Daily morphine injections and withdrawal disrupt the circadian rhythm of wheel running and expression of the clock protein PERIOD2 in the rat limbic forebrain

Running title: Morphine withdrawal disrupts PER2

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ABSTRACT

Symptoms of opiate withdrawal include disturbances in circadian rhythms. Here, we examined in male Wistar rats the effects of a daily, mid-morning morphine injection (5-40mg/kg, i.p.) and the subsequent withdrawal of morphine on 24-h patterns of wheel running and expression of the clock protein, PERIOD2 (PER2), in the master circadian clock, the suprachiasmatic nucleus (SCN), and regions of the limbic forebrain. Rats were killed either within 24 h of the last morphine injection or 2 days later. Nighttime wheel running was suppressed during daily morphine injections and following the withdrawal of morphine. Daily morphine injections and their subsequent withdrawal did not affect PER2 expression in the SCN, but blunted the normal daily peak of PER2 in the dorsal striatum, oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and dentate gyrus of the hippocampus (DG). We then examined the effect of injecting the D2/3 dopamine agonist, quinpirole (1 mg/kg, i.p.), or the alpha 2 adrenergic agonist, clonidine (0.1 mg/kg, i.p.), two drugs that alleviate opiate withdrawal symptoms, following withdrawal of the daily morphine injection. Quinpirole restored the daily PER2 pattern in the BNSTov and CEA, whereas clonidine restored and entrained a new PER2 pattern in the striatum, BLA, and DG. Together, these findings suggest that disruption of daily PER2 patterns in the forebrain might contribute to the circadian symptoms observed in opiate withdrawal. Furthermore, pharmacological treatments for withdrawal can restore PER2 patterns in regions of the limbic forebrain.

KEYWORDS

clock gene, opiate, dopamine, quinpirole, clonidine, striatum.

INTRODUCTION

Opiate withdrawal is characterized by profound physiological and motivational deficits that include disturbances in circadian rhythms of activity and hormone secretion (Li *et al*, 2009a; Li *et al*, 2009b). Disruptions in sleeping and diurnal activity patterns are common symptoms reported in humans undergoing withdrawal and are thought to contribute to increased risk of relapse (Howe *et al*, 1980; Shi *et al*, 2007; Stinus *et al*, 1998).

At the molecular level, circadian timing relies on a group of clock genes that regulate their own transcription and translation through negative and positive feedback loops (Reppert and Weaver, 2002). The master circadian clock in mammals is housed in the suprachiasmatic nucleus (SCN) of the hypothalamus and light is the primary stimulus to entrain the timing of the molecular clockwork in the SCN. Clock genes are also rhythmically expressed in a number of other brain regions and peripheral tissues, although synchronizing signals from the SCN are required to maintain and coordinate rhythmic activity across tissues. It is believed that these local rhythms regulate the daily variations in cellular and metabolic functions of individual tissues.

In addition to light, a variety of non-photic stimuli, including events having motivational salience, can entrain the rhythms of clock gene expression in other tissues independently of the SCN. These stimuli include the presentation of food to rodents maintained on a restricted feeding schedule (Waddington Lamont *et al*, 2007), stressors (Takahashi *et al*, 2001), and drugs of abuse (Masubuchi *et al*, 2000; Uz *et al*, 2005). The precise mechanisms by which each of these signals modifies the timing of the circadian clock are unknown; however, increasing evidence demonstrates that

dopamine (DA) signaling can modulate the expression of clock genes and proteins in brain regions associated with motivated behaviors, such as the striatum. In particular, we and others have identified an important role of DA activity at D2 receptors in the regulation of clock gene expression in the striatum (Hood *et al*, 2010; Imbesi *et al*, 2009; Sahar *et al*).

Many abused drugs, including opiates, increase extracellular concentrations of DA within terminal regions of the mesolimbic system, including dorsal and ventral striatum (Di Chiara and Imperato, 1988), prefrontal cortex (Moghaddam and Bunney, 1989), BNST (Carboni *et al*, 2000), and amygdala (Harmer *et al*, 1997), whereas early withdrawal from repeated drug use is associated with a reduction in the activity of mesolimbic dopamine neurons and in extracellular dopamine levels (Acquas *et al*, 1991; Diana *et al*, 1999; Pothos *et al*, 1991; Rossetti *et al*, 1992; Tran-Nguyen *et al*, 1998). Interestingly, recent findings suggest that opiate exposure and withdrawal profoundly affect transcription rhythms of the clock genes, *Period1* and *Period2 (Per2)* for at least one month in recently abstinent heroin users (Li *et al*, 2009a) and in rats undergoing spontaneous withdrawal from morphine (Li *et al*, 2009a; Li *et al*, 2010). Thus, the circadian disturbances that arise during withdrawal from chronic use of opiates could be attributable to changes in the expression of clock genes and their proteins as a result of aberrant dopamine signaling patterns.

We examined this issue in the present study by monitoring in rats the effect of a daily morphine injection and its subsequent withdrawal on 24-h patterns of wheel-running activity and expression of the clock protein, PERIOD2 (PER2), in the SCN and a number of regions in the limbic forebrain. These regions – namely, dorsal striatum,

oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and dentate gyrus of the hippocampus (DG) – are associated with the regulation of motivational states. Furthermore, rhythms of clock gene expression in these regions are responsive to motivationally salient stimuli, such as restricted feeding (Waddington Lamont *et al*, 2007). We also examined whether treatments previously demonstrated to alleviate some symptoms of opiate withdrawal through modulation of midbrain DA activity would mitigate the effects of morphine withdrawal on wheel running and on PER2 patterns in the limbic forebrain. To this end, we administered the dopamine D2/3 agonist, quinpirole (Harris and Aston-Jones, 1994), or the alpha 2 adrenergic agonist, clonidine, which at low doses increases DA tone by inhibiting the overactivity of noradrenergic neurons in opiate withdrawal (Delfs *et al*, 2000; Dumont and Williams, 2004; Taylor *et al*, 1988), to rats for two days following the last morphine injection.

MATERIALS AND METHODS

Subjects

48 male Wistar rats (Charles River, St. Constant, QC) weighing 250-275 g at the start of the experiment were housed singly in shoebox cages with running wheels and kept in individual lightproof and sound-attenuated cabinets. Within each cabinet, an overhead fluorescent tube provided light on a 12:12 light-dark (LD) schedule (light intensity within the cage was approximately 300 lux). Wheel-running activity was monitored continuously by computer (VitalView, Minimitter, Sunview, OR, USA). Throughout the experiment, rats had ad libitum access to laboratory chow (Purina

Foods) and tap water. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University.

Drugs

Morphine hydrochloride (Sigma, Oakville, Ont.) was dissolved in saline. Quinpirole hydrochloride and clonidine hydrochloride (Sigma) were dissolved in distilled water.

Immunohistochemistry

At the end of the experiment, rats were given an overdose of sodium pentobarbital (Somnotol, 100 mg/kg) at their assigned zeitgeber time (ZT) (ZT1, 1 h after lights on, or ZT13, 1 h after lights off) and perfused intracardially with 300 ml of cold 0.09% saline, followed by 300 ml of cold 4 % paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in 4 % paraformaldehyde solution overnight at 4 ° C, then sectioned at 50 µm using a vibratome. Sections were stored in Watson's cryoprotectant (Watson *et al*, 1986) at –20 ° C until immunohistochemistry was performed. Immunohistochemical staining for PER2 protein was carried out according to a protocol described previously (Hood *et al*, 2010) using an affinity-purified polyclonal antibody raised in rabbit against PER2, 1:4000 (Santa Cruz Biotechnology, USA).

Microscopy

Stained brain sections were examined under a light microscope using a 20x objective and 400 x 400 µm images of unilateral SCN, dorsal striatum, BNSTov, CEA, BLA, and DG were captured from each hemisphere using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S. D. Barrett, http://www.ImageSXM.org.uk). The number of cells with nuclei immunoreactive (IR) for PER2 was then counted in each image captured. For each brain region of interest, the mean number of PER2-IR nuclei was calculated for each group by finding for each rat the mean cell count from six images having the highest number of labeled nuclei.

Assay for plasma corticosterone

To measure the effect of the daily morphine injection and its subsequent withdrawal on plasma levels of corticosterone, tail blood samples were collected from each rat approximately 10 min before it was killed. Blood was centrifuged at 10000 rpm for 10 min at 4 ° C and the extracted plasma was stored at –80 ° C. Corticosterone level in each plasma sample was measured using enzyme-linked immunosorbent assay (ELISA; Assay Designs, Ann Arbour, Michigan) according to manufacturer's instructions.

