The impact of chronic daily nicotine exposure and its overnight withdrawal on the structure of anxiety-related behaviors in rats: Role of the Lateral Habenula

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Abstract:

Tobacco smoking is a serious health problem worldwide and a leading cause of mortality. Nicotine, the addictive component of tobacco, affects a range of emotional responses, including anxiety-related behaviors. Although perceived by smokers to be anxiolytic, evidence suggests that smoking increases anxiety and that mood fluctuates with nicotine intake. Thus, nicotine addiction may depend on easing the psychobiological distress caused by its abuse. The lateral habenula (LHb) has been implicated as a neural substrate for acute nicotine-induced anxiety, but its role in anxiety-like behaviors associated with chronic nicotine exposure has not been explored. Here, we assessed the effect of chronic nicotine exposure and its subsequent overnight withdrawal on anxiety-like behavior using both quantitative and multivariate T-pattern analysis in rats tested the hole-board apparatus. Additionally, we explored the role of the LHb by comparing the behavioral effects of short-term nicotine withdrawal in chronically treated LHb-lesioned rats. Quantitative analysis revealed increased anxiety-like behavior in chronically treated overnight nicotine-deprived rats, as manifested in reduced general and focused exploratory behaviors, which was eased in animals that received nicotine. Quantitative analysis failed to reveal a role of the LHb in short-term chronic nicotine deprivation-induced anxiety. Conversely, T-pattern analysis of behavioral outcomes revealed that chronic nicotine-treated rats following nicotine still show anxiety-behavior. Moreover, it demonstrated that the LHb lesion induced a stronger anxiolytic-like response to the acute challenge of nicotine in chronically nicotine-exposed animals, implicating the LHb in the anxiogenic effect of chronic nicotine exposure. These data further highlight the LHb as a promising target for smoking cessation therapies and support the importance of T-pattern analysis for behavioral analysis.

Keywords: Serotonin, T-pattern analysis, behavior, withdrawal symptoms, Rats

Highlights

- Nicotine (1 mg/kg, i.p.) administration did not induce appreciable alteration in anxiety-like behavior in chronic nicotine-treated rats (6 mg/kg/day i.p., for 14 days) in the hole-board
- Overnight nicotine deprivation (saline, 1 ml/kg, i.p.) induced a higher level of anxiety-like behavior in chronic nicotine-treated rats
- Quantitative analysis failed to reveal a role for the LHb in nicotine-induced changes of emotional state
- T-pattern analysis instead revealed anxiety in chronic nicotine-treated rats following nicotine challenge and a hidden LHb permissive role in anxiogenic properties of nicotine
- Performing both quantitative and qualitative approaches to behavioral research is advised

1. Introduction

Worldwide, more than 20,000 people die from smoking-related illnesses every day (CDC, 2020, Di Giovanni, 2012, Leone et al., 2010, WHO, 2017). Although the negative health consequences of nicotine addiction are well known (Di Matteo et al., 2007, Stolerman and Jarvis, 1995, Rose, 2006), it is hard to quit and the chance of success of any given attempt is low, with more than 95% of smokers relapsing within 12 months (Tobacco, 2008). One of the reasons for such dependence and failure to quit, as perceived by smokers, is the anxiolytic effect of chronic nicotine (ab)use while its abstinence is highly anxiogenic and dysphoric (Parrott and Murphy, 2012). In reality, scientific evidence shows that the link between anxiety and nicotine is more complex and even suggests an opposite scenario (Picciotto et al., 2002, Picciotto et al., 2015, Besson and Forget, 2016). Indeed, we have recently shown that acute exposure to medium-high doses of nicotine induced clear anxiogenic-like behavior in rats (Casarrubea et al., 2015a) in agreement with some studies (Ouagazzal et al., 1999b, Zarrindast et al., 2010, Hayase, 2007, Nasehi et al., 2011) but not with others that showed anxiolytic effects (O'Neill and Brioni, 1994, McGranahan et al., 2011, Villégier et al., 2010). Moreover, tolerance to nicotine's effect on anxiety did not develop over time in animal studies using hole-board (HB) experiments with a range of nicotine doses (Casarrubea et al., 2020), however this was not consistent with studies using elevated plus maze (EPM) (Irvine et al., 2001, Biala and Kruk, 2009, Ericson et al., 2000, Ijomone et al., 2015). Consistently, smokers show a higher level of anxiety and stress compared to non-smokers (Parrott and Murphy, 2012) and are at increased risk of anxiety disorders and panic attacks (Bruijnzeel, 2012, Moylan et al., 2013). Consequently, quitting smoking has been associated with a moderate reduction in anxiety levels (McDermott et al., 2013, Taylor et al., 2014). Interestingly, smokers show mood fluctuations in relation to nicotine intake with negative feelings preceding a cigarette (i.e., in abstinence/craving) (Bares et al., 2018, Parrott, 1994). Therefore, the *mood gain* after a cigarette that is reported by smokers would be more a *mood normalization* to the level of non-smokers. Thus, like for other drugs of abuse, nicotine addiction may depend on easing the psychobiological distress caused by its abuse (Parrott, 2015). This hypothesis has not been experimentally tested yet and we, therefore, wanted to clarify the effect of chronic nicotine exposure on anxiety-like behavior and possible fluctuations during its daily intermittent exposure in rats. Thus, we compared the effect of morning acute nicotine challenge (1 mg/kg, i.p.; equivalent to the first cigarette of the day) to saline (equivalent to overnight nicotine deprivation) in rats chronically exposed to high nicotine dose (6 mg/kg/day) and tested in the hole-board (HB) apparatus.

This dose of nicotine provides plasma levels approximately in the range of moderate (1 pack of cigarette per day) smokers (Lichtensteiger et al., 1988, Trauth et al., 2000).

We chose HB behavioral test since it has been useful for studying general and focused exploration in relation to anxiety under normal conditions (File and Wardill, 1975, Casarrubea et al., 2009a, Casarrubea et al., 2009b) and nicotine exposure in rodents (Casarrubea et al., 2015a, Casarrubea et al., 2020). T-pattern analysis (TPA) has been employed because it is an approach able to detect even subtle characteristics (and changes) in behaviour (Magnusson, 2000, Casarrubea et al., 2015a, Casarrubea et al., 2018).

