Graded photochemical spinal cord injury results in chronic hyperalgesia and depression-like behaviour but no anxiety exacerbation in female BALB/c mice

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ABSTRACT

Neuropathic pain (NP) is present in 40-to-50% of spinal cord injured patients. It tends to chronicity and correlates with lower quality-of-life. Moreover, the role of NP in the eventual exacerbation of anxiety- and depression-like behaviours during its development and chronification in genetically susceptible individuals remains unclear. Thus, although solely few animal models are available, new specific models are needed to complete the array of chances to assay new therapeutic strategies with the aim of treating chronic NT and its associated mood disorders. The present study was conceived to evaluate hyperalgesic responses and anxiety- and depression-like behaviours after graded photochemical spinal cord injury (SCI) up to chronic phase. BALB/c strain was used: it expresses a phenotype characterized by high innate anxiety levels, allowing to elucidate whether NP may exacerbate mood disorders at SCI chronic phase. After different photoinduction-times on exposed spinal cord, the mice developed a graded chronic hyperalgesia with minor to non-existent motor dysfunction. Behavioural data suggest that whilst hyperalgesia associated to SCI does not exacerbate BALB/c anxiety-like behaviours, it may result in depression-like behaviour at SCI chronic phase. Our study demonstrates that chronic central hyperalgesia may exacerbate despair-like behaviour at the SCI chronic phase in a mouse model of high anxiety-related behaviour. This implies that photochemical-SCI may be a suitable model to study the comorbidity between chronic NP and mood disorders.

Key words: behavioural disturbances; immunohistochemistry; mice; neuropathic pain; photochemical spinal cord injury

1. Introduction

Up to two-thirds of subjects affected by spinal cord injury (SCI) develop central neuropathic pain (NP) [1,2], which implies a high likelihood of severe daily-life disabilities [3]. Moreover, NP is commonly associated with a higher vulnerability to develop emotional disorders such as depression and anxiety [4], especially when becomes chronic pain [5]. The co-occurrence of chronic pain and mental disorders implicates that both conditions often coexist reciprocally exacerbating each other and sharing physiological pathways and neurotransmitter alterations [6]. Therefore, a better understanding of the surrounding psychiatric aspects might be an important factor in the diagnosis and treatment of NT.

Even though several studies have already suggested the comorbidity of NP and emotional disorders [6], it remains unclear whether NP might exacerbate anxiety- and depression-like behaviours during its development and chronification in genetically susceptible individuals. Moreover, this health concern is especially important to be elucidated in females since epidemiological data highlights a higher prevalence of chronic pain and vulnerability in the development of comorbid pain and depression in female [7,8]. To this end, the present study was developed with the aim of evaluating anxiety- and depression-like behaviours associated with chronic central hyperalgesia in female BALB/c, which is a mice strain with specific phenotype characterized by high basal anxiety levels [9], besides being one of the most widely used inbred strains in biomedical research [9].

In order to develop central chronic hyperalgesia, BALB/c mice were subjected to SCI up to chronic phase, by means of photochemical insults performed with Rose Bengal (RB; Sigma-Aldrich, MO, USA) photosensitive dye, which may be a complement or an alternative to a usual spinal cord contusion model. RB is an effective generator of singlet oxygen that causes an injured area with macrophage infiltration, axonal demyelination and reactive astrocytes and microglial cells [10], which are similar findings observed after traumatic SCI [11]. While SCI-photochemical rat model has been successfully used in neurotraumatic research [10,12], the mice model has been lesser explored [13,14] and none of them has still been studied up to the chronic SCI-phase. Therefore, a better understanding of SCI model in the chronic phase could be useful since NP usually develops within the first year and tends to become chronic [3] increasing the risk to develop associated mood disorders. The lack of efficient treatments to relieve NP [15,16] justifies the need for animal models suitable to perform chronic NT, especially if they take into account the genetic background, which may influence the progress of neuronal diseases [17,18], such as NT.

Overall, the main objectives of the present study were to confirm the hyperalgesic responses of BALB/c mice due to graded photochemical SCI model, analyse the SCI-associated locomotor dysfunction and evaluate the exacerbation of chronic pain-related mood disorders.

2. Materials and methods

2.1. Animal husbandry and Ethics

Fifty adult female BALB/c mice (22-25 gr) were obtained from Charles River Laboratories (France).. Mice were housed in groups of 5-6 animals per cage, with access to food and water *ad libitum* in a room kept at 19-22ºC and 40-60% humidity, under a 12:12 hour's light/dark cycle. Four animals died during the

surgical procedures. The testing order was chosen randomly across all the procedures to prevent an order effect. All experimental procedures adhered to the recommendations of the EU and the US Department of Health for the care and use of laboratory animals and they were approved by the Animal Ethics Committee of the Generalitat de Catalunya (Spain) and Universitat de Barcelona (DARP6308).

