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Research report

Place preferences induced by electrical stimulation of the external lateral parabrachial subnucleus in a sequential learning task
Place preferences induced by NLPBe stimulation

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ABSTRACT

It is known that electrical stimulation of the external lateral parabrachial nucleus (NLPBe) can sustain concurrent taste and place learning. Place preferences can be learned through different procedures. Previous studies demonstrated that electrical stimulation of the PBNLe can generate aversive and preference place learning using concurrent procedures. In the concurrent procedure, the animals can move freely in the maze, and intracranial electrical stimulation is associated with their voluntary stay in one of the two maze compartments. However, the rewarding properties of most stimuli, whether natural or drugs of abuse, have usually been investigated using the sequential procedure, in which animals are confined while receiving the unconditioned stimulus and then undergo a choice test without stimulation in a later phase. This study examined whether this stimulation can sustain place preference learning in sequential tasks. Results demonstrated that place preferences can also be induced by the electrical stimulation of the NLBe using sequential procedures. These findings suggest that the NLPBe may form part of a brain reward axis that shares certain characteristics with those observed in the processing of natural rewarding agents and especially of drugs of abuse.

1. Introduction

The parabrachial complex has been associated with various rewarding behavioral processes, including those related to nutrient intake [1–4]. Several rewarding processes have been related to the external lateral parabrachial nucleus (NLPBe), which is activated after the administration of rewarding nutrients such as glucose, lactose, or sucrose [1–3] or of drugs of abuse [5–8].

Electrical stimulation of the NLPBe can induce taste preferences for associated flavors [9] and can generate place preferences in concurrent learning tasks [10–13]. In these studies, place preferences were induced using a concurrent procedure, in which the animals could move freely in the maze and intracranial electrical stimulation was associated with their voluntary stay in one of the two maze compartments. It has been observed that place preferences induced by NLPBe stimulation by means of this procedure are blocked by naloxone administration [9,13], reflecting the high density of opioid receptors in this region [6,8,14–16]. Interestingly, preferences induced by stimulating the lateral hypothalamus are not inhibited by naloxone administration [17]. Furthermore, it has not yet been established whether electrical stimulation of the NLPBe can sustain self-stimulation behaviors [9].

Dopaminergic antagonists, specifically tiapride, do not block place preferences induced by NLPBe electrical stimulation [18]. It has been observed that electrical intracranial self-stimulation (ICSS) of the lateral hypothalamus can be affected by the administration of antipsychotics such as pimozide [19].

Repeated NLPBe stimulation produces tolerance to its rewarding effects, evidenced by a progressive loss of the capacity of stimulation to generate place preferences when repeatedly applied [20], again recalling the effect of drugs of abuse [21–23].

It has also been proposed that the key factor in developing tolerance to the rewarding effects of NLPBe stimulation is that its administration is not contingent on the behavior of the animal, unlike stimulation of the lateral hypothalamus, which does not produce tolerance regardless of its contingent (concurrent) or sequential administration [24].

All of the above findings have led to the proposal of a potential axis related to brain reward that includes the NLPBe and appears to differ from the axis that includes the lateral hypothalamus.

However, it has not been demonstrated that the electrical stimulation of these regions can generate place preference learning in non-

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contingent (sequential) tasks, in which animals are confined while receiving intracranial electrical stimulation and then undergo a choice test without stimulation in a later phase (as in the above studies). In this way, the sequential procedure involves an acquisition phase with the simultaneous presentation of rewarding stimulus and associated maze compartment, followed by a delay before the learning of the animal is tested.

It is well documented that the sequential procedure can be used to establish place preferences induced by the administration of natural rewards and especially drugs of abuse [25–37]. Thus, it has been widely reported that place preference conditioning is a highly sensitive tool to measure the rewarding properties of morphine [29,31,33,38–44].

However, rewarding electrical stimulation of the NLPBe shares some characteristics with this type of associated reward (e.g., from drugs of abuse) but differs from the reward produced by LH stimulation (see above), which can be blocked by the administration of naloxone [9,13] but not tiapride [18]. It is also possible to develop tolerance towards the rewarding properties of NLPBe stimulation when its administration is repeated [20] or not contingent on the behavior of the animal [24]. These differentiating characteristics of PNLBe stimulation-induced reward have only been tested using concurrent procedures, and it has yet to be demonstrated that this stimulation can sustain sequential place learning.

