Published in final edited form as:

Neuroscience. 2018 July 15; 383: 191–204. doi:10.1016/j.neuroscience.2018.05.008.

Brain Stimulation Differentially Modulates Nociception and Inflammation in Aversive and Non-aversive Behavioral Conditions

G. S. Bassi^{a,b,*}, A. Kanashiro^c, G. J. Rodrigues^c, F. Q. Cunha^d, N. C. Coimbra^{d,e,*}, and L. Ulloa^{b,f,*}

^aDepartment of Immunology, Ribeirão Preto Medical School of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^bYueyang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China

^cDepartment of Physiological Sciences, Federal University of São Carlos, São Carlos, São Paulo, Brazil

^dDepartment of Pharmacology, Ribeirão Preto Medical School of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^eNAP-USP-Neurobiology of Emotions Research Centre (NuPNE), Ribeirão Preto Medical School of the University of São Paulo, São Paulo, Brazil

^fDepartment of Surgery, Centre for Immunology and Inflammation, Rutgers – New Jersey Medical School, Rutgers University, Newark, NJ 07103, USA

Abstract

Inflammation and pain are major clinical burdens contributing to multiple disorders and limiting the quality of life of patients. We previously reported that brain electrical stimulation can attenuate joint inflammation in experimental arthritis. Here, we report that non-aversive electrical stimulation of the locus coeruleus (LC), the paraventricular hypothalamic nucleus (PVN) or the ventrolateral column of the periaqueductal gray matter (vlPAG) decreases thermal pain sensitivity, knee inflammation and synovial neutrophilic infiltration in rats with intra-articular zymosan. We also analyzed the modulation of pain and inflammation during aversive neuronal stimulation, which produces defensive behavioral responses such as freezing immobility to avoid predator detection. Electrical stimulation with higher intensity to induce freezing immobility in rats further reduces pain but not inflammation. However, tonic immobility further reduces pain, knee inflammation and synovial neutrophilic infiltration in guinea pigs. The duration of the tonic immobility increases the control of pain and inflammation. These results reveal survival behavioral

*Corresponding authors. Address: Yueyang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China (G. S. Bassi, L. Ulloa). Department of Pharmacology, Ribeirão Preto Medical School of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil (N. C. Coimbra). shimizug@gmail.com (G. S. Bassi); nccoimbra@fmrp.usp.br (N. C. Coimbra); Luis.Ulloa@Rutgers.edu (L. Ulloa).

AUTHOR CONTRIBUTIONS

G.S.B. designed the study; G.S.B. performed central brain stimulation and tonic immobility procedures; G.S.B. prepared the Figures; G.S.B., L.U., N.C.C. and A.K. wrote and reviewed the manuscript; G.J.R., F.Q.C., and A.K. provided financial support.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

and neuromodulatory mechanisms conserved in different species to control pain and inflammation in aversive life-threatening conditions. Our results also suggest that activation of the LC, PVN, or vIPAG by non-invasive methods, such as physical exercise, meditation, psychological interventions or placebo treatments may reduce pain and joint inflammation in arthritis without inducing motor or behavioral alterations.

Keywords

inflammation; pain; behavior; neuroimmunology; brain; stimulation

INTRODUCTION

Chronic pain is one of the most common disabling factors contributing to cognitive impairments, morbidity, and mortality in multiple clinical disorders including arthritis (Hewlett et al., 2011; Upchurch and Kay, 2012; Boyden et al., 2016; Castañeda et al., 2016). The best current treatments for arthritis are based on disease-modifying anti-rheumatic drugs (DMARDs) that neutralize inflammatory cytokines such as tumor necrosis factor (TNF) and thereby reduce inflammation and leucocytes activation (Ramiro et al., 2011; Upchurch and Kay, 2012). However, these treatments are very expensive and can induce severe side effects increasing the risk of infections and immunosuppression (Inanc and Direskeneli, 2006; Favalli et al., 2009; Ramiro et al., 2011; Inui and Koike, 2016).

Recent studies on alternative therapies to control inflammation showed a bidirectional interaction between nervous and the immune systems (Olofsson et al., 2012; Torres-Rosas et al., 2014; Bassi et al., 2015, 2017; Ulloa et al., 2017). Electrical nerve stimulation can represent a promising strategy to control inflammation without the effects of current pharmacological treatments (Olofsson et al., 2012; Bassi et al., 2015, 2017; Ulloa and Deitch, 2009). Clinical and experimental studies indicate that peripheral or central neural stimulation attenuates joint inflammation (Miao et al., 2003; Kox et al., 2014; Bassi et al., 2015, 2017; Koopman et al., 2016) and pain sensitivity (Mayer and Liebeskind, 1974; Basbaum and Fields, 1984; Ren et al., 1988; de Luca et al., 2003; Busch et al., 2013) in diverse models of experimental arthritis. We recently reported that electrical stimulation of brain structures, including the locus coeruleus (LC) or the paraventricular nucleus (PVN), decreases joint inflammation without affecting rat behavior (Bassi et al., 2017). Other studies reported that stimulation of these brain areas can decrease nociception and pain sensitivity (Panksepp, 1971; Fuchs et al., 1985; West et al., 1993; Hickey et al., 2014) in aversive defensive responses such as that displayed by prey during a predator attack (Gallup, 1977; Yardley and Hilton, 1986; Coimbra et al., 2017). However, it is unknown whether the potential to control both pain and inflammation is due to different neuronal stimulation or different networks activation by defensive mechanisms in aversive conditions. Here, we analyzed whether neural stimulation of central areas regulating pain and behavior can modulate nociception and inflammation in conscious, non-anesthetized animals both in aversive and non-aversive conditions.

EXPERIMENTAL PROCEDURES

Animal experiments

Eighty eight male Wistar *Rattus norvegicus* (Rodentia, Muridae) (250–300 g) and 22 *Cavia porcellus* (Rodentia, Caviidae) (400–500 g) were obtained from the main Animal Facility of the Ribeirão Preto Medical School of the University of São Paulo, and housed at the animal facility in plastic cages (four in a cage) under a 12-h light/dark cycle (lights on at 7am) at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The animals had unrestricted access to food and tap water. The number of animals used was the minimum required to ensure reliability of the results, and every effort was made to minimize animal discomfort. All animals were anesthetized with a mixture of ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) administered into the right posterior calf muscle through a 30-G needle. The experimental protocols comply with the recommendations of the SBNeC (Brazilian Society of Neuroscience and Behavior), the Ethical Principles of the National Council for Animal Experimentation Control (CONCEA) (Protocol 137/2013), the US National Institutes of Health Guide for The Care and Use of Laboratory Animals, and the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals (Zimmerman, 1983).

Stereotaxic surgery

Anesthetized rats were placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) and underwent surgical implant of stainless steel bipolar electrodes or a guide cannula using coordinates extracted from Rat Brain in Stereotaxic Coordinates Atlas (Paxinos and Watson, 2007). The interaural line served as the reference for each plane and the upper incisor bar was set at 2.5 mm below the interaural line, so the skull was horizontal between the bregma and lambda. LC: anteroposterior = -1.04 mm, mediolateral = 0.9 mm, dorsoventral = 7.9 mm; PVN: anteroposterior = 7.1 mm, mediolateral = 0.2 mm, dorso ventral = 8.0 mm; ventrolateral PAG column (vlPAG): anteroposterior = -0.2 mm, mediolateral = 1.4 mm, dorsoventral = 4.2 mm. After surgery, electrodes or guide-cannulas were fixed to the skull with acrylic resin and two stainless steel screws. Then, all animals received an intramuscular injection (0.2 mL) of antibiotic Pentabiotico (0.5 mL/kg, Fort Dodge, Campinas, SP, Brazil), and analgesic flunixin meglumine (Banamine, 2.5 mg/kg, Schering-Plough, Cotia, SP, Brazil).

Brain nuclei stimulation in non-anesthetized rats

Seven to ten days after the stereotaxic surgery, the animals were individually placed in a circular arena (60 cm in diameter and 50 cm high) and the stimulation cable was connected to the bipolar electrode. Rats were allowed a 10-min period of free exploration of the experimental environment. Afterward, the LC, PVN or vlPAG was electrically stimulated for 2 min with a square wave stimulator (1MIC, AVS Project, Sao Carlos, SP, Brazil) as previously reported: LC: 20 Hz, 1 ms, 100 μA (Kannan et al., 1986); PVN: 20 Hz, 0.5 ms, 50 μA (Jones and Gebhart, 1989); vlPAG: 20 Hz, 1 ms, 50 μA (Fardin et al., 1984). Animals showed no alertness, freezing, escape behaviors, or seizure during these stimulations. For the tail-flick tests, we first determined the thermal pain threshold baseline. The animals were then stimulated and underwent the tail-flick test. Twenty-four h later, rats were subjected to the same stimulation and then to the elevated plus-maze test (Fig. 1J). The animals were re-

stimulated for the immunological experiments at 24 h after the behavioral experiment (Fig. 1J). Sham-stimulated animals (control) did not receive electrical stimulation.