Nicotine's powerful addictive and aversive effects are mainly mediated by modulating dopamine (DA) (Corrigall et al., 1992, Di Chiara, 2000) and other neurotransmitters such as serotonin (5-HT), glutamate, nitric oxide and gamma-aminobutyric acid in different brain areas (Di Giovanni, 2012, Di Matteo et al., 2010, Pierucci et al., 2014, Bombardi et al., 2020). The lateral habenula (LHb), a small epithalamic structure, seems to be crucial in both the rewarding and aversive effects of drugs of abuse including nicotine (Mathis and Kenny, 2019, Baldwin et al., 2011, Fowler and Kenny, 2014) by modulating DA and 5-HT systems (Proulx et al., 2014). LHb hyperactivity is observed in depression (Cerniauskas et al., 2019), ethanol-induced conditioned test aversion (Tandon et al., 2017) and ethanol withdrawal anxiety (Kang et al., 2018). Nicotine is also capable of increasing the firing rate of the LHb neurons *in vitro* (Zuo et al., 2016a) and *in vivo* (Pierucci et al., 2011), and LHb lesion counteracts acute nicotine-induced anxiety (Casarrubea et al., 2015a). Considering such evidence, it is possible to infer that nicotine-induced LHb excitation is a pivotal part of the neural substrate for nicotine-induced anxiety.

The present study has also been designed to investigate whether the LHb plays a role in chronic nicotine-induced anxiety by comparing the behavioral effects of a challenge of 1 mg/kg nicotine in LHb-sham-lesioned and LHb-lesioned rats exposed to chronic nicotine (6 mg/kg/day). Moreover, we compared these effects with those induced by chronic saline and chronic nicotine (6 mg/kg/day) for 2 weeks in unlesioned animals.

We found that chronically treated overnight nicotine-deprived rats (those that received the morning saline) displayed higher anxiety-like behavior compared to those receiving nicotine challenge. T-pattern, but not quantitative analysis, revealed a LHb permissive role in the anxiogenic properties of nicotine.

The effect of the LHb in nicotine addiction development and the comprehension of behavioral dynamics underlying chronic nicotine dependence may be crucial for the study of new therapeutic approaches aimed at the interruption of this harmful addiction and, more generally, for the treatment of anxiety disorders.

2. Methods

2.1. Experimental apparatus

The HB equipment consisted of a square (50×50 cm) open-field arena made of white opaque Plexiglas with a raised floor, containing four equidistant holes 4 cm in diameter. Each hole center was 10 cm distance from the two adjacent walls. The HB floor was positioned 5 cm above a white opaque Plexiglas sub-floor. The square arena was surrounded by three white opaque Plexiglas walls (50×50 cm) and a front transparent wall (50×50 cm). A digital camera (Toshiba HD-DV camcorder P10) was placed in front of the transparent wall to record the behavior of each subject. Video files were stored on a personal computer for the following analyses.

2.2. Animals and housing

Sprague-Dawley male rats, weighing 300 ± 50 g, were bred and housed at the animal house of the Department of Physiology and Biochemistry, University of Malta, with a room temperature constantly monitored and maintained at a temperature of 21 ± 1 °C and a humidity of 60 ± 5%. A light-dark cycle of 12 h with lights on at 7:00 am and off at 7:00 pm was provided. All subjects received free access to food and water. Procedures involving animals and their care were conducted in conformity with European Law (EU Directive 2010/63/EU) and the Institutional Animal Use and Care Committee (IAUCC) at the University of Malta. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. LHb lesioning procedure, testing procedure and drug administration

Animals were randomly assigned to groups, each encompassing 10 subjects. Twenty rats received bilateral electrolytic LHb lesion according to a protocol developed in our lab (Casarrubea et al., 2015a). Animals were anesthetized with chloral hydrate (Sigma; 8% w.v.; 400 μg/kg i.p.) and mounted onto a stereotaxic frame. Body temperature was maintained with a homeothermic blanket (~37°C; Harvard Apparatus, Cambridge, UK). Two bipolar electrodes, made from stainless steel bifilar wire (California Fine Wire, Grover Beach, CA, USA) with their tips exposed and separated 0.5 mm, were attached to a micromanipulator (angled 10° to the coronal plane) and aimed bilaterally

at the LHb using stereotaxic coordinates; 3.6 mm posterior to bregma; 1.8 mm lateral to the midline; 5.0 mm ventral to the dura (Paxinos and Watson, 2007). Electrodes were manipulated into position and a 500 µA current applied for 30 sec using an optically isolated stimulator (DS3 Digitimer, Hertfordshire, UK). Electrodes were left in place for 2 min before removal. A further 20 Sham-lesioned animals were treated as above, however, electrodes were lowered only 3.5 mm ventral to the dura and no current was applied. Upon completion, rats received carprofen (5 mg/kg, s.c.) and were allowed to recover for 2 h under observation until anesthesia had dissipated. Animals were allowed to recover for 7-10 days before experimentation. After this period, all animals were chronically treated with 6 mg/kg/day of nicotine (3 daily injections of 2 mg/kg i.p., at 08:00, 14:00 and 18:00) for two weeks. A further group of 10 unlesioned animals was also included, receiving 6 mg/kg/day of nicotine with a similar schedule for 2 weeks (see supplementary material).

On the day of the experiment, rats were allowed to acclimate to the testing room for 30 min, placed in the center of HB and allowed to explore for 10 min while being video recorded. The apparatus was cleaned with EtOH between rats. Rats were treated with saline (nicotine's vehicle) or nicotine (1 mg/kg), administered intraperitoneally in 1 ml/kg volume, 30 min before testing in the HB. Nicotine dose was selected based on previous data (Casarrubea et al., 2015a, File et al., 1998, Ouagazzal et al., 1999a, Casarrubea et al., 2016).

The following compounds were used: (-)-nicotine hydrogen tartrate salt ((−)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt (Sigma Aldrich, St. Louis, MO, USA) and carprofen. Carprofen and nicotine were dissolved in saline and pH was adjusted to 7.4 when required. The dose of nicotine (expressed as base form) used in this study was 2.10 mg/kg/day.