Procedure

All rats were allowed to entrain to the LD cycle for 14 days before injections began. Wheel running during the 12-h light and dark phases of the entrainment period was used to assign rats to either the saline or morphine injection condition to match the activity level of the groups before the course of daily injections began. One group then received a daily intraperitoneal (i.p.) injection of morphine, between ZT3-4 (3-4 hours after lights turned on), whereas the other group received a daily saline injection. The

dose of morphine began at 5 mg/kg and increased to 40 mg/kg in the following sequence over days: 5, 5, 10, 10, 20, 30, 40. The 40 mg/kg dose was maintained for an additional 5 days for a total of 12 days of injections.

Within the morphine and saline groups, rats were randomly assigned to one of four conditions in a 2 (withdrawal day 1 (WD-1) or withdrawal day 2 (WD-2)) by 2 (ZT1 or ZT13) design (n = 3 per group). Rats assigned to the WD-1 condition were killed within 24 h of the final morphine or saline injection, at either ZT13 (10 h after the last injection) or ZT1 (22 h after the last injection). Rats assigned to the WD-2 condition received a saline injection at ZT13 in the evening after the last morphine or saline injection (as a control for the injections of quinpirole and clonidine given during withdrawal, see below). The following day, they received no morphine or saline injection between ZT3-4, but as on the previous day, they received a saline injection in the evening at ZT13. They were then killed the following day either at ZT1 (46 h after the last morphine or saline injection at ZT3-4) or at ZT13 (58 h after the last injection at ZT3-4).

We chose to kill the rats at either ZT1 or ZT13 because these times represent the points at which the 24-h rhythms of PER2 in each forebrain region studied reach their peak and trough. Examining PER2 expression at these two times provided a ‘snapshot’ of the effects of each manipulation on the normal maximum and minimum levels of PER2-IR in each brain region of interest.

Effect of quinpirole following morphine withdrawal.

To examine the effects of quinpirole on wheel running and PER2 expression following withdrawal of the daily morphine injection, rats received a daily injection of

saline or morphine at ZT3-4 for 12 days, as described above. In the evening of the last morphine or saline injection day, rats received an injection of quinpirole (1 mg/kg, i.p.) at ZT13. The next day, no injection of morphine or saline was given between ZT3-4, but as on the previous day, an injection of quinpirole was given at ZT13. Rats were then killed the following day at either ZT1 (46 h after the last morphine or saline injection) or ZT13 (58 h after the last morphine or saline injection) (n = 3 per group).

Effect of clonidine following morphine withdrawal.

To examine the effects of clonidine on wheel running and PER2 expression following withdrawal of the daily morphine injection, rats received a daily injection of morphine or saline at ZT3-4 for 12 days, as described above. In the evening of the last morphine or saline injection day, two injections of clonidine (0.1 mg/kg per injection, i.p.) were given – the first at ZT11 and the second at ZT13. The next day, no injection of morphine or saline was given between ZT3-4, but as on the previous day, two injections of clonidine were given at ZT11 and ZT13. Rats were then killed the following day at either ZT1 (46 h after the last morphine or saline injection) or ZT13 (58 h after the last morphine or saline injection) (n = 3 per group).

Statistical analyses

Independent samples t-tests were used to compare wheel running in the morphine and saline groups during the 12-h light and 12-h dark periods during the 12-day injection period, the 3 h before the daily morphine or saline injection was given (ZT0-3), and the hour after the daily injection (ZT4-5). To test the effect of withdrawing the daily morphine or saline injection on wheel running, on corticosterone levels, and on PER2-IR nuclei in each brain region of interest, two-way analyses of variance (ANOVA)

were carried out using daily injection condition (saline or morphine) and withdrawal time (WD-1 or WD-2) as factors. Additional two-way ANOVA were used to test the effect of either quinpirole or clonidine treatment following morphine withdrawal on each dependent variable of interest using daily injection condition (saline or morphine) and withdrawal treatment ((quinpirole or clonidine) or saline) as factors. Significant interactions were further analyzed using independent samples t-tests. Alpha level for all tests was set at 0.05.

RESULTS

Wheel running during daily morphine injections

Figure 1a shows average (+/- SEM) wheel-running activity in the 12-h light and dark phases from the 12-day period of daily morphine or saline injections. It can be seen that a daily injection of morphine significantly suppressed activity in the wheel during the 12-h dark phase, when rats are normally most active ($t(46) = 3.24$, $p < 0.01$), whereas morphine- and saline-injected groups did not differ in wheel-running activity in the 12-h light phase ($t(46) = 1.04$, n.s.). To examine whether this suppression of nighttime activity by morphine was dependent on the dose of morphine, we examined wheel running in the 12-h dark phase during the first two days of morphine injection (5 mg/kg) and during the first two days of injections of the highest morphine dose (40 mg/kg) (data not shown). Although nighttime wheel running was decreased within the first two days of morphine injections, activity in the morphine group at the 5 mg/kg dose was not significantly different from the saline-injected group. At the 40 mg/kg dose of morphine, however, nighttime wheel running was significantly suppressed ($t(46) = 3.84$,

$p < 0.01$). These results indicate that morphine dose-dependently reduces wheel-running activity in the dark phase.

Previous reports indicate that rats given a daily injection of a drug of abuse, such as nicotine or amphetamine, develop increased activity in a running wheel in the hours preceding the daily injection (Kosobud *et al*, 2007; Nikaido *et al*, 2001; Shibata *et al*, 1994). To determine whether rats receiving a daily injection of morphine developed a similar increase, we examined activity in the wheel during the three hours preceding the daily injection (between ZT0-3) from the last 6 days of the injection period (when the 40 mg/kg morphine dose was injected). Morphine-injected rats ran somewhat less than saline-injected rats during the 3 h before the daily injection, although this difference was not significant ($t(46) = 1.74$, n.s.) (data not shown). To establish whether morphine differentially affected wheel-running activity after the injection, we examined wheel running in the hour following the daily injection (ZT4-5). Morphine- and saline-injected rats did not differ in wheel running during this hour (data not shown). Taken together, these findings indicate that the effects of a daily morphine injection on wheel running were evident only in the dark phase, which began 9 h after the time of injection.

Wheel running after withdrawal of morphine and treatment with quinpirole or clonidine

Figure 1b shows wheel running during the 12-h light and dark phases on the day when no morphine or saline injection was given at ZT3-4. It can be seen that rats previously injected with morphine (morphine WD-2 + sal) ran significantly less during the dark phase than did rats previously injected with saline (saline WD-2 + sal) ($t(10) =$

3.62, $p < 0.01$). Groups did not differ in wheel running during the light phase on this day.

Figure 1b also shows the effect of quinpirole and clonidine treatment, injected in the evenings following the last daytime morphine injection, on wheel-running activity. It can be seen that neither quinpirole treatment following the last morphine exposure (morphine WD-2 + Q) nor clonidine (morphine WD-2 + CL) restored nighttime activity in the running wheel.

The effects of quinpirole and clonidine injections alone on wheel running were examined in rats previously given daytime injections of saline. These data are shown in Figure 1c. Interestingly, quinpirole (saline WD-2 + Q) significantly suppressed nighttime wheel running in comparison to saline control (saline WD-2 + saline) ($t(10) = 3.95$, $p < 0.01$). Quinpirole injections did not affect wheel running during the light phase. Clonidine treatment had no effect on wheel running in the light or dark phases in rats previously injected with saline (saline WD-2 + CL). Taken together, these results indicate that although neither quinpirole nor clonidine significantly affected the reduction in nighttime wheel running observed following withdrawal of the daily morphine injection, quinpirole itself decreased nighttime activity in rats previously injected with saline.

Plasma corticosterone levels

In rats, circulating levels of corticosterone fluctuate in a circadian rhythm that peaks just before the transition from the inactive (or light) phase to the active (or dark) phase (Oster *et al*, 2006). Previous reports indicate that basal corticosterone levels in rodents increase following the cessation of long-term opiate treatment (Pechnick, 1993).

In the present experiment, we measured corticosterone at ZT1 (when corticosterone is normally low) and at ZT13 (when corticosterone is normally high) in rats given daily injections of saline or morphine and killed either within 24 h of the last injection or two days later. In rats killed within 24 h of the final morphine injection, the daily fluctuation in corticosterone levels was no different from saline-injected rats (data not shown). In rats killed two days after withdrawal of morphine (morphine WD-2), corticosterone levels at ZT1 were significantly elevated in comparison to saline-injected rats ($t(4) = -6.95$, $p < 0.05$), such that the normal fluctuation in corticosterone between ZT1 and ZT13 in this group was lost ($t(4) = -0.29$, n.s.). These results are consistent with previous reports that corticosterone levels increase following withdrawal of repeated opiate exposure and that the normal rhythm of secretion of this hormone is disrupted in withdrawal (Li *et al*, 2010; Pechnick, 1993).

Corticosterone levels were also measured in groups given quinpirole. Quinpirole did not affect the elevation of corticosterone at ZT1 seen in rats given saline following the withdrawal of morphine. At ZT13, however, quinpirole significantly decreased the normal rise in corticosterone levels in both morphine- and saline-injected groups (main effect of quinpirole, $F(1, 8) = 8.37$, $p < 0.05$).

