Evidence for lack of direct causality between pain and affective disturbances in a rat peripheral neuropathy model

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Abstract

Chronic pain is frequently accompanied by the manifestation of emotional disturbances and cognitive deficits. While a causality relation between pain and emotional/cognitive disturbances is generally assumed, several observations suggest a temporal dissociation and independent mechanisms. We therefore studied Sprague-Dawley rats that presented a natural resistance to pain manifestation in a neuropathy model (spared nerve injury; SNI) and compared their performance in a battery of behavioral paradigms – anxiety, depression and fear memory – with animals that presented a pain phenotype. Afterwards, we performed an extensive volumetric analysis across prefrontal, orbitofrontal and insular cortical areas. The majority of SNI animals manifested mechanical allodynia (low threshold, LT) but 13% were similar to Sham controls (high threshold, HT). Readouts of spontaneous hypersensivity (paw flinches) were also significantly reduced in HT and correlated with allodynia. To increase the specificity of our findings we segregated the SNI animals in those with left (SNI-L) and right (SNI-R) lesions and the lack of association between pain and behavior still remains. Left- lesioned animals, independently of the LT or HT phenotype, presented increased anxiety-like behaviors and decreased wellbeing. In contrast, we found that the insular cortex (agranular division) was significantly smaller in HT than in LT.

To conclude, pain and emotional disturbances observed following nerve injury are to some extent segregated phenomena. Also, HT and LT SNI presented differences in insular volumes, an area vastly implicated in pain perception, suggesting a supraspinal involvement in the manifestation of these phenotypes.

1. Introduction

Chronic pain (CP) is frequently accompanied by altered emotional behavior and impaired cognitive function [1-3]. Such comorbidities have been largely demonstrated in preclinical models – see for instances [4-9] – additionally for review [10-13]. However, substantial evidence has been accumulating, particularly in models of peripheral neuropathies, suggesting that hypersensitivity and the behavioral impairments can to a certain extent dissociate. Keay's laboratory for instance observed that some neuropathic animals presented stable impairments in social and sleep behaviors (30%), while in others these were transient (25%) or even absent (45%) [14]; importantly, these 3 group were indistinct in their manifestation of allodynia. The proportion of affected animals was show to be constant and associated with particular morphological, endocrine, inflammatory and genetic outcomes – see [15-23]. Also, previous studies from our group demonstrated that left- and right-sided injuries are differentially associated with specific behavioral deficits but no differences were observed in their algogenic quality [5,6].

A temporal mismatch between the manifestation of mechanical allodynia – appearing soon after the neuropathic lesion – and the manifestations of anxiety- and depression-like behaviors – 4-6 or 6-8 weeks after pain onset, respectively – has also been observed [4,24]. Additional evidence comes from a recent work using the sciatic nerve cuff model, in which it was shown that anxiety-like behaviors associated with the neuropathy were maintained upon cuff displacement and recovery of pain thresholds to nearly basal levels [25]. Altogether, data suggest that the nerve injury triggers relatively independent processes that eventually culminate in the manifestation of pain, affective/cognitive disorders or both.

In a large retrospective study, De Felice and colleagues identified that a considerable number of neuropathic Sprague-Dawley (SD) and Holtzman rats did not manifest signs of ongoing allodynia [26]. These animals provide therefore an adequate model to study potential functional impacts of silent neuropathies, peripheral nerve lesions with reduced impact in sensorial part. Indeed, these authors

demonstrated that in silent neuropathies, pain could manifest transiently when rostral ventromedial medulla (RVM) activity was blocked [26] providing evidence that (i) the nerve lesion was effective and that (ii) the latent character of the silent neuropathy depended on an effective inhibition from brainstem descending controls.

In the present work, we took advantage of the natural resistance of SD rats to manifest allodynia after nerve injury to test the hypothesis that silent neuropathies could impact on emotional behavior in a manner akin to the painful condition.

2. Materials and methods

2.1 Experimental subjects

Ninety-six 2-month-old male Sprague-Dawley (SD) rats (two independent cohorts; Charles River Laboratories, Barcelona, Spain) were used in the experiment. Animals were housed in groups of 3 in plastic cages with food (4RF21; Mucedola, SRL, Settimo Milanese, Italy) and water available *ad libitum*, in a room with controlled temperature (22°C ± 1°C) and humidity (55-60%), 12-hour light/dark cycle (lights on at 8 am). During the initial handling period (1 week), animals were randomly labelled from 1 to 3 in a latin square manner across consecutive boxes (1-2-3; 2-3-1; 3-1-2). Animal 1 from box 1 was then randomly ascribed to left-sided surgery and from there, consecutive animals were operated in opposite hindpaws (eg. L; R; L; R; L; R…) until the last box. In mid-numbered boxes the nerves were used as sham operated control (see below). Our starting pool comprised 87 SNI and 9 Sham controls.

All procedures involving animals were approved by the respective local organizations and the experiments were performed according to the European Community Council Directive 2010/63/EU guidelines.

2.2 Neuropathic pain model – Spared Nerve Injury

The spared nerve injury (SNI) model was used in all experiments to model neuropathic pain [27]. The model consists in the unilateral ligation and subsequent

distal axotomy of the tibial and common peroneal nerves - the sural nerve remained intact. Control animals underwent a sham surgery, identical in all aspects except no nerve lesions were performed. Half of the SNI animals were operated in the left side (SNI-L) and the remaining in the right (SNI-R) in a randomly manner. The surgical procedures were performed under deep anesthesia with a 1.5:1.0 mixture of ketamine (Imalgene®, 100 mg/mL – Merial, Lyon, France) and medetomidine (Dormitor®, 1 mg/mL – Orion Pharma, Espoo, Finland) at a dose of 1 mg/kg (intraperitoneal - i.p).

