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RESEARCH ARTICLE

Recurrent antinociception induced by intrathecal or peripheral oxytocin in a neuropathic pain rat model

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Abstract

The search for new ligands to treat neuropathic pain remains a challenge. Recently, oxytocin has emerged as an interesting molecule modulating nociception at central and peripheral levels, but no attempt has been made to evaluate the effect of recurrent oxytocin administration in neuropathic pain. Using male Wistar rats with spinal nerve ligation, we evaluated the effects of recurrent spinal (1 nmol; given by lumbar puncture) or peripheral (31 nmol; given by intraplantar injection in the ipsilateral paw to spinal nerve ligation) oxytocin administration on pain-like behavior in several nociceptive tests (tactile allodynia and thermal and mechanical hyperalgesia) on different days. Furthermore, we used an electrophysiological approach to analyze the effect of spinal 1 nmol oxytocin on the activity of spinal dorsal horn wide dynamic range cells. In neuropathic rats, spinal or peripheral oxytocin partially restored the nociceptive threshold measured with the von Frey filaments (tactile allodynia), Hargreaves (thermal hyperalgesia) and Randall–Selitto (mechanical hyperalgesia) tests for 12 days. These results agree with electrophysiological data showing that spinal oxytocin diminishes the neuronal firing of the WDR neurons evoked by peripheral stimulation. This effect was associated with a decline in the activity of primary afferent Aδ- and C-fibers. The above findings show that repeated spinal or peripheral oxytocin administration attenuates the pain-like behavior in a well-established model of neuropathic pain. This study provides a basis for addressing the therapeutic relevance of oxytocin in chronic pain conditions.

Keywords Pain · Neuropathic · Oxytocin

Introduction

Neuropathic pain is a sensory chronic maladaptive and pathological disorder caused by a lesion or disease of the somatosensory system. This type of pain, affecting about 7–10% of the population, differs from physiological pain and is characterized by allodynia (a painful experience by a non-painful stimulus) and hyperalgesia (an exaggerated sensitivity to painful stimuli) (Colloca et al. 2017). Although several kinds of drugs exist to treat neuropathic pain, their overall efficacy is low, and in some cases their side effects are high, which stresses the importance of searching for new agents with anti-neuropathic effects (Yaksh et al. 2015; Yekkirala et al. 2017).

Extensive evidence suggests that central oxytocin (OXT) could play a key role as an analgesic molecule (Arletti et al. 1993; Condés-Lara et al. 2003, 2005, 2006, 2012; Eliava et al. 2016; Han and Yu 2009; Lundeberg et al. 1993; Mazzuca et al. 2011; Miranda-Cardenas et al. 2006; Petersson et al. 2001; Rojas-Piloni et al. 2007; Wang et al. 2003; Yang et al. 2007; Yu et al. 2003). Seminal studies showed that OXT-containing neurons in the hypothalamic paraventricular nucleus (PVN) send axons to the spinal cord (Sawchenko and Swanson 1982) and upon PVN stimulation induce analgesia (Condés-Lara et al. 2006; Eliava et al. 2016; Shiraishi et al. 1995; Yang et al. 2006). More recently, studies using behavioral, electrophysiological and optogenetic approaches have reported that a peripheral OXT-specific mechanism also seems to be present (Boada et al. 2019; de Araujo et al.

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2014; Eliava et al. 2016; González-Hernández et al. 2017; Juif et al. 2013).

Indeed, a previous report in humans with intractable cancer pain showed that epidural OXT has analgesic effects (Condés-Lara et al. 2016), reinforcing the therapeutic potential of this neuropeptide. Nevertheless, in the case of chronic neuropathic pain models tested, it is interesting to note that in most cases OXT administration schedule was acute (single administration), but the effect of multiple OXT administrations on nociceptive transmission in a pathological condition remains unknown.

The present study was designed to test the analgesic effect of OXT by repeated electrical stimulation of the PVN and/or recurrent spinal or peripheral OXT administration. We quantified the stimulus-evoked pain-like behaviors (von Frey test, Hargreaves test and Randall–Selitto pressure test) or nociceptive electrophysiological responses [extracellular unitary recording of spinal dorsal horn wide dynamic range (WDR) cells] in a well-established rat model of neuropathic pain (spinal nerve ligation, SNL) (Chung et al. 2004; Kim and Chung 1992). We must emphasize that our experimental protocols were designed to test in the same animal at different days the effects of OXT (given peripherally or spinally) on different stimulus-evoked nociceptive responses (see Fig. 1). This approach was followed, considering that during neuropathic injury several kinds of stimulus evoke pain, and consequently we tried to use several nociceptive tests in the same animal to partially mimic what happens in a real-set condition. Our results show that recurrent OXT administration produced a reversal in: (i) mechanical allodynia; and (ii) thermal and mechanical hyperalgesia.

Materials and methods

Experimental animals and ethical standards

A total of 43 male Wistar rats (250–320 *g*) were used. The animals were maintained in a 12-/12-h light/dark cycle (light beginning at 7:00 a.m.) and housed at constant temperature (22 ± 2 °C) and humidity (50%) with food and water ad libitum. The NIH Guide for the Care and Use of

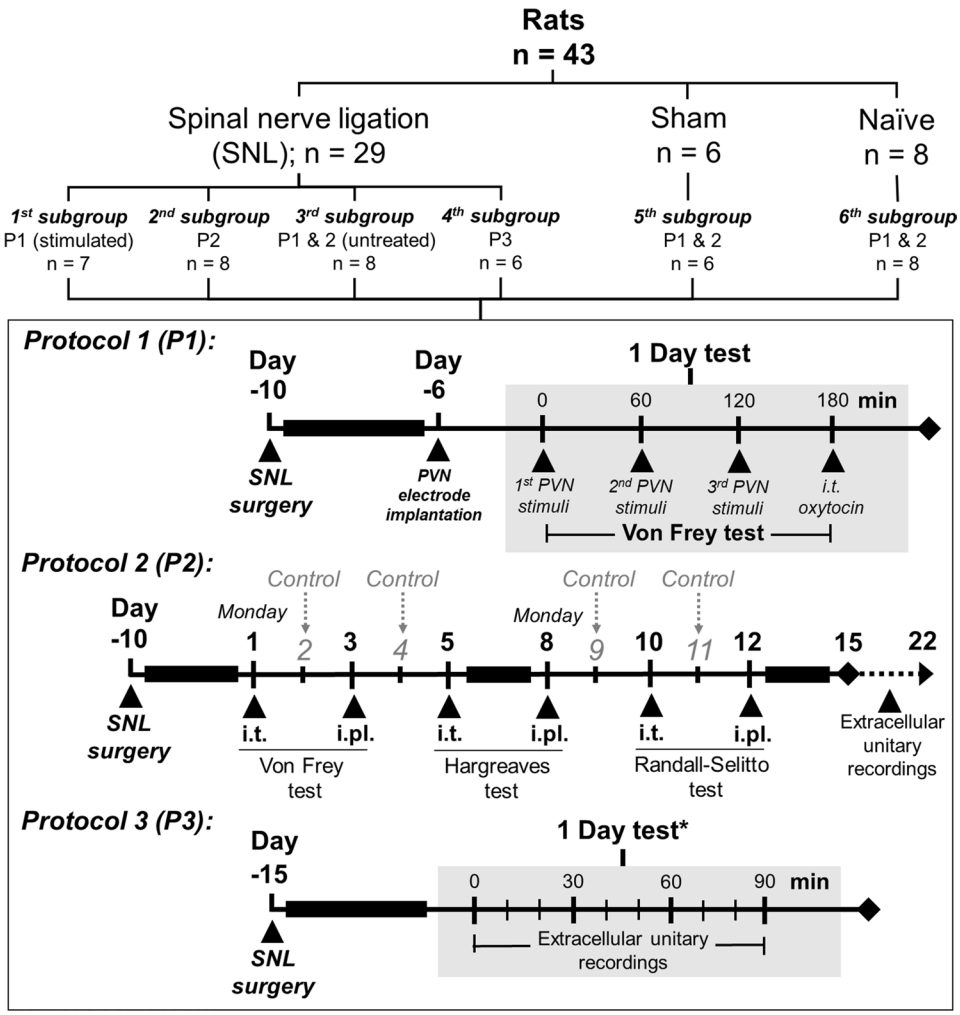

Fig. 1 Experimental protocols used in the present report. The animals were divided into three main sets (SNL, sham and naïve). The SNL animals were compared against sham and naïve groups. Protocols 1, 2 and 3 (P1, P2 and P3) show on a temporal scale the different procedures applied. For P1 and P2, SNL surgery was performed at − 10 days before the respective behavioral test. In P1, SNL was performed at − 10 days and PVN electrode implantation was done at − 6 days before the von Frey test; note that in the case of P2, electrophysiological experiments were performed after behavioral experiments were completed (after 25 days post-SNL surgery). Furthermore, in the case of P3, electrophysiological experiments were performed 15 days post-SNL surgery. *SNL* spinal nerve ligation, *PVN* hypothalamic paraventricular nuclei, *i.t.* intrathecal, *i.pl.* intraplantar

Laboratory Animals (NIH Publication 86–23), IASP Ethical Guidelines (Zimmermann 1983) and INB-UNAM Ethics Committee recommendations were followed. Three experimental protocols (see Fig. 1; P1, P2 and P3) were followed to reach the purpose of this research. *Protocol 1* (P1) was performed to study the effect of repetitive electrical stimulation of the PVN on the mechanical nociceptive responses. *Protocol 2* (P2) allowed us to analyze the chronic effects of intrathecal (i.t.) or intraplantar (i.pl.) OXT administration over three somatosensory tests: von Frey, Hargreaves and Randall–Selitto (see Fig. 1). *Protocol 3* (P3) was designed to measure the spinal nociceptive electrophysiological responses in SNL animals. The number of rats used was the minimum required to obtain reliable results, and efforts were made to minimize animal distress and the doses used of oxytocin was chosen because it significantly reduced nociception in behavioral and electrophysiological experiments (González-Hernández et al. 2017; Rojas-Piloni et al. 2010). It is important to point out that these doses do not have any effect on the locomotor performance or ambulatory activity (data not shown).

