Electrical stimulation of the posterior insular cortex induces opioid and cannabinoid-dependent antinociception and regulates glial cells in the spinal cord

A estimulação elétrica do córtex insular posterior induz antinocicepção opioide e canabinoide dependente e regula células da glia na medula espinal

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ABSTRACT

BACKGROUND AND OBJECTIVES: Half of neuropathic pain patients still end up failing clinical treatments. Electrical stimulation of the posterior insular cortex (ESI) modulates sensory and nociceptive circuits. This study evaluated the effects of a range of frequencies of ESI proposed to improve neuropathic pain.

METHODS: Male Sprague Dawley rats, 280-340 g, submitted to the chronic constriction of the right sciatic nerve were tested for mechanical sensitivity using the paw pressure and von Frey filaments tests, and for thermal sensitivity using the hot plate test. The rats were submitted to ESI 10, 60 or 100 Hz (one, five or seven ESI, 15 min, 210 µs, 1V), applied to the posterior insular cortex, and were evaluated in the tests before and after ESI, or in follow-up of 48, 72 and 168h. The open field evaluated general activity after ESI 5. The involvment of opioid and cannabinoid testes were evaluated through treatment with naloxone and SR1416A - antagonist and inverse agonist/antagonist of the receptors, respectively, after ESI 5, while activation of astrocytes, marked by glial fibrillary acid protein (GFAP), and of microglia, marked by IBA-1 (glial marker), in the spinal cord evaluated by immunohistochemistry.

RESULTS: Data demonstrate that 10, 60, and 100 Hz ESIs modulate mechanical and thermal sensitivity. ESI 5 increased immunoreactivity of GFAP in the spinal cord, without altering IBA-1 (glial marker). Naloxone and SR141716A reversed the antinociception of 60 Hz ESI 5. 60 Hz ESI 7 induced antinociception up to 72h.

CONCLUSION: 60 Hz ESI induces opioid and cannabinoid-dependent antinociception and regulates glia.

Keywords: Chronic pain, Electric stimulation, Neuroglia.

RESUMO

JUSTIFICATIVA E OBJETIVOS: Metade dos pacientes com dor neuropática são refratários aos tratamentos. A estimulação elétrica do córtex insular (EECI) posterior modula circuitos sensoriais e nociceptivos. Assim, este estudo avaliou os efeitos de uma faixa de frequências de EECI como tratamento em modelo animal de dor neuropática.

MÉTODOS: Ratos machos, Sprague Dawley, 280-340 g, submetidos a cirurgia para indução de constrição crônica (ICC) do nervo isquiático direito, foram avaliados em relação à sensibilidade mecânica com a utilização do teste de pressão de pata e de filamentos de von Frey, e sensibilidade térmica usando o teste de placa quente. Os ratos foram submetidos a EECI de 10, 60 ou 100 Hz (uma, cinco ou sete EECI, 15 min, 210 µs, 1V), aplicada ao córtex insular posterior esquerdo, e avaliados nos testes antes e após EECI, ou em *follow up* de 48, 72 e 168 horas. Por meio do teste de campo aberto, avaliou-se a atividade geral após a EECI 5. O envolvimento de receptores opioides e canabinoides foi avaliado por meio da administração de naloxona e SR141716A - antagonista e agonista/antagonista inverso dos receptores, respectivamente - após a EECI 5, enquanto a ativação de astrócitos - marcada por proteína ácida fibrilar glial (GFAP), e de micróglia - marcada por IBA-1 - na medula espinal foi avaliada por imuno-histoquímica.

RESULTADOS: Os dados mostraram que EECI em 10, 60 e 100 Hz modulam a sensibilidade mecânica e térmica dos ani-

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HIGHLIGHTS

- 60 Hz-delivered ESI was the best analgesic protocol for the insular stimulation.
- Data showed a prolonged analgesic effect up to 72h after repetitive ESI.
- ESI regulates glia activation in pain modulatory system.

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mais. A EECI 5 aumentou a imunorreatividade de GFAP na medula espinhal, sem alterar IBA-1 (marcador glial). Naloxona e SR141716A reverteram a antinocicepção produzida por EECI 5 de 60 Hz. EECI 7 de 60 Hz induziu antinocicepção por até 72 horas.
CONCLUSÃO: A EECI 60 Hz produz antinocicepção dependente de opioides e canabinoides e regula a glia.
Descritores: Dor crônica, Estimulação elétrica, Neuroglia.

INTRODUCTION

The insular cortex is a pain-matrix center[1] comprised of anterior and posterior subdivisions that modulate both sensory and emotional aspects of pain[2]. Though effective, pharmacological treatments often do not completely alleviate painful symptoms in chronic neuropathic conditions. In addition, some of these therapies lead to adverse effects and treatment discontinuation[3,4]. Different modalities of both invasive and non-invasive electrical stimulation targeting the insula have been proposed, exerting analgesic effects by functionally influencing either the insular cortex or associated pathways[5,6]. Previous reports[7,8] described protocols using single or repetitive electrical stimulation at 60 Hz in the insular cortex (ESI) to induce antinociception in experimental neuropathic pain-rats.

Projections from the insular cortex to the dorsolateral and ventrolateral periaqueductal gray (PAG) and the pain-related dorsal horn spinal cord laminae[9,10] have been proposed to modulate nociceptive input. In the dorsal horn, opioidergic and cannabinoidergic signaling promotes antinociception[11]. Neuroplastic changes in this region have been reported in chronic pain conditions, where glial-derived processes of astrocytes and microglia contribute to the development and chronification of pathological pain processes[12,13]. Glial activation of astrocytes (i.e., reactive astrogliosis) and microglial responses in the descending pain pathway are considered an early mechanism associated with the maintenance of chronic neuropathic pain[14-17].

From there this study hypothesized that diverse ESI protocols based on a range of stimulation frequencies from low frequency, 10 Hz; to high frequency, 60 Hz and 100 Hz result in different efficacies on the control of neuropathic pain symptoms, as well as are capable to influence paramount aspects of the endogenous pain modulatory signaling and glial activation, which may constitute powerful regulatory targets to improve neurostimulation efficacy and the patient's chronic pain treatment. This is of substantial importance due to the great number of chronic neuropathic pain patients while no currently available treatment reaches completely 100% effectiveness on producing analgesia. The study used the chronic constriction of the sciatic nerve (CCI), a standard model in literature to induce neuropathic pain, to assess mechanical and thermal antinociceptive effects of different frequencies of repetitive ESI and its long-term duration. The study also investigated the involvement of opioid and cannabinoid type 1 receptors, besides their effects over astrocytes and microglia activation in the spinal cord of neuropathic pain rats.