With this background, the objective of this study was to explore the possibility of inducing place preferences or aversions by electrical stimulation of the NLPBe in a sequential task. This parabrachial region was previously related to concurrent taste learning alone [45,46] but is now associated with learning tasks in which the stimulation is not contingent on the behavior of the animal in the test phase.

2. Method

2.1. Subjects and surgical procedure

Thirty-three male Wistar rats from the breeding colony at the University of Granada, weighing 280 − 350 g at baseline, were used in this study. Animals were housed in methacrylate cages with water and food ad libitum (A-04, Panlab Diets S.L., Barcelona, Spain). The laboratory was maintained at 20 − 24 °C with a 12:12 h light/dark cycle. All experimental procedures were conducted during light periods with white noise.

The animals remained under these conditions for an adaptation period of at least seven days before surgery. All behavioral procedures and surgical techniques complied with the relevant Spanish regulation (Royal Law 23/1988) and European Community Council Directive (86/609/EEC).

Animals were implanted with a stainless-steel monopolar electrode (00) in the NLPBe (Coordinates: AP = - 0.16; V = 3.0; L = + 2.5 in the atlas of Paxinos and Watson [47] using a stereotaxic apparatus (Stoelting Co. Stereotaxic 511.600) under general anesthesia (sodium thiopental, 50 mg/kg, B. Braun Medical S.A. Barcelona, Spain). As prophylactic measures, 0.1 cc penicillin (Penilevel, Level Laboratory, S.A., Barcelona, Spain) was intramuscularly injected and an antiseptic solution was applied around the implant (Betadine, Povidone-Iodine, Asta Médica, Madrid, Spain). There was a post-surgery recovery period of at least 7 days.

2.2. Equipment

For the electrical stimulation, a continuous current (range, 80 − 220 μA) with rectangular cathodic pulses at 66.6 Hz and 0.1 ms pulse duration was supplied by a CS-20 stimulator (Cibertec, Madrid, Spain) connected to an ISU 165 isolation unit (Cibertec, Madrid, Spain) and HM 404-2 oscilloscope (HAMEG Instrument GMBH, Frankfurt, Germany). The current intensity was individually established for each animal, avoiding levels that could generate involuntary movements or pain [48].

During all experimental phases, we used a rectangular three-chamber maze (50 × 25 × 30 cm) oriented North-South, in which the walls of the two lateral compartments were painted with 1-cm wide black and white stripes that were vertical in one compartment and horizontal in the other. The floor was brown cork in both compartments, with transverse incisions in one compartment and vertical incisions in the other. The floor and walls of the central area (8 × 25 cm) were white methacrylate (described in other studies, e.g., [9].

2.3. Behavioral procedure

All animals underwent acquisition tests in accordance with the two procedural learning modalities. The study comprised two parts. In the first part, animals were subjected to a “blinded” sequential place learning task without considering their positive, negative, or neutral character, thereby avoiding any bias in the development of this type of learning. In the second part, they underwent a concurrent place conditioning test in which the receipt of electrical stimulation or not depended on the voluntary stay of animals in one or other maze compartment, permitting their distribution into groups. Based on the data obtained in the concurrent test, the animals were distributed into groups as a function of their preference/aversion for intracranial electrical stimulation, and these results were considered in our analysis of the sequential task outcomes.

Before the behavioral procedure, the maze compartment to be associated with stimulation was established for each animal in a randomized and counterbalanced manner. For each animal, the same compartment was associated with stimulation in both parts of the study. The procedure was identical for intact animals except for the absence of surgery and electrical stimulation, selecting in a randomized manner the compartment considered to be associated with stimulation for statistical purposes.

2.3.1. Part one: sequential procedure

Phase 1: Baseline

All animals (26 intervened and 7 intact) were allowed to move freely around the maze for 10 min, recording the stay in each compartment. The objective of this phase was to accustom the animals to the contextual cues in the maze. No animal received intracranial electrical stimulation during this phase. Throughout the experiment, an animal was recorded as being in one of the lateral compartments when its head and front legs could be seen within it.