General procedures

The segmental spinal nerve L5 and L6 ligatures (SNL; $n=29$) were done under general anesthesia (ketamine/xylazine, 45/12 mg/kg; intraperitoneal, i.p.). The L5 and L6 left nerves were tightly ligated with 4-0 silk sutures distal to the dorsal root ganglion (DRG) as described by Kim and Chung (1992). All behavioral experiments were performed during the light phase (10:00–15:00 h). The animals with SNL were divided into four subgroups ($n=7$, 8, 8 and 6 rats, respectively) and participated in *Protocols 1*, *2* or *3* (Fig. 1). Sham surgery was performed in an identical manner omitting nerve ligation.

In the first SNL subgroup ($n=7$; submitted to *Protocol 1*), 4 days after SNL surgery, a small craniotomy was performed to place a concentric stainless steel stimulation electrode (1 MΩ) in the PVN [7.0 mm AP interaural, 0.2 mm lateral and 2 mm height; Paxinos and Watson (2006)]. Briefly, SNL rats were anesthetized with ketamine/xylazine (at the above doses) and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) for electrode implantation (stainless-steel Teflon-isolated microwire, Ø 279 µm, Cat. no. 7920; A-M Systems, Inc. Carlsberg, WA, USA). Six days after PVN implantation, tactile allodynia was evaluated using von Frey filaments before and after electrical stimulation of the PVN (see "Other procedures applying to the experimental protocols" for details) or i.t. OXT. It is important to point out that the animals of this group were only tested for 1 day.

The second SNL subgroup ($n=8$) was submitted to *Protocol 2* to test tactile allodynia or hyperalgesia on different days using three behavioral nociceptive tests, namely: (i) von Frey withdrawal threshold (to evaluate tactile allodynia); (ii) Hargreaves test (to evaluate thermal hyperalgesia); and (iii) Randall–Selitto test (to evaluate mechanical hyperalgesia). In this case, *Protocol 2* was used to test the effects of i.pl. or i.t. OXT on different days (at least 1-day intervals between treatments). At the end of the behavioral tests, this group of animals was used to study the spinal dorsal horn wide dynamic range (WDR) neuronal responses evoked by peripheral stimuli (see below for details).

The raw data collected in the behavioral nociceptive tests were compared with the data acquired from SNL untreated ($n=8$; 3rd subgroup), sham ($n=6$; 5th subgroup) and naïve ($n=8$; 6th subgroup) rats. In addition, one subgroup ($n=6$; 4th subgroup) with SNL was used to perform only electrophysiological experiments (see below for details).

Behavioral nociceptive tests

Tactile allodynia (von Frey withdrawal threshold)

Punctate mechanical tactile allodynia was quantitatively measured as previously reported (Chaplan et al. 1994), using the "up-and-down" method (Dixon 1965). This method determines the mechanical force required to elicit a paw withdrawal response in 50% of animals. A recent description of this method to evaluate mechanical allodynia can be found in Deuis et al. (2017). In short, rats were placed in individual transparent cages with a metal mesh floor and allowed to acclimatize for at least 30 min (on 3 different days) before the test. We used trials of 1.4, 2.0, 4.0, 6.0, 8.0, 10.0 and 15.0 *g* von Frey filaments (North Coast, USA) to estimate the 50% paw withdrawal threshold using the "up-and-down" method. A series of filaments were presented in ascending order of strength to the plantar surface of the paw (ipsilateral to SNL). Each hair was pressed (for 5 s) perpendicularly against the paw with sufficient force to cause slight bending. A positive response was noted if the paw was sharply withdrawn; at this point we used the next lower filament to ensure that we had reached the threshold. If the response was negative, the filament size was increased to be sure of the threshold value. This paradigm continued until four more measurements had been made after the initial change of the behavioral response or until five consecutive negative (i.e., 15 *g*) or four consecutive positive (i.e., 0.25 *g*) responses had occurred. The scores were used to calculate the 50% withdrawal threshold using the statistical formula described by Chaplan et al. (1994):

$$50\%\text{g threshold} = 10^{(Xf+\kappa\partial)}/10,000,$$

where X*f* is the value of the final filament used; κ the tabular value of response [see Appendix 1 in Chaplan et al. (1994)]; and ∂ the mean difference between stimuli. This formula considers the number of positive and negative responses

to have an indicator of the withdrawal threshold. Tactile allodynia was considered present with paw withdrawal thresholds ≤ 4 *g*.

Using *Protocol 1* for the first subgroup of rats (Fig. 1), we tested the effect of repeated PVN stimuli (given at 60-min intervals) and one i.t. OXT administration on the evoked nocifensive behavior. We measured in a temporal course (0–240 min) the antinociceptive action induced by PVN electrical stimuli given immediately after basal measurement, time 0 and at 60 and 120 min. Furthermore, i.t. OXT was given (at $t = 180$ min). The PVN was electrically stimulated for 6 s (60 Hz, 1 ms pulse duration, 300 µA) using a Grass Stimulator (Mod. S11A) attached to isolator unit (Mod. PSIU6). These parameters induce specific antinociceptive effects (Condés-Lara et al. 2006) and none of the stimulated animals presented alertness, freezing, escape or seizure behaviors during the stimulation.

In the second SNL subgroup (*Protocol 2*), the von Frey withdrawal threshold test was done at basal (B) (immediately before i.pl. 31 nmol or i.t. 1 nmol OXT administration) and at 5, 15, 30, 45, 60, 80, 100 and 120 min after OXT. In the following days, this subgroup was submitted to the Hargreaves and Randall–Selitto tests.

In both cases (1st and 2nd subgroup), the results obtained were compared against SNL untreated (3rd subgroup), sham (5th subgroup) and naïve rats (6th subgroup).

Thermal hyperalgesia (Hargreaves test)

We evaluated thermal hyperalgesia (Dirig et al. 1997) using the Hargreaves test (Hargreaves et al. 1988). Briefly, rats were placed in individual transparent boxes with a glass floor that allowed us to apply a thermal stimulus using an infrared generator on the glabrous skin of the foot (Hargreaves Apparatus, Cat. No. 37370; Ugo Basile). All animals were familiarized with the box for 30 min before the test. The device gave us the latency of the withdrawal response (time for abrupt removal of the paw); a cutoff at 20 s was set to avoid tissue damage. The test was repeated twice, and the average paw withdrawal latency was calculated. We determined the withdrawal responses to the left paw (ipsilateral to SNL) after (10 min) i.t. (1 nmol; day 5) or i.pl. (31 nmol, ipsilateral to SNL; day 8) OXT. The OXT effect was compared against the SNL untreated, naïve and sham subgroups.

Mechanical nociceptive threshold testing (Randall–Selitto pressure test)

Static hyperalgesia measured as a mechanical nociceptive threshold (Randall and Selitto 1957) was quantified using an Ugo Basile Paw Pressure Analgesia Meter (Cat. No. 57215; Stoelting), which applies a steadily increasing force to the dorsum of a rat's hind paw. Cotton tissue was used to immobilize the animals for measuring the threshold, defined as the force at which the animal withdraws its paw. The test was repeated twice, and the average was calculated. The left paw (ipsilateral to SNL) withdrawal responses after (10 min) i.t. (1 nmol; day 10) or i.pl. (31 nmol, ipsilateral to SNL; day 12) OXT were determined. The OXT effect was compared against the SNL untreated, naïve and sham rats.

In vivo electrophysiological recording of wide dynamic range (WDR) cells

For in vivo electrophysiological experiments, the second, third, fourth and fifth subgroups were used. In the case of the second and third SNL subgroups, these animals were used at the end of the behavioral experiments above described (> 25 postoperative days; see Fig. 1). It is important to emphasize that in the case of the fourth subgroup, the electrophysiological experiments were performed at postoperative (SNL surgery) days 15–17.