METHODS

Male Sprague Dawley rats (280-340 g) obtained from the Rats Production Center (Institute of Biomedical Sciences - Animal Facility Network of University of São Paulo) were used. The study was approved by the Ethics Committee on Animal Experimentation of University of São Paulo (CEUA No 5874130618; No 3447131117) and followed the guidelines of the Institutional Animal Care and Use Committee. All protocols were conducted blinded for the same researcher and animals randomly distributed in the groups. In this study, male rats were chosen as the main animal model because of the frequent use throughout the neuromodulation literature, allowing reproducibility of protocols that supported the hypothesis. Furthermore, the use of male rats intended to standardize the best frequency-based protocol of ESI treatment that resulted in analgesia, excluding differences in pain responses that could be observed according to the female rat estrous cycle[18]. This work was elaborated according to the ARRIVE guidelines[19].

Experimental design

Animals were habituated to the apparatuses (except for the open field) one day before the experiments to reduce stress[20]. Baseline measurements were evaluated before any animal manipulation. The mechanical sensitivity was assessed through the paw pressure and von Frey filaments tests, and thermal sensitivity through the hot plate test. Animals were submitted to the CCI in the right hind paw. Seven days later, an electrode was implanted in the insula as better described in "Electrode implantation and electrical stimulation of insular cortex (ESI)" section below[8].

Fourteen days after CCI, animals were submitted to either a single electrical stimulation of the insular cortex (ESI 1) or ESI repeated 5 times (ESI 5) performed at 10, 60 or 100 Hz. Animal groups: (A) 10 Hz ESI (n = 8), (B) 60 Hz ESI (n = 8), (C) 100 Hz ESI (n = 5); and Sham (n = 4 to 5). 60 Hz ESI (n=7) was also given for 7 days (ESI 7). Behavioral evaluations were performed before any manipulations (baseline), prior (Pre) and after (Post) ESI.

Naloxone or SR141716A (animal groups: ESI+Sal n= 6; ESI+NLX n= 5; ESI+DMSO n= 4; ESI+ SR141716 n= 4) were administered before ESI 5 and animals were re-evaluated in the paw pressure test 30 min and 40 min later, respectively. To study long-lasting antinociception, animals were evaluated 48h, 72h and 168h after ESI 7 (Figure 1). Fixed samples of spinal cord were collected after ESI 5 to analyze glial fibrillary acidic protein (GFAP)-marked astrocyte activation and IBA-1-marked microglia activation using immunohistochemistry assays. Researchers who performed all experiments and surgeries were blinded to the stimulation frequency.

Figure 1 illustrates, in days and in hours, post ESI 7, the behavior assessment, CCI surgery, electrode implanting, ESI days of stimulation and pain-like behavior follow up of the protocol applied in this work. Black arrows: ESI stimulation; dark gray arrows: behavior assessment of thermal and/or mechanical sensi-

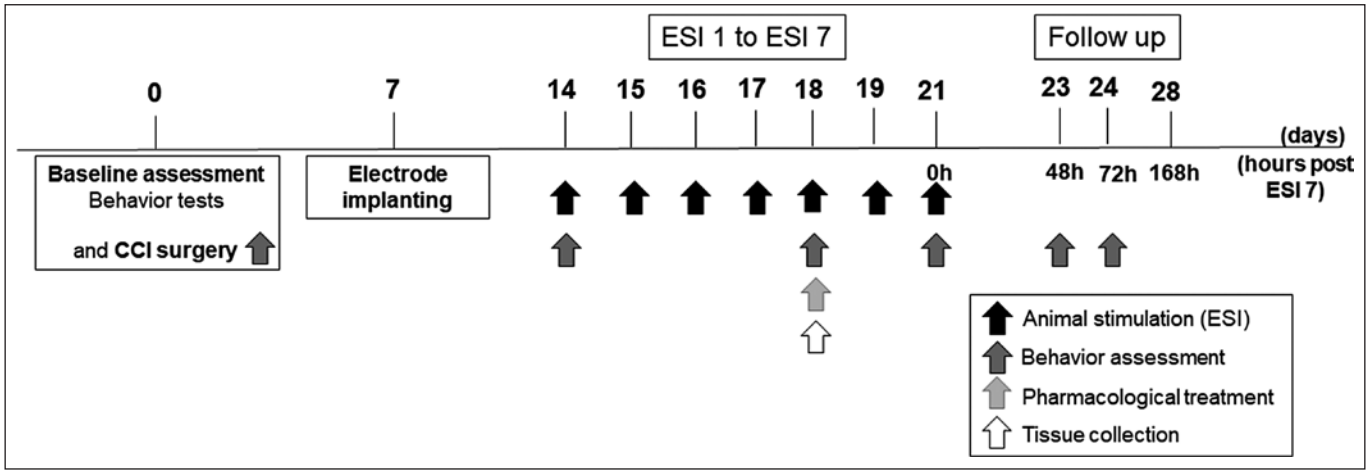

Figure 1. Experimental design for single and repetitive ESI

tivity and/or general activity; light gray arrows: pharmacological treatment with naloxone or SR141716; white arrows: fixed sample collection.

Chronic constriction injury (CCI)

CCI was performed according the authors[21]. Under 2.5% isoflurane (Isoforine™, Cristália, São Paulo, SP, Brazil), four ligatures were loosely tied around the right sciatic nerve with 5.0 chromic catgut (Ethicon™, Raritan, Nova Jersey, USA). The incision was sutured with 4.0 silk suture wire and animals were monitored during post-surgical recovery.

Electrode implantation and electrical stimulation of insular cortex (ESI)

A concentric electrode (D039035408 Spes Medica™, Genova, GE, Italy) was implanted in the left posterior agranular insula, as previously described by the authors[8]. Rats received pre-anesthesia with acepromazine (2.75 mg/kg, Acepram™, Vetnil, Louveira, SP, Brazil) via intraperitoneal, and anesthesia with ketamine hydrochloride (82 mg/kg, Dopalen™, Paulínia, SP, Brazil, Sespo) intraperitoneal, xylazine hydrochloride (7.5 mg/kg, Anasedan™, Paulínia, SP, Brazil, Sespo) via intraperitoneal and local anesthesia (3% mepivacaine hydrochloride,100 µL/animal, MEPISV™, DFL, Taquara, RJ, Brazil).