2.3. Spontaneous paw flinches and mechanical allodynia

Two researchers performed the assessment of paw flinches and mechanical allodynia. Animals were placed in an elevated grid and left to acclimatized to the experimental conditions for 5 minutes. In the last 2 minutes of this period spontaneous paw flinches were measured by a researcher. Mechanical allodynia was then assessed by second researcher using the up-and-down method [28] as described in previous studies of the group [29]. Briefly, the sural dermatome was probed with a series of von Frey calibrated monofilaments: 15.0g, 8.0g, 6.0g, 4.0g, 2.0g, 1.0g, 0.6g and 0.4g (North Coast Medical Inc., USA). Starting with the 2.0g filament, the test would advance upward if no response was elicited (=0) or downward if a brisk withdrawn of the limb was produced (=X) until 6 measurements were obtained around the threshold point according to the model developed by Dixon [30]. Paw movements, associated with locomotion or weight shifting, were not counted as a response. The 50% response threshold was then calculated using the following formula

$$50\%g\_threshold = \frac{(10^{Xf+K.\delta})}{10000}$$

where X_f= value (in log units) of the final von Frey filament; k= tabular value corresponding to pattern of positive and negative responses (X and 0 sequence;

consult [28]); δ = mean difference (in log units) between stimuli (0.224). If no response was obtained up to maximal force (15.0g) or conversely, if all filaments elicited a response down to the minimal force (0.4g), the values 15 and 0,25 were assumed as the 50% withdrawal threshold, respectively.

SNI animals were classified as low- (LT) and high-thresholds (HT) SNI if thresholds to mechanical stimuli were < 7.0 and ≥7.0 g, respectively. This value was chosen to ensure that there is a clear segregation between LT and HT animals. HT animals corresponded to 12,6%, which agrees with previous studies [26]. For practical reasons and to avoid cage-related bias, HT and the respective LT cage mates were included in the subsequent protocols; the remaining LT SNI animals (N=39) were not included in this study (Fig. 1A) and they were used in other studies of the group. In the subsequent 30 days, animals were left undisturbed to avoid possible interferences except for regular weighting and gentle handling (Figs. 1B and 1C).

2.4. Battery of behavioral paradigms

The battery of behavioral paradigms was performed by two researchers (different gender). We started the behavioral analysis by the dark/light test (D/L, anxiety-like behavior [31]; a day after we performed the forced swimming test (FST, learned helplessness – 2 days) [32]; followed by fear-conditioning (FC, contextual/cued fear memory – 3 days) [33]. Finally, we assessed spontaneous burrowing behavior (SBB, general well-being/pain – 5 days) [34,35] see Fig. 1C. All behavioral experiments were performed during the dark period starting 1 hour after lights off. To avoid possible bias related to the testing period in the long protocols, animals belonging to different groups were tested alternately.

2.4.1. Dark/light paradigm (D/L; anxiety-like behavior)

The DL [31] was performed in a squared open field arena (43.2 × 43.2 cm) that had transparent acrylic walls and a white floor (model ENV-515, Med Associates Inc, St. Albans, VT 05478). The protocol was previously described by our group [36]. Briefly,

the open field arena was divided equally (light and dark sides) with a black acrylic box with an aperture allowing the free movement of the animals between compartments. The light chamber was illuminated by a single light above the arena producing 235 lux. Each animal was initially placed in the center of the light side and horizontal activity and instant position were registered using a system of two 16-beam infrared arrays connected to a computer during 10 min. The ratio between the times spent in the dark and in the light chambers was used as an index of anxiety-like behavior. In addition, the total distance traveled was used as an index of locomotor activity.

2.4.2. Forced swimming test (FST; learned helplessness)

The FST [32] was performed in a glass cylinder filled with water (21-23°C) to a depth of 40 cm, such that the animals could not lay their rear paws on the bottom without being totally submersed, and with a height of 30 cm above the water level, thus preventing escaping [5,37]. Rats were placed in the cylinder for 5 minutes in two sessions in consecutive days; the second session was video recorded and later analyzed by an experimenter blind to the experimental condition of the animals using *Etholog 2.2* software [38]. Latency to first immobilization, immobility and active swimming were quoted.

2.4.3. Fear-conditioning (FC; contextual fear memory)

The FC was performed in a startle response box (SR-LAB™, San Diego Instruments, San Diego, CA, USA) containing a non-restrictive plexiglas cylinder (diameter 8.8 cm, length 22.2 cm) and a steel grid through which an electric current could be passed, as previously described by the group [36,39,40]. In the first day of the protocol (habituation) each animal was placed in the plexiglas cylinder within FC box for 11 minutes; no stimulus was presented. In the second day (conditioning), each rat was left in the cylinder/box for 3 minutes without any stimulation followed by 8 minutes in which 6 shocks (unconditioned stimulus; US) (0.4±0.1 mA) and light (conditioned stimulus; CS) pairs were presented with an inter-stimulus-interval of 60

seconds. In each US/CS pair, the light was on for 20 seconds; the shock was given immediately after light was turned off. The same protocol used for conditioning is used in the last day (test) except that no shock was given. Freezing behavior was analyzed *a posteriori* in the videos by an observer blind to the experimental conditions using Etholog 2.2 software [38]. Freezing was defined as the complete absence of non-respiratory voluntary movements.

2.4.4. Spontaneous burrowing behavior (SBB; general wellbeing)

The SBB evaluates the general wellbeing of the animal [34] and it also reflects the presence of ongoing pain [35]. The SBB protocol was performed as described by Deacon [34], except for some minor alterations detailed below. Gray and opaque PVC tubes (diameter: 90 mm, length: 210 mm) closed in one side and elevated in the open side 70 mm above the cage floor and filled with approximately 2 kg of gravel were used as digging media. In the first day of habituation, empty tubes were left for one day in the rats' home cages. In the second day of habituation, rats were placed, in pairs, in regular rat cages specifically used for SBB (no water and no bedding) for 2 hours (1h with each cage mate), with the PVC tube loaded with gravel. On days 3-5 each animal was placed alone in the respective (same cages used in day before) SBB cages for 2h. At the end of each session the amount of displaced gravel was weighted. Results are shown as the percentage of initial gravel burrowed by the animals in test day. The SBB cages contained no bedding and no food or water was available.

