**Somatosensory Thalamic Activity Modulation by Posterior Insular Stimulation: Cues to clinical effects from comparison of Frequencies in a Cat Model**

**Running title:** Thalamic modulation by insular stimulation

**Authors:** Hiba-Douja Chehade MSc, Sandra Kobaïter-Maarrawi PhD, Fares Komboz MD, Jean-Paul Farhat BSc, Michel Magnin PhD, Luis Garcia-Larrea MD-PhD, Joseph Maarrawi MD-PhD

**This study was carried out at the** Laboratory of Research in Neuroscience – Pôle technologie santé - Faculty of Medicine – Saint Joseph University – Beirut, Lebanon

**Funding:** This work was supported by grants from the Research Council of Saint Joseph University of Beirut (FM 294) and the National Council for Scientific Research of Lebanon (CNRS-L). It was also granted a doctoral fellowship to PhD Candidate Hiba Douja Chehade from the CNRS-L. The funding sources had no involvement in study design, in the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the article for publication.

**Authorship Statement:** Ms. Hiba Douja Chehade conducted the study including data collection and analysis, conducted statistical analysis and interpretation of the data and prepared the manuscript draft. Dr. Fares Komboz participated in the data collection and interpretation, and in writing the manuscript draft. Mr. Jean-Paul Farhat participated in the data collection and manuscript editing. Dr. Sandra Kobaïter-Maarrawi and Dr. Joseph Maarrawi worked on the conceptualization of this study, validated the methodology, acquired funding, supervised the data collection and analysis, and reviewed and edited the manuscript. Dr. Luis Garcia-Larrea and Dr. Michel Magnin worked on the conceptualization of the study and reviewed the final version of the manuscript. Ms. Hiba Douja Chehade, Dr. Sandra Kobaïter-Maarrawi, Dr. Fares Komboz and Dr. Joseph Maaarrawi had complete access to the study data. All authors approved the final manuscript.

**Conflict of interest statement:** The authors have no conflicts of interest to declare.

**Corresponding author:** Joseph Maarrawi

Address: Laboratory of Research in Neuroscience (PTS) - Faculty of Medicine,

P.O. Box 16-6830 – Beirut, Lebanon

Tel: +961 3 742374 or +961 1 210200 Fax: +961 1 615300 ext. 9512

E-mail: [joseph.maarrawi@usj.edu.lb](mailto:joseph.maarrawi@usj.edu.lb) or [jomaarrawi@hotmail.com](mailto:jomaarrawi@hotmail.com)

**Abstract**

**Background:** The posterior insula (PI) has been proposed as a potential neurostimulation target for neuropathic pain relief as it represents a key-structure of the pain matrix. However, currently available data remain inconclusive as to efficient stimulation parameters. **Objective:** As frequency was shown to be the most correlated parameter to pain relief, this study aims to evaluate the potential modulatory effects of low frequency (LF-IS, 50 Hz) and high frequency (HF-IS, 150 Hz) posterior insular stimulation on the activity of somatosensory thalamic nuclei. **Methods:** Epidural bipolar electrodes were placed over the PI of healthy adult cats, and extracellular single-unit activities of nociceptive (NS), non-nociceptive (NN) and wide dynamic range (WDR) thalamic cells were recorded within the ventral posterolateral nucleus and the medial division of the thalamic posterior complex. Mean discharge frequency and burst firing mode were analyzed before and after either LF-IS or HF-IS. **Results:** LF-IS showed significant thalamic modulatory effects increasing the firing rate of NN cells (p≤0.03) and decreasing the burst firing of NS cells (p≤0.03), independently of the thalamic nucleus. Conversely, HF-IS did not induce any change in firing properties of the three recorded cell types. **Conclusion:** These data indicate that 50 Hz IS could be a better candidate to control neuropathic pain.

