·kg^{-1}) either during the day at ZT7 (A, C) or at night at ZT15 (B, D) and sampled 30 min and 1, 2 and 4 h later (grey columns) (controls; black columns). Levels of pGSK3β were assessed separately for the ventrolateral (A, B) and dorsomedial SCN (C, D). Each column represents the mean of six to eight values ± SEM. ***P < 0. 01. Representative photomicrographs of coronal sections of the SCN demonstrate the intensity and distribution of pGSK3β in control and morphine-treated animals 2 h after morphine at ZT7 (E) or at ZT15 (F). Black and white photomicrographs are used to highlight the spatial distribution of the immunopositive signals. Scale bar = 200 μm.

The effect of morphine on the pGSK3β level was slower as compared with pERK1/2. Morphine applied at ZT7 significantly induced pGSK3β in the vlSCN after 2 h [$F(11)$ = 22.4; P < 0.01], and more strongly after 4 h [$F(11)$ = 244.5; P < 0.01] (Figure 3A). As with pERK1/2, the induction of pGSK3β occurred at the ventral border of the vlSCN (Figure 3E). In the dmSCN, a significant increase was measured after only 4 h from morphine application at ZT7 [$F(11)$ = 52.1; P < 0.01] (Figure 3C). Morphine application at ZT15 significantly reduced pGSK3β in the vlSCN within 2 and 4 h [$F(11)$ = 19.1; P < 0.01] respectively [$F(11)$ = 112.9; P < 0.01] (Figure 3B). Morphine did not affect the high endogenous level of pGSK3β in the dmSCN (Figure 3D). Contrary to pERK1/2, after morphine application at ZT15, pGSK3β disappeared from the vlSCN, including its central part (Figure 3F).

The least significant effect of acute morphine concerned the c-Fos level in the SCN. Contrary to pERK1/2 and pGSK3β, morphine applied at ZT7 significantly reduced endogenous c-Fos in the dmSCN after 2 h [$F(11)$ = 17.2; P < 0.05] and after 4 h [$F(11)$ = 20.6; P < 0.05]. Morphine applied at ZT15 had no effect (Figure 4).

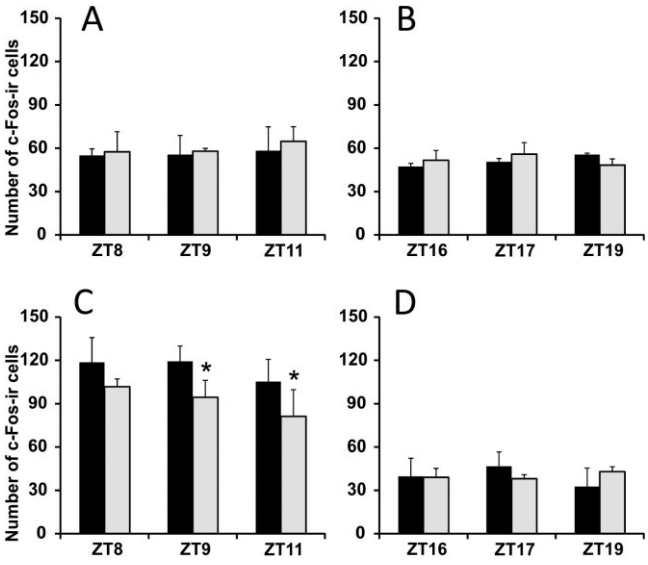

Figure 4

Effect of acute morphine on c-Fos level in rat SCN. Adult rats were injected with morphine (1 mg·kg^{-1}) either during the day at ZT7 (A, C) or at night at ZT15 (B, D) and sampled 30 min and 1, 2 and 4 h later (grey columns) (controls; black columns). Levels of c-Fos were assessed separately for the ventrolateral (A, B) and dorsomedial SCN (C, D). Each column represents the mean of six to eight values ± SEM. *P < 0.05.