2.5. Sciatic nerve

All the animals were perfused transcardially with ice-cold fixative 4% paraformaldehyde (PFA, in 1x PBS (pH 7,4) under deep sodium pentobarbital anesthesia (200 mg/kg i.p.; Eutasil®, Ceva Saúde Animal). Sciatic nerves were collected and immediately frozen (-80ºC) until use. Coronal cryosections (20 μm) were obtained and then stained for myelin [1:300; FluoroMyelin™Green Fluorescent

Myelin Stain, 1h at room temperature (RT), ThermoFisher, USA], followed with 4',6'-diamidino-2-phenylindole (DAPI, 1μg/ml, 30 min at RT, Sigma-aldrich). The sciatic nerves were analyzed right after the bifurcation. Fluorescence images from sciatic nerve sections were obtained under an Olympus BX61 motorized upright microscope with fluorescence and under the *Olympus cellSens* platform.

2.6. Morphological analysis of prefrontal nuclei

Together with sciatic nerves, brains were removed briefly, placed in PFA 4% for 2 weeks and then were embedded in glycol methacrylate (Tecnovit 7100; Hearus Kulzer, Werheim, Germany). Cut sections (30 μm) were performed in microtome and the brain slices were stained with Giemsa and mounted with Entellan New (Merck, Darmstadt, Germany) [41]. From the brain slices, we analyzed eight frontal areas: medial prefrontal cortex (mPFC) [cingulate (CgL), prelimbic (PrL), and infralimbic (IL) cortices], the orbital frontal cortex [ventral (VO) and lateral (LO) cortices], agranular (Ag-Insula) and granular (Gra-Insula) insular, medial orbital cortex (MO) and primary motor cortex (M1); borders were defined based on Paxinos and Watson atlas [42]. From each hemisphere, volumes from each brain region were estimated by area and cut brain depth (30 μm) from 8 brain sections using *StereoInvestigator* software (Microbrightfield, Williston, VT) and a camera (DXC-390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany). Visualization of the brain volumes was done using BrainNet viewer (https://www.nitrc.org/projects/bnv/) [43] and an in-house created surface mesh and the following regions of interest (ROI) extracted from the Tohoku atlas [44]: Agranular and Dysgranular and Agranular Insular Cortex; PreLimbic System; Cingular Cortex; and Primary Motor Cortex. The prelimbic from the atlas was further subdivided into infralimbic, ventral orbital and medial orbital cortex. The volume of each region was color coded using a blue-red-yellow scale with hotter colors representing higher volume values.

2.6. Statistical analysis

Statistical analysis was performed in *IBM® SPSS® Statistics version 23*. One-way and two-way ANOVA analysis were used in behavioral tasks concerning side of the spared nerve injury installation (SNI-L vs SNI-R) and the threshold observed in these animals (LT vs HT). To correlate spontaneous paw flinches with threshold of mechanical allodynia of SNI animals, in early and late assessment, the Pearson correlation coefficient test was performed. For histological analysis, a three-way ANOVA analysis was performed since another dimension were added and analyzed (hemisphere side – Left v Right). $P<0.05$ was considered to represent a significant difference.

3. Results

3.1. Behavior

3.1.1. Pain measurements

One month after SNI establishment, mechanical allodynia was assessed with Von Frey monofilaments using the up-and-down method; to increase the specificity of our analysis, we further segregated the SNI animals in those with left or right lesions (Fig. 2). One day before the initiation of the battery of behavioral paradigms (early assessment), we measured mechanical allodynia and approximately 13% of SNI animals presented a threshold above 7 to VF monofilaments stimulation and were considered High-Threshold (HT) SNI ($F_{(4,55)}$=306,892, $P<0.001$). Pos-hoc analysis demonstrated a statistically significant difference between Sham and LT animals ($P<0.01$) and no differences were observed in mechanical allodynia from contralateral injury hindpaw (Fig. 2A). The HT animals also presented a decreased number of non-evoked pain manifestations, spontaneous paw flinches ($F_{(4,55)}$=5.333, $P<0.001$). Additionally, LT animals presented a higher number of spontaneous paw flinches (Sham vs SNI LT; $P<0.001$) (Fig. 2B). Independently of threshold, these parameters were statistically correlated (Pearson correlation=-0,480; $P<0.01$) (Fig. 2C). SNI LT and SNI HT show no differences regarding lesion side comparison

($F_{(1,46)}$=0.002, n.s.). Later assessment, after the application of the battery of behavioral tests and the day before sacrifice, a similar pattern was observed for evoked ($F_{(4,55)}$=368.006, P<0.001), spontaneous pain-like behaviors ($F_{(4,55)}$=3,760, P<0.01;Sham vs SNI LT P<0.01) and for their correlation (Pearson correlation=-0,444; P=0.01) (Fig. 2D-F). In this later assessment, the mechanical allodynia of contralateral hindpaw remains equal such as early assessment and LT and HT became even more segregated. Animals with same values (threshold and spontaneous paw flinches) are represented in same point. The staining of ipsilateral nerves reveals that ipsilateral sciatic nerves from LT and HT present a reduction in myelin, showing that SNI was equally performed in lesioned animals and the results obtained are not due to operator manipulation (Fig. 2G).

3.1.2 Anxiety-like behavior

No significant differences were found between LT and HT animals regarding anxiety-like behavior ($F_{(1,46)}$=2,190, n.s.). As previously shown [5], in the D/L paradigm we observed that SNI-L animals presented an increased anxiety-like behavior when compared to SNI-R animals ($F_{(1,43)}$=4.108, P<0.05). No interactions between threshold and side were found (Fig.3). Subsequent analysis demonstrated a statistically significant difference between Sham and SNI-L ($F_{(2,\ 52)}$=5.561, P<0.01) but not between Sham and SNI-R (Fig. 3A). Concerning, distance traveled in the arena, no significant effects of lesion side ($F_{(1,46)}$=0,164, n.s.) or threshold ($F_{(1,\ 46)}$=0.864, n.s.) were found in SNI animals. However, both SNI-L and SNI-R travelled a statistically significant shorter distance than sham animals ($F_{(2,\ 53)}$=6.315, P<0.01; Sham x SNI-L P<0.05 and sham x SNI-R P<0.01) (Fig 3B).