Surgical procedures

Animals were introduced into a chamber and deeply anesthetized with 5% sevoflurane (in a mixture of ¾ N_2O and ¼ O_2) delivered through a vaporizer. Under these conditions, an intra-tracheal cannula was inserted for artificial ventilation (60 strokes/min) and to maintain the anesthesia. The stroke volume was adjusted to maintain a normal acid–base equilibrium throughout the experiment. Then, rats were mounted in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with a spinal cord restrained frame. To record the evoked unitary neuronal activity of the spinal WDR cells, the spinal cord was exposed at the lumbar vertebrae level to perform a laminectomy (at L2–L4 segments). End-tidal CO_2 was monitored with a Capstar-100 CO_2 analyzer (CWI Inc., Ardmore, PA, USA) and kept between 2.5 and 3.2%. Core body temperature was maintained at 38 °C using a circulating water pad. Furthermore, a small craniotomy was performed to place a concentric stainless steel stimulation electrode in the PVN (as described above). Subsequent experimental protocols were performed under 2.5% sevoflurane.

Extracellular unitary recordings

Glass or quartz–Pt–W microelectrodes (4–10 MΩ) mounted in a microdrive Eckhorn system (Thomas Recording GmbH, Giessen, Germany) were used to record extracellular unitary activity. The glass microelectrodes were filled with 2% pontamine (1 M KCl) and were lowered into the superficial laminae of the left spinal dorsal horn (SDH) segments (200–1500 µm from the surface) to obtain single-unit discharges. The specific somatic receptive field (RF) was located by tapping on the ipsilateral hind paw;

then, electrical stimulation was applied by two electrodes inserted into the RF (see "Mechanical or Electrical stimulation of the somatic receptive field"). Neuronal activity was recorded (for offline analysis), amplified, digitalized and discriminated by a CED 1401 interface coupled to *Spike2* V5.15 software (Cambridge Electronic Design, UK).

The evoked activity of the spinal WDR cells was analyzed as cumulative frequency and peri-stimulus time histograms (PSTH) to detect the existence of statistically significant neuronal responses. Considering the distance between the RF and the recording electrode, the recorded latency ranges match the activation of Aβ- (0–20 ms), Aδ- (21–90 ms) and C-fibers (90–350 ms) (Urch and Dickenson 2003). Thus, the number of action potentials that occurred in response to 20 RF stimuli was compared before (basal) and after PVN electrical stimulation or spinal topical OXT (1 nmol/10 µl) administration.

Mechanical or electrical stimulation of the somatic receptive field

Two fine needles (27 G) attached to a stimulus isolator unit (Mod. PSIU6, Grass Instruments Co., Warwick, RI, USA) were inserted subcutaneously (sc) into the RF of the recorded neuron. Then, electrical test stimulation was performed; this test consisted of 20 stimuli at 0.5 Hz, with 1-ms pulse duration at 1.5 times the threshold (0.1–3 mA) intensity required to evoke a C-fiber response (Mod. S88 Stimulator, Grass Instruments Co., Warwick, RI, USA). Under these conditions, we recorded the electrophysiological responses of WDR neurons in the basal situation. Then, in the case of protocol 2 (P2), the effect of spinal topical OXT (1 nmol/10 µl) administration on the neuronal nociceptive evoked responses was evaluated at time (t) = 5, 10 and 20 min.

In the case of protocol 3 (P3), after measurement of WDR response in the basal situation, the neuronal responses induced by von Frey filaments (2, 4, 10 and 15 g) were measured. Immediately after, the neuronal WDR responses induced by electrical stimulation of the RF were evaluated before and after PVN electrical stimulation (see Fig. 2a–c. The PVN was electrically stimulated for 6 s at 60 Hz with a pulse duration of 1 ms and 300 µA. Under this condition we constructed peri-stimulus time histograms (PSTHs) and raster plots to measure the WDR responses before and after PVN stimulation (Fig. 2d). Furthermore, when the neuronal activity return to baseline, we analyze the effect of spinal topical OXT (1 nmol/10 µl) administration on the neuronal nociceptive evoked responses at time (t) = 5, 10 and 20 min.

Histological reconstruction of the electrode position in the PVN and/or in the spinal dorsal horn tissue

At the end of the experiments, rats with an electrode in the PVN received an overdose of pentobarbital (80 mg/kg) and were perfused transcardially with isotonic saline solution (0.9% NaCl) followed by 10% formaldehyde (~ 200 ml of each). In the case of electrophysiological experiments performed with glass microelectrodes, before perfusion, the position of the recorded WDR cells was marked by iontophoretic injection of pontamine sky blue (cathodic current 10–15 µA, ~ 20 min). The brains and spinal cord (lumbar segments) were carefully excised and post-fixed in 10% formaldehyde. The tissue was cut into 40-µm serial coronal sections with a freezing microtome (Leica Instruments BmbH, Nussloch, Germany). Stimulated (at PVN) or recording sites (at SDH) were observed with a light microscope (Fig. 2e, f).

Other procedures applying to the experimental protocols

The i.pl. OXT (31 nmol) was administered in a volume of 50 µl using a 30 G needle. For i.t. (10 µl) OXT administration (1 nmol), a direct lumbar puncture (22 G) in the L5–L6 intervertebral space was performed (Mestre et al. 1994). To do this procedure, the rats were briefly anesthetized in a hermetic box with a mixture of 6% sevoflurane, 40% O_2 and 40% N_2O; when motor reflexes were lost, a nose cone was used to maintain the anesthetic level. The anesthesia outcome lasted less than 3 min. We must retain in mind that this procedure requires an expert to adequately perform the lumbar puncture; in this case, we measured the success index (~ 95%) to deliver i.t. lidocaine and to observe a motor dysfunction of the hindquarters. It is important to point out that in the case of OXT administration, we performed all behavioral experiments in the same animals, but to exclude any residual antinociceptive effect due to the previous OXT treatment, we measured the basal response to vehicle (saline) 1 day before the respective test. Each OXT administration was separated by a minimum washout period of 48 h to ensure drug clearance (Hicks et al. 2014). Furthermore, animals showing motor deficits 10 days after SNL surgery were excluded from the experiments.

Drugs

In addition to the anesthetic (sevoflurane; Piramal Critical Care Inc. Bethlehem, PA, USA) for electrophysiological experiments, this study used oxytocin acetate salt hydrate (CAS No. 50-56-6) purchased from Sigma Chemical Co. (St Louis, MO, USA). This peptide was dissolved in

