Highlights

This article provides evidences of the influence of sociability, anxiety and depression traits on neuropathic pain. These results may help in the understanding of mechanisms that could explain the inter-individual variation of responses to neuropathic pain, which could provide an additional step for the development of efficient personalized treatments.

Abstract

Neuropathic pain is a complex disorder associated with emotional and cognitive deficits that may impair nociceptive manifestations. There is high inter-individual variability in the manifestations of human neuropathic pain, which largely depends on personality traits. We aim to identify the influence of different behavioral traits in the inter-individual vulnerability to neuropathic pain manifestations using behavioral, electrophysiological and genetic approaches. We first selected mice with extreme social and emotional traits and look for correlation with the spontaneous neuronal activity in the central amygdala. Neuropathic pain was induced to these mice to evaluate the influence of behavioral traits on nociceptive manifestations and gene expression profiles in the amygdala. Our results show an association of the spontaneous central amygdala neuronal activity with the sociability behavior. We demonstrate that low sociable, high anxious and low depressive phenotypes develop enhanced nociceptive hypersensitivity after nerve injury. However, greater emotional alterations and cognitive impairment are observed in high sociable, anxious-like and depressive-like mice, indicating that nociceptive, emotional and cognitive manifestations of neuropathic pain do not correlate with each other. Gene analyses identify high *Pdyn* and *Il6* levels in the amygdala as indicative of enhanced nociceptive hypersensitivity and reveal an association between high *Gadd45* expression and attenuated emotional and cognitive manifestations of neuropathic pain.

Influence of behavioral traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain

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1. Introduction

Chronic neuropathic pain is a complex disorder that includes nociceptive, emotional and cognitive manifestations (Apkarian et al., 2004; La Porta et al., 2016). Several reports have established its association with emotional alterations, such as anxiety and depression (La Porta et al., 2016; Neugebauer et al., 2004), as well as with cognitive deficits, including memory, learning and decision making impairment (Apkarian et al., 2004; Conrad et al., 2007). Nociceptive, emotional and cognitive alterations could aggravate each other leading to an impairment of the quality of life of patients suffering neuropathic pain (Apkarian et al., 2004; Conrad et al., 2007). Therefore, the therapeutic approach for the treatment of these patients should consider these three dimensions of chronic pain.

The manifestations of neuropathic pain show a high inter-individual variability that depends on multiple factors, including the personality traits of patients (Asghari and Nicholas, 2006). It has been well documented that emotional, cognitive and social personality traits are important factors to modulate pain perception (D'Amato and Pavone, 2012; Rhudy et al., 2008). Clinical studies revealed that people with high anxiety sensitivity (Keogh and Mansoor, 2001) or anxiety disorders (Defrin et al., 2008) displayed amplified pain intensity. Conversely, social support has been associated with lower pain intensity in response to experimental stimuli and in chronic pain conditions (Montoya et al., 2004). According to human studies, social relationships may improve coping responses and overall function in chronic pain, promoting pain-specific resilience (Sturgeon and Zautra, 2016). The influence of depression modulating pain

intensity is still not conclusive, since both pain attenuating (Bär et al., 2006; Schwier et al., 2010) and enhancing (Chiu et al., 2005) effects of depressive disorders have been reported.

The brain areas responsible for such influences are not well known, although several evidences strongly support a crucial role of the amygdala in the emotional-affective dimension of pain (Ikeda et al., 2007; Neugebauer et al., 2009, 2004). The amygdala plays a key role in the formation of fear-related memories and emotional processing (Phelps and LeDoux, 2005) and contains several nuclei, including the lateral (LA), basolateral (BLA) and central (CeA) nuclei, which are important for sensory processing (Neugebauer et al., 2009). Strong neuronal responses to peripheral nociceptive stimuli have been reported in the CeA, which has been defined as the 'nociceptive amygdala' (Neugebauer et al., 2004). Indeed, increased excitability of CeA neurons has been reported in arthritic (Neugebauer et al., 2003), visceral (Han and Neugebauer, 2004) and neuropathic pain models (Gonçalves and Dickenson, 2012; Ikeda et al., 2007), as well as in patients with generalized anxiety, social phobia, panic and posttraumatic stress disorder (Etkin and Wager, 2007).

In this study, we evaluated the influence of sociability, anxiety-like and depressive-like behavioral traits on the nociceptive, emotional and cognitive manifestations of neuropathic pain, using an out-bred mouse line that resembles human genetic heterogeneity. We analyzed the possible correlation between spontaneous CeA activity and behavioral traits using mice displaying extreme phenotypes on social and emotional responses. Neuropathic pain was induced in these mice to evaluate the influence of behavioral traits on the inter-individual variability of pain manifestations. Gene expression profiles in the amygdala were also studied to elucidate its contribution to the molecular mechanisms associated with chronic neuropathic pain.

2. Methods

2.1. Animals

Swiss albino male mice with an initial body weight between 20-22g (Charles River, Lyon, France) were used in these experiments. Mice were housed in groups of 2 to 4 with free access to water and food. The housing conditions were maintained at 22 ± 1˚C and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 8:00 A.M. and 8:00 P.M.). Animals were handled for 5 days before starting the experimental sequence. 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. All the experiments were performed under blinded conditions.

2.2. Experimental protocol

Two hundred and fifty mice were exposed to locomotion, sociability, anxiety-like and depressive-like behavioral tests as indicated in Figure 1. Animals displaying high, intermediate and low social, anxious- and depressive-like responses were chosen for further experiments (see Results, 'Selection of the extreme phenotypes' for details of the selection procedure). The selected animals were homogeneously distributed in two experimental cohorts with representation of all the phenotypic groups. Spontaneous CeA neuronal activities were recorded in mice selected for each phenotype of the first cohort. Animals from the second cohort were exposed to a partial sciatic nerve ligation or sham surgery to induce neuropathic pain. Nociceptive responses were assessed under basal conditions and on days 3, 6, 11, 16 and 21 after nerve injury. Anhedonic state, anxiety-like behavior and cognitive performance were evaluated on day 10, 15 and 20 post-surgery, respectively, using different paradigms than in the initial screening step to

avoid double exposition of mice to the same behavioral model (Fig. 1). Finally, amygdala samples were freshly dissected at day 41 after neuropathic pain induction from animals used for the behavioral study. Transcriptional modifications in this area were examined.

2.3. Behavioral tests

2.3.1. Locomotion activity

Locomotor activity was evaluated as previously described (Martin et al., 2000) by using actimetry boxes (9 × 20 × 11 cm) (Imetronic, Lyon France) in a low luminosity room (5 lux), and with white noise. Each box contained two lines of photocells located 2 cm and 6 cm above the floor to measure horizontal and vertical movements, respectively. Mice were individually placed in the boxes and the number of activity counts was recorded for a period of 30 min.

2.3.2. Sociability behavior

Sociability test was performed the day after the locomotor activity evaluation to determine the extreme phenotypes. A black Plexiglas V-maze was used with 15 cm bars of transparent Plexiglas placed at 6.5 cm of the end of each arm that separate both sides, although allowing exploration (Panlab). The mouse was first habituated to the empty maze during 5 min. In a second step, sociability behavior was evaluated during 5 min by placing one stranger animal in the maze, behind the Plexiglas bars. A sociability index was calculated as the difference between the time spent exploring either the stranger mouse or the empty space divided by the total exploration time, onwards considered as "social preference".

2.3.3. Anxiety-like behavior

Three experimental paradigms were used. The elevated plus maze (EPM) and light/dark box (LDB) tests were used to determine the extreme phenotypes, whereas the elevated zero maze (EZM) was performed after sciatic nerve injury.

EPM test was performed 3 days after social behavior evaluation using a black Plexiglas apparatus with 2 open (45 lux) and 2 closed (5 lux) arms (29 cm long x 5 cm wide), set in cross from a neutral central square (5x5 cm) that was elevated 40 cm above the floor. The percentage of entries and time spent in the open arms were determined during 5 min, as previously reported (Busquets-Garcia et al., 2011).

LDB test was carried out 3 days after the EPM, as previously described (Filliol et al., 2000). A Plexiglas box composed of a small dark compartment (15×20×25 cm, 10 lux) and a large light compartment (30×20×25 cm, 500 lux) separated by a connecting 4 cm long tunnel was used. Floor lines separated the light compartment into three equal zones, from the tunnel to the opposite wall, designated as proximal, median and distal zones. The percentage of distal entries, the time in the light compartment was hand scored during 5 min.

EZM was performed 15 days after nerve injury, as previously described (Valverde et al., 2004), using a circular black Plexiglas apparatus (5.5 cm wide and with inner diameter of 46 cm) with 2 open (100 lux) and 2 wall-enclosed sections (10 lux) elevated above the floor (50 cm). The percentage of entries and time in open arm was measured during 5 min.

2.3.4. Depressive-like behavior

Three experimental paradigms were used: the tail suspension test (TST) and forced swimming test (FST) to determine the extreme phenotypes and the sucrose preference test (SPT) was performed after sciatic nerve injury.

TST was performed 3 days after the LDB, as previously described (Steru et al., 1985). Mice were suspended by their tails with tape, in such a position that escape or hold on to nearby surfaces were not allowed during 6 min. The immobility time was recorded during the last 4 min of the test, when mice show a sufficiently stable level of immobility.

FST was performed 5 days after the TST. Mice were placed in a narrow (17.5 x 12.5 cm) Plexiglas cylinder containing water to a depth of 15 cm (22 °C ± 0.2 °C) (Porsolt et al., 1977). Each animal was subjected to a forced swimming during 6 min and the total duration of immobility, disregarding small maintenance movements, was measured during the last 4 min, when mice show a sufficiently stable level of immobility.

SPT was performed 10 days after nerve injury, using an extremely high sensitivity (0.02 g) monitoring system (Phecomp, Panlab, ES), recently validated in our laboratory (Bura et al., 2013). Two-bottle choice procedure allows for a comparison between behavioral preference for sucrose solution (2%) in drinking water compared to water only. Three days before the test day, a 24 h session was performed to habituate the mice to the environment and the different drink solutions. During a test session of 24 h, preference is measured by volume of liquid consumed, which is then converted to a percent preference calculated as the ratio of the sucrose solution intake to total liquid intake x 100. Sucrose is a natural reinforcer and sucrose preference is attenuated by a diversity of chronic stressors, which is indicative of anhedonic-like state (i.e., inability to feel

pleasure). Thus, SPT is useful to investigate anhedonia, a commonly-accepted symptom of depressive-like behavior.

2.3.5. Cognitive evaluation

The novel object recognition (NOR) test was performed 20 days after nerve injury as previously described (Puighermanal et al., 2009) in the same V-maze used for sociability behavior evaluation without the transparent Plexiglas bars. Three phases of 9-min were performed on consecutive days. Mice were first habituated to the V-maze. On the second day, 2 identical objects (chess pieces) were presented to the mice, and the time that they spent exploring each object was recorded. The third day, 1 of the familiar objects was replaced with a novel object (a different chess piece), and the time spent exploring each object (novel and familiar) was computed. A discrimination index was calculated as the difference between the times that the animal spent exploring the novel (Tn) and familiar (Tf) object divided by the total time of object exploration: (Tn-Tf)/(Tn+Tf).

2.4. Electrophysiological procedures

Extracellular single-cell in vivo recordings were made from single neurons in the right CeA after the behavioral test used to select extreme phenotype mice. Parylene coated tungsten electrodes were applied (A-M Systems, USA) using the following stereotaxic coordinates (Franklin and Paxinos, 2008): 4.4 mm dorsoventral, 2.4 mm lateral and 1.06 mm caudal to bregma. The animals were anesthetized with isofluroane (1.5–1.7%) delivered in a gaseous mix of N2O (66%) and O2 (33%). Under anesthesia, animals were fixed in the stereotaxic device, the skull was exposed and the CeA coordinates found. A small craniotomy was performed and the dura mater taken, allowing access to

the brain. Anesthesia was maintained with isofluroane (1.5–1.7%) delivered in a gaseous mix of N2O (66%) and O2 (33%) for the entire duration of the recordings. All the neurons found in the CeA that fired spontaneously for at least 20 min were recorded (2-5 neurons/animal). Besides spontaneous activity, neuronal firing evoked by von Frey filaments (0.008g, 1g, 4g, 8g, 15g, 26g and 60g), pinch, heat (48^{o}C) and cold (4^{o}C) applied to both paws as well as by pinch, heat (48^{o}C) and cold (4^{o}C) applied to the tail and both ears was recorded. Each stimulus was applied continuously during 5 seconds. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions). At the end of each experiment, after a lethal level of isoflurane had been delivered, the brains were extracted and sliced, the recording sites verified through the placement of the electrode and plotted on a standardized section from the mouse brain atlas (Franklin and Paxinos, 2008). All neurons included were located within the CeA (Fig. S1).