3.1.3. Learned helplessness

No statistically significant side ($F_{(1,\ 46)}$=0.041, P=0.841) and threshold ($F_{(1,\ 47)}$=1.016, n.s.) effects in immobility time were observed. Also in time to latency, no significant side ($F_{(1,\ 46)}$=0.004, n.s.) and threshold ($F_{(1,\ 46)}$=1,525, n.s.) effects were observed in the forced swimming test (Fig.4 A and B).

3.1.4. Contextual fear memory

In the contextual fear paradigm, freezing behavior was measured over 3 days – habituation, US/CS pairing and CS. Freezing in the last session was used as an index of fear contextual memory. No side and no threshold effects were observed in baseline ($F_{(1,46)}$=0.140, n.s.); ($F_{(1,46)}$=0.611, n.s.) (Fig. 5A), CS ($F_{(1,46)}$=0.678, n.s.); ($F_{(1,46)}$=0.013, n.s.) (Fig. 5B) and in the variation between the two conditions ($F_{(1,46)}$=0.097, n.s.); ($F_{(1,46)}$=1.763, P=0.191) (Fig. 5C). Similarly, Sham and SNI animals presented no differences in habituation, US/CS pairing and CS [($F_{(1,46)}$=0.384, n.s.) (Fig. 5A); ($F_{(1,46)}$=0.666, n.s.) (Fig. 5B) and ($F_{(1,46)}$=0.467, n.s.) (Fig. 5C)] in this behavioral paradigm, meaning that contextual fear memory was not impaired.

3.1.5. General Wellbeing

We assessed the general wellbeing using the Spontaneous Burrowing Behavior (SBB) (Fig. 6) protocol. We observed that SNI-L animals dig lesser than Sham and SNI-R animals ($F_{(2,52)}$=6,544, P<0.01) but no thresholds ($F_{(1,46)}$=0.200, n.s.) or interactions between the two factors were observed meaning that the SNI-L animals present a decrease wellbeing compared with controls and right-lesioned animals.

3.2. Cortical morphometry

Volumes from the CgL, PrL, IL, VO, LO, MO, M1, Ag-Insula and Gra-Insula nuclei were analyzed in both hemispheres (Fig. 7). From the different brain regions analyzed and concerning the threshold factor only the Ag-Insula from LT animals presented higher volume than HT animals (LT>HT; $F_{(1,37)}$=4.942, P<0.05) independently of the hemisphere side. On the contrary, no differences were found in the IL volume regardless hemisphere, lesion side and threshold. On the remained brain regions included in the study CgL, PrL, VO, LO, MO, M1 and Gra-Insula, all presented a hemisphere effect but not lesion side or threshold effect; with the

exception of the M1 and Gra-Insula, in these areas the left hemisphere was significantly bigger than the right hemisphere.

4. Discussion

In the present study, we tested the hypothesis that "silent" peripheral neuropathic lesions can impact in behavior despite the absence of pain. In summary, we observed that (i) 12,6% of SNI lesioned animals (11 out of 87 SNI animals) presented thresholds to evoked mechanical stimuli close to sham controls; (ii) these HT animals also presented decreased spontaneous flinches reinforcing the interpretation that the algogenic impact of the lesion was effectively smaller than in LT; (iii) the phenotype evolved with time and the difference between LT and HT becomes more clear in a later assessment; (iv) left-sided SNI injuries were respectively associated with increased anxiety independent of the algogenic quality of the lesion and in accordance with previous studies of our laboratory [5]; (v) SNI-L (LT and HT) but not SNI-R presented decreased burrowing; finally, (vi) we observed a decreased volume of the insula (agranular division) of SNI HT animals.

Rodents are heterogeneous in many behavioral dimensions – presenting different resistance/susceptibility profiles to a number of challenges (e.g. chronic stress), even in highly controlled laboratorial settings – see a recent review from Einat and colleagues [45]. Regarding the specific case of pain, factors underlying resistance and/or susceptibility to its manifestation after a neuropathic lesion are not entirely understood. Genetic background seems to play a role in pain manifestation in both mice [46] and rats [26]. Also, high trait anxiety is associated with pain outcomes in peripheral neuropathy models [47,48]; furthermore, anxiogenic conditions like chronic stress have also been associated with increased pain manifestations [49-51]. On the other hand, potential differences in the SNI lesion are unlikely as the model is simple and reproducible (a tight ligation followed by a distal sectioning). Indeed, analysis of the sciatic nerves revealed a decrease in myelin content in HT and LT SNI in accordance with previous studies [52-55] as well as increased cellularity probably associated with the presence close to the injury site of macrophages, and other cells – see for instances [55-58]. Differences between LT an HT in our study are also unlikely

to be related with the evaluation of pain as in addition to the thresholds to light mechanical probing, LT and HT also differed in the amount of spontaneous paw flinches and the former presented a tendency for increased depressive-like behavior. This type of manifestation was shown to have a late onset which might explain our observation – see on this topic [4,24]. Finally, we searched for distinctive endophenotypes and we observed that insular cortex (agranular division) volume – an area with a well-established role in pain perception [59-61] – was smaller in LT. Brain morphology has been extensively studied in chronic pain, particularly in humans – see for review [62,63] – but also in rats [64]. In this experimental study, also using the SNI model, a reduction of the prefrontal cortex volume was observable by the 9th week post-surgery becoming statistically significant by the 4th month [64], i.e. outside the time window of our study. Indeed, we have not observed evident signs of atrophy in any of the areas analyzed.

