Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice

Running head: Pregabalin and behavioural outcomes of neuropathic pain


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Chronic neuropathic pain in rodents can be associated with anxiety-like and depressive-like behaviour alterations, and cognitive function impairment. In this study, we have validated different behavioural outcomes allowing a reliable measurement of the emotional and cognitive manifestations of neuropathic pain induced in mice by partial sciatic nerve ligation. These results underline the relevance to evaluate these multiple pain-related alterations to improve the predictive value of preclinical drug discovery.

The authors report no conflicts of interest.
Abstract

**Background:** Preclinical drug discovery for the treatment of chronic pain is at present challenged by the difficulty to study behaviours comparable to the complex human pain experience in animals. Several reports have demonstrated a frequent association of chronic pain in humans with affective disorders, such as anxiety and depression, and impaired cognitive functions, including memory and decision-making, and motivation for goal-directed behaviours. In this study, we validated different behavioural outcomes to measure the emotional and cognitive manifestations of neuropathic pain induced in mice by partial sciatic nerve ligation.

**Methods:** In these mice, we evaluated at different time points the nociceptive responses, the anxiety- and depressive-like behaviours, the anhedonic state, object recognition memory and the operant responding maintained by food, and the effects of the repeated administration of pregabalin on these manifestations.

**Results:** Our results demonstrated that the presence of allodynia and hyperalgesia in neuropathic pain mice was associated with increased anxiety- and depressive-like behaviours, reduced memory functions, development of an anhedonic state and impaired motivation to obtain food in the operant task. Chronic pregabalin treatment improved the nociceptive, anxiety-like and anhedonic responses, as well as the memory deficit, but did not modify the depressive-like alterations and the decreased motivation in these mice.

**Conclusions:** These results indicate that some emotional manifestations of chronic pain do not necessarily resolve when pain is relieved, and underline the relevance to evaluate multiple behavioural responses associated with chronic pain, including the affective-motivational and cognitive behaviours, to increase the predictive value of preclinical drug discovery.
1. Introduction
Neuropathic pain is defined by the International Association for the Study of Pain as a clinical entity initiated or caused by a primary lesion or dysfunction in the nervous system, which is associated with spontaneous pain, hyperalgesia and allodynia. Only a minority of patients receives an appropriate treatment for their neuropathic pain that is further limited by important side effects associated with the drugs currently available (Attal and Bouhassira, 2015). Many analgesic targets emerged from preclinical studies in the past decades, although most of them failed in the early phases of clinical trials. These disappointing results raised the question of the preclinical validity of basic pain tests used in animals compared to the complexity of chronic pain in humans. Previous preclinical animal studies to assess the efficacy of analgesics have mainly focused on the sensory-discriminative component of pain by evaluating behavioural responses to external mechanical or thermal stimuli (Ewan and Martin, 2013). However, ongoing spontaneous pain could be unrelated to external stimulation and may also impair other behaviours, such as emotional and cognitive responses. Neuropathic pain in humans is frequently associated with affective disorders, such as anxiety and depression, and impaired cognitive functions, including memory (Knaster et al., 2012; Moriarty et al., 2011; Radat et al., 2013). These emotional and cognitive alterations have been recently investigated in preclinical studies (Liu and Chen, 2014). Moreover, chronic pain also impairs several aspects of reward processing (Becker et al., 2012) and has been associated with anhedonia (i.e. the inability to feel pleasure) (Andersen et al., 2009; Bura et al., 2013) and decreased reward sensitivity and motivation (Ozaki et al., 2002; Schwartz et al., 2014). Pain and the related emotional and cognitive alterations could aggravate each other leading to a vicious cycle that negatively affects the quality of life of patients. Therefore, the design of experimental approaches that allow evaluating the
different nociceptive, emotional and cognitive manifestations of neuropathic pain would be essential to increase the preclinical validity for future analgesic discovery. So far, studies in rodent neuropathic pain models have produced contradictory results when investigating these pain-associated alterations, depending on differences in the strain, time point of measurements, behavioural paradigms and surgical procedures used (Liu and Chen, 2014). Moreover, most of these preclinical studies analyzed only a small part of the myriad of pain-associated behaviours, providing mere partial information on the complexity of pain experience.

In the present study, we evaluated the nociceptive, emotional and cognitive changes promoted at different time points after partial sciatic nerve ligation (PSNL) in mice, a widely used model of neuropathic pain (Malmberg and Basbaum, 1998). Pregabalin, a first line therapeutic agent for neuropathic pain, has demonstrated its effectiveness in relieving allodynia and hyperalgesia in animal models of neuropathic pain (Verma et al., 2014), although no information is available on other pain-related manifestations. We have validated our model of affective and cognitive manifestations of neuropathic pain by evaluating the effects induced by the repeated administration of pregabalin.

2. Materials and methods

2.1 Animal experimental conditions

Swiss albino male mice (Charles River, Lyon, France) were used in all the experiments. Mice were 8-12 weeks old at the beginning of the experiments and were housed in groups of 3 to 4 with free access to water and food. The housing conditions were maintained at 21 ± 1°C and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 8:00 AM and 8:00 PM). A light/dark reverse cycle (light off at 08:00 AM and on at 08:00 PM) was used in experiments 2 and 3 (see next sections). All
experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and were approved by the local ethical committee (Comité Etico Experimental Animal, Instituto Municipal de Asistencia Sanitaria/Universitat Pompeu Fabra). All the experiments were performed under blind conditions and the treatments randomized between groups.

2.2 Drugs and treatments

Pregabalin (generously provided by Laboratorios Dr. Esteve, Barcelona, Spain) was dissolved in physiological sterile saline solution (0.9%) and administered intraperitoneally (ip) twice daily at the dose of 20 mg/kg. Control mice received the ip administration of physiological sterile saline solution (0.9%). In all the experiments, the pregabalin treatment started one week after the PSNL surgery when the majority of pain-related alterations are already established. The duration of this treatment was dependent on the specific experimental sequence (see next sections).

