frontiers in
CELLULAR NEUROSCIENCE
IMPACT 4.2 FACTOR

Lateral Habenula Regulates Temporal Pattern Organization of Rat Exploratory Behavior and Acute Nicotine-Induced Anxiety in Hole Board

Maurizio Casarrubea, Caitlin Davies, Fabiana Faulisi, Lucy Partdrige, Stephanie Chambers, Massimo Pierucci, Roberto Colangeli, Daniel Cassar, Mario Valentino, Richard Muscat, Arcangelo Benigno, Giuseppe Crescimanno and Giuseppe Di_Giovanni

Journal Name:	Frontiers in Cellular Neuroscience
ISSN:	1662-5102
Article type:	Original Research Article
First received on:	23 Jan 2013
Frontiers website link:	www.frontiersin.org

Lateral Habenula Regulates Temporal Pattern Organization of Rat Exploratory Behavior and Acute Nicotine-Induced Anxiety in Hole-Board

Maurizio Casarrubea[1]*, Caitlin Davies[2,3], Fabiana Faulisi[1], Lucy Partridge[2,3], Stephanie Chambers[2], Massimo Pierucci[2], Roberto Colangeli[2], Daniel Cassar[2], Mario Valentino[2], Richard Muscat[2], Arcangelo Benigno[1], Giuseppe Crescimanno[1], Giuseppe Di Giovanni[2,3]*

[1]Department of Experimental Biomedicine and Clinical Neurosciences, Human Physiology Section "Giuseppe Pagano", Laboratory of Behavioral Physiology, University of Palermo - Palermo, Italy. [2]Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta - Msida, Malta. [3]School of Biosciences, Cardiff University - Cardiff, UK.

(*) Corresponding Authors

Maurizio Casarrubea: maurizio.casarrubea@unipa.it
Giuseppe Di Giovanni: giuseppe.digiovanni@um.edu.mt; digiovannig@cardiff.ac.uk

Abstract

Nicotine is one of the most addictive drugs of abuse. Tobacco smoking is a major cause of many health problems worldwide, and is the first preventable cause of death. Several findings show that nicotine exerts significant aversive as well as the well-known rewarding motivational effects. Less certain is the anatomical substrate that mediates or enables nicotine aversion. Here we have focused on nicotine-induced anxiety-like behavior in unlesioned and lesioned lateral habenula (LHb) rats. Firstly, we showed that acute nicotine induces anxiogenic effects in rats at the doses investigated (0.1, 0.5, and 1.0 mg/kg, i.p.) as measured by the hole-board apparatus, and manifested in behaviors such as decreased rearing and head-dipping and increased grooming. No changes in locomotor behavior were observed at any of the nicotine doses given. T-pattern analysis of the behavioral outcomes revealed a drastic reduction and disruption of complex behavioral patterns induced by all three nicotine doses, with the maximum effect for 1 mg/kg. Lesion of the LHb induced a significant anxiogenic effect, reduced the mean occurrences of T-patterns detected, and strikingly reverted the nicotine-induced anxiety to an anxiolytic effect. We suggest that LHb is critically involved in emotional behavior states and in nicotine-induced anxiety, most likely through modulating serotonergic/dopaminergic nuclei.

Key words: anxiety; serotonin; dopamine; lateral habenula, nicotine, T-pattern analysis

Introduction

Tobacco smoking is a serious health problem worldwide and is well known to be one of the major causes of death in developed countries. Due to a multitude of disadvantageous health effects, tobacco smoking imposes a large financial burden on health services worldwide. Despite the known nicotine-induced health risks (Doll et al., 2004;Di Matteo et al., 2007;Pierucci et al., 2014) pushing smokers to stop, few are successful (relapse rate of about 85%) suggesting the potency of smoking addiction, and the need or a better understanding and treatments to aid smoking cessation (see volumes by (Di Giovanni, 2012;Lester, 2014)).

Nicotine is the psychotropic agent in tobacco responsible for its addictive properties, re-enforcing the continued consumption of tobacco smoke (containing mainly harmful chemicals) (Di Matteo et al., 2007). The reinforcing properties of nicotine (Corrigall et al., 1992) and locomotor stimulatory action seen preferentially after chronic administration (Clarke et al., 1988) are thought to be due to increased dopamine (DA) release in the mesolimbic DA system (Di Chiara, 2000). Nicotine exerts its action by binding to nicotinic acetylcholine receptors (nAChRs), which are heterogenous, pentameric channels consisting of a possible combination six alpha (2-7) and three beta (2-4) subunits. Of particular interest are the ventral tegmental area (VTA) and *substantia nigra pars compacta* (SNc) and their projections to the nucleus accumbens (NAc) and striatum due to their high expression of nAChRs (Clarke and Pert, 1985;Clarke et al., 1985;Di Matteo et al., 2007). Besides DA, nAChRs mediate the release of a wide range of neurotransmitters within the central nervous system (CNS), including, serotonin (5-HT), *gamma*-aminobutyric acid (GABA), glutamate (GLU) and nitric oxide (Pierucci et al., 2004;Di Matteo et al., 2010;Di Giovanni, 2012;Lester, 2014;Pierucci et al., 2014).

In addition to its rewarding effects, nicotine is also highly noxious (Fowler and Kenny, 2014). Most first time smokers report their initial experiences as unpleasant with aversive reactions, including anxiety, occurring after initial exposure to nicotine. This initial aversion to nicotine can decrease the likelihood of developing a tobacco addiction (Sartor et al., 2010). The relationship between smoking and anxiety is complex and conflicting results exists (Picciotto et al., 2002). Some epidemiological studies suggest that anxiety disorders can decrease the risk for developing a tobacco addiction, while nicotine dependence increases the risk of anxiety disorder and panic attacks (Bruijnzeel, 2012). Smokers report higher level of anxiety and stress compered to non-smokers (see (Parrott and Murphy, 2012) for a recent review). Moreover, quitting or failing to quit smoking at six months has been associated with a moderate reduction or increase in anxiety levels, respectively (McDermott et al., 2013). Nicotine acts on a

heterogeneous population of nAChR subtypes in different neuronal circuits that mediate rewarding and aversive effects including anxiety, and the lateral habenula (LHb), a small epithalamic structure that conveys negative motivational signals (Bianco and Wilson, 2009), might mediate the latter (Fowler and Kenny, 2014).

The LHb has indeed recently attracted significant attention in nicotine action. Nicotine might indirectly influence the DAergic, GABAergic and serotonergic systems (Pierucci et al., 2011;Lecca et al., 2014), directly activating habenualar neurons (Pierucci et al., 2011;Dao et al., 2014;Velasquez et al., 2014) probably via α3α5β4-containing nAChRs highly expressed therein (Salas et al., 2009). Habenular β4* receptors have been shown to be necessary for nicotine intake and withdrawal symptoms (Salas et al., 2009;Fowler et al., 2011). Finally, D3 antagonism within the LHb decreased cue-induced nicotine reinstatement suggesting that this area is also involved in nicotine seeking (Khaled et al., 2014). Besides its role in nicotine and in general drugs of addiction, LHb is important for the regulation of behavior and the pathogenesis of psychiatric disorders such as depression and schizophrenia (Cui et al., 2014;Lecca et al., 2014).

Few studies have investigated the association between LHb and anxiety, while the role of the LHb in nicotine-induced anxiety has not been investigated to date. We have therefore carried out a study on the anxiety state of animals following administration of a wide range of nicotine doses in the unfamiliar hole-board (HB) environment. Moreover, we tested the effect of the most effective nicotine dose in LHb-lesioned animals. The HB is a proven measure to test the anxiety state in rodents (Boissier and Simon, 1962;File and Wardill, 1975), and is a useful tool in understanding the effects of a drug in an aversive situation. The head-dipping (HDing) behavior is indicative of the anxiolytic or anxiogenic state, as it is viewed as an exploratory behavior (Takeda et al., 1998). In this investigation we studied the role of the LHb in exploratory behavior, HDing behavior primarily, but also encompassing motor activities such as walking, rearing and grooming (Takeda et al., 1998). Furthermore, we used both quantitative and multivariate T-pattern analysis (for a recent review see (Casarrubea et al., 2015)) to interpret the activity of the unlesioned and LHb-lesioned rats in the HB, under basal conditions and after nicotine administration. A T-pattern is, basically, a sequence of events organized on the basis of significant constraints on the interval lengths separating them. T-pattern analysis of rodent behavior in the HB has previously been performed in normal conditions (Casarrubea et al., 2010a) and after diazepam administration (Casarrubea et al., 2011), and it represents a useful tool to detect even small induced behavioral changes and evaluate and compare different classes of anti-anxiety molecules.

The LHb control of behavior is complex and the majority of the evidence focuses on motivated/punishment behavior. LHb is likely essential for survival by promoting learning and subsequent activities to avoid stimuli associated with negative consequences. For instance, optogenetic activation of the LHb promotes active and passive avoidance behavior in mice (Stamatakis and Stuber, 2012) while bilateral lesions of the LHb reduced escape and avoidance latencies in rats (Pobbe and Zangrossi Jr, 2008b). In addition, under stressful conditions (i.e., induced by yohimbine) the anxiogenic response in rats was diminished following inactivation of the LHb (Gill et al., 2013). Moreover, LHb mediates the aversive effects of alcohol in suppressing voluntary ethanol consumption (Haack et al., 2014). Therefore, the LHb might also be a key area of interest in nicotine-induced anxiety. Here we showed, for the first time that the LHb induces complex behavioral responses to a novel environment that correlates with stress reactivity, anxiety-like behavior and emotionality. Firstly, we showed nicotine-induced changes in the animal behavior studied by use of the HB test and T-patter analysis. Successively, we found that the selective electrolytic bilateral lesion of the LHb induced anxiety-like behavior but strikingly counteracted the anxiogenic effect of 1 mg/kg of nicotine. Consequently, our results indicate that LHb is an important area of the anxiety circuitry and necessary for the expression of nicotine-induced anxiety.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (Charles River, Margate, UK) weighing between 250-350 g were used in the HB experiments. Rats were housed in a room kept at a constant temperature of 21 ± 1°C, a relative humidity of 60 ± 5% and under a light: dark cycle of 12 hr: 12 hr with the lights being turned on at 7 am. Food and water was provided to the animals ad libitum. Procedures involving animals and their care were conducted in conformity with European Law and the institutional guidelines, approved by the University of Malta, Faculty of Medicine and Surgery, Animal Welfare Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Hole-board

The HB apparatus is an open field square enclosure measuring 50 cm^3 consisting of four Plexiglas walls, three of which had polystyrene attached to them to make them opaque. The fourth wall was clear Plexiglas in order to record the animals via a video camera mounted on a tripod and placed a short distance from the HB. The walls were attached to a raised floor of 5 cm above the ground surface (the apparatus was placed on a white countertop). The floor was divided into 9 equal squares (each 16.6 cm^2). The raised floor had four holes, 4 cm in diameter and positioned equidistant from one another. Additionally to ensure that each hole was equidistant from their adjacent corners they were drilled 10 cm from their two neighboring walls (Casarrubea et al., 2009b).

2.3. LHb lesioning procedure

Twenty rats were bilaterally electrolytic lesioned at the LHb levels. Two holes were made in the dura, 1.6 mm lateral to the midline, one on the left lateral side and one on the right lateral side. Two bipolar twisted electrodes were used (stainless steel and coated with Teflon), these comprised of two pieces of wire twisted together (Advend Research Materials Ltd., Oxford, UK). The two ends of the electrodes were spaced a few hundred micrometers apart. Electrodes (angled at 10 degrees from the coronal plane) were attached to a micromanipulator and lowered into the right and left LHb (depth of 4.7 mm from the surface of the dura). A 1 micro ampere

(μA) current was applied for 30 seconds using an optically isolated stimulator (DS3 Digitimer, Hertfordshire, UK). The electrodes were left in place for a few minutes before removing. The rat was then left to recover from the anesthesia for approximately 1-2 hours. A subcutaneous (s.c.) injection of the non-steroidal anti-inflammatory, carparofen, at a dose of 5.0 mg/kg was given to the rat, and it was left for at least 1 week to recover before testing in the hole-board. The animals were killed at the end of the experiments by decapitation and the brains were removed. To histologically verify the extent of the lesion, the brains were freeze-sectioned in a cryostat. Slices (25 μm) were taken through the entire habenula and mounted on slides. Lesions of the lateral habenula were considered acceptable when surrounding regions (i.e., medial habenula, dorsal hippocampus and thalamic nuclei) were spared (Fig. 1). Eight animals (four for each saline and nicotine group) whose lesions did not meet these criteria were excluded from the study.

2.4. Drugs and treatments

The treatment groups were saline (control), nicotine 0.1 mg/kg, 0.5 mg/kg and 1 mg/kg, all administered i.p. acutely for the first experiment. Moreover, unlesioned and LHb-lesioned rats were treated with saline or nicotine 1 mg/kg, i.p.. The nicotine solutions used for the i.p. injections were made up using (-)-nicotine hydrogen tartrate salt ($C_{18}H_{26}N_2O_{12}$) diluted in physiological saline (0.9% NaCl) and adjusted to pH 7.4. All drug doses refer to the weight of the salt. All rats were drug naive at the start of the experiment and were randomly assigned to a treatment group (n =10 per group). Saline (control) or nicotine was given in 0.5 ml volume. Three different nicotine groups received, respectively, one i.p. injection of 0.1, 0.5, and 1.0 mg/kg of nicotine 30 min before the test.