We also analyzed whether aversive brain stimulation in non-anesthetized rats induces freezing immobility and control inflammation. Freezing behavior, also called “attentive immobility”, is a common adaptive defensive behavior characterized by physical immobility followed by neurovegetative responses to avoid predator detection (Gallup, 1977; Marks, 1987; Roelofs, 2017; Coimbra et al., 2017; Paschoalin-Maurin et al., 2018). To induce freezing immobility, rats were placed in the circular arena (60 cm in diameter and 50 cm high) and after 10-min of free exploration, the PVN, LC, or vIPAG was electrically stimulated for 2-min intervals with the electrical intensity beginning at 50 μ A and increasing it at intervals of 10 μ A to induce freezing immobility. Freezing behavior was defined as the production of physical immobility except for the respiration movements accompanied by at least two of the following responses: arching back, piloerection, defecation, micturition, exophthalmia and ear retraction during the period of brain stimulation as previously reported (Gallup, 1977). Then, the electrical stimulation was immediately stopped and the animals underwent the tail-flick nociceptive test (Fig. 6B). On the next day, animals were subjected to the same electrical stimulation followed by intra-articular injection of zymosan under anesthesia. Sham-stimulated animals (control) did not receive electrical stimulation.

Tail-flick test performed in rats

The tail-flick tests were used to analyze the modulation of pain sensitivity with electrical central stimulation. The tail-flick test was based on D'Amour and Smith's (1941) method. Rats were placed in a restraining tube, with their tails protruded. Radiant heat was focused on the lower third section of the tail (Ugo Basile, Varese, Italy). Withdrawal reflex-induced movements of the tail activate a photocell, turning off both the light and a motor reaction timer. The light intensity was adjusted to achieve baseline latencies of 2.5 and 3.5 s (Coimbra et al., 2006, 2017). A maximum latency of 6 s (i.e., the cut off) was established to minimize tail damage. The baseline trial consisted in determining the average of three individual tests separated by a 5-min interval. After the determination of baseline tail-flick latency, animals underwent stimulation of the PVN, LC or vIPAG and then the tail-flick tests. Six other tail-flick latencies were recorded across the experiments at 5-min intervals.

Behavioral analysis at the elevated plus-maze test in rats

Twenty-four hours after the tail-flick test, the animals were re-stimulated and their behavior was evaluated in the elevated plus-maze (EPM) test, a reliable ethological tool to measure anxiety in rodents (Anseloni and Brandão, 1997; File et al., 2001). The elevated plus maze was made of wood and had two open arms (50 $\times$ 10 cm) perpendicular to two enclosed arms of the same size with 50-cm-high walls, with the exception of the central part (10 $\times$ 10 cm), where the arms crossed. The apparatus was elevated 50 cm above the floor (File et al., 2001). The behavior of the animals was analyzed using a video camera (Sony Handycam HDR-CX350, Konan, Minato-ku, Tokyo, Japan) positioned 100 cm above the maze. The signal was relayed to a monitor in another room via a closed-circuit television camera to discriminate all behavioral responses. Luminosity at the level of the open arms of the elevated plus maze was 20 lx. A total of 5-min free exploration of the maze was allowed to

the rats. The maze was thoroughly cleaned after each test using damp and dry cloths embed in 5% alcoholic solution. An arm entry or exit was defined as all four paws entering into or out of a section, respectively and risk assessment behaviors were defined by the sum of the following behaviors: when the animal reached the end of the open arm (end-arm-exploration) or stretched to its full length with its forepaws, keeping its hind paws in the same place, and turning back to the anterior position (stretched-attend posture) or dipped the head below the level of the maze floor (head dipping) (Anseloni and Brandão, 1997; File et al., 2001). Twenty-four hours after the EPM test, the animals were re-stimulated and underwent the immunological experiments.

Tonic immobility recordings performed in guinea pigs

Tonic immobility (TI) is a defensive behavior characterized by profound motor inactivity, analgesia and unresponsiveness to external stimuli (Gallup, 1977; Leite-Panissi et al., 2001). Tonic immobility is considered the last defensive behavioral resource against predators within a sequence of defensive responses: freezing, flight/fight and tonic immobility (Marks, 1987). The induction of tonic immobility (TI) was attempted in a quiet environment under a dim light (30 lx) and by holding the guinea pig around the thorax with the hands, then quickly inverting and pressing its back against a padded V-shaped plywood (25 cm long $\times$ 15 cm high) proportionally to the resistance offered by the animal during the restraining maneuver (Leite-Panissi et al., 2001). When the guinea pig stopped moving, the experimenter slowly withdrew his hand and the duration of the TI behavior was counted. A TI behavior was considered to be finished when the upright position is reassumed. Immediately after the return to the upright position, the guinea pigs were anesthetized and underwent the immunological experiments (Fig. 8A).

Zymosan-induced arthritis

Zymosan suspension (100 μ g in 50 μ l) in sterile physiological saline (0.9% NaCl; vehicle) was injected into the femorotibial joint (intra-articular; i.a.) of both knees of rats and guinea pigs under isoflurane anesthesia (1.5%) through a 30-G needle (Keystone et al., 1977; Gegout et al., 1994). Six hours later, the animals were killed by decapitation and the following inflammatory parameters were evaluated. *Joint diameter*: the knee was flexed in 30 degrees and tissue thickness was measured by measuring joint width through a digital calliper. The result was depicted in millimeters (mm). *Knee neutrophil recruitment*: immediately after the joint measurements, the knees were opened by a surgical incision to expose the synovial cavity. The synovial fluids were carefully washed 3 times with PBS with EDTA (1 mM) by a micropipette in a total volume of 250 μ l. The aliquots of the resulting washes were then diluted in Turk solution (1:5) and leukocytes were counted with the aid of a Neubauer chamber and an optical microscope (400 $\times$). Differential cell counts were stained with hematoxylin-eosin and also counted under a light microscope. The number of differentiated cells was calculated by the percentage found in the total number of cells (100 cells in total) (Bassi et al., 2015, 2017). According to ethical bounds, we avoided knee inflammation for more than 48 h. Results were expressed as the mean $\pm$ SEM of the number of neutrophils per knee joint.

Brain histology

We first confirmed that the electrode tip was implanted into the LC, PVN or vIPAG with histological analyses. At the end of the experiments, the animals were anesthetized and perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde and 1 mM potassium ferrocyanide in PBS. A direct current of 1 mA was applied through the tip of the electrodes for 10 s to allow the identification of the stimulation site by Prussian blue reaction. The encephalon was removed and immersed in paraformaldehyde/potassium ferrocyanide solution for 5 h and then stored in 40% sucrose in 0.1 M PBS for cryoprotection for 48 h. Serial 40- μ m brain sections were cut using a cryostat (Leica, Wetzlar, Germany), thaw-mounted on gelatinized slides and stained with hematoxylin–eosin technique in order to localize the sites of microinjection with reference to Paxinos and Watson's Rat Brain in Stereotaxic Coordinates Atlas (2007).

Statistical analysis

The investigators were blinded to the conditions. Normality and homogeneity in the sampled distributions were confirmed with Shapiro–Wilk's test of normality and the Bartlett's test of homogeneity of variances. Statistical analyses were performed using Prism 6.0 (GraphPad) software. Neutrophil recruitment to the knee joint and behavioral data were statistically analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. The time course results of the knee joint diameter and the experimental score were analyzed with the repeated measures ANOVA followed by Bonferroni's post hoc test. Tail-flick latencies were analyzed by repeated measures ANOVA. The group factor refers to treatments, and the condition factor refers to the time before and after tail-flick latency recordings. Relationships between neutrophil migration, joint edema and tonic immobility time were examined by Spearman's rank-order correlation. The experimental sample n refers to the sample size and number of animals per experimental group and is indicated inside each graph bar. Data are expressed as mean + standard error of mean (S.E.M.). Differences were considered statistically significant when $P < 0.05$.