2.2. Surgical procedure

BALB/c mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed prone on a heating pad to maintain constant levels of body temperature. After back disinfection with povidone iodide, the spinal cord was exposed by a dorsal laminectomy at T8-T9 vertebrae. The dura was carefully cut and RB (1.5% in saline; Sigma-Aldrich, Spain) was applied directly on the exposed SCI for 10 min. The lesion was induced by illuminating the exposed dorsal surface of the spinal cord by means of one optic fibre positioned 10 mm on top of the cord. The optic fibre was connected to a halogen lamp (100 W), and the spinal cord was illuminated at a light intensity of 95 kLux for 1 min (RB1, n=9), 2 min (RB2, n=8), 5 min (RB5, n=10) and 10 min (RB10, n=10) respectively. To obtain reproducible light intensity, the irradiation power at the fibre outlet was measured using a lux-tester [10]. Furthermore, to prevent any eventual damage caused by the halogen lamp calorific source, the spinal cord was bathed with saline solution during the illumination time [7,8,10]. In sham animals (n=9), the exposed spinal cord was bathed with RB for 10 min and rinsed with saline, but it was not illuminated. Animals were randomly allocated to experimental groups (sham, RB1, RB2, RB5 or RB10) once no differences between subjects in anxiety were observed prior to the surgery procedures.

2.3. Thermal hyperalgesia

The plantar algesimetry technique (Hargreaves test) was used to evaluate nociceptive sensitivity [19] at 1, 4 and 8 weeks after SCI photoinduction. As previously described [20], mice were placed into a plastic box with an elevated glass floor (Plantar Test Algesimeter, model # 37370; Ugo Basile, Comerio, Italy) and the light of a projection lamp was focused onto the plantar surface of the hindpaw with a time-limit of 30 sec to avoid skin damage. The withdrawal latency of the heated paw was measured through a time-meter coupled with infrared detectors directed to the plantar surface of the right paw. The value for a test was the mean of three consecutive trials, separated by 5 min resting periods.

2.4. Locomotor activity

Locomotor activity was evaluated by means of the Basso Mouse Scale for locomotion (BMS) test [21] and a Gait topography analysis was also assessed, according to Parker and Clarke [22]. Both tests were performed at 0 (before surgery), 1, 4 and 8 weeks post-injury. At the same time-points, locomotor activity was also evaluated in OF tests, as explained further below.

2.4.1. Basso Mouse Scale for locomotion (BMS)

BMS test was performed as described elsewhere [20]. Briefly, one animal at a time was allowed to move freely inside a circular plastic tray (70-cm diameter x 30-cm wall height) and two independent examiners observed the hind-limb movements of the mouse and scored the locomotor function according to the Basso Mouse Scale [21], which ranges from 0 (no hind-limb movement) to 9 (normal movement-coordinated gait). The final score of each animal was the mean value of both examiners.

2.4.2. Gait topography

Steeping movements' evaluation was based on Parker and Clarke [22] description as in previous study [23]. After marking the mouse hind and forefeet with a non-toxic ink (blue and red ink, respectively), the animal walked across chart paper in a pathway, leaving a permanent record of its footprints. Stride length and stride width were measured.

2.5. Behavioural evaluation

The anxiety-like behaviour of mice was assessed by using the Open Field and the Dark/light box tests at 0 (before surgery), 1, 4 and 8 weeks' post-injury. On the other hand, despair-like behaviour was evaluated by means of the Forced Swim test, at 8 weeks' post-injury.

2.5.1. Open Field (OF)

OF test was performed on the basis of a previously described methodology [24]. Briefly, the mice were placed in the corner of a white quadratic box (50 × 50 × 45 cm) in which its arena had been previously divided into 5 × 5-cm squares by black lines. Mice were then allowed to move freely for 5 min under dim and dispersed light conditions (room light 45–50 lx) while the number of square crossings were counted by an observer in a blind manner. The behaviour during the whole session was recorded with a video camera and then analysed. The percentage of time spent in the wall and the centre (10 × 10-cm square in the middle of the OF) regions, and the latency to enter the centre zone were interpreted as anxiety-like behaviours. Additionally, the number of square crossings was used as an index of locomotor activity, as explained below.

2.5.2. Dark/light box (DLb)

DLb was performed as described elsewhere [24]. In summary, a 27×27×26-cm lit (room light 45–50 lx) white compartment with open top was connected through an opening entrance (5×5 cm) to a 27×27×26-cm black box compartment covered with a lid. The mice were placed in the centre of the dark compartment and were therefore allowed to freely explore the apparatus for 10 min. The time spent in the light compartment and the latency to enter the light compartment were both considered as indicators of anxiety-like behaviours.

2.5.3. Forced swimming test (FST)

Despair behaviour levels were evaluated using the FST, on the basis of procedures described elsewhere [8,25]. Globally, mice were individually forced to swim in open cylinders (40 cm height x 15 cm diameter) containing 30 cm of water at 25±1ºC. Their behaviour during the whole 6-min test was recorded with a video

camera and then analysed. The immobility time was determined whenever no additional activity other than the necessary movements to keep the mice head above the water was observed. FST it is a useful procedure for evaluating depression-like behaviours [26].

2.6. Spinal cord immunohistochemistry analysis

At the end of the tests, the spinal cord of mice was processed by immunohistochemical techniques as previously described [20]. Briefly, animals were anesthetized and trans-cardially perfused with 4% paraformaldehyde in phosphate-buffer saline solution (PBS, 0.1M, pH 7.4). The spinal cords were removed, fixed in the same solution overnight, divided in several segments, cryoprotected in PBS containing 30% sucrose and stored at 4ºC. Subsequently, segments from the spinal cord injured site were serially cut in the transverse plane (40 µm thickness) in a cryostat and collected in order to analyse the immunoreactivity of astrocytes (Glial fibrillary acidic protein or GFAP; 1:200; ab7260, ABCAM, UK) and peptidergic afferent pain fibres (Calcitonin gene related peptide or CGRP; 1:200; AB36001, ABCAM, UK). As a specificity control, some spinal cord sections were incubated without primary antibody. Samples were observed under a microscopy equipped with epifluorescence using the appropriate filters.