2.3 Neuropathic pain induction

PSNL was performed to induce neuropathic pain, as previously described (Castañé et al., 2006; Malmberg and Basbaum, 1998). Mice were anaesthetized with isoflurane and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the sciatic nerve using an 18-in. (9–0) non-absorbable virgin silk suture (Alcon® Surgical Inc., TX, USA), leaving the rest of the nerve “undamaged”. Control mice underwent sham surgery, consisting in the same procedure used for PSNL, but in this case the sciatic nerve was not ligated.
2.4 Nociceptive behaviour

*Mechanical allodynia* was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation through the up–down paradigm, as previously reported (Chaplan et al., 1994).

*Heat hyperalgesia* was assessed by evaluating the hind paw withdrawal latency in response to radiant heat with the plantar test apparatus (Ugo Basile, Italy), as previously reported (Hargreaves et al., 1988).

*Cold allodynia* was assessed with the hot/cold plate analgesia meter (Columbus, USA), as previously described (Bennett and Xie, 1988). The number of elevations of each hind paw was recorded for 5 min on the cold surface of the hot/cold plate analgesia meter, which was maintained at a temperature of 5 ± 0.5°C (Bura et al., 2013). A score was calculated for each animal as the difference of number of elevations between ipsilateral and contralateral paws (Bura et al., 2013).

2.5 Anxiety-like behaviour

The elevated plus maze test (EPM) was used to evaluate anxiety-like behaviour and performed in a black Plexiglas apparatus with four arms, two open and two closed, set in cross from a neutral central square elevated 40 cm above the floor. Five-min test sessions were performed and the percentage of entries and time spent in the open arms was determined, as previously reported (Busquets-Garcia et al., 2011).

2.6 Cognitive behaviour

Object recognition memory (ORM) was performed in the V-maze (Panlab, Spain) to measure cognitive performance and a discrimination index was calculated, as previously described (Puighermanal et al., 2009) (supplemental methods).
2.7 Depressive-like behaviour

Forced swimming test (FST) was performed to evaluate the depressive-like behaviour (Porsolt et al., 1977). Mice were gently lowered into a plastic cylinder containing water (23°–25°C), deep enough to prevent touching the bottom of the cylinder and forcing the mouse to swim. Test duration was 6 min. The mouse was considered immobile when it floated in an upright position and made only small movements to keep its head above water. Because little immobility was observed during the first 2 min, the duration of immobility was quantified over the last 4 min of the 6-min test period.

2.8 Anhedonia model

The anhedonic state was evaluated in metabolic boxes (Phecomp, Panlab, Spain) by measuring the preference for a 2% sucrose solution over water during a test session of 24h (supplemental methods).

2.9 Operant behaviour maintained by food

**Operant chambers**

Sessions of operant responding maintained by food were performed in mouse operant chambers (model ENV-307A-CT, Med Associates Inc., Georgia, VT, USA), as previously described (Guegan et al., 2013) (supplemental methods).

**Food pellets**

Two types of 20 mg precision food pellets were used: standard pellets (TestDiet, IN, USA) or highly palatable chocolate-flavored pellets (TestDiet, IN, USA) (supplemental methods).

2.10 Experimental protocol

**First experimental sequence.** The effects of a chronic treatment with pregabalin were evaluated in the nociceptive, affective and cognitive manifestations of neuropathic pain 30 min after the injection of pregabalin or saline on the corresponding experimental day.
We chose specific “acute” models to analyze nociception (von Frey filament stimulation, plantar test, cold plate test), anxiety-like behaviour (EPM), depressive-like behaviour (FST) and memory (ORM), as previously reported (Liu and Chen, 2014). These tests were performed in parallel in the same cohort of mice because they require nearly the same housing and experimental conditions, including a non-inverted day–night cycle where the behavioural measurements were taken during a short time over a limited time window during the day, when rodents are naturally less active. Two different experimental groups were used to evaluate these responses at two different time points (one and three weeks after surgery) to reduce the adaptation of mice to these paradigms, as previously reported (Yalcin et al., 2011). Briefly, after the measurement of nociceptive baseline responses (day -1), PSNL or sham surgery was performed and nociceptive responses assessed again on days 3, 6, 16 and 27 in both experimental sequences. In the first set of mice (group 1) the affective (EPM, FST) and cognitive (ORM) behaviours were evaluated one week after the PSNL or sham surgery. Another set of mice (group 2) received the chronic administration of pregabalin (20 mg/kg) or saline (control), twice daily, from day 8 until the end of the experiments (day 27), and the affective and cognitive behaviours were evaluated three weeks after PSNL when pain-related alterations are fully established. The effects of chronic pregabalin treatment in comparison with saline treatment were only evaluated in this second experimental group. Mice of group 1 also received chronic saline administration to maintain the same experimental conditions.

Second experimental sequence. The effects of pregabalin treatment on the anhedonic state associated with neuropathic pain were evaluated in the metabolic boxes (Phecomp, Panlab, Spain) (supplemental methods, Fig.S1b). In this experiment, we considered long-term day and night measurements (24h test sessions), which are important for the
analysis of such spontaneous behaviours that reflect the natural wellbeing of rodents during chronic pain states. After one week of habituation in the reversed light/dark cycle, mice were individually trained on 24 h sessions in these cages every second day during one week to be familiarized to the new environment and drink taste. After each training session, mice were placed again in the home cages where they were kept housed in cohort to minimally interfere with the social activity that is important for rodent spontaneous behaviour. Baseline values of drink intake were measured after this habituation (day -2). Two days later, mice were exposed to the PSNL or sham-surgery followed by 24 h sessions in the monitoring boxes on days 1, 5, 10, 15 and 20 after surgery. The chronic treatment with pregabalin (20 mg/kg) or saline, twice daily, started on day 7 and ended on day 17 after PSNL surgery. Mice were placed in the monitory boxes immediately after treatment on day 10 and 15. The nociceptive responses were also evaluated in the von Frey and plantar models before PSNL surgery (day -1) and on days 3, 6, 11, 17 and 21 post-surgery (Fig.S2). The cold plate test was not performed in order to minimize the possible influence of these nociceptive measurements on the highly sensitive anhedonic responses (the same applies to the third experimental sequence).