RESULTS

Non-aversive stimulation of the LC, PVN or vIPAG increases thermal pain threshold in conscious rats

First, histological analyses confirmed that the electrode tips were functional (Prussian blue technique) and located inside the paraventricular nucleus of the hypothalamus (PVN, Fig. 1A–C), locus coeruleus (LC, Fig. 1D–F) or the ventrolateral part of the periaqueductal gray matter (vIPAG, Fig. 1G–I). Given that previous studies suggested the PVN, LC and vIPAG can modulate nociception (Sandberg and Segal, 1978; Fardin et al., 1984; Yirmiya et al., 1990), we analyzed whether electrical stimulation of these structures increases thermal pain threshold in the tail-flick test. Electrical stimulation of the PVN (20 Hz, 1 ms, 50 μ A) (Jones and Gebhart, 1989), LC (20 Hz, 1 ms, 100 μ A) (Kannan et al., 1986) or vIPAG: (20 Hz, 1ms, 50 μ A) (Fardin et al., 1984) increases thermal pain threshold for up to 20 min post-stimulation (Figs. 2A, 3A, 4A). Repeated measures ANOVAs revealed significant effects of treatment (PVN: $F_{3,153} = 6.2$; LC: $F_{3,171} = 12.54$; vIPAG: $F_{3,126} = 6.55$; $p = 0.0001$ for all groups), time (PVN: $F_{8,153} = 1.02$; LC: $F_{8,171} = 2.3$; vIPAG: $F_{8,126} = 1.85$; $p = 0.0001$ for all

groups) and a significant interaction between treatment and time (PVN: $F_{24,153} = 1.79$; LC: $F_{24,171} = 2.18$; vIPAG: $F_{24,126} = 1.67$; $p = 0.0001$ for all groups). Post-hoc pairwise comparisons indicated that the analgesic effects peaked right at the end of the stimulation, falling down to basal levels within 20 min post-stimulation.

In the elevated plus-maze test, one-way ANOVA tests did not reveal any significant effects of the electrical stimulation of the PVN, LC or vIPAG on the frequency of open arm entries (PVN: $F_{3,17} = 0.55$, $p = 0.65$; LC: $F_{3,20} = 0.6$, $p = 0.62$; vIPAG: $F_{3,15} = 0.36$, $p = 0.77$), time spent (PVN: $F_{3,17} = 0.41$, $p = 0.74$; LC: $F_{3,20} = 0.16$, $p = 0.91$; vIPAG: $F_{3,15} = 0.34$, $p = 0.79$) and number of entries into the closed arms (PVN: $F_{3,17} = 1.22$, $p = 0.33$; LC: $F_{3,20} = 0.61$, $p = 0.61$; vIPAG: $F_{3,15} = 0.25$, $p = 0.85$) (Figs. 2B–D, 3B–D, 4B–D). For the risk assessment behaviors, the one-way ANOVA statistical test did not reveal any significant effect of the PVN, LC, or vIPAG stimulation (PVN: $F_{3,17} = 1.68$, $p = 0.20$; LC: $F_{3,20} = 1.70$, $p = 0.19$; vIPAG: $F_{3,15} = 0.37$, $p = 0.77$) (Figs. 2E, 3E, 4E) on the EPM test. These results indicate that central electrical stimulation of the PVN, LC or vIPAG in conscious rats decreases nociception without inducing behavioral or motor alterations.

Non-aversive stimulation of the LC, PVN or vIPAG reduces joint inflammation in conscious rats

Next, we analyzed whether central stimulation of these nociceptive structures in non-anesthetized rats can control joint inflammation in zymosan-induced arthritis (Bassi et al., 2017). First, we analyzed whether central neural stimulation modulates inflammation in non-anesthetized rats. For the joint edema, multiple comparison ANOVA tests revealed significant effects of PVN, LC, or vIPAG stimulation depending on treatment (PVN: $F_{3,76} = 18.43$, LC: $F_{3,88} = 32.46$, vIPAG: $F_{3,68} = 16.51$; $p = 0.0001$ for all groups as compared to control group without stimulation), time (PVN: $F_{1,76} = 376.8$, LC: $F_{1,88} = 563.1$, vIPAG: $F_{1,68} = 279.5$; $p = 0.0001$ for all groups), and there was a significant correlation between treatment and time (PVN: $F_{3,76} = 11.51$; LC: $F_{3,88} = 23.37$; vIPAG: $F_{3,68} = 12.83$; $p = 0.0001$ for all groups). Post-hoc pairwise analyses indicated that these differences were attributable to the groups with stimulation inside the LC, PVN, or vIPAG (Figs. 2F, 3F, 4F). Stimulation of the PVN, LC or vIPAG in non-anesthetized rats significantly reduces neutrophilic infiltration into the knee joint as shown by the one-way ANOVA tests (PVN: $F_{3,38} = 41.41$; LC: $F_{3,44} = 44.19$; vIPAG: $F_{3,34} = 54.05$; $p = 0.0001$ for all groups). Post-hoc analyses showed that these differences were attributable to the groups with stimulation inside the LC, PVN, or vIPAG (Figs. 2G, 3G, 4G). Together, these results indicate that stimulation of the PVN, LC or vIPAG in non-anesthetized rats attenuates both pain sensitivity and joint inflammation without inducing anxiety or motor alterations.

Non-aversive stimulation of the LC, PVN or vIPAG produces similar effects on immunity and pain perception

Next, we evaluated whether stimulation of the LC, PVN or vIPAG induces similar effects in inflammation and the tail-flick test. For the tail-flick test, the two-way repeated measures ANOVA tests revealed significant effects depending on the treatment ($F_{3,288} = 146.1$, $p = 0.0001$), time ($F_{8,288} = 179.3$, $p = 0.0001$), and treatment $\times$ time interaction ($F_{24,288} = 32.46$, $p = 0.0001$). However, these differences were only significant at 10 (PVN vs. LC) and 20

(PVN vs. LC, and LC vs. vIPAG) minutes. Considering the anti-edema effect, the two-way repeated measures ANOVAs also revealed significant effects of treatment ($F_{3,68} = 24.31$, $p = 0.0001$), time ($F_{1,68} = 282.8$, $p = 0.0001$), and treatment $\times$ time interaction ($F_{3,68} = 29.51$, $p = 0.0001$). Post-hoc pairwise comparisons indicated that these differences were not attributable to LC, PVN, or vIPAG stimulation (Fig. 5B). One-way ANOVAs also revealed significant effects of the stimulation on the neutrophilic infiltration ($F_{3,68} = 71.93$, $p = 0.0001$), but no significant differences were found among the stimulated groups (Fig. 5C). These results indicate that stimulation of the LC, PVN or vIPAG induces similar anti-inflammatory and analgesic effects.

Aversive stimulation of the LC, PVN or vIPAG reduces both nociception and joint inflammation in conscious rats

Next, we evaluated whether the analgesic and anti-inflammatory effects of brain stimulation could be physiological defensive responses against aversive threatening stimuli. Thus, we analyzed the electrical parameters needed to induce aversive freezing immobility in non-anesthetized, conscious animals. Freezing immobility, also called “attentive immobility”, is an innate adaptive defensive behavior characterized by profound motor immobility to avoid predator detection in life-threatening conditions (Marks, 1987; Roelofs, 2017). Animals were allowed 10-min free exploration of the circular arena. Then, the PVN, LC or vIPAG was electrically stimulated at 20-min intervals with 50 μ A as starting electrical current. Then, the electrical current was increased by intervals of 10 μ A until the induction of freezing immobility. Freezing threshold is defined as the electrical current needed in the specific brain structure to induce freezing immobility. The average freezing threshold stimulation for LC and vIPAG (279 μ A and 220 μ A, respectively) was statistically similar and lower than that required for PVN stimulation (410 μ A) (Fig. 6A). These results concur with previous studies showing the direct involvement of LC and PAG in the innate survival responses while hypothalamic structures coordinate goal-oriented behaviors (Markgraf et al., 1991; Motta et al., 2009; LeDoux, 2012; Ullah et al., 2015, 2017).