2.5. Procedure

All recordings took place between 9 am and 1 pm and none of the rats had previously been exposed to the HB before experimentation. Each rat was brought into the testing room and left for 30 minutes to acclimatize. They then received the drug treatment as described previously. The animals were subsequently placed in the center of the HB and allowed to freely explore for 10 minutes, whilst being recorded by the video camera. After each recording the HB was cleaned with ethanol (70%) to remove all scent traces and faces. The video recordings were blind analyzed off-line.

2.6. Data analysis

The ethogram utilized in the present investigation (fig. 2) is the same that we employed in our previous studies (Casarrubea et al., 2009b;c;Casarrubea et al., 2010a;Casarrubea et al., 2011). Video files were coded by means of a software coder (*The Observer*, Noldus Information Technology bv, The Netherlands) and event log files generated for each subject. To detect temporal relationships among behavioral elements, event log files were processed with *Theme software* (PatternVision Ltd, Iceland; Noldus Information Technology, The Netherlands). *Theme* is a specific software able to detect repeated sequences of events on the basis of statistically significant constraints on the intervals separating them (Magnusson, 2000). In brief, an algorithm compares the distributions of each pair of the behavioral elements A and B searching for a time window so that, more often than expected by chance, A is followed by B within that time window. In this case, a statistically significant relationships exists between A and B and are by definition a t-pattern indicated as (A B). Then, such 1st level t-patterns are considered as potential A or B terms in higher order patterns, e.g. ((A B) C). And so on, up to any level. A more detailed description of concepts, theories and procedures behind t-pattern analysis can be found in our previous articles (Casarrubea et al., 2009a;Casarrubea et al., 2010a;Casarrubea et al., 2011;Casarrubea et al., 2013b;a;Casarrubea et al., 2014;Casarrubea et al., 2015).

The following parameters of the behavioral response were analyzed: 1) mean durations of each behavioral element, for each subject; 2) mean occurrences of each behavioral element, for each subject; 3) overall number of different t-patterns detected for each group both in real and random generated data; 4) structure of all the different T-patterns detected for each group (strings); 5) overall occurrences and 6) mean occurrences of T-patterns detected, for each subject. 7) percent distribution of T-patterns including behaviors of hole-exploration, namely, head dip and edge sniff.

2.7. Statistics

One-way ANOVA, followed by Newman-Keuls post-hoc test for multiple comparisons, was carried out to assess possible drug-induced modifications of the mean occurrences and mean durations of behavioral elements in saline and nicotine (0.1, 0.5 and 1 mg/kg) administered groups.

Two-way ANOVA (treatment by lesion) were used to analyze differences among saline in unlesioned animals, saline in LHb-lesioned rats, nicotine 1 mg/kg, i.p. in unlesioned rats and nicotine 1 mg/kg i.p. in LHb-lesioned rats, with post hoc Fisher's PLSD test to assess individual group comparisons on most behavioral variables. In the case of a significant effect of lesion group or a significant lesion group×drug treatment interaction, the data of the unlesioned and lesioned groups, comparisons of nicotine to the vehicle control condition were made by paired t-tests. Differences were considered significant at $p < 0.05$.

Concerning T-pattern analysis, albeit all detected T-patterns imply a statistical significance among critical intervals separating their events, the enormous amount of possible relationships raises the question whether the number of different detected t-patterns is different by chance. The software used for T-patterns detection deals with such a crucial issue by repeatedly randomizing and analyzing the original data. In brief, for each group, the mean number of t-patterns + 1 SD detected in random generated data is compared with the actual number of T-patterns detected in real data. Two-way ANOVA (treatment x lesion) were used to analyze differences among saline in unlesioned animals, saline in LHb-lesioned rats, nicotine 1 mg/kg i.p. in unlesioned rats and nicotine 1 mg/kg i.p. in LHb-lesioned rats. Finally, chi-square test was carried out to compare possible significant differences in the percent distribution of T-patterns.

3. Results

3.1. Effects of acute nicotine administration on different behavioral components in HB

Mean durations ±SEM of each behavioral component in saline and nicotine (0.1, 0.5 and 1 mg/kg) treated groups are presented in Tab. 1. One-way ANOVA revealed significant nicotine-related changes for climbing ($F_{3,39} = 3.19$, $p < 0.035$), rearing ($F_{3,39} = 3.28$, $p < 0.032$), head-dip ($F_{3,39} = 9.58$, $p < 0.0001$), front paw licking ($F_{3,39} = 6.07$, $p < 0.002$), hind paw licking ($F_{3,39} = 2.87$, $p < 0.05$), face grooming ($F_{3,39} = 3.58$, $p < 0.023$) and body grooming ($F_{3,39} = 5.69$, $p < 0.003$). Newman-Keuls post-hoc test showed significant ($p < 0.05$) nicotine-induced decrease, in comparison with saline, for head dip at all nicotine doses, for rearing at 0.5 and 1 mg/kg and for climbing, front paw licking, face grooming and body grooming only at 0.1 mg/kg.

Mean occurrences ±SEM of each behavioral component in saline and nicotine (0.1, 0.5 and 1 mg/kg) injected groups are illustrated in Tab. 2. One-way ANOVA showed significant drug-related changes for climbing ($F_{3,39} = 4.23$, $p < 0.012$), rearing ($F_{3,39} = 3.61$, $p < 0.022$), head-dip ($F_{3,39} = 6.53$, $p < 0.001$), front paw licking ($F_{3,39} = 4.23$, $p < 0.012$) and immobility ($F_{3,39} = 3.72$, $p < 0.020$). Newman-Keuls post-hoc test highlighted significant ($p < 0.05$) decrease, in comparison with saline, for climbing, rearing, head-dip and immobility at all nicotine doses.

3.2. Effects of bilateral LHb lesion on different behavioral components and on nicotine effects in HB

Of the 20 rats that underwent LHb lesion, four in both saline and nicotine groups (1 mg/kg) did not have a satisfactory lesion, and were not included in the statistical analysis. Otherwise, no data were excluded from analysis.

Mean durations ± SEM of each behavioral component are illustrated in fig. 3 while mean occurrences ± SEM of each component are presented in fig. 4.

Walking

Two-factor ANOVA showed no difference between unlesioned and LHb-lesioned groups ($F_{1,28} = 1.835$; $p = 0.0675$), no significant effect of treatment (saline or nicotine) $F_{1,28} = 15.98$; $p = 0.1863$), and a lack of interaction between groups (unlesioned vs LHb-lesioned) and treatment (nicotine or saline) ($F_{1,28} = 1.010$; $p = 0.3236$) on walking mean duration (Fig. 3). Similarly, there was no effect of lesion group on the mean occurrences of walking behavior ($F_{1,28} = 2.3$; $p = 0.1$). Neither there was a significant effect of drug treatment ($F_{1,28} = 1.5$; $p = 0.2$) nor any significant interaction of lesion group by drug treatment ($F_{1,28} = 1.4$; $p = 0.5$) (Fig. 4).

Climbing

An ANOVA revealed a significant effect of group (unlesioned vs LHb-lesioned) ($F_{1,28} = 21$; $p < 0.001$) and a significant main effect of treatment (nicotine or saline) ($F_{1,28} = 4.3$; $p < 0.05$) on climbing mean duration. However, no significant interaction of the two factors was observed ($F_{1,28} = 0.1$; $p = 0.8$) (Fig. 3). On the other hand, there was a significant effect of lesion group ($F_{1,28} = 14.7$; $p < 0.001$) and a significant effect of drug treatment ($F_{1,28} = 7.2$; $p < 0.001$) on the frequency of climbing behavior. Conversely, no significant interaction of lesion group by drug treatment ($F_{1,28} = 2.1$-5; $p = 0.9$) was observed on the frequency of climbing behavior (Fig. 4).

Rearing

There was a significant effect of lesion group on the duration of rearing behavior ($F_{1,28} = 8.4$; $p < 0.001$), and a significant effect of drug treatment ($F_{1,28} = 4.4$; $p < 0.05$), but no significant interaction of lesion group by drug treatment ($F_{1,28} = 3.8$; $p = 0.06$) (Fig. 3). As for occurrence, there was a significant effect of lesion group ($F_{1,28} = 7.6$; $p < 0.05$), drug treatment ($F_{1,28} = 4.3$; $p < 0.05$) and no interaction of these factors ($F_{1,28} = 0.005$; $p = 0.08$) (Fig. 4).

Immobile-Sniffing

There was no significant effect of lesion group on the duration of immobile sniffing ($F_{1,28} = 4.0$; $p = 0.054$), and a significant effect of drug treatment ($F_{1,28} = 5.1$; $p < 0.05$), but no significant interaction of lesion group by drug treatment ($F_{1,28} = 0.4$; $p = 0.5$) (Fig. 3). As for occurrence, there was a significant effect of lesion group ($F_{1,28} = 7.1$; $p < 0.05$), no significant effect of drug treatment ($F_{1,28} = 2.3$; $p = 0.14$) but a significant interaction of these factors ($F_{1,28} = 12.9$; $p < 0.005$) was revealed by two-way ANOVA. Post hoc analysis revealed that LHb lesion did induce a significant decrease of the occurrence ($p < 0.005$) of immobile sniffing. Nicotine reduced the mean occurrence ($p < 0.05$) in LHb-lesioned animals and was ineffective in unlesioned animals.

Edge Sniff

There was no effect of lesion group on the duration ($F_{1,28} = 3.8$; $p = 0.06$) nor on mean occurrence ($F_{1,28} = 3.6$; $p = 0.07$) of edge sniff. Neither was there a significant effect of drug treatment on duration ($F_{1,28} = 0.1$; $p = 0.9$) and mean occurrence ($F_{1,28} = 0.2$; $p = 0.6$) nor any significant interaction of lesion group×drug treatment ($F_{1,28} = 0.6$; $p = 0.4$) (Fig. 3, 4).

Head-Dip

There was a significant effect of lesion group on the duration of head dipping behavior ($F_{1,28}$ = 10.1; $p < 0.005$), and a significant effect of drug treatment ($F_{1,28}$ = 7.7; $p < 0.01$), but no significant interaction of lesion group×drug treatment ($F_{1,28}$ = 3.2; p = 0.08) (Fig. 3). As for occurrence, there was no significant effect of lesion group ($F_{1,28}$ = 1.2; p = 0.2), drug treatment ($F_{1,28}$ = 2.6; p = 0.1) and no interaction of these factors ($F_{1,28}$ = 0. 5; p = 0.5).

Front Paw Licking

There was a significant effect of lesion group on the duration of front paw licking behavior ($F_{1,28}$ = 10.5; $p < 0.005$), but no significant effect of drug treatment ($F_{1,28}$ = 2.6; p = 0. 1), and a significant interaction of lesion group×drug treatment ($F_{1,28}$ = 7.7; $p < 0.001$) (Fig. 3). As for occurrence, there was a significant effect of lesion group ($F_{1,28}$ = 11.5; $p < 0.05$), a no significant effect of drug treatment ($F_{1,28}$ = 0.3; p = 0.6) and no interaction of these factors ($F_{1,28}$ = 2. 8; p = 0.1). Post hoc analysis revealed that LHb lesion induced a significant decrease of the duration ($p < 0.005$) and occurrence ($p < 0.005$) of front paw licking. Nicotine did not change duration and occurrence in the unlesioned animals (p = 0.3 for both groups) but increased duration in LHb-lesioned rats ($p < 0.05$).

Hind Paw Licking

There was no effect of lesion group on the duration ($F_{1,28}$ = 0.8; p = 0.4) nor on mean occurrence ($F_{1,28}$ = 0.8; p = 0.8) of hind paw licking. Neither was there a significant effect of drug treatment on duration ($F_{1,28}$ = 0.2; p = 0.6) and mean occurrence ($F_{1,28}$ = 0.1; p = 0.1) nor any significant interaction of lesion group×drug treatment for ($F_{1,28}$ = 0.6; p = 0.4) and occurrences ($F_{1,28}$ = 0.5; p = 0.5) (Fig. 3, 4).

Face Grooming

There was a significant effect of lesion group on the duration ($F_{1,28}$ = 9.3; $p < 0.05$) and on mean occurrence ($F_{1,28}$ = 6.9; $p < 0.05$) of face grooming. However, there was no significant effect of drug treatment on duration ($F_{1,28}$ = 1.5; p = 0.2) and mean occurrence ($F_{1,28}$ = 2.1; p = 0.1) nor any significant interaction of lesion group by drug treatment for duration ($F_{1,28}$ = 0.8; p = 0.4) and occurrences ($F_{1,28}$ = 2.3; p = 0.2) (Fig. 3, 4).

Body Grooming

There was no significant effect of lesion group on the duration of body grooming behavior ($F_{1,28}$ = 1.5; p = 0.2), nor significant effect of drug treatment ($F_{1,28}$ = 3.4; p = 0. 07), and neither was there an interaction of lesion group×drug treatment ($F_{1,28}$ = 0.3; p = 0.5) (Fig. 3). As for occurrence, there was a significant effect of lesion group ($F_{1,28}$ = 4.7; p < 0.05), a significant effect of drug treatment ($F_{1,28}$ = 5.1; p < 0.05) but no interaction of these factors ($F_{1,28}$ = 1. 4; p = 0.3).

Immobility

There was no significant effect of lesion group on the duration ($F_{1,28}$ = 0.1; p = 0.9) and mean occurrence ($F_{1,28}$ = 0.6; p = 0.5) of immobility. No significant effect of drug treatment on the duration ($F_{1,28}$ = 3.3; p = 0. 08) and mean occurrence ($F_{1,28}$ = 3.0; p = 0.09), and neither a significant interaction of lesion group×drug treatment on the duration ($F_{1,28}$ = 1.9; p = 0.2) and mean occurrence ($F_{1,28}$ = 3.2; p = 0.08) (Fig. 3).