Painful and painless neuropathies have also been studied in clinical conditions. Increased expression of the pro-inflammatory cytokines IL-2 and TNF- alpha were shown to be associated with painful neuropathies, while increased expression of the anti-inflammatory IL-10 was observed in patients with painless neuropathies [65,66]. Patients with painful neuropathies also presented increased depression scores. It is nevertheless difficult to establish a causal relation between these observations. Depression, as well as other psychosocial constructs like anxiety and catastrophization, can be predictive of chronic (post-surgical) pain [67,68], i.e. they might precede and affect pain evolution. Also, the association between depression and cytokines is well-stablished [69,70] – see for review [71,72] – including in neuropathic pain conditions [73] – see for review [74]. Finally, it is not clear if cytokine values observed in the above-mentioned are the cause of or the result from differences in the pathophysiology between the 2 groups [65].

Our study demonstrates that these questions can be tackled in controlled conditions using a model where the phenotype painful/painless is not experimentally

conditioned. Furthermore, it raises a question with potentially relevant clinical impact regarding the "silent" character of the painless neuropathies. Indeed, we demonstrate that despite the absence of pain the neuropathic lesion is still effective and potentially deleterious. Indeed, HT animals manifested anxiety-like behavior similar to that observed in LT animals. Curiously, this impairment manifested in a side-specific manner, left-sided SNI being most affected, in accordance with previous observations of the group [5,6]. Such reinforces the role of the lesion and its segregation from pain-related effects (no differences were observed between left- and right-sided SNI regarding thresholds to light mechanical stimulation or spontaneous paw flinches). Similarly, only SNI-L (HL and LT) were affected in spontaneous burrowing. SBB is a part of rodents' behavioral repertoire and has been used as a proxy of well-being [34]. Pain has also been shown to reduce burrowing in several experimental models [35,75-77] – see also [13,78,79] – though some inter-animal [76] and inter-laboratory [77] variability has been reported. Another evidence from segregation between sensory and affective impacts of neuropathies comes from a work using the cuff model reporting that upon cuff removal thresholds to mechanical stimulus evolved to near baseline values while anxiety-like behavior maintained elevated [25]. Finally, De Felice and colleagues showed that the administration of lidocaine in the rostral ventromedial medulla (RVM) can momentously shifts HT to LT profile providing an additional evidence that the "silent" neuropathy is in fact active [26].

In summary, we took advantage of the natural variability of an outbred strain we were able to demonstrate that heightened anxiety observed in SNI animals is to some extent independent of pain/hypersensivity. Rather, our results suggest independent mechanists with specific kinetics (anxiety manifestation is substantially delayed in relation to allodynia; see above) that are triggered by a common event, the nerve injury.

5. Conflict of interest statement

None of the authors report a conflict of interest.

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Figure legends

Figure 1. Experimental organization. The SNI model was installed in 87 Sprague-Dawley rats. From this batch, high-threshold (HT) and respective low-threshold (LT) cage-mates were included in subsequent experiments (A). Animals were regularly monitored throughout the protocol (B) and were tested according to the following sequence of behavioral paradigms: D/L, FST, FC and SBB (C). D/L, Dark Light; FC, Fear Conditioning; FST, Forced Swimming Test; HT, High Threshold; LT, Low Threshold; SBB, Spontaneous Burrowing Behavior; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury; VF, Von Frey (mechanical allodynia).

Figure 2. Pain-related behaviors. In an early assessment, LT and HT SNI animals can be clearly distinguished based on both evoked (A) and spontaneous (B) pain-like manifestations. These 2 parameters presented a statistically significant correlation (C). Similar results were obtained in a later assessment, after the behavioral testing (D-F). The 2 groups, LT and HT, became more segregated in this later assessment (F). Representative coronal sections of ipsilateral sciatic nerves from both SNI groups, LT and HT, stained for fluoromyelin (in green) and 4',6'- diamidino-2-phenylindole (DAPI) in blue (G); a marked reduction of myelin and increased cellularity can be observed in the both SNI groups. HT, High Threshold; LT, Low Threshold; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury; VF, Von Frey (mechanical allodynia). *P<0,05; **P<0,01; ***P<0,001; Data presented as mean ± SEM.

Figure 3. Dark/light paradigm. SNI side, but not threshold, was associated with dark/light, ratio outcome, particularly SNI-L presented an increased anxiety-like behavior (A). Locomotor activity did not differ within SNI groups although sham animals explored more the arena (both dark and light sides) (B). HT, High Threshold; LT, Low Threshold; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury. *P<0,05; **P<0.01 Data presented as mean ± SEM.