To perform these analyses, a set of spinal cord injured sections from each experimental group was analysed using NIH Image software (Image-J; version 1.37; National Institutes of Health, USA). Spinal cord images (x200) from GFAP labelling histological samples were taken from the dorsal and ventral horns. For each image, the appropriate threshold value was set up manually at the level allowing to save most of the astroglial processes from the background, and glial reactivity was expressed as the area of immunoreactivity to GFAP [20]. In addition, spinal cord images (x100) from CGRP labelling histological samples were also taken from the dorsal horns but, in this specific case, the intensity of labelling was measured on a 0 (no fluorescence) to 255 (maximal fluorescence) range [20].

2.7. Statistical analysis

All functional, behavioural and histological measurements were performed in a blinded manner. Data are shown as mean ± SEM. Data were analysed using repeated measures MANOVA (Wilks' criterion) and analysis of variance (ANOVA) followed by Duncan's test, when applicable. The α level was set at 0.05 (SPSS v23.0).

3. Results

3.1. General observations

The MANOVA analysis of the mice weight indicated significant effects on week factor ($F_{(3,39)}$= 7836.7, $p<0.001$) but no significant differences in group factor were recorded ($F_{(4,41)}$=0.941, p=0.450). Although significant interaction for week×group ($F_{(12,103)}$=6.567, $p<0.001$) was also recorded, no statistically significant loss of weight was observed between groups ($p>0.05$) at any experimental day, as ANOVA analysis revealed (all F's$_{(4,45)}$>0.165, p's<0.122). Furthermore, although RB5 and RB10 showed a slower

weight gain in comparison to the other groups, all animals showed an increased weight 8 weeks after the surgery (Sham 27.64±0.43; RB1 27.69±0.42; RB2 27.47±0.44; RB5 27.03±0.35; RB10 26.81±0.30).

3.2. Chronic thermal hyperalgesia assessment

The MANOVA analysis of thermal hyperalgesia indicated significant effects on week ($F_{(3,39)}$= 33.63, p<0.001) and group ($F_{(4,41)}$= 19.84, p<0.001) factors, and significant interaction for week×group ($F_{(12,103)}$ = 3.82, p<0.001). The results stemming from the Hargreaves test contrasted by means of the ANOVA analysis revealed significant group differences at post-photoinduction experimental weeks 1, 4 and 8 (all F's$_{(4,45)}$>4,784, p's<0.003). One week after intervention, the different photoinduction times resulted in an accretive hyperalgesia from sham to RB10 groups (Fig. 1). RB10 mice showed the lowest withdrawal latency to heat stimulus and shams the highest in comparison to other groups. Results obtained at 4 weeks after operation revealed a slight recovery across all experimental groups but all of them showed significant lower withdrawal latencies to heat stimulus than the sham-treated group. Finally, at 8 weeks after SCI, RB10 and RB5 groups showed significant increased hyperalgesia in comparison to mice from RB1 and the sham group. These results suggest that inducing SCI for a length of time between 1 and 10 minutes causes hyperalgesia up to 4 weeks after injury. In addition, if the SCI is performed either for 5 or 10 minutes, it leads to chronic hyperalgesia up to 8 weeks after intervention.

3.3. BMS-scores and locomotor activity after graded spinal cord injury

The MANOVA analysis of BMS-scores indicated significant effects on week ($F_{(3,39)}$= 397.125, p<0.001) and group ($F_{(4,41)}$ = 23.02, p<0.001) factors, and significant interaction week×group ($F_{(12,103)}$= 6.762, p<0.001). On further ANOVA analysis, significant group differences were detected at post-photoinduction experimental weeks 1, 4 and 8 (all F's$_{(4,45)}$>4,868, p's<0.001). Results revealed that while 1 week after injury all experimental groups showed significant reduced BMS-scores in comparison to the sham group, at 8 weeks after injury only RB5 and RB10 groups showed significant lower BMS-scores with respect to all other groups (Fig. 2). Specifically, mice from the sham group gradually achieved normal BMS-scores along the experimental period up to 8 weeks after injury. At 4 weeks after SCI, mice from RB1 and RB2 -but not RB5 or RB10- showed levels of locomotion recovery approaching the sham BMS-scores. Finally, while no significant differences were found between RB1 and RB2 groups at 8 weeks after injury in comparison with either sham or the other groups, RB5 and RB10 BMS-scores remained significant lower with respect to all other groups. Overall, although these latest groups showed significant BMS-scores, their final scores at 8 weeks after injury were associated with consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift off.

On another note, both the gait topography analysis and the OF test revealed no significant differences across the experimental groups at any time-point along the study period. The gait topography analysis revealed that neither stride length nor stride width differed among groups, either for hind limbs or fore limbs, at 1, 4 or 8 weeks after SCI (Fig. 3). Additionally, no differences in locomotor activity were detected

between groups neither at 1, 4 nor 8 weeks after SCI as the number of squares crossed in the OF did not differ among groups at any of these time-points (all $F's_{(4,45)} > 0.210$, $p's > 0.07$). (Table 1).