3.3. T-pattern analysis

Fig. 5 shows the structure of all T-patterns detected in saline and nicotine (0.1, 0.5 and 1mg/kg) administered groups. For each T-pattern, its terminal string (i.e., events in T-pattern's structural sequence) and occurrences are indicated. 17 different T-patterns were detected in the saline-administered group. Nicotine 0.1, 0.5 and 1 mg/kg groups revealed 7, 12 and 4 different T-patterns, respectively. Fig. 5 also shows, for each group, T-patterns length distribution in real data and in random generated data ± SD. For all groups, T-patterns search run performed on random *vs* real data demonstrated that the largest amount of different T-patterns detected is present, by far, in real data (fig. 5, dark bars) rather than in random generated data (fig. 5, white bars). Finally, the mean number of T-patterns shows a clear-cut reduction in all nicotine-administered groups (fig. 5, bottom left of each panel). ANOVA ($F_{3,39}$ = 19.03, p < 0.0001), followed by Newman-Keuls post-hoc test for multiple comparisons revealed, in comparison with saline, significant reductions of T-patterns in all nicotine administered groups.

Fig. 6 shows the structure of all T-patterns detected in subjects with lesion in lateral habenula and injected with saline and nicotine 1mg/kg. As for fig. 5 (see above), for each T-pattern, its terminal string and occurrences are indicated. 7 different T-patterns have been detected in LHb + saline administered group; 15 different T-patterns have been found in nicotine 1 mg/kg administered group. For both groups, T-patterns search run performed on random *vs* real data demonstrated that the largest amount of different T-patterns detected is present, by far, in real

data (fig. 6, dark bars) rather than that which is random generated (fig. 6, white bars). The mean number of T-patterns did not show significant changes between the two groups.

Fig. 7 shows mean occurrences ± SEM of all T-patterns detected in unlesioned saline, nicotine 1 mg/kg, LHb-lesioned + saline and LHb-lesioned +nicotine 1 mg/kg groups. There was no significant effect of the lesion group on the T-pattern mean occurrence ($F_{1,28} = 2.4$; $p = 0.1$), but a significant effect of drug treatment ($F_{1,28} = 10.6$; $p < 0.05$), and a significant interaction of lesion group by drug treatment ($F_{1,28} = 13.6$; $p < 0.001$) (Fig. 7). Post hoc analysis revealed that LHb lesion induced a significant decrease of the T-pattern occurrence ($p < 0.01$) and also counteracted the effect of nicotine ($p = 0.8$). On the other hand, nicotine significantly decreased the T-pattern occurrence in unlesioned animals ($p < 0.001$).

Finally, fig. 8 illustrates percent distributions of T-patterns containing hole-exploratory behavioral components (edge sniff and/or head dip) both in unlesioned and LHb-lesioned groups. In comparison with saline group where 48.2% of T-patterns contained edge sniff and/or head dip, significant ($p < 0.0001$) reductions were detected following nicotine administration at all doses, ranging from 39.3% in nicotine 0.1 mg/kg, to 39.5% in nicotine 0.5 mg/kg, to 18.5% in nicotine 1mg/kg. With regard to LHb-lesioned subjects, the saline group, with a value of 29.7%, showed a significant reduction in comparison with the unlesioned saline one. On the contrary, LHb-lesioned + nicotine 1 mg/kg, with a percentage of 77.2%, showed a significant clear-cut increase of T-patterns containing edge sniff and head dip, in comparison both with the unlesioned saline and LHb-lesioned saline groups.

Discussion

Nicotine addiction is a global health concern and is a very real issue for many smokers who are unable to quit, despite knowledge of the deleterious effects on their health. A large body of data has been produced in the field of nicotine research; nevertheless,we still do not have a clear picture with regards to its action in the brain. Once this has been achieved, we will be able to produce more effective therapies for the treatment of nicotine addiction. In this regard, the link between anxiety and nicotine dependence, if elucidated, might represent an interesting strategy in smoking cessation. Indeed, some epidemiological studies suggest that anxiety disorders can decrease the risk of developing a tobacco addiction, while nicotine dependence increases the risk of anxiety disorder and panic attacks (Bruijnzeel, 2012). Smokers report higher levels of anxiety and stress compered to non-smokers (Parrott and Murphy, 2012). Moreover, a moderate reduction in anxiety levels has been observed six months after quitting smoking (McDermott et al., 2013). Nicotine has been reported to be both an anxiolytic and an anxiogenic, dependent upon the route of nicotine administration, the treatment length (acute, chronic, chronic withdrawal), the dose and the time of the pretreatment (File et al., 1998;Irvine et al., 1999;Varani et al., 2012) and behavioral tests such as the elevated plus maze (EPM) (Olausson et al., 1999;Varani et al., 2012) and social interaction (SI) test (File et al., 1998;Irvine et al., 1999;Irvine et al., 2001;Tucci et al., 2003a) used to investigate the anxiety state of animals. It has been shown that acute nicotine administration induced an anxiolytic effect at low doses and an anxiogenic effect at higher doses in both in the EPM (Balerio et al., 2005;Hayase, 2007;Varani et al., 2012;Anderson and Brunzell, 2015) and in the SI test (File et al., 1998;Tucci et al., 2003a;Tucci et al., 2003b). Nicotine induced only anxiety-like responses in mice when tested in the black and white box at different doses (Trigo et al., 2009). The contradictory evidence of nicotine on anxiety might be explained by regional nAChR subunit configuration (File et al., 2000). Indeed, α4-nAChRs knock out (KO) have decreased anxiety-like behavior (Ross et al., 2000;McGranahan et al., 2011), while α7- (Paylor et al., 1998) β3- (Booker et al., 2007), β4-nAChRs KO mice (Salas et al., 2003) seem to present an increase in anxiety-related behavior. Interestingly, elimination of α4β2-nAChRs specifically from DAergic neurons decreased sensitivity to the anxiolytic effects of nicotine (McGranahan et al., 2011). Recently, it has been suggested that low dose nicotine inhibits β2*nAChRs inducing the anxiolytic-like effects, while high doses stimulate them leading to the anxiogenic-like effects of nicotine (Anderson and Brunzell, 2015). Hence, due to these many confounding elements the nexus between smoking and anxiety is not clear. Here we investigated the role of nicotine-

induced anxiety behavior and the influence of LHb on the response to nicotine by using HB and medium-high doses of nicotine (0.1-1 mg/kg). The first aim of this study was, therefore, to further enrich this debate through an examination of a wide range of nicotine doses on anxiety behaviors of adult rats. We performed HB behavior test, one of the numerous tests for the identification of anxiolytic or anxiogenic-like drug effects (Boissier and Simon, 1962;File and Wardill, 1975) that we have previously extensively used (Casarrubea et al., 2009b;c;Casarrubea et al., 2010a;Casarrubea et al., 2010b). Rats are well known to be extremely cautious in unfamiliar and potentially dangerous environments. Risk assessment is crucial, and has been described as the balance between the propensity to investigate a novel stimulus and the fear it causes (Augustsson et al., 2005). Such an equilibrium strongly depends on the subject's anxiety level. Indeed, the rationale of experimental assays employed to study anxiety such as the open field, the HB or the EPM, centers upon these aspects.

Our results, obtained from the analysis of rat behavior in the HB apparatus, demonstrated that acute administration of medium-high doses of nicotine, by modifying important indicators of anxiety such as various parameters associated with the exploration of the ground holes, induced clear anxiogenic effects. Specifically, the HB findings show an anxiogenic profile of all doses of nicotine when compared to control, observed 30 min after injection. The total time HDing was statistically decreased for all the doses (0.1-1 mg/kg, i.p.) treatments compared to acute saline. Strikingly, the more anxiogenic nicotine effect was due to the lower dose. A similar scenario was induced by nicotine in HD mean occurrences with 0.1 mg/kg nicotine treatment producing the highest decrease. The amounts of rearing and climbing were also significantly reduced following doses of 0.5 and 1 mg/kg, while the occurrences were reduced by all the doses compared to control. This effect following acute nicotine treatment is coherent with previous studies, which showed an anxiogenic effect following the acute administration of nicotine at 0.25-0.5 (Zarrindast et al., 2000), 0.5-1.0 mg/kg (Ouagazzal et al., 1999a;Hayase, 2007;Zarrindast et al., 2010) doses in the EPM in rats and mice and 0.5 mg/kg measured by HD in mice (Nasehi et al., 2011). On the other hand, an anxiolytic nicotine response has been observed with lower nicotine doses (0.01, 0.05 and 0.1 mg/kg) (File et al., 1998;Ouagazzal et al., 1999a;Picciotto et al., 2002;Zarrindast et al., 2010;Varani et al., 2012). In agreement with previous evidence (Zarrindast et al., 2000;Zarrindast et al., 2010;Nasehi et al., 2011), the mean walking duration and the occurrence were not significantly different between treatment groups, indicating that the nicotine-induced anxiogenic reductions in HDing and rearing were not due to change in locomotory activity. Grooming is another useful behavioral parameter to consider,

as it is indicative of anxiety levels and it is thought to be initiated in response to changes occurring in the animal as a result of anxiogenic stimuli (Spruijt et al., 1992;Kalueff and Tuohimaa, 2005). Consistent with the changes observed on HDing and rearing, grooming duration for the different behavioral components (FP, HPL, FG and BG) appeared also to be significantly affected by nicotine treatments. Overall, our behavioral results support the aversive effects of acute nicotine. In order to study changes in the emotional state and exploratory behavior of rats we performed T-pattern analysis. T-pattern analysis revealed that more t-patterns arise in real data than in randomized data for all doses of nicotine, suggesting firstly that the outcome number of t-patterns for all treatments was not just due to chance alone (Casarrubea et al., 2011). Secondly, it appears that the number of different T-patterns (that is the number of different sequences detected), their overall occurrences and their mean number are significantly reduced in all nicotine-administered groups, with a maximum effect observed at the higher 1 mg/kg dose, suggesting that more complex behavior arises in drug naive rats and nicotine strongly affected it (Fig. 5). Thus, it is possible to conclude that acute nicotine administration has a dramatic negative impact in terms of behavioral variability and organization. On the other hand, our data suggest that the acute administration of nicotine induces an increase of the anxiety level in the animal as indicated, for instance, by the consistent reduction of HDing duration, important index of anxiety (Takeda et al., 1998). Thus, it might be inferred that the simplification of temporal characteristics of behavior is linked to an increased anxiety condition induced by the acute nicotine administration. However, the simple assessment of T-patterns quantitative features, such as length and occurrences, is not sufficient to assess whether, following nicotine administration, the animal behavior modifications are coherent with anxiety. The assessment of the qualitative characteristics of T-patterns detected in nicotine administered groups is necessary. Therefore, the evaluation of the sequential structure of T-patterns detected, including edge sniff and head dip is important (i.e., anxiety-related components of environmental exploration in the HB) (Casarrubea et al., 2009b;c). The percentage of T-patterns containing edge sniff and head dip, following nicotine administration, is reduced in a significant and almost dose-dependent fashion. Hence, behavior structure is significantly reorganized in terms of a reduced exploratory approach, consistent with an increased anxiety level.

The behavioral output following nicotine administration is complex and it is an obvious sum of the effects of i) the activation of different nAChRs in the brain, ii) the different brain areas involved in anxiety together and iii) the neurotransmitter systems regulated by nAChRs all taken together. Hence, nicotine can activate the release of stimulatory (glutamate), inhibitory

(GABA) and modulatory (DA, norepinephrine, 5-HT) neurotransmitters in different brain regions with different time courses, as well as stress hormone levels in the periphery (Di Giovanni, 2012;Lester, 2014). Local administration studies in animals have identified different brain areas that may be involved in the modulation of anxiety by nicotine and endogenous ACh. Bilateral administration of nicotine (in the µg range) into the central amygdala (intra-CeA) (Zarrindast et al., 2008;Zarrindast et al., 2013), into the dorsal raphe nucleus (DRN) (Cheeta et al., 2001), lateral septal nucleus (LSN) (Ouagazzal et al., 1999b) and hippocampus (Ouagazzal et al., 1999a;Kenny et al., 2000), or applied in different sites of the mesolimbic DA system (Picciotto et al., 2002;Zarrindast et al., 2013) has been shown to induce an anxiogenic-like effect. It is likely that intra-CeA injection of nicotine induces an anxiogenic effect indirectly through the activation of the VTA (Zarrindast et al., 2013) and the nucleus accumbens (Zarrindast et al., 2012) since anxiety levels are decreased by local application of D1/D2 receptors antagonists in these different areas. Moreover, excitation of CeA neurons induced anxiety, is blocked by itra-amygdaloid application of D1 receptor antagonist (de la Mora et al., 2010) suggesting a DAergic modulation feed-back.

Recently, the LHb has been implicated in both reinforcing and aversive nicotine effects (Lecca et al., 2014;Proulx et al., 2014;Stopper and Floresco, 2014;Velasquez et al., 2014) due to its connectivity in the anti-reward systems (Proulx et al., 2014) but its role in nicotine-induced anxiety has not yet been investigated. The LHb is a key player in reward processing, particularly in coding for negative value signals to the DAergic and serotonergic systems (Matsumoto and Hikosaka, 2007;Proulx et al., 2014). Furthermore, it has been shown that the LHb is actually a "preference" center. Indeed, inactivation of the LHb disrupted the decision making circuitry necessary to evaluate the long-term cost and benefit of actions (Stopper and Floresco, 2014). Based on its anatomical connectivity, the LHb seems to be an important hub for controlling emotional behaviors, particularly during an aversive and stressful situation (Proulx et al., 2014). Optogenetic activation of the LHb promotes active and passive avoidance behavior in mice (Stamatakis and Stuber, 2012) while bilateral lesions of the LHb reduced escape and avoidance latencies in rats (Pobbe and Zangrossi Jr, 2008b). Moreover, LHb mediated the aversive effects of alcohol in suppressing voluntary ethanol consumption (Haack et al., 2014), and LHb/rostromedial tegmental nucleus (RMTg) pathways contribute critically to cocaine-induced avoidance behaviors (Jhou et al., 2013).