Phase 2: NLPBe electrical stimulation

At 48 h after phase 1, all animals underwent four confined trials in one of the two compartments of the maze in a counterbalanced manner, as follows. During the first trial, one group of animals was left in the stimulation-associated compartment, administering electrical stimulation to the NLPBe (intervened group). After 24 h, these animals were placed in the other maze compartment, and no electrical stimulation was applied. The same confinement procedure was repeated. In all cases, the animals remained confined in the corresponding compartment for 20 min in each trial. In other words, the animals were confined, alternately, for two days in the compartment in which they received electrical stimulation for 20 min and for two days in the compartment in which they received no stimulation.

Phase 3: Test

At 48 h after the second phase, animals were placed in the central area and allowed to move freely around the maze for 10 min, recording the time of stay in each compartment. None of the animals received electrical stimulation during this phase.

This procedure (phases 1, 2, and 3) is frequently used for place preference conditioning (sequential modality) in animal research on natural reinforcers [25,49–52] and drugs of abuse [31,33,35,36,38,39,41,43,44,53–56].

Table 1
Experimental protocol.

Part One. Sequential Procedure			
Baseline. A single 10-min trial without electrical stimulation			
Electrical Stimulation during Confinement:			
- Two 20-minute trials in the stimulated compartment, on alternate days			
- Two 20-minute trials in the non-stimulated compartment, on alternate days			
Test. A single 10-minute test without electrical stimulation			
Part Two. Concurrent Procedure to establish different groups			
A single 10-minute trial with electrical stimulation, allowing the animals to move freely in the maze			
POSITIVE	NEGATIVE	IMPLANTED CONTROL	INTACT CONTROL
More than 300 seconds in stimulated compartment	Fewer than 180 seconds in stimulated compartment	Between 180 and 300 seconds in stimulated compartment	Animals with no surgical intervention or electrical stimulation.

2.3.2. Part two: concurrent procedure for classification of the animals

The sole objective of this second part was to classify the animals in groups according to the effect of stimulation, applying behavioral criteria widely used in previous studies [9–13,57–59].

At 48 h after the previous phase, the animals underwent a concurrent place learning test in the same maze. It was performed after phases 1, 2 and 3 to ensure that the animals had no previous experience of concurrent place conditioning tasks when tested in the sequential task.

In this trial, animals were again placed in the central area of the rectangular maze and allowed to wander freely for 10 min. However, when they entered one of the compartments, selected in a random and counterbalanced manner at the start of the behavioral procedure, they received NLPBe electrical stimulation, which was not applied while in the central area or other compartment. The time of their stay in each area of the maze was recorded.

A summary of the behavioral procedure is depicted in Table 1.

2.4. Statistical analysis

Statistical 6.0 (Statsoft Inc., Tulsa, OK) was used for the statistical analysis. When the groups had been established according to the sequential procedure, a between-group ANOVA was performed, followed by application of the Newman-Keuls test for post-hoc comparisons. Finally, a correlation analysis was carried out between the results obtained with the two learning procedures, including all stimulated animals.

2.5. Histology

After the behavioral tests, animals were deeply anesthetized with an overdose of sodium pentothal and a small electrolytic lesion (0.3 mA/5 s) was performed, followed by intracardiac perfusion with a solution of isotonic saline and 10 % formaldehyde. Brains were removed and stored in formaldehyde for at least one week and sectioned with a freezing microtome into 70-μm coronal slices (1320 M microtome-freezer, Leitz, Wetzlar, Germany). Electrode placement was verified by examining and photographing Cresyl Violet staining under a stereoscopic magnifying glass (VMZ-4 F stereoscopic magnifying glass, PM-6 camera, Olympus, Tokyo, Japan) (Fig. 1).

3. Results

Given that analysis of sequential procedure results (Part One) took account of the concurrent procedure results (Part Two), the latter are reported first Table 2.

3.1. Concurrent place preferences

In Part Two, the animals were distributed into the following three groups according to previously published behavioral criteria [9–13,57–59]: Positive group of animals that stayed in the stimulated compartment for > 50 % of the available time, i.e., more than 5 min (n = 11); Negative group of animals that stayed in the stimulated compartment for < 30 % of the available time, i.e., fewer than 3 min (n = 10); and Neutral group of animals that remained in the stimulated compartment for 30–50 % of the time, i.e., between 3 and 5 min (n = 5). In addition, an Intact (non-implanted) Control group (n = 7) was studied. Average stay times in the stimulated compartment (maximum session of 10 min) for each group were: Positive group = 494.27 s; Negative group = 78.80 s; Neutral group, henceforth "Implanted Control group", = 231.80 s; and Intact Control group = 262.57 s.