Injections of clonidine decreased the elevation in corticosterone levels seen at ZT1 following the withdrawal of morphine, although this effect was not significant. At ZT13, clonidine significantly decreased corticosterone in both morphine- and saline-injected groups (main effect of clonidine, $F(1, 8) = 5.87$, $p < 0.05$). Thus, these results indicate that the elevation of daytime corticosterone levels following withdrawal of morphine can be partially decreased by clonidine injections. Both quinpirole and

clonidine injections themselves, given in the evening, however, significantly blunt the normal nighttime rise in corticosterone.

PER2 expression

SCN

In the SCN, PER2-IR nuclei reach their lowest levels at the beginning of the light phase (ZT1) and are most abundant just after the transition from the light to dark phase (ZT13). Figure 2a shows the number of PER2-IR nuclei expressed in the SCN at each of these timepoints in rats killed within 24 h of the final injection of morphine or saline at ZT3-4. Figure 2b shows PER2-IR nuclei in rats killed two days after the final injection. In both figures, it can be seen that daily injections of morphine and their subsequent withdrawal had no effect on the normal pattern of PER2 expression in the SCN, indicating that morphine withdrawal does not influence the activity of the master clock.

Figure 2b also shows the effects of administering quinpirole (morphine WD-2 + Q) or clonidine (morphine WD-2 + CL) in the evenings following the last morphine injection on PER2 in the SCN. Neither treatment significantly affected the normal PER2 pattern in morphine-injected rats. Furthermore, as shown in Figure 2c, in saline-injected rats neither quinpirole (saline WD-2 + Q) nor clonidine treatment (saline WD-2 + CL) had any effect on the normal pattern of PER2 expression.

Dorsal striatum

PER2 expression in the dorsal striatum is lowest at the beginning of the dark phase and greatest at the beginning of the light; thus, its daily PER2 pattern is opposite to that in the SCN. As shown in Figure 3a, in rats killed within 24 h of the last daily

injection of morphine, PER2 was significantly increased at ZT13 (10 h after the last morphine injection) ($t(4) = -3.66$, $p < 0.05$), whereas PER2 expression at ZT1 (22 h after the last injection) was suppressed ($t(4) = 5.63$, $p < 0.01$).

Figure 3b shows the pattern of PER2 expression in rats killed two days after the last daily injection at ZT3-4. In rats previously injected with morphine (morphine WD-2 + sal), it can be seen that the normal peak of PER2 at ZT1 (46 h after the last injection) was blunted compared to rats previously injected with saline (saline WD-2 + sal) ($t(4) = 4.49$, $p < 0.05$). Figure 3b also illustrates the effect of quinpirole or clonidine treatment following withdrawal of morphine injections on PER2 expression in the striatum. Evening injections of quinpirole (morphine WD-2 + Q) did not restore PER2 expression at ZT1, but increased PER2 at ZT13 ($t(4) = -2.99$, $p < 0.04$) in comparison to saline treatment. Clonidine treatment (morphine WD-2 + CL) also did not restore PER2 expression at ZT1 in rats previously injected with morphine, but significantly increased PER2 at ZT13 ($t(4) = -8.01$, $p < 0.01$).

Figure 3c shows the effect of quinpirole or clonidine alone on PER2 expression in rats previously given daily injections of saline at ZT3-4. Quinpirole itself (saline WD-2 + Q) significantly suppressed the normal peak of PER2 at ZT1 ($t(4) = 3.20$, $p < 0.05$). In contrast, clonidine (saline WD-2 + CL) did not affect the normal pattern of PER2 in the striatum. Taken together, these results indicate that withdrawal of daily morphine injections blunts the normal peak of PER2 expression in the dorsal striatum, and that a daily increase in PER2 expression can be reinstated partially by quinpirole or fully by clonidine. Quinpirole itself, however, blunts the normal peak of PER2 expression in the striatum.

BNSTov

In the BNSTov, the daily fluctuation of PER2 expression rises toward the beginning of the dark phase (ZT13) and falls during the light (ZT1), and thus coincides with the PER2 pattern in the SCN. As shown in Figure 4a, in rats killed within 24 h of the last morphine injection, PER2 expression at ZT13 (10 h after the last injection) was significantly decreased ($t(4) = 3.11$, $p < 0.05$).

Figure 4b shows the pattern of PER2 expression in rats killed two days after the last daily injection at ZT3-4. In rats killed two days after the final morphine injection (morphine WD-2 + sal), PER2 at ZT13 (58 h after the last injection) was significantly decreased ($t(3) = 4.79$, $p < 0.05$). Figure 4b also shows the effect on PER2 of quinpirole and clonidine treatment following the last morphine injection. Quinpirole (morphine WD-2 + Q) increased PER2 expression at ZT13, although this effect was not significant. Clonidine (morphine WD-2 + CL) significantly increased PER2 at ZT1 ($t(4) = -5.65$, $p < 0.05$), but did not affect the blunted expression of PER2 at ZT13.

Figure 4c shows the effects of quinpirole and clonidine treatments alone on PER2 expression in rats previously injected with saline at ZT3-4. Quinpirole itself (saline WD-2 + Q) did not affect the normal PER2 pattern. Clonidine (saline WD-2 + CL) significantly increased PER2 expression at ZT1 ($t(3) = -5.79$, $p < 0.01$) and suppressed the normal peak of PER2 at ZT13 ($t(3) = 4.25$, $p < 0.05$). Thus, these results indicate that withdrawal of morphine blunts the normal PER2 peak in the BNSTov, and that evening injections of quinpirole can increase PER2 expression at ZT13. Clonidine

treatment, either alone or in combination with morphine withdrawal, establishes a new pattern of PER2 expression in the BNSTov.

CEA

As shown in Figure 5a, the normal pattern of PER2 expression in the CEA peaks at ZT13 and falls near ZT1, and is thus in phase with the BNSTov and SCN. In rats killed within 24 h of the last morphine injection (Figure 5a), PER2 was significantly increased at ZT1 (22 h after the last injection) ($t(4) = -4.54$, $p < 0.01$), the time at which PER2 is normally lowest in this region, and was reduced at ZT13 (10 h after the injection).

Figure 5b shows the pattern of PER2 expression in rats killed two days after the last daily injection at ZT3-4. It can be seen that in rats previously injected with morphine (morphine WD-2 + sal), PER2 expression at ZT1 (46 h after the last injection) was unaffected, but the normal peak of PER2 at ZT13 (58 h after the last injection) was blunted ($t(4) = 2.67$, $p = 0.05$). Figure 5b also shows the effects of quinpirole or clonidine treatment following morphine withdrawal. Quinpirole (morphine WD-2 + Q) increased PER2 at ZT13, although this increase was not statistically significant. Clonidine treatment (morphine WD-2 + CL) following withdrawal of morphine non-significantly increased PER2 expression at ZT1, and had no effect on the blunted expression of PER2 at ZT13.

Figure 5c shows the effect of quinpirole and clonidine alone in saline-injected rats. Quinpirole itself (saline WD-2 + Q) did not affect the normal PER2 pattern in this region, whereas clonidine (saline WD-2 + CL) increased PER2 expression at ZT1 ($t(4) = -10.56$, $p < 0.01$) and decreased PER2 at ZT13 ($t(4) = 3.55$, $p < 0.05$). Together,

these results show that the normal daily PER2 pattern in the CEA is disrupted by daily morphine injection and withdrawal. Quinpirole treatment following morphine withdrawal increased PER2 at ZT13, the time at which its expression normally peaks, whereas clonidine treatment did not significantly affect the pattern of PER2 following morphine withdrawal. Clonidine alone, however, disrupted the normal pattern of PER2 expression in saline-injected rats.

BLA

In the BLA, PER2 expression peaks near ZT1 and falls to a trough level near ZT13, thereby resembling the pattern observed in the dorsal striatum. As shown in Figure 6a, in rats killed within 24 h of the last morphine injection, PER2 expression at ZT13 (10 h after the last injection) was significantly increased ($t(4) = -5.07$, $p < 0.01$) whereas PER2 at ZT1 (22 h after the last injection) was significantly blunted ($t(4) = 4.97$, $p < 0.01$).

Figure 6b shows the pattern of PER2 expression in rats killed two days after the last daily injection at ZT3-4. It can be seen that in rats killed following the last morphine injection (morphine WD-2 + sal), PER2 at ZT1 (46 h after the last injection) was blunted ($t(4) = 3.03$, $p < 0.05$). Quinpirole treatment following the last morphine injection (morphine WD-2 +Q) had no effect on this decrease in PER2 expression at ZT1. Clonidine (morphine WD-2 + CL), however, restored and induced a new pattern of PER2 expression in rats previously injected with morphine, such that PER2 was significantly increased at ZT13 ($t(4) = -5.16$, $p < 0.01$).