The role of the LHb in regulating the anxiety state has only recently been revisited (Pobbe and Zangrossi Jr, 2008b;Gill et al., 2013), although the exact nature of this modulation is not clear.

The fact that there is an anatomical connection with all the areas involved in stress and/or emotional response (Proulx et al., 2014) and the evidence that anxiety and stress induces c-FOS expression in LHb as well as in the classical anxiety-related areas (Wirtshafter et al., 1994;Kurumaji et al., 2003) supports the important role of the habenular in anxiety. An early study showed that bilateral lesions of the habenular nuclei did not affect exploratory behavior such as HDing, time spent immobile and locomotory activity but reduced rearing in HB and open arena (Thornton and Evans, 1982). Conversely, lateral habenular lesion significantly augmented locomotor activity, rearing and wall hole-poke without enhancing general motor function on the rotarod in rats. The same authors also showed the LHb lesion *per se* and stress induced increase in central region activity of the arena; however, LHb lesion abolished this stress effect (Lee and Huang, 1988). In addition, he lesion of the fasciculus retroflexus, the habenula efferent pathway, was ineffective in not-stressed rats in EPM and open field, but increased plasma levels of corticosterone and produced attenuation of anxiety induced by isolation and food deprivation (Murphy et al., 1996). Consistently, only under stressful conditions (i.e., induced by yohimbine treatment) was the anxiogenic response such as the time spent in the EPM open arms and the time spent burying marble balls diminished following reversible inactivation of the LHb in rats (Gill et al., 2013). In addition, bilateral electrolytic lesions of the LHb or its chemical stimulation impaired and facilitated inhibitory avoidance, respectively, a behavior that has been related in terms of psychopathology to generalized anxiety (Pobbe and Zangrossi Jr, 2008a). In general, therefore, it seems that LHb is responsible for phasic modulation of emotional behavior especially under stress (Amat et al., 2001) and in states of heightened anxiety.

In contrast to these findings, our results clearly show that bilateral electrolytic LHb lesion induces anxiety-like behaviours with significant differences in the behavioral parameters compared to unlesioned animals treated with saline without any confounding locomotor effects. Indeed, here we did not detect a significant change in the locomotor activity previously observed in other studies after LHb lesion (Nielson and McIver, 1966;Wang et al., 2013;Jean-Richard Dit Bressel and McNally, 2014), although an increasing trend of duration and occurrence of walking in LHb-lesioned animals was detected. Consistently, duration and occurrence of climbing and rearing, the total but not the occurrences of HDing significantly decreased in the lesioned animals. Moreover, the occurrence of grooming (FPL and FG) that is known to be induced by mild stress such as exposure to novel environment (Moody et al., 1988) increased following LHb lesioning. Nicotine 1.0 mg/kg in lesioned animals was unable to produce the same anxiogenic effects compared to 1.0 mg/kg acute nicotine treatment alone

(in unlesioned animals). While climbing and rearing were further inhibited, grooming was increased by nicotine in lesioned animals. Interestingly, the lesion changed the direction of nicotine effect on FPL increasing it compared to the lesioned animals that receive saline.

Concerning T-pattern analysis, Fig. 7 depicts well the highly significant changes induced both by nicotine administration in unlesioned and in lesioned rats. Such an outcome demonstrates that lesion of the LHb has an evident impact in terms of behavioral organization.

Animals with lesions in the LHb are characterized by a clear modification of anxiety-related behavior. Actually, strings (fig. 6) and percentage of T-patterns containing edge-sniff and/or head dip (fig. 8) describe, in lesioned animals, a situation essentially consistent with an increased anxiety level. Such an outcome is coherent with existing literature demonstrating the important role of the habenular formation in the regulation of emotional states (Murphy et al., 1996;Andrade et al., 2013).

The above discussed condition of increased anxiety, in rats with a lesion in the LHb, radically changes if nicotine is acutely administered. Fig. 6 (bottom panel) and fig. 8 clearly demonstrate that following nicotine administration the number of T-patterns containing head dip and edge sniff is strongly increased. Values of patterns containing edge sniff and head dip in unlesioned+saline and lesioned+saline animals are 48% and 30%, respectively, while LHb lesion rats present the largest extent of patterns, about 77%, containing edge sniff and head dip. Hence, in comparison with both unlesioned and with lesioned subjects, it appears that acutely nicotine injected animals with lesion in the LHb do explore the holes significantly more. Such evidence, coherent with a low anxiety-related profile, is suggestive of the important role of the LHb in the behavioral organization of the animal following pharmacological modulation (i.e., nicotine) of its emotional reactivity (i.e., anxiety).

From our study it can be noted how standard quantitative analyses (such as durations, frequencies, latencies etc.) provide a reductive portrait of animal behavior. The reason is that these approaches describe the behavior in terms of individual components, separate from the comprehensive behavioral architecture. On the other hand, our results using a multivariate approach providing information concerning the structural relationships among each component of the rat behavioral repertoire, show that T-pattern analysis is capable of revealing effects that otherwise would have been neglected. The case of head dip duration is explicative. LHb lesion produces a highly significant reduction of head dip duration; on the other hand, nicotine administration does not affect HDing compared to its vehicle in lesioned animals. As we have discussed in the preceding section, this would be a wrong conclusion. In reality, when the relationships of head dip with the other components of the behavior are analyzed, a completely

different scenario emerges. The numbers of head dip and edge sniff become components of the largest amount of behavioral sequences performed by the lesioned animals following nicotine administration. In these animals, the environmental exploration becomes significantly more organized, in comparison with the saline administered (both unlesioned and lesioned) groups.

Altogether, the present results demonstrate that although nicotine and LHb lesion both induce anxiogenic behavior, they have antagonistic actions when given together. These observations are consistent with the evidence that LHb lesion limits and abolishes certain behaviors shown under highlighted anxiety state (Pobbe and Zangrossi Jr, 2008a;Gill et al., 2013). Our current findings support and extend these prior studies by showing that the inactivation of the LHb *per se* induces anxiety-like traits and decreases the complexity of behavioral functions (from t-pattern results) in rats, an effect never observed before. Nevertheless, we cannot rule out the interference of different factors, such as type of test, the lesion versus chemical inactivation, strain or level of basal anxiety differences of the rats used in our research in this anxiogenic LHb tonic effect.

It still remains to be explained why a lesion in this small epithalamic formation reverts acute nicotine-induced anxiety. Actually, the lateral habenula, through the stria medullaris, receives inputs mainly from the basal ganglia and from the limbic system (Hikosaka et al., 2008;Hikosaka, 2010). The output, through the fasciculus retroflexus, is directed to brain structures containing dopaminergic neurons (e.g., substantia nigra pars compacta, VTA) and serotonergic neurons (e.g., DRN, medial raphe nucleus (MRN)); also, indirect connections take place through the GABA-ergic rostromedial tegmental nucleus (RMTg) (Hikosaka, 2010;Proulx et al., 2014). Thus, it is evident that the LHb occupies a key position among pathways involved in the transmission of information concerning emotional processes (limbic input) and motor behavior decision-making processes (basal ganglia input). Indeed, LHb-lesioned rats showed for instance a deficit in escape behavior indicating a role for the habenula in the selection of correct behavioral strategies and innate motor programs (Thornton and Evans, 1982). Thus, the increased anxiety observed in animals with lesion in the LHb and the anxiolysis observed following nicotine administration may depend on the imbalance between DA and 5-HT produced by the disruption of specific bidirectional pathways toward DAergic and serotoninergic systems both essential in the homeostasis of anxiety levels (Zweifel et al., 2011;Zangrossi and Graeff, 2014).

Specifically, one possible explanation of the present findings is that nicotine, activating the nACh receptors located within or outside the LHb, may eventually increase the LHb activity

(Pierucci et al., 2011;Dao et al., 2014) this would indirectly cause a reduction in activity of DAergic systems, by strongly increasing the RMTg GABAergic input to the VTA neurons projecting to the NAc lateral shell (Hong et al., 2010;Lecca et al., 2011;Lammel et al., 2012) decreasing nicotine rewarding effects. A direct LHb-VTA excitatory input also exists toward a neuronal subpopulation of the medial VTA that mediates aversion and projects to the medial prefrontal cortex (mPFC) (Lammel et al., 2012). mPFC is part of the anxiety network and it has been shown to modulate amygdale, bed nucleus of the stria terminals and ventral hippocampal neuronal activity synchronizing them on the theta band during high state of anxiety (Adhikari, 2014). The evidence that the LHb spontaneously generates theta oscillations in phase with hippocampus (Goutagny et al., 2013) further suggests that LHb might also be considered part of the anxiety brain network. The LHb couples the DA and 5-HT systems, and nicotine activating the LHb may modulate 5-HT neuronal activity of the raphe nuclei, directly and indirectly via the RMTg (Sego et al., 2014;Zhao et al., 2015). LHb-RMTg projection is inhibitory on a DRN subpopulation of presumptive glutamatergic neurons, while the direct LHb-DRN is excitatory on distinctive 5-HT-containing neurons area (Sego et al., 2014). Therefore, nicotine acting on the LHb would increase 5-HT neuronal activity and its release in several brain regions (Pierucci et al., 2014), including mPFC, hippocampus and amygdale leading to the development of anxiety state. Strikingly, in our conditions the LHb lesion revert the anxiogenic-like effect mediated by 1 mg/kg of nicotine into an anxiolytic effect. The LHb lesion might produce some neurochemical (i.e., DA, 5-HT, glutamate, GABA) or hormonal (e.g., corticosterone) changes which indirectly antagonize the anxiety state induced by nicotine treatment. The nature of such an interaction is far from being simple. Firstly, it is very difficult to tease apart the different contributions of the single LHb projections and the consequences of removing the LHb in modulating nicotine effects. Secondly, nAChRs are highly represented in all the areas of the anxiety network, including DA and 5-HT areas.

Since some evidence suggests that excitation of the DRN, and the resulting increase of 5-HT release into the dorsal hippocampus, mediates some of the nicotine-induced anxiogenic effects (Cheeta et al., 2001;Tucci et al., 2003b), the anxiogenic effect of LHb lesion seen in our study should lead to the excitation of the DRN-hippocampus pathway.

Our data are in agreement with early findings, which showed that lesioning of the fasciculus retroflexus improved the behavioral response of depressed rats by increasing the 5-HT level in the DRN (Yang et al., 2008), while its kainic-stimulation impaired inhibitory avoidance acquisition, in the EPM, indicating an anxiolytic-like effect (Pobbe and Zangrossi Jr, 2008b).

Further investigations with specific lateral and medial habenular lesions together with measurements of differential neurochemical and behavioral alterations under normal and stressful situations are needed to clarify the nature of the function of the habenular complex.

In conclusion, this study demonstrates that nicotine itself leads to anxiety, and this requires the LHb for its expression. Interestingly, most smokers report their initial smoking experiences as unpleasant, and aversive reactions such as anxiety to nicotine after initial exposure can decrease the likelihood of developing a tobacco habit (Sartor et al., 2010). Nicotine acts on a heterogeneous population of nAChR subtypes in different neuronal circuits that mediate reward and aversive effects and the LHb might mediate the latter (Fowler and Kenny, 2014).
Another important finding of this study is that LHb has a role in modulating emotionality also under moderate/low anxiety conditions such as during exploratory behavior, which has never been shown before. Moreover, from a methodological point of view, an important output of our research is the evidence of the necessity of a synergic use of both quantitative and multivariate analyses to gain a precise description of the effects induced by one or more independent variables in behavior analysis.
Much work still remains to be done in order to understand specifically how the aversive anxiety effects of nicotine are encoded in the brain by the LHb, and how aversion-related circuits may interact with reward circuits to control nicotine intake. Nevertheless, our data support the interesting possibility that increasing the noxious properties of nicotine acting at the level of the LHb may serve as a novel strategy for the development of efficacious smoking cessation agents.

Acknowledgements

This project was supported by University of Malta funding scheme (GDG) and EU COST CM1103. CD, LP and SC received a grant from the British Council.