*Keywords: Posterior Insula, Somatosensory thalamus, Neurostimulation, Nociception, Neuropathic pain, Cat*

**Introduction**

Neuropathic pain (NP) is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system (1). NP may become refractory to pharmacological treatment in up to 50% of cases (2), hence the recent surge of new therapeutic methods based on neurostimulation techniques that target the central or the peripheral nervous system (3). Prominent neurostimulation methods include spinal cord stimulation, motor cortex stimulation (MCS) and deep brain stimulation of the ventral posterolateral thalamic nucleus (VPL) (4). Even though these techniques provide pain relief in a proportion of drug-resistant NP patients, a signiﬁcant fraction of these patients still fail to treatment (5), hence the need to find new targets for neurostimulation.

Strong evidence supports the key role of the posterior insula (PI) in pain processing. It is the major cortical recipient of spinothalamic tract endings in felines (6) and primates, including humans (7,8). It is most consistently activated by noxious stimulation (9), with a lateralization to the right PI (10), its activation is correlated to stimulus intensity (11-13), and is somatotopically organized (14). The PI is the single cortical region where direct electrical stimulation and epileptic seizures can induce contralateral pain in humans (10,15-19), while lesions to the PI were found to increase pain threshold (20,21) or to induce central pain with allodynia in the long term (22).

PI have recently been suggested as a potential target of neurostimulation for NP treatment. Transcranial magnetic stimulation applied to the PI seemed to induce a bilateral reduction of heat pain detection mediated by Aδ fibers in healthy human volunteers (23) and an increase of heat pain threshold in NP patients (24). Besides, stimulation at 60 Hz on a rat model of chronic constriction injury significantly increased the mechanical hypersensitivity threshold (25). A 125Hz stimulation frequency was able to increase heat pain threshold in epileptic patients when the PI was stimulated through implanted electrodes for stereo-electroencephalogram (26). Thus, it appears that two different frequencies, one considered as low (25) and the other as high (26), applied invasively to the same target structure – though in two different species –, both induced antinociceptive effects. However, although it did induce antinociceptive effects in NP patients, IS could not achieve pain relief in these patients (24).

Since it has been shown that pain relief is mostly correlated to the frequency of neurostimulation, whether it is applied to the motor cortex (27) or to subcortical targets (28), and taking into consideration the above-mentioned discrepancies in the literature as to PI stimulation, this study compares the effects of two frequencies under the same experimental conditions. In line with analgesic MCS (27) and recent rodent study of IS (25), our first frequency, hereinafter referred to as low-frequency IS (LF-IS), was set at 50 Hz. Our high-frequency IS (HF-IS) was set at 150 Hz, based on one human IS study (26) and recent evidence of NP relieving anterior cingulate cortex stimulation (29).

Given the fact that restoration of thalamic activity – regardless of the analgesic procedure – is essential for relief of NP (30-33) this study aims to evaluate the effects of LF-IS and HF-IS on the extracellular unitary activity of two somatosensory and nociceptive thalamic nuclei; the VPL and the medial nucleus of the posterior complex (PoM), in healthy adult cats.

A cat model was chosen based on the important similitude of its spinothalamic tract to that of humans (34), as well as the anatomy and cytoarchitecture of its PI (6,35). Moreover, GABAergic thalamic interneurons – thought to play a major role in the analgesic effect of neurostimulation techniques (36) – are present in both cat and human somatosensory thalami (37), while absent in rodents (38).

**Methods**

This study included forty-six healthy adult cats (mean weight 2.48 ± 0.58 kg). All procedures were in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee at the Saint-Joseph University (*protocol number 2012/25*). Cats were housed in groups, according to in-house regulations, in a constant ambient room with controlled temperature. Dry cat food and water were available ad libitum, in addition to which a daily portion of whole moist diet was provided.

Cats were left on an empty stomach twelve hours prior to experimentation, isolated in a cage equipped with a bed and a litter box. Each cat underwent up to four experiments, separated by at least two-week intervals, after thorough evaluation of the quality of neurological and behavioral recovery.