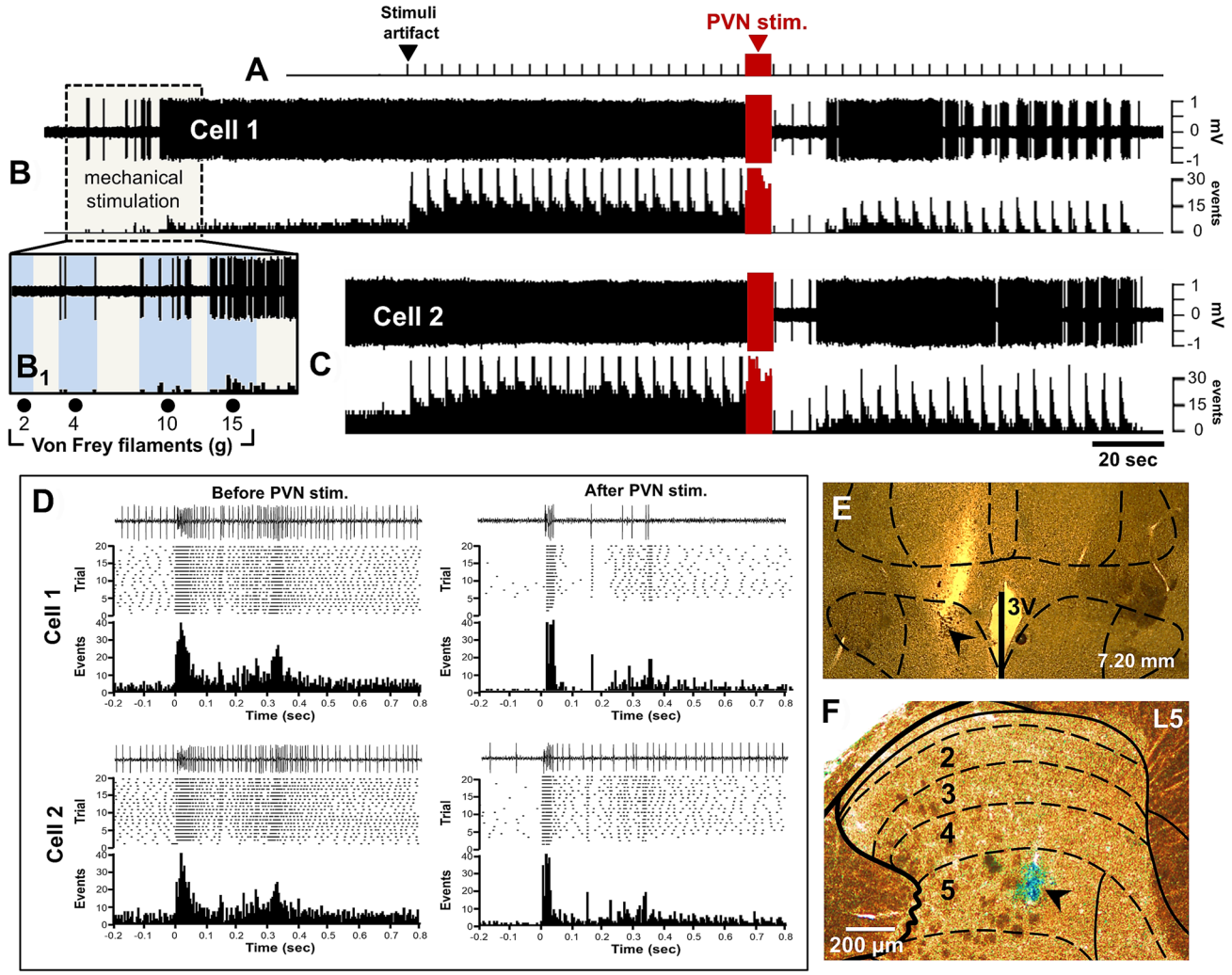

Fig. 2 Effect of PVN electrical stimulation on the neuronal activity of WDR cells of rats under SNL and histological analysis in rats with SNL. **a** Artifacts of RF (arrow black) and PVN electrical stimulation (in red); 20 stimuli to the RF were given before and after PVN stimuli. **b** The electrophysiological recording of WDR cell (cell 1; above), and the histogram of the instantaneous frequency discharge (below) was observed. Notice the reduction of evoked neuronal responses after PVN stimulation. It is interesting to notice the increase of spontaneous neuronal discharge after mechanical stimulation with von Frey filaments (B1). **c** Another WDR cell (cell 2) presents similar changes after PVN stimulation. **d** We can observe for Cell 1 and Cell 2 the electrophysiological recording of single electrical stimulation of the RF (upper channel) and the raster plot (middle) and PSTH (below) generated by 20 stimuli before (left) and after (right) PVN electrical stimulation. Note that a selective diminution of neuronal nociceptive (Aδ- and C-fibers)-evoked responses between 0.05 and 0.4 s. **e** A histological electrolytic lesion in the rostral part of PVN (black arrow). **f** A spinal cord histological section showed the location of recorded cell using blue pontamine (black arrow). *SNL* spinal nerve ligation, *PVN* hypothalamic paraventricular nuclei, *WDR cell* wide dynamic range cell

physiological saline (0.9% NaCl, *w*/*v*). The vehicle had no effect on the baseline values of behavioral responses or on the neuronal-evoked activity.

Statistical analysis

In all cases, we checked that our data set followed a normal distribution (Shapiro–Wilk test) and the power of the performed test (α was set out at 0.8 minimum). In all cases to exclude outliers in a sample group, we performed the Grubb's test. In the experiments using the von Frey withdrawal threshold, curves were constructed [mean ± standard error of the mean (SEM)] plotting the 50% withdrawal threshold as a function of time. However, since the calculated threshold did not yield a mathematical continuum (i.e., non-parametrical data), we performed a Friedman repeated measures analysis of variance (RM ANOVA) on ranks to analyze the intra-group stability of the measurements across time. Furthermore, to analyze the effect of the different treatments on the von Frey measurements, the

data were normalized by calculating the area under the 50% withdrawal threshold against time curves (AUC) and then by performing a one-way ANOVA. The AUC, which is an expression of the duration and intensity of the effect, was calculated by the trapezoidal rule. The data obtained from the Hargreaves and Randall–Selitto tests were represented using box-and-whiskers plots and analyzed with a one-way ANOVA. For electrophysiological experiments, the number of evoked potentials induced by electrical stimulation was normalized and expressed as percentage change from the baseline. This baseline response was established after an identified neuron had a ≤ 10% variation in the neuronal responses induced by RF stimulation during three consecutive tests (5 min between each test). To compare the values of neuronal Aβ-, Aδ- or C-fiber responses before (basal response) and after spinal OXT administration, we used a two-way RM ANOVA. The one-way and two-way ANOVAs were followed, if applicable, by the Tukey post hoc test for multiple comparisons. Statistical significance was accepted at $p < 0.05$.

Results

Behavioral experiments

Protocol 1. Electrical PVN stimulation or i.t. OXT reverts the induced tactile allodynia

During the 4-h test (Fig. 3a), the behavioral responses to von Frey filaments were stable for naïve ($\chi^2 = 1.287$, $n = 8$; $p = 0.936$), sham ($\chi^2 = 4.043$, $n = 6$; $p = 0.671$) and SNL (untreated) ($\chi^2 = 12.917$, $n = 8$; $p = 0.074$) rats. Indeed, naïve and sham rats showed a stable withdrawal threshold rounding 13 *g*, whereas the SNL rats showed a reduction in this threshold (< 4 *g*). Together, these data show that no time-dependent changes in the paw withdrawal threshold responses occurred during our experimental protocols.

Certainly, 10 days after the SNL surgery, the basal values of the 50% paw withdrawal threshold of SNL (treated and untreated) rats was diminished (< 4 *g*) (Fig. 3a), as compared to naïve or sham rats (> 12 *g*), indicating that SNL induced tactile allodynia. Furthermore, tactile allodynia evoked by the von Frey filaments in SNL rats was briefly reversed by PVN electrical stimulation or OXT administration (Fig. 3a). In both cases, the maximal anti-allodynic effect occurred 30 min after PVN electrical stimulation or i.t. neuropeptide treatment. By analyzing these data as AUC (Fig. 3b), we found that the nocifensive behavior induced by the von Frey filaments was reduced in the SNL rats treated with

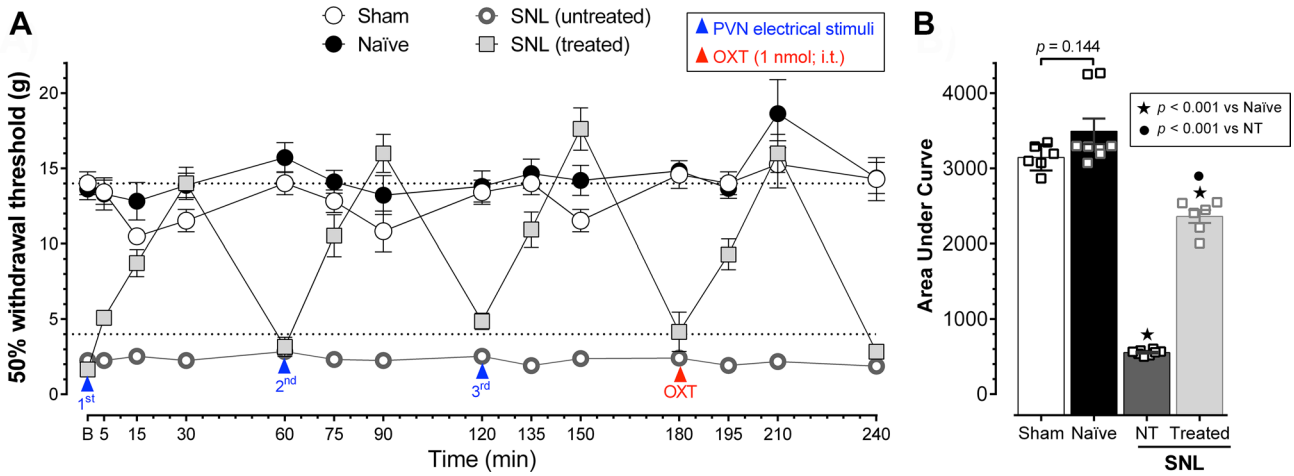

Fig. 3 Repetitive electrical stimulation of the PVN (blue arrow) or spinal OXT administration (red arrow) increases the nociceptive threshold of SNL rats (squares). **a** Time course (240 min) of the withdrawal threshold measured with von Frey filaments in naïve, shams and rats with SNL (treated and untreated). Note that electrical stimulation of the PVN (given three times at $t = 0$, 60 and 120 min; blue arrows) reverses the punctate mechanical allodynia induced by SNL (compared with the SNL untreated groups) and similar effects were observed with an i.t. OXT administration given at $t = 180$ min (red arrow). **b** The global effect of treatments (PVN stimulation and i.t. OXT administration) on the 50% withdrawal threshold. Data are expressed as mean ± SEM. Statistical analysis in (**b**) was performed using a one-way ANOVA followed by a Tukey post hoc test. *ANOVA* analysis of variance, *B* basal response, *NT* untreated, *SNL* spinal nerve ligation, *OXT* oxytocin, *PVN* hypothalamic paraventricular nucleus, *SEM* standard error of the mean

PVN electrical stimulation and i.t. OXT ($F_{3,24}=160.364$; $p<0.001$) (using the one-way RM ANOVA followed by the Tukey post hoc test).

