Article

Hypothalamic A11 Nuclei Regulate the Circadian Rhythm of the Mechanonociception and the Spinal Clock Gene Transcription through Dopamine Receptor Activation

Celia Piña-Leyva[1,5], Manuel Lara-Lozano[1,5], Marina Rodríguez Sánchez[1], Guadalupe C. Vidal-Cantú[2], Erika Zavalza Barrientos[4], Ismael Jiménez-Estrada[1], Rodolfo Delgado-Lezama[1], Leonardo Rodríguez-Sosa[3], Vinicio Granados-Soto[2], Benjamín Florán-Garduño[1] and Juan Antonio González-Barrios[5,*]

[1] Department of Physiology, Biophysics, and Neurosciences, CINVESTAV, Av. No. 2508 National Polytechnic Institute, Mexico City, 06760, Mexico.
[2] Neurobiology of Pain Laboratory, Departamento de Farmacología, Cinvestav, Sede Sur, Mexico City, Mexico.
[3] Department of Physiology, Medicine Faculty, National Autonomous University of Mexico. University City. 04510 Mexico City, Mexico
[4] Doctorado en Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Iztapalapa. Mexico City, Mexico.
[5] Genomic Medicine Laboratory, Regional Hospital "October 1st", ISSSTE, Av. No. 1669 National Polytechnic Institute, Mexico City, 07760, Mexico
*Correspondence: jantgonzalez69@gmail.com; Tel.: +52-55-81077971

Abstract: Patients with degenerative diseases refer to feeling more pain during the night. However, it is unknown whether spinal nociception can be circadian and how is it controlled. We investigated whether the paw withdrawal threshold (PWT) could exhibit physiological circadian behavior as well as the contribution of the dopaminergic A11 nucleus and the spinal dopamine (DA) receptors (DRs) on the circadian PWT and the spinal clock gene transcription. Results revealed that control rats present a circadian PWT. Injecting 6-hydroxidopamine (6-OHDA) into the dopaminergic A11 nucleus reduced DA tissue content in the lumbar spinal cord, abolished the circadian PWT, induced allodynia, and reduced Period 1 and 2 (Per1 and 2), retinoid-related orphan receptor α (Rorα), Cryptochrome 1 (Cry1), and brain and muscle aryl-hydrocarbon receptor nuclear translocator-like protein (Bmal) mRNA. Likewise, administration of D1-like and D2-like DR antagonists blunted circadian PWT, producing allodynia, and altered the clock genes mRNA. In contrast, administration of D1-like or D2-like DR agonists blocked 6-OHDA-induced allodynia. This study shows that the spinal cord has physiological circadian PWT, which is modulated by the descending dopaminergic A11 through differential activation of the spinal DRs. Also, A11 nuclei and spinal DRs can regulate the clock gene transcription, which can likely modulate the circadian PWT.

Keywords: Pain; spinal cord; dopaminergic receptors; clock gene; dopaminergic A11 nucleus; paw withdrawal threshold

1. Introduction

Understanding nociception modulation is vital since chronic pain is a health problem around the world [1, 2]. Chronic pain is present in patients with diabetes [3, 4], cancer [5], and Parkinson´s disease [6], among others diseases. All these patients have desynchronization of circadian rhythms and refer to a differential intensity of pain throughout the day. Accordingly, there is evidence that mice and rats are slightly more sensitive to thermal stimuli during the afternoon/night than during the night/morning [7]. In contrast, other studies found that mice are more sensitive to a chemical stimulus (1% formalin test) during the night [8-10]. These facts suggest that the nociceptive process at the spinal cord could have a circadian rhythm. However, until now, it has been unknown if some nociceptive mechanism of the spinal cord has a circadian rhythm and how it is regulated.

Nociception is a complex process integrated by the primary afferent fibers, dorsal horn of the spinal cord, ascending and descending supraspinal pathways, and higher brain centers [2, 11-13]. In recent years, there has been an increased interest in analyzing the descending modulatory pathways of nociception at the spinal cord, particularly noradrenergic, serotonergic, and dopaminergic fibers [2, 14-18]. To date, it is well known that several regions of the spinal cord receive dopaminergic input from the hypothalamic dopaminergic A11 nucleus [2, 18-22]. Moreover, D1- and D2-like receptor families are present at the dorsal and ventral spinal cord [23]. Evidence indicates the participation of the A11 nuclei and the dopaminergic receptors (DRs) in nociception [20, 24-27]. There is also evidence supporting the participation of DRs on clock gene expression in retinal ganglion cells [28] and striatum primary cultures [29].

The clock genes are a family of transcription factors that regulate molecular, cellular, and physiological circadian function [30-32]. Moreover, these genes have been shown in different tissues [33-35]. However, the studies about the function and control of the clock genes are reduced. Until now, it is known that the clock genes, such as Period (Per1, Per2, and Per3), brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (Bmal), and Cryptochrome 1 and 2 (Cry1 and Cry2) follow a circadian rhythm in control rats, but this rhythmicity is suppressed after nerve injury [36]. However, the involved mechanisms are unknown.

This study used the evaluation of paw withdrawal threshold (PWT) using the von Frey hairs as a tool for the study of mechanisms of cutaneous stimulation-induced sensory input [37-39], allowing us to evaluate spinal cord nociception.

The purpose of this work was four-fold. The first aim was to demonstrate a circadian rhythm in the PWT in control rats. The second was to study the participation of descending dopaminergic A11 nuclei on the PWT. The next was to determine whether spinal DRs modulate circadian rhythm of the PWT in control rats and 6-hydroxydopamine (6-OHDA) into the A11 nucleus. Finally, we studied the participation of descending dopaminergic A11 nuclei and spinal DRs on spinal clock gene transcription.

2. Results

2.1. Circadian rhythm of PWT in control rats

To determine a possible circadian rhythm in the PWT of control rats, we used two groups of animals. One group of control rats was maintained in light–dark cycles (LD; 12 h–12 h), and the other was maintained in continuous dark (DD) for 15 days. This second group was necessary to determinate if the PWT has intrinsic circadian behavior. The evaluation of the PWT was carried out every 4 hours for 24 hours in both groups (the experiment started at 8:00 AM and finished at the same time the next day). The temporally data were evaluated with Chronos-Fit software to determinate the rhythmic parameters. The rhythmic parameters to the sinusoidal curve were mesor (midline estimating statistic of rhythm), amplitude (amplitude of sinewave), acrophase (time of the maximum value of the sine wave), and period (first-period length 20 h–28 h). Results indicated that rats in the LD group had PWT values of about 8 g at 8:00 AM. Interestingly, this value increased to about 19 g at 12:00 PM and after that decreased to reach the lowest level of PWT (about 7 g) at 12:00 AM. PWT then returned to a level similar to that obtained 24 h before (8:00 AM) (Fig. 1A). This experiment was repeated in two different laboratories in Mexico City, and the results were similar in the two laboratories. Further rhythm analysis of the time course of the PWT revealed a period of 28 h ($n=13$, $F_{6.94}=12.42$, $P<0.05$), an acrophase at 14.92 h, an amplitude of 3.21 g, and a mesor of 9.87 g (Fig. 1A).

The DD group of rats exhibited PWT values of about 5 g at 8:00 AM. PWT increased to about 10 g at 2:00 PM and after that decreased to reach the lowest level of PWT (about 4 g) at 4:00 AM. PWT then returned to a baseline similar (about 5 g) to that obtained 24 h before (at 8:00 AM). The rhythmic analysis of the time course of this group presented a period of 20 h ($n=8$, $F_{9.55}=43.14$, $P<0.05$), an acrophase at 13 h, an amplitude of 2.9 g, and a mesor of 5.8 g (Fig. 1B).

Both the LD and DD groups presented rhythmic behavior, but the analysis of the measured rhythmic parameters between the groups revealed that the DD group had a reduction of the mesor (36.5%, $F_{11.7}=9.58$, $P=0.0004$), acrophase (5.4%, $F_{7.12}=17.20$, $P=0.0285$), and period (24%, $F_{8.8}=1.361$, $P<0.0001$) with respect to the LD group of rats (Figs. 1 C-D). In contrast, the amplitude of the DD group was not different than that of the LD group (Figs. 1 C-D).