2.4. Histology

Histological verification of the LHb lesion was conducted upon experimental completion. Animals were euthanized with a chloral hydrate (8% w.v.; i.v.) overdose. Brains were removed and immersed in formalin solution (10%) for 2 days. Serial coronal sections (100-120 µm) of the LHb were cut using a vibratome, mounted onto slides and photographed under a light microscope. LHb lesions were considered acceptable when surrounding areas (e.g. paraventricular nuclei and medial habenula) were left intact. The brains of sham animals were also checked to ensure the LHb remained undamaged. Only animals with satisfactory lesions were included in the study (Fig. 1).

2.5. Data analysis

A list of all the components of the behavioral repertoire and their formal description, is shown in Fig. 2. Such a list, or ethogram, is based on components and behavioral categories employed in preceding studies (Casarrubea et al., 2017, Casarrubea et al., 2020, Casarrubea et al., 2009a, Casarrubea et al., 2009b) and represents the observation instrument used in the present study. It encompasses four main categories and eleven behavioral components. - *General Exploration*: Walking (Wa), Immobile Sniffing (IS), Climbing (Cl), Rearing (Re); - *Focused Exploration*: Edge-Sniff (ES), Head-Dip (HD); - *Grooming Activity*: Front-Paw Licking (FPL), Hind-Paw Licking (HPL), Face Grooming (FG), Body Grooming (BG); the last category contains only - *Immobility* (Im).

Based on this ethogram, video files of each subject were annotated by an highly trained observer blind to treatment and lesion status using a professional software tool (The Observer, Noldus Information Technology bv, Netherlands) and event log files were generated for each subject. The propaedeutic assessment of reliability was evaluated based on five video files, randomly taken from the experimental recordings, and scored by the observer in two different moments. In a second step, after such a propaedeutic step, all video files were annotated. Once event log files of all subjects were obtained, they were re-arranged by removing non-essential information to contain exclusively performed behaviors and their onset. These files were then utilized to perform both quantitative and T-pattern analyses (TPA). Various concepts concerning observation methodology and approaches utilized in the present study can be found in (Anguera et al., 2011, Sacket, 1977). TPA is a multivariate technique able to reveal the temporal architecture of behavior. Such an approach orbits around the utilization of a software tool known as Theme (PatternVision Ltd., Iceland). In brief, this software can reveal hidden sequences of events, called T-patterns, based on the detection of significant constraints on the intervals separating them.

In brief, given a distribution of events occurring within a T0-Tx observation period (e.g. various behavioral components “a”…”b”…”c”… “x”… “y”… “z”… etc.), a search algorithm compares the distributions of each pair of the behavioral events, e.g. “a” and “b”, searching for a time window so that “a” is followed by “b” within such a window. If this condition between event “a” and event “b” is verified, a first-level T-pattern encompassing only two events, that is (a b), is detected; then such first level T-pattern is considered as “a” or “b” terms for the detection of higher-order patterns, e.g., ((a b) c)... and so on up to any level. When no more patterns are detected the search stops. That said, an important feature of TPA is its ability to describe behavioral dynamics under a qualitative perspective. On this subject, three important qualitative features of T-patterns are represented by their (i) *Variability* (i.e. the number of T-patterns of different composition detected), (ii) *Complexity* (i.e.

the length of T-patterns, namely, the number of events in sequence) and, finally, (iii) *Recursivity* (i.e. the number of times each T-pattern does occur) (Casarrubea et al., 2019). As discussed elsewhere (Anguera et al., 2018, Teddie and Tashakkori, 2010), the synergic utilization of qualitative and quantitative approaches is able to offer an enhanced and more comprehensive view of the investigated behavior.

Concepts, theories and procedures concerning the detection and analysis of T-patterns can be found in a recent monography (Magnusson et al., 2016), in a comprehensive review (Casarrubea et al., 2015b) and various articles (Magnusson, 1996, Magnusson, 2000, Magnusson, 2004, Magnusson et al., 2016). In the present study, to maintain results of TPA comparable with previous ones, we used the same rigorous detection parameters employed in previous studies (Casarrubea et al., 2020, Casarrubea et al., 2015a) i.e., minimum occurrences = 6; significance level = 0.0001; lumping factor = 0.90; minimum percent of samples = 60.

For each group, the following parameters of the behavioral responses were analyzed: mean duration and percent distributions of behavioral components; the structure of all the different T-patterns (terminal strings and related tree structures); length distribution of different T-patterns, both in real and randomly generated data; mean length of T-patterns; mean occurrences of T-patterns and, finally, percent distribution of T-patterns encompassing behavioral components of hole-exploration.

2.6. Statistics

As to mean durations and occurrences, possible significant results among groups were assessed using Two-Way Analysis of Variance for independent samples; $p < 0.05$ was considered a significant value. As to T-pattern analysis, even if each sequence orbits around the detection of a significant relationship among the events in patterns, in data with hundreds or thousands of events, an exceedingly high number of possible relationships might be possible. Such an aspect could raise an important issue: whether the T-patterns are detected only by chance. Theme, the software tool used for T-pattern detection, deals with such an important aspect by repeatedly randomizing and reanalyzing the original data, using the same search parameters utilized in the detection process performed in the real data. Then, the mean number of T-patterns of each length detected in the randomized data is compared with the number of patterns identified in the original data. Mean occurrences and mean length of T-patterns detected in real data were assessed using the Two-Way Analysis of Variance for independent samples; $p < 0.05$ was considered significant. Comparison with unlesioned animals was performed with 1-way ANOVA analysis followed by Bonferroni's post-hoc test (see supplementary material).

Finally, percent distribution of T-patterns encompassing behavioral components of hole-exploration was assessed using the Chi-square test; $p < 0.05$ was considered significant.

3. Results

3.1. Quantitative analyses

3.1.1. Mean durations

Concerning mean durations (Fig. 3), two-way ANOVA revealed significant main effects of nicotine for Walking, Immobility and Immobile-Sniffing. No significant results were detected for the remaining components of the behavioral repertoire. A lesion x treatment ANOVA showed no significant effects or interactions in any of the behaviors observed.