Next, we analyzed whether the stimulations needed to induce freezing immobility affects nociception and pain sensitivity (Fig. 6B). Similar to non-aversive stimulation, repeated measures ANOVA revealed that aversive stimulation of the PVN, LC or vIPAG also attenuates pain sensitivity depending on the treatment (PVN: $F_{8,72} = 45.16$; LC: $F_{8,72} = 24.29$; vIPAG: $F_{8,72} = 40.89$; $p = 0.0001$ for all groups), time (PVN: $F_{1,72} = 385.4$; LC: $F_{1,72} = 146.5$; vIPAG: $F_{8,72} = 269.1$, $p = 0.0001$ for all groups), and there was a significant correlation between treatment and time (PVN: $F_{8,72} = 57.77$; LC: $F_{8,72} = 19.35$; vIPAG: $F_{8,72} = 35.74$, $p = 0.0001$ for all groups). Post-hoc pairwise comparisons indicated that these effects were attributable to all groups and lasted up to 20 min after the stimulation (Fig. 6C, F, I). Next, we analyzed whether these stimulations also attenuate joint inflammation. The next day, the animals underwent the same electrical stimulation used above to induce freezing immobility. Then, the animals were anesthetized and subjected to intra-articular injection of zymosan, and six hours later inflammation was assessed as determined above (Fig. 6B). Aversive stimulation of the PVN, LC, or vIPAG attenuates knee inflammation. For the articular edema, multiple comparison ANOVA tests revealed significant effects of PVN, LC, or vIPAG stimulation on treatment (PVN: $F_{1,28} = 17.76$, $p < 0.0002$; LC: $F_{1,28} = 6.67$, p

< 0.014 ; vIPAG: $F_{1,28} = 13.21$, $p = 0.001$), time (PVN: $F_{1,28} = 134.9$; LC: $F_{1,28} = 190.3$; vIPAG: $F_{1,28} = 131.3$; $p = 0.0001$ for all groups), and interaction between treatment and time (PVN: $F_{1,28} = 8.57$, $p = 0.0067$; LC: $F_{1,28} = 9.94$, $p = 0.0038$; vIPAG: $F_{1,28} = 19.33$, $p = 0.0001$). Post-hoc pairwise analyses indicated that aversive electrical stimulation of these nuclei significantly reduced joint inflammation (Fig. 6D, G, J). Likewise, Student's t -test revealed that aversive electrical stimulation of PVN, LC, or vIPAG also attenuates neutrophilic infiltration into the synovial capsule (PVN: $t_{1,14} = 13.00$, $p = 0.0067$; LC: $t_{1,14} = 13/15$, $p = 0.0038$; vIPAG: $t_{1,14} = 18.09$, $p = 0.0001$ for all groups as compared to control without stimulation) (Fig. 6E, H, K). These results show that central stimulation of the PVN, LC, or vIPAG to induce aversive life-threatening challenges includes neuromodulation of both nociception and inflammation.

Aversive brain stimulation further reduces nociception but not joint inflammation in conscious rats

We next compared whether the aversive life-threatening stimulations could further decrease nociception and inflammation as compared to neutral stimuli. Previous animals' data were placed in either aversive or non-aversive stimulation procedures. Both aversive and non-aversive brain stimulation significantly reduces nociception in the tail-flick tests, as revealed by the multiple comparisons ANOVA on the treatment ($F_{1,30} = 21.35$), time ($F_{8,240} = 313.16$) and there is a significant correlation of treatment and time ($F_{8,240} = 5.02$) ($p = 0.0001$ for all groups as compared to control without stimulation). Post-hoc pairwise analyses indicated that aversive encephalic stimulation was more effective than non-aversive encephalic stimulation in reducing nociception for up to 20 min after the stimulation (Fig. 7A). Likewise, multiple comparisons ANOVA revealed that both aversive and non-aversive encephalic stimulation significantly reduces neutrophilic infiltration ($F_{2,109} = 191.3$, $p = 0.0001$). However, post hoc pairwise analyses showed no significant difference between the aversive and non-aversive-stimulated groups (Fig. 7B). These results show that aversive encephalic stimulation further reduces nociception but not joint inflammation in conscious rats.

Tonic immobility in guinea pigs attenuates inflammation in experimental arthritis

To determine whether these neuronal stimulations during life-threatening conditions can control inflammation and nociception are physiological mechanisms conserved in different species, we studied whether tonic immobility (TI) in guinea pigs controls inflammation to intra-articular zymosan (Fig. 8A). Tonic immobility is a well-known innate defensive behavior that causes immobility and unconditioned fear-induced analgesia in guinea pigs and in other species (e.g., confrontation with predators) (Gallup, 1977; Leite-Panissi et al., 2001; Moskowitz, 2004). In our study, Spearman's correlation tests indicate that tonic immobility significantly reduces both joint inflammation ($r = -0.58$; Fig. 8B) and neutrophil migration ($r = -0.57$; Fig. 8C) in guinea pigs challenged with intra-articular zymosan. Our previous studies showed that 2-min stimulation of peripheral or central neural networks reduces joint inflammation (Bassi et al., 2015, 2017). Thus, we investigated whether 2 min of tonic immobility can reduce knee inflammation in guinea pigs challenged with intra-articular zymosan (Fig. 8D). Considering joint inflammation edema, multiple comparison ANOVA also revealed significant effects of 2 min of tonic immobility on treatment ($F_{2,62} =$

16.82), time ($F_{1,62} = 292.1$) and there was a significant correlation of treatment and time ($F_{2,62} = 19.04$) ($p = 0.0001$ for all groups). Post-hoc pair-wise analyses indicated that these differences occurred between control (no-TI) and TI, and sham-TI and TI groups (Fig. 8E). Likewise, multiple comparison ANOVA revealed significant effects of 2 min of tonic immobility on neutrophilic infiltration ($F_{2,30} = 11.17$, $p = 0.0002$). Post-hoc pairwise analyses showed significant reduction in the TI group (TI = 2 min) when compared to the non-TI group, but this effect was not statistically significant when sham-TI (TI < 2 min) (Fig. 8F).

DISCUSSION

Our present results reveal a new physiological link between neural stimulation and neural control of inflammation and pain that may be harnessed for treating inflammatory disorders such as arthritis. Our results indicate that brain stimulation in non-aversive neutral conditions activates neuromodulatory mechanisms that attenuate both inflammation and pain. When brain stimulation is intensified to produce defensive behavioral responses, similar to that produced in aversive life-threatening conditions such as freezing immobility, it further reduced pain but not inflammation. These results suggest that nociception and inflammation are regulated through different neuromodulatory mechanisms.

Our present results are consistent with previous studies showing that peripheral (Miao et al., 2003; Bassi et al., 2015, 2017; Koopman et al., 2016; Abe et al., 2017) and central stimulation of the PVN or LC decreases joint inflammation in experimental arthritis (Bassi et al., 2017). Our current results concur with both clinical and experimental studies showing that stimulation of the LC (Jones and Gebhart, 1986; Levine et al., 1990; West et al., 1993; Hickey et al., 2014), PVN (Jones and Gebhart, 1989; Yirmiya et al., 1990; Condés-Lara et al., 2008), or vPAG (de Luca et al., 2003; Yang et al., 2007) produces analgesia. The hypothalamus is a diencephalic structure that coordinates homeostatic systems such as the body temperature, thirst, hunger, sleep, autonomic function, and emotional activity, while the LC is a nucleus in the pons involved with physiological responses to stress, panic and anti-nociception induced by aversive stimuli (Levine et al., 1990; West et al., 1993; Hickey et al., 2014). The PAG is a strategic interface between the hypothalamus and LC that integrates behavioral responses to internal (e.g.: pain) and external (e.g.: threat) stressors (Fardin et al., 1984; Bandler and Depaulis, 1991; Carrive, 1993; Millan, 2002). The central position of these brain structures can explain the analgesic and anti-inflammatory effects induced by PVN, LC, or vPAG stimulation. In fact, the PAG is considered an important pain processing center in which electrical and chemical stimulation produces analgesia (Reynolds, 1969; Basbaum and Fields, 1984; Fardin et al., 1984; Bandler and Depaulis, 1991; Behbehani, 1995; Coimbra et al., 2006). Furthermore, the potential of PVN and LC to induce analgesia involves the activation of neural pathways connecting the PAG and the spinal cord (Yang et al., 2007; Condés-Lara et al., 2008; Martínez-Lorenzana et al., 2008). Since the tail-flick response is a spinal reflex, our data concur with Miao et al. (2003) showing that stimulation of pain modulation systems under PAG control reduced bradykinin-induced plasma extravasation through the activation of lower brainstem structures, possibly between the LC and the spinal cord (Miao et al., 2003; Jänig and Green, 2014).