The electrode was placed according to -1.0 AP; +5.8 ML; -7.1 DV. Coordinates of electrode positioning were the same as previously described by the authors[7]. Sprague-Dawley rats were based on the coordinates of The Rat Brain Atlas[22]. As some minimal variations may exist between surgeries, electrode placement was continually verified through random selection of implanted animals to analyze the slices containing electrode placement.

Positioning in the insular cortex was observed in histological sections and verified under a stereoscope microscope magnifier (DI 724, DIGILAB) and coordinates were compared as to the bregma positioning with the coordinates of The Rat Brain Atlas[22], based on previously published data by the study[7]. The present research group used histological sections of the left posterior insular cortex submitted to the Nissl staining to confirm the electrode positioning compared to the atlas coordinates

Animals received amoxicillin trihydrate (100 mg/kg subcutaneously, Agemoxi L.A., Agener União™, São Paulo, SP, Brazil) and were monitored for 7 days. ESI was performed in awake, freely moving animals, with Medtronic™ Model 3625 Test Stimulator (Dublin, Ireland), through adapted connecting cables (10000NNHF3 Spes Medica™, Genova, GE, Italy)[7,8], according to the parameters: one, five or seven ESI sessions, 15 min per session, at 10, 60, or 100 Hz, 210 µs, 1V. Sham animals were implanted with electrodes but were not given stimulation. Behavioral assessments were conducted right after ending the 15min and with the animals still under stimulation (Figure 1).

Behavioral assessments

Paw pressure test

The test was performed as described by Randall and Selitto (1957). A force with increasing magnitude (16 g/s) was applied to the right hind paw with a pressure apparatus (Insight™, Ribeirão Preto, São Paulo, Brazil). Mechanical sensitivity was evaluated as the force required to induce a withdrawal response.

Von Frey filaments

Animals were individually placed in small acrylic boxes with mesh floor and habituated to the test the day before the analysis. Mechanical punctate hypersensitivity was assessed by von Frey monofilaments (Touch-Test™ Sensory Evaluators, North Coast Medical, Morgan Hill, CA, USA), according to the method described by the author[23,24]. Nine bending monofilaments with different caliber each (3.22, 3.61, 3.84, 4.08, 4.31, 4.56, 4.93, 5.18, 5.46) were applied perpendicularly to the mid-plantar surface of the posterior right paw. A positive response was considered during paw withdrawal, licking, or shaking. The 50% threshold was calculated using the formula: 50% (g) = 10 [xf + K.δ], where xf is the caliber of the tested filament, K is the standard value to the sequence of six behavior responses, and δ is the measure of the differences (in log) of the filaments used (0.28 for the sequence used)[23].

Hot plate test

To evaluate thermal sensitivity, a metal plate heated to a constant temperature (51.5 ºC) was used. The latency in seconds was observed for a positive response, including paw withdrawal, jumping, shaking, or licking the hind paw. A 15 second limit exposure was used to avoid tissue injury by the high temperature[24].

Open field test

To evaluate and confirm no ESI effects over general activity after stimulation sessions, rats were placed for three minutes in a circular arena (95 cm in diameter x 45 cm in height with floor divided in quadrants) and evaluated for locomotion (total number of quadrant entries) and raising (total number of bipedal position). The test was performed once after ESI 5.

Pharmacological treatments

Naloxone (naloxone hydrochloride, cod:0599 Tocris Bioscience™, Bristol, England, 2 mg/kg i.p. in 0.9% saline), a non-specific opioid receptors antagonist, was administered 30 min prior to paw pressure test. SR141716A (rimonabant hydrochloride/SR141716A, cod:0923 Tocris Bioscience™, Bristol, England, 1 mg/kg i.p. in DMSO 80%), a selective inverse agonist/antagonist of type 1 cannabinoid receptors was administered 40 min prior to the testing[7]. Animals were submitted to the drug treatment and behavior evaluation after ESI 5. Drug administration was conducted blinded to the main researcher who performed behavior tests and animals were randomly distributed in the groups.

Immunohistochemistry assays for GFAP and Iba-1 in spinal cord

Fixed spinal cord samples were collected after transcardiac perfusion (0.9% in 0.1 M PB plus 4% formaldehyde) and cut in a freezing microtome (30 µm). Sections were processed as free floating immunohistochemistry: a) 3 washes of 10 min in 0.1M PB; b) overnight incubation at 4ºC under agitation with GFAP (1:500; clone GA5 MAB360; MilliporeTM, Burlington, Massachusetts, USA) or Anti- Iba1/AIF-1 (1:500 MABN92, MilliporeTM, Burlington, Massachusetts, USA); c) 3 washes of 10 min in 0.1M PB; d) incubated for 2h at room temperature with biotinylated goat anti-mouse IgG antibody (1:200 Jackson Immuno Research Laboratories, INC.TM, Bar Harbor, Maine, USA,); e) incubation for 2h at room temperature with avidin-biotin kit (1:100) and 0.05% diaminobenzidine–0.01% hydrogen peroxide (Jackson Immuno Research Laboratories, INC.™, Bar Harbor, Maine, USA), mounting on gelatinized glass slides using a glycerol-based mounting medium, dehydration with graded ethanol solutions and xylene, and cover slipped with Permount solution (Fisher ScientificTM, Hampton, Nova Hampshire, USA).

Three slices of the spinal cord from each animal of each group (n per group= 4-5) were captured under an optical microscope (Aristoplan, Leitz™, Stuttgart, Germany) with final magnification of 200x. Images of spinal cord were compared with spinal cord figures of the Rat Brain in Stereotaxic Coordinates[23] to delineate the dorsal horn area of the L4-L6 lumbar segments, where the sciatic nerve was inserted in spinal cord. The quantification of GFAP-immunoreactivity was performed using a threshold-based quantification in the Image J software, in which the software identifies within a chosen area pixels between a particular threshold value defined according to its staining. This value is applied by all images and the optical density is obtained by each measure.

Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M). The 95% confidence interval (95% CI) is presented. The GraphPad Prism software version 8 (GraphPad Software Inc.™, San Diego, CA, USA) was used for statistical analyzes. Normality was assessed by the Shapiro-Wilk test. Parametric data obtained from behavioral measures, including the paw pressure, hot plate tests and von Frey filaments, were analyzed by two-way repeated Analysis of Variance (ANOVA), while the follow up of paw pressure and open field test were analyzed by one-way ANOVA, and ordinary two-way ANOVA was used for immunohistochemistry analyses, followed by Bonferroni post-hoc test. A value of $p<0.05$ was considered significant. Unless otherwise specified, values of F in the text are related to interactions treatment x time.