**Third experimental sequence.** The effects of pregabalin treatment were evaluated on an operant responding maintained by food under the neuropathic pain state (supplemental methods, Fig.S1c). Mice were individualized in a room with the reversed light/dark cycle 7 days before the beginning of the experiments to reduce the possible stress induced by the isolation of mice during each daily 1h session in the operant chamber that could bias the responses evaluated. The reversed light-dark cycle, in which mice are naturally more active, was essential to allow an appropriate acquisition of the operant tasks. The nociceptive responses were assessed in the von Frey and plantar models.
before (day -1) and after PSNL on days 3, 6 and 27 (Fig.S3). On day 6 (three days before starting the operant responding sessions) mice were food-deprived to maintain the 85% of their ad libitum initial weight adjusted for growth. This food restriction was maintained during the first 5 training days (days 9-13) to allow an appropriate acquisition of the operant task with standard pellets, whereas animals were fed ad libitum during the remaining days of training (from day 14 to 27). During the whole experiment water was available ad libitum. Chronic pregabalin (20 mg/kg) or saline treatment started on day 7 and lasted until day 27 after PSNL surgery. Mice were tested 30 min after drug administration. The operant responding sessions were divided in three phases. During the first phase (days 9-14), standard pellets were used in conditions of food deprivation to permit an appropriate responding to obtain food, and mice were trained during 5 days (days 9-13) on a fixed-ratio 1 (FR1) schedule of reinforcement. On day 14 mice were exposed to a progressive ratio (PR) schedule in which the response requirement to earn one pellet escalated according to the following series: 1-5-12-33-51-75-90-120-155-180-225-260-300-350-410-465-540-630-730-850-1000-1200-1500-1800-2100-2400-2700-3000-3400-3800-4200-4600-5000-5500. The PR session lasted 4 h or until mice stopped responding for at least 1 h. The breaking point was determined in each animal as the last response ratio completed. During the second (days 15-20) and third phases (days 21-26), the standard pellets were replaced by highly palatable chocolate-flavored pellets and mice were fed ad libitum to avoid the influence of food deprivation on the operant responses maintained by this highly palatable food. In the second phase, mice were trained for 5 additional days (days 15-19) on FR1 and a second PR was performed on day 20. In the third phase, a fixed ratio 5 (FR5) schedule was used during 5 more days (days 21-25). Finally, on day 26 mice were exposed to a third PR.
2.11 Statistical analysis
Data obtained in the EPM, FST and ORM were analyzed by one-way (surgery) or two-way ANOVA (surgery and treatment), whereas data obtained in the nociception and anhedonia models were analyzed by three-way repeated measures ANOVA (surgery and treatment as between-subject factors and day as within-subject factor). Data of PR and active responses in the operant responding paradigm were analyzed by two-way (treatment and surgery) or repeated measures four-way ANOVA (treatment and surgery as between subject factors, and day and phase as within subject factors), respectively. Post hoc analysis (Fisher’s LSD) was performed after ANOVA when appropriate. STATISTICA 6.0 software (StatSoft, Inc., OK, USA) was used. The differences were considered statistically significant when the $P$ value was below 0.05.

3. Results
3.1 First experimental sequence: effects of pregabalin chronic treatment in the nociceptive responses associated with neuropathic pain.

The nociceptive responses during neuropathic pain were evaluated in both experimental groups (group 1 and group 2), as described in the previous section. The responses in both groups were similar after saline treatment (data not shown).

Mechanical allodynia (von Frey stimulation model) (Fig. 1a). Three-way ANOVA for the ipsilateral responses to mechanical stimulation revealed a significant effect of surgery ($F_{(1,52)}= 734.07; P< 0.001$), treatment ($F_{(1,52)}= 15.24; P< 0.001$) and day ($F_{(4,208)}= 83.53; P< 0.001$), and interaction between these factors ($F_{(4,208)}= 3.89; P< 0.01$), whereas no significant effects were revealed for the contralateral responses. Subsequent post hoc analysis indicated that the baseline values for both ipsilateral and contralateral hind paws were similar in all mouse groups before PSNL or sham surgery. Sham
surgery did not modify the nociceptive responses. In contrast, PSNL significantly decreased the withdrawal threshold in the ipsilateral, but not in the contralateral paw. Saline treatment did not modify any of the nociceptive responses, whereas pregabalin treatment significantly increased the withdrawal threshold only in the ipsilateral paw of the PSNL group. However, the ipsilateral withdrawal threshold in PSNL mice was still significantly different from sham mice after pregabalin treatment. Therefore, chronic pregabalin treatment significantly improved the mechanical allodynia induced by PSNL in the ipsilateral paw.

**Heat hyperalgesia (plantar test)** (Fig. 1b). Three-way ANOVA for the ipsilateral responses revealed a significant effect of surgery ($F_{(1,45)} = 184.67; P< 0.001$), treatment ($F_{(1,45)} = 8.90; P< 0.01$) and day ($F_{(4,180)} = 18.72; P< 0.001$), and interaction between these factors ($F_{(4,180)} = 3.76; P< 0.01$), whereas no significant effects were revealed for the contralateral responses. Subsequent post hoc analysis indicated that the baseline values for both ipsilateral and contralateral hind paws were similar in all mouse groups before PSNL or sham surgery. Sham surgery did not modify the nociceptive responses. In contrast, PSNL significantly decreased the withdrawal latency in the ipsilateral, but not the contralateral paw. Saline treatment did not modify any of the nociceptive responses, whereas pregabalin treatment significantly increased the withdrawal latency only in the ipsilateral paw of the PSNL group. However, the ipsilateral withdrawal latency in PSNL mice was still significantly different from sham mice after pregabalin treatment. Therefore, chronic pregabalin treatment significantly improved the thermal hyperalgesia induced by PSNL in the ipsilateral paw.

**Cold allodynia (cold plate test)** (Fig. 1c). Three-way ANOVA for the score value revealed a significant effect of surgery ($F_{(1,52)} = 43.31; P< 0.001$) and day ($F_{(4,208)} = 20.22; P< 0.001$), and interaction between these factors ($F_{(4,208)} = 14.56; P< 0.001$).
Subsequent *post hoc* analysis indicated that the baseline score values were similar in all mouse groups before PSNL or sham surgery. Sham surgery did not modify these nociceptive responses. In contrast, PSNL significantly increased the score value. Saline treatment did not modify any of the nociceptive responses. However, no significant differences between sham and PSNL mice treated with pregabalin were revealed. Therefore, the chronic treatment with pregabalin reduced the cold allodynia induced by PSNL.