- *Walking:* There was no significant interaction between LHb lesioning and nicotine treatment for walking mean duration score, $F(1, 36) = 0.052$, $p = 0.821$. There was no significant main effect of LHb lesioning on walking mean duration score, $F(1, 36) = 0.016$, $p = 0.900$. Finally, there was a significant main effect (increase) of nicotine treatment on walking mean duration score, $F(1, 36) = 39.361$, $p < 0.001$.

- *Immobility:* There was no significant interaction between LHb lesioning and nicotine treatment for immobility mean duration score, $F(1, 36) = 0.094$, $p = 0.761$. There was no significant main effect of LHb lesioning on immobility mean duration score, $F(1, 36) = 0.031$, $p = 0.860$. Finally, there was a significant main effect (decrease) of nicotine treatment on immobility mean duration score, $F(1, 36) = 10.746$, $p = 0.002$.

- *Immobile Sniffing:* There was no significant interaction between LHb lesioning and nicotine treatment for immobile sniffing mean duration score, $F(1, 36) = 0.119$, $p = 0.733$. There was no significant main effect of LHb lesioning on immobile sniffing mean duration score, $F(1, 36) = 2.627$, $p = 0.114$. Finally, there was a significant main effect (increase) of nicotine treatment on immobile sniffing mean duration score, $F(1, 36) = 15.844$, $p < 0.001$.

3.1.2. Mean occurrences

As to mean occurrences (Fig. 4), two-way ANOVA revealed significant main effects of nicotine for Walking, Immobile-Sniffing, Edge-Sniff and Head-Dip. Significant main effects of LHb lesioning were revealed for Immobile-Sniffing, Immobility and Edge-Sniff. A lesion x treatment ANOVA showed no significant effects or interactions in any of the behaviors observed.

- *Walking:* There was no significant interaction between LHb lesioning and nicotine treatment for walking mean occurrence score, $F(1, 36) = 0.608$, $p = 0.441$. There was no significant main effect of LHb lesioning on walking mean occurrence score, $F(1, 36) = 0.683$, $p = 0.414$. There was a significant main effect (increase) of nicotine treatment on walking mean occurrence score, $F(1, 36) = 35.915$, $p < 0.001$.
- *Immobility:* There was no significant interaction between LHb lesioning and nicotine treatment for immobility mean occurrence score, $F(1, 36) = 0.719$, $p = 0.402$. There was a significant main effect (increase) of LHb lesioning on immobility mean occurrence score, $F(1, 36) = 6.098$, $p = 0.018$. There was no significant main effect of nicotine treatment on immobility mean occurrence score, $F(1, 36) = 0.123$, $p = 0.728$.
- *Immobile-Sniffing:* There was no significant interaction between LHb lesioning and nicotine treatment for immobile sniffing mean occurrence score, $F(1, 36) = 1.233$, $p = 0.274$. There was a significant main effect of LHb lesioning (increase) on immobile sniffing mean occurrence score, $F(1, 36) = 6.039$, $p = 0.019$. There was a significant main effect (increase) of nicotine treatment on immobile sniffing mean occurrence score, $F(1, 36) = 46.640$, $p < 0.001$.
- *Edge-Sniff:* There was no significant interaction between LHb lesioning and nicotine treatment for edge sniff mean occurrence score, $F(1, 36) = 0.023$, $p = 0.880$. There was a significant main effect of LHb lesioning (increase) on edge sniff mean occurrence score, $F(1, 36) = 6.109$, $p = 0.018$. There was a significant main effect (increase) of nicotine treatment on edge sniff mean occurrence score, $F(1, 36) = 10.446$, $p = 0.003$.
- *Head-Dip:* There was no significant interaction between LHb lesioning and nicotine treatment for head dip mean occurrence score, $F(1, 36) = 0.158$, $p = 0.693$. There was no significant main effect of LHb lesioning on head dip mean occurrence score, $F(1, 36) = 1.917$, $p = 0.175$. There was a significant main effect (increase) of nicotine treatment on head dip mean occurrence score, $F(1, 36) = 7.145$, $p = 0.011$.

3.2. T-pattern analyses

Results of T-pattern detection in terms of terminal strings, overall occurrences and tree structures of each T-pattern detected are presented in Fig. 5. In LHb Lesion + Saline animals, 17 different T-patterns occurred 1878 times; in LHb lesion + Nicotine animals, 17 different T-patterns occurred 2055 times; in Sham Lesion + Saline animals, 41 different T-patterns occurred 1629 times; finally, in Sham Lesion + Nicotine animals, 33 different T-patterns occurred 2769 times.

The length distribution of T-patterns (that is the number of T-patterns of different lengths detected for each group) are illustrated in Fig. 6. In LHb Lesion + Saline animals, 7 different T-patterns encompass two events, 7 contain three events and 3 contain four events in their structure; in LHb lesion + Nicotine animals, 11 T-patterns have two events, 5 have three events and 1 has four events; In Sham Lesion + Saline animals, 15 T-patterns have two events in their structure, 10 have three events, 10 have four events and 6 contain a sequence of five events; finally, in Sham Lesion + Nicotine animals, 14 T-patterns have two events in their structure, 8 have three events, 8 have four events and 3 have five events.

Mean occurrences ± SE of detected T-patterns and their mean length ± SE are illustrated in Fig. 7 and Fig. 8, respectively. Concerning mean occurrences of T-patterns (Fig. 7), there was no significant interaction between LHb lesioning and nicotine treatment, $F(1, 104) = 0.882$, $p = 0.350$. There was a significant main effect (increase) of LHb lesioning on T-pattern mean occurrence, $F(1, 104) = 8.971$, $p = 0.003$. Finally, there was no significant main effect of nicotine treatment on T-pattern mean occurrence, $F(1, 104) = 2.304$, $p = 0.132$.

As to mean length of T-patterns (Fig. 8): there was no significant interaction between LHb lesioning and nicotine treatment for T-pattern mean length, $F(1, 104) = 0.207$, $p = 0.650$. There was a significant main effect (decrease) of LHb lesioning on T-pattern mean length, $F(1, 104) = 6.151$, $p = 0.015$. Finally, there was no significant main effect of nicotine treatment on T-pattern mean length, $F(1, 104) = 1.706$, $p = 0.194$.