The general between-group ANOVA using data from the concurrent task test (Fig. 2) showed a significant effect ($F_{(3,29)} = 65.40$; $p < 0.0001$). In particular, the Newman-Keuls test for post-hoc comparisons revealed a significant difference in performance between the Positive and Negative Groups (P = 0.0002), between the Positive and Intact Control Groups (P = 0.0001), between the Positive and Implanted Control Groups (P = 0.0001), between the Negative and Intact Control Groups (P = 0.0002) and between the Negative and Implanted Control Groups (P = 0.0003). However, there were no significant differences between the Intact and Implanted Control Groups (P = 0.3926), as expected (see Fig. 2).

3.2. Sequential place preferences

As noted above, the classification of animals obtained in the concurrent procedure was considered in analysis of the sequential place learning results. The between-group ANOVA of the data obtained in the sequential place learning test revealed a main effect of both group ($F_{(3,29)} = 4.9392$; $p < 0.0068$) and experimental phase ($F_{(1,29)} = 9.0417$; $p < 0.0054$) but not of the interaction ($F_{(3,29)} = 2.5392$; $p < 0.0759$).

Likewise, the general between-group ANOVA conducted with data from the sequential task test (Fig. 3) showed a significant effect ($F_{(3,29)} = 5.47$; $p < 0.0042$). In particular, the Newman-Keuls test for post-hoc comparisons revealed a significant difference in performance between the Positive and Negative Groups (P = 0.016145), between the Positive and Intact Control Groups (P = 0.048375), and between the Positive and Implanted Control Groups (P = 0.021489). However, there were no significant differences between the Negative and Intact Control Groups (P = 0.491979), between the Negative and Implanted Control Groups (P = 0.715601), or between the two Control Groups (P = 0.440743). According to these data, "Negative" and Control animals showed the same behaviors in the sequential task test.

The Newman-Keuls test for post-hoc comparisons showed significant differences between the baseline trial and the sequential task test in the Positive Group (P = 0.006532), whereas there were no differences in the Negative Group (P = 0.762041), Implanted Control Group (P = 0.979193), or Intact Control Group (P = 0.653260). See Fig. 4.

Finally, we conducted a correlation analysis of the data obtained in the two place learning modalities (concurrent and sequential) for all

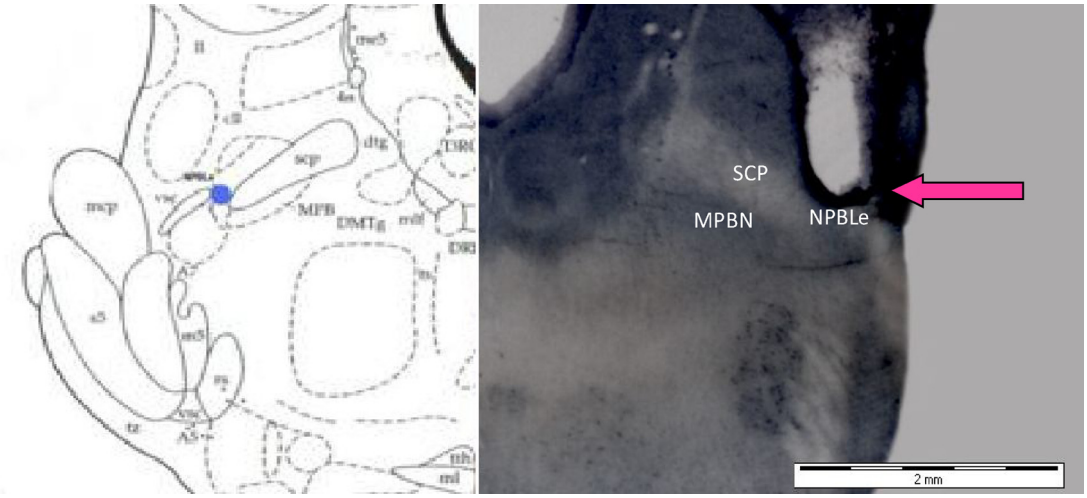

Fig. 1. Localization of the electrode in an animal from the Positive Group. Abbreviations: MPBN: Medial parabrachial nucleus; NLPBe: External lateral parabrachial nucleus; SCP: Superior cerebellar peduncle.