2.5. Neuropathic pain induction and assessment

2.5.1. Neuropathic pain model

A partial sciatic nerve ligation (PSNL) was used to induce neuropathic pain to the selected mice (Malmberg and Basbaum, 1998). Briefly, mice were anaesthetized with isoflurane (induction 5%; surgery 2%) 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., Fort Worth, TX, USA). The remaining nerve was left untouched. The muscle was stitched and the

incision was closed with wound clips. Sham mice underwent the same procedure without manipulation nor ligation of the nerve.

2.5.2. Nociceptive behaviors

Mechanical allodynia, heat hyperalgesia and cold allodynia were used as outcome measures of neuropathic pain, as previously reported (La Porta et al., 2016). Mice were tested in each paradigm at different time points (see experimental protocol), using the same sequence.

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response to von Frey filaments stimulation, after 1h of habituation period. Animals were placed in Plexiglas cylinders (20 cm high, 9 cm diameter) on a metal grid through which the von Frey calibrated filaments (North Coast Medical, USA) were applied by using the up–down paradigm. The threshold of response was then calculated using the up–down Excel program provided by Dr A. Basbaum (University of California, San Francisco, CA), which applies a Dixon non-parametric test (Chaplan et al, 1994). Clear paw withdrawal, shaking, or licking was considered as a positive nociceptive response. Both hind paws were tested.

Heat hyperalgesia was evaluated by measuring paw withdrawal latency in response to radiant heat with plantar test apparatus (Ugo Basile, Italy). Mice were placed in Plexiglas boxes (20 cm high, 9 cm diameter) on a glass surface and habituated to the environment for 30 min before testing. The mean paw withdrawal latencies for the ipsilateral and contralateral hind paws were determined from the average of 3 separate trials, taken at 5-10 min intervals to avoid thermal sensitization. A cut-off time of 20 s was used to prevent tissue damage.

Cold allodynia was assessed with the hot/cold plate analgesia meter (Columbus, USA). A glass cylinder (25 cm high, 20 cm diameter) was used to keep mice on the cold surface of the plate, which was maintained at 5±0.5º C. The number of each hind paw elevations, defined as clear paw lift without displacement, was recorded for 5 min. Walking/stepping movements were not considered. A score was calculated as the difference of number of elevations between ipsilateral and contralateral paws.

2.6. Tissue collection and RNA isolation

The animals were sacrificed 41 days after the PSNL. Brains were removed, and amygdala samples were freshly dissected. The samples were placed in individual tubes with the tissue storage reagent RNAlater (Qiagen Inc., Valencia, CA, USA) and stored at −80°C until RNA isolation. Samples were thawed at room temperature and homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA isolation was performed in accordance with the manufacturer's protocol. The total RNA concentration was measured using a NanoDrop ND- 1000 Spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE, USA). RNA quality was determined by chip-based capillary electrophoresis using an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Reverse transcription (RT) was performed using Omniscript reverse transcriptase (Qiagen Inc.) at 37°C for 60 min.

2.7. Quantitative real-time PCR analysis

The qRT-PCR reactions were performed using Assay-On-Demand TaqMan probes: Hprt1-Mm01545399_m1, Gadd45g-Mm00442225_m1, Il6-Mm00446190_m1, Nr3c1-Mm00433832_m1, Pdyn-Mm00457573_m1, Tsc22d3-Mm00726417_s1, (Applied Biosystems, Carlsbad, CA, USA) and were run on the CFX96 Touch Real-

Time PCR machine (BioRad, Hercules, CA, USA). Each template was generated from an individual animal, and the amplification efficiency for each assay was determined by running a standard dilution curve. The expression of the hypoxanthine guanine phosphoribosyltransferase 1 (Hprt1) transcript was quantified at a stable level between the experimental groups to control for variations in cDNA amounts. The cycle threshold values were calculated automatically by the CFX MANAGER v.2.1 software with default parameters. RNA abundance was calculated as $2^{-(Ct)}$. The transcript levels were normalized against the housekeeping gene, Hprt1, and interpreted using the comparative Ct method.

2.8. Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed using the Statistica 6.0 software (StatSoft, Tulsa OK, USA). For behavioral studies one or two-way ANOVA were performed followed by Bonferroni *post hoc* analysis. Electrophysiological data were analyzed with a one-way ANOVA followed by Dunn's multiple comparison test. RT-qPCR data were analyzed for PSNL and phenotype differences with one-way ANOVA followed by Bonferroni *post hoc* test. Correlation analyses between the behavioral traits and the neuropathic pain manifestations as well as between gene expression and the behavioral traits were performed with the IBM SPSS 19 (SPSS Inc., Chicago, IL, USA) software. A probability of 0.05 or less was considered statistically significant. Detailed statistical analyses are presented in Supplementary Tables S1-S3.

3. Results

3.1. Selection of extreme phenotypes and control groups

Responses of 250 mice to sociability, anxiety- and depressive-like behaviors were recorded in order to classify animals in accordance with their behavioral traits. First, mice with extreme locomotor responses were excluded (65 mice) to avoid a bias of this abnormal behavior, according to excluding criteria in Table 1. Both the exclusion and inclusion cutoffs were set *a priori* as indices of behavioral intensity. One hundred forty-eight animals were then selected considering their extreme or intermediate phenotypes and were homogeneously distributed in cohort 1 (60 mice) and 2 (88 mice) to be further studied. The remaining 37 mice were also excluded for the rest of the experimental sequence because of the lack of fulfillment of inclusion criteria. Cohort 1 was used for electrophysiological studies, whereas cohort 2 was used for behavioral evaluation of neuropathic pain manifestations and for gene transcription study. Two thirds of the mice included in the cohort 2 (55 mice) underwent PSNL, whilst one third (33 mice) was subjected to sham surgery.

Mice showing extreme sociability, anxiety- or depressive-like behavior were classified in two extreme phenotypes (high and low percentiles) for each behavioral trait. One parameter was used for sociability classification, while two independent parameters were considered for anxiety- and depressive-like categorization. The reason for using two tests to measure anxiety- and depressive-like behavior is that these rodent models are not specifically indicated for naïve conditions, but for the evaluation of antidepressant drugs or experimental manipulations that are aimed at rendering or preventing these emotional-like states. Thus, selecting the animals that correlated in both tests makes phenotyping more robust. Previous publications that aim to segregate particular populations with extreme performance in a given behavior considered that

mice above the third quartile (75th percentile) (Mancino et al., 2015) or above the even lower 66th percentile (Deroche-Gamonet et al., 2004) had extreme responses. Based on these previous publications, we defined a more restrictive 80th/20th percentiles as inclusion cutoff when only one variable was used for phenotyping (sociability trait), and 70th/30th percentiles as inclusion cutoff when two independent parameters were considered for the classification (anxiety and depressive traits). Table 1 summarizes parameters and percentiles considered for the classification of extreme phenotype animals for each behavioral trait. As expected, some mice fulfilled respective inclusion criteria for more than 1 behavioral trait, being the most frequent association high sociability and low anxiety (n=9). These mice were therefore considered as independent values in the corresponding experimental groups for which they have been selected. In the cohort 1, 17 mice reached the inclusion criteria for 2 different extreme phenotypes, whereas 1 mouse reached the criteria for 3 different extreme phenotypes (see Table 2). In the cohort 2, 26 mice reached the inclusion criteria for 2 different extreme phenotypes, 3 mice reached the criteria for 3 different extreme phenotypes and 18 mice were considered for the 3 control intermediate groups. Therefore, the total number of experiments (79 in cohort 1 and 156 in cohort 2) was higher than the total number of mice taking into account animals reaching criteria for more than 1 single experimental group. Despite this partial overlapping, these groups were only extreme for 1 particular behavioral trait when considered as whole extreme phenotype groups (Fig. 2), demonstrating they were representing mostly independent features. Each behavioral trait was analyzed independently in both cohorts.

Mice with intermediate responses for sociability, anxiety- or depressive-like behavior (see Table 1 for detailed percentiles) were selected as control animals for each respective trait and these animals were therefore not always intermediate for the other

traits. Consequently, 3 different control groups for each behavioral trait were included in cohort 1 used in electrophysiological experiments. The experimental protocol of cohort 2 included 4 different experimental interventions i.e., PSNL surgery, nociceptive measurements, emotional and cognitive evaluation. In order to minimize the variability associated to these complex surgical and behavioral interventions, mice showing intermediate responses in all the behavioral traits were assigned to a unique control group avoiding by this manner a possible bias due to this variability.

Table 1. Summary of selection and exclusion criteria for mice phenotyping

Behavioral trait	**Test**	**Parameters**	**Exclusion criteria**	**Inclusion criteria**		
				High	Control	Low
Locomotor activity	Actimetry boxes	Horizontal movement	Below10th percentile Above 90th percentile			
		Vertical movement	Below 5th percentile Above 95th percentile			
Sociability-like behavior	Sociability Test	Ratio preference mouse empty		Above 80th percentile	Between 35th and 65th percentiles	Below 20th percentile
Anxiety-like behavior	Light Dark Box Test	% White time		Both below 30th percentile	Both between 35th and 65th percentiles	Both above 70th percentile
	Elevated Plus Maze	% Time open arms				
Depressive-like behavior	Tail Suspension Test	Immobility time		Both above 70th percentile	Both between 35th and 65th percentiles	Both below 30th percentile
	Forced Swimming Test	Immobility time				

Table 2. Number of animals assigned to each phenotypic group according to criteria stated in Table 1.

Cohort 1 (60 mice = 42 + 17 + 1)				
Phenotype	***Total number of mice in each group***	***Mice selected only for 1 group***	***Mice selected for 2 groups***	***Mice selected for 3 groups***
	60 mice =	***42 mice*** +	***17 mice*** +	***1 mouse***
LS	10	6	4	0
CTRL	10	6	4	0
HS	10	5	4	1
LA	10	5	4	1
CTRL	10	6	4	0
HA	7	4	3	0
LD	7	3	4	0
CTRL	7	3	3	1
HD	8	4	4	0

Cohort 2 (88 mice = 41 + 26 + 21)				
Phenotype	***Total number of mice in each group***	***Mice selected only for 1 group***	***Mice selected for 2 groups***	***Mice selected for 3 groups***
	88 mice =	***41 mice*** +	***26 mice*** +	***21 mice***
LS	16	6	9	1
CTRL	18	0	0	18
HS	23	9	12	2
LA	16	9	6	1
CTRL	18	0	0	18
HA	16	8	6	2
LD	16	3	11	2
CTRL	18	0	0	18
HD	15	6	8	1

In cohort 1, 79 experiments were considered using a total of 60 mice. Among them, 42 mice were considered only in 1 phenotypic group, 17 were considered in 2 phenotypic groups since they achieved the corresponding exclusion and inclusion criteria, and 1 mouse was considered in 3 phenotypic groups accordingly with these exclusion and inclusion criteria. Therefore, the total numbers are 42 + 34 (17x2) + 3 (1x3) =79.

In cohort 2, a total of 156 experiments were considered using 88 mice. Among them, 41 were considered in 1 group, 26 were considered in 2 and 21 mice were considered in 3 groups considering the abovementioned exclusion and inclusion criteria. Therefore, the total numbers are 41 (41x1) + 52 (26x2) + 63 (21x3) =156. LS, low sociability; LA, low anxiety; LD, low depression; HS, high sociability; HA, high anxiety; HD, high depression; CTRL, control group.

3.2. CeA neuronal activity is directly proportional to social behavioral trait

We aimed to know if the activity of CeA neurons can be influenced by specific behavioral traits, and whether it can determine subsequent manifestations of neuropathic pain. For this purpose, we evaluated spontaneous and evoked electrophysiological activity of CeA neurons of extreme phenotype mice to look for any possible association with behavioral responses. Spontaneous CeA neuronal activity was directly proportional to social behavioral trait, since highly sociable mice had a significantly higher activity than low sociable animals ($p<0.001$), and the control group exhibited an intermediate response (Fig. 3A). Similar spontaneous activity of CeA neurons were recorded in the low, control and high anxiety groups (Fig. 3B). No clear relation between the depressive-like behavior and the spontaneous CeA neuronal activity was observed, with the highest activity in the control group (Fig. 3C). No significant differences between spontaneous and evoked activity by any stimulus were found within any group of mice (Fig. S2). However, the differences in spontaneous activity observed between extreme phenotypes were maintained following stimuli application (Fig. S2).