**Animal preparation**

Atropine (0.04 mg/kg, *Atropine-Sulfate-Renaudin*, Itxassou, France) premedication was administered subcutaneously to avoid vegetative effects of anesthesia. General anesthesia was induced by intramuscular (IM) injection of a mixture of ketamine (30 mg/kg, *Ketamine*, Luitré, France) and xylazine (0.6 mg/kg, *Ilium-Xylazil-20*, Glendenning, Australia). It was maintained throughout the experiment by IM ketamine doses (20 mg/kg), injected at variable intervals such that a slight withdrawal reflex could be elicited in response to noxious stimuli. Heart rate (100-220 bpm) was monitored and temperature maintained at 38±1°C (rectal) with a heating pad. Bupivacaine (<0.5 mg/kg, *Bucaine*, Amman, Jordan) local anesthesia was administered at the level of the scalp (surgical site) and at the lower orbital ridge (points of contact with the stereotaxic head holder). Lidocaine spray (*Xylocaine*, Södertälje, Sweden) was applied to the external auditory meatus for the placement of the metallic rods of the stereotaxic apparatus. An antibiotic ophthalmic ointment was applied to the cornea to prevent corneal desiccation and/or infection. All surgical procedures followed a strict aseptic protocol. After the end of the experiment – which lasts overall around five hours – anti-inflammatory analgesics (tolfenamic acid, 4 mg/kg, *Tolfédine-4%*, Lure, France) and antibiotics (gentamicine 4.4 mg/kg, every 12 hours on 48 hours, *Pan-Gentamicine*, Fougères, France) were administered subcutaneously and cats were left to recover in isolated cages, each equipped with a bed, a heated pad, a litter box and water. Postoperative recovery was closely evaluated daily (*4Avet scale for postoperative pain*) and an additional dose of tolfenamic acid was administered if needed.

**Epidural posterior insular stimulation**

Cats were placed in a stereotaxic apparatus and a median axial scalp incision was performed from the occiput to the fronto-nasal suture, exposing the periosteum. Two burr holes were drilled at the right PI stereotaxic coordinates (y=17.5 mm, z=11.5 mm) (39) and a pair of Ag/AgCl epidural electrodes (cylinders of 1 mm diameter;*EP1, World Precision Instruments, Inc. – USA*) was placed orthogonally to the cortex, the cathode posterior to the anode. Epidural placement was used in order to be closer to clinical conditions – in which epidural implantation, compared to subdural, reduces the risk of induced seizure and post-surgical complications (40). The electrodes were fixed with bone wax and connected to a stimulator (*AM Systems, Isolated Pulse Stimulator, Model 2100*). Pulse duration (250 µs) and amplitude (2.5 V) were in line with those in use in the literature to avoid tissue damage (41). Frequency was set at 50 Hz to study the effect of LF-IS and at 150 Hz to study that of HF-IS. At the end of the experiment, the electrodes were removed, the holes filled with sterile bone wax and the skin sutured. The same skin incision was reopened when a cat underwent another experiment.

**Thalamic extracellular single-unit recording**

A 1-cm craniotomy centered at 8.5 mm caudorostrally and 4.5 mm mediolaterally exposed the dura above the ipsilateral thalamic VPL and PoM nuclei. A deep-brain tungsten microelectrode (average impedance 0.21 Ω, FHC, USA) was stereotaxically lowered to the VPL (x = 7.5, y = 8.5, z = 3.5 mm) (42) or the PoM (x = 5, y = 7, z = 3 mm) (42) until a single-unit signal was well isolated. The signal was amplified, filtered (band-pass: 500Hz – 2 kHz) and processed by data acquisition system (*AD Instruments, PowerLab 4/30*).

Once a single-unit signal was isolated, the nature of the firing cell was identified by receptive field stimulation. Cells only responding to brushing, light touching, tapping or light pressure were identified as non-nociceptive cells (NN), those responding exclusively to hard pressure, noxious pinches or squeezing were identified as nociceptive specific (NS), and those responding gradually to both types of stimuli were identified as wide dynamic range (WDR).