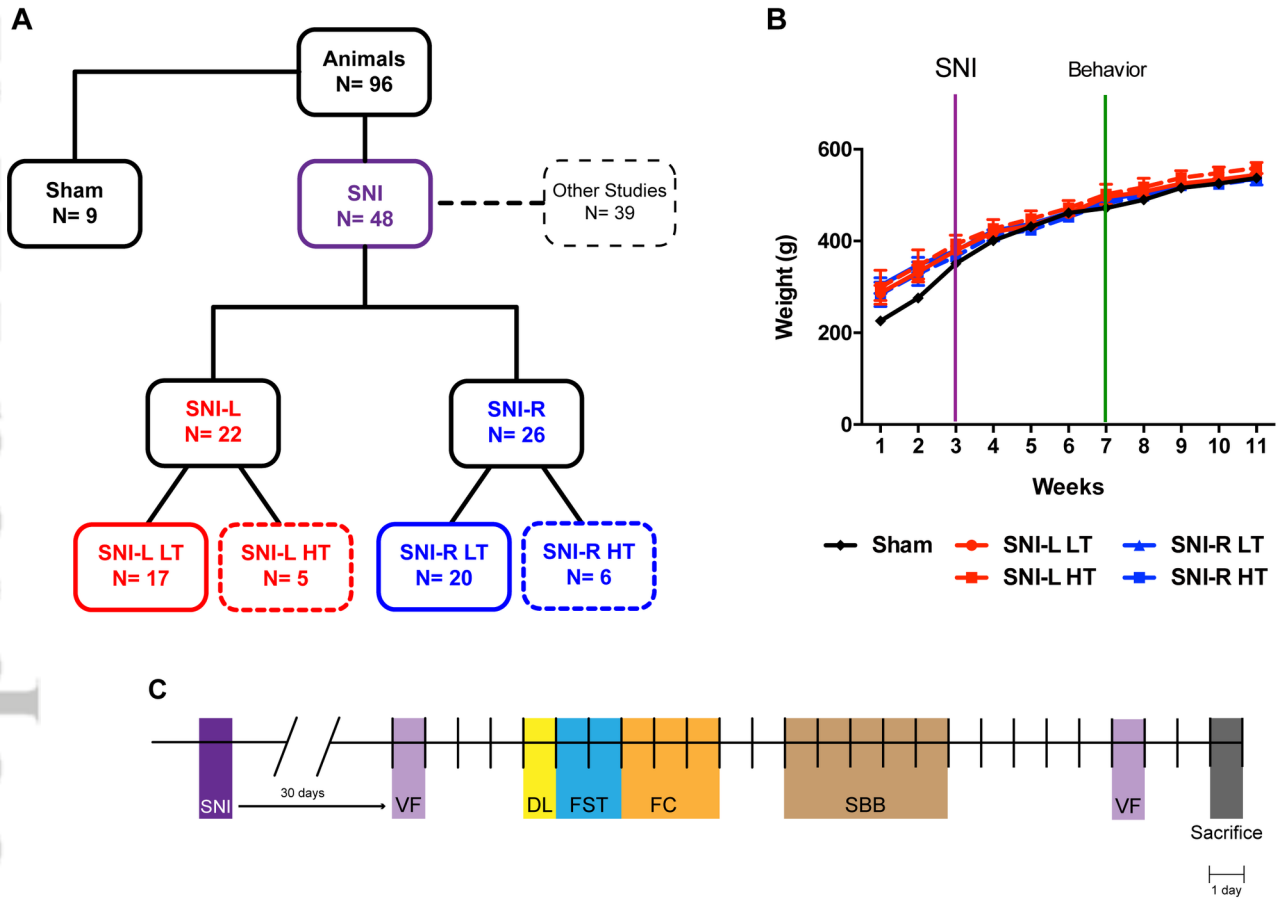
Figure 4. Learned helplessness. No side or threshold of SNI lesion were observed in the forced-swimming test parameters total immobility time (A) and latency to immobility (B) indicating that depressive-like behavior is not affected. HT, High Threshold; LT, Low Threshold; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury. Data presented as mean ± SEM.

Figure 5. Contextual fear memory. Freezing behavior was used as a proxy of fear in a basal condition (A) and after the presentation of the CS/light (B) previously paired with a noxious US/foot shock. All groups presented a similar increment in freezing behavior upon the presentation of the CS (C) indicating no differences in contextual fear memory. CS, conditioned stimulus; HT, High Threshold; LT, Low Threshold; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury; US, unconditioned stimulus. Data presented as mean ± SEM.

Figure 6. General wellbeing of the Sham and SNI animals. Left-lesioned animals present decreased burrowed behavior than Sham and right-lesioned animals. HT, High Threshold; LT, Low Threshold; SNI-L, Left Spared Nerve Injury; SNI-R, Right Spared Nerve Injury; *P<0,05; **P<0,01. Data presented as mean ± SEM.

Figure 7. Cortical morphometry. Volumes were estimated in coronal sections and obtained volumes are represented in axial top, axial bottom and frontal coronal brain representations (A) and in graphics by region of interest (B). No differences were observed in overall volumes of the nine areas analyzed, however we detected a statistically significant threshold effect on the Ag_Insula (LT>HT) and several volumetric asymmetries: L>R (CgL, PrL, VO, LO, MO) and L<R (Gra_Insula and M1). Ag_Insula, Agranular Insula; CgL, cingulate cortex; Gra_Insula, Granular Insula; HT, High Threshold; IL, Infralimbic cortex; LO, Lateral orbital cortex; LT, Low Threshold; M1, Primary Motor Cortex; PrL, Prelimbic cortex; SNI-L, Left Spared

Nerve Injury; SNI-R, Right Spared Nerve Injury; VO, Ventral orbital cortex. Data presented as mean ± SEM.



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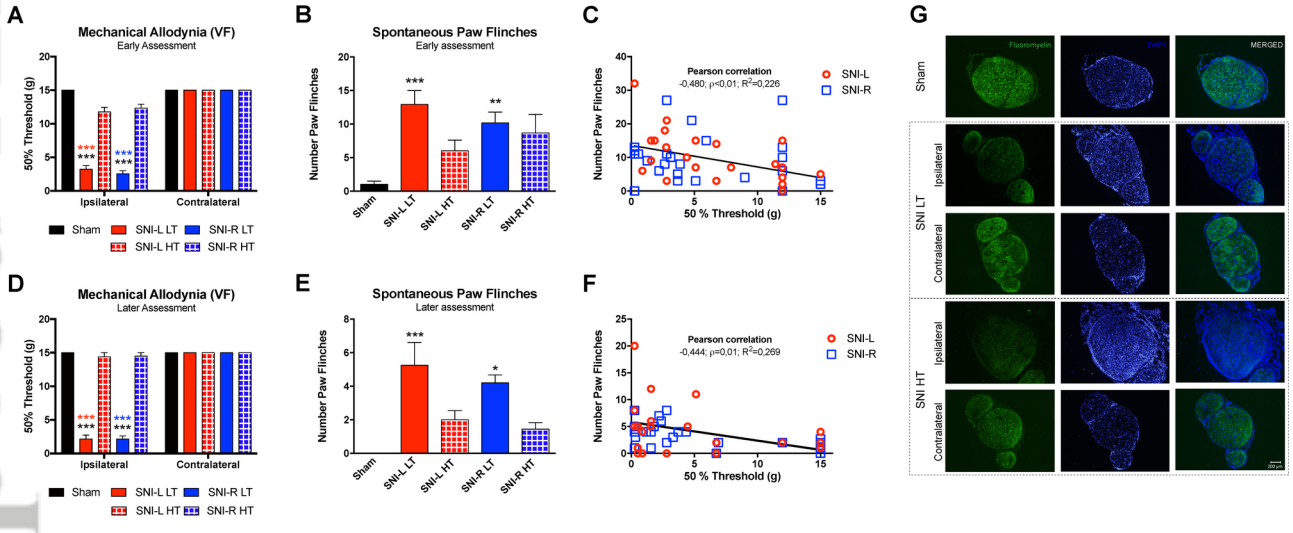