3.3. Extreme phenotypes influence nociceptive behavior

The development of mechanical and cold allodynia as well as thermal hyperalgesia in nerve-injured mice was first confirmed, as revealed by the significant differences when comparing sham and PSNL mice ($p<0.001$) (Fig. 4A-I). Next, we examined whether extreme phenotypes of sociability, anxiety- and depressive-like behaviors affect nociceptive responses (sham-operated groups) and influence the magnitude of neuropathic pain-induced allodynia and hyperalgesia (PSNL groups).

Sociability trait significantly influenced responsiveness to mechanical stimuli. Low-sociable animals displayed enhanced mechanical sensitivity compared to high-sociable

and control group, both under sham ($p<0.001$ vs control; $p<0.001$ vs high-sociable) and nerve-injured conditions ($p<0.05$ vs control; $p<0.01$ vs high-sociable) (Fig. 4A). In addition, the low sociable phenotype potentiated cold allodynia in early stages of neuropathic pain ($p<0.01$), but the differences in cold allodynia compared to high sociable and control groups disappeared in later stages (Fig. 4C). No influence of sociability trait was observed on cold nociception in the absence of nerve lesion (Fig. 4C), nor in heat sensitivity under sham and nerve-injured conditions (Fig. 4B).

Anxiety trait had an impact on mechanical sensitivity only under neuropathic pain conditions. Mice with high anxiety-like behavior showed enhanced mechanical allodynia compared to those with low anxiety-like behavior ($p<0.01$), while the control group elicited an intermediate response (Fig. 4D). This relationship was confirmed by a significant positive correlation between both anxiety-related parameters (time in open arms and time in white compartment) and the area under the curve (AUC) of mechanical thresholds of nerve-injured mice (Table 3). This result translates into a positive correlation of anxiety trait (higher times mean less anxiety-like behavior) with mechanical allodynia (higher AUC means less mechanical pain). In contrast, responses to mechanical stimuli were similar in all the sham groups. A significant effect of anxiety trait on cold allodynia was also observed since low-anxious animals showed enhanced cold allodynia compared to high-anxious mice ($p<0.05$) (Fig. 4F). No influence of anxiety trait on heat sensitivity was observed neither under sham nor neuropathic pain conditions (Fig. 4E).

Depression trait also influenced nociceptive responses to mechanical stimulation under neuropathic pain conditions. A positive correlation between both depression-related parameters (time immobility in the tail suspension and the forced swimming) and the AUC of mechanical thresholds of nerve-injured mice was shown (Table 3). This result

indicated a negative correlation between depression trait (higher immobility times mean more depressive-like behavior) and mechanical allodynia (higher AUC mean less mechanical pain). In this sense, nerve-injured mice with low depressive-like behavior developed more intense mechanical allodynia than those with high depressive-like behavior ($p<0.01$) and control groups ($p<0.01$) (Fig. 4G). No effect of depression trait was revealed on mechanical nociceptive manifestations of sham-operated animals (Fig. 4G). Animals displaying low depressive-like behavior showed higher cold sensitivity both under sham and nerve-injury conditions compared to high depression phenotype ($p<0.01$) and control group ($p<0.01$) (Fig. 4I). Mice with low depression also showed higher heat nociception than control group in sham conditions ($p<0.01$) (Fig. 4H). Therefore, mice with low depressive-like behavior consistently showed enhanced nociceptive behavior compared to the opposite phenotype and/or the control group after sham surgery and/or nerve injury conditions.

Table 3. Correlation analysis between behavioral traits and neuropathic pain manifestations

			AUC of mechanical thesholds (von Frey)	AUC of heat thesholds (plantar)	AUC of cold thresholds (cold plate)	Anhedonia (sucrose preference)	Anxiety (EZM, time open arms)	Memory
Sociability	Preference mouse/empty	Pearson Correlation	0,326	-0,006	-0,185	-0,302	-0,329	0,103
		Sig. (2-tailed)	0,064	0,973	0,304	0,088	0,061	0,568
Anxiety	EPM (time open arms)	Pearson Correlation	0,453*	-0,108	0,303	-0,114	0,604**	0,292
		Sig. (2-tailed)	**0,014**	0,577	0,11	0,556	**0,001**	0,124
	LDB (white time)	Pearson Correlation	0,434*	-0,343	0,259	-0,176	0,526**	0,182
		Sig. (2-tailed)	**0,019**	0,069	0,175	0,362	**0,003**	0,344
Depression	TST (time of immobility)	Pearson Correlation	0,396*	0,167	-0,15	0,186	-0,261	-0,226
		Sig. (2-tailed)	**0,023**	0,354	0,406	0,301	0,143	0,206
	FST (time of immobility)	Pearson Correlation	0,389*	0,262	-0,15	0,284	-0,218	-0,16
		Sig. (2-tailed)	**0,025**	0,141	0,405	0,109	0,222	0,374

* $p<0.05$, ** $p<0.01$ significant correlation

3.4. Extreme phenotypes influence emotional and cognitive manifestations of neuropathic pain

We confirmed the previously reported (La Porta et al., 2016) emotional and cognitive symptoms in nerve-injured mice compared to sham-operated ones as shown by decreased percentage of sucrose intake ($p<0.05$) (Fig. 5A), decreased time spent in open arms in the EZM ($p<0.05$) (Fig. 5E) and decreased discrimination index in the NOR ($p<0.05$) (Fig. 5I). We next evaluated the influence of extreme phenotypes of sociability, anxiety- and depressive-like behavior on these manifestations associated to neuropathic pain. No clear effect of the behavioral traits was observed on the depressive-like manifestations of neuropathic pain revealed in the sucrose preference test (Fig. 5B-D). In contrast, an effect of the anxiety trait on the time spent in the open arms of the EZM (anxiety-like behavior) was revealed ($p<0.001$). Mice with low and high anxiety-like behavior prior to the injury had the same phenotypic traits 15 days after the sham or PSNL surgery, while the control group showed an intermediate response (Fig. 5G). These results were further confirmed by a significant correlation between the elevated zero maze and both the elevated plus maze and light/dark box (Table 3). Among animals selected for sociability and depression, neuropathic pain induced anxiety-related behavior in those mice with high sociability and high depression prior to PSNL ($p<0.05$ in both cases), but not in low and CTRL phenotypes. Highly sociable and highly depressed-like animals behave similar to highly anxious-like mice after the nerve lesion. In addition, the development of anxiety following nerve injury was exacerbated in mice selected for high sociability and high anxiety compared to their respective low phenotypes (high vs low sociability PSNL $p<0.05$; high vs low anxiety PSNL $p<0.001$). Therefore, mice with high sociability, high anxiety and high depression prior to the lesion showed enhanced anxiety-like behavior following the

nerve injury (Fig. 5F-H). In turn, memory deficits induced by neuropathic pain were aggravated in mice with high anxiety ($p<0.01$) and high depression ($p<0.05$) prior to the injury (Fig. 5J-L).

3.5. Extreme phenotypes and partial sciatic nerve ligation (PSNL) alter the expression of selected genes in the amygdala

We next evaluated the presence of gene expression patterns in the amygdala associated to extreme behavioral phenotypes, and if they could be considered underlying factors of the influence of behavioral traits on neuropathic pain manifestations. This brain area was selected considering its crucial role in regulating pain and emotional behaviors and its involvement in the harmful effects of stressors. As an indicator of amygdala activation, we checked the expression level of the activity gene *Npas4*. As tracers of neuroinflammatory and stress responses, we evaluated transcript levels of the *Il6* and *Gadd45*, respectively. We were also interested in the expression profile of the neuropeptide precursor prodynorphin (*Pdyn)*, whose expression in this structure is highly implicated in negative mood states (Knoll et al., 2011; Knoll and Carlezon, 2010; Koob, 2009). Finally, we assessed the expression level of two stress-related genes, the glucocorticoid receptor gene *Nr3c1* and the *Tsc22d3* gene encoding the TSC22 domain family protein 3, a glucocorticoid-induced leucine zipper protein that functions as transcriptional regulator.

Behavioral traits were associated to the expression profiles of some of these genes in the amygdala (sham conditions). A negative correlation between sociability trait (preference mouse/empty) and *Pdyn* expression in the amygdala was revealed (Table 4, sham). Both anxiety-related parameters (time in open arms and time in white compartment) also correlated negatively with *Pdyn* expression (Table 4, sham). These results indirectly indicate a positive correlation of anxiety trait (higher times mean less anxiety-like

behavior) and *Pdyn* expression profiles in the amygdala. High level of *Pdyn* mRNA was associated to low sociability and high anxiety ($p<0.05$), while low *Pdyn* level was associated to high sociability ($p<0.05$) and low anxiety ($p<0.05$, Fig. 6B). No clear pattern of *Pdyn* expression was associated to depressive-like phenotypes (Fig. 6B).

A negative correlation between depression trait and glucocorticoid receptor *Nr3c1* gene expression was found (Table 4, sham). High level of *Nr3c1* transcripts was found in the amygdala of mice with low depressive-like behavior ($p<0.05$), while the highly depressed-like mice and the control group showed lower levels ($p<0.05$, Fig. 6D). *Nr3c1* mRNA levels were unchanged in the different sociability- and anxiety-like phenotypes (Fig. 6D).

Gadd45 expression in the amygdala was positively correlated with depressive trait (Table 4, sham). Thus, mice with high depressive-like behavior had higher Gadd45 levels ($p<0.001$, Fig. 6F). *Gadd45* transcript levels were not significantly affected by sociability and anxiety traits in sham conditions (Fig. 6F). No effect of the examined behavioral traits on *Il6* expression was observed in the absence of neuropathic pain (Fig. 6H). *Npas4* and *Tsc22d3* expression in the amygdala was not modified by any behavioral trait (Fig. 6J, 6L).

Table 4. Correlation analysis between gene expression in the amygdala and behavioral traits

		Sociability	Anxiety		Depression	
		Preference mouse/empty	EPM (time open arms)	LDB (white time)	TST (time of immobility)	FST (time of immobility)
Sham-operated mice						
Pdyn	Pearson Correlation	-0,639**	-0,648*	-0,582*	0,166	-0,054
	Sig. (2-tailed)	**0,004**	**0,012**	**0,029**	0,572	0,855
Nr3c1	Pearson Correlation	-0,168	-0,067	0,006	-0,677*	-0,646*
	Sig. (2-tailed)	0,468	0,821	0,984	**0,032**	**0,044**
Gadd45	Pearson Correlation	-0,49	0,265	0,396	0,653*	0,575*
	Sig. (2-tailed)	0,075	0,381	0,18	**0,016**	**0,04**
Il6	Pearson Correlation	0,387	-0,148	0,116	-0,189	-0,398
	Sig. (2-tailed)	0,215	0,665	0,733	0,556	0,201
Npas4	Pearson Correlation	-0,296	-0,347	-0,203	-0,012	-0,28
	Sig. (2-tailed)	0,304	0,245	0,507	0,968	0,354
Tsc22d3	Pearson Correlation	-0,098	-0,271	-0,129	0,125	-0,186
	Sig. (2-tailed)	0,751	0,371	0,675	0,683	0,544
Nerve-injured mice (PSNL)						
Pdyn	Pearson Correlation	-0,355*	-0,425*	-0,354	-0,037	0,063
	Sig. (bilateral)	**0,046**	**0,027**	0,07	0,839	0,731
Nr3c1	Pearson Correlation	0,054	0,132	0,322	-0,479*	-0,379
	Sig. (bilateral)	0,768	0,503	0,094	**0,011**	0,051
Gadd45	Pearson Correlation	-0,037	0,648*	0,603*	-0,127	-0,05
	Sig. (bilateral)	0,885	**0,017**	**0,029**	0,574	0,826
Il6	Pearson Correlation	0,078	-0,585*	-0,745**	-0,609**	-0,582**
	Sig. (bilateral)	0,767	**0,028**	**0,002**	**0,006**	**0,009**
Npas4	Pearson Correlation	0,089	0,565*	0,695**	-0,201	-0,18
	Sig. (bilateral)	0,708	**0,022**	**0,003**	0,37	0,423
Tsc22d3	Pearson Correlation	0,135	0,38	0,505*	-0,17	-0,128
	Sig. (bilateral)	0,571	0,146	**0,046**	0,45	0,571

* $p<0.05$, ** $p<0.01$ significant correlation

The development of neuropathic pain also altered gene expression in the amygdala. Indeed, nerve injury significantly increased *Pdyn* ($p<0.05$, Fig. 6A), *Gadd45* ($p<0.01$,

Fig. 6E) *Npas4* ($p<0.05$, Fig. 6I) and *Tsc22d3* ($p<0.05$, Fig. 6K) expression in the amygdala, while it did not modify *Nr3c1* (Fig. 6C) and *Il6* (Fig. 6G) expression levels. This modulatory effect of the nerve lesion depended on the behavioral traits. Thus, the increase of *Pdyn* expression after PSNL was enhanced in low sociable and high anxious mice ($p<0.05$ in both cases, Fig. 6B). As in sham conditions, correlation of *Pdyn* expression with sociability and anxiety traits were also significant following nerve injury (Table 4, PSNL). The expression levels of *Nr3c1* according to extreme depression phenotypes in sham conditions were similar after PSNL, and negative correlation between *Nr3c1* and immobility time in the tail suspension was also significant in nerve-injured mice (Table 4, PSNL). Thus, mice with low depressive-like behavior showed the highest *Nr3c1* expression in both conditions ($p<0.05$, Fig. 6D). The increase of *Gadd45* expression following PSNL was restricted to mice with low anxiety- and low depressive-like behavior (Fig. 6F). A negative correlation between anxiety trait and *Gadd45* became significant following PSNL (Table 4, PSNL), when *Gadd45* transcript levels were significantly higher in mice with low than in those with high anxiety-like behavior ($p<0.05$). The different *Gadd45* expression profiles between mice with low and high depressive-like behavior in sham-operated mice disappeared following the nerve injury (Fig. 6F). Finally, anxiety- and depressive-like behavior showed clear regulatory effects of *Il6* gene expression in the amygdala only under neuropathic pain conditions. Although correlation of *Il6* expression with anxiety and depressive traits was not significant in sham-operated animals, they became significant under neuropathic pain conditions (Table 4, PSNL). Thereby, anxiety trait enhanced *Il6* expression, whereas depression trait reduced it after PSNL (Fig. 6H). Table 5 summarizes the previously described influence of extreme phenotypes and PSNL on the amygdala gene expression.