The recording protocol consisted of a 5-min recording session of the spontaneous activity of the identified cell, followed by a 10-min recording under posterior insular stimulation (either LF-IS or HF-IS, depending on randomization) and finally by a 30-min session of post-stimulation recording of the spontaneous activity of the same cell (***Figure 1***). The shape and duration of action potentials were monitored to ensure recording stability, and spike discrimination was conducted in real time through the Spike Histogram module of LabChart 7 Pro (*ADInstruments*, NSW, Australia).

**Signal analysis**

Analysis of single-unit activities was performed offline with LabChart and NeuroExplorer (*Nex Technologies*, Colby, KS) softwares. Isolation of single spikes was done using the spike histogram module in LabChart by the method of root mean square deviation and fit tolerance. Analysis of spontaneous activity included the mean firing rate and the frequency of burst occurrence. Bursts were identified as at least 3 contained spikes, with a maximum interval of 2 milliseconds between first 2 spikes and a maximum interspike interval of 10 milliseconds between the following spikes (43) and must be preceded or followed by a period (>10 ms) of discharge quiescence to be considered as separate bursts.

**Statistical analysis**

Recordings of the cells receiving LF-IS and those receiving HF-IS were analyzed separately. The spontaneous variations of the firing rate in the absence of stimulation were quantified to establish a threshold above or under which stimulation after-effects could be considered significant. The mean value of spontaneous activity recorded before stimulation was calculated. Consequently, for a given cell, the threshold to consider as a significant variation in its firing rate after stimulation was defined as 2 SD above or below its mean spontaneous firing rate during the baseline period of spontaneous activity.

One-way repeated-measures ANOVAs and post-hoc paired *t*-tests were applied to evaluate effect of IS on the different cell types. If the assumption of normality was not completely met, the non-parametric Wilcoxon test was applied instead.

To confirm the univariate analysis, stimulation after-effect on the spontaneous firing rate of the global cell population recorded through the entire protocol was assessed using a mixed-design analysis of variance (ANOVA) with two “between” – separating the 3 types of cells (NN, NS and WDR) and the 2 studied thalamic nuclei (VPL, PoM) – and one “within” factors – distinguishing pre- and post-stimulation periods. The dependent variable was either the mean firing rate or the spike burst occurrence. Mauchly’s sphericity tests for the repeated measures variable showed that the main effect of both IS did not meet the assumption of sphericity. Therefore, the *F-*value for the main effect of IS and its interaction with the between-subject variables were corrected with Greenhouse-Geisser. Statistical analyses were generated using *SPSS Statistics 26* (IBM SPSS Statistics, Chicago, USA) with a significant level set at a 2-tailed p<0.05 corrected with Greenhouse-Geisser when needed.

**Results**

Sixty-three cells were recorded in the VPL. Receptive fields were identified all over the contralateral hemibody. Due to occasional signal loss, forty-four of these recorded cells were submitted for statistical analysis. Sixteen responded exclusively to non-noxious mechanical stimuli and were considered as NN cells, thirteen responded exclusively to noxious mechanical stimuli and were considered as NS cells, and fifteen responded gradually to both types of stimuli and were considered as WDR cells. Forty-seven cells were recorded in the PoM, forty of which were submitted to statistical analysis. Receptive fields were identified bilaterally on the body. Twenty-one cells were considered as NS cells and nineteen cells were considered as WDR cells. Two of the NS cells were characterized as outliers (data points were more than two interquartile ranges above the third quartile) and were excluded from further analysis. The LF-IS protocol included seventeen NS cells, sixteen WDR cells and eight NN cells. The HF-IS protocol included fifteen NS cells, eighteen WDR cells and eight NN cells **(*Table 1*)**.

***Figure 2*** shows an example of the firing rate recording of two NN cells, one studied under LF-IS protocol and the second under HF-IS protocol.