### 3.2 First experimental sequence: effects of pregabalin chronic treatment in the affective and cognitive manifestations of neuropathic pain.

One week after PSNL, one-way ANOVA revealed a significant effect of surgery in the percentage of time spent in the EPM open arms ($F_{(1,25)} = 13.21; P < 0.01$) and in the ORM discrimination index ($F_{(1,26)} = 29.61; P < 0.001$), whereas no significant effect was revealed in the FST immobility time. Subsequent *post hoc* analysis indicated that PSNL significantly increased the anxiety-like behaviour, as revealed by a reduction in the percentage of time spent in the EPM open arms (Fig. 2a), and impaired memory, as indicated by a reduction in the ORM discrimination index in PSNL mice compared to sham at this time point (Fig. 2b). PSNL only induced a trend to develop depressive-like behaviour at this time point, as indicated by the immobility time in the FST (Fig. 2c).

Three weeks after PSNL, two-way ANOVA revealed a significant effect of surgery ($F_{(1,52)} = 18.04; P < 0.001$) and treatment ($F_{(1,52)} = 18.04; P < 0.001$) for the percentage of time spent in the EPM open arms, and a significant effect of surgery for the ORM discrimination index ($F_{(1,50)} = 19.18; P < 0.001$) and the FST immobility time ($F_{(1,52)} = 13.44; P < 0.001$). Subsequent *post hoc* analysis indicated that saline treatment did not modify any of the responses in these paradigms. Pregabalin treatment did not change the responses of sham mice compared to saline. In contrast, pregabalin treatment
significantly increased the percentage of time spent in the EPM open arms (Fig. 2d) and the ORM discrimination index (Fig. 2e) in PSNL mice compared to saline treatment. However, these responses remained significantly lower in PSNL mice compared to sham mice after pregabalin treatment (Fig. 2d, e) and this treatment did not reduce the increased immobility time of PSNL mice in FST (Fig. 2f).

Thus, chronic pregabalin treatment significantly improved the increased anxiety-like behaviour and the memory impairment induced by PSNL, but did not modify the increased depressive-like behaviour.

3.3 Second experimental sequence: effects of pregabalin chronic treatment on the anhedonic state associated with neuropathic pain

Three-way ANOVA for the percentage of sucrose preference revealed a significant effect of surgery ($F_{(5,220)}= 2.92; P< 0.05$) and day ($F_{(5,220)}= 3.186; P< 0.01$). Subsequent post hoc analysis indicated that baseline values of the percentage of sucrose preference were similar in all groups before PSNL or sham surgery. Sham surgery did not modify sucrose preference, whereas PSNL developed an anhedonic-like state, revealed by a significant decrease in the sucrose preference in PSNL compared to sham mice (Fig. 3). Saline treatment did not modify the sucrose preference in any of the groups and pregabalin treatment did not modify this sucrose preference in the sham group. In contrast, pregabalin treatment reversed the anhedonic-like state in PSNL mice, as revealed by a significant increase of the sucrose preference in this group compared to saline (Fig. 3).

Therefore, in parallel with the attenuation of the allodynic and hyperalgesic responses (supplemental results, Fig.S2), chronic pregabalin treatment completely reversed the anhedonic-like state induced by PSNL.
3.4 Third experimental sequence: effects of pregabalin chronic treatment on the operant responding to obtain food during neuropathic pain

Four-way ANOVA for the active responses in the operant task revealed a significant effect of surgery \( (F_{(1,40)}= 6.74; \ P< 0.05) \), day \( (F_{(4,160)}= 16.98; \ P< 0.001) \) and phase \( (F_{(2,80)}= 138.51; \ P< 0.001) \) and interaction between surgery, treatment and phase \( (F_{(2,80)}= 4.46; \ P< 0.05) \), whereas no significant effects were revealed for the inactive responses. Subsequent post hoc analysis indicated that no differences were observed between the different groups in the operant responding to obtain standard or highly palatable chocolate-flavored pellets on a FR1 schedule during the first and second phases, respectively (Fig. 4a). In contrast, PSNL impaired the operant responding to obtain chocolate-flavored pellets under FR5 in the third phase, as revealed by a significant decrease in the active responses in the saline PSNL group compared to sham (Fig. 4a). Saline treatment did not modify the active responses in any of the groups and pregabalin treatment did not modify these responses in the sham group. In contrast, pregabalin treatment completely reversed the decreased operant responding of PSNL mice during this phase, as revealed by a significant increase of the active responses in this group compared to saline (Fig. 4a).

Two-way ANOVA for the breaking point revealed a significant effect of surgery \( (F_{(1,40)}= 10.08; \ P< 0.01) \) and treatment \( (F_{(1,40)}= 4.85; \ P< 0.05) \), and interaction between these factors \( (F_{(1,40)}= 7.65; \ P< 0.01) \) in the PR at the end of the first phase. A significant effect of surgery \( (F_{(1,40)}= 7.75; \ P< 0.01) \) and treatment \( (F_{(1,40)}= 6.17; \ P< 0.05) \), and interaction between these factors \( (F_{(1,40)}= 16.88; \ P< 0.001) \) were revealed in the PR at the end of the second phase. A significant effect of surgery \( (F_{(1,40)}= 3.92; \ P< 0.05) \) and treatment \( (F_{(1,40)}= 4.66; \ P< 0.05) \), and interaction between these factors \( (F_{(1,40)}= 9.26; \ P< 0.01) \) were revealed in the PR at the end of the third phase. Subsequent post hoc
analysis indicated that PSNL impaired motivation, as revealed by a significant decrease of the breaking point in the saline PSNL group compared to sham in all the three PRs (Fig. 4b). Saline treatment did not modify the breaking point in any of the groups. In contrast, pregabalin treatment significantly reduced the breaking point in the sham group, although it did not further modify this breaking point in the PSNL group compared to saline in all the three PRs (Fig. 4b).