Table 2
Results obtained in phases 1 and 3, showing the mean time in s that each group remained in the intracranial stimulation-associated compartment before and after the confinement phase in the sequential learning test.

Group	At Baseline Sequential task	In Test Sequential task
Positive Group	217.36	364.36
Negative Group	174.80	203.80
Implanted Control Group	199.00	222.20
Intact Control Group	213.43	261.29

implanted animals, revealing a significant correlation ($r = 0.53$, $p < 0.05$). See Fig. 5.

4. Discussion

The results of this study demonstrate that place preference behaviors can be induced by electrical stimulation of the NLPBe, using a sequential procedure that involves confinement of the animals while receiving the stimulation. However, the same was not observed for place aversion induced by NLPBe stimulation, which can be achieved

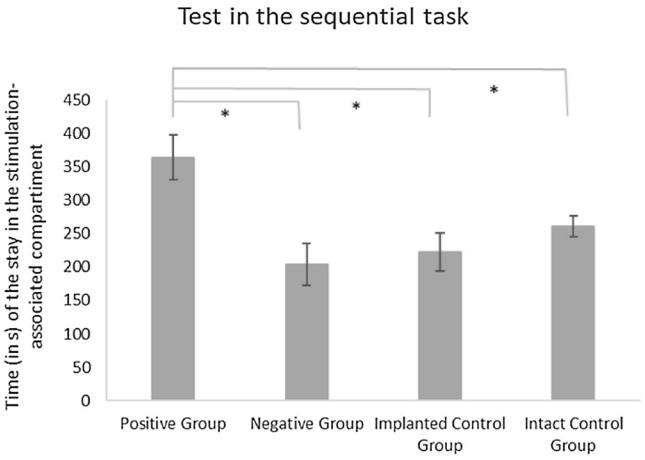

Fig. 3. Graphic representation of results obtained in the sequential task test. *: $p < 0.05$.

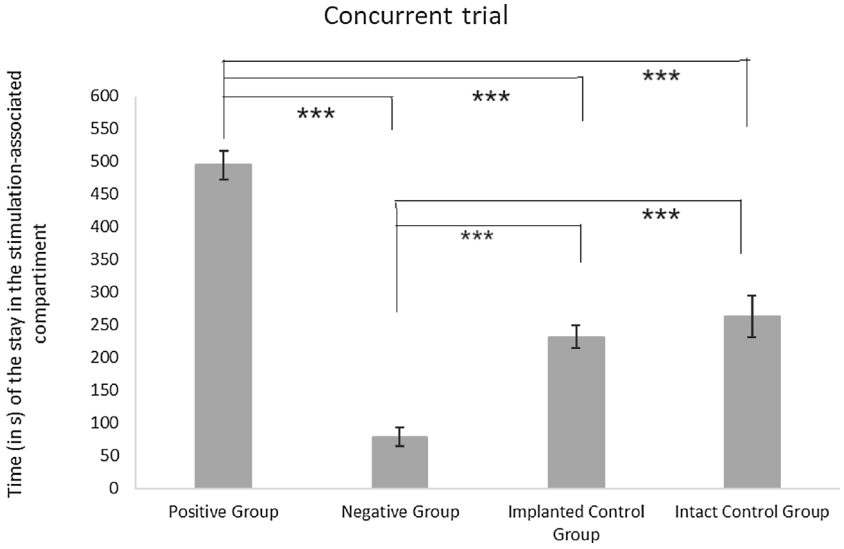

Fig. 2. Graphic representation of results obtained in the concurrent task. ***: $p < 0.01$.