The mean firing rate (***Figures 3 and 5***) and the burst occurrence(***Figures 4 and 6***) were evaluated before and after stimulation. The 30 min post-stimulation period was divided into six intervals of 5 min each to better evaluate the evolution of the effect induced by the stimulation. These intervals are labeled hereafter as PostIS1, PostIS2, PostIS3, PostIS4, PostIS5 and PostIS6.

1. **LF-IS**
   1. **Global firing rate analysis (*Figure 3*)**

A one-way ANOVA evaluating the overall effect of LF-IS among the different cell types (NS: n = 17, WDR: n = 16, NN: n = 8) independently of the nucleus in which they were recorded, revealed a statistically significant effect for NN cells (*F* (1.4, 9.5) = 6.1; p = 0.028). Such an effect was not statistically significant neither for WDR (*F* (1.9, 25.3) = 1.5; p = 0.25) nor for NS cells (*F* (1.9, 30.2) = 1.7; p = 0.20). A paired t-test for NN cells global firing rate confirmed that the increased activity induced by LF-IS is statistically significant starting immediately after the stimulation (PreIS: 5.12 ± 0.84 *vs* PostIS1: 8.97 ± 1.74; *t(7)* = -2.77, p = 0.03) and remains so until 30 min post-stimulation (PostIS2: 9.17 ± 1.77; *t(7)* = -2.72, p = 0.03 / PostIS3: 9.31 ± 5.35; *t(7)* = -2.66, p = 0.03 / PostIS4 : 9.87 ± 1.96; *t(7)* = -2.85, p = 0.03 / PostIS5 : 9.97 ± 2.05; *t(7)* = -2.73, p = 0.03 / PostIS6 : 10.11 ± 2.23; *t(7)* = -2.46, p = 0.04) (***Figure 3,D***).

A mixed-design ANOVA confirmed these observations as it showed that the main effect of LF-IS is statistically significant: (*F* (2.08, 70.77) = 4.57; p = 0.013). The main cell type effect was also statistically significant (*F* (4.16, 70.77) = 5.4; p = 0.009). However, there was no statistically significant main effect of the nucleus recorded (*F* (2.08, 70.77) = 5.4; p = 0.33).

* 1. **Burst analysis (*Figure 4*)**

A one-way ANOVA showed a statistically significant effect for NS cells (*F* (1, 16) = 3.3; p = 0.028), but not for WDR cells (*F* (1, 13) = 2.44; p = 0.14) or NN cells (*F* (1, 16) = 4.67; p = 0.068). Wilcoxon non-parametric test confirmed that the decrease in burst rate induced by LF-IS was statistically significant for NS cells starting 15 min after the interruption of stimulation (PreIS: 0.06 ± 0.03 *vs* PostIS4: 0.02 ± 0.01; *Z* = -1.99, p = 0.05, and PostIS6: 0.02 ± 0.01; *Z* = -2.19, p = 0.03) (***Figure 4, B***).

A mixed-design ANOVA revealed a significant main effect of LF-IS on burst firing (*F* (1.75, 59.48) = 4.75; p = 0.016). However, neither nucleus type (*F* (1.75, 59.48) = 0.12; p = 0.86) nor cell type (*F* (3.50, 59.48) = 1.64; p = 0.18) factors showed a significant main effect.

**2. HF-IS**

**2.1 Global firing rate (*Figure 5*)**

A One-Way ANOVA showed no statistically significant overall effect for any cell type (NS: *F* (1.76, 22.86) = 1.00; p = 0.37. WDR: *F* (2.05, 34.85) = 0.78; p = 0.47. NN: *F* (2.20,15.36) = 0.72; p = 0.52). Thus, no post-hoc analysis was made.

A mixed-design ANOVA confirmed the univariate analysis as it showed no main effect of HF-IS (*F* (2.26, 78.97) = 0.41 and p = 0.69) nor main effects of nucleus type (*F* (1, 35) = 0.90; p = 0.35) or cell type (*F* (2, 35) = 0.96; p = 0.39).