The effect of quinpirole and clonidine treatments in saline-injected rats is shown in Figure 6c. Quinpirole itself (saline WD-2 + Q) significantly suppressed the normal peak of PER2 expression at ZT1 ($t(4) = 7.55$, $p < 0.01$). Clonidine (saline WD-2 + CL) did not affect PER2 expression at ZT1, but increased PER2 expression at ZT13 ($t(4) = -4.60$, $p < 0.05$). Thus, these results indicate that daily morphine injection and withdrawal blunts the normal rise in PER2 in the BLA. Quinpirole treatment following withdrawal does not restore a normal pattern of PER2 expression, whereas clonidine establishes a new pattern of PER2 expression in withdrawal. Each of these two treatments also significantly disrupts the normal pattern of PER2 in saline-injected rats.

DG

The daily pattern of PER2 expression in the DG peaks near ZT1 and falls to a trough level near ZT13, and is thus in phase with the patterns in the BLA and dorsal striatum. As shown in Figure 7a, in rats killed within 24 h of the last morphine injection PER2 expression at ZT1 (22 h after the last injection) was significantly blunted ($t(4) = 3.27$, $p < 0.01$).

Figure 7b shows the pattern of PER2 expression in rats killed 2 days after the last daily injection at ZT3-4. It can be seen that in rats killed following the withdrawal of morphine (morphine WD-2 + sal), PER2 expression at ZT1 (46 h after the last injection) was blunted ($t(4) = 7.41$, $p < 0.01$). The effect of quinpirole or clonidine following morphine withdrawal is also shown in Figure 7b. In rats given quinpirole in the evening following the last morphine injection (morphine WD-2 + Q), PER2 expression was increased at both ZT1 ($t(4) = -2.78$, $p = 0.05$) and ZT13 ($t(4) = -3.58$, $p < 0.05$). Clonidine treatment (morphine WD-2 + CL) following morphine withdrawal induced a

new pattern of PER2 expression, whereby PER2 was increased at ZT13 ($t(4) = -13.39$, $p < 0.01$).

Figure 7c shows the effects of quinpirole and clonidine alone in saline-injected rats. Quinpirole itself (saline WD-2 + Q) significantly blunted the normal peak of PER2 expression at ZT1 ($t(4) = 7.67$, $p < 0.01$). Clonidine (saline WD-2 + CL) appeared to disrupt the normal pattern of PER2, although these effects were not significant.

DISCUSSION

The present findings show that in rats, housed in 12:12 LD, scheduled daily injections of morphine given early in the light phase disrupt the normal circadian pattern of wheel-running activity, such that running in the dark phase (beginning 9 h after the daily injections) is completely suppressed at the higher doses of morphine. Daily morphine injections and their subsequent withdrawal also markedly disrupt the daily pattern of PER2 expression in several regions of the limbic forebrain including dorsal striatum, BNSTov, CEA, BLA, and DG without affecting the PER2 rhythm in the SCN, itself. Injections of the D2/3 DA agonist, quinpirole, following withdrawal of morphine restored normal patterns of PER2 in the BNSTov and CEA, whereas injections of the alpha 2 adrenergic agonist, clonidine, restored PER2 expression in the dorsal striatum, BLA, and DG.

Nighttime wheel-running activity

To our knowledge, the pronounced effect of daily morphine injections on wheel-running activity during the dark phase has not been reported previously. Other studies using different methods of drug delivery and different light schedules have shown that

opiate receptor agonists can alter circadian activity rhythms, for example by phase shifting the rhythm of wheel running in mice housed in constant dim light (Marchant and Mistlberger, 1995; Vansteensel *et al*, 2005). Interestingly, we have given daily injections of morphine (5 – 40 mg/kg) in the middle of the inactive phase to rats housed in constant darkness (DD) and have not observed any shift in the phase of the wheel-running rhythm (unpublished observations). Consistent with the present findings under 12:12 LD conditions, however, in these rats housed in DD the amplitude of wheel running during the subjective night was reduced.

One possible explanation for the reduction in nighttime wheel-running activity could be the development of withdrawal symptoms over the 24 h following the morning morphine injection. Indeed, nighttime wheel running was significantly decreased only when the higher doses of morphine were given and remained suppressed the day after the last morphine injection was given. Withdrawal symptoms have been reported to emerge relatively rapidly (within 14 h after a final injection) in rats given twice-daily morphine injections over one week (Li *et al*, 2009a). Furthermore, spontaneous withdrawal from chronic morphine treatment, delivered via subcutaneous pellet or twice-daily injections, has been found to diminish nighttime locomotor activity in rats (Georges *et al*, 1999; Stinus *et al*, 1998). Given that our injections were made eight to nine hours before lights were turned off, the emergence of some withdrawal symptoms over the course of the dark period could have interfered with the ability or motivation to run.

An alternative hypothesis is that the effect of morphine on nighttime wheel running is associated with its effects on the daily pattern of PER2 expression in the dorsal striatum. As shown in Figures 3a and 3b, the normal peak of PER2 expression in

the dorsal striatum was suppressed both in rats killed within 24 h after the last morphine injection and 2 days after the last injection. Similarly, injections of quinpirole in saline-treated rats were found to both suppress nighttime activity in the wheel (Figure 1c) and blunt the normal peak of PER2 at ZT1 in the striatum (Figure 3c). Given the prominent role of the dorsal striatum in the regulation of movement, it is possible that rhythmic clock gene expression in this region could be functionally related to the circadian modulation of locomotor activity. Consistent with this, we have found that by one week after the last morphine injection, both a high level of wheel-running activity in the dark phase and the normal daily pattern of PER2 in the dorsal striatum are re-established (unpublished observations). Additional evidence from the literature lends support to this argument. For example, Masubuchi and colleagues (2000) have shown that in rats housed on a 12:12 LD cycle and given ad libitum access to methamphetamine (in the drinking water), rhythms of wheel-running activity and clock gene expression in the striatum gradually desynchronize from the LD schedule and from clock gene expression in the SCN, but remain in phase with each other.

Effects of morphine on daytime wheel-running activity

As noted above, several reports in the literature suggest that rats develop anticipatory locomotor activity to a predictable daily injection of a drug of abuse, such as methamphetamine and nicotine (Iijima *et al*, 2002; Kosobud *et al*, 2007; Shibata *et al*, 1994), but not to other drugs that lack abuse potential, such as haloperidol (Kosobud *et al*, 2007). This phenomenon has been compared to the food anticipatory activity exhibited by rats maintained on a daytime restricted feeding schedule. In the present

study, however, rats receiving a daily injection of morphine showed no evidence of increased wheel running during the 3 h preceding the daily injection. Thus, these findings do not support the claim that a daily injection of an abused drug entrains a bout of increased wheel-running activity.

It is possible, however, that morphine-treated rats demonstrated signs of anticipation of the daily morphine injection in rhythmic functions other than wheel running that were not monitored in the present study. For example, Eikelboom and Stewart (1979) reported that in rats given injections of morphine at the same time of day over many days, body temperature decreased in a 1-h period in advance of the injection. This hypothermia was found to occur whether or not rats were tested in the same environment that predicted drug injections, suggesting that the decrease in temperature was mediated via a circadian mechanism.

Effect of morphine withdrawal on PER2 expression

Our findings reveal that morphine withdrawal profoundly affects the daily fluctuation of PER2 expression in several limbic forebrain regions without disrupting the rhythm of PER2 expression in the SCN. This latter finding in the SCN is consistent with previous findings showing that rhythmic activity in the adult rat SCN is unresponsive to exposure to drugs of abuse (Masubuchi *et al*, 2000), and to manipulations of catecholamine levels in the forebrain (Hood *et al*, 2010).

In contrast, however, in each of the forebrain regions examined, the normal daily peak of PER2 expression was suppressed in rats killed 2 days after the withdrawal of morphine. Although the specific mechanisms underlying this suppression remain unclear, changes in the normal daily rhythms of extracellular catecholamine levels, and

specifically DA, as a result of the daily morphine injections could play a role in this effect. We have demonstrated previously that the rhythm of extracellular DA in the striatum, which rises during the dark or active phase (Castaneda *et al*, 2004; Paulson and Robinson, 1994), regulates the daily PER2 pattern in this region (Hood *et al*, 2010). Loss of normal DA tone blunts the normal peak of PER2 in the striatum, whereas timed stimulation of D2 receptors using injections of quinpirole entrains a new pattern of PER2 expression. Given that acute injections of morphine, and several other abused drugs, stimulate release of DA in terminal regions of the mesolimbic system, whereas withdrawal of morphine is associated with a reduction in basal DA levels in several regions including the striatum (Pothos *et al*, 1991; Rossetti *et al*, 1992) and amygdala (Tran-Nguyen *et al*, 1998), it is likely that the present schedule of morphine injections in the light phase and their subsequent termination disrupted the normal rhythm of extracellular DA.