Adhikari, A. (2014). Distributed circuits underlying anxiety. *Front Behav Neurosci* 8**,** 112.
Amat, J., Sparks, P.D., Matus-Amat, P., Griggs, J., Watkins, L.R., and Maier, S.F. (2001). The role of the habenular complex in the elevation of dorsal raphe nucleus serotonin and the changes in the behavioral responses produced by uncontrollable stress. *Brain Res* 917**,** 118-126.
Anderson, S.M., and Brunzell, D.H. (2015). Anxiolytic-like and anxiogenic-like effects of nicotine are regulated via diverse action at beta2*nAChRs. *Br J Pharmacol* In Press.
Andrade, T.G., Zangrossi, H., Jr., and Graeff, F.G. (2013). The median raphe nucleus in anxiety revisited. *J Psychopharmacol* 27**,** 1107-1115.
Augustsson, H., Dahlborn, K., and Meyerson, B.J. (2005). Exploration and risk assessment in female wild house mice (Mus musculus musculus) and two laboratory strains. *Physiol Behav* 84**,** 265-277.
Balerio, G.N., Aso, E., and Maldonado, R. (2005). Involvement of the opioid system in the effects induced by nicotine on anxiety-like behaviour in mice. *Psychopharmacology (Berl)* 181**,** 260-269.
Bianco, I.H., and Wilson, S.W. (2009). The habenular nuclei: a conserved asymmetric relay station in the vertebrate brain. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 364**,** 1005-1020.
Boissier, J.R., and Simon, P. (1962). [The exploration reaction in the mouse. Preliminary note]. *Therapie* 17**,** 1225-1232.
Booker, T.K., Butt, C.M., Wehner, J.M., Heinemann, S.F., and Collins, A.C. (2007). Decreased anxiety-like behavior in beta3 nicotinic receptor subunit knockout mice. *Pharmacol Biochem Behav* 87**,** 146-157.
Bruijnzeel, A.W. (2012). Tobacco addiction and the dysregulation of brain stress systems. *Neurosci Biobehav Rev* 36**,** 1418-1441.
Casarrubea, M., Jonsson, G.K., Faulisi, F., Sorbera, F., Di Giovanni, G., Benigno, A., Crescimanno, G., and Magnusson, M.S. (2015). T-pattern analysis for the study of temporal structure of animal and human behavior: a comprehensive review. *J Neurosci Methods* 239**,** 34-46.
Casarrubea, M., Magnusson, M.S., Roy, V., Arabo, A., Sorbera, F., Santangelo, A., Faulisi, F., and Crescimanno, G. (2014). Multivariate temporal pattern analysis applied to the study of rat behavior in the elevated plus maze: methodological and conceptual highlights. *J Neurosci Methods* 234**,** 116-126.
Casarrubea, M., Roy, V., Sorbera, F., Magnusson, M.S., Santangelo, A., Arabo, A., and Crescimanno, G. (2013a). Significant divergences between the temporal structure of the behavior in Wistar and in the spontaneously more anxious DA/Han strain of rats tested in elevated plus maze. *Behav Brain Res* 250**,** 166-173.
Casarrubea, M., Roy, V., Sorbera, F., Magnusson, M.S., Santangelo, A., Arabo, A., and Crescimanno, G. (2013b). Temporal structure of the rat's behavior in elevated plus maze test. *Behav Brain Res* 237**,** 290-299.
Casarrubea, M., Sorbera, F., and Crescimanno, G. (2009a). Multivariate data handling in the study of rat behavior: an integrated approach. *Behav Res Methods* 41**,** 772-781.
Casarrubea, M., Sorbera, F., and Crescimanno, G. (2009b). Structure of rat behavior in hole-board: I) multivariate analysis of response to anxiety. *Physiol Behav* 96**,** 174-179.
Casarrubea, M., Sorbera, F., and Crescimanno, G. (2009c). Structure of rat behavior in hole-board: II) multivariate analysis of modifications induced by diazepam. *Physiol Behav* 96**,** 683-692.

Casarrubea, M., Sorbera, F., Magnusson, M., and Crescimanno, G. (2010a). Temporal patterns analysis of rat behavior in hole-board. *Behav Brain Res* 208**,** 124-131.

Casarrubea, M., Sorbera, F., Magnusson, M.S., and Crescimanno, G. (2011). T-pattern analysis of diazepam-induced modifications on the temporal organization of rat behavioral response to anxiety in hole board. *Psychopharmacology (Berl)* 215**,** 177-189.

Casarrubea, M., Sorbera, F., Santangelo, A., and Crescimanno, G. (2010b). Microstructure of rat behavioral response to anxiety in hole-board. *Neurosci Lett* 481**,** 82-87.

Cheeta, S., Irvine, E.E., Kenny, P.J., and File, S.E. (2001). The dorsal raphe nucleus is a crucial structure mediating nicotine's anxiolytic effects and the development of tolerance and withdrawal responses. *Psychopharmacology (Berl)* 155**,** 78-85.

Clarke, P.B., and Pert, A. (1985). Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons. *Brain Res* 348**,** 355-358.

Clarke, P.B.S., Hommer, D.W., Pert, A., and Skirboll, L.R. (1985). Electrophysiological actions of nicotine on substantia nigra single units. *British Journal of Pharmacology* 85**,** 827-835.

Corrigall, W.A., Franklin, K.B., Coen, K.M., and Clarke, P.B. (1992). The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology (Berl)* 107**,** 285-289.

Cui, W., Mizukami, H., Yanagisawa, M., Aida, T., Nomura, M., Isomura, Y., Takayanagi, R., Ozawa, K., Tanaka, K., and Aizawa, H. (2014). Glial dysfunction in the mouse habenula causes depressive-like behaviors and sleep disturbance. *J Neurosci* 34**,** 16273-16285.

Dao, D.Q., Perez, E.E., Teng, Y., Dani, J.A., and De Biasi, M. (2014). Nicotine enhances excitability of medial habenular neurons via facilitation of neurokinin signaling. *J Neurosci* 34**,** 4273-4284.

De La Mora, M.P., Gallegos-Cari, A., Arizmendi-Garcia, Y., Marcellino, D., and Fuxe, K. (2010). Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: Structural and functional analysis. *Prog Neurobiol* 90**,** 198-216.

Di Chiara, G. (2000). Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur J Pharmacol* 393**,** 295-314.

Di Giovanni, G. (2012). *Nicotine Addiction: Prevention, Health Effects and Treatment Options.* New York: Nova Science Publishers, Inc.

Di Matteo, V., Pierucci, M., Benigno, A., Esposito, E., Crescimanno, G., and Di Giovanni, G. (2010). Critical role of nitric oxide on nicotine-induced hyperactivation of dopaminergic nigrostriatal system: Electrophysiological and neurochemical evidence in rats. *CNS Neurosci Ther* 16**,** 127-136.

Di Matteo, V., Pierucci, M., Di Giovanni, G., Benigno, A., and Esposito, E. (2007). The neurobiological bases for the pharmacotherapy of nicotine addiction. *Curr Pharm Des* 13**,** 1269-1284.

Doll, R., Peto, R., Boreham, J., and Sutherland, I. (2004). Mortality in relation to smoking: 50 years' observations on male British doctors. *Bmj* 328**,** 1519.

File, S.E., Cheeta, S., and Kenny, P.J. (2000). Neurobiological mechanisms by which nicotine mediates different types of anxiety. *Eur J Pharmacol* 393**,** 231-236.

File, S.E., Kenny, P.J., and Ouagazzal, A.M. (1998). Bimodal modulation by nicotine of anxiety in the social interaction test: role of the dorsal hippocampus. *Behav Neurosci* 112**,** 1423-1429.

File, S.E., and Wardill, A.G. (1975). The reliability of the hole-board apparatus. *Psychopharmacologia* 44**,** 47-51.

Fowler, C.D., and Kenny, P.J. (2014). Nicotine aversion: Neurobiological mechanisms and relevance to tobacco dependence vulnerability. *Neuropharmacology* 76 Pt B**,** 533-544.

Fowler, C.D., Lu, Q., Johnson, P.M., Marks, M.J., and Kenny, P.J. (2011). Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. *Nature* 471**,** 597-601.

Gill, M.J., Ghee, S.M., Harper, S.M., and See, R.E. (2013). Inactivation of the lateral habenula reduces anxiogenic behavior and cocaine seeking under conditions of heightened stress. *Pharmacology Biochemistry and Behavior* 111**,** 24-29.

Goutagny, R., Loureiro, M., Jackson, J., Chaumont, J., Williams, S., Isope, P., Kelche, C., Cassel, J.C., and Lecourtier, L. (2013). Interactions between the lateral habenula and the hippocampus: implication for spatial memory processes. *Neuropsychopharmacology* 38**,** 2418-2426.

Haack, A.K., Sheth, C., Schwager, A.L., Sinclair, M.S., Tandon, S., and Taha, S.A. (2014). Lesions of the lateral habenula increase voluntary ethanol consumption and operant self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate ethanol-induced conditioned taste aversion. *PLoS One* 9**,** e92701.

Hayase, T. (2007). Chronologically overlapping occurrences of nicotine-induced anxiety- and depression-related behavioral symptoms: effects of anxiolytic and cannabinoid drugs. *BMC Neurosci* 8**,** 76.

Hikosaka, O. (2010). The habenula: from stress evasion to value-based decision-making. *Nat Rev Neurosci* 11**,** 503-513.

Hikosaka, O., Sesack, S.R., Lecourtier, L., and Shepard, P.D. (2008). Habenula: crossroad between the basal ganglia and the limbic system. *J Neurosci* 28**,** 11825-11829.

Hong, S., Jhou, T.C., Smith, M., Saleem, K.S., and Hikosaka, O. (2010). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *Journal of Neuroscience* 31**,** 11457-11471.

Irvine, E.E., Bagnalasta, M., Marcon, C., Motta, C., Tessari, M., File, S.E., and Chiamulera, C. (2001). Nicotine self-administration and withdrawal: modulation of anxiety in the social interaction test in rats. *Psychopharmacology (Berl)* 153**,** 315-320.

Irvine, E.E., Cheeta, S., and File, S.E. (1999). Time-course of changes in the social interaction test of anxiety following acute and chronic administration of nicotine. *Behav Pharmacol* 10**,** 691-697.

Jean-Richard Dit Bressel, P., and Mcnally, G.P. (2014). The role of the lateral habenula in punishment. *PLoS One* 9**,** e111699.

Jhou, T.C., Good, C.H., Rowley, C.S., Xu, S.P., Wang, H., Burnham, N.W., Hoffman, A.F., Lupica, C.R., and Ikemoto, S. (2013). Cocaine drives aversive conditioning via delayed activation of dopamine-responsive habenular and midbrain pathways. *J Neurosci* 33**,** 7501-7512.

Kalueff, A.V., and Tuohimaa, P. (2005). The grooming analysis algorithm discriminates between different levels of anxiety in rats: potential utility for neurobehavioural stress research. *J Neurosci Methods* 143**,** 169-177.

Kenny, P.J., Cheeta, S., and File, S.E. (2000). Anxiogenic effects of nicotine in the dorsal hippocampus are mediated by 5-HT1A and not by muscarinic M1 receptors. *Neuropharmacology* 39**,** 300-307.

Khaled, M.A., Pushparaj, A., Di Ciano, P., Diaz, J., and Le Foll, B. (2014). Dopamine D3 Receptors in the Basolateral Amygdala and the Lateral Habenula Modulate Cue-Induced Reinstatement of Nicotine Seeking. *Neuropsychopharmacology*.

Kurumaji, A., Umino, A., Tanami, M., Ito, A., Asakawa, M., and Nishikawa, T. (2003). Distribution of anxiogenic-induced c-Fos in the forebrain regions of developing rats. *Journal of Neural Transmission* 110**,** 1161-1168.

Lammel, S., Lim, B.K., and Malenka, R.C. (2012). Reward and aversion in a heterogeneous midbrain dopamine system. *Neuropharmacology* 76**,** 351-359.

Lecca, S., Melis, M., Luchicchi, A., Ennas, M.G., Castelli, M.P., Muntoni, A.L., and Pistis, M. (2011). Effects of drugs of abuse on putative rostromedial tegmental neurons, inhibitory afferents to midbrain dopamine cells. *Neuropsychopharmacology* 36**,** 589-602.

Lecca, S., Meye, F.J., and Mameli, M. (2014). The lateral habenula in addiction and depression: An anatomical, synaptic and behavioral overview. *European Journal of Neuroscience* 39**,** 1170-1178.

Lee, E.H.Y., and Huang, S.L. (1988). Role of lateral habenula in the regulation of exploratory behavior and its relationship to stress in rats. *Behavioural Brain Research* 30**,** 265-271.

Lester, R.J. (ed.). (2014). *Nicotinic Receptors.* Springer New York.

Magnusson, M.S. (2000). Discovering hidden time patterns in behavior: T-patterns and their detection. *Behav Res Methods Instrum Comput* 32**,** 93-110.

Matsumoto, M., and Hikosaka, O. (2007). Lateral habenula as a source of negative reward signals in dopamine neurons. *Nature* 447**,** 1111-1115.

Mcdermott, M.S., Marteau, T.M., Hollands, G.J., Hankins, M., and Aveyard, P. (2013). Change in anxiety following successful and unsuccessful attempts at smoking cessation: cohort study. *Br J Psychiatry* 202**,** 62-67.

Mcgranahan, T.M., Patzlaff, N.E., Grady, S.R., Heinemann, S.F., and Booker, T.K. (2011). alpha4beta2 nicotinic acetylcholine receptors on dopaminergic neurons mediate nicotine reward and anxiety relief. *J Neurosci* 31**,** 10891-10902.

Moody, T.W., Merali, Z., and Crawley, J.N. (1988). The effects of anxiolytics and other agents on rat grooming behavior. *Annals of the New York Academy of Sciences* 525**,** 281-290.

Murphy, C.A., Dicamillo, A.M., Haun, F., and Murray, M. (1996). Lesion of the habenular efferent pathway produces anxiety and locomotor hyperactivity in rats: a comparison of the effects of neonatal and adult lesions. *Behav Brain Res* 81**,** 43-52.

Nasehi, M., Mafi, F., Oryan, S., Nasri, S., and Zarrindast, M.R. (2011). The effects of dopaminergic drugs in the dorsal hippocampus of mice in the nicotine-induced anxiogenic-like response. *Pharmacol Biochem Behav* 98**,** 468-473.

Nielson, H.C., and Mciver, A.H. (1966). Cold stress and habenular lesion effects on rat behaviors. *J Appl Physiol* 21**,** 655-660.

Olausson, P., Engel, J.A., and Soderpalm, B. (1999). Behavioral sensitization to nicotine is associated with behavioral disinhibition; counteraction by citalopram. *Psychopharmacology (Berl)* 142**,** 111-119.