Protocol 2. Repeated OXT administration reverts tactile allodynia and thermal and mechanical hyperalgesia

Results of the von Frey test showed that ligature of L5–L6 spinal nerves reduced the 50% paw withdrawal threshold response (< 4 *g*) when compared with naïve rats (> 12 *g*), indicating tactile allodynia (Fig. 4a). In contrast, the SNL subgroup treated with OXT (i.t. or i.pl.) exhibited a marked recovery of the withdrawal threshold, with a maximal effect at 45 min after OXT administration (reaching almost 12 *g*) with similar values to those observed in the naïve subgroup. This anti-allodynic effect is clearly depicted in Fig. 4b. By analyzing these data as AUC using the one-way RM ANOVA, we found that i.t. or i.pl. OXT significantly reduced ($F_{4,33}=197.007$; $p<0.001$) nocifensive behavior.

In the case of the Hargreaves test for thermal hyperalgesia, Fig. 5a shows that the withdrawal latency in the naïve subgroup was 13.1 ± 0.6 s and 14.0 ± 0.9 s for non-treated and vehicle administration groups, respectively. On the other hand, the withdrawal latency after SNL surgery in this group fell drastically to 4.0 ± 0.3 s (control SNL) and 3.5 ± 0.2 s (vehicle SNL). In contrast, thermal hyperalgesia was partially reversed by i.t. (7.8 ± 0.4 s) or i.pl. (8.9 ± 0.5 s) OXT. Interestingly, the withdrawal threshold between the i.t. or i.pl. OXT administration days was 4.18 ± 0.33 s, a similar value to those obtained in the SNL untreated rats. When we analyzed the withdrawal latency (s) using the one-way RM ANOVA ($F_{6,86}=59.804$; $p<0.001$), we found that withdrawal latency was partially recovered in the animals treated with i.t. or i.pl. OXT.

Using the Randall–Selitto test to measure mechanical hyperalgesia, we observed that the average withdrawal threshold for naïve rats was 185.6 ± 5.2 *g* and 196.3 ± 7.2 *g* for control and vehicle administration, respectively (see Fig. 5b). In contrast, the average withdrawal threshold for SNL rats was 71.3 ± 4.6 *g* and 86.3 ± 4.9 *g* for control and vehicle administration, respectively. The diminution of withdrawal threshold was partially reversed in the SNL rats treated with i.t. (170.6 ± 5.4 *g*) or i.pl. (144.4 ± 3.4 *g*) OXT. Furthermore, the withdrawal threshold measured between i.t. or i.pl. OXT administration days was 90.7 ± 6.0 *g*, a similar value to those obtained in the SNL untreated rats. Analyzing the withdrawal threshold (*g*) with the one-way RM ANOVA ($F_{6,86}=88.114$; $p<0.001$), we found that the withdrawal threshold was partially recovered in the animals treated with i.t. or i.pl. OXT.

Electrophysiological experiments

Although electrophysiological experiments were performed in a total of 28 rats, successful recordings were made from a total of 15 units in 15 rats (2nd subgroup = 3 cells; 3rd subgroup = 2

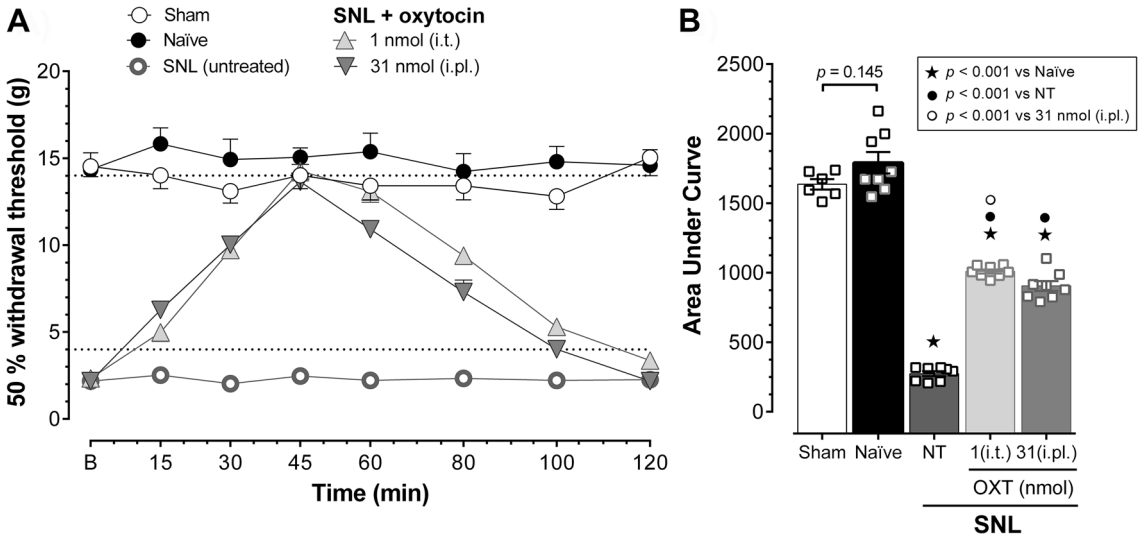

Fig. 4 Spinal or peripheral OXT reverses the tactile allodynia induced by SNL. Time course (**a**) or area under the curve (**b**) of the anti-allodynic effect observed after i.t. (1 nmol) or i.pl. (31 nmol) injection of OXT in rats subjected to L5-L6 SNL. **a**, **b** The values of naïve, sham, SNL (untreated) and SNL *plus* OXT. The 50% withdrawal threshold was assessed before and after OXT treatment at 15, 30, 45, 60, 80 and 100 min. **a** Notice the temporal evolution of sensitive changes after OXT treatment. **b** The area under the curve values is expressed with statistic comparisons. Data are expressed as mean ± SEM. Statistical analysis was performed using a one-way ANOVA followed by a Tukey post hoc test. *ANOVA* analysis of variance, *B* basal response, *i.pl* intraplantar, *i.t.* intrathecal, *NT* untreated, *OXT* oxytocin, *SNL* spinal nerve ligation, *SEM* standard error of the mean

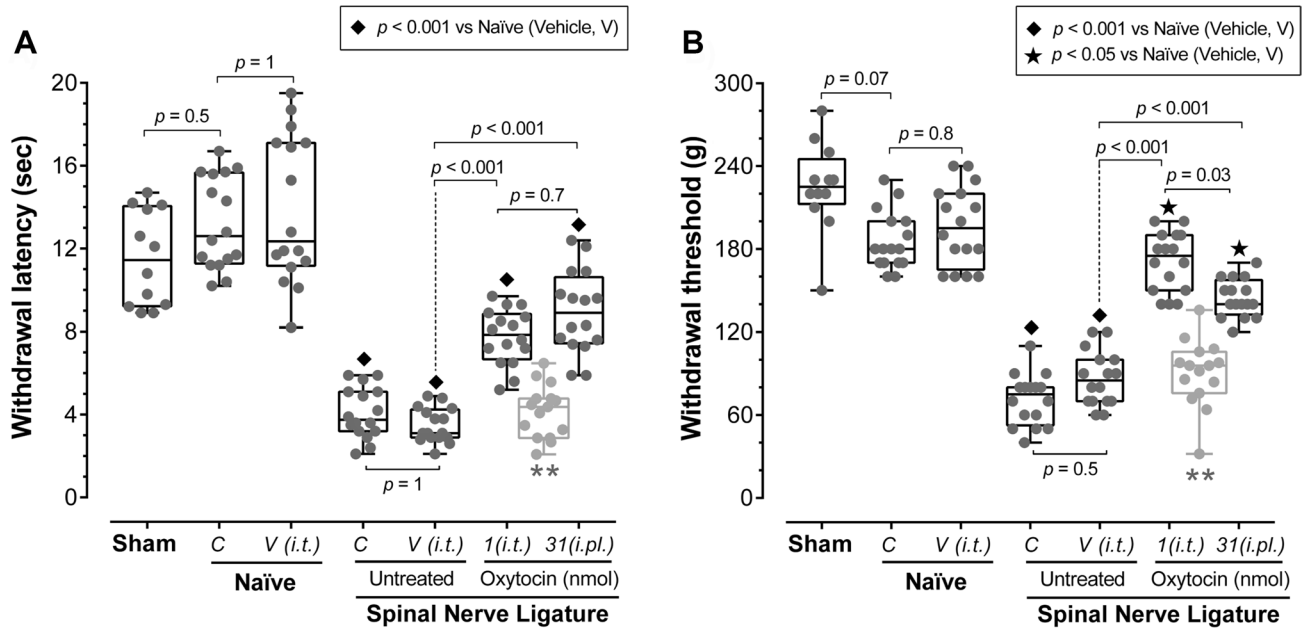

Fig. 5 Spinal or peripheral OXT reverses thermal (**a**) and mechanical (**b**) hyperalgesia induced by SNL. Box-plots showing the results obtained from the Hargreaves test (**a**) and Randall–Selitto pressure test (**b**). **a** The withdrawal latency values obtained using the Hargreaves test and **b** the withdrawal threshold values obtained from the Randall–Selitto test. I.t. or i.pl. OXT treatment partially reverses the neuropathic pain-like symptoms induced by SNL. Both tests were performed on different days and on the same rats. *To avoid possible bias, an internal control test in the OXT-treated group was performed between treatments (see gray box plot); the results show that no residual antinociceptive effects were found 1 day before the next OXT administration. Furthermore, in the case of vehicle treatment in the naïve subgroup, the administration was i.t. Statistical analysis was performed using a one-way ANOVA for repeated measures (with Greenhouse–Geisser correction) followed by a Tukey post hoc test. *ANOVA* analysis of variance, *i.pl* intraplantar, *i.t.* intrathecal