In support of the proposal that changes in DA signaling mediate the observed changes in PER2 expression, we found that injections of two different drugs known to modulate the activity of midbrain DA systems could restore a daily fluctuation in PER2 following morphine withdrawal. Injections of the D2/3 DA agonist, quinpirole, have been found to alleviate several somatic symptoms of opiate withdrawal in rats such as wet dog shakes and teeth chattering (Harris *et al*, 1994). Here, we found that quinpirole restored PER2 expression in the BNSTov and CEA, and more modestly increased PER2 around the time of its injection (ZT13) in the striatum and DG. Interestingly, quinpirole blunted the normal peak of PER2 expression at ZT1 in the dorsal striatum, BLA, and DG in rats previously given daily injections of saline. Although not directly

tested here, this effect in the dorsal striatum is likely attributable to the inhibitory effects of quinpirole at low doses on the activity of DA neurons via activation of D2 autoreceptors (Jeziorski and White, 1989; Lacey *et al*, 1987). In the present study, quinpirole was injected at the beginning of the dark phase, when extracellular DA levels are normally rising; as such, one likely effect of quinpirole given at this time in morphine-naïve rats is a reduction in evening DA levels and, in turn, a blunting of the normal peak of PER2 in these regions.

Injections of the alpha 2 adrenergic agonist, clonidine, have also been demonstrated to modulate the activity of midbrain DA neurons in opiate withdrawal (Pothos *et al*, 1991). In rats, clonidine injections increase DA levels in terminal regions of the mesolimbic system during opiate withdrawal by inhibiting a noradrenaline-mediated suppression of midbrain DA neuron activity (Dumont *et al*, 2004). Here, we found that evening injections of clonidine restored and entrained a new pattern of PER2 expression in the striatum, BNSTov, BLA, and DG following morphine withdrawal. These results suggest that administration of drugs that stimulate DA signaling at the beginning of the dark phase, when dopamine levels would normally increase in terminal regions of the mesolimbic dopamine system, can reverse the loss of PER2 expression in morphine withdrawal. Given that quinpirole and clonidine had differential effects on PER2 expression according to the forebrain region examined, however, additional mechanisms unique to each treatment and perhaps to each region are likely contribute to the pattern of results obtained here.

The effects of morphine and withdrawal on catecholamine signaling are not limited to DA, however, and thus the changes in PER2 expression observed in

morphine-treated rats could also be mediated by changes in other neurotransmitters, such as noradrenaline. Noradrenaline has been shown previously to modulate clock gene expression in the brain and periphery (Simonneaux *et al*, 2004; Terazono *et al*, 2003; Warnecke *et al*, 2005). In the present study we saw that clonidine injections given to saline-treated rats had effects on patterns of PER2 in some regions of the brain that were different from the effects of the direct DA agonist.

Finally, it is interesting to speculate on the relation between the disturbances in rhythms of clock protein expression in the limbic forebrain and the physiological and motivational symptoms associated with opiate withdrawal. For example, loss of normal rhythmic activity in the BNSTov, CEA, and DG might contribute to the disrupted corticosterone rhythm following morphine withdrawal, as these regions play important roles in the regulation of HPA axis activity (Forray and Gysling, 2004; Herman and Cullinan, 1997; Xu *et al*, 1999). Disturbances in motivational states, including dysphoria and anxiety, are also frequently reported during withdrawal from chronic opiate use (Wikler, 1973). Given that each of the forebrain regions examined in the present study is implicated in the regulation of motivated behavior, the disruption of normal clock activity (and presumably normal cellular functioning) in these tissues as a result of morphine withdrawal might contribute to these aversive motivational states.

CONCLUSION

Taken together, our results show dramatic effects of daily morphine injections, given in the light phase, on circadian rhythms of wheel running and expression of PER2 in the limbic forebrain. Morphine-treated rats exhibit profound suppression of normal

nighttime wheel-running activity and major changes in daily patterns of PER2 expression in the dorsal striatum, BNSTov, CEA, BLA, and DG, although no effects were seen on the pattern of PER2 expression in the SCN. Administration of quinpirole or clonidine following the last morphine injection restored a daily fluctuation in PER2 expression in these forebrain nuclei, although the effects of each drug varied as a function of region. Together, these findings implicate disruptions in normal daily patterns of clock protein expression in the circadian features of opiate withdrawal. Furthermore, they suggest that treatments known to alleviate opiate withdrawal symptoms might do so in part by modulating clock protein expression patterns in the limbic forebrain.

Disclosure/Conflict of Interest

The authors have no conflicts of interest to disclose.

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Figure Captions

Figure 1. (a) Wheel-running activity in the 12-h light (open bars) and 12-h dark (filled bars) phases by rats given a daily injection of either saline or morphine at ZT3-4. Bars represent the average (± SEM) of wheel running in each phase over the 12 days of injections. (b) Mean wheel-running activity (± SEM) in the light and dark phases on the day after the final saline or morphine injection at ZT3-4. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean wheel running (± SEM) in the light and dark phases by rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

Figure 2. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the SCN. (a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c)

Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

Figure 3. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the dorsal striatum. (a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

Figure 4. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the BNSTov. (a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the

evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

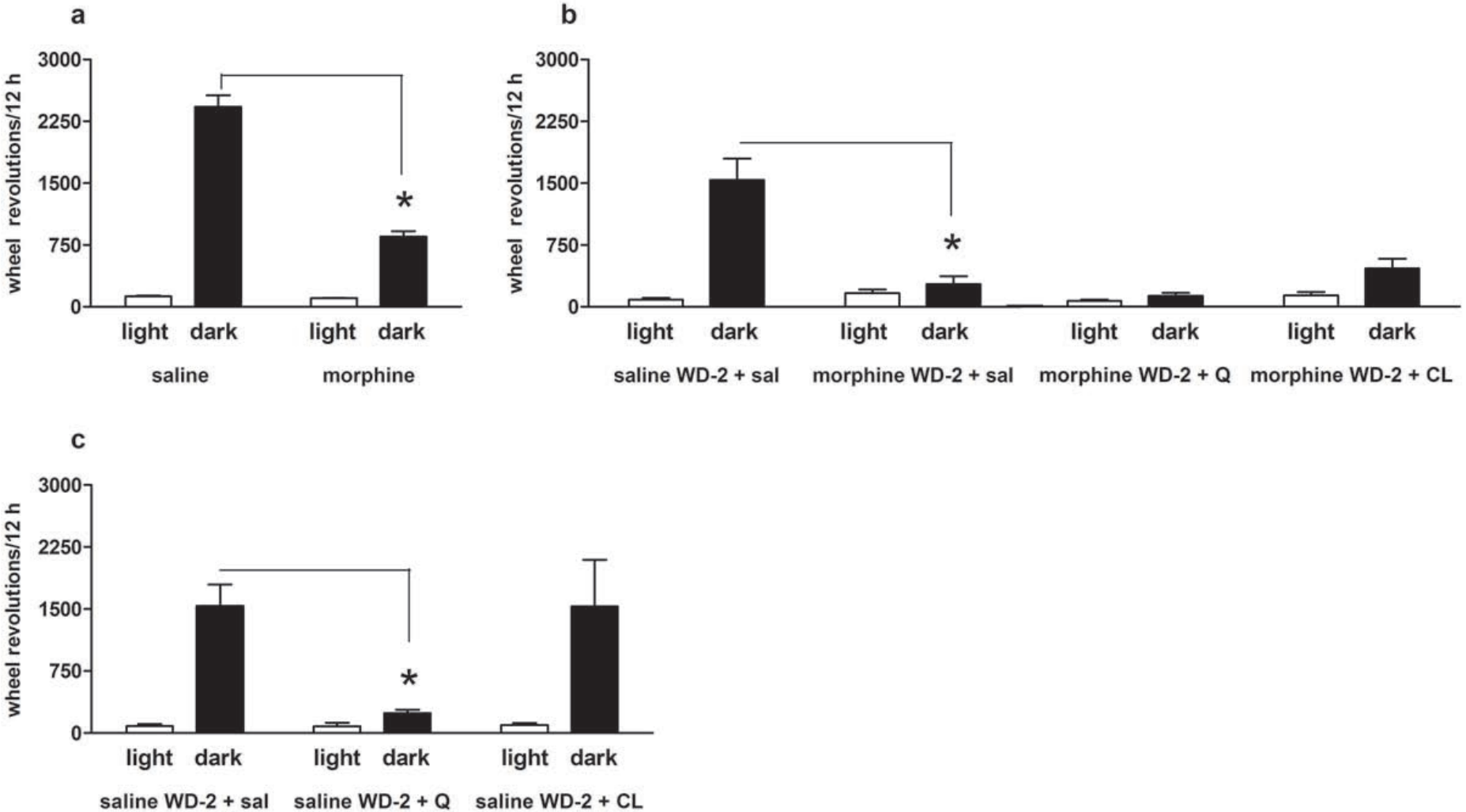
Figure 5. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the CEA.
(a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an

injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

Figure 6. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the BLA.
(a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.