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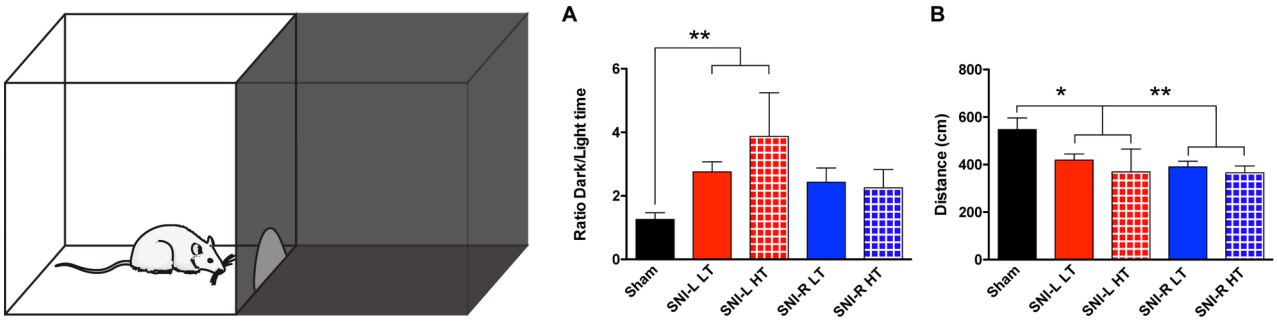

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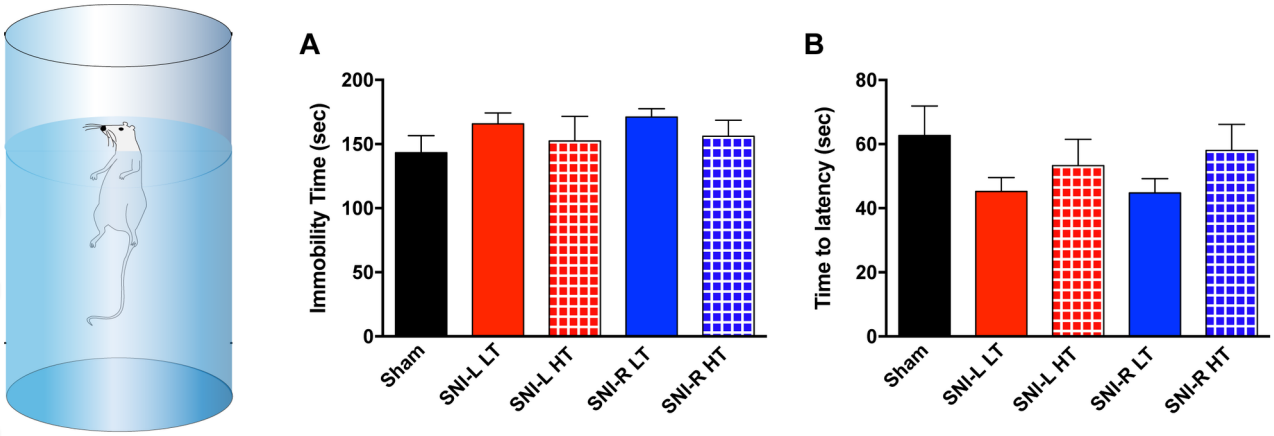

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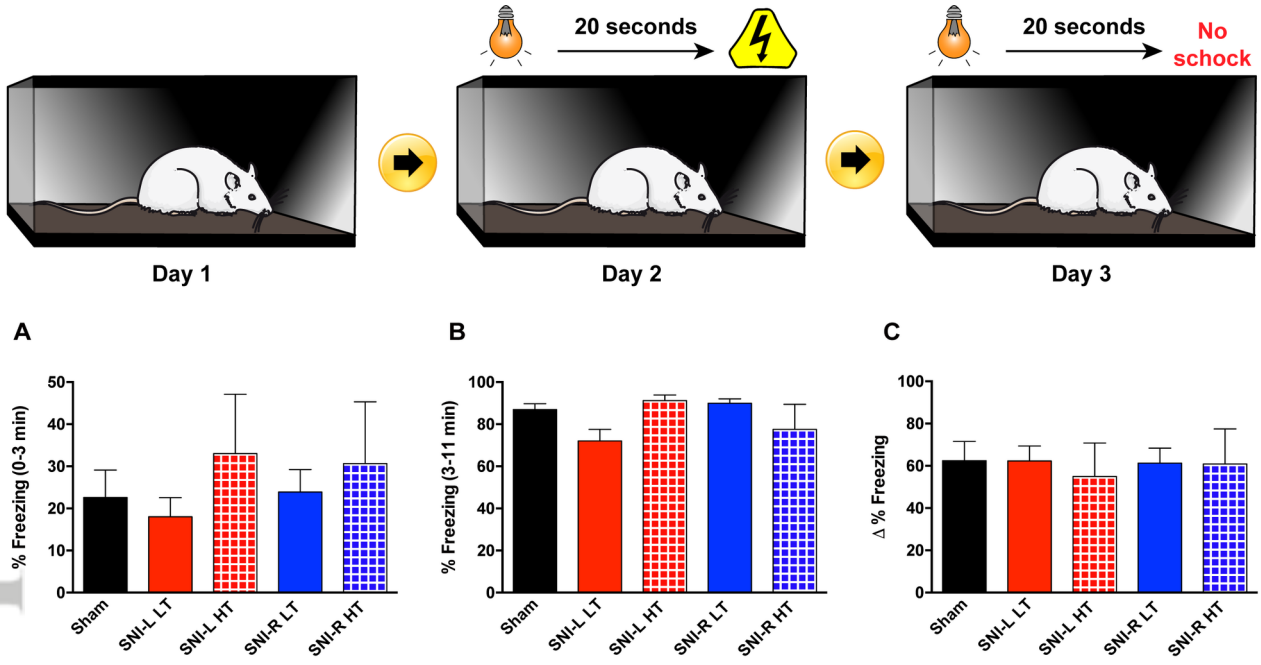