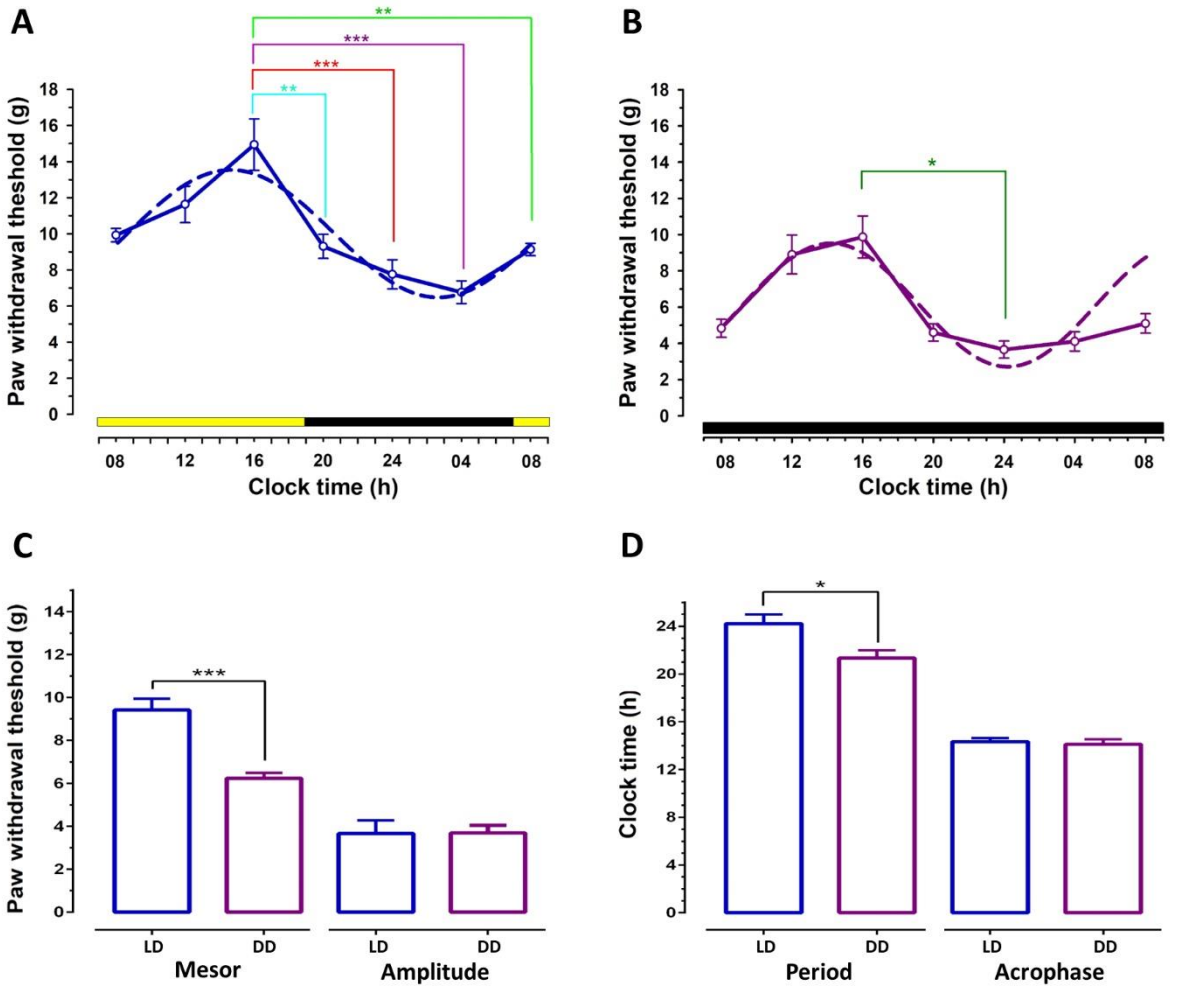


Figure 1. Time course of the paw withdrawal threshold (g). A) Light–Dark (LD) group, control rats maintained in light–dark cycles (12 h–12 h, n=13); B) DarkDark (DD) group, control rats maintained in continuous darkness condition (for 15 days, n=8); C) Mesor and amplitude rhythmic parameters of PWT of both LD and DD groups; D) Period and acrophase rhythmic parameters of PWT of both LD and DD groups. All rats were evaluated every 4 hours for 24 hours. Yellow and black bars represent the period of the day; continuous black bar represents constant darkness. The discontinuous line represents a sinusoid function. Data in all panels are presented as mean±SEM. In A and B, *P<0.05, ***P<0.001, by non-parametric one-way repeated measures ANOVA (Friedman test) followed by Dunn's test. In C and D, *P<0.05, ***P<0.001 by Student's t-test.

To determine a possible circadian rhythm in a chronic pain model, we evaluated the PWT of formalin-treated rats. We used a group of rats injected with 1% formalin. Animals were tested for tactile allodynia 6 days after injection. The evaluation of the PWT was carried out every 4 hours for 24 hours (experiment started at 8:00 AM and finished at the same time the next day). This group of rats was maintained in light–dark cycles (LD; 12 h–12 h). As expected, rats in the formalin-treated group demonstrated PWT values of about 4 g at 8:00 AM. This value increased to about 8 g at 4:00 PM and after that diminished to reach the lowest level of PWT (about 4 g) at 4:00 AM. PWT then returned to a baseline of about 6 g (8:00 AM) (Suppl Fig. 1). Also, the rhythmic analysis of this group had a rhythm PWT with a period of 24 h (n=8, $F_{6.94}$=198, P<0.05), an acrophase at 14 h, a PWT amplitude of 1.6 g, and a PWT mesor of 5.2 g (Suppl Fig. 1).

2.2. Effect of 6-OHDA on PWT of control rats

To determine whether the dopaminergic A11 nuclei participate by maintaining circadian PWT in control rats, we used three groups of animals to evaluate PWT: one control group of rats and two A11 lesion groups that were bilaterally injected with 6-OHDA (10 nM/μl) or vehicle. Previously we standardized the coordinates of the hypothalamic A11 nucleus (AP -3.6 mm, ML ± 0.6 mm, VL -7.5 mm) [40] (Fig. 2A). All groups were maintained in light–dark cycles (LD; 12 h–12 h). The evaluation of the PWT was carried out every 4 hours for 24 hour in all groups.

As an internal control, we evaluated the depletion of dopaminergic neurons of the A11 nucleus 8 days after injection of 6-OHDA. The injection of 6-OHDA into the A11 nucleus reduced the percent of positive tyrosine hydrolase (TH; n=3, 48%, $P<0.001$), compared with the control group (Fig. 2B). Likewise, we used four groups of animals—1) control group, sacrificed at 10:00 AM (n=4), 2) A11 lesion group, bilaterally injected with 6-OHDA and sacrificed at 10:00 AM (n=4); 3) control group, sacrificed at 10:00 PM (n=4); and 4) A11 lesion group, bilaterally injected with 6-OHDA and sacrificed at 10:00 PM (n=4)—to determinate whether administration of 6-OHDA reduces tissue content of DA at the lumbar region of the spinal cord. Administration of 6-OHDA into the A11 nuclei of the control rats reduced DA tissue content in the lumbar section of the spinal cord at both day (41.6%, $P<0.001$) and night (33.9%, $P<0.05$) compared to their respective controls (Fig. 2C).

Intrahypothalamic A11 nucleus injection of 6-OHDA but not vehicle (Suppl Fig. 2) reduced PWT to allodynic values (about 5 g) after 8 days of the 6-OHDA injection at 12:00 and 4:00 PM (n=9, $P<0.05$), compared with the control group (Fig. 2D). Moreover, intrahypothalamic injection of 6-OHDA abolished the circadian rhythm of PWT in the control rats (Fig. 2D).

We also determined whether intrathecal (IT) injection (between the L4 and L5 vertebrae) of 6-OHDA (10 nM/μl) in the lumbar spinal cord can alter the circadian PWT. We used two groups of rats: a) IT lesion group, with IT injection of 6-OHDA in the lumbar spinal cord; and b) A11 lesion + IT lesion group, with bilateral injection of 6-OHDA in A11 nucleus plus IT injection in the lumbar spinal cord. Both groups were maintained in light–dark cycles (LD; 12 h–12 h) and were evaluated for PWT 15 days after administration of 6-OHDA. The evaluation of PWT was carried out every 4 hours for 24 hours in each group.

The IT lesion and IT lesion + A11 lesion groups (Suppl Fig. 3), but not vehicle (Suppl Fig. 4), also diminished PWT to allodynic values (about 4 g) respect with the control group (n=11, $P<0.05$; n=5, $P<0.05$, respectively).