Finally, as to percent distributions of T-patterns encompassing behavioral components of hole-exploration (Fig. 9), comparisons between LHb Lesion + Saline (Fig. 9A) and LHb Lesion + Nicotine (Fig. 9B) revealed significant changes for Edge-Sniff (36.42% Vs 33.38%, $p < 0.05$) and Head-Dip (42.44% Vs 23.70%, $p < 0.0001$); comparisons between Sham Lesion + Saline (Fig. 9C) and Sham Lesion + Nicotine (Fig. 9D) revealed significant changes for Edge-Sniff (56.35% Vs 46.26%, $p < 0.0001$).

Comparisons between LHb Lesion + Saline (Fig. 9A) and Sham Lesion + Saline (Fig. 9C) revealed significant changes for Edge-Sniff (36.42% Vs 56.35%, $p < 0.0001$) and Head-Dip (42.44% Vs 33.58%, $p < 0.0001$); finally, comparison between LHb Lesion + Nicotine (Fig. 9B) and Sham Lesion + Nicotine (Fig. 9D) revealed significant changes for Edge-Sniff (33.38% Vs 46.26%, $p < 0.0001$) and Head-Dip (23.70% Vs 37.31%, $p < 0.0001$).

Comparisons with unlesioned animals, for both quantitative and qualitative data, can be found in the Supplementary material annex.

4. Discussion

The main findings of this investigation were manifold. The quantitative analysis showed a high anxiety-like state in chronically treated rats deprived of nicotine overnight, while nicotine challenge eased anxiety-like behaviors to the chronic saline-treated animals. Second, it did not reveal any LHb role in such an effect. On the other hand, TPA revealed that the LHb lesion, independently from the treatment, induced a reduction in the complexity of behavior that was accompanied by a significant increase in the repetitiveness of less variable behavioral sequences. Finally, and more importantly, TPA analysis showed that LHb-lesioned rats had a stronger anxiolytic-like response to nicotine, suggesting that the LHb is involved in the anxiogenic effect of chronic nicotine exposure.

The effect of cigarette smoking on anxiety is still a matter of debate, although their association is undeniable. This bidirectional relationship is complex, likely due to the complexity of anxiety disorders, the multifaceted effects of nicotine in the brain and possible common neuropathological mechanisms (Morissette et al., 2007, Kutlu and Gould, 2015). Indeed, smoking has been reported to be anxiolytic and its abstinence is perceived to heighten anxiety. Moreover, smoking is often used as "self-medication" to alleviate anxiety symptoms in patients with generalized anxiety, post-traumatic stress and other neuropsychiatric disorders (Parrott and Murphy, 2012, Bruijnzeel, 2012, Moylan et al., 2013) who show a higher smoking rate compared to the general population (Lawrence et al., 2010, Moylan et al., 2013). On the other hand, nicotine abuse can lead to increased anxiety (Parrott and Murphy, 2012) and worsens fear-related symptoms in anxiety disorders (Kutlu and Gould, 2015). Consistently, anxiety levels are reduced in quitting smokers (McDermott et al., 2013, Taylor et al., 2014). Nicotine reinforcing efficacy is lower compared to the other drugs of abuse (Le Foll and Goldberg, 2009) and relieving the psychobiological distress caused by its abuse (Parrott, 2015) might contribute to its addictive potential.

4.1. Quantitative analyses

Overall, our quantitative results in rats chronically exposed to nicotine seem to support this scenario. Taking into consideration the duration and frequency of Walking and Immobile-Sniffing, overnight nicotine-deprived subjects spent much less time exploring and did so much less frequently compared to those challenged with nicotine, in both lesioned and unlesioned animals (see supplementary material). Of note, locomotor hyperactivity induced by LHb lesion (Jean-Richard Dit Bressel and McNally, 2014, Wang et al., 2013, Nielson and McIver, 1966, Lecourtier et al., 2008, Gifuni et al.,

2012), likely due to the strong inhibitory control over midbrain DA neurons exerted by the LHb (Matsumoto and Hikosaka, 2007), is not modified by acute 1 mg/kg nicotine (Casarrubea et al., 2015a). Our chronic nicotine regime instead maintained locomotion in both LHb-lesioned and Sham-lesioned animals, preserving locomotory activity in line with that of unlesioned animals chronically treated with saline or nicotine. This indicates that the locomotory depressant response to nicotine observed at lower doses (Casarrubea et al., 2020) in the same experimental conditions develops tolerance (see supplementary material) after chronic treatment with the higher dose used in this study. In overnight nicotine-deprived rats, a depression of locomotion was instead observed in both LHb-lesioned and Sham-lesioned animals that was probably correlated to the high anxiety-like state associated with nicotine withdrawal. Therefore, LHb-lesion was not capable of altering the locomotor activity in chronic nicotine exposure rats, differently from its effect in nicotine acute-treated rats (Casarrubea et al., 2015b). The reason of such a lack of effect it is unclear, LHb lesion for example enhanced the locomotor response to morphine (Funk and Stewart, 1992), apomorphine (Heldt and Ressler, 2006) and amphetamine (Gifuni et al., 2012), pointing out a different LHb control of nicotine-induced locomotion compared to other psychostimulants.

In animals subject to short-term nicotine withdrawal, a reduction in behaviors aimed at the focused exploration of the holes i.e., occurrences of Edge-Sniff and Head-Dip were evident, highly indicative of heightened anxiety (Casarrubea et al., 2017, Casarrubea et al., 2009b). Thus, the overnight abstinence from chronic administration of nicotine (2 weeks, 2 mg/kg, 3 times/day), known to produce plasma nicotine levels similar to those observed in heavy smokers (Benowitz et al., 1990, Matta et al., 2007), led to a considerable aggravation of anxiety-related behaviors. Conversely, a single administration of 1 mg/kg nicotine, which induces anxiety in naïve rats in the same experimental conditions (Casarrubea et al., 2015a), reverted this condition, bringing behavioral values toward that recorded after chronic saline administration (Casarrubea et al., 2020), equivalent to non-smoker subjects (see supplementary material).

From our evidence, it remains unclear whether nicotine has a direct effect on alleviating anxious mood, or whether it is merely relieving its short-term withdrawal (about 12 h).