Therefore, chronic pregabalin treatment completely abolished the impairment in the FR5 operant responses induced by PSNL in parallel with the attenuation of the allodynic and hyperalgesic responses (supplemental results, Fig.S2). This treatment did not improve the decreased motivation for food in PSNL mice and decreased this motivation in sham mice.

4. Discussion

We characterized different nociceptive, emotional, cognitive and motivational manifestations of neuropathic pain produced by PSNL in mice and the effects of pregabalin treatment on these manifestations. Allodynia and hyperalgesia in this neuropathic pain model were associated with increased anxiety- and depressive-like behaviours, reduced memory functions, the development of an anhedonic-like state and impaired responses and motivation in operant tasks. Most of these pain-related alterations were significantly improved after a chronic pregabalin treatment.

The increased anxiety-like behaviour and impaired memory functions already appeared by week one and persisted at week three after PSNL, suggesting that the early presence of pain is sufficient for the development of these affective and cognitive alterations. In contrast, the increased depressive-like behaviour only appeared at week three after PSNL, suggesting that the depressive symptoms are manifested only once pain persists.
along time probably underlying different mechanisms than for anxiety and cognitive symptoms. Depressive-like behaviour usually appears later than anxiety-like behaviour after neuropathic pain induction (Yalcin et al., 2011). However, the affective responses are highly dependent on the behavioural paradigm used (Liu and Chen, 2014). As demonstrated in the present and previous studies, recognition and working memory are cognitive aspects particularly sensitive to be disrupted under neuropathic pain states (Dimitrov et al., 2014; Grégoire et al., 2012; Kodama et al., 2011). The mechanisms involved in this cognitive impairment have not been yet clarified. However, pain-related modifications in functional and structural plasticity have been reported in several brain regions during chronic pain (Kim et al., 2012; Liu and Chen, 2014; Luo et al., 2014; Tan and Waxman, 2014).

Pain and reward are opponent responses that interact and influence each other (Becker et al., 2012). Indeed, rewarding stimuli decrease pain sensitivity (Leknes and Tracey, 2008) and pain impairs reward processing, as demonstrated by the association of chronic pain with anhedonia (Marbach et al., 1983) and altered reward responsiveness (Becker et al., 2012; Elvemo et al., 2015). The reduced preference for highly palatable sweet solutions has been previously used to reveal anhedonia in different chronic pain models (Andersen et al., 2009; Bura et al., 2013). Nutritional intake is mediated by taste-derived enjoyment and post-ingestion satisfactory experiences and their absence can reflect the development of anhedonia (Andersen et al., 2009). Therefore, the anhedonic-like state observed in PSNL mice seems to be associated with reduced reward responsiveness. We have further investigated if chronic neuropathic pain could alter reward responsiveness and motivation by using an operant paradigm. PSNL mice showed a reduction in the operant responding only when the efforts required to obtain highly palatable food were increased under the FR5 schedule, whereas the responses to
seek for standard or highly palatable pellets under FR1 were not affected. This behaviour was not due to motor activity impairment since the responses in the inactive lever were similar in sham and PSNL mice. The results obtained under FR5 were also mirrored by the reduced motivation to work for reward revealed in PSNL mice under PR schedules requiring progressively more effort to earn each subsequent food pellet. This reduced motivation did not depend on the type of reinforcement since a similar decrease in the breaking point was observed with standard and highly palatable pellets. In agreement with our results, the responding maintained by standard food as evaluated in the PR schedule was also reduced in another neuropathic pain model (Schwartz et al., 2014). The altered responses found in operant difficult tasks (FR5 and PR) may also be partially attributed to a learning impairment since operant responding highly depends on proper cognitive functions. Large overlaps exist in the anatomical and neurochemical substrates of pain and reward, which could explain the mutual influence between these processes (Leknes and Tracey, 2008). Notably, changes in the opioid and dopamine systems accompanied by alterations in neuronal activity and connectivity in the nucleus accumbens have been proposed as potential mechanisms of this altered reward processing during chronic pain in patients and animal models (Baliki et al., 2012; Becker et al., 2012; Chang et al., 2014; Elvemo et al., 2015; Schwartz et al., 2014).