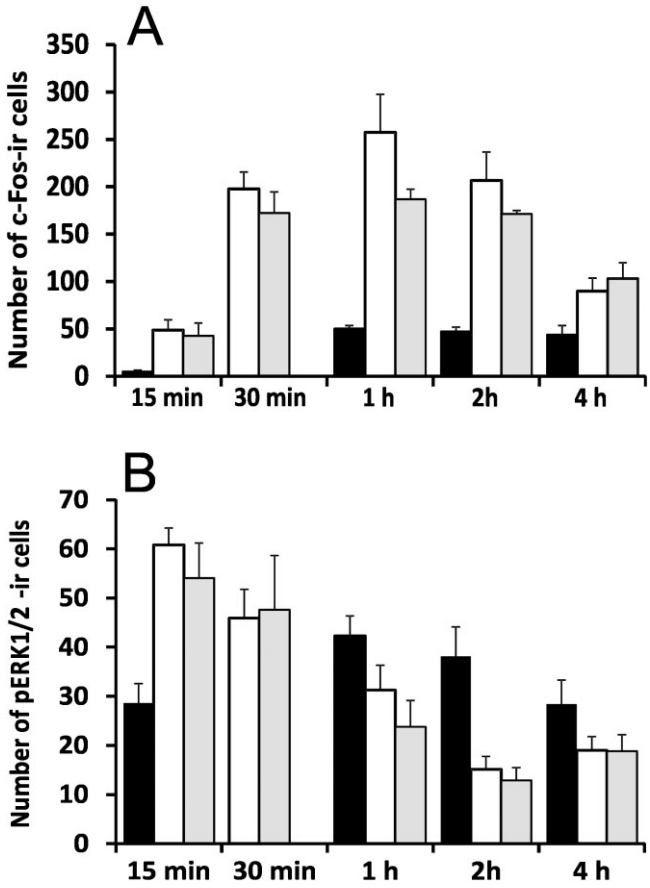

Figure 5

Effect of morphine on light-induced c-Fos (A) and pERK1/2 (B) within the rat vlSCN. Rats were released into constant darkness and either injected with morphine (1 mg·kg^{-1}) or saline at CT14. Control animals were divided into two groups. Whereas one group was left in darkness (black columns), the other control group (white columns), together with the morphine-treated group (grey columns), was exposed to a 30 min light pulse during the subjective night at CT15. Animals were sampled 15 and 30 min and 1, 2 and 4 h later. Each column represents the mean of four values ± SEM.

Acute morphine does not affect light-induced levels of c-Fos and pERK1/2

To determine the possible interaction of photic and morphine-activated pathways within the SCN, we investigated the effect of acute morphine on light-induced elevation of pERK1/2 and c-Fos levels within the vlSCN. As shown in previous studies, a light pulse delivered at CT15 induced an increase in the c-Fos level at a maximum of 1 h from the beginning of the light pulse, and an increase in the level of pERK1/2 within 15–30 min. In our experiment, morphine applied 1 h before the light pulse did not mimic the light-induced c-Fos and pERK1/2 in the SCN (Figure 5).

Acute morphine applied at night induces Per1 *but not* Per2 *in rat SCN*

To reveal the effect of acute morphine on the clockwork mechanism, animals released into constant darkness were injected with morphine at CT3 to measure the response at the time of maximum *Per1* and *Per2* endogenous expression, and at CT15 – the time of minimum *Per* genes expression in the SCN. As shown in Figure 6B and E, the substantial induction of *Per1* mRNA was observed in the central part of the vlSCN 1 h after morphine application at night [$F(7) = 60.5$; $P < 0.01$]. Morphine applied at CT3 caused only a minor increase in *Per1* mRNA in the vlSCN [$F(7) = 6.5$; $P < 0.05$] (Figure 6A; Supporting Information Fig. S2). No difference between control groups and morphine-treated animals was detected for *Per2* expression in the SCN (Supporting Information Fig. S3).

Activity of κ and μ receptors but not δ receptors changes between day and night

To dissect the possible signalling mechanism mediating the bidirectional effect of acute morphine on the SCN, we performed [^{35}S]-GTPγS binding assay to assess the activity of opioid receptors in the SCN. [^{35}S]-GTPγS binding stimulated by the κ receptor agonist U-50488 was significantly higher during the day than at night [$F(7) = 19.7$; $P < 0.01$] (Figure 7A, B; Supporting Information Fig. S4). The significant difference between day and night [^{35}S]-GTPγS binding was observed also after stimulation with the μ receptor agonist DAMGO [$F(7) = 11.2$; $P < 0.01$]. No difference between day and night in [^{35}S]-GTPγS binding was detected in the SCN after stimulation with DADLE (δ receptor agonist).