Table 5. Influence of extreme behavioral phenotypes and PSNL on the amygdala gene expression

	Gene		Surgery	Sociability		Anxiety		Depression	
				Sham	PSNL	Sham	PSNL	Sham	PSNL
Activity	*Npas4*	neuronal PAS domain protein 4	★↑	-	-	-	-	-	-
Stress	*Tsc22d3*	TSC22 domain family protein 3	★↑	-	-	-	-	-	-
	Nr3c1/gr	nuclear receptor subfamily 3, group C, member 1	-	-	-	-	-	☆LD>HD	☆LD>CTRL
Inflammation	*Il6*	interleukin 6	-	-	-	-	☆LA<HA	-	☆LD>HD
	Gadd45	growth arrest and DNA-damage-inducible, gamma	★↑	-	-	-	☆LA>HA	☆☆☆LD<HD ☆☆☆CTRL<HD	☆LD>CTRL
Neuropeptide	*Pdyn*	prodynorphin	★↑	☆LS>HS	☆LS>HS	☆☆LA<HA	☆LA<HA	-	-

Gene expression in the amygdala of mice displaying extreme phenotypes (sociability, anxiety and depressive-like) following partial sciatic nerve injury (PSNL) or sham surgery was performed. Arrows indicate elevated mRNA level in nerve-injured mice. ★$p<0.05$ vs sham surgery (One-way ANOVA, Bonferroni); ☆$p<0.05$, ☆☆☆$p<0.001$ between the indicated extreme phenotypes (One-way ANOVA, Bonferroni). LS, low sociability; LA, low anxiety; LD, low depression; HS, high sociability; HA, high anxiety; HD, high depression; CTRL, control group. Detailed statistical analyses are presented in Supplementary Table S3.

4. Discussion

Our study reveals that some specific behavioral traits may influence spontaneous and evoked CeA neuronal activity and basal nociceptive responses, and seem to be crucial for the nociceptive, emotional and cognitive manifestations of neuropathic pain. Our results also show that these behavioral traits may be linked to gene expression changes in the amygdala.

The amygdala is a critical integrator for affective processing. Indeed, alterations in amygdala activation have been found in a variety of neuropsychiatric disorders, including autism and social phobia (Wellman et al., 2016). Both amygdala hyperactivity

and hypoactivity have been associated with altered social processing (Becker et al., 2012; Sladky et al., 2012), which have placed the amygdala at the center of the social brain. Our results revealed a direct association between spontaneous and evoked CeA activity and sociability behavior, so higher CeA function was observed in high sociable mice. In agreement with a prosocial role of the amygdala, many previous studies reported loss of social interactions following permanent damage to the amygdala in nonhuman primates. These deficits in social behavior included loss of social status, decreased affiliative interactions, and decreased response to threats following amygdala ablation (Kalin et al., 2004; Meunier and Bachevalier, 2002), and less severe behavioral alterations after more circumscribed excitotoxic amygdala lesions (Machado et al., 2008; Machado and Bachevalier, 2006). In our experimental settings, CeA function was unrelated to the anxiety- and depressive-like traits. Several studies agreed that the CeA has a crucial role in fear, but not in anxiety and depression control (Davis et al., 2010, 1997).

We revealed that low sociability was associated to enhanced mechanical sensitivity, while low depression trait increased responding to heat and cold stimulation in sham mice. Therefore, low sociability and low depression phenotypes could represent vulnerability factors to enhance nociceptive responses. Decreased pain sensitivity was previously demonstrated by social interaction with conspecifics in rodents (D'Amato and Pavone, 2012), and social relationships were suggested to promote pain-specific resilience in humans (Sturgeon and Zautra, 2016). Thus, greater social support was associated with lower nociceptive manifestations to painful experimental stimuli (Montoya et al., 2004). In agreement, depressive-like behavior decreased the perceived intensity of painful stimulation in rats (Shi et al., 2010), and individuals with depressive

disorders showed decreased sensitivity to noxious stimulation (Bär et al., 2006; Schwier et al., 2010).

Our behavioral results also revealed that sociability, anxiety and depression traits modulate nociceptive manifestations after PSNL. Low sociability trait was associated to enhanced mechanical and cold allodynia. The role of social variables affecting neuropathic pain was previously described in a neuroma rat model (Raber and Devor, 2002). Clinical studies also revealed that social factors may improve coping responses and overall function in chronic pain (Sturgeon and Zautra, 2016). Meaningful social ties may play a protective role by engaging neural networks associated with more adaptive responses to pain (Younger et al., 2010), and social support protects patients against pain-related exacerbations in negative mood (Onoda et al., 2009).

We have demonstrated that anxiety-like behavior has a modulatory effect on nociception after PSNL that depends on the modality of the stimuli. A positive correlation between anxiety trait and mechanical allodynia was revealed. However, mice displaying low anxiety-like behavior showed higher cold sensitivity. Different noxious sensory modalities are transduced by distinct nociceptive primary fibers. Therefore, anxiety could have a particular impact in specific sensory pathways. In agreement, opposite effects of the anxiety trait depending on the nociceptive modality were previously reported in animals. Indeed, high anxiety increased mechanical hypersensitivity in neuropathic rats (Roeska et al., 2009), but decreased thermal pain sensitivity (Jochum et al., 2007), whereas patients with high anxiety exhibited higher rates of pain (Asmundson and Norton, 1995; Defrin et al., 2008; Keogh and Mansoor, 2001).

Depression trait negatively correlated with mechanical allodynia and similarly, mice with low depressive-like behavior also showed enhanced cold allodynia in our

experimental conditions. The consistent influence of low depression phenotype enhancing different pain modalities suggests that depression trait is not directly related to pain severity. In agreement, decreased mechanical allodynia was previously reported in neuropathic rats with depressive-like behavior (Shi et al., 2010). Patients with depressive disorders often report pain, but they are less sensitive to experimental pain (Bär et al., 2006; Graff-Guerrero et al., 2008), and our findings also suggest that depression might decrease neuropathic pain-induced hypersensitivity. Avoidance of noxious stimulation is considered a motivation-driven behavior. Therefore, the inhibitory effect of depression on the stimulus-evoked pain, may be related to the loss of motivation, a key symptom of depression reported in neuropathic mice (La Porta et al., 2016).

We further evaluated the influence of behavioral traits on emotional and cognitive dimensions after PSNL. We first confirmed different behavioral outcomes used in our laboratory as reliable measurements of emotional and cognitive manifestations of neuropathic pain. The post-surgery evaluation of emotional behaviors revealed the stability of previously selected extreme phenotypes. The retention of extreme anxiety traits in sham-operated mice 3 weeks after phenotyping demonstrated that the defined extreme phenotypes referred to actually extreme behavioral traits. The lack of consistency between the extreme depression phenotypes and the responses in sham-operated mice may be related to the different behavioral responses evaluated in each paradigm. The immobility measured in the forced swimming and tail suspension reflect a behavioral despair, directly related to the reduced motivation to maintain effort in an inescapable situation, whereas the sucrose preference test includes different components of the reward processing that are related to the pleasure cycle (Thomsen, 2015). We found that high sociable mice developed enhanced anxiety and cognitive manifestations

of neuropathic pain, while low sociable mice developed nociceptive hypersensitivity. In contrast to the enhancer effect of low depression trait on nociceptive manifestations, high depressive neuropathic mice were the most anxious and had the worst memory index. Anxiety trait modulated emotional and cognitive neuropathic pain manifestations in the same direction as mechanical nociception, since mice with high anxiety prior to the lesion were the most anxious and showed the most severe memory impairment after PSNL. These results indicate that high sociability, high anxiety and high depression play a crucial role in anxiety manifestations related to neuropathic pain, while high anxiety and high depression are also crucial factors for neuropathy-induced cognitive impairment. Collectively, our findings show that certain behavioral traits in mice, which can be translated into human personality traits, are crucial factors in modulating sensory processes and affective and cognitive comorbidities of neuropathic pain that do not need to be proportional to allodynia and hyperalgesia. These findings support once more the importance of evaluating not only simple nociceptive endpoints, but also complex behavioral manifestations of pain in animal models of neuropathies.

We also revealed that extreme sociability, anxiety and depression traits influence gene expression in the amygdala in the absence of pain. *Pdyn* expression correlated negatively with sociability and positively with anxiety trait. In agreement, several studies have shown that low sociability is related to higher levels of anxiety (Kudryavtseva et al., 2004; Tõnissaar et al., 2008). *Pdyn* deletion and blockade of kappa opioid receptor (KOR) enhanced social memory (Bilkei-Gorzo et al., 2014). The prodynorphin system may play a role in anxiety (Knoll et al., 2011), but present data do not provide a consistent picture of the *Pdyn* functions in anxiety. Consistent with our results, *Pdyn* deletion and KOR blockade decreased anxiety in mice, and treatment of *Pdyn* knockouts with a KOR agonist reversed their anxiolytic phenotype(Wittmann et

al., 2009). However, increased anxiety-like behaviors was also observed in *Pdyn* knockouts (Femenía et al., 2011).

The depression trait was negatively correlated with *Nr3c1* levels in the amygdala. This observation agrees with the association of glucocorticoid receptor with depressive disorders. Thus, high levels of *Nr3c1* promoter methylation have been associated with major depressive disorder (Nantharat et al., 2015). As DNA methylation usually represses gene transcription, our results support the hypothesis that decreased *Nr3c1* receptor level could be an indicative factor for depressive-like behavior.

Gadd45 expression in the amygdala showed a positive correlation with depression trait. GADD45 protein is considered a molecular player for active DNA demethylation under stressful situations, which may suggest that GADD45 is inducing stable changes in amygdala gene expression, neural circuit function, and ultimately behavior in mice with depression. Indeed, aberrant epigenetic regulation induced by environmental factors and subsequent transcriptional dysregulation is a unifying topic in psychiatric disorders, including depression (Bagot et al., 2014).

Changes in the amygdala gene expression profiles were also observed after PSNL. An up-regulation of spinal dynorphin and its precursor (PDYN) expression is a common critical feature in neuropathic pain involved in the transition to chronic pain (Laughlin et al., 2001; Wang et al., 2001). Here we show that *Pdyn* up-regulation after PSNL also takes place in the amygdala, which suggests a role of dynorphin in the negative affective component of pain, in agreement with a recent study showing dynorphinergic system alterations in other brain regions (Palmisano et al., 2018). Increased *Gadd45* expression in the spinal cord and the dorsal root ganglia have also been reported during neuropathic pain (Lacroix-Fralish et al., 2011; Perkins et al., 2014). Our results revealed that these changes can be spread to more distant brain areas, such as the amygdala.