We recently showed that electrical stimulation of the PVN or LC decreases knee inflammation (Bassi et al., 2017), and other groups also showed that stimulation of these nuclei can also reduce nociception (Jones and Gebhart, 1989; Levine et al., 1990; Yirmiya et al., 1990; West et al., 1993; Condés-Lara et al., 2008; Hickey et al., 2014; Jones and Gebhart, 1986). However, our study is the first report showing that LC, PVN, or vIPAG stimulation reduces both inflammation and nociception in the same animals under the same behavioral conditions. Noteworthy, these effects were achieved with low-intensity stimulation that did not induce motor or behavioral alterations. These results are significant for two reasons: first because low-intensity non-invasive stimulation can be clinically used to control pain and inflammation without inducing motor or behavioral alterations. Second, physiological mechanisms such as physical exercise, meditation, psychological interventions or placebo treatments can induce a low-intensity stimulation able to modulate nociception and inflammation (Ulloa and Deitch 2009; Foley and Kirschbaum, 2010; Kaptchuk et al., 2010; Kox et al., 2012, 2014; Scheef et al., 2012; Singleton et al., 2014; Craggs et al., 2014; Middendorp et al., 2015; Sevel et al., 2015; Grech et al., 2016).

Freezing behavior is a reversible innate limbic reaction in response to life-threatening stimuli that includes behavioral motor immobility, to avoid predator detection but it also includes autonomic modifications and neuromodulatory mechanisms to prepare the body for a potential attack (Arduino and Gould, 1984; Leite-Panissi et al., 2001; Korte et al., 2005; Coimbra et al., 2017; Paschoalin-Maurin et al., 2018). It is proposed that tonic immobility can increase the chances of escaping by making the predator believe that its prey is dead (Gallup, 1977; Arduino and Gould, 1984; Korte et al., 2005). On the other hand, the behavioral mechanism also induces strong analgesia, decreases responses to external stimuli and lack of motor activity to prevent reflex movements induced by painful stimuli (e.g., predator attack) (Klemm, 1971; Gallup, 1977; Fanselow and Sigmundi, 1986). Previous studies showed that defensive behaviors and analgesia are elicited by increasing the intensity of the electrical stimulation of the vIPAG, PVN, and LC (Bandler and Depaulis, 1991; Markgraf et al., 1991; Monnikes et al., 1992; Randich and Gebhart, 1992; Carrive, 1993; Ossipov et al., 2010). Higher electrical stimulation of the LC, PVN, or vIPAG induces freezing immobility in rats; it produces greater analgesia than that produced by lower intensity (neutral) stimulation. This higher electrical stimulation also reduces inflammation, but this effect was statistically similar to that produced with lower intensity (neutral) stimulation. This anti-inflammatory effect induced by aversive stimuli in rats was also found by inducing tonic immobility in guinea pigs. Actually, both freezing immobility induced by aversive brain stimulation in rats and tonic immobility in guinea pigs simulate life-threatening conditions with a strong analgesia. Our current results suggest that the control of inflammation is another behavioral expression conserved through different species during life-threatening situations.

Recent studies showed that TI behavior increases the neural activity in the PAG (Vieira et al., 2011) and is dependent on vIPAG mechanisms (Monassi et al., 1994; Leite-Panissi et al., 2001). Thus, both inflammation and pain control are part of the innate behavioral defense repertoire to aversive, life-threatening, stimuli. Noteworthy, TI in animals is related with the gradual activation of different brain structures involved with neurovegetative, motor, and cognitive responses (Carli, 1968; Klemm, 1971; Leite-Panissi et al., 2001; Leite-Panissi and

Menescal-de-Oliveira, 2002; Vieira et al., 2011). Thus, the progressive anti-inflammatory effect induced during TI in guinea pigs can be due to the gradual and cumulative stimulation of different brain areas controlling pain and inflammation, such as the LC, PVN, and PAG.

To date, defensive behaviors like TI are mostly characterized by analgesia, motor immobility (Gallup, 1977; Leite-Panissi et al., 2001; Moskowitz, 2004) and freezing responses (Marks, 1987; Roelofs, 2017). Our present results show that the physiological networks involved in defensive behaviors also modulate the immune system. As constant injuries caused by immobility or escaping strategies can increase cytokine production (as observed in endotoxemia) and impair cognitive and motor performance, we hypothesized that neuronal control of inflammation improves survival during life-threatening situations because inflammatory factors: (a) increase body energy expenditure by injured tissues; (b) redirect blood flow to non-essential organs such as the gastro-intestinal system; (c) increase nociceptive neurons activity (hyperalgesia); and (d) increase the production of cytokines, such as TNF- α and IL-6, that impair cognitive and physical performance (Aubert et al., 1997; Bassi et al., 2011; Dantzer, 2006; Hart, 1988; Elenkov and Chrousos, 2006). Collectively, these mechanisms of neuromodulation are activated to preserve body integrity and to improve survival in life-threatening situations.

CONCLUSION

Neutral stimulation of the PVN, LC or vIPAG attenuates joint inflammation and pain sensitivity. Furthermore, freezing immobility induced by aversive stimulation of these brain structures further increases analgesia, but not inflammation. Similar effects were also observed after inducing tonic immobility in guinea pigs. Our results suggest that defensive behaviors in aversive events include neuromodulatory mechanisms of inflammation and pain conserved through different species to improve survival in life-threatening events.

ACKNOWLEDGEMENTS

The authors want to thank Prof. Dr. Ilia Elenkov for skilful suggestions and revising the manuscript. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) project grants (11/20343-4, 12/04237-2, 13/20549-7 and 13/08216-2). GSB holds a PhD scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (142068/2012-8). AK holds a PD scholarship from CNPq (118636/2017-0).

Abbreviations:

LC	locus coeruleus
EPM	elevated-plus maze
ES	electrical stimulation
IA	inter-aural
i.a	intra-articular
PVN	paraventricular hypothalamic nucleus
TI	tonic immobility

vIPAG	ventrolateral periaqueductal gray matter
VNS	vagus nerve stimulation

REFERENCES

- Abe C, Inoue T, Inglis MA, Viar KE, Huang L, Ye H, Rosin DL, Stornetta RL, et al. (2017) C1 neurons mediate a stress-induced anti-inflammatory reflex in mice. *Nat Neurosci* 20(5):700–707. [PubMed: 28288124]
- Anseloni VZ, Brandão ML (1997) Ethopharmacological analysis of behaviour of rats using variations of the elevated plus-maze. *Behav Pharmacol* 8(6):533–540. [PubMed: 9832967]
- Arduino PJ, Gould JL (1984) Is tonic immobility adaptive? *Animal Behav* 32(3):921–923.
- Aubert A, Goodall G, Dantzer R, Gheusi G (1997) Differential effects of lipopolysaccharide on pup retrieving and nest building in lactating mice. *Brain Behav Immun* 11(2):107–118. [PubMed: 9299060]
- Bandler R, Depaulis A (1991) Midbrain periaqueductal gray control of defensive behavior in the cat and the rat In: Depaulis A, Bandler R, editors. *The midbrain periaqueductal gray matter*. New York: Springer: Plenum Press p. 175–198.
- Bandler R, Shipley MT (1994) Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci* 17(9):379–389. [PubMed: 7817403]
- Basbaum AI, Fields HL (1984) Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Ann Rev Neurosci* 7(1):309–338. [PubMed: 6143527]
- Bassi GS, Brognara F, Castania JA, Talbot J, Cunha TM, Cunha FQ, Ulloa L, Kanashiro A, et al. (2015) Baroreflex activation in conscious rats modulates the joint inflammatory response via sympathetic function. *Brain Behav Immun* 49:140–147. [PubMed: 25986215]
- Bassi GS, Kanashiro A, Santin FM, de Souza GE, Nobre MJ, Coimbra NC (2011) Lipopolysaccharide-induced sickness behaviour evaluated in different models of anxiety and innate fear in rats. *Basic Clin Pharmacol Toxicol* 110(4):359–369. [PubMed: 22059515]
- Bassi GS, Dias DPM, Franchin M, Talbot J, Reis DG, Menezes GB, Castania JA, Garcia-Cairasco N, et al. (2017) Modulation of experimental arthritis by vagal sensory and central brain stimulation. *Brain Behav Immun* 64:330–343. [PubMed: 28392428]
- Behbehani MM (1995) Functional characteristics of the midbrain periaqueductal gray. *Prog Neurobiol* 46(6):575–605. [PubMed: 8545545]
- Boyden SD, Hossain IN, Wohlfahrt A, Lee YC (2016) Non-inflammatory causes of pain in patients with rheumatoid arthritis. *Curr Rheumatol Rep* 18(6):30. [PubMed: 27097817]
- Busch V, Zeman F, Heckel A, Menne F, Ellrich J, Eichhammer P (2013) The effect of transcutaneous vagus nerve stimulation on pain perception – an experimental study. *Brain Stimul* 6 (2):202–209. [PubMed: 22621941]
- Carli G (1968) Depression of somatic reflexes during rabbit hypnosis. *Brain Res* 11(2):453–456. [PubMed: 5701209]
- Carrive P (1993) The periaqueductal gray and defensive behavior: functional representation and neuronal organization. *Behav Brain Res* 58(1–2):27–47. [PubMed: 8136048]
- Castañeda S, Nurmohamed MT, González-Gay MA (2016) Cardiovascular disease in inflammatory rheumatic diseases. *Best Pract Res Clin Rheumatol* 30(5):851–869. [PubMed: 27964792]
- Coimbra NC, De Oliveira R, Freitas RL, Ribeiro SJ, Borelli KG, Pacagnella RC, Moreira JE, da Silva LA, et al. (2006) Neuroanatomical approaches of the tectum-reticular pathways and immunohistochemical evidence for serotonin-positive perikarya on neuronal substrates of the superior colliculus and periaqueductal gray matter involved in the elaboration of the defensive behavior and fear-induced analgesia. *Exp Neurol* 197 (1):93–112. [PubMed: 16303128]
- Coimbra NC, Calvo F, Almada RC, Freitas RL, Paschoalin-Maurin T, Dos Anjos-Garcia T, Elias-Filho DH, Ubiali WA, et al. (2017) Opioid neurotransmission modulates defensive behavior and fear-induced antinociception in dangerous environments. *Neuroscience* 354:178–195. [PubMed: 28457818]