**2.2 Burst analysis (*Figure 6*)**

A One-Way ANOVA showed no statistically significant overall effect for any cell type (NS: *F* (1.71, 22.24) = 1.01; p = 0.37. WDR: *F* (2.41, 38.58) = 2.50; p = 0.09. NN: *F* (2.20, 15.38) = 3.43, p = 0.06). Thus, no post-hoc analysis was made.

A mixed-design ANOVA confirmed the univariate analysis as it showed a main effect of HF-IS (*F* (2.65, 90.04) = 0.41; p = 0.003). However, there was no main effect of nucleus type (*F* (1, 34) = 1.00; p = 0.32) nor of cell type (*F* (2, 34) = 0.49; p = 0.61) for the burst rate.

**Discussion**

This study shows that LF-IS (50 Hz) has a significant modulatory effect on somatosensory thalamic activities increasing the firing rate of NN cells (p≤0.03) and decreasing the burst firing of NS cells (p≤0.03). In contrast, HF-IS (150 Hz) does not seem to induce any change in the firing properties of the recorded cells.

**Effect of LF-IS on NN cells firing rate and NS cells burst firing**

MCS was shown to increase VPL NN cells activity in cats (44). These results were in line with a previous clinical study where effective analgesic MCS could enhance peripheral NN sensory thresholds in NP patients, suggesting that MCS may act by strengthening the action of NN sensory inputs on nociceptive systems (45). Similarly, the increased activity of VPL NN cells observed in our study could contribute to a potential analgesic effect of posterior IS. Indeed, studies show that the insular cortex is an interface for the interaction of pain and other bottom-up sensory modalities like touch, such that tactile stimuli could attenuate nociceptive perception (46). Enhancement of firing of NN cells at the thalamic level may act via these bottom-up pathways on pain perception at insular level.

Thalamic cells fire in either a tonic mode or a burst mode (47). Bursts are most importantly generated by T-type Ca2+ channels like CaV3.2 (48). In Cav3.2 knockout rodents, bursts were shown to be reduced and correlated with a reduction in nociception, hyperalgesia and allodynia (49). Thalamic hyperexcitability with increased burst firing was observed in rats with spinothalamic lesion and was hypothesized to underlie the alteration of their pain perception (50). In rodents with spinal cord injury, increased burst firing was recorded in the VPL suggesting the contribution of somatosensory thalamic burst to chronic NP (51), whether the animals presented associated allodynia or not (52). An excess of burst firing was recorded as well in non-human primates with deafferentation pain (53) and in the somatosensory thalamus of NP patients (54-57). Moreover, MCS was shown to reduce burst firing of neurons in the cat’s VPL, suggesting that MCS could reduce cortical sensitivity to ascending sensory input by decreasing the priming of thalamocortical loops to nociceptive information (44). The reduction of burst firing of NS cells in our study may represent the potential substratum for the effect of posterior IS on NP, similar to the effect observed during MCS.

**Posterior insular neurostimulation effects**

There are discrepancies in the literature pertaining to IS-mediated modulations of nociceptive signaling. It has been suggested that inhibiting the PI would yield analgesic effects (23,26,58) via top-down mechanisms (24) as locally increasing GABA in the insula can induce analgesia (59). However, it has also been suggested that the activation of the PI and the subsequent disinhibition of the ACC could induce analgesic effects (46,60-62).

Anatomically, the cat’s PI is reciprocally connected subcortically to the VPL periphery and PoM, principal recipients of the spinothalamic tract (6). It is also connected to somatosensory cortices and to the prefrontal cortex (63), like the human PI in the primary pain matrix (64). Nevertheless, the nature of these insulo-cortical and insulo-thalamic connections has not yet been identified.

Thus, although we were able to show that LF-IS has significant modulatory effects of thalamic activity, we cannot determine the exact mechanism underlying these observations, as this goes beyond the scope of our study. Future studies should address the signaling pathways responsible for the potentially analgesic effect of LF-IS.