cells; 4th subgroup = 5 cells; and 5th subgroup = 5 cells). In SNL rats, a larger RF was found (Suzuki et al. 2000), probably reflecting a Wallerian degeneration as previously suggested (Montoya et al. 2002; Ye et al. 2015). Nevertheless, extracellular unitary recordings in the SDH were made exclusively from WDR neurons with a cutaneous RF in the left hindpaw (ipsilateral to SNL). These neurons were characterized according to the properties of their responses to peripheral RF stimulation and were classified as WDR neurons since they were activated by non-noxious and noxious stimuli and presented Aβ-, Aδ- and C-fiber responses after peripheral RF electrical stimulation (see Figs. 2, and 6). Since in some experiments the units were recorded (using the Microdrive Eckhorn system) at a mean depth of 1235 ± 247 µm below the surface, we consider that the cells were primarily found in the lamina V of the dorsal horn. Indeed, in the case of WDR cells recorded with glass microelectrodes, the cells were iontophoretically marked in the lamina V (see Fig. 2f).

Protocol 2. Spinal OXT seems to diminish the evoked neuronal activity of WDR cells of animals 25 days (or more) post-SNL surgery

With this protocol, the number of animals with specific WDR responses was low ($n = 3$ in the 2nd and $n = 2$ in the 3rd subgroup). In general, we found a lack of spinal cord activity or a silent cell that with the recording electrode started to emit high firing rates. Although data for the WDR neuronal activity associated with Aβ-, Aδ- and C-fibers were recorded within the ranges of 0–20 ms, 21–90 ms and 90–350 ms, respectively, there was not enough power to perform statistical analysis. Regardless, Fig. 6 shows an example of the WDR neurons recorded in these animals. Note that OXT diminished the evoked neuronal responses produced by electrical stimulation of the RF. At this point, we must keep in mind that Wallerian degeneration of the primary afferent fibers (i.e., deafferentation) could be significant 25 days after SNL surgery impeding us from successfully recording WDR neurons with reliable peripheral inputs (Montoya et al. 2002; Ye et al. 2015). The validation of this hypothesis led us to perform additional electrophysiological experiments using animals at 15–17 days post-SNL surgery as reported in similar electrophysiological experiments (Chapman et al. 1998; Dalmolin et al. 2017).

Protocol 3. Spinal OXT or PVN electrical stimulation diminishes the evoked neuronal activity of WDR cells at 15–17 days post-SNL surgery

Figure 7 shows the evoked neuronal responses of spinal convergent WDR cells in animals at 15–17 days post-SNL.

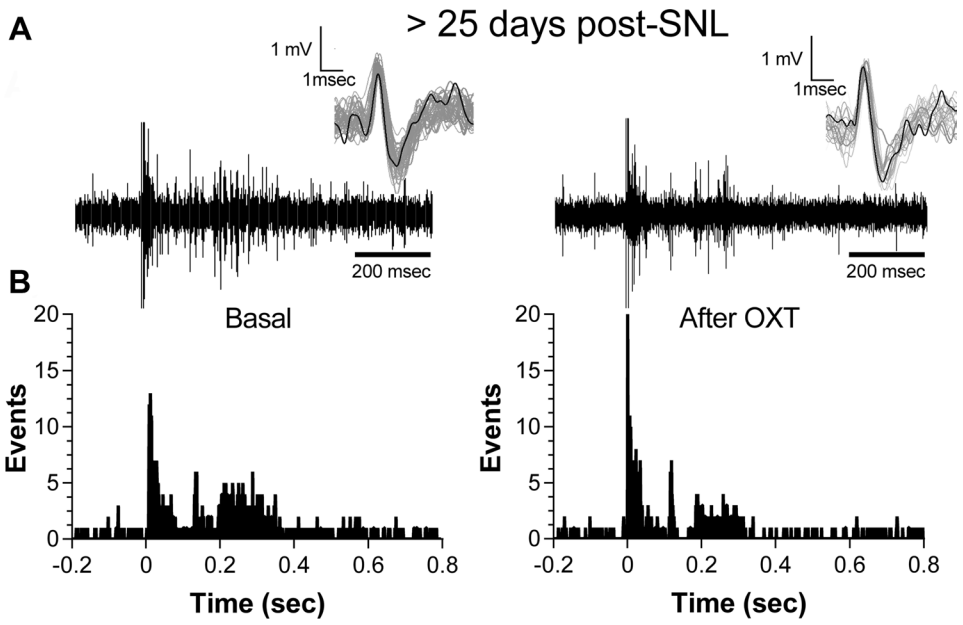

Fig. 6 Recording of WDR cells (**a**) and PSTH (**b**) in rats with more than 25 days of SNL. **a** A single evoked neuronal raw data of single peripheral stimuli (Basal) and after spinal topical OXT (1 nmol) administration. **b** PSTH constructed by 20 RF stimuli before and after OXT administration. Notice the decrease of nociceptive evoked responses between 0.2 and 0.4 s. It is important to notice that this kind of cell was very difficult to record in these rats, probably by a hyperpolarization of this kind of cell (see Fig. 8). *OXT* oxytocin, *PSTH* peri-stimulus time histogram, *SNL* spinal nerve ligation

In this set of rats, we had a better success index to record WDR cells; one that allowed us to statistically analyze the effect of topical OXT administration on the evoked WDR responses. Figure 7a shows the raw data of a single evoked WDR response obtained before and after 1 nmol OXT administration (given spinally). The PSTH constructed from 20 evoked responses (Fig. 7b) suggests that after OXT administration, the number of evoked potentials associated with Aδ- and C-fiber activation diminishes. Indeed, when we constructed a temporal course of the percentage change of neuronal activity (Fig. 7c) obtained from 10 units ($n=5$ units naïve and $n=5$ units from SNL rats), we found that (using the two-way RM ANOVA) spinal OXT selectively diminishes the neuronal activity of Aδ- ($F_{1,8}=52.192$; $p<0.001$) and C-fibers ($F_{1,8}=19.821$; $p=0.002$), but not Aβ-fibers ($F_{1,8}=2.481$; $p=0.154$).

Discussion and conclusion

General

This study demonstrates that recurrent i.t. or i.pl. OXT administration reverses tactile allodynia and thermal and mechanical hyperalgesia induced by SNL. These data reinforce previous studies suggesting the potential therapeutic usage of this neuropeptide in chronic pain conditions. Although previous reports using behavioral and electrophysiological approaches showed that i.t. OXT (~ 1–2 nmol/rat) relieves neuropathic pain (Condés-Lara et al. 2005, 2006; Miranda-Cardenas et al. 2006), these studies were designed for single dose acute testing and failed to analyze if recurrent OXT administration retained its analgesic effect. In this sense, the present study reports that the administration of OXT every other day for 12 days diminishes evoked pain-like responses in a chronic model of pain. This behavioral antinociception agrees with the electrophysiological experiments showing that at spinal level the nociceptive input of rats with SNL still responds to OXT treatment.

Recurrent oxytocin administration inhibits nociception

We chose the doses to administer OXT based on previous electrophysiological and behavioral studies showing that ≈ 1 nmol i.t. (Chow et al. 2016; Condés-Lara et al. 2005; 2006; Miranda-Cardenas et al. 2006; Rojas-Piloni et al. 2007) or 31 nmol i.pl. (González-Hernández et al. 2017) OXT administration induces antinociception. This allowed us to directly translate previous findings into a well-established model of neuropathic pain. Also, the doses used did not affect locomotor coordination (González-Hernández et al. 2017; Manzano-García et al. 2018). This point is relevant in view of that assessment of locomotor parameters gives us a better interpretation about the specific analgesic effect of a compound (Yaksh et al. 2015).