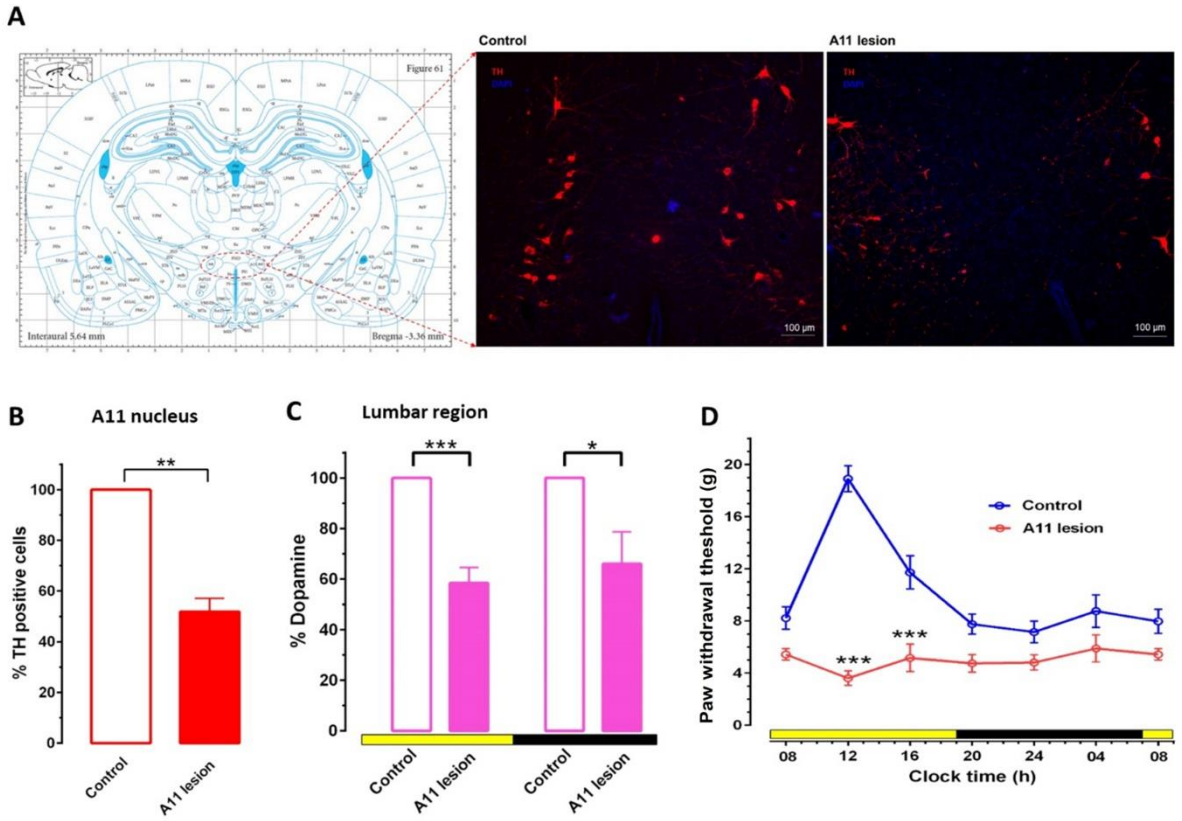


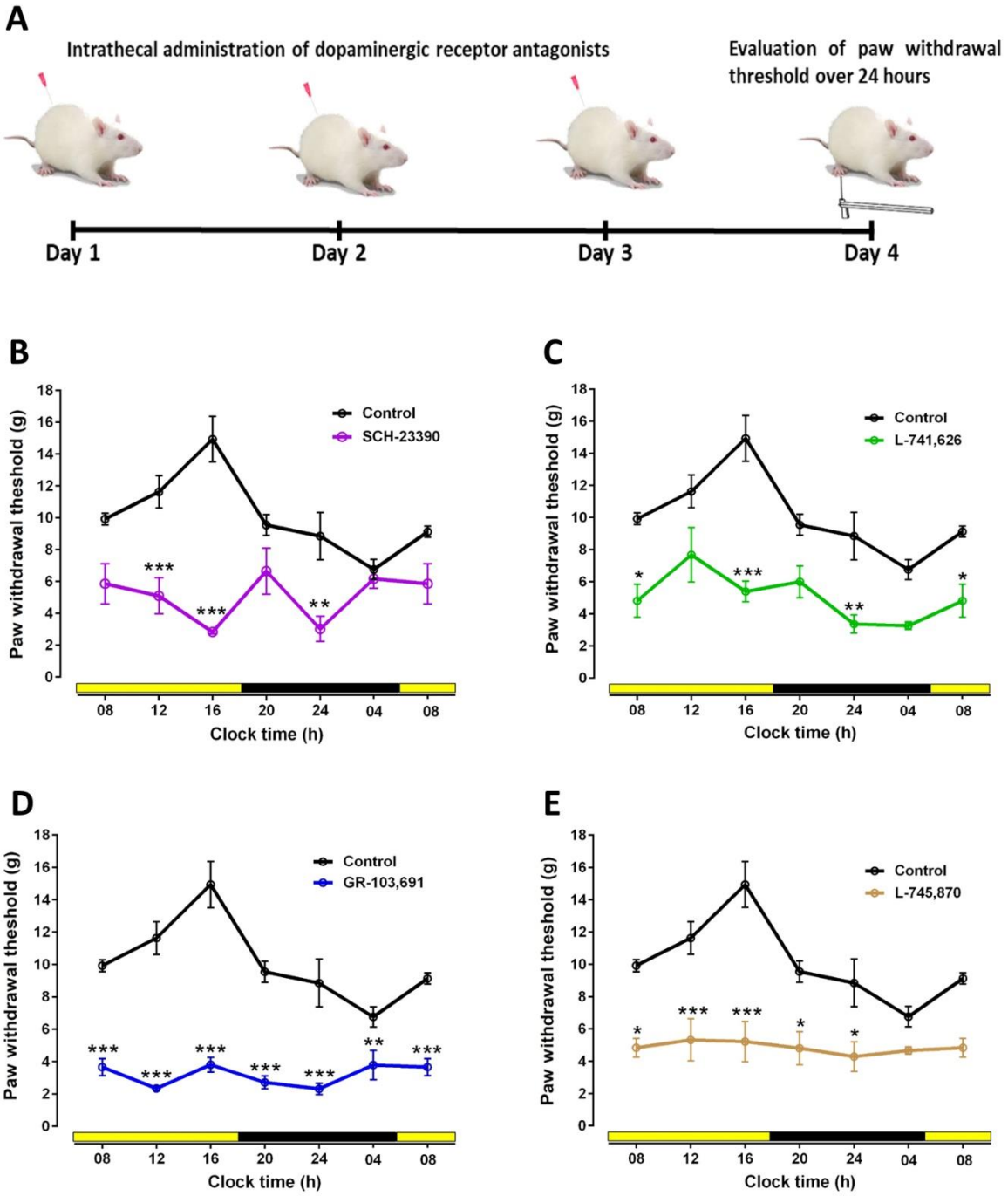
Figure 2. Effect of the administration bilateral of 6-OHDA (A11 lesion group) in the A11 nucleus. A) Localization of A11 nucleus of the atlas of Paxinos and Watson (left panel) and tyrosine hydroxylase (TH) immunoreactivity in the A11 nucleus in control rats (n=3) and A11 lesion rats (n=3, right panel) 8 days after injection of 6-OHDA; B) Quantification of the % TH-positive cells of the A11 nucleus in both control (n=3) and A11 lesion (n =3) groups 8 days after injection of 6-OHDA; C) Quantification of the tissue content of dopamine in the lumbar spinal cord, in both control (n=4) and A11 lesion (n=4) groups 8 days after injection of 6-OHDA. The samples were collected at day (10:00 AM) and night (10:00 PM); D) Effect of the administration of 6-OHDA (A11 lesion group) on the normal circadian PWT behavior was evaluated in both control (n=13) and A11 lesion (n=11) groups. All groups of rats were maintained in light–dark cycles (12 h–12 h). The yellow and dark bars show the period of the day. Data are the mean±SEM. In B and C, *P<0.05, ***P<0.001 by Student's t-test. In D, *P<0.05, by two-way ANOVA, followed by Dunnett's test, compared with the control group each hour.

2.3. Effect of intrathecal dopaminergic receptor antagonists on the circadian rhythm of the PWT in control rats

To assess the participation of spinal DRs in the modulation of circadian PWT in control rats, we used six groups of rats: 1) control group, 2) SCH-23390 group (D1/D5 receptor antagonist), 3) L-741,626 group (D2 receptor antagonist, 10 nM/5 µl), 4) GR-103,691 (D3 receptor antagonist, 10 nM/5 µl), 5) L-745,870 (D4 receptor antagonist, 10 nM/5 µl), and 6) vehicle group (injectable water, 5 µl). Animals (n=6, all groups) received a daily injection of each drug for three consecutive days; on the fourth day, the time course of PWT was evaluated every 4 hours for 24 hours (Fig. 3A). All drugs abolished the circadian rhythm (Figs. 3 B-E), as determined by the mean of all values of PWT in 24 hour of all antagonist groups. In support of this finding, the Cosinor model did not find a circadian rhythm. Moreover, all intrathecal DR antagonists reduced the PWT to values considered as tactile allodynia about 4 g (n=6, P>0.0001) compared to their respective control (Figs. 3 B-E) but was not affected the vehicle

202 group (Suppl. Fig. 4). Of note, the order of efficacy to induce tactile allodynia was
203 D3>D1/D5=D4>D2 (Suppl. Fig. 5).

204



205

206 **Figure 3**. Effect of the intrathecal injection of the dopaminergic receptor (DR) antagonists on circadian paw withdrawal
207 threshold (g). A) Experimental protocol of intrathecal administration of each DR antagonist separately injected; B) Effect
208 of the SCH-23390 (D1/D5 dopamine receptor antagonist); C) Effect of the L-741,626 (D2 dopamine receptor antagonist);
209 D) Effect of the GR-103,691 (D3 dopamine receptor antagonist); E) Effect of the L-745,870 (D4 dopamine receptor
210 antagonist). All rats were evaluated every 4 hours for 24 hours. Rats were maintained in light–dark cycles (12 h–12 h). The
211 yellow and dark bars show the period of the day. Data are the mean±SEM of 6 independents experiments. *P<0.05,
212 **P<0.001, ***P<0.0001, by two-way ANOVA, followed by Dunnett's test, compared with control group each hour.

213

214 *2.4. Effect of intrathecal dopaminergic receptor agonists on 6-OHDA-induced tactile allodynia*

Since the administration of 6-OHDA into the A11 nucleus induced tactile allodynia (8 days later) in control rats, we wished to understand the role of spinal DR in this effect. For this, we used the following groups: two control groups; one A11 lesion + SKF-38393 group, bilaterally injected with 6-OHDA (10 nM/μl) and, after 8 days, given one dose of SKF-38393 (D1 and D5 receptor agonist, 10 nM/5 μl); and one A11 lesion + quinpirole group, bilaterally injected with 6-OHDA (10 nM/μl), after 8 days, given one dose of quinpirole (D2, D3, and D4 receptor agonist, 10 nM/5 μl). Each agonist was intrathecally administered between the L4 and L5 vertebral space of the rats (Fig. 4A). Intrathecal administration of SKF-38393 and quinpirole enhanced the PWT (n=6, P<0.05 and n=6, P<0.05, respectively) abolished with the administration of 6-OHDA (n=6, P<0.05 and n=6, P<0.05, respectively), therefore SKF-38393 and quinpirole indicated antiallodynic effect (Figs. 4 B-C).