RESULTS

Different frequencies of repetitive ESI interfere with mechanical sensitivity

Animals developed mechanical hypersensitivity (i.e decreased mechanical threshold or 50% threshold, respectively) evaluated by both paw pressure (Figure 2) and von Frey filaments (Figure 2) tests 14 days after CCI. ESI 1 and ESI 5 induced antinociception (i.e increase on mechanical threshold) only when stimulated by using 60 and 100 Hz (Figure 2, B and C) compared to Sham, which maintained a low mechanical threshold (60 Hz ESI and Sham, n=5 to 7, $F(4,40) = 60.51$, $p<0.0001$; Post ESI 5 Sham 24.2± 1.46; ESI: 92.1± 2.88; 95% IC= -78,56 to -57,32); (100 Hz ESI and Sham, n=4 to 5, $F(4,16) = 284.4$, $p<0.0001$; Post ESI 5 Sham 30.75± 2.13; ESI: 74.6± 2.56; 95% CI= -53,27 to -34,43).

10 Hz stimulation induced antinociception after ESI 5 when compared to Sham treatment, (Figure 2, A) (10 Hz ESI and Sham, n=5 to 8, $F(4,44) = 1.41$, $p=0.246$; Post ESI 5 Sham 24.2± 14.6; ESI: 37.37± 2.07; 95% CI= -23,73 to -2,621). Similar results were observed for punctate hypersensitivity, as revealed by von Frey filaments tests (Figure 2, D-F) (10 Hz ESI and Sham, n=4 to 5, $F(4,28) = 17.81$, $p<0.0001$; Post ESI 5 Sham 2.19± 0.24; ESI: 10.74± 1.08; 95% CI= -12.02 to -5.095); (60 Hz ESI and Sham, n=4 to 5, $F(4,28) = 28.83$, $p<0.0001$; Post ESI 5 Sham 2.13± 0.23; ESI: 13.28± 0.94; 95% CI= -5.443 to -1.750); (100 Hz ESI and Sham, n=4, $F(4,24) = 17.76$, $p<0.0001$; Post ESI 5 Sham 2.19± 0.24; ESI: 12.94± 1.16; 95% CI= -3.879 to 0.6244).

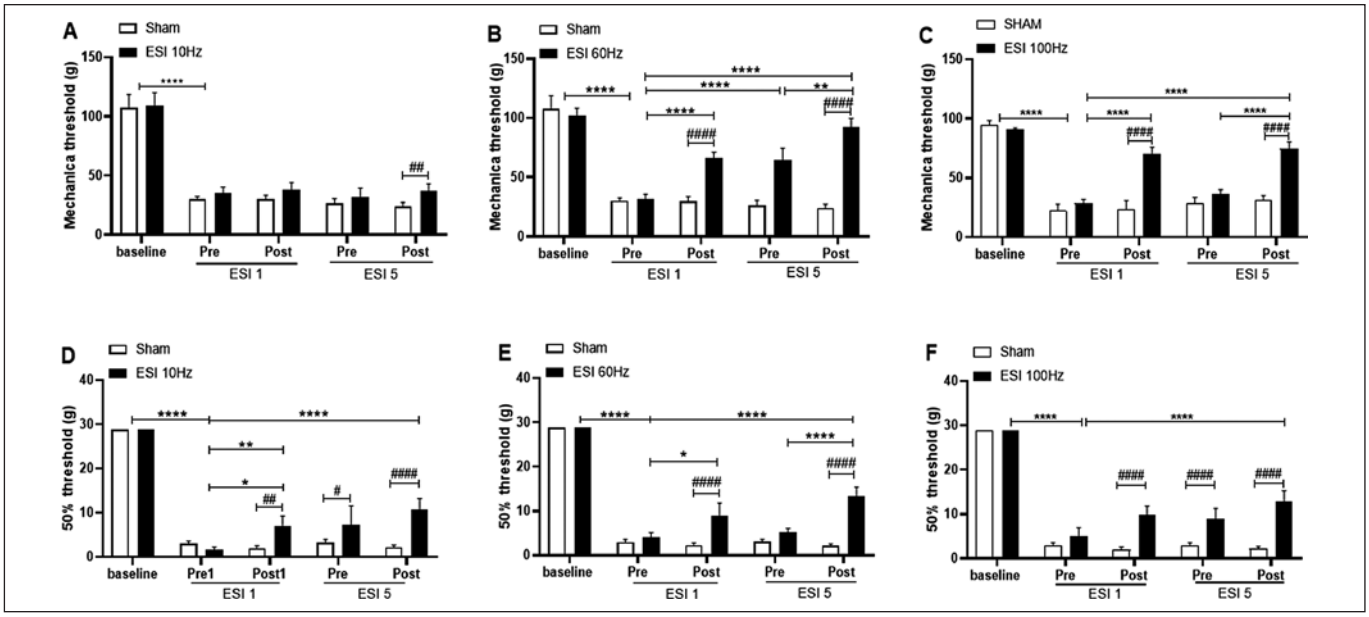

Figure 2. Effect of repetitive ESI delivered at 10, 60, and 100Hz on mechanical sensitivity.

CCI-rats with electrodes implanted in left posterior insular cortex were submitted to repetitive ESI delivered at 10, 60, and 100Hz and evaluated in the paw pressure test (A to C) and using von Frey filaments (D to F) at baseline, before (pre) and after (post) a single or five ESI sessions. Groups were separated in (A) ESI 10Hz (n = 8), (B) ESI 60Hz (n = 8), (C) ESI 100Hz (n = 5); and SHAM (n = 4 to 5). Data is presented as mean ± S.E.M. Two-way ANOVA was followed by the Bonferroni post-test. (A) baseline vs Pre ESI 1 1, ****p <0.0001. (B) Baseline vs Pre ESI 1; Pre ESI 1 vs Post ESI 1; Pre ESI 1 vs Pre ESI 5; Pre ESI 1 vs Post ESI 5, ****p <0.0001. Pre ESI 5 vs Post ESI 5; **p = 0.0048; Groups SHAM vs ESI, Post ESI 1 and Post ESI 5 ####p<0.0001. (C) Baseline vs Pre ESI 1, ****p <0.0001; Pre ESI 1 vs Post ESI 1 and Post ESI 5, ****p <0.0001; Pre ESI 5 vs Post ESI 5; ****p <0.0001; Groups SHAM vs ESI, Post ESI 1 and ESI 5 ####p<0.0001; (D) Baseline vs Pre ESI 1, ****p< 0.0001; Pre ESI 1 vs Post ESI 1, *p = 0.0211; Pre ESI 1 vs Pre ESI 5, **p = 0.0015; Pre ESI 1 vs Post ESI 5, ****p< 0.0001. Groups SHAM vs ESI, Post ESI 1, Pre ESI 5; Post ESI 5 ##p= 0.0013; #p= 0.0227; ####p<0.0001, respectively. (E) Baseline vs Pre ESI 1, ****p<0.0001; Pre ESI 1 vs Post ESI 1, **p = 0.0244; Pre ESI 1 vs Post ESI 5, ****p<0.0001. Groups SHAM vs ESI, Post ESI 1, Post ESI 5 ####p<0.0001. (F) Baseline vs Pre ESI 1; ****p< 0.0001; Pre ESI 1 vs Post ESI 1, **** p<0.0001; Groups SHAM vs ESI, Post ESI 1, Pre ESI 5, Post ESI 5 ####p<0.0001.