Gadd45 expression is induced after ischemic damage and neurodegenerative processes with anti-apoptotic properties (Chen et al., 1998; Torp et al., 1998), and *Gadd45* could therefore be induced after PSNL to maintain genomic stability. *Npas4* is an early-response transcription factor that represents a homeostatic switch regulating excitatory-inhibitory neural balance (Spiegel et al., 2014). The increase in *Npas4* following PSNL indicates an amygdala overactivation, which may contribute to the nociceptive and emotional manifestations of neuropathic pain. Our results also revealed the up-regulation of *Tsc22d3* in the amygdala after PSNL. Further studies should be performed to elucidate the biological meaning for the enhanced expression of this transcriptional regulator during neuropathic pain.

Neuropathic pain-induced gene expression changes in the amygdala varied in line with the behavioral traits. *Pdyn* expression showed a negative and a positive correlation with sociability and anxiety traits, respectively, also under conditions of neuropathic pain. Interestingly, the groups with higher *Pdyn* levels (low sociability and high anxiety) also showed enhanced nociceptive manifestations of neuropathic pain and/or enhanced related comorbidities. These findings suggest a role of amygdala *Pdyn* in aggravating neuropathic pain syndrome. *Gadd45* up-regulation following PSNL was restricted to mice with low anxiety- and low depressive-like behavior, going along with milder emotional and cognitive manifestations of neuropathic pain. Therefore, *Gadd45* induction in the amygdala after PSNL may play a protective role against emotional and cognitive chronic pain manifestations, probably by promoting genomic stability and protecting neurons from apoptosis. Although PSNL did not globally modify *Il6* expression in the amygdala, it differentially modulated *Il6* expression depending on anxiety and depression traits. Thus, under neuropathic pain conditions *Il6* transcript levels showed a positive and a negative correlation with anxiety and depression traits,

respectively. Higher levels of *Il6* were observed in nerve-injured mice with high anxiety and low depression traits, both showing enhanced nociceptive hypersensitivity. Considering this interleukin as an inflammatory marker, these findings suggest that neuroinflammatory processes in the amygdala may contribute to enhance neuropathic pain, what may be favored by anxiety and attenuated by depression. Further determination of protein level expression would strengthen the biological meaning of the observed transcriptional changes.

5. Conclusions

We have demonstrated the influence of specific behavioral traits on basal nociception and emotional, cognitive and nociceptive manifestations of neuropathic pain. Our results also revealed that behavioral traits modulate the expression of selected genes in the amygdala under normal and nerve-injured conditions. These results may help in the understanding of mechanisms that could explain the inter-individual variation of the neuropathic pain manifestations, which could provide an additional step for the development of efficient personalized treatments for chronic pain.

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Figure 1. Experimental schedule. Extreme phenotypes selection for the assessment of electrophysiological correlates and for the evaluation of the nociceptive, affective and cognitive behaviors in mice exposed to neuropathic pain.

Figure 2. Selection of the extreme phenotypes. Behavioral responses of selected extreme phenotype mice in the tests used for this selection are represented. (A) The social preference index was used to distinguish low and high sociable mice. (B) The percentage of time in open arms (elevated plus maze) and the percentage of time in white area (light/dark box) were considered together to identify animals with low and high anxiety-like behavior. (C) The time of immobility in the forced swimming test and in the tail suspension test were two independent measures used to establish low and high depressive-like mice. Unique control group including animals that did not show extreme responses in none of the tests was considered to illustrate the behavior of the average population. The selected phenotypes were not affected by each other and were similar to control group when considered for other behavioral traits. Boxplot represent mean ± SEM. LS, low sociability (n=26); LA, low anxiety (n=26); LD, low depression (n=23); HS, high sociability (n=33); HA, high anxiety (n=23); HD, high depression (n=23); CTRL, control group (n=18).

Figure 3. In-vivo electrophysiological evaluation of central amygdala (CeA) in anesthetized mice displaying extreme phenotypes. CeA spontaneous activity of mice in extreme low and high phenotypes as well as in control group for (A) sociability, (B) anxiety-like and (C) depressive-like behavior was recorded. Results from the overall population of neurons are represented in the upper graphs. The ‘n’ numbers shown in the graphs indicate the total number of neurons included in each bar histogram. Data are expressed as mean ± SEM. n=5-6 mice/group. ✶ $p<0.05$, ✶✶✶ $p<0.001$ compared to the corresponding group (one-way ANOVA, Dunn's multiple comparison test). Typical

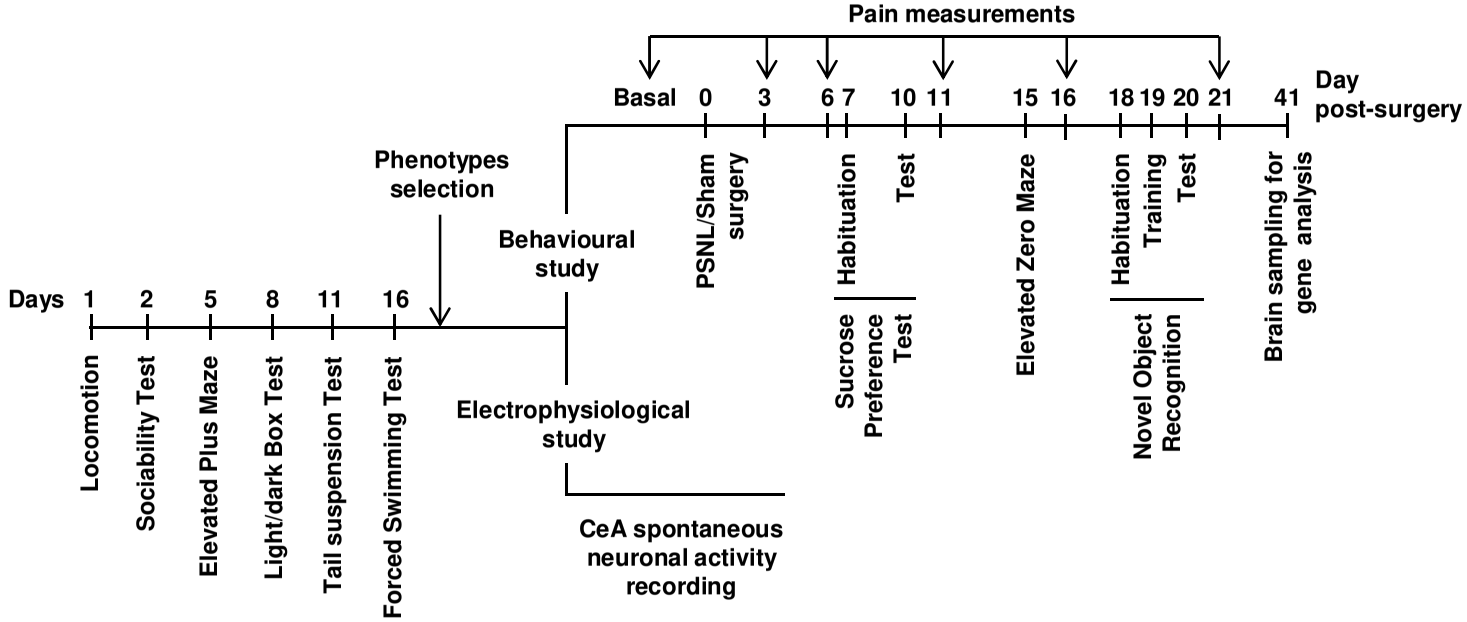
full recordings (raw data) of a representative single CeA neuron spontaneous activity are shown in (D) for sociability, (E) for anxiety-like and (F) for depressive-like behavioral groups. Values of firing per second (Hz) are represented on the y-axis and time (a fragment of 275s of the 20min recordings) is represented on the x-axis. LS, low sociability; LA, low anxiety; LD, low depression; HS, high sociability; HA, high anxiety; HD, high depression; CTRL, control groups for sociability, anxiety and depression.

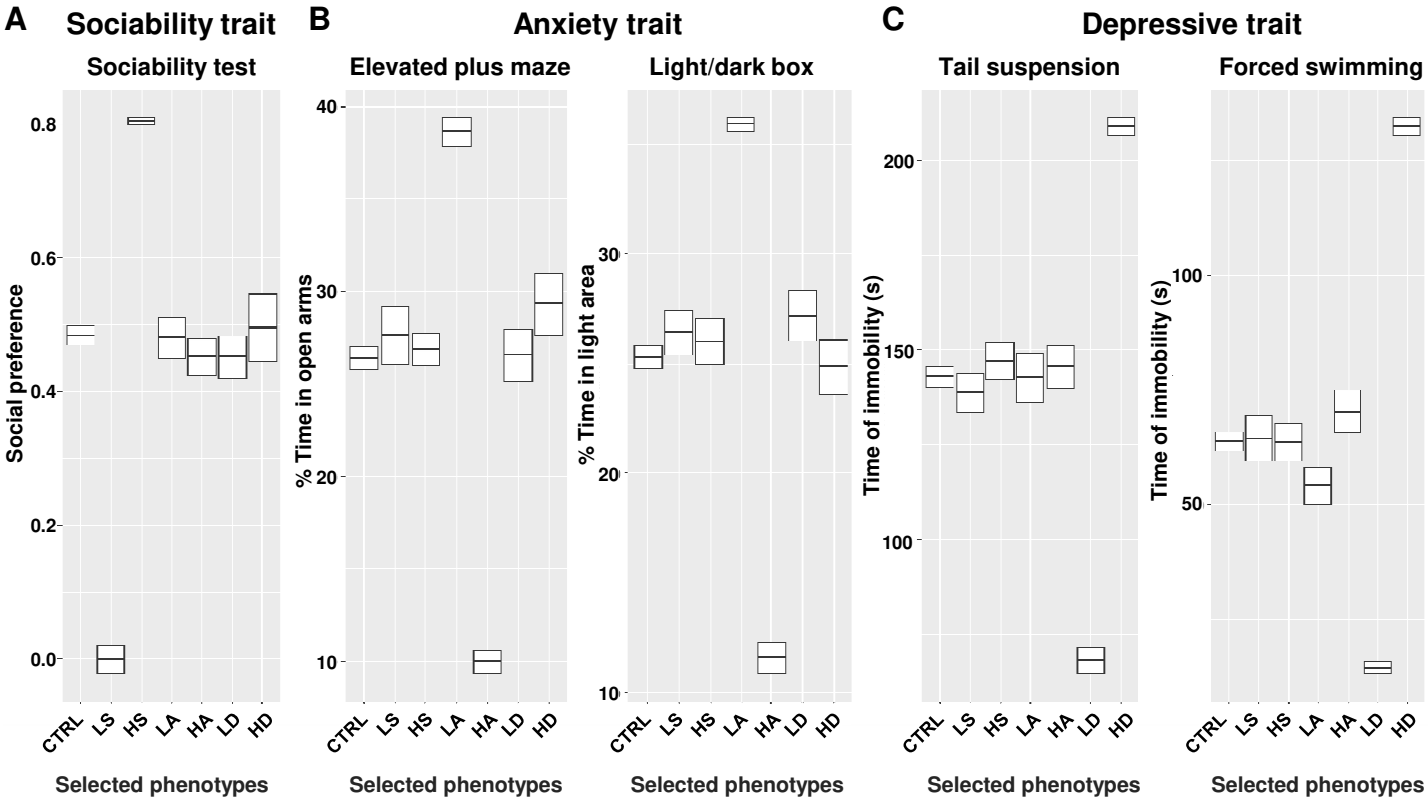
Figure 4. Nociceptive manifestations of neuropathic pain on mice displaying extreme behavioral phenotypes. The influence of extreme phenotypes for sociability (A, B, C), anxiety (D, E, F) and depression (G, H, I) on nociceptive responses was evaluated. Time course of mechanical thresholds (in grams) to von Frey filaments stimulation of the ipsilateral hind paw following sham-injury or PSNL, in mice selected for extreme (A) sociability, (D) anxiety- and (G) depressive-like phenotypes. Time course of withdrawal latencies (in sec) to radiant heat stimulation of the ipsilateral hind paw following sham-injury or PSNL, in mice selected for extreme (B) sociability, (E) anxiety- and (H) depressive-like phenotypes. Cold allodynia after sham-injury or PSNL in mice selected for extreme (C) sociability, (F) anxiety- and (I) depressive-like phenotypes was evaluated in the cold plate test and expressed as score values (difference in the number of elevations between the ipsilateral and contralateral hind paws). Nociceptive measurements were performed under basal conditions (not shown) and on days 3, 6, 11, 16, and 21 after PSNL or sham-injury. Data are expressed as mean ± SEM (n=5-10 mice/sham groups and n=9-13 mice/PSNL groups). ★★★$p<0.001$ main effect of PSNL; ## $p<0.01$, ### $p<0.001$ vs CTRL group, + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ between low and high extreme phenotypes (Two-way ANOVA, Bonferroni). Symbols placed on the right of the graphs refer to the whole experimental period. LS, low