We have demonstrated that PSNL in mice represents a suitable model to reveal different pain-associated co-morbidities in a short time period and have validated this model by evaluating the effects induced by repeated pregabalin treatment. Pregabalin is a gamma-aminobutyric acid analogue with high affinity for α2δ subunit of the P/Q type voltage-gated calcium channel that reduces the synaptic release of neurotransmitters and represents a first line compound for neuropathic pain (Micó and Prieto, 2012; Verma et al., 2014). We have shown that chronic pregabalin treatment produced antinociceptive
effects and reduced the anxiety-like and memory alterations, but not the acute depressive-like behaviour observed during neuropathic pain. Higher doses of pregabalin could not be evaluated since they induce important locomotor changes in rodents (Vartanian et al., 2006; Yokoyama et al., 2007), which would bias the behavioural responses evaluated (Liu and Chen, 2014). The anxiolytic effects of pregabalin have been widely reported in animals and humans (Micó and Prieto, 2012; Navarrete et al., 2012) and improving effects on the altered cognitive functions produced by long-term benzodiazepine treatment have been recently described (Oulis et al., 2014). In agreement with our results, chronic pregabalin treatment did not affect the depressive-like behaviour in a model of chronic inflammatory pain despite its analgesic effects (Maciel et al., 2013). These results suggest that depressive-like symptoms, once established, do not necessarily resolve when pain is treated. This conclusion is further supported by a study showing that mechanical allodynia produced by placing a plastic cuff around the sciatic nerve in mice completely resolved when the cuff was removed, whereas the affective manifestations persisted (Dimitrov et al., 2014). In contrast, the anhedonic-like state induced by PSNL in our study completely disappeared after chronic pregabalin treatment, when allodynia and hyperalgesia were only partially reversed. The different effects of pregabalin on the acute depressive-like and the anhedonic-like states could be due to the specific behavioural response evaluated in each of the two paradigms. Indeed, the immobility analysed in the forced swimming test is directly related to the inability or reduced motivation to maintain effort in an inescapable situation, whereas the sucrose preference includes different components of the reward processing (wanting, liking and learning) that are related to the appetitive, consummatory and satiety phases of a pleasure cycle (Thomsen, 2015). Pregabalin treatment also completely abolished the impairment induced by neuropathic pain in the
food-maintained operant responses under FR5 schedule. The improvement of anhedonic and operant responses by pregabalin in PSNL mice could be an indirect consequence of pain relief, although a direct effect of this drug on the reward processing cannot be excluded. Repeated pregabalin treatment reduced operant responding to obtain alcohol and cocaine, whereas the responses to obtain food pellets where not affected (de Guglielmo et al., 2013; Spencer et al., 2014; Stopponi et al., 2012) accordingly with our results in sham mice. However, we have also observed a decreased responding under PR schedule in sham mice after pregabalin treatment, indicating a specific impairing effect of this drug on motivation and not on the general intake of food in normal physiological conditions. This decrease in motivation to seek for food under PR schedule in sham mice was comparable with that of PSNL after pregabalin treatment. These similar responses found in sham and PSNL groups make difficult the interpretation of the results obtained after pregabalin treatment in PSNL mice. The mechanisms by which pregabalin could produce this impairing effect on motivation have not been explored. Similarly to its structural analogue gabapentin, pregabalin would decrease the release of several neurotransmitters, including glutamate and dopamine in brain areas regulating reward processing (Reimann, 1983; Spencer et al., 2014). This effect in brain areas controlling goal-directed behaviours could potentially contribute to the impaired responses observed in sham mice after chronic pregabalin treatment. Affective, cognitive and motivational symptoms develop as a result of ongoing pain after nerve injury and, in turn, could also influence pain manifestations. We did not investigate to which extent these different alterations could affect pain behaviour, although we cannot exclude that their relief by pregabalin could also contribute to a reduced pain perception.
In conclusion, this study validates behavioural models to evaluate multiple responses associated with chronic pain. These responses include the nociceptive, affective-motivational and cognitive dimensions, which more closely reflect human experience and potentially increase the predictive value for preclinical drug discovery. Indeed, we have demonstrated that a well validated analgesic drug, pregabalin, improved pain and several related emotional and cognitive manifestations, but is inadequate to modify the acute depressive-like manifestations. The effects of other analgesics used for neuropathic pain treatment (e.g. opioids) were not evaluated. Opioid effects on emotion, motivation, reinforcement and food intake (Bodnar, 2012) would profoundly influence the responses in the behavioural paradigms used in this study and further complicate the interpretation of the results. Our data also reveal the need to further investigate in future studies the emotional and cognitive alterations associated with chronic pain to optimize the future therapeutic approaches and minimize side-effects.
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The authors report no conflicts of interests.

Author contributions

C.L.P and I.M.L.M participated in experimental design, performed the experiments, and contributed to write the manuscript. R.N. performed a part of the experiments. R.M. conceived the study, participated in experimental design, supervised and wrote the manuscript. All authors discussed the results, commented and approved the final version of the manuscript.
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Figure legends

Figure 1. Effects of pregabalin on nociceptive behaviour of mice exposed to PSNL.
Mice were tested in the ipsilateral and contralateral paws to evaluate mechanical allodynia, heat hyperalgesia, and cold allodynia under basal conditions and on days 3, 6, 16, and 27 after PSNL or sham surgery. From day 8 to 27, mice were chronically treated with pregabalin (20 mg/kg, twice daily) or saline and the pain responses were evaluated 30 min after the administration on days 16 and 27 (n= 14 per group). (a) Mechanical allodynia evaluated by the von Frey model (withdrawal thresholds, g). (b) Heat hyperalgesia evaluated in the plantar test (paw withdrawal latencies, s). (c) Cold allodynia evaluated in the cold plate test, score values: difference in the number of elevations between the ipsilateral and contralateral paws. The dotted line indicates the test days in which the nociceptive responses are evaluated under pregabalin or saline chronic treatment. Values are expressed as mean ± SEM. ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001 vs. sham surgery (Fisher’s LSD test). ☆☆☆ P < 0.001 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation.

Figure 2. Effects of pregabalin on affective and cognitive behaviours in mice exposed to PSNL. The percentage of entries and time spent in EPM open arms (a, d), the ORM discrimination index (b, e) and the FST immobility time (c, f) were evaluated one and three weeks after the PSNL or sham surgery. The effects of the chronic pregabalin (20 mg/kg, twice daily) or saline treatment (from day 8 to 25) in these paradigms were evaluated 30 min after the administration at three weeks post-surgery. Data are expressed as mean ± SEM (n= 14 per group). ★ P < 0.05, ★★★ P < 0.001 vs. sham surgery (Fisher’s LSD test). ☆ P < 0.05, ☆☆ P < 0.01 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation; EPM, elevated plus maze; ORM, object recognition memory; FST, forced swimming test.
Figure 3. Effects of pregabalin on anhedonic-like manifestations in mice exposed to PSNL. Percentage of sucrose preference during 24 hour sessions was evaluated in the monitoring system (Phecomp boxes) before and after PSNL or sham surgery every fifth day during 20 days (day -2, 1, 5, 10, 15, 20). From day 7 to 17, mice were chronically treated with pregabalin (20 mg/kg, twice daily) or saline. The last anhedonia session (day 20) was performed in the absence of pregabalin treatment to analyze any possible residual effect of this administration procedure on these responses. The dotted line indicates the test days in which the behavioural responses are evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n= 12 per group). ★ P < 0.05 vs. sham surgery (Fisher’s LSD test). ☆ P < 0.05 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation.