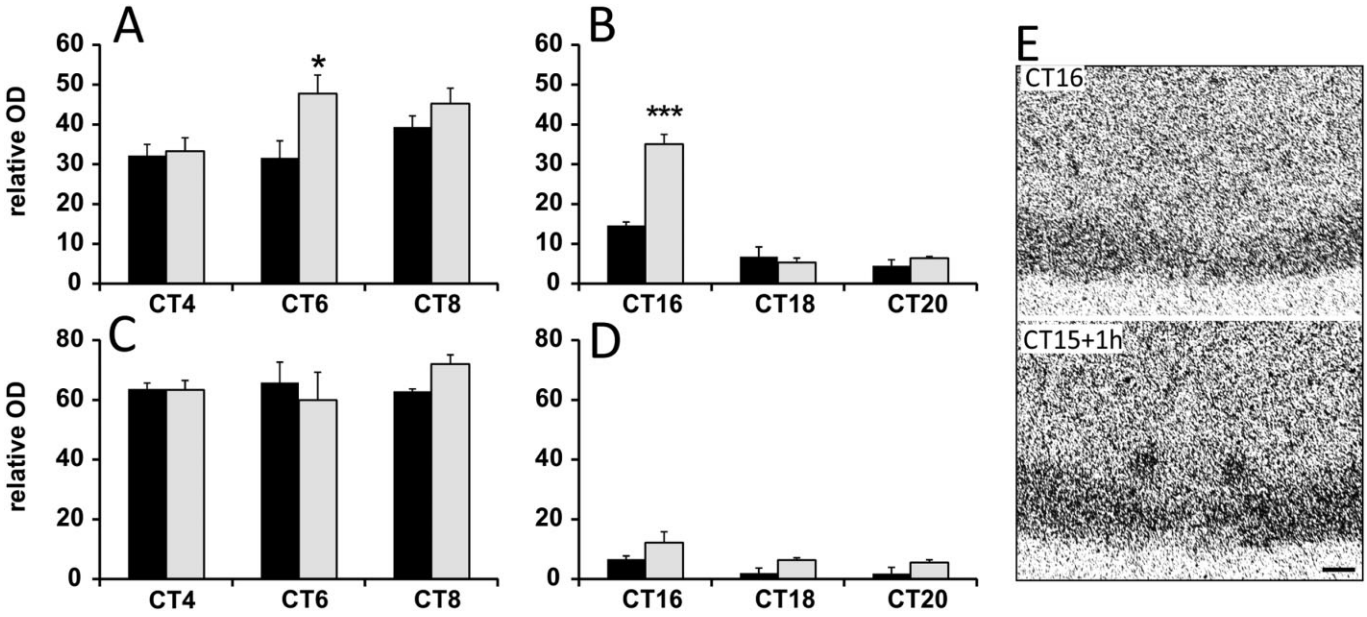

Figure 6

Effect of acute morphine on *Per1* expression in rat SCN. Adult rats were released into constant darkness and injected with morphine (1 mg·kg^{-1}) either during the subjective day at CT3 (A, C) or during the night at CT15 (B, D). Animals were sampled 1, 3 and 5 h later (grey columns) (controls; black columns). Levels of *Per1* were assessed separately for the ventrolateral (A, B) and dorsomedial SCN (C, D). Each column represents the mean of four values ± SEM. *$P < 0.05$; ***$P < 0.01$. Representative photomicrographs (E) of coronal sections of the SCN demonstrate the intensity and distribution of *Per1* in control animals (top) and morphine-treated animals (bottom) 1 h after morphine at CT15. Scale bar = 100 μm.

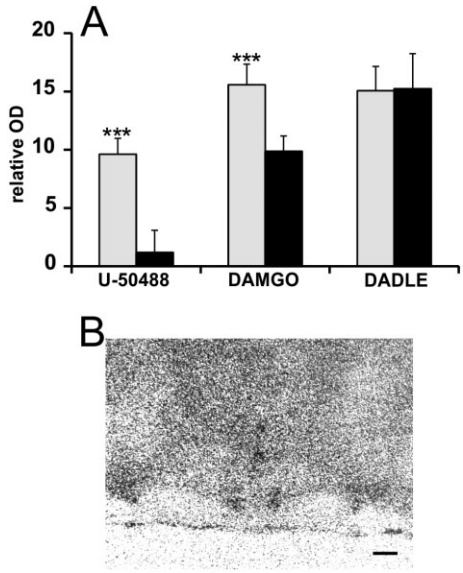

Figure 7

The day/night difference in the coupling efficacy of κ, μ and δ receptors to Gi/o proteins in rat SCN (A). Adult rats were sampled at ZT7 (white columns) or ZT15 (black columns), and agonist-induced binding of [^{35}S]-GTPγS was visualized by autoradiography. The intensity of G protein binding was quantified as relative OD. Each column represents the mean of four values ± SEM. ***$P < 0.01$. Representative autoradiograph demonstrates DAMGO-stimulated [^{35}S]-GTPγS binding in the SCN collected at ZT7 (B). Scale bar = 100 μm.