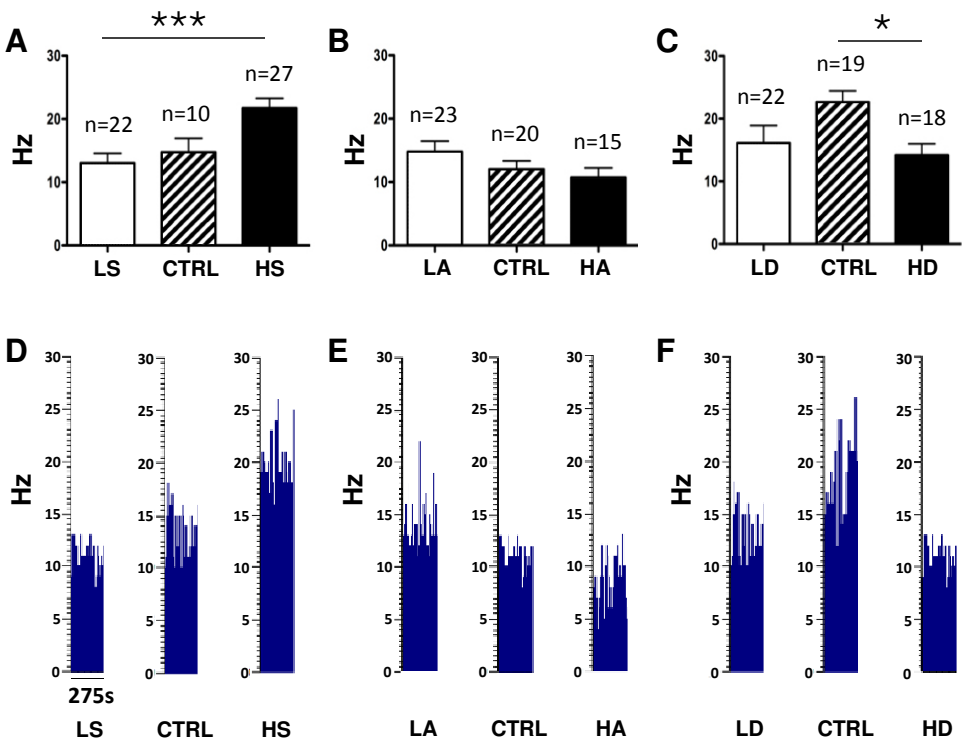
sociability; HS, high sociability; LA, low anxiety; HA, high anxiety; LD, low depression; HD, high depression; CTRL, control group. Detailed statistical analyses are shown in Supplementary Table S1.

Figure 5. Emotional and cognitive manifestations of neuropathic pain on mice displaying extreme behavioral phenotypes. The influence of sociability, anxiety and depressive-like extreme phenotypes on emotional responses and cognitive performance was evaluated. (A-D) Anhedonic responses were measured on day 10 post-PSNL or sham injury as the percentage of sucrose preference during 24-hour sessions in (A) the whole set of animals, and in mice selected for extreme (B) sociability, (C) anxiety- and (D) depressive-like phenotypes. (E-H) Anxiety-like responses associated with neuropathic pain were evaluated as the percentage of time spent in open arms in the elevated zero maze on day 15 post-PSNL or sham injury in (A) the whole set of animals, and in mice selected for extreme (B) sociability, (C) anxiety- and (D) depressive-like phenotypes. (I-L) The discrimination index between a novel and a familiar object was assessed in the novel object recognition, as an indicator of the long-term memory, on day 20 post-PSNL or sham-injury in (I) the whole set of animals, and in mice selected for extreme (J) sociability, (K) anxiety- and (L) depressive-like phenotypes. Data are expressed as mean ± SEM (n=33 mice sham and n=55 mice PSNL in the whole set of animals; n= 5-10 mice for each sham extreme phenotype and n= 9-13 mice for each PSNL extreme phenotype). ★ $p<0.05$, ★★ $p<0.01$, ★★★ $p<0.001$ versus sham; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus corresponding phenotypes (Two-way ANOVA, Bonferroni). LS, low sociability; HS, high sociability; LA, low anxiety; HA, high anxiety; LD, low depression; HD, high depression; CTRL, control group. Detailed statistical analyses are shown in Table S2.

Figure 6. Expression levels of selected mRNAs in the amygdala of mice displaying extreme behavioral phenotypes following PSNL or sham-injury. The effect of neuropathic pain exposure on the expression of (A) *Pdyn*, (C) *Nr3c1*, *(E) Gadd45*, (G) *Il6*, (I) *Npas4* and (K) *Txc22d3* in the amygdala 41 days after the nerve injury was evaluated. PSNL induced the upregulation of *Pdyn*, *Gadd45*, *Npas4* and *Txc22d3* transcripts, whereas it did not affect *Nr3c1* and *Il6* expression. Data are expressed as mean ± SEM (n=33 mice in the sham group; n=55 mice in the PSNL group). ✶$p<0.05$ versus sham (One-way ANOVA, Bonferroni). The influence of the extreme sociability, anxiety- and depressive-like phenotypes on the expression of (B) *Pdyn*, (D) *Nr3c1*, (F) *Gadd45*, (H) *Il6*, (J) *Npas4* and (L) *Txc22d3* in the amygdala 41 days after PSNL or sham-injury was also evaluated. The sociability trait down-regulated the expression of *Pdyn*, whereas the anxiety trait increased it under sham and neuropathic pain conditions. *Nr3c1* expression was higher in low depressive animals both after sham-surgery and PSNL. *Gadd45* expression was up-regulated by the high depression trait in basal conditions, but this effect disappeared following the nerve injury. Anxiety trait increased *Il6* expression in the amygdala only following nerve injury, whereas depression trait decreased *Il6* levels in the same conditions. Data are expressed as mean ± SEM (n=5-10 mice/sham groups; n=9-13 mice/PSNL groups) # $p<0.05$, ## $p<0.01$, ### $p<0.001$ between respective extreme phenotypes (One-way ANOVA, Bonferroni). Bars represent $2^{-(Ct)}$ values expressed relative to the housekeeping gene (Hprt1). Detailed statistical analyses are shown in Table S3.


Pain measurements
Basal
0
3
6
7
10
11
15
16
18
19
20
21
41
Day post-surgery
Phenotypes selection
Behavioural study
PSNL/Sham surgery
Habituation
Test
Elevated Zero Maze
Habituation
Training
Test
Brain sampling for gene analysis
Days
1
2
5
8
11
16
Sucrose Preference Test
Novel Object Recognition
Locomotion
Sociability Test
Elevated Plus Maze
Light/dark Box Test
Tail suspension Test
Forced Swimming Test
Electrophysiological study
CeA spontaneous neuronal activity recording


A
Sociability trait
Sociability test
Social preference
0.8
0.6
0.4
0.2
0.0
CTRL LS HS LA HA LD HD
Selected phenotypes
B
Anxiety trait
Elevated plus maze
% Time in open arms
40
30
20
10
CTRL LS HS LA HA LD HD
Selected phenotypes
Light/dark box
% Time in light area
30
20
10
CTRL LS HS LA HA LD HD
Selected phenotypes
C
Depressive trait
Tail suspension
Time of immobility (s)
200
150
100
CTRL LS HS LA HA LD HD
Selected phenotypes
Forced swimming
Time of immobility (s)
100
50
CTRL LS HS LA HA LD HD
Selected phenotypes