60 Hz ESI, but not 10 and 100 Hz ESI, increased thermal sensitivity of CCI-induced rats without changes in general activity of animals

Results obtained demonstrate that 60 Hz ESI induced increase in thermal latency after a single ESI and five ESI in CCI-rats (60 Hz ESI and Sham, n=4, F (4,12) = 17.08, p<0.0001; Post ESI 5 Sham 2.15± 0.05; ESI: 5.65± 0.218; 95% CI= -4.641 to -2.359), while 10 Hz (10 Hz ESI and Sham, n=4 and 5, F (4,35) = 0.971, p=0.435; ESI: 3.24± 0.294; 95% CI= -2.275 to 0.09453) and 100 Hz ESI (100 Hz ESI and Sham, n=4, F (4,12) = 2.47, p=0.1; Post ESI 5, ESI: 3.25± 0.338; 95% CI= -2,368 to 0,1683) did not affect thermal hypersensitivity in hot plate test (Figure 3, A-C), compared to Sham animals, which did maintain the thermal hypersensitivity (i.e. low latency of response) observed after CCI. Locomotion and raising evaluated by the open field test did not change after 10, 60 and 100 Hz ESI (Figure 3, D-E) (Raising: 60 Hz ESI and Sham, n=4, F (3,12) = 1.297, p=0.3294; 95% CI= -7.456 to 3.456); (Locomotion 60 Hz ESI and Sham, n=4, F (3,8) = 5.405, p=0.025; 95% CI= -37.95 to 5.285).

Repetitive 60 Hz ESI-induced mechanical threshold increase is reversed by opioidergic and cannabinoidergic antagonists

Naloxone reversed mechanical threshold increase after ESI 5 (**60 Hz ESI:** n=7; F [4,44]=4.853, p=0.0025; **Pre ESI 5**: 42.571± 4.628; **Post ESI 5:** 28.142± 3.595**; 60 Hz ESI+Sal:** n=6; **Pre ESI 5**= 44.5± 3.584; **Post ESI 5:** 53.833±7.773; Figure 4A; 95% CI= -42,17 to -9,214). Similarly, SR141716 reversed ESI 5 effects (**60 Hz ESI:** n=4; F [4,24] =7.292, p=0.0003; **Pre ESI 5**: 52.5± 3.883; **Post ESI 5:** 35.75± 3.172; **60 Hz ESI+DMSO**: n=4; **Pre ESI 5:** 58.5± 7.643; **Post ESI 5:** 74±7.047; Figure 4, B; 95% CI= -56,62 to -19,88).

Repetitive 60 Hz ESI increased GFAP, but not IBA-1, immunoreactivity in spinal cord

Qualitative analysis of immunohistochemistry images suggests an increase in GFAP-IR in the dorsal horn after 60 Hz ESI (**DHSC: ESI**; Figures 5, E and F vs **Sham** Figures 5, C and D), and no changes on IBA-1 IR (**DHSC: ESI;** Figures 5, J and K vs **Sham;** Figures 5, H and I). Quantitative analysis revealed an increase on GFAP-IR in the right dorsal horn of stimulated animals (**60 Hz ESI**; n=5, F (1,14)= 9.184, p=0.009; Right 1.250x1010 ± 3.916x1009; vs **SHAM** n=4; right 3.346x1009 ± 7.466x1008; Figure 5, G; 95% CI= -17945884820 to -365735414), but changes on IBA-1-IR (**60 Hz ESI**; n=5, F(1,16)= 0.0768, p=0.7852; Figure 5, L; 95% CI= -4782549390 to 2757797535).

Repetitive 60 Hz ESI induced a sustained mechanical threshold increase for up to 72h

Results demonstrate that CCI-rats had an increase in mechanical threshold maintained for 72h after the offset of