Under our experimental conditions and 10 days after SNL surgery, the tactile allodynia measured with von Frey filaments was well established. Moreover, repeated PVN stimulation or OXT administration (spinal or peripheral) reduced the nociceptive pain-like behavior tested with von Frey filaments (Figs. 3 and 4), Hargreaves test (Fig. 5a) and Randall–Selitto pressure test (Fig. 5b). When the nociceptive responses were analyzed with the "up-and-down" method in animals treated with OXT or PVN stimulation, an inverted U-shaped curve was obtained (see Figs. 3 and 4); this type

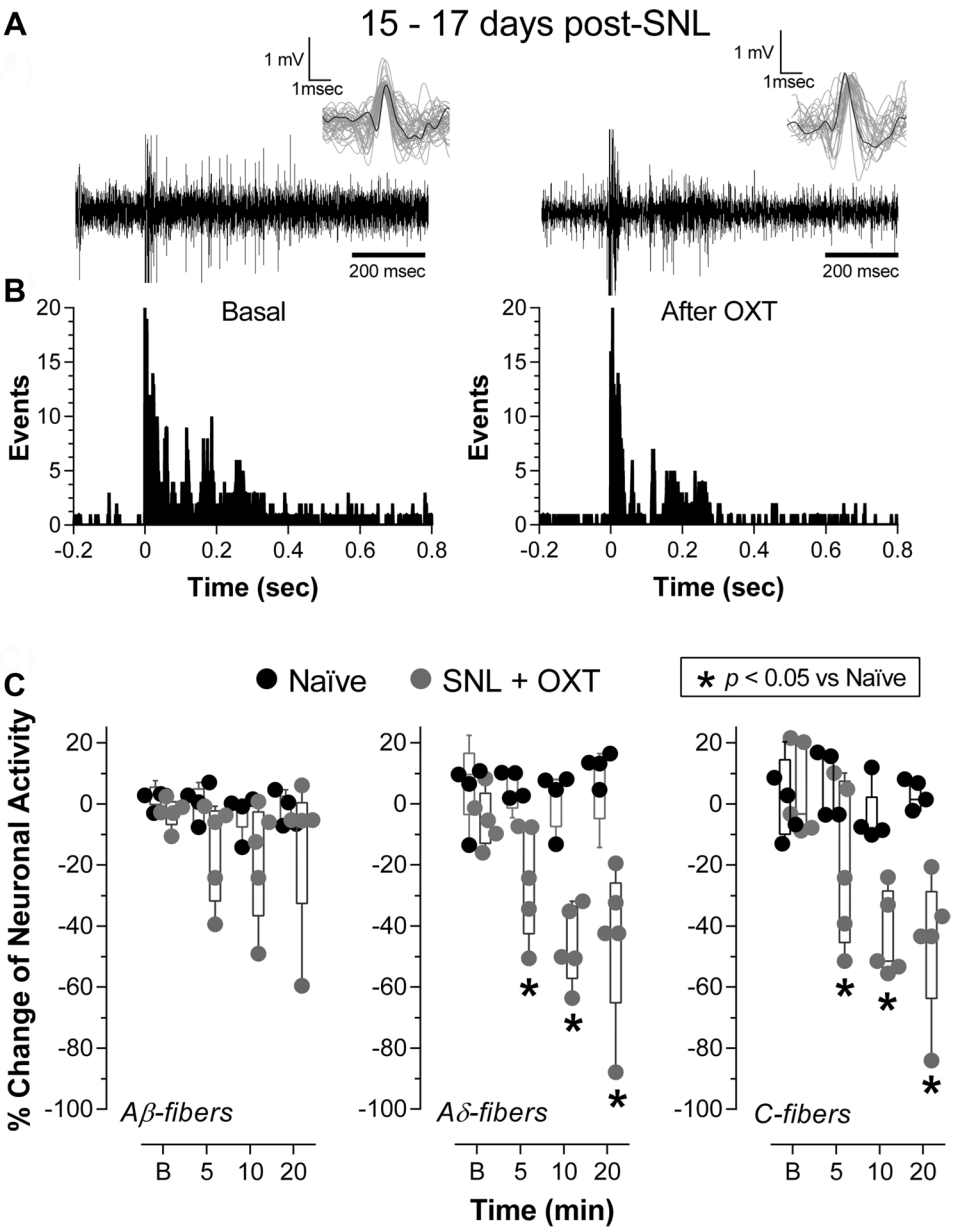

Fig. 7 Effect of spinal OXT on the neuronal activity of WDR cells in rats with 15–17 days of SNL. **a**, **b** The raw responses (upper) and the PSTH (below) obtained in the electrophysiological experiments. Notice that after 0.1 nmol OXT (given topically at spinal level) the neuronal-evoked response from electrical stimulation of the receptive field is reduced. The neuronal-evoked response is markedly minor (200 and 400 ms), corresponding to the C-fibers response. **c** The temporal effect (% change of neuronal activity) of OXT on the Aβ- Aδ- and C-fibers is illustrated in WDR cells of naïve and SNL rats. Notice that OXT does not have a statistical diminution on the neuronal activity associated to activation of Aβ-fibers. However, after OXT, the neuronal nociceptive activity associated to activation of Aδ- and C-fibers was achieved. *PSHT* peri-stimulus time histogram, *SNL* spinal nerve ligation. In (**c**) statistical analysis was performed using a two-way RM ANOVA followed by a Tukey post hoc test. *RM ANOVA* repeated measures analysis of variance, *B* basal response, *OXT* oxytocin, *SNL* spinal nerve ligation

of curve may reflect pharmacodynamic and pharmacokinetic aspects of the action elicited by OXT during neuropathic pain. A similar pattern of action was reported in a recent study (Sun et al. 2018) evaluating how spinal OXT relieves nociception in a neuropathic pain model (partial sciatic nerve ligation).

One interesting finding in the electrophysiological experiments was the fact that in rats with 25 days (or more) post-SNL surgery, the localization of suitable WDR neurons activated by peripheral electrical stimuli was low due to a lack of evoked spinal cord activity. This effect could reflect in part Wallerian degeneration (Montoya et al. 2002; Ye et al. 2015). Nevertheless, as shown in Fig. 8, some "silent" cells seem to be under powerful hyperpolarization considering that when we inject current to depolarize the membrane, the "silent" cell started to present spontaneous action potentials and responses to peripheral electrical stimuli. Furthermore, in rats with 15–17 days post-SNL surgery, we consistently found WDR neurons responding to peripheral stimuli and the neuronal nociceptive activity was inhibited by OXT (Fig. 7).

At this point, we need to keep in mind that we used several nociceptive tests in the same animal to mimic the nature of multifactorial stimulus to evoke pain during neuropathic condition. Furthermore, using several nociceptive tests in the same animal, we avoid potential learned responses (Deuis et al. 2017).

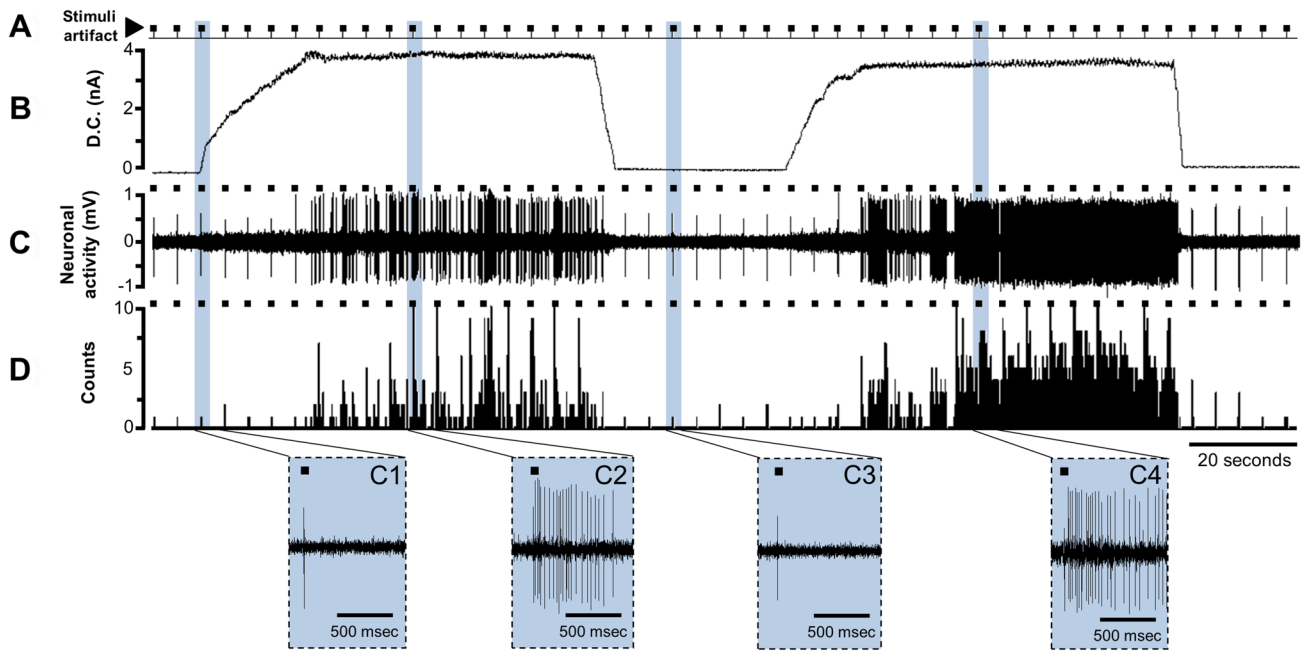

Fig. 8 Juxtacellular electrophysiological recording of silent cell after 25 days of SNL. The upper trace (**a**) shows artifacts (black squares) of electric stimulation on the specific RF. **b** The D.C. recording of the extracellular neuronal current shows the activation (depolarization) of the recorded cell by no more than 4 nA DC injection current using the glass microelectrodes. **c** The neuronal activity shows that the depolarizing current injected activates the electrophysiological recording activity of a silent WDR cell. **d** The histogram of frequency discharge is depicted. The lower traces (C1, C2, C3 and C4) are examples of neuronal activity evoked by the RF electrical stimulation (black squares); C1 and C3 illustrates the non-response to RF electric stimulation, whereas C2 and C4 panels illustrates the cell responses to the RF stimulation during the depolarization current injected. This is an example of several silent cells recorded in rats after at least 25 days of SNL. It seems that the silent cells are under a powerful hyperpolarization action