A
***
Hz
n=22
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n=27
LS
CTRL
HS
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n=23
n=20
n=15
LA
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n=19
n=18
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275s
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Hz
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Hz
LD
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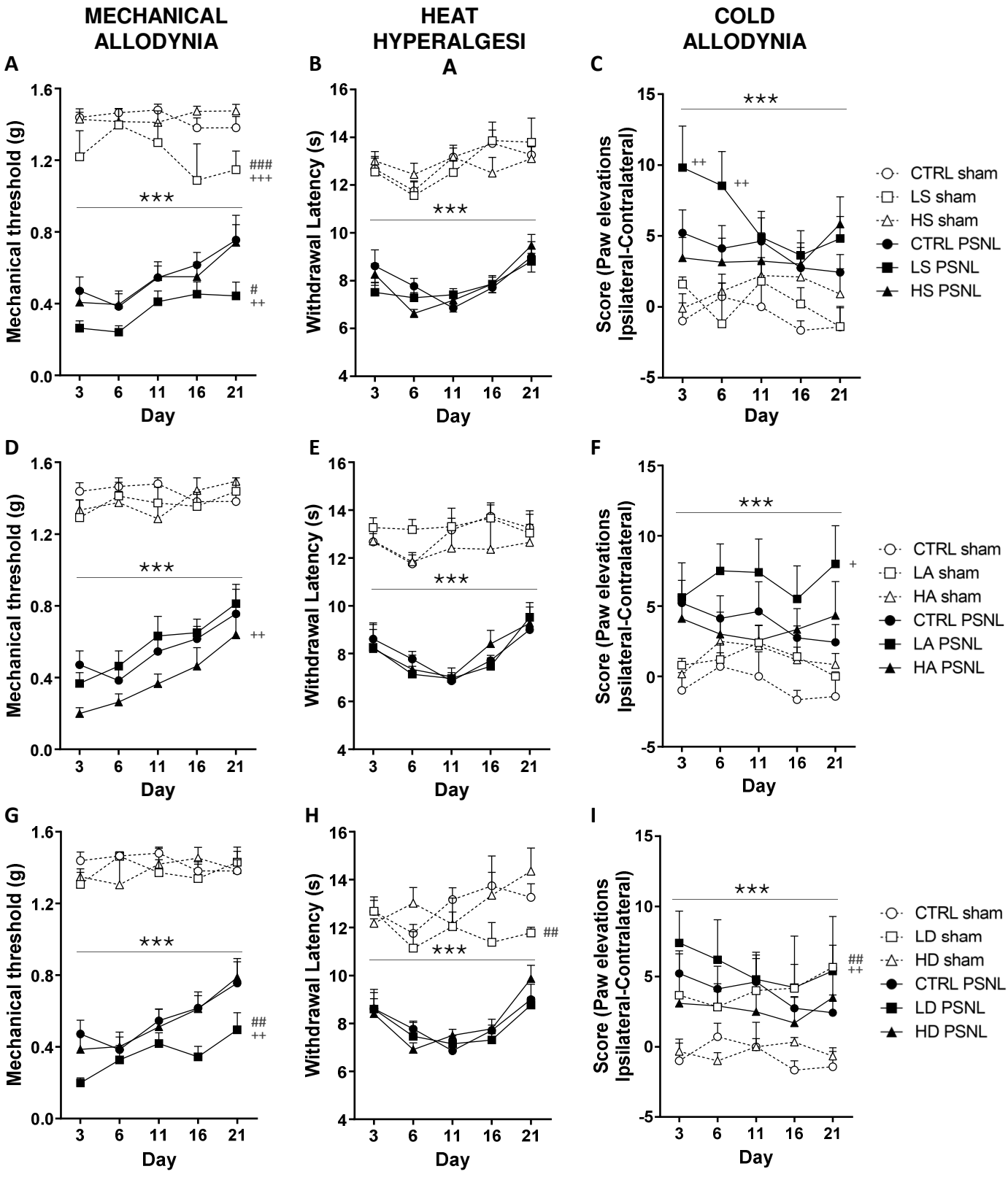
MECHANICAL ALLODYNIA
HEAT HYPERALGESIA
COLD ALLODYNIA
A
B
C
D
E
F
G
H
I
Mechanical threshold (g)
Withdrawal Latency (s)
Score (Paw elevations Ipsilateral-Contralateral)
Day
CTRL sham
LS sham
HS sham
CTRL PSNL
LS PSNL
HS PSNL
LA sham
HA sham
LA PSNL
HA PSNL
LD sham
HD sham
LD PSNL
HD PSNL
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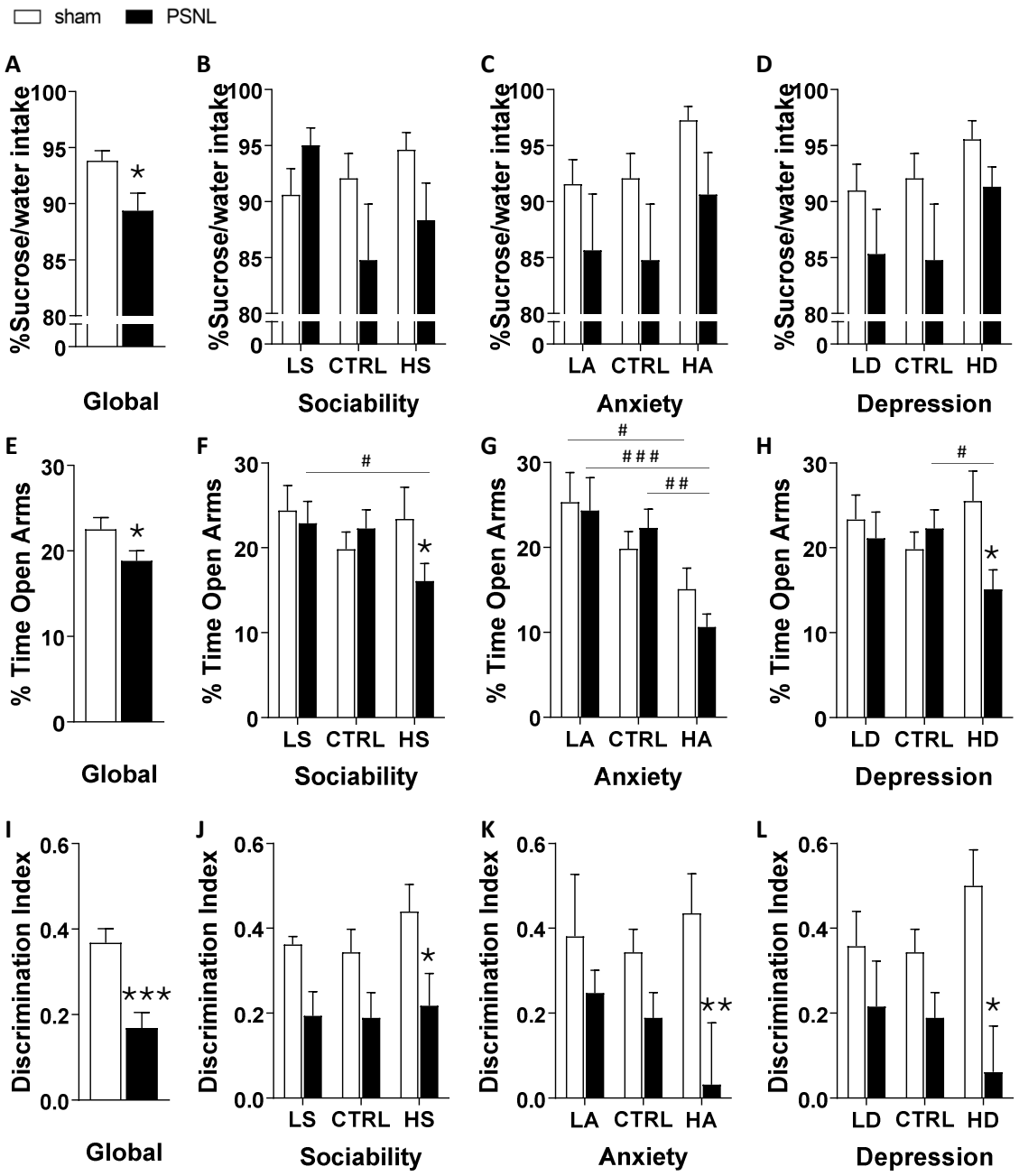
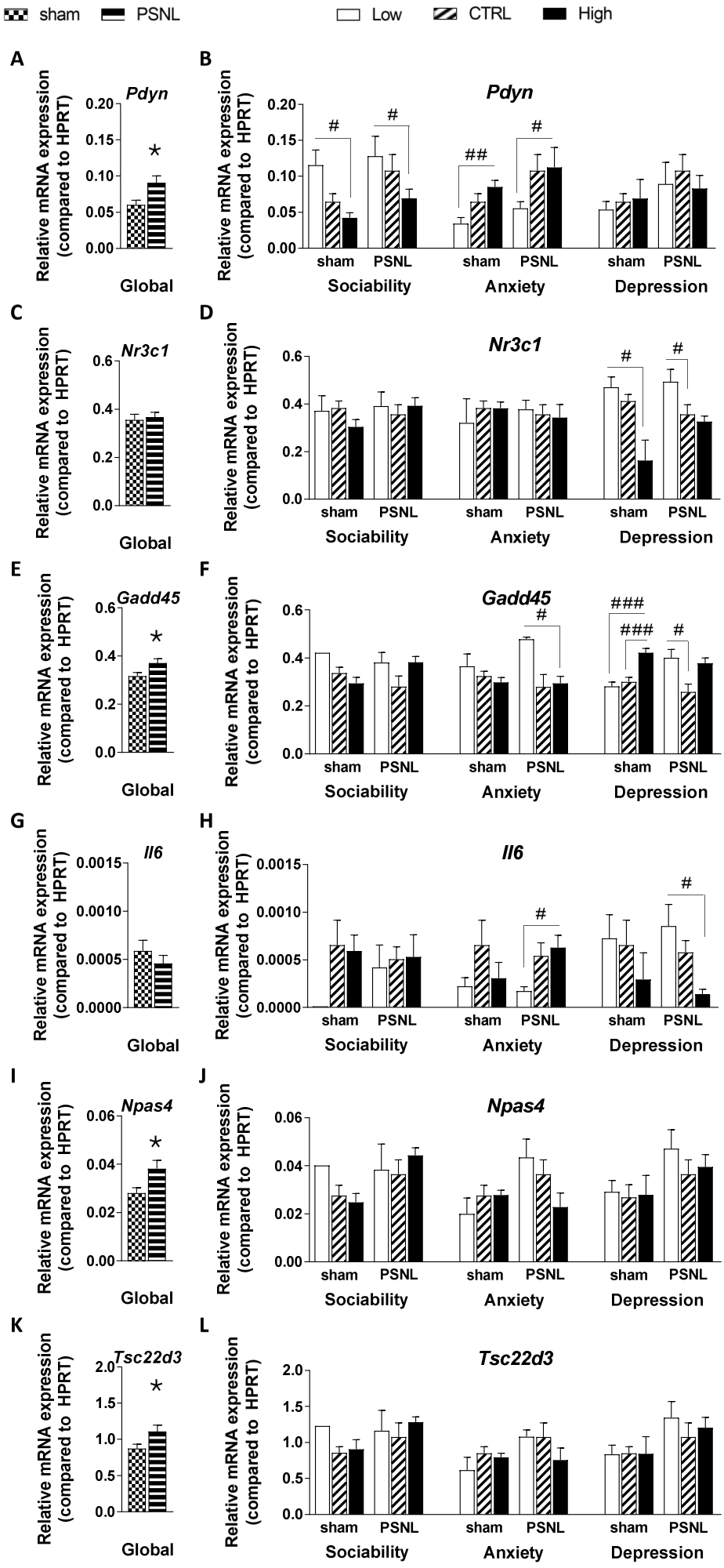

sham
PSNL
A
B
C
D
%Sucrose/water intake
100
95
90
85
80
0
*
LS CTRL HS
LA CTRL HA
LD CTRL HD
Global
Sociability
Anxiety
Depression
E
F
G
H
% Time Open Arms
30
20
10
0
#
###
##
I
J
K
L
Discrimination Index
0.6
0.4
0.2
0.0
***
**


sham
PSNL
Low
CTRL
High
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B
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D
E
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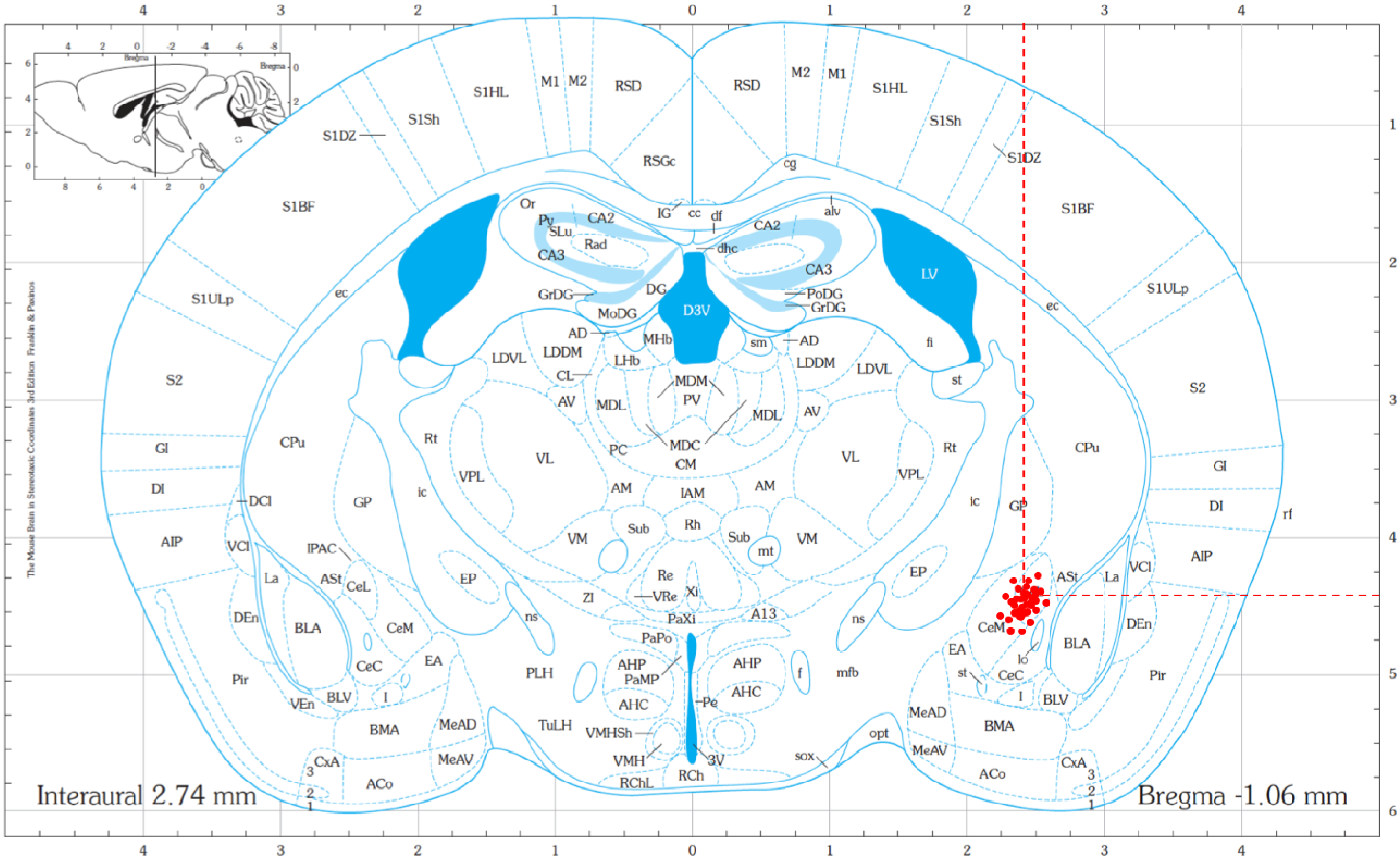
K
L
Pdyn
Nr3c1
Gadd45
Il6
Npas4
Tsc22d3
Relative mRNA expression (compared to HPRT)
Global
Sociability
Anxiety
Depression
sham
PSNL
#
##
###
*