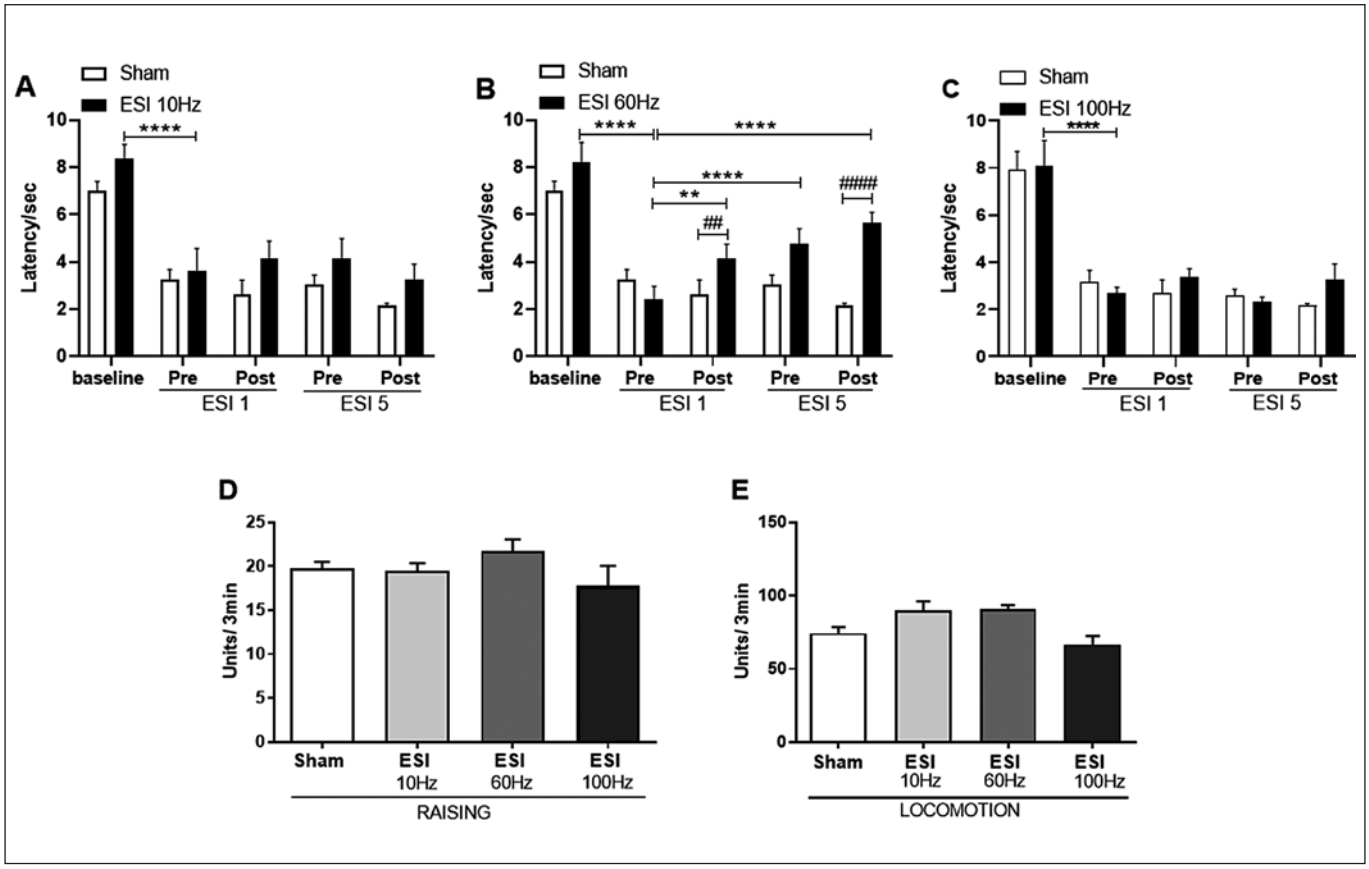

Figure 3. Effect of repetitive ESI delivered at 10, 60, and 100 Hz on thermal hypersensitivity and general activity

CCI-rats implanted with electrodes in the left posterior insular cortex were submitted to repetitive ESI delivered at 10, 60, 80 and 100 Hz and evaluated in hot plate test for thermal hypersensitivity at baseline, before (pre) and after (post) a single or five ESI sessions. For thermal sensitivity analyses, groups were separated in (A) ESI 10 Hz (n = 5), (B) ESI 60 Hz (n = 4), (C) ESI 100 Hz (n = 4); and SHAM (n = 4). General activity was measured in the open field test once after five ESI. Raising (D) and locomotion (E) were assessed in 10 Hz ESI (n=3), 60 Hz ESI (n=3), 100 Hz ESI (n=3) and SHAM (n=3) animals during three minutes. Data presented as mean ± S.E.M. In (A-C), two-way ANOVA followed by the Bonferroni post-hoc test. In (A) Baseline vs Pre ESI 1, ****p< 0.0001. (B) Pre ESI 1 vs Post ESI 1, **p=0.0066; Baseline vs Pre ESI 1, Pre ESI 1 vs Pre ESI 5 and Pre ESI 1 vs Post ESI5, ****p< 0.0001. Groups SHAM vs ESI, Post ESI 1, ##p=0.0076; Post ESI 5, ####p<0.0001. In (C) Baseline vs Pre ESI 1; ****p< 0.0001. (D-E), one-way ANOVA followed by the Bonferroni post-hoc test.

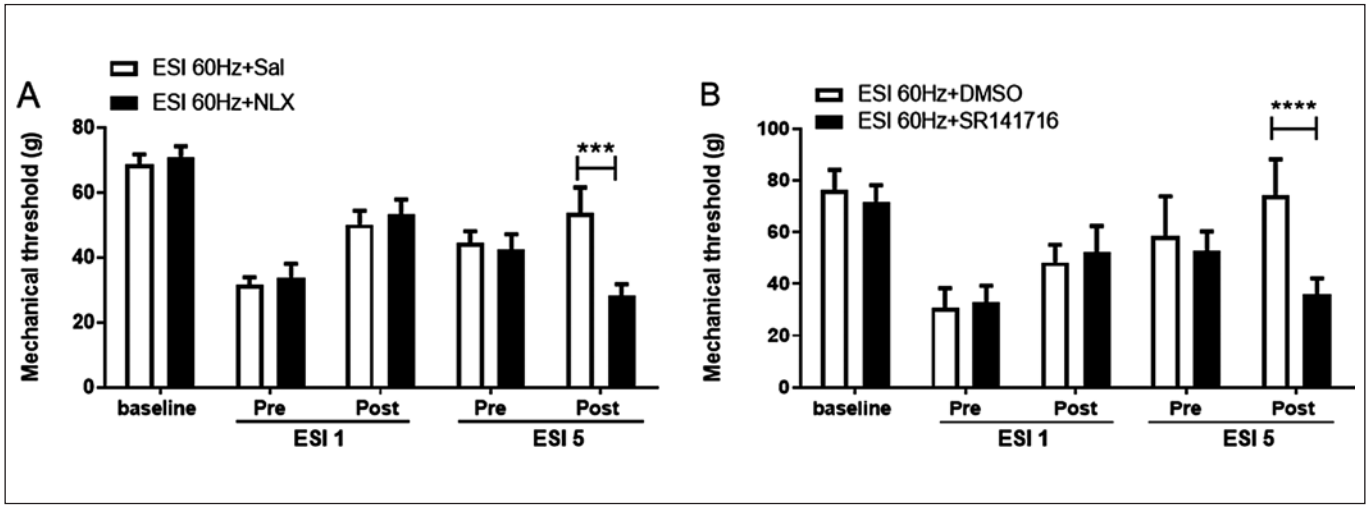

Figure 4. Pharmacological assessment of opioid and cannabinoid mechanisms of 60Hz ESI.

CCI-rats implanted with electrodes in the left posterior insular cortex submitted to repetitive 60Hz ESI were injected with (A) naloxone (2 mg/kg; i.p.) (ESI+Sal n= 6; ESI+NLX n=5) or (B) SR141716 (1 mg/kg; i.p.) (ESI+DMSO n= 4; ESI+ SR141716 n=4) 30 min and 40 min prior to mechanical nociceptive evaluation, respectively. Animals were evaluated in paw pressure test in a baseline, before (pre) and after (post) a single or five 60Hz ESI sessions. Data presented as mean ± S.E.M. Two-way ANOVA followed by the Bonferroni post-hoc test. (A) 60 HZ ESI +Sal vs 60 HZ ESI +NLX ***p=0.0006 (B) ESI 60 HZ +DMSO vs 60 HZ ESI +SR141716 ****p<0.0001. Abbreviations: Dimethyl sulfoxide (DMSO).