Further evidence supporting the anti-neuropathic effect of oxytocin

Our findings concerning antinociception (behavioral and electrophysiological) induced by spinal OXT agree with several studies reporting that acute i.t. OXT results in antinociception in experimental models of neuropathic pain (Boada et al. 2019; Condés-Lara et al. 2005; 2012; De la Torre et al. 2009; Martínez-Lorenzana et al. 2008; Miranda-Cardenas et al. 2006; Sun et al. 2018). Similar analgesic or antinociceptive effects have been reported after intracerebroventricular OXT administration in humans (Madrazo et al. 1987) and rats (Arletti et al. 1993). More recently it was reported that recurrent epidural OXT in a human patient (with chronic pain secondary to prostatic and metastatic bone cancer) relieves pain over the course of 6 weeks (Condés-Lara et al. 2016).

In fact, OXT could be a relevant endogenous analgesic molecule considering that the levels of OXT in the cerebrospinal fluid (CSF) are higher during chronic pain than during acute pain in dogs (Brown and Perkowski 1998) and rats (Sun et al. 2018). Certainly, PVN electrical stimulation increases OXT in the CSF and spinal cord in rats with SNL (Martínez-Lorenzana et al. 2008). In humans with chronic lower back pain, an increase of OXT in the CSF has been also reported (Yang 1994). These data suggest that chronic pain increases OXT levels in the CSF, probably to counterbalance the nociceptive input.

Certainly, the parameters used to stimulate the PVN not only provoked behavioral analgesia (Miranda-Cardenas et al. 2006), but also suppressed the neuronal nociceptive-evoked response associated with the activation of Aδ- and C-fibers (Condés-Lara et al. 2009). It is well documented that the main neuromediator involved in the PVN-induced antinociception at the spinal cord level is OXT. Nevertheless, we cannot exclude that after PVN stimulation, several supraspinal structures are engaged to consequently enhance descending analgesic mechanisms (Eliava et al. 2016; Godínez-Chaparro et al. 2016; Wang et al. 2003).

Since some studies suggest that OXT inhibits peripheral nociceptors (de Araujo et al. 2014; Eliava et al. 2016; González-Hernández et al. 2017; Juif and Poisbeau 2013; Kubo et al. 2017), a further aim was to investigate whether OXT exerts behavioral analgesia when given peripherally. Our results show that peripheral OXT administration also induced antinociception in SNL animals. Certainly, it has been demonstrated that OXT receptors (OTRs) are expressed in the DRG (Boada et al. 2019; Moreno-López et al. 2013)

and, accordingly, OXT produces antinociception at peripheral level (de Araujo et al. 2014; Eliava et al. 2016; Nersesyan et al. 2017), probably by activation of OTRs present in the terminal nerve endings (González-Hernández et al. 2017). In addition, OXT could directly modulate (via desensitization) the activity of TRPV1 channels (Nersesyan et al. 2017).

Likewise, OTR was found in mouse embryonic skin tissue (Hammock and Levitt 2013) and a previous study using an isolated ex vivo preparation showed that oxytocin could block the capsaicin-induced release of CGRP from dural nociceptors (Tzabazis et al. 2016). Together, these evidences support our contention about that oxytocin induces local antinociception by OTRs activation present in terminal nerve endings. Indeed, oxytocin is released by keratinocytes stimulation (Walker et al. 2017) and could explain the locally induced oxytocin effect.

Possible mechanisms of the OXT-induced antinociception during neuropathic pain

Although additional studies that fall beyond the scope of this investigation will be required to ascertain the precise mechanisms by which OXT (at peripheral or spinal level) or PVN electrical stimulation suppresses the noxious responses, one might speculate upon the possible mechanisms involved. At spinal level, regardless of the receptor (OTR or $V_{1A}R$ for a brief discussion see González-Hernández et al. [2014]), it has been demonstrated that OXT microinjection at single neuronal recorded WDR suppresses the glutamate-induced neuronal firing (Condés-Lara et al. 2003), probably by recruiting GABAergic inhibitory mechanisms at SDH neurons located in the substantia gelatinosa (Breton et al. 2008; Rojas-Piloni et al. 2007). Furthermore, spinal OXT could also recruit serotonergic (Condés-Lara et al. 2012; Godínez-Chaparro et al. 2016) and noradrenergic mechanisms (Rojas-Piloni et al. 2012) which add to its global antinociceptive effect. Also, OXT may directly (Nersesyan et al. 2017) or indirectly (Han et al. 2018; Sun et al. 2018) modulate TRPV1 channels and could potentiate the $GABA_A$ activity (Juif et al. 2013) that recruit molecular mechanisms which in turn modulate neuronal excitability. Altogether, these findings indicate the pleiotropic nature of OXT to induce antinociception.

In addition, we cannot discard the possibility that at spinal level, OXT injection could reach beyond the intrathecal space reaching peri-spinal tissue and the DRG, contributing to the antinociception induced by this peptide. Certainly, some evidence suggests that OXT exerts its antinociceptive effect by OTR activation at DRG (Hobo et al. 2012; Moreno-López et al. 2013). Hobo et al. (2012) showed that OXT inhibits intracellular Ca^{2+} increases in capsaicin-sensitive neurons. Similarly (Boada et al. 2019), during nerve injury induced by spinal L5 nerve ligature, intracellular electrophysiological recording of DRG neurons perfused with OXT showed that this neuropeptide hyperpolarized the low-threshold mechanoreceptors (which are desensitized) and desensitized the high-threshold mechanoreceptors (which are sensitized).

Some limitation and considerations on our experimental conditions

Admittedly, some limitations of our investigation could be that:

1. First, the use of the same animal to perform several nociceptive tests. Although this point could be considered as an issue, we think that the approach followed allowed us to weigh the potential analgesic effect of OXT in a real-set condition; i.e., different pain-like behaviors induced by specific activation of distinct components of neuropathic pain. This line of reasoning gains weight when considering that the electrophysiological recordings performed at the end of the behavioral experiments showed that under SNL, spinal OXT inhibits preferentially the activity of nociceptive specific input (Figs. 6, 7 and 8).
2. Second, the experiments were performed in male rats, and Uhl-Bronner et al. (2005) demonstrate that the OXT-binding sites at the spinal cord level are denser in males than in females. A recent report showed that i.t. OXT (~ 1 nmol) in mice under neuropathic injury elicited a potent analgesic effect in males, whereas in females only a weak effect was observed (Chow et al. 2018). Besides, in rats with inflammatory nociception, i.t. injection of 0.125 nmol OXT elicited antinociception in males, whereas the same effect in females was observed not until 1.25 nmol (Chow et al. 2016). Regardless of the animal's sex, OXT exerts an antinociceptive effect depending on the dose.
3. Third, intrathecal OXT administration. Although the spinal puncture procedure in the present study was performed as described by Mestre et al. (1994), we need to keep in mind that this procedure is highly invasive and stressful. Admittedly, in a context of potential clinical use, recurrent spinal injections are disregarded, but, our experimental approach was designed to allow us to investigate the effect of recurrent OXT administration in nociceptive behavior.

Conclusion

In this proof of principle study, we showed that recurrent OXT administration (given spinally or peripherally) diminishes pain-like behaviors in a model of neuropathic pain, probably by inhibiting primary afferent nociceptive activity.

Our findings provide a basis for addressing the therapeutic relevance of OXT in chronic pain conditions. Investigating the mechanisms by which OXT exerts antinociception in neuropathic pain is clearly central to understand the mechanisms by which we could block neuropathic pain.

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Author contributions AEM-Z contributed to the acquisition, analysis and interpretation of data and participated in drafting the manuscript. AG-H, GM-L and MC-L contributed to the conception and design of the study; acquisition, analysis and interpretation of data; and participated in drafting the manuscript. All authors participated in a critical review of the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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