Altogether, locomotion analysis suggested that although the BMS-scores of RB5 and RB10 groups indicated mild stability alterations, all mice groups were able to move freely without severe locomotor disturbances at 8 weeks after photochemical SCI. The relationship between BMS scores and OF locomotion at week 8 is shown in Fig. 4A. In addition, the relationship between withdrawal to heat noxious stimulus and OF locomotion at week 8, suggested that chronic hyperalgesia did not modify horizontal locomotor activity (Fig. 4B).

3.4. Anxiety- and depression–like behaviour association with chronic central hyperalgesia

MANOVA analysis on the percentage of time spent in the centre zone of the OF revealed a significant effect in the week factor ($F_{(2,40)} = 32.37$, $p<0.001$) but no significant differences for both the group factor ($F_{(4,41)} = 1.04$, $p=0.400$) and the week × group interaction ($F_{(8,80)} = 1.15$, $p=0.339$). Thus, all experimental groups, including shams, showed significant decrease in the time spent in the centre zone along the study independently to the spinal cord injury. Concerning the latency to enter the centre zone, similar results were observed. A significant effect in the week factor was detected ($F_{(2,40)} = 48.87$, $p<0.001$) but with no differences for the group factor ($F_{(4,41)} = 1.185$, $p=0.332$) and the week × group interaction ($F_{(8,80)} = 1.064$, $p=0.397$), which indicates a significant increase in the latency to enter the centre zone along the study with no-association to the SCI (Table 1)

Similarly, MANOVA analysis on the latency to light compartment of the DLb revealed a significant effect in the week factor ($F_{(2,40)} = 19.35$, $p<0.001$) but no differences for both the group factor ($F_{(4,41)} = 0.450$, $p=0.846$) and the week×group interaction ($F_{(8,80)} = 0.446$, $p=0.889$). Concerning the percentage of time spent in the light compartment, a significant effect in the week factor was detected ($F_{(2,40)} = 24.28$, $p<0.001$) but with no differences in the group factor ($F_{(4,41)} = 0.437$, $p=0.781$) and the week×group interaction ($F_{(8,80)} = 0.79$, $p=0.607$). The results suggest a significant reduction in the percentage of time spent in the light compartment along the study independently to the SCI. (Table 1)

On the other hand, statistically significant group differences were found in the FST by ANOVA analysis at 8 weeks after spinal cord photoinduction ($F_{(4,45)} = 11.384$, $p<0.001$) (Fig. 5). Interestingly, results revealed that photoinduction on the spinal cord over 5 minutes caused an increase of immobility time in RB10 and RB5 in comparison to all other groups.

Altogether, behavioural data suggested that while hyperalgesia associated to SCI did not exacerbate BALB/c anxiety-like behaviour at any time-point after photoinduction, it may result in depression-like behaviour at the SCI chronic phase. Particularly in this later phase, the relationships between emotional behaviour and hyperalgesia at 8 weeks after photochemical SCI showed that chronic central hyperalgesia associates with despair behaviour (Fig. 6A) but not modify the basal anxiety of BALB/C mice neither in OF (Fig. 6B) nor DLb (Fig. 6C).

3.5. Immunohistochemistry analysis of graded spinal cord injury

Immunohistochemistry analysis at the end of the experimental period revealed significant differences across groups concerning the CGRP-immunoreactivity of peptidergic afferent nociceptive nerve fibres in the spinal dorsal horn ($F_{(4,94)}$= 12,716, p<0.001). CGRP-immunoreactivity was significantly higher in RB10 and RB5 in comparison with the other experimental groups (Fig. 7A). Histological images of spinal cord sections immunostained against GGRP either in sham-treated or injured groups are shown in Fig. 7B.

Concerning GFAP-immunoreactivity analysis, significant differences were detected across groups 8 weeks after photoinduction ($F_{(4,144)}$= 8.818, p<0.001) (Fig. 8A). The areas (μm^2) of GFAP-immunoreactivity for both RB10 and RB5 were significantly higher with respect to the other experimental groups. Histological images of spinal cord sections immunostained against GFAP either in sham-treated or injured groups are shown in Fig. 8B.

Overall, these immunohistochemical analyses showed that spinal cord photoinduction lasting over 5 minutes caused intraspinal sprouting of afferent nociceptive nerve fibres and increased astrogliosis in the dorsal horn at the SCI chronic phase. These results may be associated with those obtained in Hargreaves test, indicating a relationship between chronic hyperalgesia and either increased CGRP sprouting (Fig. 9A) and GFAP-immunoreactivity (Fig. 9B) in the dorsal horns after graded photochemical SCI.

4. Discussion

The main objective of the present research was to determine whether chronic central hyperalgesia may exacerbate emotional disturbances in female BALB/c mice subjected to photochemical SCI, a strain known for its high basal anxiety levels [9]. While the existent photochemical SCI mice models are based in the administration of RB intraperitoneally [14] or intravenously [13], in the present model the spinal cord was directly bathed with RB, ensuring a successful lesion on the proper spinal cord segments.

The present work highlights the direct relationship between the time of photoinduction and the persistence of hyperalgesia after SCI. While 1 or 2 minutes of photoinduction triggers hyperalgesia up to 4 weeks after injury, the SCI associated with 5 or 10 minutes of photoinduction results in hyperalgesia up to the chronic phase (8 weeks after injury). In parallel, our results showed that chronic hyperalgesia associated to SCI increases the depression-like behaviour at the chronic phase of the injury.