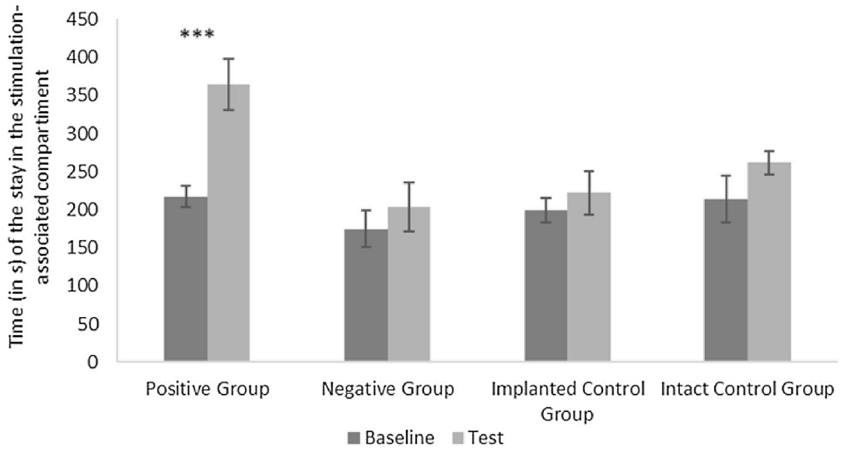

Fig. 4. Graphic representation of the results obtained. The vertical axis represents the average time of stay in s of each group in the compartment associated with NLPBe electrical stimulation at baseline and in the test using a sequential procedure. ***: $p < 0.01$.

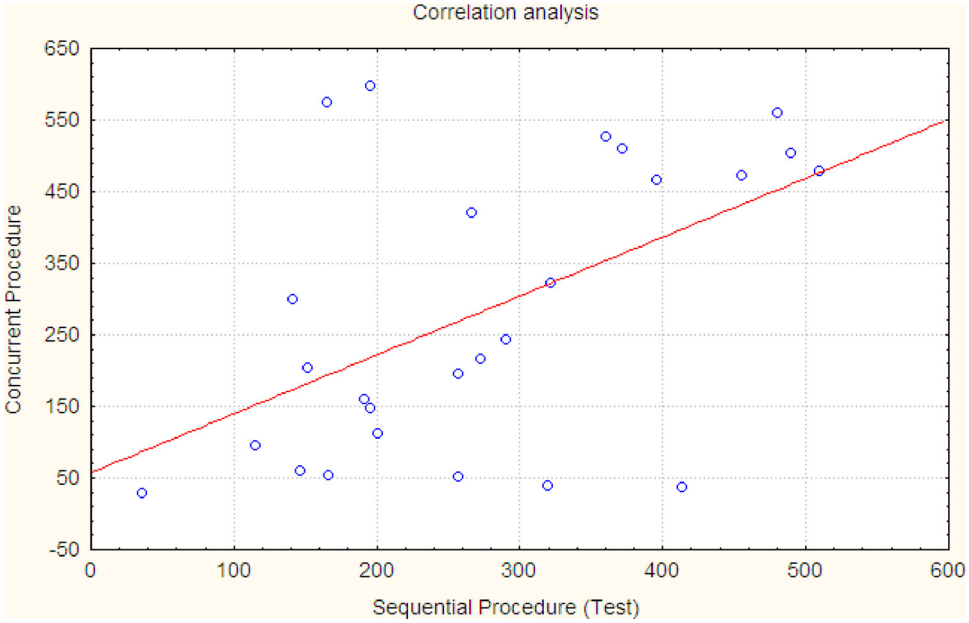

Fig. 5. Graphic representation of the results obtained in the correlation analysis. Numbers represent the average time of stay in s of each animal in the compartment associated with NLPBe electrical stimulation in the sequential procedure (horizontal axis) and in the concurrent procedure (vertical axis).

using concurrent but not sequential procedures, at least when the duration of electrical stimulation is the same.

The concurrent procedure yielded three types of animal: Positive, Negative, or Control, as in numerous previous studies [12,13,18]. The fact that electrical stimulation of the NLPBe from the same stereotaxic coordinates generates either preferences or aversions suggests that the systems processing rewarding and aversive motivational information may be anatomically very close together [60,61]. The stainless steel 00 electrodes used for electrical brain stimulation in our study can activate cell bodies, initial axon segments, and Ranvier nodules within a small spherical field of electrical influence [62]. Dissociation among different functional systems adjacent to the electrode tip [62] depends on the specific placement of the electrode within the subnucleus and may also be achieved by modifying the current parameters to activate given systems (e.g. stimulus-bound eating and self-stimulation) [63]. Specifically, electrical stimulation of the LPBe nucleus appears to be involved in opposite behavioral processes [45,46], as observed with the stimulation of other brain areas, such as the lateral hypothalamus (e.g., eating, drinking, self-stimulation, or aversion, etc.) [63,64] or the periaqueductal gray matter (pain or analgesia) [65,66]. Therefore, electrical stimulation in the "neutral" animals may have simultaneously activated cells that process appetitive and aversive information from neighboring neuronal populations, as observed in other brain regions [60,67,68].