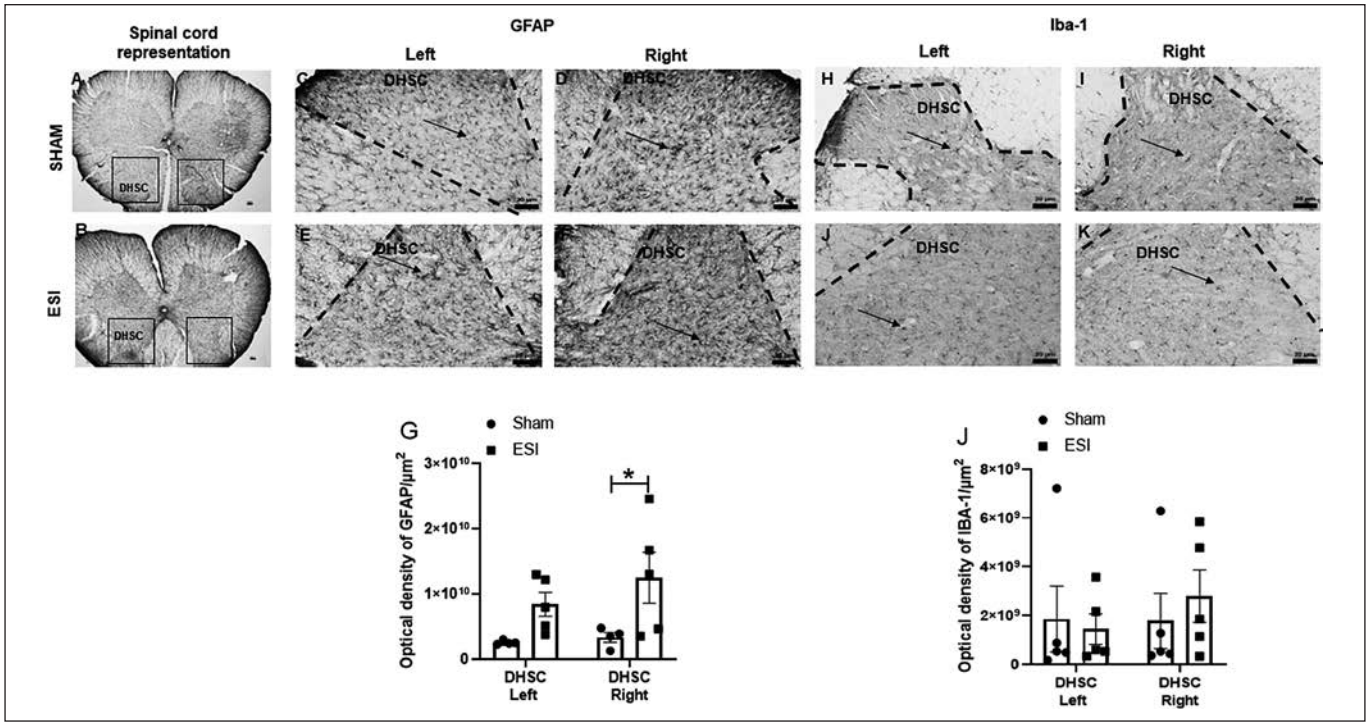

Figure 5. Effect of 60 Hz ESI on the immunoreactivity of astrocytes and microglia in spinal cord.

Fixed spinal cord slices of 60Hz ESI (n = 5) or SHAM (n = 4-5) CCI-rats were prepared for GFAP (C-F) or IBA-1 (H-K) immunohistochemistry. Representative images of the DHSC were obtained using an optical microscope, and optical density immunoreactivity analyzed by a threshold-based counting in Image J software. In A and B, horizontal bar = 100 µm; in all others, horizontal bar = 20 µm. GFAP-IR is marked by dark black points in the image and pointed by black arrows. In (H) and (L), data is presented as mean ± S.E.M. and was analyzed by two-way ANOVA followed by the Bonferroni post-hoc test. In (E) GFAP optical density SHAM vs ESI group in the right DHSC, * p = 0.0024.

ESI 7 (72h, Figure 6) and this effect was partially reversed 168h after the last ESI (168h, Figure 6) (**60 Hz ESI**, n=7, F (1.861, 11.16) = 16.89, p=0.0005; **Post ESI 7**= 66.00± 4.440; 72h: 52.00± 3.170; Day 168h= 37.43± 1.888; 95% CI= 11.58 to 45.57).

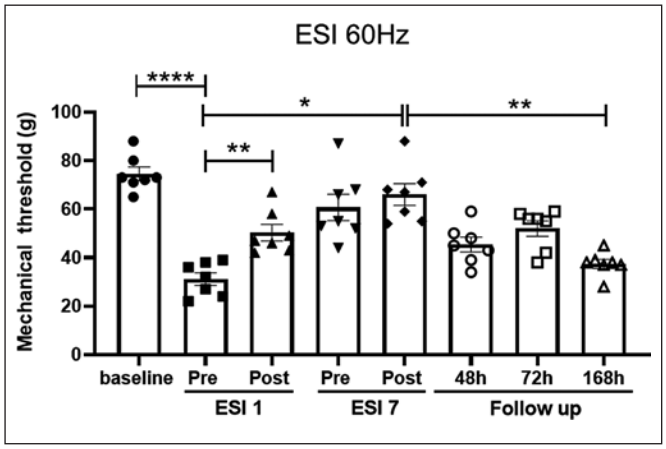

Figure 6. Long-term antinociceptive effect of 60 Hz ESI 7

CCI-rats implanted with electrodes in the left posterior insula were submitted to seven daily repetitive 60 Hz ESI (n=7) and evaluated in the paw pressure test at baseline, before (pre) and after (post) a single ESI (ESI 1), seven ESI sessions (ESI 7) and after different follow up periods (48h, 72h, 168h). Data is presented as mean ± S.E.M. One ANOVA was performed after the Bonferroni post-test. Baseline vs Pre ESI 1, ****p< 0.0001; Pre ESI 1 vs Post ESI 1, **p=0.0015; Pre ESI 1 vs Post ESI 7, *p=0.0465; Post ESI 7 vs 168h, **p=0.0030.