Discussion and conclusions

Previous studies have shown that opioids induce phase advance of circadian activity and *Per1* expression, decrease spontaneous electrical activity in the SCN and interfere with photic entrainment (Cutler *et al.*, 1999; Tierno *et al.*, 2002; Vansteensel *et al.*, 2005). To investigate the upstream signalling events mediating these actions in the SCN, we tested the effect of morphine on two principal kinases of the circadian clockwork pERK1/2 and pGSK3β, the clock genes *Per1* and *Per2*, and immediate early gene c-Fos. Contrary to the dmSCN, the vlSCN is responsive to external cues and receives strong input from other brain parts. Therefore, we expected morphine-induced changes to occur particularly in the vlSCN and intended firstly to specify the phase of minimum and maximum pERK1/2 and pGSK3β level in the vlSCN that allows precise distinguishing of induction or reduction, respectively, of both these kinases after morphine application. We found that pERK1/2 level in the vlSCN varies over the 24 h cycle in antiphase with the dmSCN, the low level being displayed during the day, which is similar to previous observations in mice and hamsters (Obrietan *et al.*, 1998; Nakaya *et al.*, 2003). We also monitored the daily changes in the pGSK3β level in both parts of the SCN, which displayed a maximum during the second part of the night. To the best of our knowledge, there is only one report based on Western blot analysis that describes the pGSK3β rhythm in the SCN with a similar waveform as in our study (Iitaka *et al.*, 2005). The phosphorylation of GSK3β at serine 9 causes its inactivation. The physiological role of GSK3β in the circadian clock

might thus be defined by the interval of its basal rather than high level. Interestingly, our data show low levels of pGSK3β only during short intervals around the light/dark transition.

Clock genes *Per1* and *Per2* are integral part of the clockwork machinery, and their expression in the SCN varies: expression is high during the day and low at night. *Per* genes regulation is the major mechanism mediating the photic or non-photic phase resetting of the circadian clock (Albrecht *et al.*, 1997; Akiyama *et al.*, 1999). Several studies have indicated a decrease in *Per1*, *Per2* and pERK1/2 levels in response to various non-photic stimuli when applied during the day (Maywood *et al.*, 1999; Horikawa *et al.*, 2000; Fukuhara *et al.*, 2001). Our data show that morphine applied at ZT7 decreased slightly the spontaneous c-Fos level in the dmSCN, but had no effect on *Per* genes expression. Likewise, little effect of the μ receptor agonist fentanyl on *Per* genes expression was reported in hamsters (Vansteensel *et al.*, 2005). Contrary to our expectations, morphine up-regulated pERK1/2 and pGSK3β levels during the day in both parts of the SCN. Thus, it seems that opioids mediate their effect via other pathways than those used by non-photic stimuli. Because ERK1/2 is considered a positive regulator of the negative feedback loop of the molecular clockwork, it is possible that its up-regulation will accelerate the inhibition of CLOCK/Bmal1-mediated transcription and thus phase advance the circadian clock. Up-regulated pGSK3β may block nuclear entry of clock genes – predominantly Rev-Erb-α, which is high during the daytime, thereby accelerating *Bmal1* transcription. Our data suggest that a phase-shifting effect of morphine applied during the day may not be caused by alteration in clock gene expression as proposed for other non-photic cues, but it can be mediated via post-translational modifications of clock proteins.