Figure 4. Effects of pregabalin on operant responses to obtain food in mice exposed to PSNL. (a) Active and inactive lever presses were evaluated after PSNL or sham surgery under FR1 with standard pellets (phase 1, days 9-13), FR1 with chocolate-flavored pellets (phase 2, days 15-19) and FR5 with chocolate-flavored pellets (phase 3, days 21-25). (b) Breaking point achieved in the PR sessions at the end of phases 1 (day 14), 2 (day 20) and 3 (day 26) in PSNL or sham mice. From day 7 to 27, mice were chronically treated with pregabalin (20 mg/kg, twice daily) or saline and the behavioural responses evaluated 30 min after the administration. The dotted line indicates the test days in which the behavioural responses are evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n= 10-12 per group). ★★★ P < 0.01, ★★★★ P < 0.001 vs. sham surgery (Fisher’s LSD test). ☆☆ P < 0.01, ☆☆☆☆ P < 0.001 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation; FR1, fixed ratio 1; FR5, fixed ratio 5; PR, progressive ratio.
**Figure 1**

(a) **Ipsilateral**

- Withdrawal threshold (g)
- Treatment: Saline sham, Pregabalin sham, Saline PSNL, Pregabalin PSNL

(b) **Contracontrolateral**

- Withdrawal latency (s)
- Treatment: Saline sham, Pregabalin sham, Saline PSNL, Pregabalin PSNL

(c) **Score**

- Basal, Day 3, Day 6, Day 16, Day 27
- Treatment: Saline sham, Pregabalin sham, Saline PSNL, Pregabalin PSNL
Figure 3

Treatment

- Sham Basal
- PSNL Day 1
- Sham Day 5
- PSNL Day 10
- Sham Day 15
- PSNL Day 20

- Saline treatment on day 10 and 15
- Pregabalin treatment on day 10 and 15
Figure 4

(a) Deprived 85% | Ad libitum

FR1 Standard food | FR1 Highly palatable food | FR5 Highly palatable food

Phase 1 | Phase 2 | Phase 3

Treatment

(b) Saline | Pregabalin

Breaking point

Phase 1

Phase 2

Phase 3

Breaking point

Active lever presses

Inactive lever presses

Day 9 | Day 13 | Day 15 | Day 19 | Day 21 | Day 25

Figure 4
Supplementary methods

Object recognition memory

This task consists of three sessions: habituation, training and test. On day 1, mice were habituated for 9 min to the V-maze. On the second day, mice were put back in the maze for 9 min, two identical objects were presented and the time that mice spent exploring each object was recorded. Mice were again placed in the maze 24 h later for 9 min, one of the familiar objects was replaced with a novel object and the total time spent exploring each of the two objects (novel and familiar) was computed, and a discrimination index was calculated as the difference between the times spent exploring either the novel or familiar object divided by the total time exploring the two objects. A higher discrimination index is considered to reflect greater memory retention for the familiar object (Puighermanal et al., 2009).

Anhedonia model

The anhedonic state was evaluated in metabolic boxes (Phecomp, Panlab, Spain). These boxes consist of a food and drink monitoring system recently validated in our laboratory to evaluate the preference for a palatable food and/or drink with an extremely high sensitivity (less than 0.02 g for both food and drink) (Bura et al., 2010, 2013). In this study, two different drink solutions were used: water and a palatable drink represented by 2% sucrose solution. The anhedonic state was evaluated in mice exposed to the PSNL by measuring the preference for the 2% sucrose solution over water during a test session of 24h. The percentage of mean sucrose preference was calculated as the ratio of the sucrose solution intake to total liquid intake x 100.
Operant behaviour maintained by food

Operant chambers

The chambers were equipped with two retractable levers, one randomly selected as the active and the other as the inactive. Pressing the active lever resulted in a pellet delivery together with a stimulus-light for 2 s (associated cue), while pressing the inactive lever had no consequences. A food dispenser equidistant between the levers permitted delivery of food pellets. The beginning of each session was signalled by turning on a house light placed on the ceiling of the box for 3 s, which was then turned off during the remaining duration of the session. The active and inactive levers were counterbalanced between animals. A time-out period of 10 s was established after each pellet delivery where no cues were presented and no reward was provided following an active response. The session was finished after 100 reinforcements were delivered or after 1 h, whichever occurred first.

Pellet composition

Two types of 20 mg precision food pellets were used: standard pellets (TestDiet, IN, USA) or highly palatable chocolate-flavoured pellets (TestDiet, IN, USA). The standard pellet formula was similar to the standard maintenance diet provided to mice in their home cage: 24.1% protein, 10.4% fat, 65.5% carbohydrate (sucrose content was 3.09% of the total quantity of carbohydrates), with a caloric value of 3.30 kcal/g. Highly palatable chocolate-flavoured pellets contain 2% pure unsweetened cocoa and are composed of 20.5% protein, 12.7% fat, 66.8% carbohydrate (sucrose content was 50.11% of the total carbohydrates), with a caloric value of 3.48 kcal/g. This different composition, together with the cocoa content, makes these chocolate-flavoured pellets much more palatable than standard pellets.
Rationale of the experimental procedures

In the present work, we aimed to characterize different behavioural models for the study of neuropathic pain in mice that span long periods of time and involve multiple measurements. Therefore, we combined different behavioural tests addressing several pain components and pain-related co-morbidities and evaluated the effects of pregabalin treatment on these manifestations.

In the first set of experiments, we chose specific “acute” models (elevated plus maze, object recognition and forced swimming tests) to evaluate the emotional (anxiety- and depressive-like responses) and cognitive (memory) manifestations of chronic pain, as previously reported (Liu and Chen, 2014; La Porta et al., 2015). These tests were performed in parallel in the same cohort of mice because they require nearly the same housing and experimental conditions. The chronological sequence for these tests was chosen depending on the level of stress produced on the animal by each experimental paradigm that could affect the behavioural responses in the subsequent one (e.g. forced swimming test was the last test to be performed since it represents the most stressful experimental condition). The same tests could not be carried out repetitively in the same animal due to the rapid mouse adaptation to these paradigms (Yalcin et al., 2011). Therefore, two different mouse groups were required to study the time-dependent patterns of these “acute” emotional and cognitive pain-associated responses during the first and the third weeks after surgery.