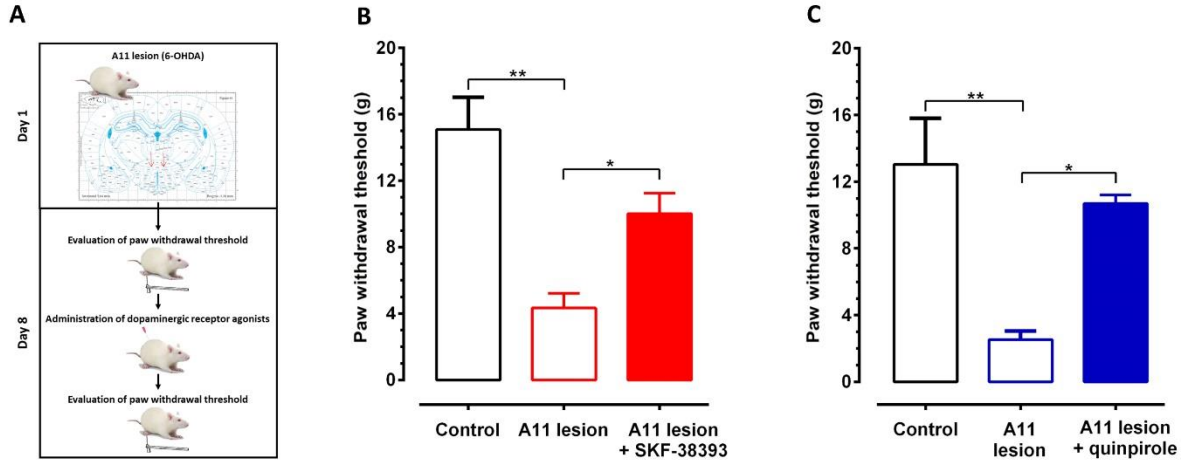


Figure 4. Effect of the intrathecal injection of the dopaminergic receptor (DR) agonists on 6-OHDA-induced tactile allodynia in rats. A) Experimental protocol of intrathecal administration of each DR agonist separately injected in the A11 lesioned rats; B) Effect of the SKF-38393 (D1/D5 dopamine receptor agonist); C) Effect of the quinpirole (D2, D3, and D4 dopamine receptor agonist). All rats were maintained in light–dark cycles (12 h–12 h). Data are presented as mean±SEM of 6 independent experiments. *P<0.05, **P<0.005, by one-way ANOVA, followed by the Tukey test.

2.5. Effect of 6-OHDA on the spinal clock genes

To evaluate whether 6-OHDA (10 nM/μl) injected into the A11 nucleus modulates the clock gene transcription in the spinal cord, rats were killed 8 days (at 10:00 AM and 10:00 PM) after 6-OHDA injection to harvest the lumbar spinal cord. The results indicated that 6-OHDA reduced Per1 and retinoid-related orphan receptor β (Rorβ) transcript levels during the day (54%, P<0.0001, and 23%, P=0.041, respectively) compared with the respective control. In contrast, 6-OHDA diminished Bmal (68%; P<0.0001), Rorα (70%, P<0.0001), Per1 (50%, P<0.0001), Per2 (50%, P=0.018), and Cry1 (58%, P<0.0001) mRNA levels at night (Fig. 5).

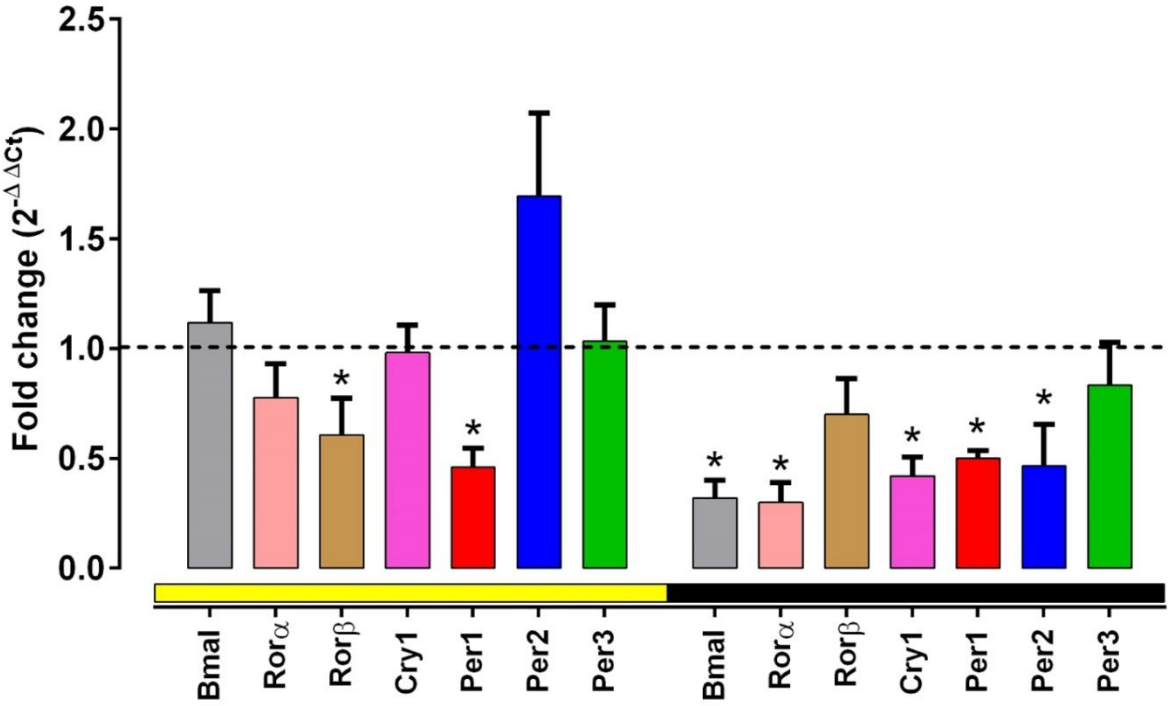


Figure 5. Effect of the intrathecal injection of 6-OHDA in the A11 nucleus on clock gene transcription of the lumbar spinal cord. Rats were maintained in light–dark cycles (12 h–12 h). The black discontinuous line represents the basal transcription for each gene in control conditions. The samples were collected from the lumbosacral spinal cord at day (10:00 AM) and night (10:00 PM). Data are presented as mean±SEM of 6 independent experiments. *P<0.05, by Student's t-test.

2.6. Effect of dopaminergic receptor antagonists on spinal clock genes in control rats

To determine the role of DA receptors on clock gene transcription in the lumbar spinal cord of control rats, we intrathecally injected individual specific dopaminergic antagonists (one daily injection for consecutive three days); then, on the fourth day, the control and with antagonist groups of animals were killed at 10:00 AM and other control and with-antagonist groups at 10:00 PM to obtain the lumbar spinal cord (Fig. 6A). Intrathecal administration of SCH-23390 (D1 and D5 receptor antagonist, 10 nM/5 µl) enhanced Bmal (25%, P=0.0082) and reduced Rorα (67%, P=0.00027) mRNA level at day (Fig. 6B). In contrast, it decreased Bmal (60%, P=0.0080), Rorα (70%, P=0.00075) and Cry2 (50%, P=0.0018) mRNA level and enhanced Rorβ (180%, P<0.0001) mRNA level expression at night (Fig. 6B). Spinal injection of L-741,626 (D2 receptor antagonist, 10 nM/5 µl) increased Bmal (100%, P=0.00012), Rorα (103%, P=0.0045), Rorβ (150%, P=0.0067), and Per2 (86%, P=0.0008) transcription at day (Fig. 6C). Moreover, it increased Bmal (500%, P<0.0001), Rorα (286%, P=0.00064), Rorβ (340%, P<0.001), Per2 (130%, P=0.0002), and Per3 (120%, P=0.002) at night (Fig. 6C). Intrathecal administration of GR-103,691 (D3 receptor antagonist, 10 nM/5 µl) increased Bmal (13%, P=0.0049) and decreased Rorα (40%, P=0.0089) and Per2 (33%, P=0.02) transcripts at day; it also decreased Rorα (73%, P=0.00017) mRNA transcription at night (Fig. 6D). Finally, the spinal administration of L-745,870 (DR4 receptor antagonist, 10 nM/5 µl) only increased Rorβ (62%, P=0.0024) transcript at day. Moreover, it increased Bmal (160%, P=0.0001), Rorβ (250%, P=0.0022), and Per3 (82%, P=0.021) mRNA levels and decreased Per1 (40%, P=0.0089) at night (Fig. 6E).