DISCUSSION

Neurostimulation techniques have been promising non-pharmacological therapies in both experimental and clinical studies, with several trials showing their efficacy in treating neuropathic pain patients that did not respond to conventional analgesic therapies[27]. Insular cortex is a complex cortical area with intra and extra-insular reciprocal connections, projecting to limbic, sensory and associative cortical essential areas to create pain perception, besides a delimitated posterior somatosensory area which regulates sensory inputs, intimately involved in mechanisms of nociception and pain[2,25,28]. As a consequence, insular neurostimulation has been recently proposed in many clinical studies[6].

Stimulation efficacy can vary greatly according to stimulation parameters[29]. This indicates and reaffirms that, according to the frequency and even to other parameter settings, antinociceptive responses may vary and urge to be adjusted according to the desired therapeutic mechanisms. While low frequencies activate neural elements, high frequency DBS reduces cell firing and drives axonal projections in the vicinity of the electrodes[30,31]. Evoked pain can be observed after insular stimulation using 1 to 50 Hz in short-term (5s) and modulated following interventions in brain pathways associated to pain[32-35]. Accordingly, 60 and 100 Hz were frequencies deemed to be potentially more efficacious in the present study. Results from this study are similar to those found with 60 Hz motor cortex stimulation,

which also induced antinociception in CCI rats[36] in the same behavioral paradigms applied in our study.

It was previously shown that ESI affects nociception in neuropathic pain animals[7,8]. The present work corroborates those findings and provide a more detailed appraisal on stimulation protocols and mechanisms through which ESI modulates neuropathic pain. The study found that, while the Sham group kept hypersensitivity, a range of frequencies of ESI was able to induce mechanical antinociception in both tests after the ESI 5, potentially through the modulation of pain-related circuits[8].

In contrast with mechanical nociception, only 60 Hz ESI reversed thermal hypersensitivity. This is in line with the fact that the insula modulates heat nociceptive thresholds after high frequency inhibitory stimulation in selected epileptic individuals[35]. Notwithstanding, studies standardizing a thermal pain test in experimental insular neurostimulation are still lacking. The present study provided additional evidence suggesting that the insula may reduce heat nociception when stimulated at particular settings, which may help with the design of future translational studies.

The open field test was used to confirm no ESI impairment over animal's general activity, confirmed by the measurement of two parameters, locomotion and raising, and no alterations in both parameters were observed, as previously published[7,8]. CCI is a nerve injury model to study neuropathic pain that, although may cause anxiety behaviors, did not affect the general locomotion behaviors in rats previously demonstrated by our ESI model. This supports other pain-like behaviors[7,8,37]. Also[7], naive rats demonstrated no changes in open field test parameters after single ESI, corroborating again its safety for the animals.

There might be regulation of endogenous opioid and cannabinoid systems after the CCI model, as pointed by the literature[38]. Herein, it's possible to observe that opioid and cannabinoid type 1 receptors antagonists, naloxone and SR141716 act blocking the ESI antinociception. Similar results were obtained after epidural stimulation of the posterior insular cortex in rats with neuropathic pain which was dependent of the opioidergic system[39]. Given the i.p. drug administration in the present study, their action affects different structures of the pain circuitry, including central and peripheral targets such as PAG, rostral ventromedial medulla (RVM) and spinal cord, for example. A study[8] showed less Fos-marked neuronal activation in PAG, an area known by its opioid-dependent analgesia, and no GAD65 inhibitory signaling association in the RVM after ESI. This suggests that these may not be the major areas of opioid and cannabinoid analgesia in insular stimulation.

One possibility is that the opioid system induces analgesia via post-synaptic modulation of the spinal cord. In contrast, a key mechanism of cannabinoid-induced analgesia involves the post synaptic inhibition of peripheral neurons and inflammatory processes[11]. This may be important for the implementation of the treatment and the selection of complementary modalities. On the other hand, the present study did not evaluate receptor activation or expression, what in fact may stand for differences after nerve injury and ESI.

Glial cells are crucial for the development and maintenance of neuropathic pain[17,40]. Activated glia sustains neuronal functions, regulates synapses, and facilitates nociceptive neurotransmission, while participating in the sensitization of neurons located in the dorsal horn of the spinal cord[17,40]. Activation of astrocytes in dorsal horn has been described after CCI[15,41] and has been suggested to play a role in the genesis of allodinia[42]. The present work postulates that the increase in GFAP immunoreactivity in the dorsal horn of animals given 60 Hz stimulation in the study is potentially related to selective modulation of local inhibitory circuits by glial cells. The literature demonstrates GABAergic stimulation effects on glial cells[43]. The present study observed a significant increase on glial activity on ESI-induced antinociception, suggesting that ESI modulates glial activity throughout the inhibitory GABAergic signaling[8]. This reinforces the idea of an inhibitory pathway acting to establish analgesia after ESI. Notwithstanding, astrocytes are known by their responsiveness to pain-related signals and neuroplasticity, influencing those inhibitory circuits[44].

The present study also characterized the long-lasting sustained antinociceptive effect of repetitive 60 Hz ESI in CCI-animals. Mechanical antinociception was observed from the first to the seventh ESI. It was found to be sustained for 72h with a partial antinociceptive effect still observed 168h later. Insular neuronal activity was previously demonstrated to be increased after 60 Hz ESI[8] and when the insular cortex is submitted to LTP and synaptic plasticity following a neuropathic process[45]. The present work proposed that glial neuroplasticity following ESI may contribute to the prolonged functional and structural changes that occur along with the behavior improvement observed in the long-term[46].

Finally, the study limitations work may be pointed as some behavioral results ended with a few samples, as in the open field test in figure 5 with n=3, due to the necessity of excluding data from animals whose implanting was lost and did not complete the testing or the stimulation protocols. Nevertheless, results were subjected to robust statistical analysis and allowed the most consistent conclusions.

CONCLUSION

Different frequencies of ESI are able to induce antinociception in animals with induced neuropathic pain by the CCI model without affecting general locomotor activity, suggesting 60 Hz ESI as the most likely to get the best benefits, as it also was shown to sustain antinociceptive effect up to 72h, consolidating the insula as a target to brain stimulation protocols and as a way to achieve pain relief in clinical medicine. Opioid and cannabinoid signaling and the activation of astrocytes in the spinal cord were found to be affected by ESI, which supports its capacity of modulation of pain pathway components. Future studies will be able to investigate new insights about insular stimulation.

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AUTHORS' CONTRIBUTIONS

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Writing - Preparation of the original, Supervision
Camila Squarzoni Dale
Funding acquisition, Conceptualization, Project Management, Writing - Preparation of the original, Supervision

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