In the second and third experimental sequence, we studied the effects of neuropathic pain on reward processing. We assessed the anhedonic-like state by using the sucrose preference model in the second experimental sequence, and then we further investigated the reward responses by analyzing the operant behaviour maintained by food in the third sequence. In these experiments, the behavioural responses were measured repetitively in
the same cohorts of mice to monitor the appearance and evolution of the alterations in reward processing induced by the presence of pain over the course of time. Mice were kept housed in cohort for the sucrose preference test to minimally interfere with the social structures that are important for rodent spontaneous behaviour (Tappe-Theodor and Kuner, 2014). These mice were individually tested in the metabolic cages during 24h sessions, which represent lower stress conditions than the operant training due to a longer duration of the test that was not performed every day. For the operant paradigm, we used the standard experimental conditions previously described that include mouse isolation (Guegan et al., 2013). This housing condition is mandatory to reduce the possible stress induced by the isolation of mice during each daily 1h session in the operant chamber that could bias the responses evaluated. Mice were food-deprived in the operant paradigm, but only during the first phase of training with standard pellets to allow an appropriate acquisition of the operant task, whereas animals were fed ad libitum during the remaining days of this experimental sequence to avoid the influence of food deprivation on the operant responses maintained by highly palatable pellets (Guegan et al., 2013). Beside the reward responses, the operant paradigm allowed the simultaneous evaluation of the cognitive responses because mice have to learn to obtain the food under different schedules of reinforcement.

In all the experiments, the pregabalin treatment started one week after the PSNL surgery when the majority of pain-related alterations are already established. The duration of this treatment was dependent on the specific experimental sequence. In the case of the first and third sequences, the pregabalin injections continued until the last day of experiments to maintain the same experimental conditions for each test (first experimental sequence) or each phase (third experimental sequence) evaluated. In the second set of experiments, this treatment lasted 11 days (from day 7 to 17 post-surgery)
because we already observed a significant reversion of the anhedonic responses at day 15. Therefore, the last anhedonia session (day 20) was performed in the absence of pregabalin treatment to analyze any possible residual effect of this administration procedure on these responses.

References:


Supplementary results

Second experimental sequence: effects of pregabalin chronic treatment in the nociceptive responses associated with neuropathic pain

Three-way ANOVA revealed a significant effect of surgery ($F_{(1,44)} = 55.25; P < 0.001$), treatment ($F_{(1,44)} = 12.82; P < 0.001$) and day ($F_{(5,220)} = 92.56; P < 0.001$), and interaction between these factors ($F_{(5,220)} = 9.45; P < 0.001$) for the ipsilateral responses in the von Frey model, and a significant effect of surgery ($F_{(1,44)} = 102.73; P < 0.001$), treatment ($F_{(1,44)} = 30.34; P < 0.001$) and day ($F_{(5,220)} = 186.63; P < 0.001$), and interaction between these factors ($F_{(5,220)} = 29.88; P < 0.001$) for the ipsilateral responses in the plantar model. Subsequent post hoc analysis indicated that the basal responses in both the von Frey and plantar models were similar in all groups (Fig. S2). Sham surgery did not modify the nociceptive responses. In contrast, PSNL surgery significantly increased the nociceptive responses in these models compared to sham (Fig. S2). Saline treatment did not modify any of the nociceptive responses, whereas pregabalin treatment significantly reduced these nociceptive responses in the PSNL group (Fig. S2). However, these responses in PSNL mice were still significantly different from sham mice after pregabalin treatment (Fig. S2). The nociceptive responses of the contralateral paw were not modified by either surgery or treatment (data not shown).

Third experimental sequence: effects of pregabalin chronic treatment in the nociceptive responses associated with neuropathic pain

Three-way ANOVA, revealed a significant effect of surgery ($F_{(1,44)} = 404.74; P < 0.001$), treatment ($F_{(1,44)} = 17.81; P < 0.001$) and day ($F_{(3,132)} = 69.71; P < 0.001$), and interaction between these factors ($F_{(3,132)} = 12.81; P < 0.001$) for the ipsilateral responses in the von Frey model, and a significant effect of surgery ($F_{(1,44)} = 136.55; P < 0.001$) and day...
(F(3,132)= 28.62; P< 0.001), and interaction between surgery, treatment and day (F(3,132)= 5.69; P< 0.01) for the ipsilateral responses in the plantar model. Subsequent post hoc analysis indicated that the basal responses in both the von Frey and plantar models were similar in all groups before sham or PSNL surgery (Fig. S3). Sham surgery did not modify the nociceptive responses, whereas PSNL surgery significantly increased the nociceptive responses in these models compared to sham (Fig. S3). Saline treatment did not modify any of the nociceptive responses. In contrast, pregabalin treatment significantly reduced these nociceptive responses in the PSNL group (Fig. S3). However, these responses in PSNL mice were still significantly different from sham mice after pregabalin treatment (Fig. S3). The nociceptive responses of the contralateral paw were not modified by either surgery or treatment (data not shown).
Figure S1

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Figure S1. Experimental sequence to evaluate the nociceptive, affective and cognitive behaviours (a), the anhedonic-like state (b), and the operant responding to obtain food (c) after pregabalin chronic treatment in mice exposed to PSNL. PSNL, partial sciatic nerve ligation; NR, nociceptive responses; EPM, elevated plus maze; ORM, object recognition memory; FST, forced swimming test; FR1, fixed ratio 1; FR5, fixed ratio 5; PR, progressive ratio.
Figure S2. Experiment 2: effects of pregabalin on nociceptive behaviour of mice exposed to PSNL. Mechanical allodynia (withdrawal threshold, g) and heat hyperalgesia (withdrawal latency, s) were evaluated under basal conditions and on days 3, 6, 11, 17 and 21 after PSNL or sham surgery. From day 7 to 17, mice were chronically treated with pregabalin (20 mg/kg, twice daily) or saline. The dotted line indicates the test days in which the behavioural responses are evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n= 12 per group). ★★ P < 0.01, ★★★ P < 0.001 vs. sham surgery (Fisher’s LSD test). ☆☆☆ P < 0.001 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation.
Figure S3. Experiment 3: effects of pregabalin on nociceptive behaviour of mice exposed to PSNL. Mechanical allodynia (withdrawal threshold, g) and heat hyperalgesia (withdrawal latency, s) were evaluated under basal conditions and on days 3, 6, and 27 after PSNL or sham surgery. From day 7 to 27, mice were chronically treated with pregabalin (20 mg/kg, twice daily) or saline and the behavioural responses evaluated 30 min after the administration. The dotted line indicates the test days in which the behavioural responses are evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n= 10-12 per group). ★ P < 0.05, ★★★ P < 0.001 vs. sham surgery (Fisher’s LSD test). ☆☆☆ P < 0.001 vs. saline treatment (Fisher’s LSD test). PSNL, partial sciatic nerve ligation.