Figure 7. Daily fluctuation of PER2-immunoreactive (IR) nuclei in the DG.
(a) Mean PER2-IR nuclei (± SEM) in rats killed within 24 h after the final daily saline (saline WD-1) or morphine (morphine WD-1) injection at ZT3-4. Rats were killed either in the evening after the last injection at ZT13 (10 h after the injection, filled bars) or the following morning at ZT1 (22 h after the injection, open bars). (b) Mean PER2-IR nuclei (± SEM) at ZT1 or ZT13 in rats killed 2 days after the last daily saline or morphine

injection. At the beginning of the dark phase following the final daytime injection, rats received an injection of saline (saline WD-2 + sal; morphine WD-2 + sal), quinpirole (1 mg/kg) (morphine WD-2 + Q), or clonidine (0.1 mg/kg) (morphine WD-2 + CL). (c) Mean PER2-IR nuclei (± SEM) in rats previously injected with saline at ZT3-4. At the beginning of the dark phase following the last daytime saline injection, rats received an injection of saline (saline WD-2 + sal), quinpirole (saline WD-2 + Q), or clonidine (saline WD-2 + CL). * indicates a significant difference, $p < 0.05$.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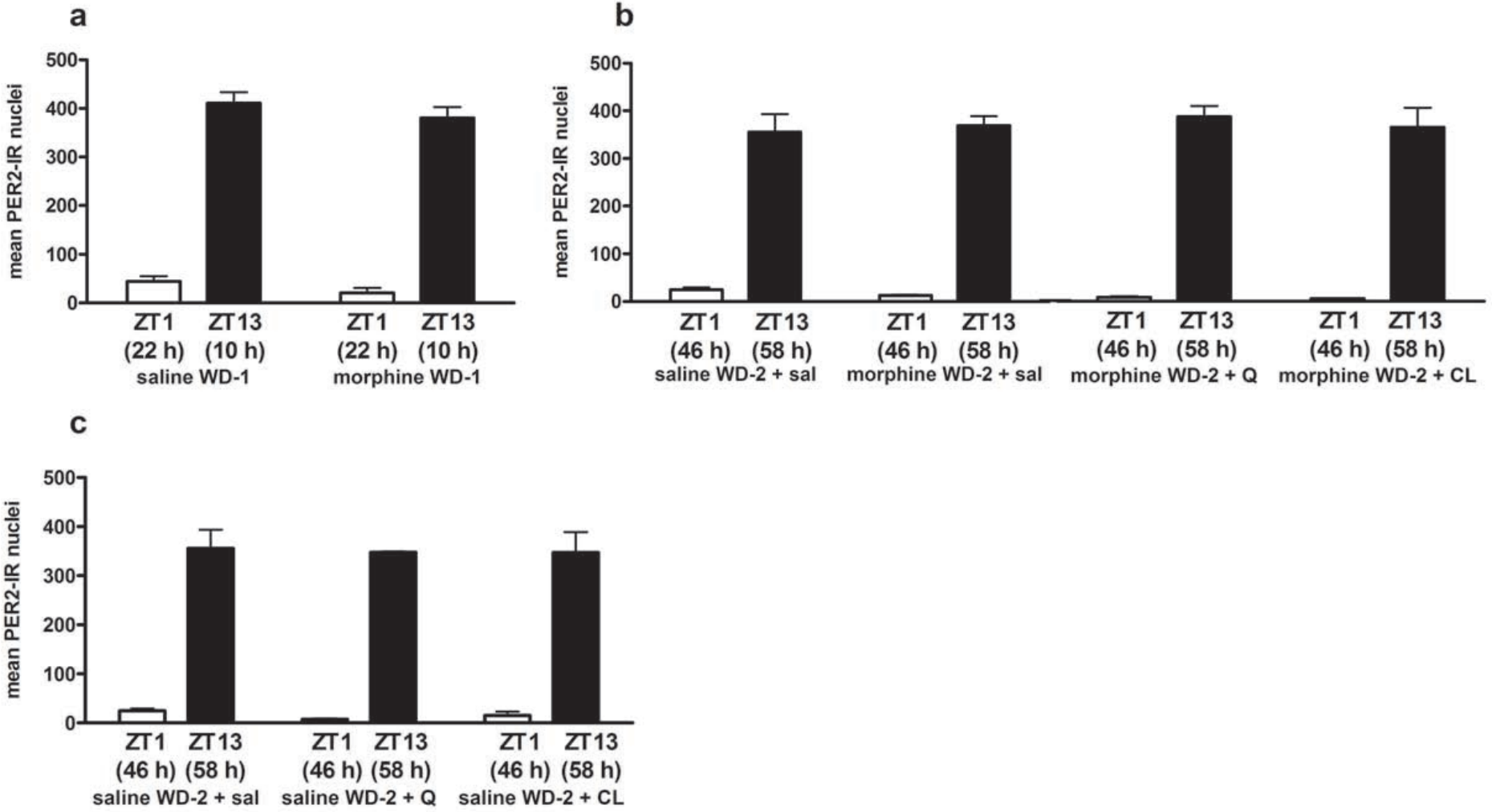
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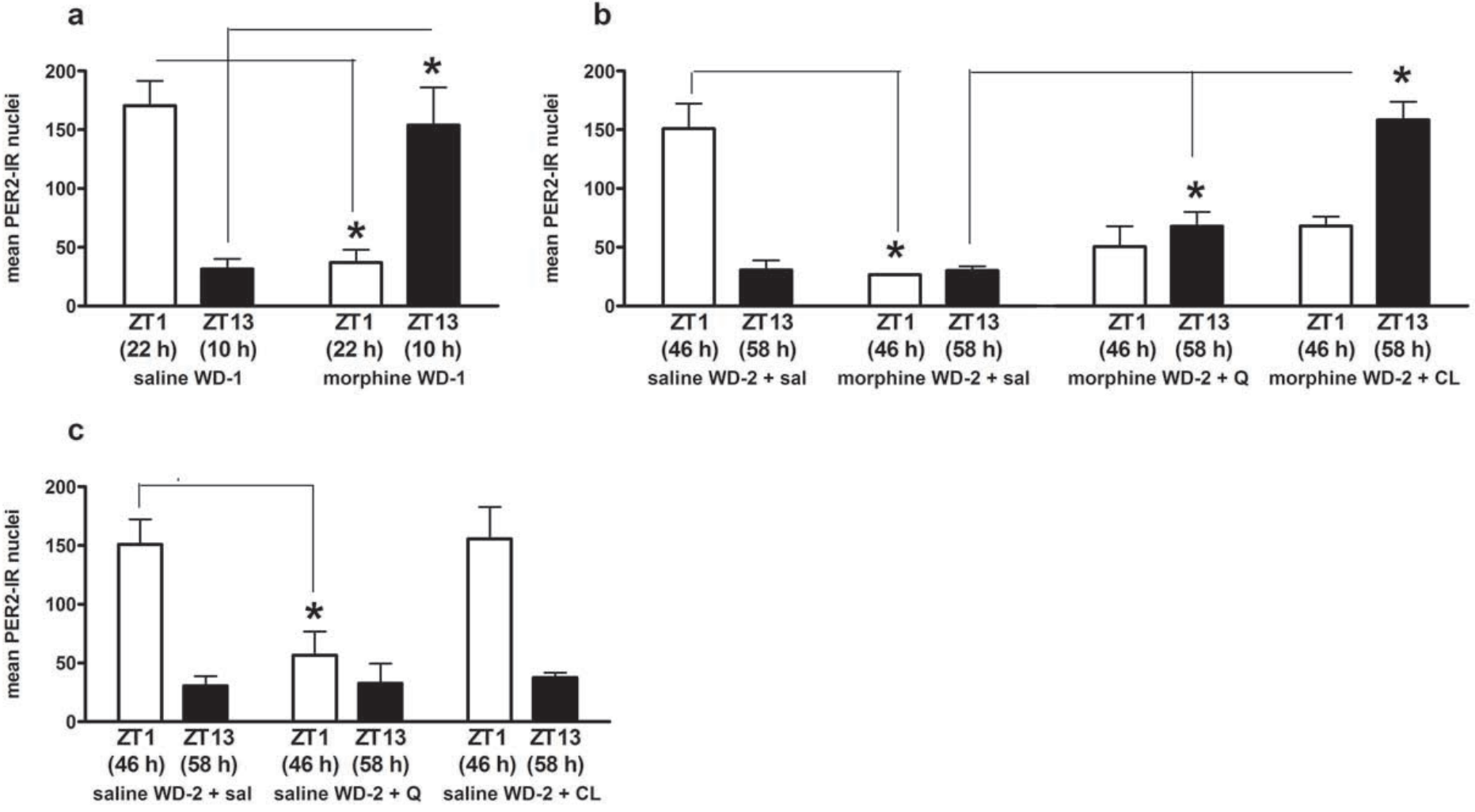
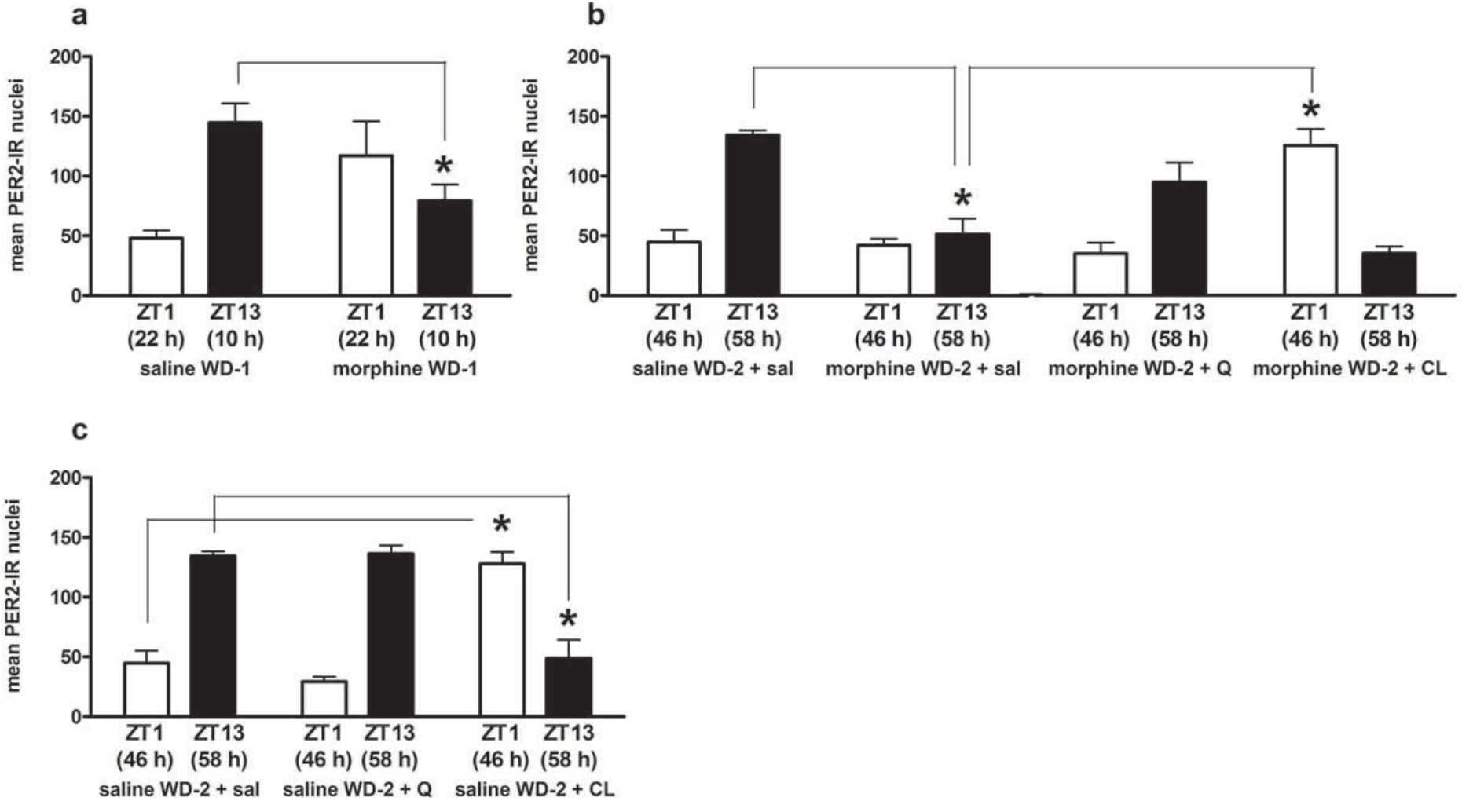
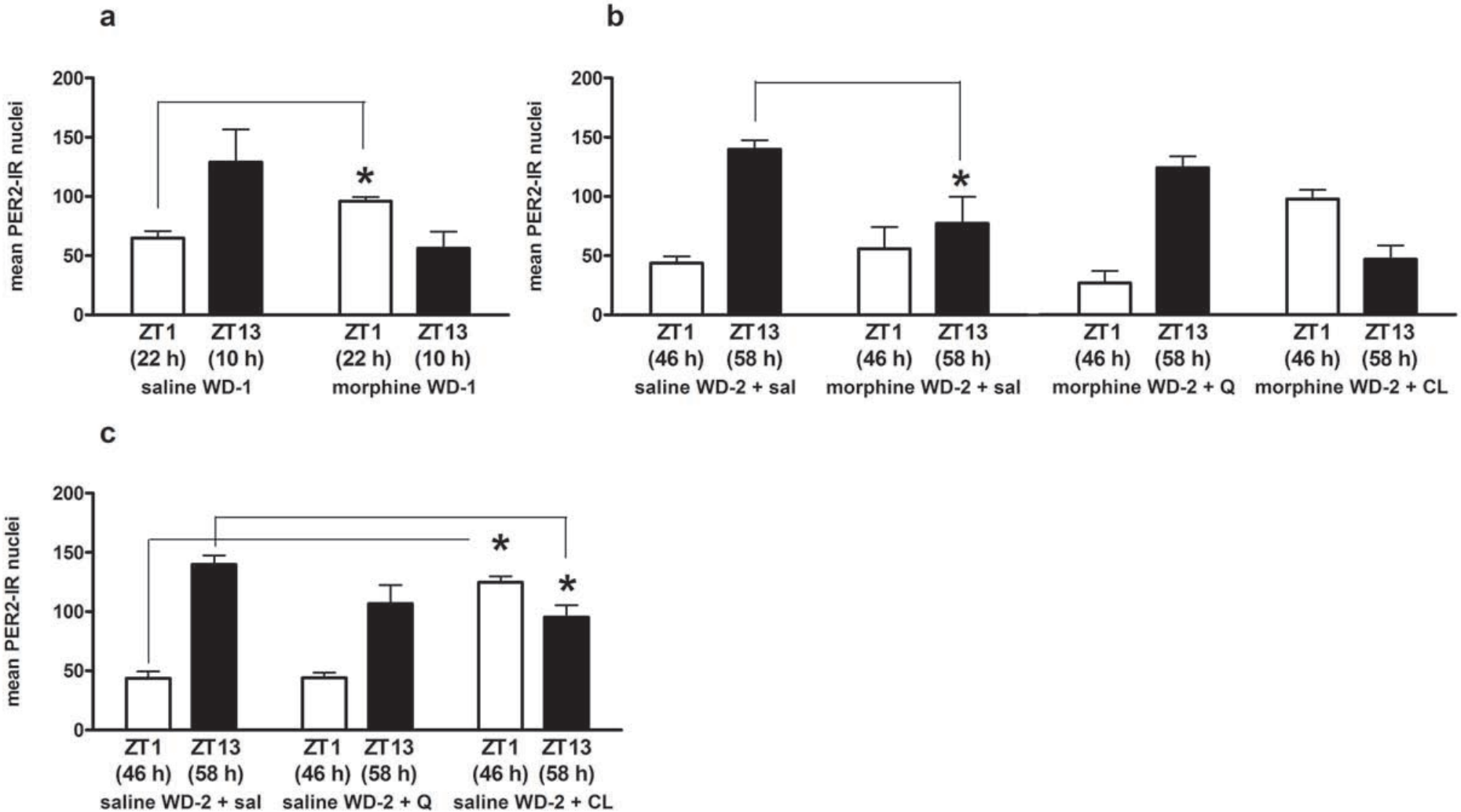
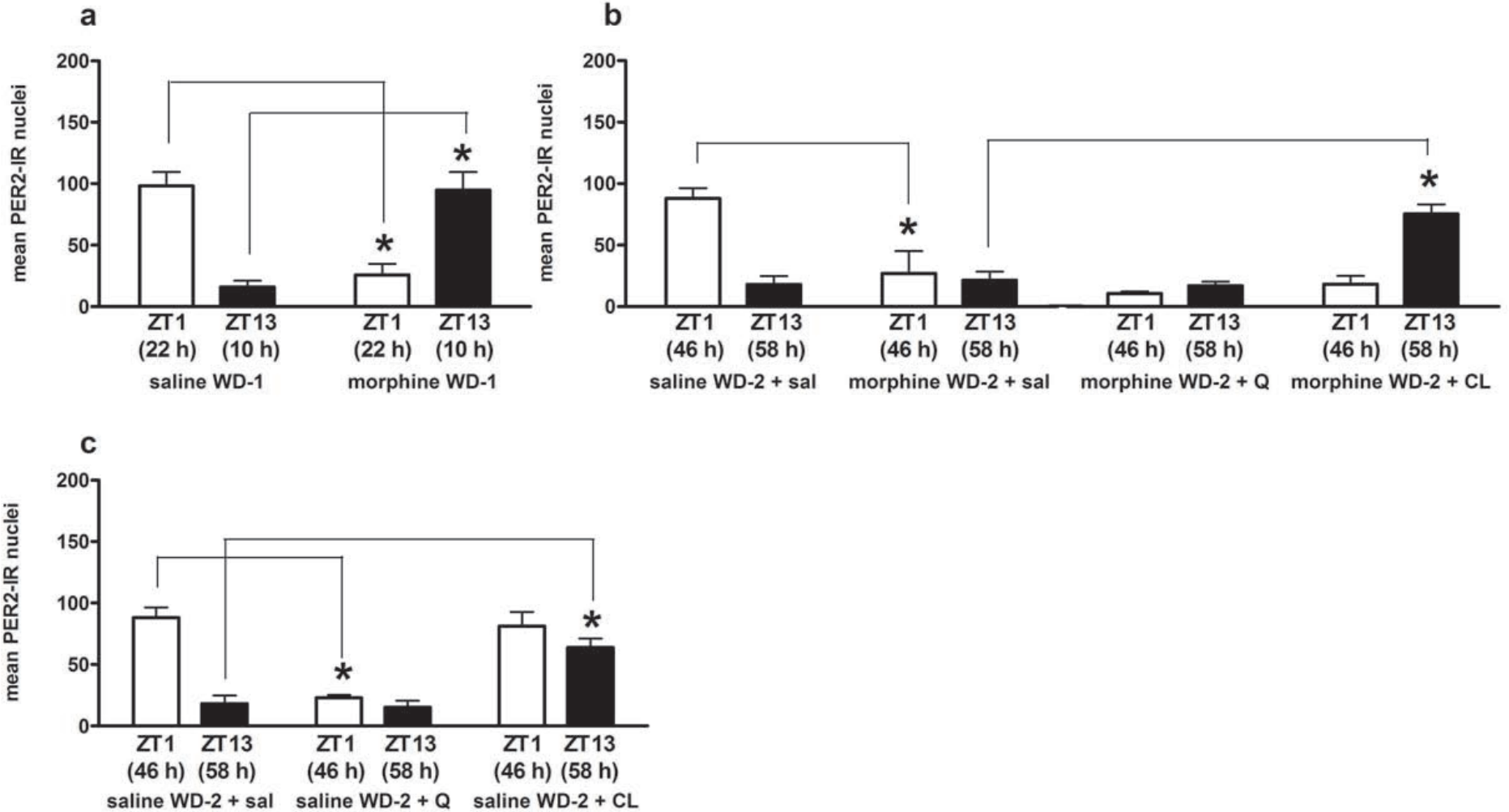
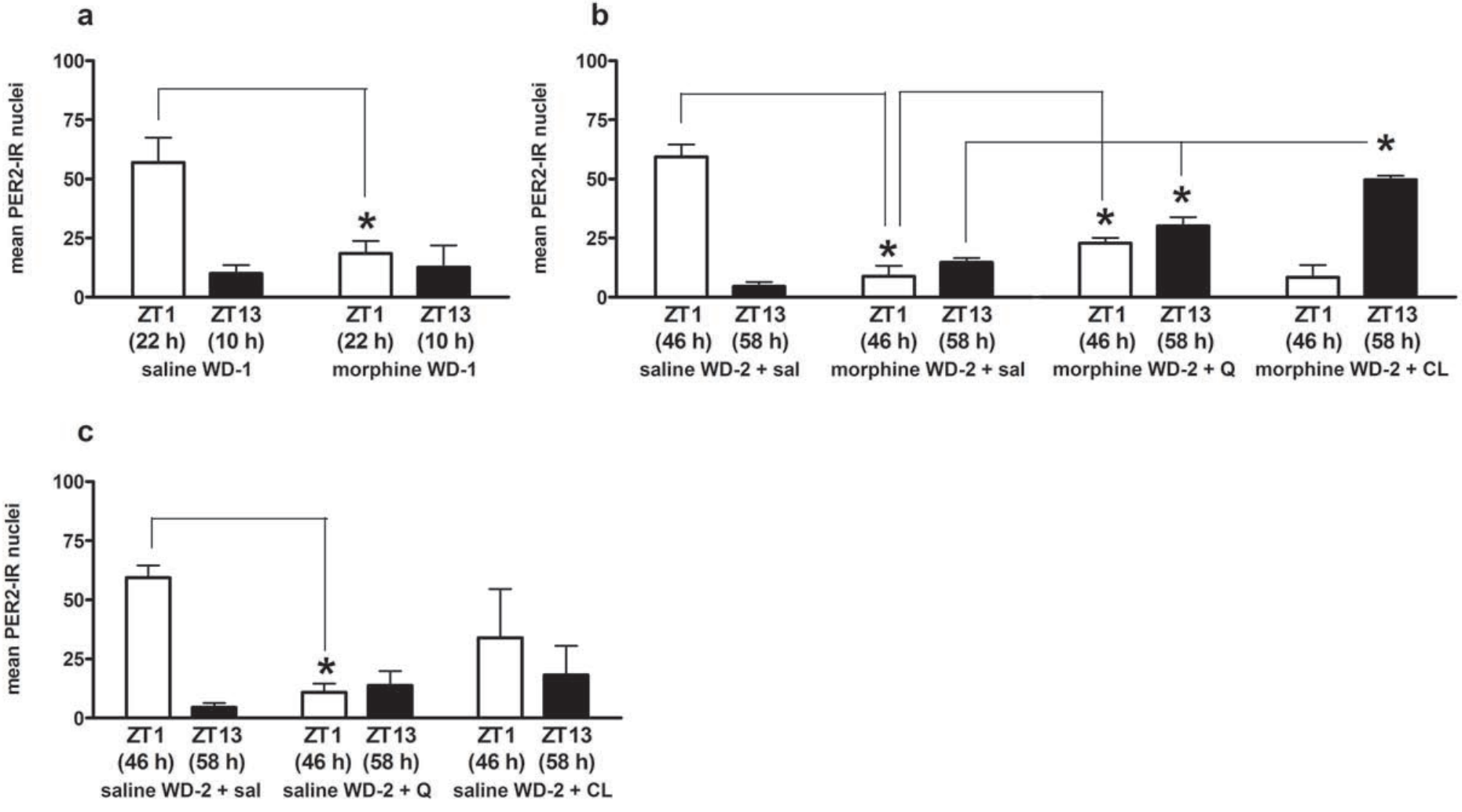
0
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
ZT1 (22 h)
ZT13 (10 h)
morphine WD-1
b
mean PER2-IR nuclei
500
400
300
200
100
0
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
ZT1 (46 h)
ZT13 (58 h)
morphine WD-2 + sal
ZT1 (46 h)
ZT13 (58 h)
morphine WD-2 + Q
ZT1 (46 h)
ZT13 (58 h)
morphine WD-2 + CL
c
mean PER2-IR nuclei
500
400
300
200
100
0
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + Q
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + CL