The reported emotional deficits cannot be attributed to reduced locomotor activity since results showed a graded motor dysfunction affecting only coordination but not horizontal activity. Concretely, while long time-photoinduction (RB5 and RB10) revealed mild significant BMS-scale alterations at 8 weeks after injury, no significant effects were detected neither on the horizontal activity in the OF nor with regard to the gait topography analysis at any time of the experiment. In comparison to other chronic SCI animal models in which NP is accompanied with significant motor dysfunctions [27], photochemical-SCI in mice may be a better suitable model to study central chronic NP and associated mood disorders without severe locomotor disturbances. In contrast with our findings on BALB/c, in female C57BL/6 mice photochemical-SCI causes motor impairment during the first 7 days' post-injury in mice irradiated 6-8 min after RB intravenous injection [13]. In rats, photochemical-SCI causes significant motor impairment at 5 and 10 min of photoinduction: the withdrawal latency to heat stimulus was abolished in RB5 and RB10 rats since the

ventrolateral and dorsolateral funiculi were completely damaged in these experimental groups [10,13]. These findings may suggest that functional outcomes in mice after photochemical-SCI might be related to either mice strain or nature of lesion.

This experimental model of graded SCI might allow assessing the effects of several pharmacological treatments against pain-associated disorders at two different severity degrees: low severity (RB1, RB2) and high severity (RB5, RB10). Contusion, compression and laceration are the main experimental models of SCI used for pharmacological treatment, since the main cause of human SCI is usually mechanical. Other experimental models of SCI, including the chemical-mediated SCI (photochemical SCI) may be useful to model specific aspects of the secondary injury that occurs after the initial traumatic SCI as well as to address questions about spinal cord circuitry [28]. For instance, it is well known that photochemical SCI also causes gliosis in rat [10], which is implicated in the development of NP after SCI [29] and the immunohistological analysis of the site of SCI-photochemical injured mice in the present work showed a graded GFAP-immunoreactivity directly linked to both photoinduction-time and chronic hyperalgesia. Moreover, at 8 weeks post-photoinduction CGRP-immunoreactivity was significantly higher in RB10 and RB5 groups, which also showed chronic hyperalgesia. These data suggest that higher severity of photochemical SCI causes sprouting of afferent CGRP-fibres in the dorsal horn and it may be associated with hyperalgesia chronification. These results are congruent with previous studies suggesting that sprouting of C-fibre, stained against CGRP, into dorsal horn laminae parallels the development of pain behaviour [30-32]. Therefore, this SCI-photochemical mice model may also be a useful strategy to assay potential drugs to downregulate both GFAP- and CGRP-immunoreactivity in order to relief chronic central NP and modify associated exacerbated emotional responses.

The present study showed, for the first time, that mice exposed to photochemical SCI exhibited an increase in despair-like behaviour during the chronic phase compared to sham mice. Previous findings on the impact of chronic pain on mood disturbances have been inconsistent, probably due to the use of different animal models to induce chronic pain, which also reveals considerable variability in emotional manifestations. C57BL/6J mice with SCI showed increased depressive-like behaviour [9,27]. Similar results were also found in Sprague-Dawley rats whose levels of depression and anxiety-like behaviour were increased by the induction of SCI [33]. In our experiment, the lack of changes concerning anxiety suggests that the basal levels of anxiety may interact with the emotional consequences of SCI. Thus, it has been previously described that BALB/c inbred strain showed high levels of anxiety and aggressive behaviours, large brain size, underdevelopment of the corpus callosum, and low levels of brain serotonin [34]. Based on measures in the OF test, BALB/c mice showed higher anxious behaviour [35]. In the DLb test, BALB/c mice displayed defensive and protective behaviours, with limited exploration of the new environment together with low locomotor activity, suggesting also higher anxious levels [36]. On the other hand, BALB/c mice showed higher immobility times in both the tail suspension test [37] and FST [38]. In all of these experimental studies BALB/c behaviour was compared to the behaviours of C57BL/6 mice. Since, these two strains clearly differ in their stress-coping strategies, BALB/c mice strain has been extensively used to test effects of anxiolytic drugs [39,40]. Moreover, BALB/C mice exhibited low levels in the expression of MAO-

A and MAO-B [41] which might be related to an increase on their stress reactivity [42]. All together these findings suggest that BALB/c mice presented vulnerability to anxiety-and depression-like behaviours. Therefore, as chronic NP after SCI is related to an increased risk for depressive mood and anxiety disorders [43,44], the use of a mouse strain with higher anxiety and depression phenotype may mask the emotional alternation due to NP after SCI due to the possibility of the ceiling effect. Nevertheless, our results indicate that, in female BALB/c mice, NP following SCI does not modify their basal anxiety, but it increases depressive-like behaviours after RB5 and RB10. To our knowledge, despite the fact that pain responses have already been reported in BALB/c mice after spinal cord contusion using the Infinite Horizon Tissue Impactor [45], this is the first study to observe and consider the emotional changes in BALB/c strain after chronic SCI. The present data may emphasize the validation of this animal model for the study of the co-occurrence of chronic pain and depression, since other models of NP including the spared nerve injury [46] and the chronic constriction injury [47] have been shown to induce depressive-like behaviours.

Overall, the female BALB/c graded photochemical-SCI may be suitable to assay new therapeutic approaches at the chronic phase of SCI, increasing the range of useful SCI pre-clinical models to study central NT. Our data show that the photoinduction-time is predictive of the extent of the pathology and their associated hyperalgesic and depression-like behaviour up to chronic phase. Thus, it may be useful to obtain a specific grade of SCI, which will involve particular motor and central pain-related behaviours increasing the chances to elucidate underling mechanism associated with pain-mood disorders exacerbation.