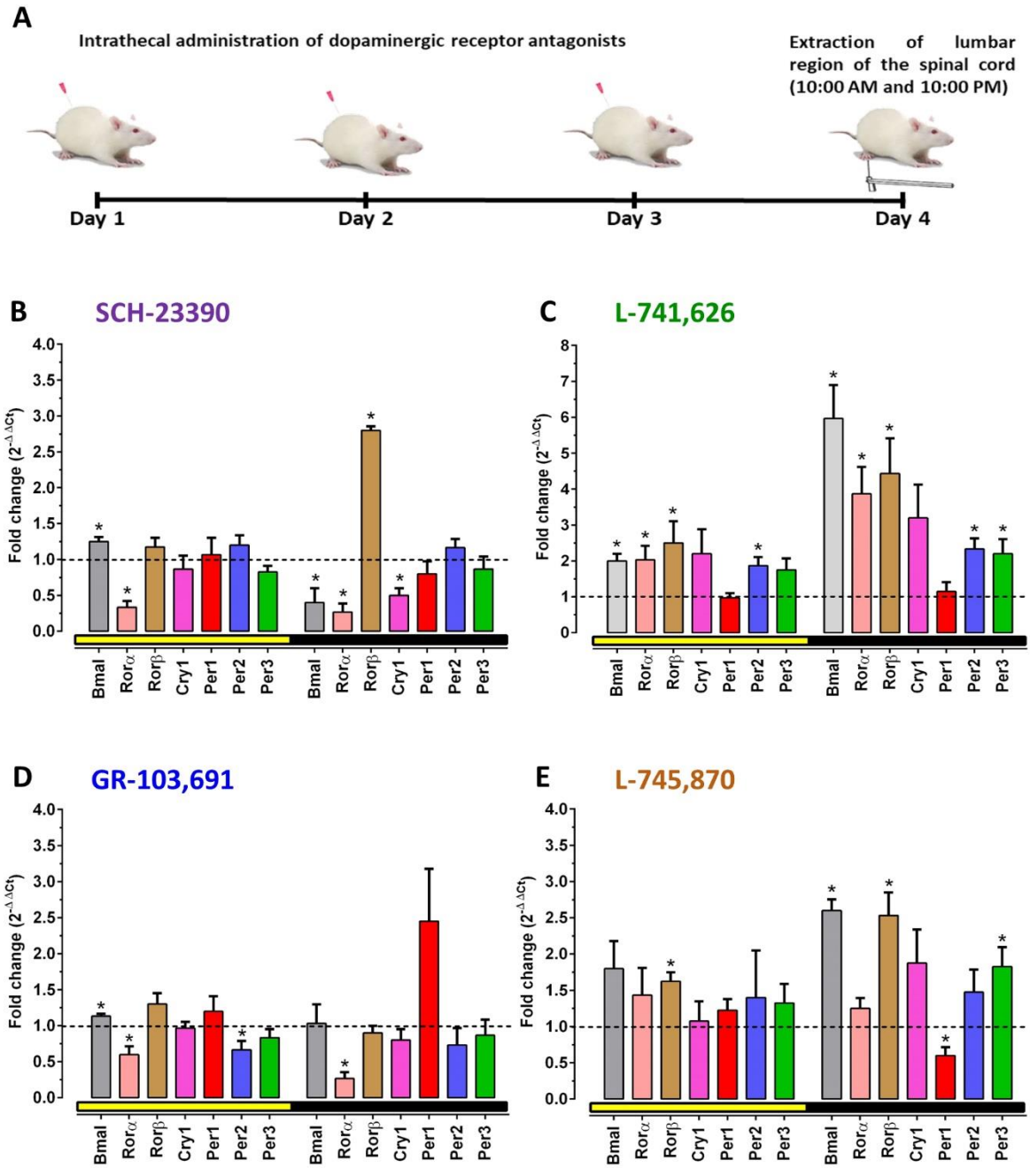


Figure 6. Effect of the intrathecal injection of dopaminergic receptor (DR) antagonists on clock gene transcription of the lumbar spinal cord. A) Experimental protocol of intrathecal administration of each DR antagonist separately injected; B) Effect of the SCH-23390 (D1/D5 dopamine receptor antagonist); C) Effect of the L-741,626 (D2 dopamine receptor antagonist); D) Effect of the GR-103,691 (D3 dopamine receptor antagonist); and E) Effect of the L-745,870 (D4 dopamine receptor antagonist). Rats were maintained in light–dark cycles (12 h–12 h). The black discontinuous line represents the basal transcription for each gene in control conditions. The samples were collected from the lumbar spinal cord at day (10:00 AM) and night (10:00 PM). Data are presented as mean±SEM of 4 independent experiments. *P<0.05, by Student's t-test.

3. Discussion

This study reveals the physiological rhythmic properties of the circadian pain behavior of the spinal cord. We demonstrated that the dopaminergic A11 nuclei modulated the physiological circadian PWT behavior through the differential

activity of the spinal DRs, and significantly, the A11 nuclei and spinal DRs have modulated the clock gene transcription at the spinal cord. Our data suggest that the spinal clock genes could be responsible for the circadian PWT.

3.1. Time course of PWT in control and formalin-treated rats

Our results reveal the existence of a circadian rhythm in PWT behavior. We found that rats are more sensitive to mechanical stimuli during the night and less sensitive during the day. Our data partially agree with a pioneer study revealing that mice are more sensitive to thermal stimuli during the night and morning (4:30 AM–11:30 AM) than during afternoon and night (3:30 PM–4:30 AM) [7]. In our conditions, control rats displayed a lower threshold to mechanical stimuli during the night period (8:00 PM–8:00 AM). Differences from the previous study may be due to the different protocols, environmental conditions, rodent species, and stimuli. However, in both studies, the higher sensitivity to thermal or mechanical stimuli was found around 4:00–8:00 AM. Likewise, others have reported more sensitivity to thermal or chemical (1% formalin) stimuli in mice during the night [9, 41, 42]. In support of this, the synaptic excitability of the hypothalamus is higher during the night than during the day [43]. Moreover, clinical studies have found that patients with osteoarthritis [44], diabetic [3] and postherpetic neuropathy [4] or Parkinson's [6] report more pain during the night.

It is important to note that in the animals under conditions of constant darkness, a circadian rhythm of the PWT was observed but with different rhythmic parameters, a finding that indicates that the circadian PWT behavior is inherent at the spinal cord. However, there is a dependency on the suprachiasmatic nucleus (SCN) (about 40%). This is a logical result because the organization of the biological systems in mammalians is mainly controlled by the SCN, which controls the peripheral clock gene present outside the SCN [31]. It has been shown that the peripheral clock gene have intrinsic activity; thus they are capable of independently controlling circadian activities [36, 45].

Further, our results indicate the permanence of a circadian rhythm in the formalin-treated rats. We found a response reduced circadian rhythm in the long-lasting mechanical hypersensitivity induced by formalin injected in rats. To our knowledge, this is the first report on the circadian rhythm in this model of pain. This result is consistent with a previous study finding that 1% formalin injection in mice induces higher acute (1 h) licking and flinching behaviors in the first and second phases of the test during the dark period (7:00–10:00 PM) [10]. Another study founded circadian expression response to intraplantar formalin injection, which was reduced through deletion on the clock gene [36]. Thus, data suggest that normal mechanical sensitivity follows a circadian rhythm that could be altered by conditions such as acute nociception and long-lasting hypersensitivity induced by formalin. Therefore, this circadian function may be regulated by intrinsic circadian components. Our data show that the spinal cord has a robust circadian rhythm of nociception-related behaviors.

329
330 *3.2. Effects of 6-OHDA on PWT of control rats*

331 Administration of 6-OHDA into the A11 nucleus abolished the circadian rhythm
332 of the PWT in control rats and reduced PWT to values that indicate tactile allodynia.
333 Moreover, lesion of the dopaminergic A11 neurons provoked a diminished tissue
334 content of DA in the lumbar spinal cord.
335 These results suggest that spinal DA coming from the A11 nucleus modulates
336 the circadian rhythm of the PWT. Since the lesion to the A11 nucleus leads to tactile
337 allodynia, data implies that spinal DA plays a tonic anti-nociceptive role at the spinal
338 cord. The reduction of spinal DA in our study is consistent with previous research
339 demonstrating that lesion of the A11 nucleus decreases DA and DR1, DR2, and DR3
340 proteins at the spinal cord [46]. Taken together, data suggest that the A11 nucleus
341 supplies DA to the spinal cord, which in turn activates DA receptors in the lumbar
342 spinal cord, which maintains a tonic antinociceptive effect.
343 Our data agree with previous observations that damage to the A11 nucleus
344 induces nociception in restless leg syndrome [47] and migraines [20]. Also, there is
345 evidence that exogenous DA, administered into the substantia gelatinosa, displays
346 inhibitory effects on noxious and innocuous stimuli to the skin, which was mimicked
347 by a selective D2-like receptor agonist, quinpirole, and by electrical stimulation of
348 the A11 nucleus [22]. Our data also suggest that DA participates in antinociception
349 and the maintenance of the circadian rhythm of the PWT of control animals.
350 However, the cellular and molecular mechanisms of this participation remain
351 mostly unknown and prompted our current investigation about the role of DA
352 receptors in the circadian PWT.
353
354 *3.3. Effect of spinal dopaminergic receptor on the circadian PWT in control rats and 6-OHDA-induced tactile*
355 *allodynia*

356 All specific DR antagonists induced tactile allodynia and abolished circadian
357 rhythm of PWT in control rats. The efficacy order of the DR antagonists to induce
358 tactile allodynia was D3>(D1/D5)=D4>D2. On the other hand, intrathecal
359 administration of the D2-like receptor agonist quinpirole partially reversed 6-
360 OHDA-induced tactile allodynia. These data imply that activation of spinal D2, D3,
361 and D4 receptors participate in the quinpirole-induced antiallodynic effect in
362 lesioned rats. Further, our results suggest that activation of spinal D2-like receptors
363 leads to antinociception; this is an expected result considering that lesion of the A11
364 nucleus leads to tactile allodynia. These results confirm that spinal DA has a tonic
365 antiallodynic role, which is disrupted by the lesion of the A11 nucleus or differential
366 inhibition of the spinal DRs.
367 In support of this, several studies using pharmacological tools have reported
368 that spinal DA, acting on D2-like receptors, diminishes formalin-, capsaicin-,
369 thermal stimulation, and carrageenan-induced nociception [11, 26, 48-50]. Also,
370 electrophysiological studies have revealed that DA application into the spinal cord
371 induces hyperpolarization of substantia gelatinosa neurons [35]. Also, spinal DA

inhibits the excitatory postsynaptic currents evoked in lamina I or IV-V projection neuron [50, 51]. Thus, data indicate that spinal DA, through D2-like receptors, is responsible for the antinociceptive effect in control animals.

Surprisingly, administration of the D1-like receptor agonist SKF-39383 also reversed 6-OHDA-induced tactile allodynia in rats. These data suggest that activation of spinal D1 and D5 receptors participates in the antiallodynic effect. Since these are stimulatory receptors, activation of D1-like receptors should lead to a pronociceptive effect [12, 25]. However, there is evidence that spinal activation of D1-like receptors by low concentrations of DA depresses ventral root potential, which is a C fiber-evoked polysynaptic response and believed to reflect nociception [25]. Thus, more experiments are needed to further understand the role of D1-like receptors in this process.

There is little evidence concerning the functionality of D1-like receptors in the spinal cord. Galbavy and collaborators demonstrated that the activation of DR1/DR5 could inhibit sodium current and reduce the number of action potentials in response to stimulus in the dorsal root ganglia. They suggest that these events can serve to adjust the sensitivity of nociceptors to noxious stimuli [52].

Our results suggest that mainly spinal D2-like and, to a lesser extent, D1-like receptors modulate the circadian rhythm of the PWT in control rats. To our knowledge, this is the first report regarding the involvement of spinal DRs in the maintenance of circadian rhythm of PWT behavior.