Our findings seem consistent with the theory that mood fluctuations in smokers occur due to intermittent nicotine intake and consequent peak-to-trough oscillations in blood levels of nicotine from cigarette to cigarette. Thus, smoking would produce a mood normalization rather than a mood gain (Bares et al., 2018, Parrott, 1994). Here, we showed that high intake of nicotine (6 mg/kg/day, corresponding to 2.1 mg nicotine free base) induced tolerance to nicotine-induced anxiety (see supplementary material) in line with other EPM evidence (Irvine et al., 2001, Biala and Kruk, 2009, Ericson et al., 2000, Ijomone et al., 2015) but in contrast to the effect of lower nicotine doses in HB

(Casarrubea et al., 2020). Interestingly, the anxiety induced by lower doses of chronic nicotine (Casarrubea et al., 2020) was different from that observed after acute nicotine treatment (Casarrubea et al., 2015a), resulting in a more defined, structured and organized anxiety-related behavior (Casarrubea et al., 2020).

LHb-lesioned rats showed changes in the frequencies but not in the durations of some behaviors, such as Edge-Sniff, Immobile-Sniffing and Immobility, compared to Sham-lesioned animals. Of these, the significant increase in Edge-Sniff is particularly interesting. This behavioral component is an assessment activity characterized by sniffing the edge of the holes which may, or may not, be followed by Head-Dip, i.e., the exploration of the hole inside. The relationship between Edge-Sniff and Head-Dip is heavily influenced by animal anxiety level that, in turn, has an important impact on the motivation to explore (Casarrubea et al., 2017, Casarrubea et al., 2009b). Therefore, the increase of Edge-Sniff not accompanied by a concomitant increase of Head-Dip must be considered strongly suggestive of increased anxiety (Casarrubea et al., 2017, Casarrubea et al., 2009b) in subjects with LHb lesion. Such an increased anxiety level is confirmed by the significant increase of Immobile-Sniffing and Immobility, behaviors that imply the absence of locomotion and the maintenance of the same position.

To summarize, quantitative outcomes discussed above show increased anxiety-related behaviors in overnight nicotine-deprived subjects compared to those receiving nicotine challenge following chronic nicotine treatment. In addition, nicotine itself is not capable of inducing anxiety-like behavior in chronic nicotine-treated animals when compared to chronic saline treatment in unlesioned animals (see supplementary material). Moreover, LHb-lesioned animals exhibit some behaviors indicative of increased anxiety, although these are subtle changes.

4.2. T-pattern detection and analysis

The analysis of T-patterns highlights behavioral dynamics, in the different groups, not appreciable through conventional quantitative approaches. This aspect should be seen as complementary to what the measurements of durations and frequencies show. When applying TPA, individual behavioral elements and their quantitative features are not taken into consideration (for example the durations or occurrences of each element of the behavioral repertoire, disjointed from the comprehensive behavioral structure). TPA is a multivariate analysis and, as such, it relates multiple elements and considers their relational structure over time. Evaluation is therefore carried out more on the *qualitative* characteristics of the behavior than on the quantitative ones (Casarrubea et al., 2019).

4.2.1. T-patterns: basic qualities

Chronic nicotine exposure and its overnight withdrawal caused similar alterations in all three basic *qualities* of the T-patterns, i.e., their variability, complexity and recursivity (Casarrubea et al., 2019). On this subject, it is worth mentioning that such an ability of TPA, namely, to describe behavioral dynamics under a qualitative perspective, do represent a great benefit of such an approach. Notably, as also underlined in introduction section, the synergic utilization of qualitative and quantitative approaches is able to offer a better and more detailed portrait of the observed behavior, as extensively highlighted and discussed in other works from different Authors (Anguera et al., 2018, Teddie and Tashakkori, 2010). More in detail, concerning the three qualities above mentioned:

- ***Variability:*** Sham-lesioned animals showed a high number of different T-patterns (41 and 33 for those treated with saline or nicotine, respectively), in line with the results obtained with chronic nicotine at lower doses (0.3, 1.5-3 mg/kg) in unlesioned rats (Casarrubea et al., 2020). On the other hand, LHb-lesioned rats showed only 17 different T-patterns (both in animals treated with saline or nicotine).
- ***Complexity:*** the aforementioned reduction in variability was accompanied by a reduction in the complexity of the behavioral sequences in subjects with LHb lesion, with no group exhibiting T-patterns containing 5 events and displayed T-patterns having shorter mean lengths.
- ***Recursivity:*** differently from the two qualitative parameters mentioned above, namely variability and complexity, here it is possible to appreciate an increase. In fact, in subjects with LHb lesion, each T-Pattern occurs more times. This datum clearly emerges when the mean occurrences are taken into consideration as well. This outcome lays down for a behavior, in subjects with LHb lesion, more recursive / repetitive.

These three aspects, considered synergistically, highlight an effect of LHb lesion, which takes the form of a less variable, less complex, and more repetitive behavior that is similar in chronic nicotine exposed rats that are deprived of and challenged with nicotine. Therefore, LHb lesion seems to counteract the changes induced by chronic nicotine exposure bringing behavioral structure towards that observed in the chronic saline group, i.e., the non-smokers (supplementary results and (Casarrubea et al., 2020)) and further away from those observed in animals treated with chronic nicotine but without any LHb lesion (these findings and (Casarrubea et al., 2020)). Our TPA, therefore, confirms an anxiogenic role for the LHb in nicotine observed after chronic exposure, similar to that seen following acute nicotine administration (Casarrubea et al., 2015a).

4.2.2. T-patterns: structural features

The further study of the structure of T-patterns takes into consideration the anxiety-related components of focused exploration (Edge-Sniff and Head-Dip), which are related to the exploration of each of the four holes (relevant cues to the subject, a potential source of danger, and for this reason a source of anxiety-related behavior) and represent an index of the rodent's anxiety condition in the HB apparatus (Casarrubea et al., 2017, Casarrubea et al., 2009b).