Ouagazzal, A.M., Kenny, P.J., and File, S.E. (1999a). Modulation of behaviour on trials 1 and 2 in the elevated plus-maze test of anxiety after systemic and hippocampal administration of nicotine. *Psychopharmacology (Berl)* 144**,** 54-60.

Ouagazzal, A.M., Kenny, P.J., and File, S.E. (1999b). Stimulation of nicotinic receptors in the lateral septal nucleus increases anxiety. *Eur J Neurosci* 11**,** 3957-3962.

Parrott, A.C., and Murphy, R.S. (2012). Explaining the stress-inducing effects of nicotine to cigarette smokers. *Hum Psychopharmacol* 27**,** 150-155.

Paylor, R., Nguyen, M., Crawley, J.N., Patrick, J., Beaudet, A., and Orr-Urtreger, A. (1998). Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. *Learn Mem* 5**,** 302-316.

Picciotto, M.R., Brunzell, D.H., and Caldarone, B.J. (2002). Effect of nicotine and nicotinic receptors on anxiety and depression. *Neuroreport* 13**,** 1097-1106.

Pierucci, M., Chambers, S., Partridge, L., De Deurwaerdère, P., and Di Giovanni, G. (2014). "Role of Central Serotonin Receptors in Nicotine Addiction," in *Nicotinic Receptors,* ed. R.a.J. Lester. Springer New York), 279-305.

Pierucci, M., Di Matteo, V., and Esposito, E. (2004). Stimulation of serotonin2C receptors blocks the hyperactivation of midbrain dopamine neurons induced by nicotine administration. *Journal of Pharmacology and Experimental Therapeutics* 309**,** 109-118.

Pierucci, M., Pitruzzella, A., Valentino, M., Zammit, C., Muscat, R., Benigno, A., and Di Giovanni, G. (2011). Lateral Habenula contribution in nicotine addiction: Focus on dopamine, GABA and serotonin interactions. *Malta Medical Journal* 23**,** 28-32.

Pobbe, R.L.H., and Zangrossi Jr, H. (2008a). Involvement of the lateral habenula in the regulation of generalized anxiety- and panic-related defensive responses in rats. *Life Sciences* 82**,** 1256-1261.

Pobbe, R.L.H., and Zangrossi Jr, H. (2008b). The lateral habenula regulates defensive behaviors through changes in 5-HT-mediated neurotransmission in the dorsal periaqueductal gray matter. *Neuroscience Letters* 479**,** 87-91.

Proulx, C.D., Hikosaka, O., and Malinow, R. (2014). Reward processing by the lateral habenula in normal and depressive behaviors. *Nature Neuroscience* 17**,** 1146-1152.

Ross, S.A., Wong, J.Y., Clifford, J.J., Kinsella, A., Massalas, J.S., Horne, M.K., Scheffer, I.E., Kola, I., Waddington, J.L., Berkovic, S.F., and Drago, J. (2000). Phenotypic characterization of an alpha 4 neuronal nicotinic acetylcholine receptor subunit knock-out mouse. *J Neurosci* 20**,** 6431-6441.

Salas, R., Pieri, F., Fung, B., Dani, J.A., and De Biasi, M. (2003). Altered anxiety-related responses in mutant mice lacking the beta4 subunit of the nicotinic receptor. *J Neurosci* 23**,** 6255-6263.

Salas, R., Sturm, R., Boulter, J., and De Biasi, M. (2009). Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. *J Neurosci* 29**,** 3014-3018.

Sartor, C.E., Lessov-Schlaggar, C.N., Scherrer, J.F., Bucholz, K.K., Madden, P.A., Pergadia, M.L., Grant, J.D., Jacob, T., and Xian, H. (2010). Initial response to cigarettes predicts rate of progression to regular smoking: findings from an offspring-of-twins design. *Addict Behav* 35**,** 771-778.

Sego, C., Goncalves, L., Lima, L., Furigo, I.C., Donato, J., and Metzger, M. (2014). Lateral habenula and the rostromedial tegmental nucleus innervate neurochemically distinct subdivisions of the dorsal raphe nucleus in the rat. *Journal of Comparative Neurology* 522**,** 1454-1484.

Spruijt, B.M., Van Hooff, J.A., and Gispen, W.H. (1992). Ethology and neurobiology of grooming behavior. *Physiol Rev* 72**,** 825-852.

Stamatakis, A.M., and Stuber, G.D. (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nature Neuroscience* 15**,** 1105-1107.

Stopper, C.M., and Floresco, S.B. (2014). What's better for me? Fundamental role for lateral habenula in promoting subjective decision biases. *Nat Neurosci* 17**,** 33-35.

Takeda, H., Tsuji, M., and Matsumiya, T. (1998). Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *Eur J Pharmacol* 350**,** 21-29.

Thornton, E.W., and Evans, J.C. (1982). The role of habenular nuclei in the selection of behavioral strategies. *Physiological Psychology* 10**,** 361-367.

Trigo, J.M., Zimmer, A., and Maldonado, R. (2009). Nicotine anxiogenic and rewarding effects are decreased in mice lacking beta-endorphin. *Neuropharmacology* 56**,** 1147-1153.

Tucci, S., Genn, R.F., Marco, E., and File, S.E. (2003a). Do different mechanisms underlie two anxiogenic effects of systemic nicotine? *Behav Pharmacol* 14**,** 323-329.

Tucci, S.A., Genn, R.F., and File, S.E. (2003b). Methyllycaconitine (MLA) blocks the nicotine evoked anxiogenic effect and 5-HT release in the dorsal hippocampus: possible role of alpha7 receptors. *Neuropharmacology* 44**,** 367-373.

Varani, A.P., Moutinho, L.M., Bettler, B., and Balerio, G.N. (2012). Acute behavioural responses to nicotine and nicotine withdrawal syndrome are modified in GABA(B1) knockout mice. *Neuropharmacology* 63**,** 863-872.

Velasquez, K.M., Molfese, D.L., and Salas, R. (2014). The role of the habenula in drug addiction. *Frontiers in Human Neuroscience* 8.

Wang, Z., Wang, L., Yamamoto, R., Sugai, T., and Kato, N. (2013). Role of the lateral habenula in shaping context-dependent locomotor activity during cognitive tasks. *NeuroReport* 24**,** 276-280.

Wirtshafter, D., Asin, K.E., and Pitzer, M.R. (1994). Dopamine agonists and stress produce different patterns of Fos-like immunoreactivity in the lateral habenula. *Brain Res* 633**,** 21-26.

Yang, L.-M., Hu, B., Xia, Y.-H., Zhang, B.-L., and Zhao, H. (2008). Lateral habenula lesions improve the behavioral response in depressed rats via increasing the serotonin level in dorsal raphe nucleus. *Behavioural Brain Research* 188**,** 84-90.

Zangrossi, H., Jr., and Graeff, F.G. (2014). Serotonin in anxiety and panic: contributions of the elevated T-maze. *Neurosci Biobehav Rev* 46 Pt 3**,** 397-406.

Zarrindast, M.R., Eslahi, N., Rezayof, A., Rostami, P., and Zahmatkesh, M. (2013). Modulation of ventral tegmental area dopamine receptors inhibit nicotine-induced anxiogenic-like behavior in the central amygdala. *Prog Neuropsychopharmacol Biol Psychiatry* 41**,** 11-17.

Zarrindast, M.R., Homayoun, H., Babaie, A., Etminani, A., and Gharib, B. (2000). Involvement of adrenergic and cholinergic systems in nicotine-induced anxiogenesis in mice. *Eur J Pharmacol* 407**,** 145-158.

Zarrindast, M.R., Khalifeh, S., Rezayof, A., Rostami, P., Aghamohammadi Sereshki, A., and Zahmatkesh, M. (2012). Involvement of rat dopaminergic system of nucleus accumbens in nicotine-induced anxiogenic-like behaviors. *Brain Res* 1460**,** 25-32.

Zarrindast, M.R., Naghdi-Sedeh, N., Nasehi, M., Sahraei, H., Bahrami, F., and Asadi, F. (2010). The effects of dopaminergic drugs in the ventral hippocampus of rats in the nicotine-induced anxiogenic-like response. *Neurosci Lett* 475**,** 156-160.

Zarrindast, M.R., Solati, J., Oryan, S., and Parivar, K. (2008). Effect of intra-amygdala injection of nicotine and GABA receptor agents on anxiety-like behaviour in rats. *Pharmacology* 82**,** 276-284.

Zhao, H., Zhang, B.L., Yang, S.J., and Rusak, B. (2015). The role of lateral habenula-dorsal raphe nucleus circuits in higher brain functions and psychiatric illness. *Behav Brain Res* 277**,** 89-98.

Zweifel, L.S., Fadok, J.P., Argilli, E., Garelick, M.G., Jones, G.L., Dickerson, T.M., Allen, J.M., Mizumori, S.J., Bonci, A., and Palmiter, R.D. (2011). Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nat Neurosci* 14**,** 620-626.

Caption to Figures

Fig. 1 – Photomicrographs (an enlargement of the box in C) demonstrate the typical location of lesions in the LHb (B), shown in contrast to the intact LHb in a unlesioned animal (A). Also shown is the corresponding diagramatic representation of the analogous coronal section (C). fr = fasciculus retroflexus; LHbM = Medial part of Lateral Habenula; LHbL = Lateral part of Lateral Habenula; MHb = Medial Habenula; sm = stria medullaris.

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

Fig. 3 - Mean durations ± SEM of behavioral components in unlesioned (Unles + Saline and Unles + Nicotine 1 mg/kg) and LHb-Lesioned (LHb-Les + Saline and LHb-Les + Nicotine 1 mg/kg) groups. *= $p < 0.05$ compared to the unlesioned group after the same drug treatment; ** = $p < 0.01$ compared to the unlesioned group after the same drug treatment; *** = $p < 0.005$ compared to the unlesioned group after the same drug treatment; **** = $p < 0.001$ compared to the unlesioned group after the same drug treatment; + = $p < 0.05$ compared to the same group saline condition (two-tailed paired t-test). Panel A = behavioral components with durations in one or more groups > 5 seconds; panel B = behavioral components with durations in one or more groups < 5 seconds. See Tab. 1 for significant differences between mean durations in saline and nicotine 1 mg/kg unlesioned groups. See fig. 1 for abbreviations.

Fig. 4 - Mean occurrences ± SEM of behavioral components in unlesioned (Unles + Saline and Unles + Nicotine 1 mg/kg) and LHb-Lesioned (LHb-Les + Saline and LHb-Les + Nicotine 1 mg/kg) groups. *= $p < 0.05$ compared to the unlesioned group after the same drug treatment; ** = $p < 0.01$ compared to the unlesioned group after the same drug treatment; *** = $p < 0.005$

compared to the unlesioned group after the same drug treatment; **** = $p < 0.001$ compared to the unlesioned group after the same drug treatment; + = $p < 0.05$ compared to the same group saline condition (two-tailed paired t-test). Panel A = behavioral components with occurrences in one or more groups > 5; panel B = behavioral components with occurrences in one or more groups < 5. See Tab. 2 for significant differences between mean occurrences in saline and nicotine 1 mg/kg unlesioned groups. See fig. 1 for abbreviations.

Fig. 5 - T-patterns detected in unlesioned groups (saline, nicotine 0.1, 0.5 and 1 mg/kg). "TP#" column: number of each different T-pattern detected; "String" column: events encompassed in T-pattern's structure; "Occs" column: occurrences of each T-pattern. Histograms: T-patterns length distribution in real data (dark bars) and random generated data + 1 SD (white bars). Bottom left of each panel: overall T-patterns detected in the group and mean number of T-patterns for each subject. * = significant difference in comparison with saline (ANOVA + Newman-Keuls post-hoc test for multiple comparisons). See fig. 1 for abbreviations.

Fig. 6 - T-patterns detected in LHb-lesioned groups (LHb-Les + Saline and LHb-Les + Nicotine 1 mg/kg). "TP#" column: number of each different T-pattern detected; "String" column: events encompassed in T-pattern's structure; "Occs" column: occurrences of each T-pattern. Histograms: T-patterns length distribution in real data (dark bars) and random generated data + 1SD (white bars). Bottom left of each panel: overall T-patterns detected in the group and mean number of T-patterns for each subject. See fig. 1 for abbreviations.

Fig. 7 - Mean occurrences ± SEM of T-patterns detected in unlesioned (Unles + Saline and Unles + Nicotine 1 mg/kg) and LHb-Lesioned (LHb-Les + Saline and LHb-Les + Nicotine 1 mg/kg) groups. *= $p < 0.05$ compared to the unlesioned group after the same drug treatment; ** = $p < 0.01$ compared to the unlesioned group after the same drug treatment; + = $p < 0.05$ compared to the same group saline condition (two-tailed paired t-test); ++++ = $p < 0.001$ compared to the same group saline condition (two-tailed paired t-test). # = $p < 0.05$ interaction of lesion group×drug treatment. #### = $p < 0.001$. See fig. 1 for abbreviations.

Fig. 8 – Percent distribution of T-patterns containing hole-exploratory behavioral components (edge sniff and/or head dip) in unlesioned (saline, nicotine 0.1, 0.5 and 1 mg/kg) and in LHb-lesioned (LHb-Les + Saline, LHb-Les + Nicotine 1 mg/kg) groups. *= $p < 0.0001$ compared to

the unlesioned saline group; + = $p < 0.0001$ compared to the same group saline condition (chi-square test).