In our experiment, however, the negative animals did not stay for a shorter time period in the maze compartment associated with aversive stimulation in the sequential task. It is also possible that the electrode may have been localized outside the NLPBe, although this is not supported by the histological study results. It is more likely that the aversion induced in these animals is not sufficient to sustain sequential task learning, because taste discrimination experiments have indicated that the NLPBe is involved solely in concurrent or ongoing learning and not in non-concurrent or explicit learning [46,69]. As in the case of taste conditioning studies, place preference conditioning tests can be conducted in various manners to generate different types of learning. In our concurrent procedure, animals could select at each session between rectangular compartments, in one of which they received intracranial electrical stimulation. In the sequential procedure, the animals were placed on alternate days in compartment A or B of the maze, where they received (in a counterbalanced manner) a test or control treatment. In

the final test, animals could choose between compartments, and the duration of their stay in each was recorded in order to assess their preferences and estimate the rewarding capacity of the treatment.

Taste aversion learning studies have demonstrated that concurrent and sequential learning modalities use different neurobiological systems as substrates. Specifically, concurrent taste aversion learning appears to depend on the vagal pathway, medial parabrachial division, NLPBe, and cerebellar circuits [9,45,46,58,59,70–72], while sequential learning seems to be independent of this pathway, requiring the integrity of structures such as the *area postrema* and lateral parabrachial division [73,74]. More recent studies demonstrated the importance of the NLPBe in concurrent taste learning, either aversive [45] or appetitive [46]. It is therefore possible that these two learning modalities are also mediated by different neurotransmitters, given that electrical stimulation is always simultaneous with the presentation of environmental cues in the place conditioning experiments conducted in our laboratory. NLPBe activation may have activated a motivational neurobiological system in which the time overlap between rewarding stimulus and environmental cues is decisive.

An important finding of this study was that NLPBe electrical stimulation induces place preference behaviors in sequential tasks, given that this parabrachial subnucleus has to date been exclusively associated with concurrent learning. This effect may be attributable to the stimulation of cells in the appetitive motivational system, which may have mobilized opioid mechanisms in the parabrachial nucleus [75]. Comparable results have been obtained with morphine administration, which frequently produces a powerful unconditioned effect in this type of learning task [29,31,33,39,41–44,53,54]. There are few data on place aversion induced by these substances [76–78], but they have given rise to evident aversive effects in taste aversion learning tasks [77,79,80].

Interestingly, although natural agents and drugs of abuse (e.g., morphine) can both induce place preferences using sequential procedures, subtle behavioral differences can be observed during the conditioning. For instance, in the case of morphine, visits to the stimulation-associated compartment are less frequent but longer, and the animals usually remain in contact with place cues for a longer time in comparison to animals stimulated by the administration of food, characterized by more frequent and shorter visits and a predominance of exploratory behaviors rather than staying next to place cues [51]. This suggests that place cues acquire a more intense rewarding value when the conditioning is induced by morphine, and it has even proven possible to establish place preferences using morphine after its utilization to induce taste aversion learning [40].

According to previous studies, there appears to be a biological predilection for reward induced by NPLBe stimulation to be associated with place cues [10]. In the present study, we show that the effect of reward induced by NLPBe stimulation can also be associated with place cues when a sequential procedure is utilized, in which the receipt of stimulation is not contingent on the animal's behavior. In this regard, previous studies revealed a tolerance effect after repeated stimulation of NLPBe when administered non-contingently to the behavior of animals [24]. These data suggest that this brain region may play a key role in processing the rewarding properties of drugs that act on the opiate system [9,17,24].

Further research is warranted on the pharmacological and physiological properties of the learning induced by this sequential procedure, especially when this can be blocked by opiate and/or dopaminergic antagonist, as in the case of natural reinforcers such as sexual stimuli [81] or food [25,49–51,82] as well as drugs of abuse [27,34,83].

In conclusion, this experiment induced place preferences by NLPBe electrical stimulation under procedural conditions similar to those used by other researchers to induce preferences with natural reinforcers [25,49–51,82,84,85] or opiate [29,31,33,39,41–44,53–55,86] and non-opiate [27,28,30,31,33–39,41,55] substances of abuse, a parallelism that deserves future study.

Author statement

The authors do not have permission to share data

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