Interaural 2.74 mm
Bregma -1.06 mm
The Mouse Brain in Stereotaxic Coordinates 3rd Edition Franklin & Paxinos
Bregma
M1
M2
RSD
RSGc
S1HL
S1Sh
S1DZ
S1BF
S1ULp
S2
GI
DI
AIP
rf
cg
IG
cc
df
alv
dhc
Or
Py
SLu
Rad
CA2
CA3
DG
GrDG
MoDG
PoDG
LV
D3V
ec
fi
st
AD
MHb
LHb
sm
LDVL
LDDM
CL
MDM
MDL
MDC
PV
AV
VL
VPL
PC
CM
IAM
AM
Rh
Sub
mt
VM
Re
Xi
VRe
PaXi
ZI
A13
Rt
ic
CPu
GP
DCl
VCl
IPAC
ASt
CeL
CeM
CeC
La
BLA
BLV
DEn
Pir
VEn
I
EA
EP
ns
PLH
PaPo
AHP
PaMP
AHC
Pe
f
mfb
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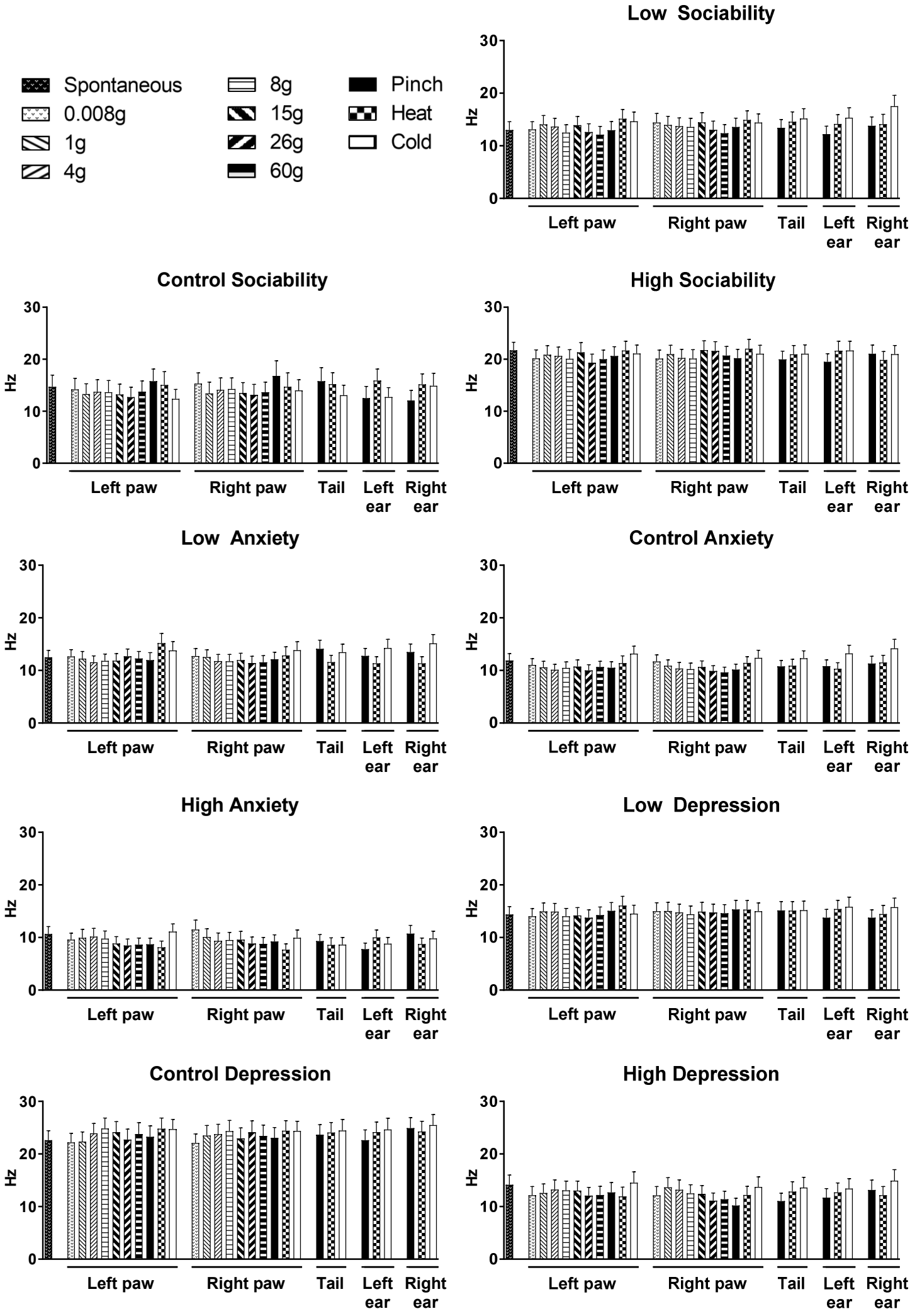
RChL
RCh
sox
opt
MeAD
MeAV
BMA
ACo
CxA
lo


Spontaneous
0.008g
1g
4g
8g
15g
26g
60g
Pinch
Heat
Cold
Low Sociability
Control Sociability
High Sociability
Low Anxiety
Control Anxiety
High Anxiety
Low Depression
Control Depression
High Depression
Hz
0
10
20
30
Left paw
Right paw
Tail
Left ear
Right ear

Conflict of interest

The authors declare no conflicts of interest.

Supporting material

Influence of behavioral traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain

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Figure S1. Atlas section representing the placement of the recordings (red dots) within the central amygdala (CeA).

Figure S2. In-vivo electrophysiological evaluation of anesthetized mice displaying extreme phenotypes. Central amygdala (CeA) neuronal firing evoked by von Frey filaments (0.008g, 1g, 4g, 8g, 15g, 26g and 60g), pinch, heat (48^{o}C) and cold (4^{o}C) applied to both paws as well as by pinch, heat (48^{o}C) and cold (4^{o}C) applied to the tail and both ears was recorded in mice with extreme low and high phenotypes as well as in control group for sociability, anxiety-like and depressive-like behavior. Data are expressed as mean ± SEM. n=5-6 mice/group. The same number of neurons than in Figure 3 were included in the recordings.

Supporting table S1. Detailed statistical evaluation for Fig. 4.

Two-way ANOVA for hyperalgesia and allodynia (whole evaluated period)

Sociability	Mechanical allodynia
Surgery	F(1,264)=816, p<0.001
Phenotype	F(2,264)=15, p<0.001
Surgery x Phenotype	F(2,264)=0.2, p=0.81

Sociability	Thermal hyperalgesia
Surgery	F(1,264)=645, p<0.001
Phenotype	F(2,264)=0.14, p=0.86
Surgery x Phenotype	F(2,264)=0.02, p=0.97

Sociability	Cold allodynia
Surgery	F(1,264)=47, p<0.001
Phenotype	F(2,264)=2.38, p=0.09
Surgery x Phenotype	F(2,264)=3.94, p<0.01

Anxiety	Mechanical allodynia
Surgery	F(1,219)=677, p<0.001
Phenotype	F(2,219)=4.77, p<0.01
Surgery x Phenotype	F(2,219)=3.17, p<0.05

Anxiety	Thermal hyperalgesia
Surgery	F(1,219)=386, p<0.001
Phenotype	F(2,219)=0.01, p=0.98
Surgery x Phenotype	F(2,219)=2.04, p=0.13

Anxiety	Cold allodynia
Surgery	F(1,219)=27.8, p<0.001
Phenotype	F(2,219)=3.32, p<0.05
Surgery x Phenotype	F(2,219)=1.94, p=0.144

Depression	Mechanical allodynia
Surgery	F(1,234)=630, p<0.001
Phenotype	F(2,234)=5.35, p<0.01
Surgery x Phenotype	F(2,234)=3.26, p<0.05

Depression	Thermal hyperalgesia
Surgery	F(1,234)=422, p<0.001
Phenotype	F(2,234)=2.55, p=0.07
Surgery x Phenotype	F(2,234)=3.8, p<0.05

Depression	Cold allodynia
Surgery	F(1,234)=13.7, p<0.001
Phenotype	F(2,234)=5.3, p<0.01
Surgery x Phenotype	F(2,234)=2.6, p=0.07

Two-way ANOVA for cold allodynia (early stage of neuropathic pain, until D6)

Sociability	Cold allodynia
Surgery	F(1,108)=21.628, p<0.001
Phenotype	F(2,108)=1.459, p=0.237
Surgery x Phenotype	F(2,108)=6,614, p<0.01

Supporting table S2. Detailed statistical evaluation for Fig. 5

One-way ANOVA for emotional and cognitive manifestations of neuropathic pain

Sham vs PSNL	
%sucrose/water intake	F(1,86)= 4.851, p<0.05
%time open arms	F(1,81)= 3.973, p<0.05
discrimination index	F(1,86)= 13.698, p<0.001

Two-way ANOVA for emotional and cognitive manifestations of neuropathic pain

Sociability	%sucrose/water intake
Surgery	F(1,51)=1.464, p=0.232
Phenotype	F(2,51)=0.936, p=0.399
Surgery x Phenotype	F(2,51)=1.798, p=0.176

Sociability	%time open arms (EZM)
Surgery	F(1,51)=0.865, p=0.357
Phenotype	F(2,51)=0.952, p=0.393
Surgery x Phenotype	F(2,51)=1.780, p=0.179

Sociability	discrimination index (NOR)
Surgery	F(1,51)=10.282, p<0.01
Phenotype	F(2,51)=0.532, p=0.591
Surgery x Phenotype	F(2,51)=0.153, p=0.858

Anxiety	%sucrose/water intake
Surgery	F(1,42)=3.455, p=0.070
Phenotype	F(2,42)=1.029, p=0.366
Surgery x Phenotype	F(2,42)=0.013, p=0.987

Anxiety	%time open arms (EZM)
Surgery	F(1,42)=0.186, p=0.668
Phenotype	F(2,42)=8.520, p<0.001
Surgery x Phenotype	F(2,42)=0.762, p=0.473

Anxiety	discrimination index (NOR)
Surgery	F(1,42)=8.447, p<0.01
Phenotype	F(2,42)=0.323, p=0.726
Surgery x Phenotype	F(2,42)=1.167, p=0.321

Depression	%sucrose/water intake
Surgery	F(1,41)=2.959, p=0.093
Phenotype	F(2,41)=0.884, p=0.421
Surgery x Phenotype	F(2,41)=0.069, p=0.934

Depression	%time open arms (EZM)
Surgery	F(1,41)=1.994, p=0.165
Phenotype	F(2,41)=0.204, p=0.817
Surgery x Phenotype	F(2,41)=2.203, p=0.123

Depression	discrimination index (NOR)
Surgery	F(1,41)=8.450, p<0.01
Phenotype	F(2,41)=0.026, p=0.974
Surgery x Phenotype	F(2,41)=1.097, p=0.344

Supporting table S3. Detailed statistical evaluation for Fig. 6.

One-way ANOVA for the influence of PSNL on gene expression

	Pdyn
Surgery	F(1,80)=6.415, p<0.05

	Nr3c1
Surgery	F(1,80)=0.318, p=0.574

	Gadd45
Surgery	F(1,51)=4.906, p<0.05

	Il6
Surgery	F(1,50)=0.953, p=0.334

	Npas4
Surgery	F(1,53)=5.224, p<0.05

	Tsc22d3
Surgery	F(1,53)=4.092, p<0.05

One-way ANOVA for the influence of behavioral traits on gene expression in sham and neuropathic pain conditions separately

Sociability trait	*Pdyn*
Sham	F(2,15)=5.785, p<0.05
PSNL	F(2,27)=3.487, p<0.05

Sociability trait	*Nr3c1*
Sham	F(2,18)=0.875, p=0.434
PSNL	F(2,29)=0.402, p=0.673

Sociability trait	*Gadd45*
Sham	F(2,10)=2.682, p=0.117
PSNL	F(2,17)=0.010, p=0.990

Sociability trait	*Il6*
Sham	F(2,10)=0.800, p=0.476
PSNL	F(2,15)=0.231, p=0.797

Sociability trait	*Npas4*
Sham	F(2,10)=0.817, p=0.469
PSNL	F(2,17)=0.239, p=0.790

Sociability trait	*Tsc22d3*
Sham	F(2,10)=0.748, p=0.498
PSNL	F(2,17)=0.176, p=0.840

Anxiety trait	*Pdyn*
Sham	F(2,11)=8.302, p<0.01
PSNL	F(2,23)=3.840, p<0.05

Anxiety trait	*Nr3c1*
Sham	F(2,11)=0.423, p=0.663
PSNL	F(2,26)=0.503, p=0.611

Anxiety trait	*Gadd45*
Sham	F(2,10)=1.404, p=0.290
PSNL	F(2,11)=6.257, p<0.05

Anxiety trait	*Il6*
Sham	F(2,9)=1.187, p=0.349
PSNL	F(2,11)=6.504, p<0.05

Anxiety trait	*Npas4*
Sham	F(2,10)=1.770, p=0.220
PSNL	F(2,13)=4.199, p<0.05

Anxiety trait	*Tsc22d3*
Sham	F(2,10)=1.932, p=0.195
PSNL	F(2,13)=2.143, p=0.157

Depressive trait	*Pdyn*
Sham	F(2,11)=0.280, p=0.761
PSNL	F(2,29)=0.035, p=0.965

Depressive trait	*Nr3c1*
Sham	F(2,7)=4.814, p<0.05
PSNL	F(2,24)=4.379, p<0.05

Depressive trait	*Gadd45*
Sham	F(2,10)=24.103, p<0.001
PSNL	F(2,19)=4.028, p<0.05

Depressive trait	*Il6*
Sham	F(2,9)=0.552, p=0.594
PSNL	F(2,16)=5.287, p<0.05

Depressive trait	*Npas4*
Sham	F(2,10)=0.074, p=0.929
PSNL	F(2,19)=2.833, p=0.084

Depressive trait	*Tsc22d3*
Sham	F(2,10)=0.006, p=0.994
PSNL	F(2,19)=1.477, p=0.253