There is evidence showing the participation of DA and the DR2-like in the modulation of circadian activities. Pozdeyev et al. revealed that the DA and DR4 regulate circadian rhythm of the protein phosphorylation state in photoreceptor cells [45], and Smit et al. demonstrated a dopaminergic regulation through DR2 of the circadian food anticipatory activity rhythms in rats [53].

Furthermore, there is evidence that the DA transporter may be responsible for the circadian rhythm, as the DA release is lower during the dark phase in wild type but not in DA transporter KO mice [51]. Moreover, it has been reported that spinal TH also follows a circadian rhythm [47]. Since TH is the rate-limiting enzyme for the production of DA, it is tempting to suggest that the circadian rhythm of PWT is due to the circadian rhythm of TH or DA transporter. However, further studies are needed to examine these possibilities.

3.4. Effect of 6-OHDA and dopaminergic receptor antagonists on the spinal clock genes

Our results indicate that lesions on dopaminergic A11 neurons modify the transcription profile of several spinal clock genes, including Cry1, Per1, Per 2, Bmal, Rorα, and Rorβ genes. These results suggest that the integrity of the A11 neurons is needed to preserve the expression of the spinal clock genes, which in turn could maintain the physiological circadian rhythm of the PWT. Our data are consistent with a previous study demonstrating that nerve injury leads to the downregulation of Per1, Per2, and Cry1 mRNA in the spinal dorsal horn of mice and knockdown of Per1 expression induced phosphorylation of c-jun N-terminal kinase (JNK), and the

upregulation of chemokine CCL2 in the lumbar spinal dorsal horn [36]. It is known that these mechanisms are associated with chronic pain. Accordingly, expression of Per1, Per2, and Cry1 mRNA displays a circadian rhythm. These genes are enhanced in the early evening, whereas Bmal1 mRNA reaches its maximal expression at midnight [54]. Furthermore, exogenous DA increases Per1 expression in cultured rat spinal astrocytes, while a mix of serotonin, glutamate, and DA enhances Per1 and Bmal1 expression [55]. Our data suggest that the 6-OHDA-induced reduction of several clock genes plays an essential role in the circadian rhythm of PWT, which leads to tactile allodynia during the night.

Having found that the depletion of DA in the spinal cord disrupts the normal rhythm of PWT, leads to nociception, and reduces several clock genes, we investigated whether blockade of either D1- or D2-like DA receptors has an effect on clock gene expression at the spinal cord. Our results indicate a differential role of D1- and D2-like receptors in the clock gene expression. SCH-23390 decreased Bmal, Rorα, and Cry1 mRNA expression at night. In contrast, L-741,626 and L-745,870 increased Bmal, Rorα, Rorβ, Cry1, Per1, and Per2 mRNA expression at night. GR-103,691 decreased Rorα mRNA expression both at day and at night. To the best of our knowledge, this is the first report about the effect of DA receptor antagonists on the mRNA expression of clock genes at the spinal cord of control rats. Our results agree with a previous observation that activation of D1- and D2-like receptors exerts a stimulatory and inhibitory effect, respectively, on the expression of Clock and Per1 genes [29]. According to these observations, we proposed that D1-like receptors participate in the up-regulation of some clock genes, while D2-like receptors could be participating in the downregulation of other clock genes in the lumbar spinal cord of the rat.

4. Conclusion

This study provides evidence that the PWT has a physiological circadian rhythm, which is regulated by the descending dopaminergic A11 nucleus and by differential activation of spinal DA receptors. Furthermore, the descending dopaminergic A11 nucleus and the differential activation of spinal DA receptors modulate the spinal clock gene transcription. Therefore, our results suggest that the processing of nociception at the spinal cord can have a circadian rhythm and that this rhythm could be regulated by the spinal clock genes. Our data could help to understand the exacerbated pain described by patients during the night and elucidate new therapeutic strategies.

5. Materials and methods

5.1. Experimental animals

Male Wistar rats (210 to 230 g) were obtained from our breeding facilities (Cinvestav). Animals were maintained in suitable animal rooms with controlled

456 temperature conditions (22 ± 3°C) and a light–dark cycle (12 h–12 h, light onset at 7:00
457 AM). Food and water were provided ad libitum. Animals were anesthetized using a
458 mixture of ketamine and xylazine (50 mg/kg–10 mg/kg). Lesion of the dopaminergic
459 A11 neurons was induced by the bilateral injection of the 6-OHDA (10 μg/μl) toxin in
460 rats previously (30 min earlier) injected with imipramine (10 mg/kg, i.p.) to protect
461 noradrenergic fibers [56]. All experimental procedures were conducted according to
462 the current Mexican legislation (NOM-062-ZOO-1999, SAGARPA), the Guide for the
463 Care and Use of Laboratory Animals (NIH), and the Guidelines on Ethical Standards
464 for Investigation of Experimental Pain in Animals [57]. These experiments were
465 approved by our local Ethics Committee (Protocol 0146-15, Cinvestav, Mexico City,
466 Mexico). Efforts were made to minimize the number of animals used and their
467 suffering.

468 *5.2. Paw withdrawal threshold*

469 Paw withdrawal threshold (PWT) was determined as previously described [38].
470 Briefly, rats were placed in cages with a mesh grid floor and allowed to acclimate for
471 at least 30 min before experimentation. Von Frey filaments (Stoelting, Wood Dale, IL)
472 were then used to determine the 50% paw withdrawal threshold [37, 38]. The PWT
473 was evaluated in 1) a control group (n = 13) maintained in a light–dark (LD) cycle (12
474 h–12 h, light onset at 7:00 AM), 2) a darkness (DD) group (n = 8) of rats maintained in
475 constant darkness for 15 days to determine whether PWT could be an intrinsic
476 circadian behavior, 3) a formalin group (n = 8), as a model of chronic nociception, 4)
477 an A11 lesion group (n = 9), with bilateral administration of 6-OHDA into the
478 dopaminergic A11 nucleus, 5) a SCH-23390 group (n = 6), DR antagonist to DR1/DR5,
479 6) an L-741,626 group (n = 6), DR antagonist to DR2, 7) a GR-103,691 group (n = 6), DR
480 antagonist to DR3, 8) an L-745,870 group (n = 6), DR antagonist to DR4, 9) an A11
481 lesion + SKF-38393 group (n = 6), bilateral administration of 6-OHDA into the
482 dopaminergic A11 nucleus plus DR agonist to DR1/DR5, and 10) an A11 lesion +
483 quinpirole group (n = 6), with bilateral administration of 6-OHDA into the
484 dopaminergic A11 nucleus plus DR agonist to DR2, DR3, and DR4.

485 *5.3. Formalin-induced long-lasting allodynia*

486 Rats were briefly immobilized to obtain open access to the hind limbs. Animals
487 then received a subcutaneous injection of formalin (1%, 50 μl) into the dorsal surface
488 on the right hind paw with a 30-gauge needle. The nociceptive hypersensitivity
489 induced by formalin was tested 6 days after the injection. Previous studies have
490 demonstrated that allodynia is fully established at this time [58].

491 *5.4. Determination of DA content in the lumbar spinal cord of rats*

492 DA content was quantified with a high-pressure liquid chromatography (HPLC)
493 system with electrochemical detection (ECD, Intro Antec Leyden) in both control
494 and 6-OHDA-treated rats in A11 nuclei in rats. The samples were obtained from the
495 lumbar spinal cord and processed, according to Quiroz et al. [59]. The separation of
496 DA was achieved with a C18 column (4 x 3.0 mm, Phenomenex) using the mobile

phase: 100 mM disodium hydrogen phosphate, 20% methanol, 3.5% acetonitrile, pH 6.7, adjusted with phosphoric acid.

5.5. Determination of TH in the A11 nucleus

Each animal was anesthetized and perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde solution; then, the brain was collected in 30% sterile phosphate-buffered sucrose for one day. These samples were sliced with a cryostat (30 μm) and collected free-floating in PBS. Tissue sections were blocked for 2 h in 0.1% Tween-PBS containing 1% bovine serum albumin (BSA) and incubated overnight at 4°C with anti-tyrosine hydroxylase antibody (1:500, Abcam, USA). After washing in 0.1% Tween-PBS, sections were incubated for 2 h with an anti-sheep IgG-conjugated Alexa 599 antibody (1:500, Abcam, USA). The sections were observed using a Leica microscope (Leica Microsystem, Mannheim, Germany).

5.6. Retrotranscription

Total RNA was extracted from the rat lumbosacral spinal cord region by using Trizol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer-suggested protocol, quantified by spectrophotometry at 260 nm, and analyzed by 1% agarose gel electrophoresis. cDNA was obtained from 5 μg of total RNA using 1 μl of SuperScript III reverse transcriptase kit (Catalog 18080093, Invitrogen; Carlsbad, CA), 1 μl of Oligo dT 50 μM, 1 μl of dNTP mix 10 mM, and molecular biology grade water to 13 μl. Retrotranscription conditions were denaturation at 70°C for 10 min, hybridization at 42°C for 5 min, synthesis of cDNA at 55°C for 50 min and then 70°C for 15 min, and removal of RNA at 37°C for 20 min. Finally, 1 μl of RNase H (Invitrogen, Carlsbad, CA) was added, and samples were incubated at 37°C for 20 min.