	Saline		Nicotine 0.1 mg/kg		Nicotine 0.5 mg/kg		Nicotine 1 mg/kg	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wa	100	5.66	89.6	3.65	110	9.24	95.8	13.1
Cl (#)	70.4	6.41	**37.2 (*)**	4.7	55.2	7.9	54.7	10.3
Re (#)	17.3	4.84	12.2	4.54	**6.16 (*)**	1.24	**3.41 (*)**	1.26
IS	230	12.7	271	13.6	249	15.8	260	13.6
ES	44.6	6.05	62.5	5.24	41.6	4.63	56.4	8.2
HD (#)	97.4	9.41	**42.6 (*)**	5.47	**52.9 (*)**	8.72	**56.8 (*)**	6.86
FPL (#)	3.71	1.62	**9.48 (*)**	2.19	1.44	0.77	1.89	1
HPL (#)	1.11	0.75	14.5	5.9	0.76	0.57	5.05	4.59
FG (#)	2.69	0.99	**13.8 (*)**	3.93	5.14	2.72	3.36	1.68
BG (#)	0.66	0.45	**15.5 (*)**	5.15	3.06	2.26	1.95	1.2
Imm	15.1	2.62	47.9	10.4	56.9	30	51	13.8

Tab. 1 - Mean durations ± SE (in seconds) of behavioral components. # = significant ($p < 0.05$) ANOVA result; * = significant ($p < 0.05$) difference in comparison with saline (Newman-Keuls post-hoc test for multiple comparisons). See fig. 1 for abbreviations.

	Saline		Nicotine 0.1 mg/kg		Nicotine 0.5 mg/kg		Nicotine 1 mg/kg	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Wa	76.3	7.06	64.8	5.45	69.9	6.79	70.4	9.95
Cl (#)	30.2	3.34	**16.2 (*)**	1.98	**19.7 (*)**	2.46	**20.4 (*)**	3.60
Re (#)	10.7	3.21	**4.7 (*)**	2.04	**3.4 (*)**	0.67	**2.3 (*)**	0.80
IS	98.6	4.82	109	4.20	100	4.92	111	5.87
ES	42.4	6.11	48	4.09	36.8	3.51	44.5	4.08
HD (#)	33.1	3	**17.6 (*)**	1.75	**23 (*)**	2.34	**25 (*)**	2.79
FPL (#)	1	0.37	**2.4 (*)**	0.78	0.3	0.15	0.6	0.22
HPL	0.5	0.22	2.7	1.01	0.3	0.21	1.5	1.39
FG	0.8	0.33	2.8	1.40	1.4	0.72	0.8	0.42
BG	0.2	0.13	2.3	0.98	1.0	0.61	0.7	0.42
Imm (#)	9.6	2.12	**23.9 (*)**	4.34	**22 (*)**	4.27	**25.4 (*)**	3.80

Tab. 2 - Mean occurrences ± SE of behavioral components. # = significant ($p < 0.05$) ANOVA result; * = significant ($p < 0.05$) difference in comparison with saline (Newman-Keuls post-hoc test for multiple comparisons). See fig. 1 for abbreviations.

Figure 1.TIF

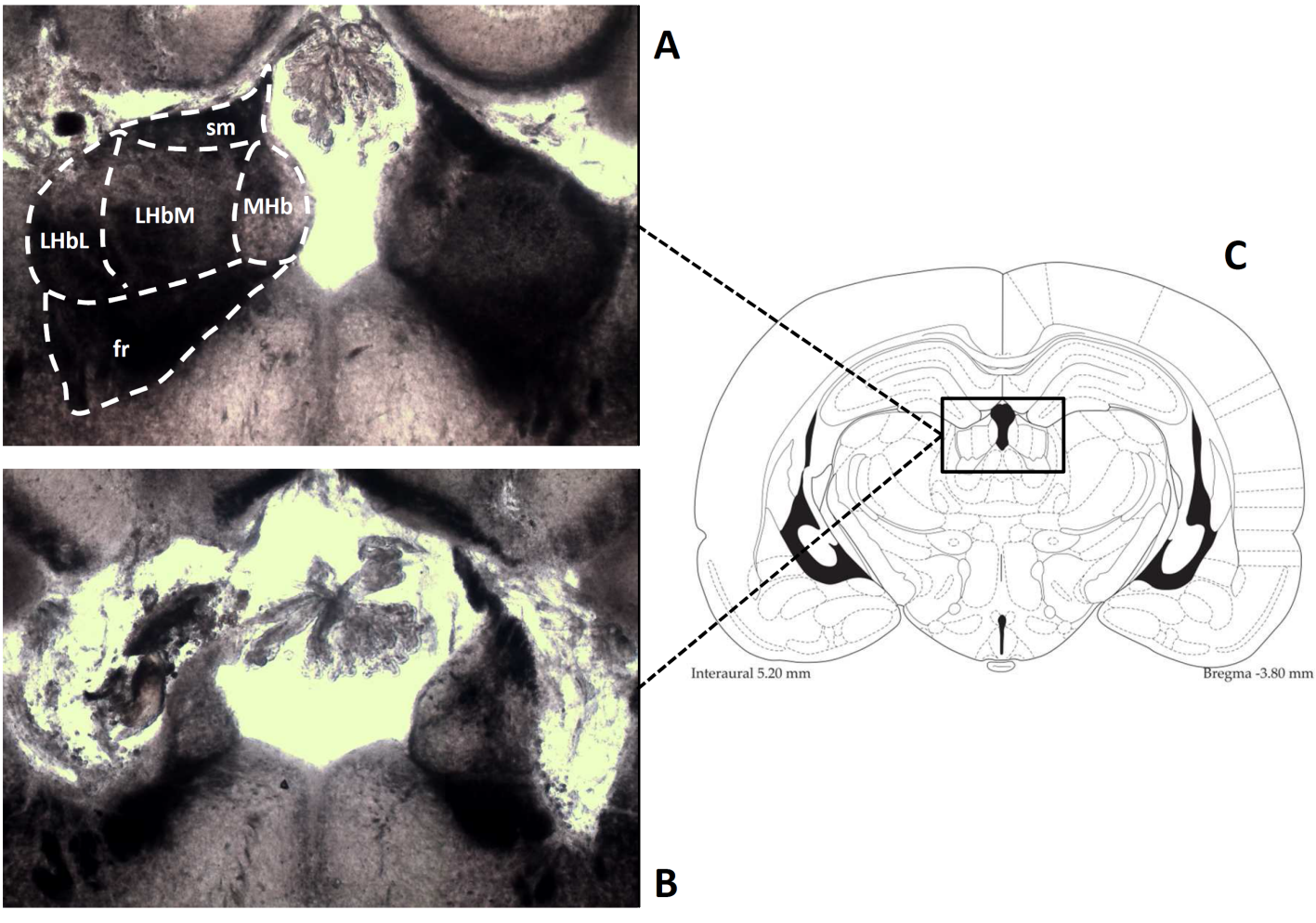


Figure 2.TIF

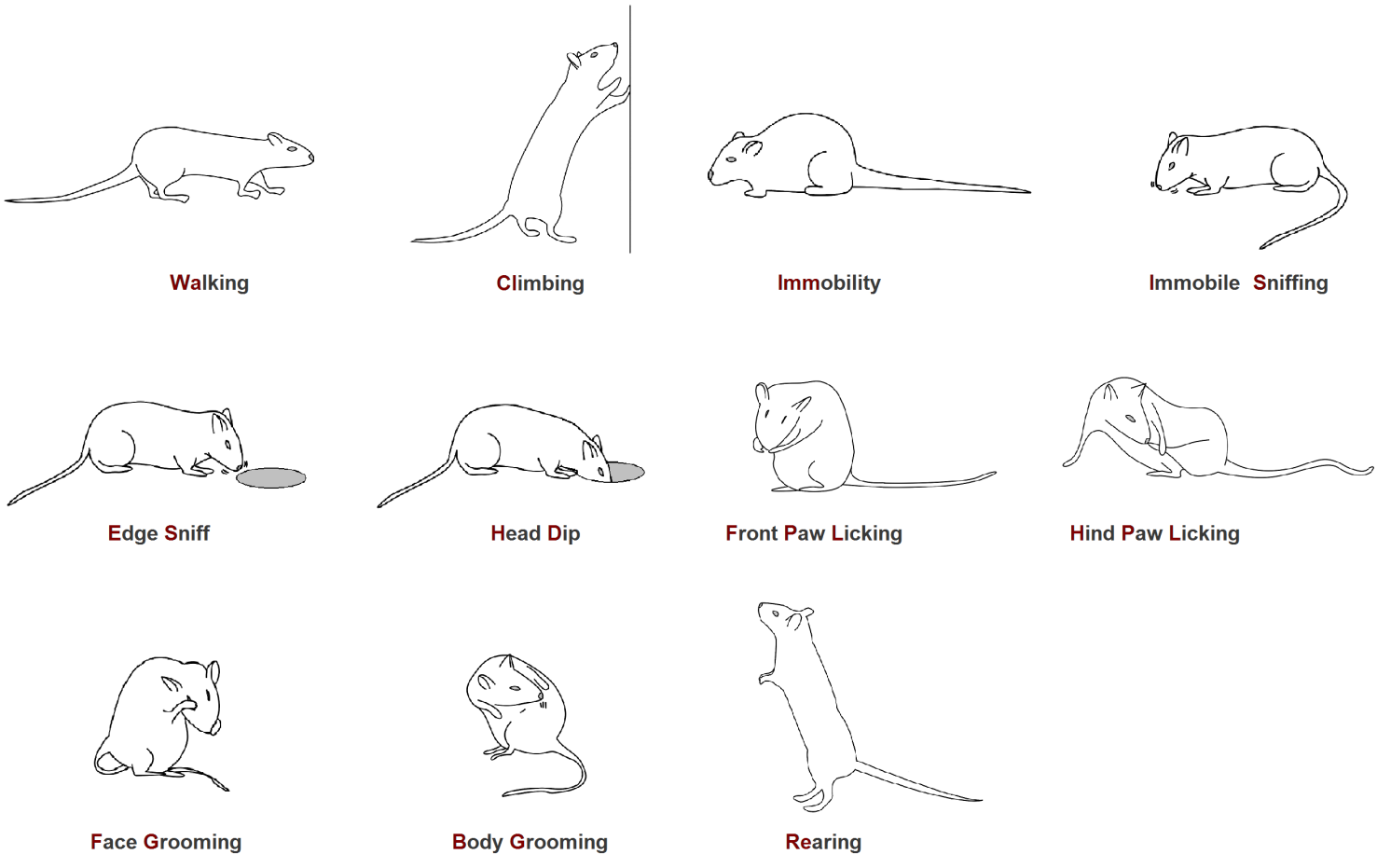

Walking
Climbing
Immobility
Immobile Sniffing
Edge Sniff
Head Dip
Front Paw Licking
Hind Paw Licking
Face Grooming
Body Grooming
Rearing