**Stimulation parameters affecting neuromodulatory effects**

Low-frequency electrical stimulation (0.5 to 25 Hz) was shown to activate stimulated axons but as the frequency was increased above 50 Hz *in vitro* and 125 Hz *in vivo*, it locally produced inhibition of cortical excitability and axonal block (65,66). Such observations led to a dichotomous view suggesting that, *in vivo*, low-frequency stimulation (<100 Hz) would enhance the excitability of the stimulated region whereas high-frequency stimulation (>100 Hz) would induce its inhibition (67). However, a MCS study using a frequency range of 25 to 40 Hz showed decreased regional cerebral blood flow in the motor cortex, indicating the inhibition of neuronal excitability underneath the electrodes (60). Except that it has been shown that a decreased regional cerebral blood flow could originate from an actual increase of neuronal activity (68,69). A recent study in rats evaluating the effect of 60 Hz IS showed that this stimulation could increase pain thresholds and attributed this effect to the local inhibition of the insular cortex (25). In contrast, the effects of IS at 66.6 Hz on the reward system was attributed to an activation of the insula and the reward circuitry (70-72).

Evaluating the effect of two different frequencies, one most often qualified as low (50 Hz) and the other as high (150 Hz), in the same experimental conditions, was the aim of our study in order to shed more light on the discrepancies seen in the neurostimulation literature. Here, only LF-IS induced modulatory effects, whereas HF-IS did not. This data highlights the importance of the frequency parameter when developing a neurostimulation paradigm.

The low- and high-frequency dichotomous view is to be taken with caution as the frequency parameter is not the only one affecting the stimulation effect (67). For instance, in MCS the intensity of stimulation is adjusted in order to set it just below motor threshold (4). In IS, such criterion is not available and intensity is chosen almost randomly at a level that avoids tissue damage. Moreover, in a study that showed that repetitive IS eventually had its effects decreased, the authors show that increasing the intensity of stimulation could restore these effects (72). Electrodes positions also affect stimulation effects: in MCS cathodal stimulation would preferentially activate fibers parallel to the cortex, whereas anodal stimulation would preferentially activate fibers perpendicular to the cortex (73). Besides, anodal stimulation can produce a hyperpolarization of the proximal axon and depolarization in the distal axon, and the more the anode and cathode are separated, the more distal can the depolarization be (74). Moreover, the effect of the epidural electrical stimulation depends on neural elements underneath the electrode, mainly the thickness of cerebrospinal fluid layer and cortical layer cell composition (75).

**Limitations**

First, we could not determine histologically the placement of the electrodes over the PI because of ethical restrictions concerning experimental procedures in cats. However, the used design ensures a good spatial specificity to stimulate the target structure (76). Besides, we used a unique strain of cats, the same as the one used in the stereotaxic atlas (39), and a study comparing cat brain gyration showed only minor individual variability among the animals (77).

Second, we used ketamine anesthesia. Ketamine has antinociceptive effects mediated by endogenous opioids and opioid receptors (78). The opioid system was also shown to be involved in IS antinociception (25). Accordingly, it is possible that the antinociceptive effect of IS in this study was masked by the antinociceptive effect of ketamine, the latter attenuating the spontaneous firing of nociceptive cells from the beginning and all over the experimentation.

Finally, our study used a healthy model. Studies have shown that the functional connectivity of the PI in pain conditions is different from the resting state, whether the pain is experimentally induced (79) or pathological (81,82). The observed effects here may not directly reflect the potential yield of IS effects in pain conditions.

**Conclusion**

We showed that the stimulation of the PI of the cat modulates the somatosensory thalamic activity, when using low-frequency stimulation. This data supports the hypothesis that the PI could be a potential neuromodulation target for the treatment of refractory NP.

Future studies should address IS in NP models, both at behavioral and electrophysiological levels. Assessing the effect of this stimulation underneath the electrodes for a better understanding of the underlying mechanisms of potential analgesic effects should be addressed as well. It would also be interesting to investigate the stimulation of the left PI.

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