- Condés-Lara M, Martínez-Lorenzana G, Rodríguez-Jiménez J, Rojas-Piloni G (2008) Paraventricular hypothalamic nucleus stimulation modulates nociceptive responses in dorsal horn wide dynamic range neurons. *Neurosci Lett* 444(2):199–202. [PubMed: 18721859]
- Craggs JG, Price DD, Robinson ME (2014) Enhancing the placebo response: functional magnetic resonance imaging evidence of memory and semantic processing in placebo analgesia. *J Pain* 15(4):435–446. [PubMed: 24412799]
- D'Amour FE, Smith DL (1941) A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72(1):74–79.
- Dantzer R (2006) Cytokines, stress, and depression. *Neurol Clin* 24(3):441–460. [PubMed: 16877117]
- Elenkov IJ, Chrousos GP (2006) Stress system – organization. *Physiol Immunoregulation Neuroimmunomodulat* 13(5–6):257–267.
- Fanselow MS, Sigmundi RA (1986) Species-specific danger signals, endogenous opioid analgesia, and defensive behavior. *J Exp Psychol: Anim Behav Proc* 12(3):301–309.
- Fardin V, Oliveras JL, Besson JM (1984) A reinvestigation of the analgesic effects induced by stimulation of the periaqueductal gray matter in the rat. I. The production of behavioral side effects together with analgesia. *Brain Res* 306(1–2):105–123. [PubMed: 6540613]
- Favalli EG, Desiati F, Atzeni F, Sarzi-Puttini P, Caporali R, Pallavicini FB, Gorla R, Filippini M, et al. (2009) Serious infections during anti-TNF treatment in rheumatoid arthritis patients. *Autoimmun Rev* 8(3):266–273. [PubMed: 19022409]
- File ES, Lippa AS, Beer B, Lippa MT (2001) Animal tests of anxiety. *Curr Prot Neurosci* 26(1):8.3.1–8.3.22.
- Foley P, Kirschbaum C (2010) Human hypothalamus–pituitary–adrenal axis responses to acute psychosocial stress in laboratory settings. *Neurosci Biobehav Rev* 35(1):91–96. [PubMed: 20109491]
- Fuchs SAG, Edinger HM, Siegel A (1985) The organization of the hypothalamic pathways mediating affective defense behavior in the cat. *Brain Res* 330(1):77–92. [PubMed: 4039213]
- Gallup GG (1977) Tonic immobility: the role of fear and predation. *Psychol Rec* 27(S1):41–61.
- Gegout P, Gillet P, Chevrier D, Guingamp C, Terlain B, Netter P (1994) Characterization of zymosan-induced arthritis in the rat: effects on joint inflammation and cartilage metabolism. *Life Sci* 55(17):PL321–PL326. [PubMed: 7934634]
- Grech P, Li Z, Morcillo P, Kalyoussef E, Kim DD, Bekker A, Ulloa L (2016) Intraoperative low-frequency electroacupuncture under general anesthesia improves postoperative recovery in a randomized trial. *J Acup Merid Stud* 9(5):234–241.
- Hart BL (1988) Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev* 12(2):123–137. [PubMed: 3050629]
- Hewlett S, Sanderson T, May J, Alten R, Bingham CO, 3rd, Cross M, March L, Pohl C, et al. (2011) “I’m hurting, I want to kill myself”: rheumatoid arthritis flare is more than a high joint count—an international patient perspective on flare where medical help is sought. *Rheumatology* 51(1):69–76. [PubMed: 21565901]
- Hickey L, Li Y, Fyson SJ, Watson TC, Perrins R, Hewinson J, Teschemacher AG, Furue H, et al. (2014) Optoactivation of locus ceruleus neurons evokes bidirectional changes in thermal nociception in rats. *J Neurosci* 34(12):4148–4160. [PubMed: 24647936]
- Inanc N, Direskeneli H (2006) Serious infections under treatment with TNF- antagonists compared to traditional DMARDs in patients with rheumatoid arthritis. *Rheumatol Int* 27(1):67–71. [PubMed: 16896990]
- Inui K, Koike T (2016) Combination therapy with biologic agents in rheumatic diseases: current and future prospects. *Ther Adv Musculoskelet Dis* 8(5):192–202. [PubMed: 27721905]
- Jänig W, Green PG (2014) Acute inflammation in the joint: its control by the sympathetic nervous system and by neuroendocrine systems. *Auton Neurosci* 182:42–54. [PubMed: 24530113]
- Jones SL, Gebhart GF (1989) Increase in sympathetic outflow by paraventricular nucleus stimulation in awake rats. *Am J Physiol-Reg, Int Comp Physiol* 256(6):R1325–R1330.
- Jones SL, Gebhart GF (1986) Quantitative characterization of ceruleospinal inhibition of nociceptive transmission in the rat. *J Neurophysiol* 56(5):1397–1410. [PubMed: 3025380]