5. Conclusion

To our knowledge, this is the first study to show that mice graded photochemical SCI results in an accretive chronic hyperalgesia accompanied with graded emotional disturbances without severe locomotor alterations. Concretely, chronic central hyperalgesia was associated with despair-like behaviour at the SCI chronic phase but did not increase basal female BALB/c anxiety-like behaviour. Our results add further evidence to consider the present model particularly suitable to perform new therapeutic strategies to treat depression-like disorders associated with chronic NT, specifically focused on subjects with genetic susceptibility to mood disorders. The latter is an important issue, since NP is present in 40-to-50% of patients affected by SCI, and it is also associated with higher depression ratings [48], which may exacerbate in individuals with anxiety-like phenotype.

Conflict of interest statement

The authors declare that they have no competing interests.

Authors' contributions

P.B-V. and E.V. conceived the experiments and obtained funding for the study. P.B-V., J.H., B.A-P., M.D. and E.V. conducted the experiments. P.B-V., J.H., M.P-T and E.V. analysed the results. P.B-V, M.P-T and E.V. wrote the manuscript. All authors read and approved the final manuscript.

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References

[1] N.B. Finnerup, T.S. Jensen, Spinal cord injury pain-mechanisms and treatment, Eur. J. Neurol. 11 (2004) 73–82.

[2] L. Werhagen, C.N. Budh, C. Hultling, C. Molander, Neuropathic pain after traumatic spinal cord injury-relations to gender, spinal level, completeness, and age at the time of injury. Spinal Cord 42 (2004) 665–673.

[3] N.B. Finnerup, Pain in patients with spinal cord injury, Pain 154 (2013) 71–76.

[4] G. Blackburn-Munro, R.E. Blackburn-Munro, Chronic pain, chronic stress and depression: coincidence or consequence?, J. Neuroendocrinol. 13(2001) 1009–1023.

[5] L. Doan, T. Manders, J. Wang, Neuroplasticity underlying the comorbidity of pain and depression, Neural. Plast. (2015) 504691.

[6] I. Yalcin, M. Barrot, The anxiodepressive comorbidity in chronic pain, Curr. Opin. Anaesthesiol. 27 (2014) 520–527.

[7] L.R. Miller, A. Cano, Comorbid chronic pain and depression: who is at risk?, J. Pain 10 (2009) 619–27.

[8] J. Goesling, D.J. Clauw, A.L. Hassett, Pain and depression: an integrative review of neurobiological and psychological factors, Curr. Psychiatry Rep. 15 (2013) 421.

[9] S. Buitrago, T.E. Martin, J. Tetens-Woodring, A. Belicha-Villanueva, G.E. Wilding, Safety and efficacy of various combinations of injectable anesthetics in BALB/c mice, J. Am. Assoc. Lab. Anim. Sci. 47 (2008) 1–17.

[10] E. Verdú, G. García-Alías, J. Forés, M. Vela, J. Cuadras, R. López-Vales, X. Navarro, Morphological characterization of photochemical graded spinal cord injury in the rat, J. Neurotrauma 20 (2003) 483–99.

[11] Y. Taoka, K. Okajima, Spinal cord injury in the rat, Prog. Neurobiol. 56 (1998) 341-358.

[12] E. Verdú, G. García-Alías, J. Forés, G. Gudiño-Cabrera, V.C. Muñetón, M. Nieto-Sampedro, X. Navarro, Effects of ensheathing cells transplanted into photochemically damaged spinal cord, Neuroreport 12(2001) 2303–2309.

[13] M. Gaviria, H. Haton, F. Sandillon, A. Privat, A mouse model of acute ischemic spinal cord injury, J. Neurotrauma 19 (2002) 205–521.

[14] M.S. Piao, J.K. Lee, J.W. Jang, S.H. Kim, H.S. Kim, A mouse model of photochemically induced spinal cord injury, J. Korean Neurosurg. Soc. 46 (2009) 479–483.

[15] N.B. Finnerup, N. Attal, S. Haroutounian, E. McNicol, R. Baron, R.H. Dworkin, I. Gilron, M. Haanpää, P. Hansson, T.S. Jensen, P.R. Kamerman, K. Lund, A. Moore, S.N. Raja, A.S. Rice, M. Rowbotham, E.

Sena, P. Siddall, B.H. Smith, M. Wallace, Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis, Lancet Neurol. 14 (2015) 162–173.

[16] Y.S. Gwak, H.Y. Kim, B.H. Lee, C.H. Yang, Combined approaches for the relief of spinal cord injury-induced neuropathic pain, Complement Ther. Med. 25 (2016) 27–33.

[17] S.S. Moy, J.J. Nadler, N.B. Young, A. Perez, L.P. Holloway, R.P. Barbaro, J.R. Barbaro, L.M. Wilson, D.W. Threadgill, J.M. Lauder, T.R. Magnuson, J.N. Crawley, Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains, Behav. Brain Res. 176 (2007) 4-20.

[18] S. Oriel, O. Kofman, Strain dependent effects of conditioned fear in adult C57Bl/6 and Balb/C mice following postnatal exposure to chlorpyrifos: relation to expression of brain acetylcholinesterase mRNA, Front. Behav. Neurosci. 9 (2015) 110.