a
mean PER2-IR nuclei
200
150
100
50
0
*
*
ZT1 (22 h)
ZT13 (10 h)
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
morphine WD-1
b
mean PER2-IR nuclei
200
150
100
50
0
*
*
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
morphine WD-2 + sal
morphine WD-2 + Q
morphine WD-2 + CL
c
mean PER2-IR nuclei
200
150
100
50
0
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
saline WD-2 + Q
saline WD-2 + CL


a
b
c
mean PER2-IR nuclei
200
150
100
50
0
*
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
morphine WD-1
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
morphine WD-2 + sal
morphine WD-2 + Q
morphine WD-2 + CL
saline WD-2 + Q
saline WD-2 + CL


a
mean PER2-IR nuclei
200
150
100
50
0
*
ZT1 (22 h)
ZT13 (10 h)
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
morphine WD-1
b
mean PER2-IR nuclei
200
150
100
50
0
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
morphine WD-2 + sal
morphine WD-2 + Q
morphine WD-2 + CL
c
mean PER2-IR nuclei
200
150
100
50
0
*
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
saline WD-2 + Q
saline WD-2 + CL


a
mean PER2-IR nuclei
200
150
100
50
0
*
*
ZT1 (22 h)
ZT13 (10 h)
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
morphine WD-1
b
mean PER2-IR nuclei
200
150
100
50
0
*
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
morphine WD-2 + sal
morphine WD-2 + Q
morphine WD-2 + CL
c
mean PER2-IR nuclei
200
150
100
50
0
*
*
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
saline WD-2 + Q
saline WD-2 + CL


a
b
c
mean PER2-IR nuclei
100
75
50
25
0
*
ZT1 (22 h)
ZT13 (10 h)
ZT1 (22 h)
ZT13 (10 h)
saline WD-1
morphine WD-1
ZT1 (46 h)
ZT13 (58 h)
saline WD-2 + sal
morphine WD-2 + sal
morphine WD-2 + Q
morphine WD-2 + CL
saline WD-2 + Q
saline WD-2 + CL