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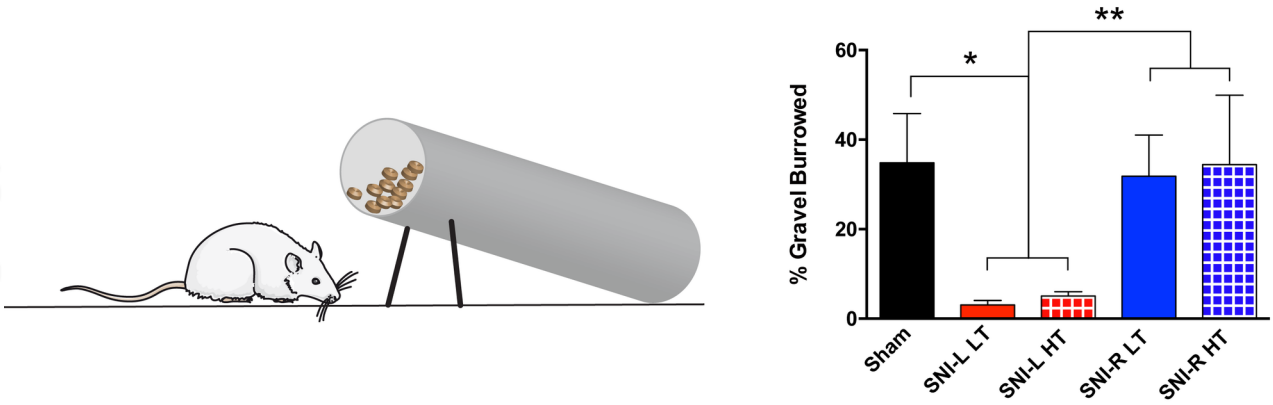

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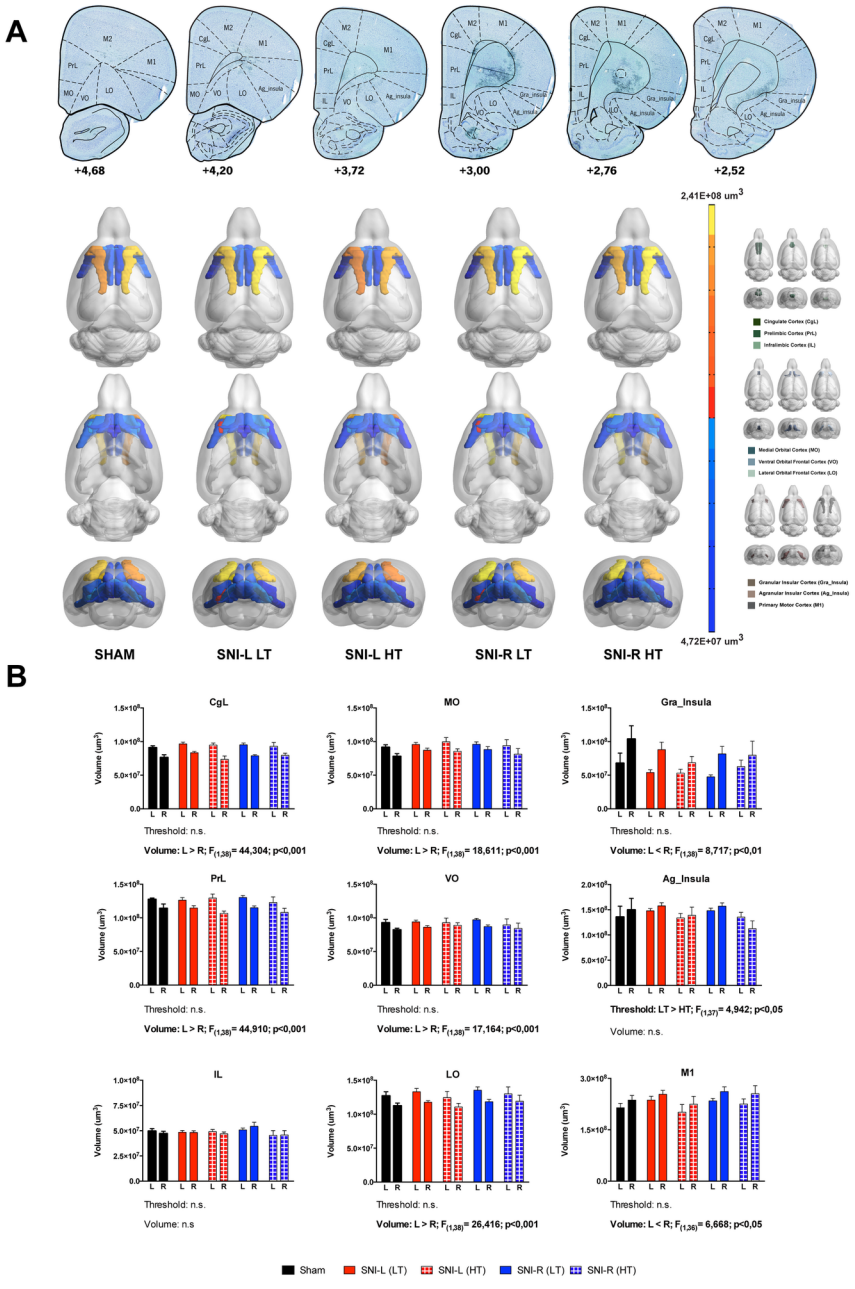

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