[19] K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, Pain 32 (1988) 77–88.

[20] B. Álvarez-Pérez, J. Homs, M. Bosch-Mola, T. Puig, F. Reina, E. Verdú, P. Boadas-Vaello, Epigallocatechin-3-gallate treatment reduces thermal hyperalgesia after spinal cord injury by down-regulating RhoA expression in mice, Eur. J. Pain 20 (2016) 341–352.

[21] D.M. Basso, L.C. Fisher, A.J. Anderson, L.B. Jakeman, D.M. McTigue, P.G. Popovich, Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains, J Neurotrauma 23 (2006) 635–659.

[22] A.J. Parker, K.A. Clarke, Gait topography in rat locomotion, Physiol. Behav. 48 (1990) 41–7.

[23] C. Soler-Martín, P. Boadas-Vaello, E. Verdú, N. Garcia, J. Llorens, Chronic proximal axonopathy in rats is associated with long-standing neurofilament depletion in neuromuscular junctions and behavioral deficits, J. Neuropathol. Exp. Neurol. 73 (2014) 568–579.

[24] R. Bisaz, P. Boadas-Vaello, D. Genoux, C. Sandi, Age-related cognitive impairments in mice with a conditional ablation of the neural cell adhesion molecule, Learn. Mem. 20 (2013) 183–93.

[25] R.D. Porsolt, A. Bertin, M. Jalfre, Behavioral despair in mice: a primary screening test for antidepressants, Arch. Int. Pharmacodyn. Ther. 229 (1977) 327-36.

[26] B.J. Caldarone, V. Zachariou, S.L. King, Rodent models of treatment-resistant depression, Eur. J. Pharmacol. 753 (2015) 51–65.

[27] J. Wu, Z. Zhao, B. Sabirzhanov, B.A. Stoica, A. Kumar, T. Luo, J. Skovira J, A.I. Faden, Spinal cord injury causes brain inflammation associated with cognitive and affective changes: role of cell cycle pathways, J. Neurosci. 34 (2015) 10989–11006.

[28] S.M. Onifer, A.G. Rabchevsky, S.W. Scheff, Rat models of traumatic spinal cord injury to assess motor recovery, ILAR J. 48(2007) 385–395.

[29] Y.S. Gwak, J. Kang, G.C. Unabia, C.E. Hulsebosch, Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats, Exp. Neurol. 234 (2012) 362–372.

[30] M.D. Christensen, C.E. Hulsebosch, Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat, Exp. Neurol. 147 (1997) 463–475.

[31] A.B. Ondarza, Z. Ye, C.E. Hulsebosch, Direct evidence of primary afferent sprouting in distant segments following spinal cord injury in the rat: colocalization of GAP-43 and CGRP, Exp. Neurol. 184 (2003) 373–380.

[32] T.A. Nees, A. Tappe-Theodor, C. Sliwinski, M. Motsch, R. Rupp, R. Kuner, N. Weidner, A. Blesch, Early-onset treadmill training reduces mechanical allodynia and modulates calcitonin gene-related peptide fiber density in lamina III/IV in a mouse model of spinal cord contusion injury, Pain 157 (2016) 687–697.

[33] S. Maldonado-Bouchard, K. Peters, S.A. Woller, B. Madahian, U. Faghihi, S. Patel, S. Bake, M.A. Hook, Inflammation is increased with anxiety- and depression-like signs in a rat model of spinal cord injury, Brain Behav. Immun. 51 (2016) 176–195.

[34] E.S. Brodkin, BALB/c mice: low sociability and other phenotypes that may be relevant to autism, Behav. Brain Res. 176 (2007) 53–65.

[35] X. Tan, S.M. Orchard, L.D. Sanford, Home cage activity and behavioral performance in inbred and hybrid mice, Behav. Brain Res. 136 (2002) 555–69.

[36] E.M. Lepicard, C. Joubert, I. Hagneau, F. Perez-Diaz, G. Chapouthier, Differences in anxiety-related behavior and response to diazepam in BALB/cByJ and C57BL/6J strains of mice, Pharmacol. Biochem. Behav. 67 (2000) 739–748.

[37] R. Trullas, B. Jackson, P. Skolnick, Genetic differences in a tail suspension test for evaluating antidepressant activity, Psychopharmacology (Berl) 9 (1989) 287–288.

[38] Y. Sugimoto, Y. Kajiwara, K. Hirano, S. Yamada, N. Tagawa, Y. Kobayashi, Y. Hotta, J. Yamada, Mouse strain differences in immobility and sensitivity to fluvoxamine and desipramine in the forced swimming test: analysis of serotonin and noradrenaline transporter binding, Eur. J. Pharmacol. 592 (2008) 116–22.

[39] G. Griebel, C. Belzung, G. Perrault, D.J. Sanger, Differences in anxiety-related behaviours and sensitivity to diazepam in inbred and outbred strains of mice, Psychopharmacology 148 (2000) 164–70.

[40] S.C. Fowler, T.J. Zarcone, E. Vorontsova, Haloperiodol-induced microcatalepsy differs in CD-1, BALB/c, and C57BL/6 mice, Exp. Clin. Psychopharmacol. 9 (2001) 277–84.

[41] W.B. Siesser, X. Zhang, J.P. Jacobsen, T.D. Sotnikova, R.R. Gainetdinov, M.G. Caron, Tryptophan hydroxylase 2 genotype determines brain serotonin synthesis but not tissue content in C57Bl/6 and BALB/c congenic mice, Neurosci. Lett. 481 (2010) 6–11.