- Kannan H, Hayashida Y, Yamashita H (1986) Quantitative characterization of ceruleospinal inhibition of nociceptive transmission in the rat. *J Neurophysiol* 56(5):1397–1410. [PubMed: 3025380]
- Kapchuk TJ, Friedlander E, Kelley JM, Sanchez MN, Kokkotou E, Singer JP, Kowalczykowski M, Miller FG, et al. (2010) Placebos without deception: a randomized controlled trial in irritable bowel syndrome. *PLoS ONE* 5(12):e15591. [PubMed: 21203519]
- Keystone EC, Schorlemmer HU, Pope C, Allison AC (1977) Zymosan-induced arthritis: a model of chronic proliferative arthritis following activation of the alternative pathway of complement. *Arthritis Rheum* 20(7):1396–1401. [PubMed: 911357]
- Klemm W (1971) Neurophysiologic studies of the immobility reflex (“animal hypnosis”). *Neurosci Res* 4(165):165–212. [PubMed: 4948604]
- Koopman FA, Chavan SS, Miljko S, Grazio S, Sokolovic S, Schuurman PR, Mehta AD, Levine YA, et al. (2016) Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis. *Proc Natl Acad Sci USA* 113(29):8284–8289. [PubMed: 27382171]
- Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci Biobehav Rev* 29(1):3–38. [PubMed: 15652252]
- Kox M, Stoffels M, Smeekens SP, van Alfen N, Gomes M, Eijssvogels TM, Hopman MT, van der Hoeven JG, et al. (2012) The influence of concentration/meditation on autonomic nervous system activity and the innate immune response. *Psychosom Med* 74 (5):489–494. [PubMed: 22685240]
- Kox M, van Eijk LT, Zwaag J, van den Wildenberg J, Sweep FC, van der Hoeven JG, Pickkers P (2014) Voluntary activation of the sympathetic nervous system and attenuation of the innate immune response in humans. *Proc Natl Acad Sci USA* 111 (20):7379–7384. [PubMed: 24799686]
- LeDoux J (2012) Rethinking the emotional brain. *Neuron* 73 (4):653–676. [PubMed: 22365542]
- Leite-Panissi CR, Rodrigues CL, Brentegani MR, Menescal-De-Oliveira L (2001) Endogenous opiate analgesia induced by tonic immobility in guinea pigs. *Braz J Med Biol Res* 34(2):245–250. [PubMed: 11175501]
- Leite-Panissi CR, Menescal-de-Oliveira L (2002) Central nucleus of the amygdala and the control of tonic immobility in guinea pigs. *Brain Res Bull* 58(1):13–19. [PubMed: 12121807]
- Levine ES, Litto WJ, Jacobs BL (1990) Activity of cat locus coeruleus noradrenergic neurons during the defense reaction. *Brain Res* 531(1–2):189–195. [PubMed: 2289120]
- de Luca MC, Brandão ML, Motta VA, Landeira-Fernandez J (2003) Antinociception induced by stimulation of ventrolateral periaqueductal gray at the freezing threshold is regulated by opioid and 5-HT_{2A} receptors as assessed by the tail-flick and formalin tests. *Pharmacol Biochem Behav* 75(2):459–466. [PubMed: 12873638]
- Markgraf CG, Winters RW, Liskowsky DR, McCabe PM, Green EJ, Schneiderman N (1991) Hypothalamic, midbrain and bulbar areas involved in the defense reaction in rabbits. *Physiol Behav* 49 (3):493–500. [PubMed: 2062925]
- Marks I (1987). *Fears, phobias, and rituals: panic, anxiety, and their disorders* (Marks I, ed), Oxford University Press.
- Martínez-Lorenzana G, Espinosa-López L, Carranza M, Aramburo C, Paz-Tres C, Rojas-Piloni G, Condés-Lara M (2008) PVN electrical stimulation prolongs withdrawal latencies and releases oxytocin in cerebrospinal fluid, plasma, and spinal cord tissue in intact and neuropathic rats. *Pain* 140(2):265–273. [PubMed: 18823708]
- Mayer DJ, Liebeskind JC (1974) Pain reduction by focal electrical stimulation of the brain: an anatomical and behavioral analysis. *Brain Res* 68(1):73–93. [PubMed: 4549764]
- van Middendorp H, Kox M, Pickkers P, Evers AW (2015) The role of outcome expectancies for a training program consisting of meditation, breathing exercises, and cold exposure on the response to endotoxin administration: a proof-of-principle study. *Clin Rheumatol* 35(4):1081–1085. [PubMed: 26194270]
- Miao FJ, Jänig W, Jasmin L, Levine JD (2003) Blockade of nociceptive inhibition of plasma extravasation by opioid stimulation of the periaqueductal gray and its interaction with vagus-induced inhibition in the rat. *Neuroscience* 119(3):875–885. [PubMed: 12809707]
- Millan MJ (2002) Descending control of pain. *Prog Neurobiol* 66 (6):355–474. [PubMed: 12034378]

- Monassi CR, Hoffmann A, Menescal-de-Oliveira L (1994) Participation of the periaqueductal gray matter in the modulation of tonic immobility in the guinea pig. *Braz J Med Biol Res* 27 (5):1243–1248. [PubMed: 8000346]
- Mönnikes H, Heymann-Mönnikes I, Taché Y (1992) CRF in the paraventricular nucleus of the hypothalamus induces dose-related behavioral profile in rats. *Brain Res* 574(1–2):70–76. [PubMed: 1638411]
- Moskowitz AK (2004) Scared Stiff: catatonia as an evolutionary-based fear response. *Psychol Rev* 111(4):984–1002. [PubMed: 15482070]
- Motta SC, Goto M, Gouveia FV, Baldo MV, Canteras NS, Swanson LW (2009) Dissecting the brain's fear system reveals the hypothalamus is critical for responding in subordinate conspecific intruders. *Proc Natl Acad Sci USA* 106 (12):4870–4875. [PubMed: 19273843]
- Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ (2012) Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol Rev* 248(1):188–204. [PubMed: 22725962]
- Ossipov MH, Dussor GO, Porreca F (2010) Central modulation of pain. *J Clin Invest* 120(11):3779–3787. [PubMed: 21041960]
- Panksepp J (1971) Aggression elicited by electrical stimulation of the hypothalamus in albino rats. *Physiol Behav* 6(4):321–329. [PubMed: 4948212]
- Paschoalin-Maurin T, Dos Anjos-Garcia T, Falconi-Sobrinho LL, de Freitas RL, Coimbra JPC, Laure CJ, Coimbra NC (2018) The rodent-versus-wild snake paradigm as a model for studying anxiety- and panic-like behaviors: face, construct and predictive validities. *Neuroscience* 369:336–349. [PubMed: 29183829]
- Paxinos G, Watson C (2007), *The rat brain in stereotaxic coordinates* (Paxinos G, Watson C, eds), Amsterdam Boston: Elsevier.
- Ramiro S, Radner H, van der Heijde D, van Tubergen A, Buchbinder R, Aletaha D, Landewé RB (2011) Combination therapy for pain management in inflammatory arthritis (rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, other spondyloarthritis). *Cochrane Database Syst Rev* 10 CD008886.
- Randich A, Gebhart GF (1992) Vagal afferent modulation of nociception. *Brain Res Rev* 17(2):77–99. [PubMed: 1327371]
- Ren K, Randich A, Gebhart GF (1988) Vagal afferent modulation of a nociceptive reflex in rats: involvement of spinal opioid and monoamine receptors. *Brain Res* 446(2):285–294. [PubMed: 2836031]
- Reynolds DV (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science* 164(3878): 444–445. [PubMed: 4887743]
- Roelofs K (2017) Freeze for action: neurobiological mechanisms in animal and human freezing. *Philos T R Soc B: Biol Sci* 372 (1718):20160206.
- Sandberg DE, Segal M (1978) Pharmacological analysis of analgesia and self-stimulation elicited by electrical stimulation of catecholamine nuclei in the rat brain. *Brain Res* 152(3):529–542. [PubMed: 210879]
- Scheef L, Jankowski J, Daamen M, Weyer G, Klingenberg M, Renner J, Mueckter S, Schürmann B, et al. (2012) An fMRI study on the acute effects of exercise on pain processing in trained athletes. *Pain* 153(8):1702–1714. [PubMed: 22704853]
- Sevel LS, Craggs JG, Price DD, Staud R, Robinson ME (2015) Placebo analgesia enhances descending pain-related effective connectivity: a dynamic causal modeling study of endogenous pain modulation. *J Pain* 16(8):760–768. [PubMed: 26022539]
- Singleton O, Hölzel BK, Vangel M, Brach N, Carmody J, Lazar SW (2014) Change in brainstem gray matter concentration following a mindfulness-based intervention is correlated with improvement in psychological well-being. *Front Hum Neurosci* 8:33. [PubMed: 24600370]
- Torres-Rosas R, Yehia G, Peña G, Mishra P, del Rocio Thomps-Bonilla M, Moreno-Eutimio MA, Arriaga-Pizano LA, Isibasi A, et al. (2014) Dopamine mediates vagal modulation of the immune system by electroacupuncture. *Nat Med* 20(3):291–295. [PubMed: 24562381]
- Ulloa L, Quiroz-Gonzalez S, Torres-Rosas R (2017) Nerve stimulation: immunomodulation and control of inflammation. *Trends Mol Med* 12:1103–1120.