Short-term nicotine withdrawal, in both LHb- and Sham-lesioned rats, induced a higher proportion of TP behaviors containing Edge-Sniff and Head-Dip compared to nicotine-treated groups. Thus, if on the one hand Edge-Sniff and Head-Dip are reduced from a quantitative point of view (which is indicative of clear increased anxiety), the TPA indicates reorganization of anxiety-related behavior, which is evidenced by the greater presence of these two exploration patterns in the animals subjected to overnight nicotine withdrawal (i.e., those that received saline challenge). This is a similar situation to that observed in chronically-treated unlesioned animals, where chronic nicotine treatment induced a more defined, structured, and organized anxiety-related behavior following the context in which the behavior occurs, i.e., the holes (Casarrubea et al., 2020).

These differences were less evident in Sham-lesioned subjects, in which there was no significant variation in the percentage of T-patterns containing Head-Dip. Thus, we might infer that LHb lesion worsened anxiety-like behavior in the withdrawal group. Notably, these issues are consistent with the results we have described regarding the delicate balance that exists between Edge-Sniff and Head-Dip following pharmacological manipulation of anxiety levels (Casarrubea et al., 2017, Casarrubea et al., 2009b).

Concerning the role of the LHb on the effect of nicotine challenge on these TP structures, LHb lesion induced a reduction of Edge-Sniff and a Head-Dip compared to Sham-lesioned rats. Therefore, this last group of data suggests that LHb-lesioned rats under chronic nicotine exposure and following nicotine challenge present a lower level of a less defined and organized anxiety-related behavior in comparison with Sham-lesioned subjects. Interestingly, the same dose of 1 mg/kg of nicotine, delivered acutely to naïve unlesioned animals, induced a decrease in the proportion of T-patterns containing HD and/or edge sniff that was reversed upon LHb lesion (Casarrubea et al., 2015a).

The lower anxiety induced by nicotine challenge following its chronic exposure is likely due to plastic changes in the CNS, ranging from molecular to structural levels. For instance, chronic exposure to nicotine produces desensitization and upregulation of nAChRs in different areas (Picciotto et al., 2015, Cohen and George, 2013, Slotkin, 2004).

Chronic nicotine also changes other heteroreceptor signals such as serotonin (5-HT) 5-HT_{2A} receptors (Bombardi et al., 2020, Pierucci et al., 2014), which are known to be altered in anxiety (Jiang et al., 2009, Moulédous et al., 2018) and are important in controlling brain areas that are involved in anxiety circuitry (Bombardi and Di Giovanni, 2013). Moreover, nicotine intake likely contributes to the alleviation of anxiety induced by tobacco dependence by modifying connectivity among anxiety circuitry brain areas such as the hippocampus, prefrontal cortex and amygdala (Robinson et al., 2019). Indeed, it has been shown that overnight nicotine withdrawal induces functional changes such as increased resting-state functional connectivity of the amygdala-insula circuit and the insula-default mode network circuit (Sutherland et al., 2013).

We recently showed that the anxiety induced by acute nicotine is alleviated by LHb lesion (Casarrubea et al., 2015a) and nicotine induced excitation of LHb neurons *in vivo* (Pierucci et al., 2011) and *in vitro* via the activation of α6*-nAChRs on LHb neurons and glutamatergic synapses (Zuo et al., 2016a). The LHb is extremely responsive to the aversive effects of nicotine, being excited at concentrations that are ineffective in modifying dopaminergic neuron activity (Zuo et al., 2016a). LHb hyperactivity can also be observed in ethanol aversion (Tandon et al., 2017) and during drug withdrawal symptoms such as ethanol withdrawal-induced anxiety (Kang et al., 2018) and anxiety induced by cocaine reinstatement (Gill et al., 2013).

Identifying the exact brain circuitry responsible for nicotine-induced anxiety is not an easy task, but likely does not involve the downstream LHb input to the ventral tegmental area or the upstream entopeduncular nucleus projections to the LHb since their optogenetic and chemogenic activation reduced immobility and motivation but not anxiety in chronically stressed mice (Cerniauskas et al., 2019). Instead, it is plausible that anxiety induced by nicotine involves the serotonergic projections to the LHb. Indeed, 5-HT is crucially involved in anxiety (den Boer et al., 1987) and nicotine affects 5-HT function (Pierucci et al., 2014). Moreover, optogenetic activation of 5-HT projections to the LHb modulates the excitability of LHb neurons (Zhang et al., 2018) and increases anxiety-like behavior in the EPM and dark/bright maze tests in mice (Ohmura et al., 2014). On the other hand, LHb lesion increased 5-HT in the dorsal raphe nucleus (DRN) (Yang et al., 2008), while optogenetic silencing of the LHb increased 5-HT in the amygdala and blocked the typical anxiety-like behaviors produced by inescapable tail shock (Dolzani et al., 2016).

In line with this evidence, 5-$HT_{2A/2C}$ receptor activation induces strong modulations of LHb neuronal activity (Delicata et al., 2018, Bombardi et al., 2020, Zuo et al., 2016b) and their LHb blockade alleviated anxiety-like behavior induced by withdrawal from alcohol consumption (Fu et al., 2020). Therefore, it is possible to infer that LHb excitation via dysfunction of 5-$HT_{2A/2C}$ receptor signaling might be the neural substrate for overnight nicotine withdrawal-induced anxiety in chronically

exposed rats that we observed in our study. Furthermore, tolerance to this nicotine anxiogenic effect may develop over time with nicotine being incapable of exciting the LHb/5-$HT_{2A/2C}$ receptor system and re-establishing a low anxiety state, an interesting hypothesis that remains to be experimentally proved. Nevertheless, many other mechanisms can be involved, including the unresponsiveness of the hypothalamic-pituitary axis and corticosterone release observed for instance in LHb-lesioned animals during stress (Jacinto et al., 2017).

Of note, from our quantitative data, there was no observed interaction between nicotine treatment and lesion status, suggesting that LHb lesion had no role in the anxiety-related effects of nicotine, although LHb-lesioned animals had a higher level of anxiety. On the other hand, our results using a multivariate approach show that T-pattern analysis was capable of revealing effects that otherwise would have been neglected, i.e., the anxiolytic nicotine activity in LHb-lesioned rats. Therefore, the LHb may be a key component of the circuitry of nicotine-induced anxiety for both acute and chronic nicotine exposure, although with a different modality.