The night effect of opioids on the circadian clock has been studied mostly in the context of the modification of light-induced phase shifts or *Per* gene induction. Phase advance of locomotor activity in hamsters induced by a light pulse at night was shown to be almost completely blocked by fentanyl or agonists of δ receptors (Tierno *et al.*, 2002; Vansteensel *et al.*, 2003). Fentanyl also blocked the light-induced *Per1* expression (Vansteensel *et al.*, 2005). In our experiments, we did not observe any significant attenuation of light-induced changes in c-Fos or pERK1/2 levels. The general agreement exists for opioids affecting light-induced changes in the SCN via presynaptic modulation of the retinal input into the vlSCN (Cutler *et al.*, 1999; Tierno *et al.*, 2002; Vansteensel *et al.*, 2005). Reduction of glutamate release from the retino-hypothalamic tract should unequivocally affect the calcium-sensitive signalling components such as pERK1/2 and c-Fos. It is possible that the relatively low dose of morphine used in our study was not sufficient to significantly interfere with strong signalling activation by a light pulse at night. Still, this dose is high enough to significantly suppress a high level of pERK1/2 and pGSK3β and to induce a transient increase in *Per1* mRNA in the vlSCN at night. Although the reduction in pGSK3β occurs within the entire vlSCN, pERK1/2-positive cells disappear from most of it but remain in its central part. The phosphorylation state of ERK1/2 in the central SCN has been the topic of several reports (Nakaya *et al.*, 2003; Antle *et al.*, 2005a; 2008; Guillaumond *et al.*, 2007). In the rat, a cell population expressing a high night level of pERK1/2 in the central SCN has been associated with gastrin-releasing peptide and their involvement in transition of photic information into the dmSCN has been suggested (Guillaumond *et al.*, 2007). Interestingly, sleep deprivation activates the analogic central region in hamster SCN and induces pERK1/2 there during the subjective day (Antle *et al.*, 2008). Assuming that activated ERK1/2 is essential for *Per* gene induction (Dziema *et al.*, 2003), the morphine-induced *Per1* in the vlSCN seems to contradict reduction of pERK1/2. Closer observation of autoradiographs, however, suggests that up-regulated *Per1* mRNA occurs also in the central SCN. It can be speculated that silencing of neurons in the vlSCN may disconnect central SCN from some inhibitory networks, and *Per1* can be induced via the ERK1/2 signalling pathway. In support of such a hypothesis, the constant high level of pERK1/2 within individual cells as measured by OD suggests that an intercellular network is affected by morphine rather than an intracellular ERK signalling pathway. Alternatively, morphine can only postpone natural *Per1* decrease. Spontaneous rhythm in *Per1* expression declines in the evening and during early night, and its last signal before disappearance occurs only in the central SCN (Lambert *et al.*, 2005). In our control animals, there was no existing *Per1* expression in the SCN at CT16. Therefore, an alternative hypothesis may arise that *Per1* in the central SCN is not induced *de novo*, and morphine may rather phase delay on its natural decrease.

Morphine is a potent μ receptor agonist but is able to bind also to κ and δ receptors (Chen *et al.*, 1993). All types of opioid receptors have been identified in the rat SCN, although some discrepancies exist about μ and κ receptors in hamsters and mice (Desjardins *et al.*, 1990; Mansour *et al.*, 1994; Ding *et al.*, 1996; Byku *et al.*, 2000). Our [^{35}S]-GTPγS binding data from *in situ* experiments suggest that the effect of morphine at night can be mediated predominantly by δ receptors and to a lesser extent by μ receptors. During the day, all receptor subtypes may function equally. Constitutive activity of δ receptors in the SCN may explain why agonists of δ receptors provide the most consistent data across all studies concerning its effect on circadian system (Byku and Gannon, 2000a,b; Byku *et al.*, 2000; Tierno *et al.*, 2002). Also, the low night activity of κ receptors may explain why its agonist U-50488 had no effect on light-induced phase advances of circadian activity rhythm induced during the late subjective night (Tierno *et al.*, 2002).

In summary, our data show that morphine at a dose of 1 mg·kg^{-1} shows a bidirectional effect on the SCN depending on time of application. We suggest that the opioids may exert their impact on the circadian system via modification of ERK1/2 and GSK3β that may lead to subsequent post-translational modification of clock proteins.

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Author contributions

D. P., B. V., K. Č. and L. H. performed the research. J. N. substantially contributed to the design of the research study and contributed essential reagents. Z. B. designed the research study, analysed the data and wrote the paper.

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effect of acute morphine on pERK1/2 (A, B, C, D) and pGSK3β (E, F, G, H) expression within the cells of rat SCN.
Figure S2 Effect of acute morphine on *Per1* expression in rat SCN.
Figure S3 Effect of acute morphine on *Per2* expression in rat SCN.
Figure S4 The day/night difference in the coupling efficacy of κ-OR, μ-OR and δ-OR to Gi/o proteins in the rat SCN.