- Ulloa L, Deitch EA (2009) Neuroimmune perspectives in sepsis. *Crit Care* 13(2):133. [PubMed: 19439052]
- Ullah F, dos Anjos-Garcia T, dos Santos IR, Biagioni AF, Coimbra NC (2015) Relevance of dorsomedial hypothalamus, dorsomedial division of the ventromedial hypothalamus and the dorsal periaqueductal gray matter in the organization of freezing or oriented and non-oriented escape emotional behaviors. *Behav Brain Res* 293:143–152. [PubMed: 26205826]
- Ullah F, Dos Anjos-Garcia T, Mendes-Gomes J, Elias-Filho DH, Falconi-Sobrinho LL, Freitas RL, Khan AU, Oliveira R, et al. (2017) Connexions between the dorsomedial division of the ventromedial hypothalamus and the dorsal periaqueductal grey matter are critical in the elaboration of hypothalamically mediated panic-like behaviour. *Behav Brain Res* 319:135–147. [PubMed: 27856260]
- Upchurch KS, Kay J (2012) Evolution of treatment for rheumatoid arthritis. *Rheumatology suppl* 51(6):vi28–vi36.
- Vieira EB, Menescal-de-Oliveira L, Leite-Panissi CR (2011) Functional mapping of the periaqueductal gray matter involved in organizing tonic immobility behavior in guinea pigs. *Behav Brain Res* 216(1):94–99. [PubMed: 20654655]
- West WL, Yeomans DC, Proudfit HK (1993) The function of noradrenergic neurons in mediating antinociception induced by electrical stimulation of the locus coeruleus in two different sources of Sprague-Dawley rats. *Brain Res* 626(1–2):127–135. [PubMed: 7904225]
- Yang J, Yang Y, Xu HT, Chen JM, Liu WY, Lin BC (2007) Arginine vasopressin induces periaqueductal gray release of enkephalin and endorphin relating to pain modulation in the rat. *Reg Peptides* 142(1–2):29–36.
- Yardley CP, Hilton SM (1986) The hypothalamic and brainstem areas from which the cardiovascular and behavioural components of the defence reaction are elicited in the rat. *J Auton Nerv Syst* 15(3):227–244. [PubMed: 3958439]
- Yirmiya R, Ben-Eliyahu S, Shavit Y, Marek P, Liebeskind JC (1990) Stimulation of the hypothalamic paraventricular nucleus produces analgesia not mediated by vasopressin or endogenous opioids. *Brain Res* 537(1–2):169–174. [PubMed: 1982239]
- Zimmerman M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16(2):109–110. [PubMed: 6877845]

Fig. 1.

A schematic representation of histologically confirmed sites of electrical stimulation performed inside (circles) or outside (triangles) the (A–C) paraventricular hypothalamic nucleus (PVN), (D–F) the locus coeruleus (LC), or (G–I) the ventrolateral column of the periaqueductal gray matter (vIPAG) depicted in modified anagrams of transverse sections of Wistar rats brain, according to Paxinos and Watson's atlas (2007). (J) Time course of the experiments in animals that underwent non-aversive electrical stimulation (ES) of the above-mentioned areas. IA: inter-aural.

Fig. 2.

PVN stimulation (20 Hz, 0.5 ms, 50 μ A) on rat behavior and joint inflammation. (A) Time course of tail-flick latencies during each 5-min period across the baseline period (–10, –5, and 0 min) and up to 30 min after electrical stimulation of the PVN; Mean (+ SEM) number of entries into the (B) open and (C) closed arms of the elevated plus maze, (D) percentage of time spent in the open arms, and (E) risk assessment behaviors in rats immediately after the stimulation of the PVN; (F) Joint diameter and (G) neutrophilic infiltration into the knee synovia 6 h after PVN stimulation followed by intra-articular zymosan. Data are expressed

as mean $\pm$ S.E.M. Tail-flick test and joint edema were analyzed through repeated measures ANOVA with Bonferroni's post hoc test. Elevated plus-maze test and neutrophilic infiltration were analyzed through a one-way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ from the control group. The sample size is shown at the bottom of the graph.

Fig. 3.

LC stimulation (20 Hz, 1 ms, 100 μ A) on rat behavior and joint inflammation. (A) Time course of tail-flick latencies during each 5-min period across the baseline period (–10, –5, and 0 min) and up to 30 min after electrical stimulation of the LC; Mean (+ SEM) number of entries into the (B) open and (C) closed arms of the elevated plus maze, (D) percentage of time spent in the open arms, and (E) risk assessment behaviors in rats immediately after the stimulation of the LC; (F) Joint diameter and (G) neutrophilic infiltration into the knee synovia 6 h after LC stimulation followed by intra-articular zymosan. Data are expressed as

mean $\pm$ S.E.M. *** $p < 0.001$ from the control group. Data are expressed as mean $\pm$ S.E.M. Tail-flick test and joint edema were analyzed through repeated measures ANOVA followed by Bonferroni's post hoc test. Elevated plus-maze test and neutrophilic infiltration were analyzed through a one-way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ from the control group. The sample size is shown at the bottom of the graph.

Fig. 4.

IPAG stimulation (20 Hz, 1 ms, 50 μ A) on rat behavior and joint inflammation. (A) Time course of tail-flick latencies during each 5-min period across the baseline period (–10, –5, and 0 min) and up to 30 min after electrical stimulation of the vIPAG; Mean (+ SEM) number of entries into the (B) open and (C) closed arms of the elevated plus maze, (D) percentage of time spent in the open arms, and (E) risk assessment behaviors in rats immediately after the stimulation of the vIPAG; (F) Joint diameter and (G) neutrophilic infiltration into the knee synovia 6 h after vIPAG stimulation followed by intra-articular

zymosan. Data are expressed as mean $\pm$ S.E.M. Tail-flick test and joint edema were analyzed though repeated measures ANOVA followed by Bonferroni's post hoc test. Elevated plus maze test and neutrophilic infiltration were analyzed through a one-way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ from the control group. The sample size is shown at the bottom of the graph.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

Analysis of the pain response and inflammatory parameters among the PVN-, LC-, and vIPAG-stimulated (ES) groups. Comparison of the (A) time course of tail-flick latencies, (B) joint edema and (C) neutrophilic infiltration into the knee synovia in animals that underwent electrical stimulation of the PVN, LC, or vIPAG. The control group is the sum of all control animals from PVN, LC, and vIPAG groups. Data are expressed as mean $\pm$ S.E.M. Tail-flick test and joint edema were analyzed through repeated measures ANOVA followed by Bonferroni's post hoc test. Neutrophilic infiltration was analyzed through a one-way

ANOVA followed by Tukey's multiple comparison test. ***, ###, \$\$\$: $p < 0.001$. *: Compared between PVN, LC, and vIPAG groups and the control group. #: Compared between LC and PVN groups. \$: Compared between LC and vIPAG groups. The sample size is shown at the bottom of the graph.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6.

Aversive stimulation of the PVN, LC or vIPAG on rat behavior and joint inflammation. (A) Freezing threshold (μA) produced by the electrical stimulation of the PVN, LC, or vIPAG. The sample size is shown at the bottom of the graph. (B) Time course of the experiments carried in animals that underwent aversive electrical stimulation (ES) of the above mentioned areas. Time course of tail-flick latencies during each 5-min period across the baseline period (-10 , -5 , and 0 min) and up to 30 min after aversive electrical stimulation (arrow) of the PVN, LC, or vIPAG in rats (C, F, I). Aversive stimulation of the PVN, LC, or

vIPAG decreases joint edema (D, G, J) and neutrophilic infiltration into knee synovia (E, H, K). Data are expressed as mean $\pm$ SEM. *** $p < 0.001$. Tail-flick test and joint edema were analyzed through repeated measures ANOVA followed by Bonferroni's post hoc test. Neutrophilic infiltration was analyzed with a one-way ANOVA followed by Tukey's multiple comparison test.

Fig. 7.

Analysis of the pain response (A) and neutrophilic infiltration (B) between animals that underwent aversive (freezing) or non-aversive (non-freezing) stimulation (ES) groups. Data are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Tail-flick test was analyzed through repeated measures ANOVA followed by Bonferroni's post hoc test. Neutrophilic infiltration was analyzed through a one-way ANOVA followed by Tukey's multiple comparison test. The sample size is shown at the bottom of the graph.

Fig. 8. Time course of the experiments carried in guinea pigs that underwent tonic immobility (A). Spearman's correlation coefficient between joint edema and tonic immobility (TI) time and (B) joint neutrophil infiltration and TI time in guinea pigs subjected to TI ($n = 12$ for each group). (C) TI time considered for TI-sham (below 2 min) and TI groups (above 2 min) (D). TI response induced in guinea pigs decreased joint diameter and (E) inhibited neutrophil migration (F) in the articular synovial fluid. Relationship between neutrophil migration and tonic immobility time were examined by Spearman's rank-order correlation. Experimental

score and joint diameter data were analyzed using a two-way ANOVA followed by Bonferroni's multiple comparison test comparing individual time point. Neutrophilic infiltration data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed as mean + SEM (E) and $\pm$ SEM (CD). $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The sample size is shown at the bottom of the graph.