5.7. Real-time PCR assay

cDNA was used to amplify each gene using TaqMan probes (Thermo Fisher Scientific, Waltham, MA). The amplification reactions contained 0.25 μl of the respective TaqMan probe: β-actin (Rn00667869_m1), Cry1 (Rn01503063_m1), Per1 (Rn01496757_m1), Per2 (Rn01427704_m1), Per3 (Rn00709499_m1), Bmal (Rn00577590_m1), Rorα (Rn01173769_m1), and Rorβ (Rn01451215_m1); 2.5 μl of Master Mix (TaqMan Universal Master Mix, Life Technologies, Carlsbad, CA); and 2.25 μl of cDNA in a final volume of 5 μl. The qPCR conditions were: 10 min for denaturation at 9°C, followed by 45 cycles of amplification of 15 s at 95°C and 1 min at 60°C. Rat β-actin was used as an internal control and for normalization. The amplification assays were made using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative transcript levels expressed as fold change for gene expression.

5.8. Drugs

6-Hydroxydopamine hydrochloride (6-OHDA), imipramine hydrochloride, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine

hydrochloride (SCH-23390), (±)-3-[4-(4-chlorophenyl)-4-hydroxypiperidinyl]methylindole (L-741,626), 4′-acetyl-N-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl]-[1,1′-biphenyl]-4-carboxamide (GR-103,691), (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF-38393) and trans-(−)-(4aR)-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline monohydrochloride ([−]-quinpirole monohydrochloride) were obtained from Sigma-Aldrich (St. Louis, MO). 3-(4-[4-chlorophenyl]piperazin-1-yl)-methyl-1H-pyrrolo[2,3-b]pyridine trihydrochloride (L-745,870) was purchased from Tocris Bioscience (Bio-Techne Corporation, Minneapolis, MN). 6-OHDA was dissolved in 0.02% ascorbic acid. Imipramine, SCH-23390, SKF-3839, and L-745,870 were dissolved in injectable water. L-741,626 and GR-103,691 were dissolved in dimethyl sulfoxide (DMSO). All drugs were prepared on the day of the experiment. Drugs were intrathecally injected by direct puncture using a 30-gauge needle according to a reported method [60].

The dopaminergic drugs mentioned above were selected based on relevant receptor selectivity and efficacy. These included: (i) selective D1 (pKi 6.2-6.8) and D5 (pKi 7) receptor agonist SKF-38393 [61, 62], and D2 (pKi 7.7), D3 (pKi 7.3-7.8), and D4 (pKi 7.5) agonist quinpirole [63-65]; (ii) selective D1 (pKi 7.4-9.5) and D5 (pKi 7.5-9.5) receptor antagonist SCH-2339 [61, 62], D2 (pKi 7.9-8.5) receptor antagonist L-741,62625 [66], D3 (Ki 0.3 nM) receptor antagonist GR-103,69 [47], and D4 (pKi 9.4) receptor antagonist L-745,870 [66].

5.9. Circadian analysis

The time-course data were expressed as the mean ± standard error. These data were analyzed by the Friedman test, followed by Dunn's test. The data were then examined by the rhythm analysis using the Chronos-Fit software v1.0. This software includes a linear analysis and rhythm analysis, which calculate the parameters of the sine curve with the partial Fourier series. The corresponding parameters are mesor (midline estimating statistic of rhythm), amplitude (amplitude of sinewave), acrophase (time of the maximum value of the sine wave), and period (first-period length 20 h–28 h) [67, 68].

5.10. Statistical analysis

The values are expressed as the mean ± standard error. Unpaired Student´s t-test was performed for comparison between two groups. Statistical comparisons among three or more groups were analyzed by one- or two-way ANOVA followed by Dunnett´s or Tukey's test. A p-value less than 0.05 was considered significant.

Author Contributions: Conceptualization, P.L.C. and G.B.J.A.; methodology, P.L.C., D.L.R., G.S.V., R.S.M., Z.B.E., and V.C.G.C.; software, R.S.L.; validation, P.L.C., F.G.B., and G.B.J.A.; formal analysis, P.L.C. and L.L.M.; investigation, P.L.C.; resources, F.G.B.; data curation, R.S.L.; writing-original draft preparation, P.L.C.; writing-review and editing, R.S.L., F.G.B., J.E.I., VG-S., and G.B.J.A.; visualization, C.P.L; supervision, F.G.B and G.B.J.A.; project administration, G.B.J.A.; funding acquisition G.B.J.A.

Funding: ISSSTE funded the APC with the grant number RPI-033.2015.

Acknowledgments: The authors thankfully acknowledge Samuel Gabriel Horta Mendoza (Head of Hospital Regional "Primero de Octubre") for providing the necessary laboratory facilities for the study, Guadalupe C. Vidal Cantú M.Sc., for her assistance in mechanical allodynia experiments. Celia Piña-Leyva and Manuel Lara-Lozano was a recipient of scholarships from CONACYT (Mexico). This work was partly supported by the ISSSTE grant 831/15 to Juan Antonio González-Barrios.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ANOVA	Analysis of Variance
Bmal	brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein
Cry1	Cryptochrome circadian regulator 1
DA	Dopamine
DRs	Dopaminergic Receptors
D1	Dopamine Receptor D1
D2	Dopamine Receptor D2
D3	Dopamine Receptor D3
D4	Dopamine Receptor D4
D5	Dopamine Receptor D5
GABA	γ-Aminobutyric Acid
GR-103,691	4′-Acetyl-N-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl]-[1,1′-biphenyl]-4-carboxamide
L-745,870	3-(4-[4-Chlorophenyl]piperazin-1-yl)-methyl-1H-pyrrolo[2,3-b]pyridine trihydrochloride
L-741,626	3-[[4-(4-Chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1H-indole
NPAS	neuronal PAS domain protein 2
Per1	Period circadian regulator 1
Per2	Period circadian regulator 2
Per3	Period circadian regulator 3
PWT	Paw Withdrawal Threshold
Rorα	Retinoid-Related Orphan Receptor alpha
Rorβ	Retinoid-Related Orphan Receptor beta
SCH23390	(R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride
SG	Substantia Gelatinosa
SKF38393	(±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrobromide
TH	Tyrosine Hydroxylase
WT	Wild Type
6-OHDA	6-Hydroxydopamine

References

1. Murphy, K.R., et al., *Prevalence of Specific Types of Pain Diagnoses in a Sample of United States Adults.* Pain Physician, 2017. **20**(2): p. E257-E268.
2. Puopolo, M., *The hypothalamic-spinal dopaminergic system: a target for pain modulation.* Neural Regen Res, 2019. **14**(6): p. 925-930.
3. Gilron, I. and N. Ghasemlou, *Chronobiology of chronic pain: focus on diurnal rhythmicity of neuropathic pain.* Curr Opin Support Palliat Care, 2014. **8**(4): p. 429-36.

4. Odrcich, M., et al., *Chronobiological characteristics of painful diabetic neuropathy and postherpetic neuralgia: diurnal pain variation and effects of analgesic therapy.* Pain, 2006. **120**(1-2): p. 207-12.
5. van den Beuken-van Everdingen, M.H., et al., *Update on Prevalence of Pain in Patients With Cancer: Systematic Review and Meta-Analysis.* J Pain Symptom Manage, 2016. **51**(6): p. 1070-1090 e9.
6. Adewusi, J.K., et al., *Peripheral neuropathic pain in idiopathic Parkinson's disease: Prevalence and impact on quality of life; a case controlled study.* J Neurol Sci, 2018. **392**: p. 3-7.
7. Frederickson, R.C., V. Burgis, and J.D. Edwards, *Hyperalgesia induced by naloxone follows diurnal rhythm in responsivity to painful stimuli.* Science, 1977. **198**(4318): p. 756-8.
8. Brewer, K.L., et al., *Dopamine D3 receptor dysfunction prevents anti-nociceptive effects of morphine in the spinal cord.* Front Neural Circuits, 2014. **8**: p. 62.
9. Castellano, C., et al., *Genetic differences in daily rhythms of pain sensitivity in mice.* Pharmacol Biochem Behav, 1985. **23**(1): p. 91-2.
10. Perissin, L., et al., *Diurnal changes of tonic nociceptive responses in mice: evidence for a proalgesic role of melatonin.* Pain, 2004. **110**(1-2): p. 250-8.
11. Frias, B. and A. Merighi, *Capsaicin, Nociception and Pain.* Molecules, 2016. **21**(6).
12. Lu, Y., et al., *Presynaptic Inhibition of Primary Nociceptive Signals to Dorsal Horn Lamina I Neurons by Dopamine.* J Neurosci, 2018. **38**(41): p. 8809-8821.
13. Ossipov, M.H., G.O. Dussor, and F. Porreca, *Central modulation of pain.* J Clin Invest, 2010. **120**(11): p. 3779-87.
14. Bannister, K. and A.H. Dickenson, *What do monoamines do in pain modulation?* Curr Opin Support Palliat Care, 2016. **10**(2): p. 143-8.
15. Benarroch, E.E., *Descending monoaminergic pain modulation: bidirectional control and clinical relevance.* Neurology, 2008. **71**(3): p. 217-21.
16. Chen, Q., et al., *Optogenetic Evidence for a Direct Circuit Linking Nociceptive Transmission through the Parabrachial Complex with Pain-Modulating Neurons of the Rostral Ventromedial Medulla (RVM).* eNeuro, 2017. **4**(3).
17. Fleetwood-Walker, S.M., P.J. Hope, and R. Mitchell, *Antinociceptive actions of descending dopaminergic tracts on cat and rat dorsal horn somatosensory neurones.* J Physiol, 1988. **399**: p. 335-48.
18. Pappas, S.S., et al., *Lack of D2 receptor mediated regulation of dopamine synthesis in A11 diencephalospinal neurons in male and female mice.* Brain Res, 2008. **1214**: p. 1-10.
19. Barraud, Q., et al., *Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate.* PLoS One, 2010. **5**(10): p. e13306.
20. Charbit, A.R., et al., *Neurons of the dopaminergic/calcitonin gene-related peptide A11 cell group modulate neuronal firing in the trigeminocervical complex: an electrophysiological and immunohistochemical study.* J Neurosci, 2009. **29**(40): p. 12532-41.