[42] N.K. Popova, L.N. Maslova, E.A. Morosova, V.V. Bulygina, I. Seif, MAO-A knockout attenuates adrenocortical response to various kinds of stress, Psychoneuroendocrinology 31 (2006) 179-86.

[43] M.M. Gerrits, N. Vogelzangs, P. van Oppen, H.W. van Marwijk, H. van der Horst, B.W. Penninx, Impact of pain on the course of depressive and anxiety disorders, Pain 153 (2012) 429–436.

[44] M. Zhuo, Neural Mechanisms Underlying Anxiety-Chronic Pain Interactions, Trends Neurosci. 39 (2016) 136–145.

[45] B.J. Ker, S. David, Pain behaviors after spinal cord contusion injury in two commonly used mouse strains, Exp. Neurol. 206 (2007) 240–247.

[46] G.J. Norman, N. Zhang, J.S. Morris, K. Karelina, G.G. Berntson, A.C. DeVries, Social interaction modulates autonomic, inflammatory, and depressive-like responses to cardiac arrest and cardiopulmonary resuscitation, Proc. Natl. Acad. Sci. 107 (2010) 16342–16347.

[47] O. Caspani, M.C. Reitz, A. Ceci, A. Kremer, R.D. Treede, Tramadol reduces anxiety-related and depression-associated behaviors presumably induced by pain in the chronic constriction injury model of neuropathic pain in rats, Pharmacol. Biochem. Behav. 124 (2014) 290–296.

[48] E. Ataoğlu, T. Tiftik, M. Kara, H. Tunç, M. Ersöz, S. Akkuş, Effects of chronic pain on quality of life and depression in patients with spinal cord injury, Spinal Cord 51 (2013) 23-26.

Figure legends

Figure 1. Withdrawal latency to heat noxious stimulus at 1, 4 and 8 weeks after photochemical SCI. Results are the mean ± SEM. a,b,c: groups not sharing a letter are significantly different, $p<0.05$.

Figure 2. BMS scores of each experimental group at 1, 4 and 8 weeks after photochemical SCI. Results are the mean ± SEM. Dashed line represents the maximum score in a BMS scale and the value scored by all animals before surgery. a,b,c: groups not sharing a letter are significantly different, $p<0.05$.

Figure 3. Gait topography performance of experimental groups at 1, 4 and 8 weeks after photochemical SCI. (A) Forelimb stride width (B) Forelimb stride length (C) Hindlimb stride width (D) Hindlimb stride length. The results are the mean ± SEM (Sham, n=9; RB1, n=9; RB2, n=8; RB5 n=10; RB10 n=10). $p>0.05$ in all groups for each time point.

Figure 4. Relationship between locomotor activity and hyperalgesia at 8 weeks after photochemical SCI. (A) Relationship between time BMS scores and horizontal activity in the OF test; letters above the symbols correspond to BMS, and letters to the right of the symbol refer to squares crossed in the OF. (B) Relationship between the horizontal activity in the OF test and hyperalgesia; letters above the symbols correspond to the squares crossed in the OF, and letters to the right of the symbol refer to withdrawal to heat noxious stimulus. a,b: groups not sharing a letter are significantly different, $p<0.05$.

Figure 5. The average times of immobility behaviour in FST at 8 weeks after photochemical SCI. Results are the mean ± SEM. a,b,c: groups not sharing a letter are significantly different, $p<0.05$.

Figure 6. Relationship between hyperalgesia and emotional behaviour at 8 weeks after photochemical SCI. (A) Relationship between time of immobility behaviour in FST and hyperalgesia. (B) Relationship between the percentages of time spent in the OF wall zone and hyperalgesia. (C) Relationship between the percentages of time spent in the light compartment of DLb and

hyperalgesia. a,b: groups not sharing a letter are significantly different, $p<0.05$, letters above the symbols correspond to emotional behaviour data, and letters to the right of the symbol refer to withdrawal to heat noxious stimulus.

Figure 7. Graded sprouting of CGRP afferent nerve fibres in the spinal dorsal horn after photochemical SCI. (A) CGRP-immunoreactivity. Results are the mean ± SEM. a,b,c: groups not sharing a letter are significantly different, $p<0.05$. **(B)** Representative histological images of the dorsal horn immunostained against CGRP of each group, at 8 weeks after photoinduction. Barr= 100 μm.

Figure 8. Astrogliosis in the spinal dorsal horn after photochemical SCI. (A) GFAP-immunoreactivity area (μm^2). Results are the mean ± SEM (Sham, n=9; RB1, n=9; RB2, n=9; RB5 n=10; RB10 n=10). a,b: groups not sharing a letter are significantly different, $p<0.05$. (B) Representative confocal images of astrocytes immunolabelled against GFAP in the spinal cord grey of each group at 8 weeks after photoinduction. Barr= 100 μm.

Figure 9. Relationship between hyperalgesia and spinal cord immunohistochemistry at 8 weeks after photochemical injury. (A) Relationship between CGRP-immunoreactivity in the dorsal horn and hyperalgesia. (B) Relationship between GFAP-immunoreactivity in the dorsal horn and hyperalgesia. a,b,c: groups not sharing a letter are significantly different, $p<0.05$, letters above the symbols correspond to immunoreactivity, and letters to the right of the symbol refer to withdrawal to heat noxious stimulus.