Although we obtained interesting results, the study encounters some limitations. For instance, the drug administration route. Here we used passive, non-contingent exposure paradigms in vivo (experimenter-administered) exposure to nicotine, while perhaps self-administered drug delivery may produce different effects (see for example the effect of different routes on nicotinic receptors in medial habenula (Jin et al., 2020). Moreover, the effects seen here are dependent on the dose of 6 mg/kg/day nicotine corresponding to about 2 mg/kg/day of freebase (moderate smokers), therefore a dose-response more comprehensive study would be advisable. Finally, we cannot completely exclude the involvement of the MHb in the effects of the LHb lesion on nicotine seen in our study.

In conclusion, while the LHb has a clear role in acute nicotine-induced anxiety, with its lesion capable of counteracting the anxiety-like and the structure of behavioral changes induced by its acute administration (Casarrubea et al., 2015a), here we observed a more complex LHb role under chronic nicotine exposure. Notably, we confirmed that overnight nicotine withdrawal induced higher levels of anxiety state that decreased after nicotine administration toward non-smoker levels, in line with evidence indicating that nicotine addiction is sustained by alleviating withdrawal symptoms (Bares et al., 2018, Parrott, 1994, Parrott and Murphy, 2012, Parrott, 2015). However, TPA indicates that in our heavy smoking-like regimen, 1 mg/kg nicotine challenge, although less intense, is still anxiogenic compared to chronic saline treatment representative of non-smokers (supplementary material and (Casarrubea et al., 2020). Therefore, under both acute and chronic high-nicotine exposure, the LHb appears to have a permissive role in related anxiety-like behavior. The difference in intensity between the effect the LHb lesion on acute and chronic nicotine-induced behavioral changes may be determined by the fact that LHb-lesion attenuated anxiogenic behavior preferentially during

heightened anxiety (Gill et al., 2013) (i.e., during acute nicotine exposure or nicotine withdrawal). Future works should clarify the complex role of the LHb in different aspects of nicotine addiction in relation to anxiety state.

Based on the current results, it appears that a desirable characteristic of future pharmacological agents for smoking cessation would be to decrease LHb hyperactivity and contextual anxiety during early nicotine withdrawal, facilitating the ability of smokers to quit. More generally, our findings indicate that modification of the LHb function may also be a good pharmacological target for treating anxiety disorders.

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Conflicts of Interest

The authors declare no conflict of interest.

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Captions of figures

- **Fig.1.** Representative photomicrograph of the LHb lesion (**A**) and sham lesion (**B**) at the level of the interaural 5.20 mm (an enlargement of the box in **C**, taken from (Paxinos and Watson, 2007)). Also shown is the corresponding diagrammatic representation of the analogous coronal section (**C**). LHbM, Medial part of Lateral Habenula; LHbL, Lateral part of Lateral Habenula; MHb, Medial Habenula; sm, stria medullaris.

- **Fig. 2**. Ethogram of rat's behavior in the hole-board apparatus. **General exploration**: Walking (**Wa**) = rat walks around sniffing the environment; Immobile-Sniffing (**IS**): rat sniffs the environment standing on the ground; Rearing (**Re**) = rat maintains an erect posture without leaning against the Plexiglas box; Climbing (**Cl**) = rat maintains an erect posture leaning against the Plexiglas wall. **Focused exploration**: Edge-Sniff (**ES**): rat sniffs the border of one of the four holes; Head-Dip (**HD**) = rat puts its head into one of the four holes. **Grooming activity**: Front-Paw Licking (**FPL**): rat licks or grooms its forepaws; Hind-Paw Licking (**HPL**): rat licks or grooms its hind paws; Face-Grooming (**FG**): rat rubs its face (ears, mouth, vibrissae, eyes) with rapid circular movements of its forepaws; Body-Grooming (**BG**): rat licks its body combing its fur with fast movements of incisors. **Immobility** (**Im**): rat maintains a fixed posture. No movements are produced.

- **Fig. 3**. Mean durations ± SE (Y-axis) in seconds (s) of all the behavioral components of the behavioral repertoire (X-axis). * = significant ($p < 0.05$) main effect of pharmacological treatment (Saline Vs Nicotine, two-way ANOVA). Data obtained from the analysis of four groups (n = 10 subjects in each group). For abbreviations see fig.1.

- **Fig. 4**. Mean occurrences ± SE (Y-axis) of all the components of the behavioral repertoire (X-axis). * = significant ($p < 0.05$) main effect of pharmacological treatment (Saline Vs Nicotine, two-way ANOVA); # = significant main effect surgery (LHb Lesion Vs Sham Lesion, two-way ANOVA). Data obtained from the analysis of four groups (n = 10 subjects in each group). For abbreviations see fig.1.

- **Fig. 5**. Results of T-pattern detection. Terminal strings and tree structures of T-patterns detected in each group. The number on the left of each string indicates the corresponding tree structure illustrated on the right. Numbers on the right of each string indicate their overall

occurrences (Occs) and length. Data obtained from the analysis of four groups (n = 10 subjects in each group). For abbreviations see fig.1.

- **Fig. 6**. The overall number of different T-patterns (Y-axis) detected on the basis of their different length (X-axis) for each group. Filled bars = number of patterns detected in real data; white/empty bars = mean+1SD number of patterns detected in randomized data.

- **Fig. 7**. Mean occurrences ± SE (Y-axis) of T-patterns in each group (X-axis). # = significant ($p < 0.01$) main effect of lesion (LHb lesion Vs Sham Lesion, two-way ANOVA). Data obtained from the analysis of four groups (n = 10 subjects in each group).

- **Fig. 8**. Mean length ± SE (Y-axis) of T-patterns in each group (X-axis). # = significant ($p < 0.01$) main effect of lesion (LHb lesion Vs Sham Lesion, two-way ANOVA). Data obtained from the analysis of four groups (n = 10 subjects in each group).

- **Fig. 9**. Percent distribution of T-patterns containing ES and HD. * = significant ($p < 0.05$) difference between saline treatments and nicotine treatments (A Vs B and C Vs D), same lesion status (Chi-Square test); # = significant ($p < 0.05$) difference between LHb lesion and Sham lesions (A Vs C and B Vs D), same pharmacological treatment (Chi-Square test). Data obtained from the analysis of four groups (n = 10 subjects in each group). For abbreviations, see fig. 1.