21. Koblinger, K., et al., *Characterization of A11 neurons projecting to the spinal cord of mice.* PLoS One, 2014. **9**(10): p. e109636.
22. Taniguchi, W., et al., *In vivo patch-clamp analysis of dopaminergic antinociceptive actions on substantia gelatinosa neurons in the spinal cord.* Pain, 2011. **152**(1): p. 95-105.
23. Zhu, H., et al., *Expression and distribution of all dopamine receptor subtypes (D(1)-D(5)) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic in situ hybridization study.* Neuroscience, 2007. **149**(4): p. 885-97.
24. Bergerot, A., R.J. Storer, and P.J. Goadsby, *Dopamine inhibits trigeminovascular transmission in the rat.* Ann Neurol, 2007. **61**(3): p. 251-62.
25. Kawamoto, K., et al., *Inhibitory effects of dopamine on spinal synaptic transmission via dopamine D1-like receptors in neonatal rats.* Br J Pharmacol, 2012. **166**(2): p. 788-800.
26. Lapirot, O., et al., *Tonic and phasic descending dopaminergic controls of nociceptive transmission in the medullary dorsal horn.* Pain, 2011. **152**(8): p. 1821-31.
27. Megat, S., et al., *A Critical Role for Dopamine D5 Receptors in Pain Chronicity in Male Mice.* J Neurosci, 2018. **38**(2): p. 379-397.
28. Yujnovsky, I., et al., *Signaling mediated by the dopamine D2 receptor potentiates circadian regulation by CLOCK:BMAL1.* Proc Natl Acad Sci U S A, 2006. **103**(16): p. 6386-91.
29. Imbesi, M., et al., *Dopamine receptor-mediated regulation of neuronal "clock" gene expression.* Neuroscience, 2009. **158**(2): p. 537-44.
30. Golombek, D.A. and R.E. Rosenstein, *Physiology of circadian entrainment.* Physiol Rev, 2010. **90**(3): p. 1063-102.
31. Kwon, I., et al., *Mammalian molecular clocks.* Exp Neurobiol, 2011. **20**(1): p. 18-28.
32. Mazzoccoli, G., V. Pazienza, and M. Vinciguerra, *Clock genes and clock-controlled genes in the regulation of metabolic rhythms.* Chronobiol Int, 2012. **29**(3): p. 227-51.
33. Hasakova, K., et al., *The expression of clock genes cry1 and cry2 in human colorectal cancer and tumor adjacent tissues correlates differently dependent on tumor location.* Neoplasma, 2018. **65**(6): p. 986-992.
34. Hashimoto, A., et al., *Changes in Clock Genes Expression in Esophagus in Rat Reflux Esophagitis.* Dig Dis Sci, 2019. **64**(8): p. 2132-2139.
35. Tognini, P., et al., *Distinct Circadian Signatures in Liver and Gut Clocks Revealed by Ketogenic Diet.* Cell Metab, 2017. **26**(3): p. 523-538 e5.
36. Morioka, N., et al., *Downregulation of the spinal dorsal horn clock gene Per1 expression leads to mechanical hypersensitivity via c-jun N-terminal kinase and CCL2 production in mice.* Mol Cell Neurosci, 2016. **72**: p. 72-83.
37. Bonin, R.P., C. Bories, and Y. De Koninck, *A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments.* Mol Pain, 2014. **10**: p. 26.

682 38. Chaplan, S.R., et al., *Quantitative assessment of tactile allodynia in the rat paw.* J
683 Neurosci Methods, 1994. **53**(1): p. 55-63.
684 39. Lolignier, S., N. Eijkelkamp, and J.N. Wood, *Mechanical allodynia.* Pflugers
685 Arch, 2015. **467**(1): p. 133-9.
686 40. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates*. 6th ed. 2007,
687 Amsterdam ; Boston ;: Academic Press/Elsevier.
688 41. Kubynin, A.N. and D. Ignatov Iu, *[Study of chronobiological parameters of pain*
689 *sensitivity in rats and mice].* Biull Eksp Biol Med, 1995. **119**(5): p. 537-40.
690 42. Rosenfeld, J.P. and P.E. Rice, *Diurnal rhythms in nociceptive thresholds of rats.*
691 Physiol Behav, 1979. **23**(2): p. 419-20.
692 43. Barnes, C.A., et al., *Circadian rhythm of synaptic excitability in rat and monkey*
693 *central nervous system*. Science, 1977. **197**(4298): p. 91-2.
694 44. Bellamy, N., et al., *Rhythmic variations in pain, stiffness, and manual dexterity in*
695 *hand osteoarthritis.* Ann Rheum Dis, 2002. **61**(12): p. 1075-80.
696 45. Pozdeyev, N., et al., *Dopamine modulates diurnal and circadian rhythms of protein*
697 *phosphorylation in photoreceptor cells of mouse retina.* Eur J Neurosci, 2008.
698 **27**(10): p. 2691-700.
699 46. Zhao, H., et al., *Spinal cord dopamine receptor expression and function in mice with*
700 *6-OHDA lesion of the A11 nucleus and dietary iron deprivation.* J Neurosci Res,
701 2007. **85**(5): p. 1065-76.
702 47. Clemens, S., M.A. Sawchuk, and S. Hochman, *Reversal of the circadian*
703 *expression of tyrosine-hydroxylase but not nitric oxide synthase levels in the spinal*
704 *cord of dopamine D3 receptor knockout mice.* Neuroscience, 2005. **133**(2): p. 353-
705 7.
706 48. Almanza, A., et al., *A D2-like receptor family agonist produces analgesia in*
707 *mechanonociception but not in thermonociception at the spinal cord level in rats.*
708 Pharmacol Biochem Behav, 2015. **137**: p. 119-25.
709 49. Gao, X., Y. Zhang, and G. Wu, *Effects of dopaminergic agents on carrageenan*
710 *hyperalgesia after intrathecal administration to rats.* Eur J Pharmacol, 2001. **418**(1-
711 2): p. 73-7.
712 50. Liu, Q.S., J.T. Qiao, and N. Dafny, *D2 dopamine receptor involvement in spinal*
713 *dopamine-produced antinociception.* Life Sci, 1992. **51**(19): p. 1485-92.
714 51. Ferris, M.J., et al., *Dopamine transporters govern diurnal variation in extracellular*
715 *dopamine tone.* Proc Natl Acad Sci U S A, 2014. **111**(26): p. E2751-9.
716 52. Galbavy, W., et al., *Inhibition of tetrodotoxin-resistant sodium current in dorsal*
717 *root ganglia neurons mediated by D1/D5 dopamine receptors.* Mol Pain, 2013. **9**: p.
718 60.
719 53. Smit, A.N., et al., *Dopaminergic regulation of circadian food anticipatory activity*
720 *rhythms in the rat.* PLoS One, 2013. **8**(11): p. e82381.
721 54. Morioka, N., et al., *Spinal astrocytes contribute to the circadian oscillation of*
722 *glutamine synthase, cyclooxygenase-1 and clock genes in the lumbar spinal cord of*
723 *mice.* Neurochem Int, 2012. **60**(8): p. 817-26.

55. Morioka, N., et al., *The induction of Per1 expression by the combined treatment with glutamate, 5-hydroxytriptamine and dopamine initiates a ripple effect on Bmal1 and Cry1 mRNA expression via the ERK signaling pathway in cultured rat spinal astrocytes.* Neurochem Int, 2015. **90**: p. 9-19.
56. Slattery, D.A., A.L. Hudson, and D.J. Nutt, *Invited review: the evolution of antidepressant mechanisms.* Fundam Clin Pharmacol, 2004. **18**(1): p. 1-21.
57. Zimmermann, M., *Ethical guidelines for investigations of experimental pain in conscious animals.* Pain, 1983. **16**(2): p. 109-10.
58. Ambriz-Tututi, M., et al., *Role of opioid receptors in the reduction of formalin-induced secondary allodynia and hyperalgesia in rats.* Eur J Pharmacol, 2009. **619**(1-3): p. 25-32.
59. Quiroz-Gonzalez, S., et al., *Endogenous content and release of [(3)H]-GABA and [(3)H]-glutamate in the spinal cord of chronically undernourished rat.* Neurochem Res, 2013. **38**(1): p. 23-31.
60. Mestre, C., et al., *A method to perform direct transcutaneous intrathecal injection in rats.* J Pharmacol Toxicol Methods, 1994. **32**(4): p. 197-200.
61. Sunahara, R.K., et al., *Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1.* Nature, 1991. **350**(6319): p. 614-9.
62. Tiberi, M. and M.G. Caron, *High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype.* J Biol Chem, 1994. **269**(45): p. 27925-31.
63. Burris, K.D., et al., *Lack of discrimination by agonists for D2 and D3 dopamine receptors.* Neuropsychopharmacology, 1995. **12**(4): p. 335-45.
64. Sokoloff, P., et al., *Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics.* Nature, 1990. **347**(6289): p. 146-51.
65. Van Tol, H.H., et al., *Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine.* Nature, 1991. **350**(6319): p. 610-4.
66. Kulagowski, J.J., et al., *3-((4-(4-Chlorophenyl)piperazin-1-yl)-methyl)-1H-pyrrolo-2,3-b-pyridine: an antagonist with high affinity and selectivity for the human dopamine D4 receptor.* J Med Chem, 1996. **39**(10): p. 1941-2.
67. Rodriguez-Sosa, L., et al., *Dopaminergic modulation of the caudal photoreceptor in crayfish.* Synapse, 2011. **65**(6): p. 497-504.
68. Rodriguez-Sosa, L., et al., *Serotonin modulation of caudal photoreceptor in crayfish.* Comp Biochem Physiol C Toxicol Pharmacol, 2006. **142**(3-4): p. 220-30.