Figure 3.TIF

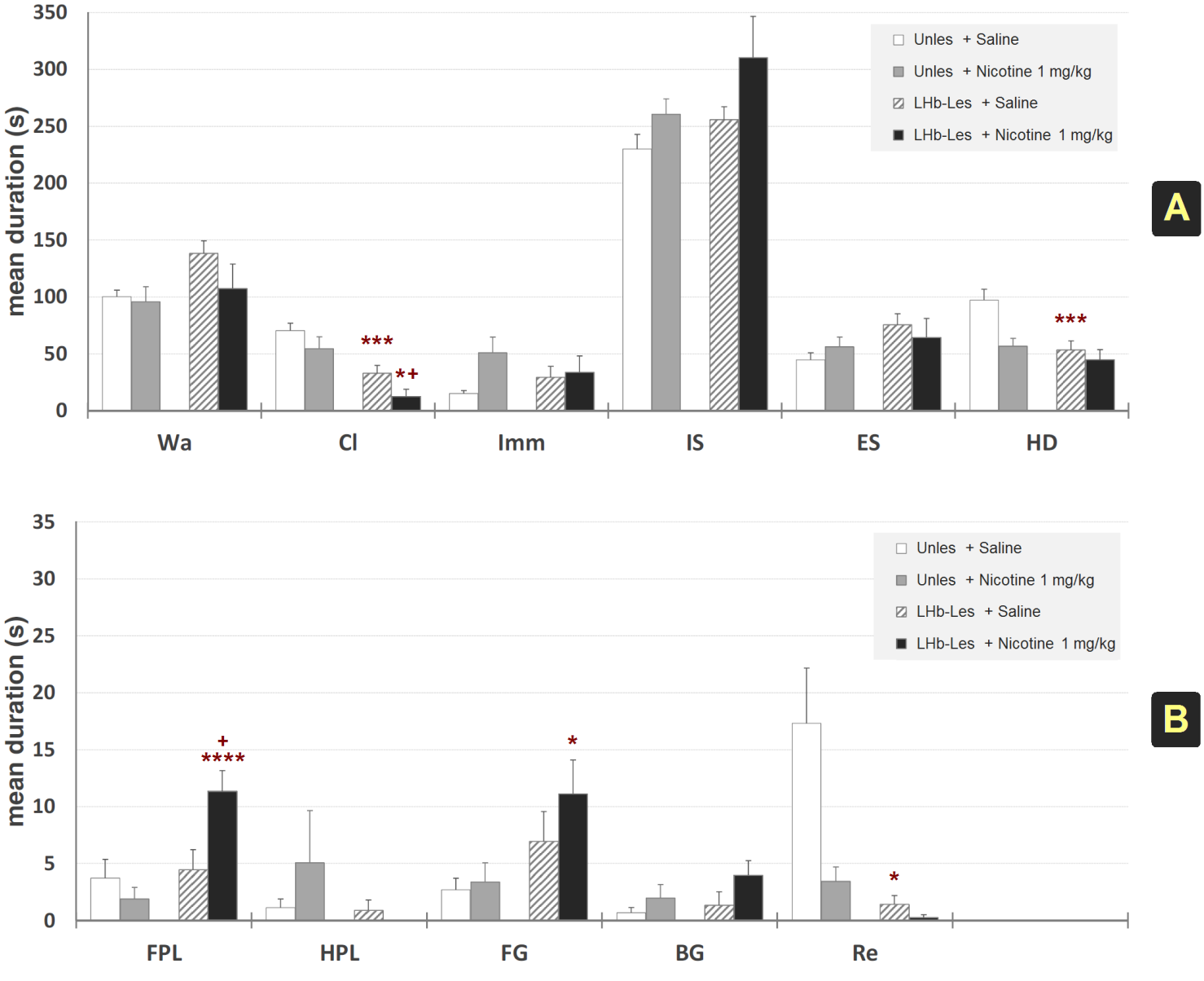
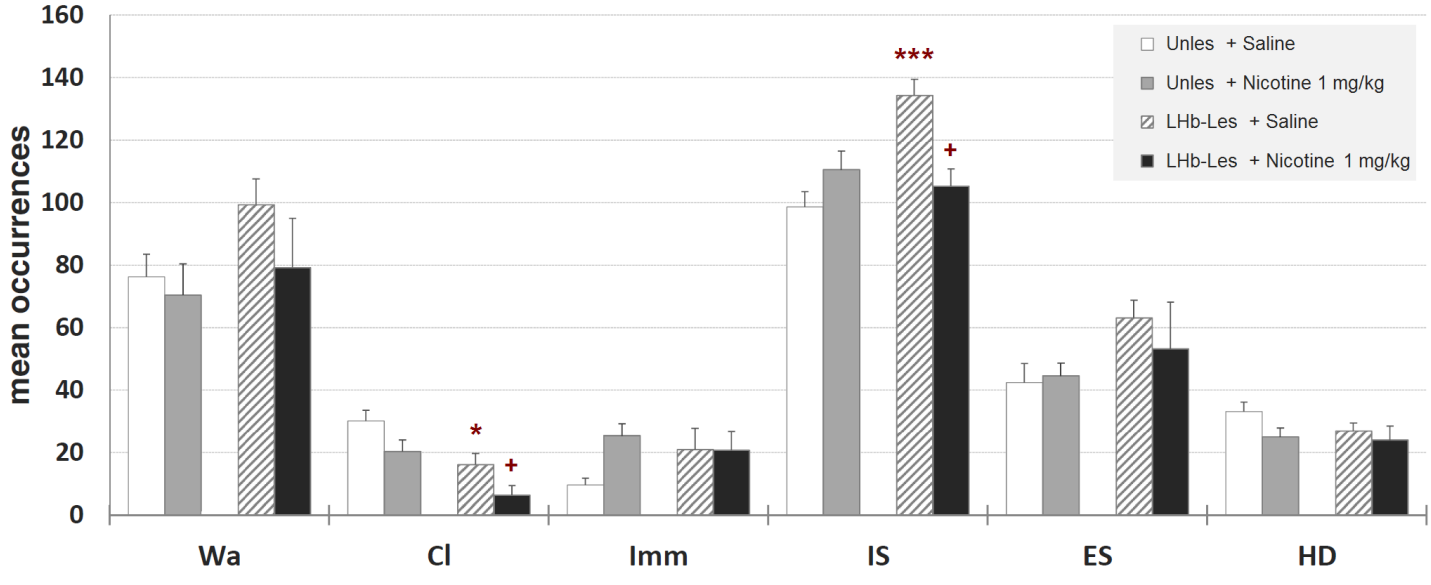

mean duration (s)
350
300
250
200
150
100
50
0
Unles + Saline
Unles + Nicotine 1 mg/kg
LHb-Les + Saline
LHb-Les + Nicotine 1 mg/kg
***
*+
***
Wa
Cl
Imm
IS
ES
HD
A
mean duration (s)
35
30
25
20
15
10
5
0
Unles + Saline
Unles + Nicotine 1 mg/kg
LHb-Les + Saline
LHb-Les + Nicotine 1 mg/kg
+
****
*
*
FPL
HPL
FG
BG
Re
B

Figure 4.TIF


mean occurrences
160
140
120
100
80
60
40
20
0
Unles + Saline
Unles + Nicotine 1 mg/kg
LHb-Les + Saline
LHb-Les + Nicotine 1 mg/kg
***
+
*
+
Wa
Cl
Imm
IS
ES
HD
A

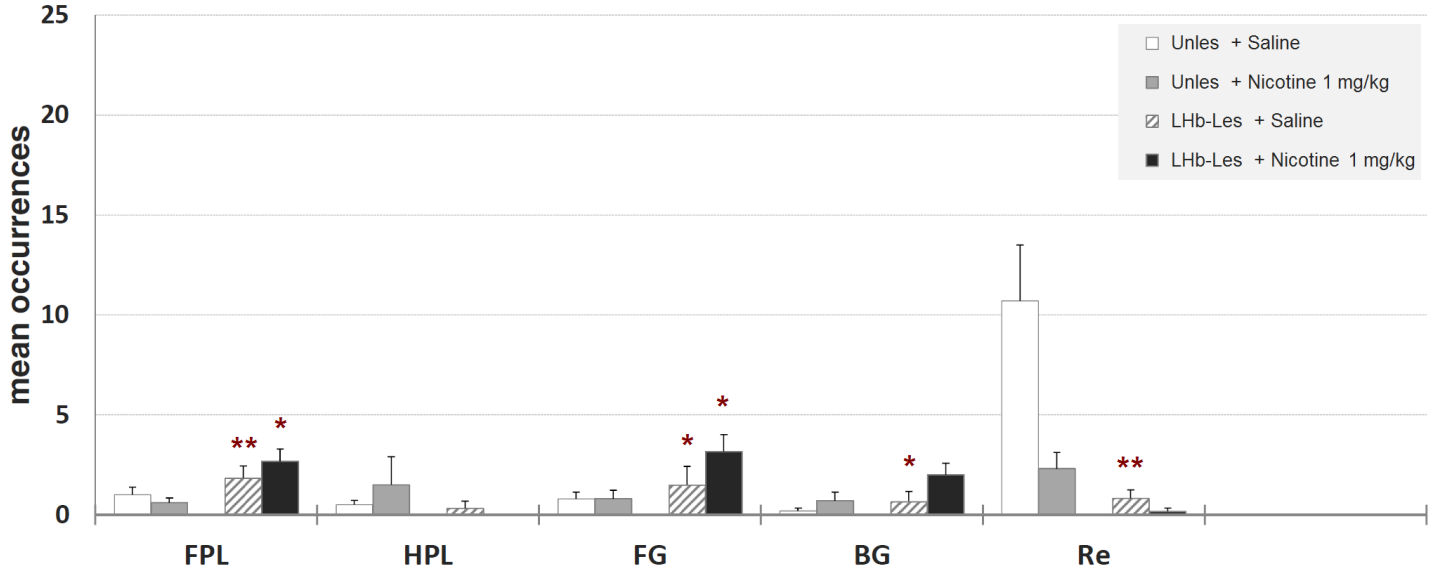

mean occurrences
25
20
15
10
5
0
Unles + Saline
Unles + Nicotine 1 mg/kg
LHb-Les + Saline
LHb-Les + Nicotine 1 mg/kg
**
*
*
*
*
*
**
FPL
HPL
FG
BG
Re
B

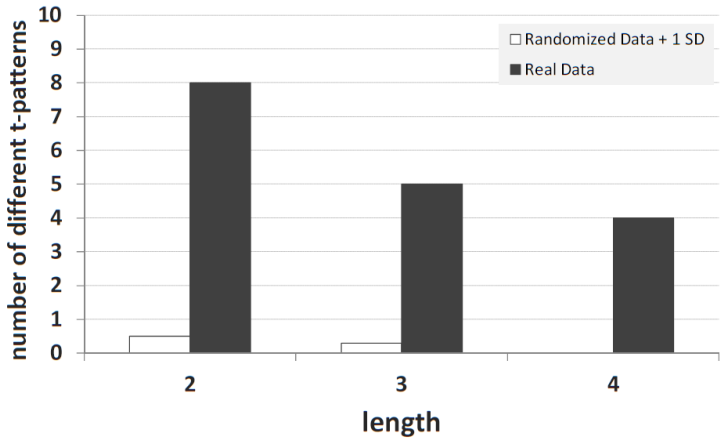
Figure 5.TIF

Saline

TP#	String	Occs
1	(CI IS)	266
2	(CI Wa)	215
3	(ES HD)	218
4	(ES IS)	292
5	(Im IS)	84
6	(IS Wa)	538
7	(Wa ES)	191
8	(Wa IS)	607
9	(ES (Wa IS))	86
10	(Wa (ES IS))	144
11	((CI Wa) ES)	84
12	((CI Wa)(ES IS))	79
13	((Wa ES) HD)	108
14	((Wa ES) IS)	168
15	(CI (Wa (ES IS)))	59
16	((Wa (ES IS)) CI)	83
17	(((CI Wa) ES) IS)	78

Overall number of T-patterns detected: 3300

Mean number for each subject: 330 ± 44.75

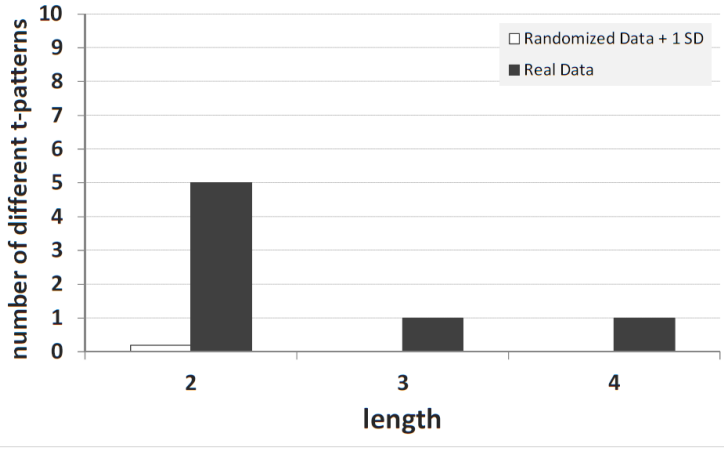


Nicotine 0.1 mg/kg

TP#	String	Occs
1	(CI IS)	153
2	(ES HD)	138
3	(FPL FG)	14
4	(HD ES)	152
5	(IS Wa)	474
6	((CI IS) ES)	90
7	(((CI IS) ES) HD)	35

Overall number of T-patterns detected: 1056

Mean number for each subject: 105.6 ± 6.79 *

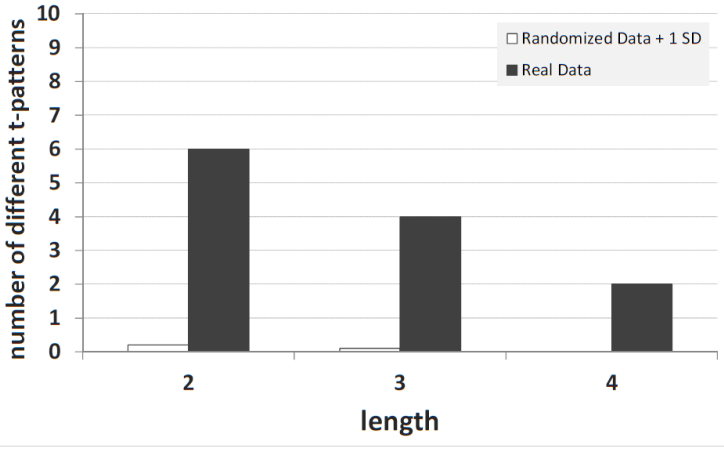


Nicotine 0.5 mg/kg

TP#	String	Occs
1	(CI IS)	191
2	(ES HD)	158
3	(ES Wa)	117
4	(HD ES)	126
5	(IS CI)	128
6	(IS Wa)	469
7	(ES (IS Wa))	95
8	(HD (IS Wa))	91
9	(Wa (IS CI))	84
10	((ES Wa)(IS CI))	21
11	((IS CI) Wa)	103
12	(HD (ES (IS Wa)))	27

Overall number of T-patterns detected: 1610

Mean number for each subject: 161 ± 15.41 *



Nicotine 1 mg/kg

TP#	String	Occs
1	(CI IS)	195
2	(ES HD)	179
3	(IS Wa)	476
4	(Wa CI)	116

Overall number of T-patterns detected: 966

Mean number for each subject: 96.6 ± 13.33 *

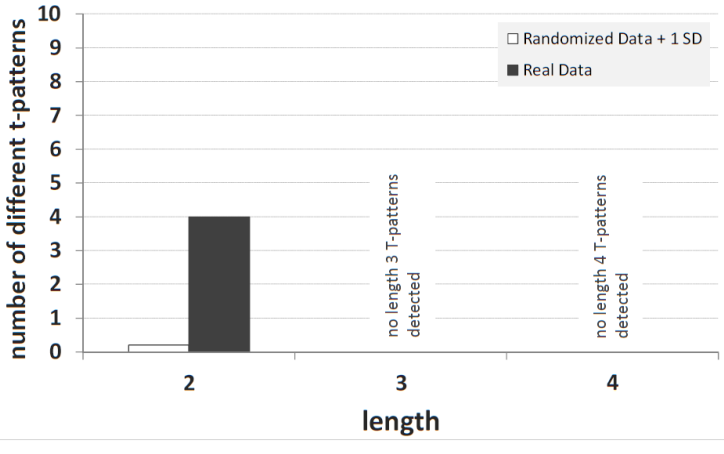


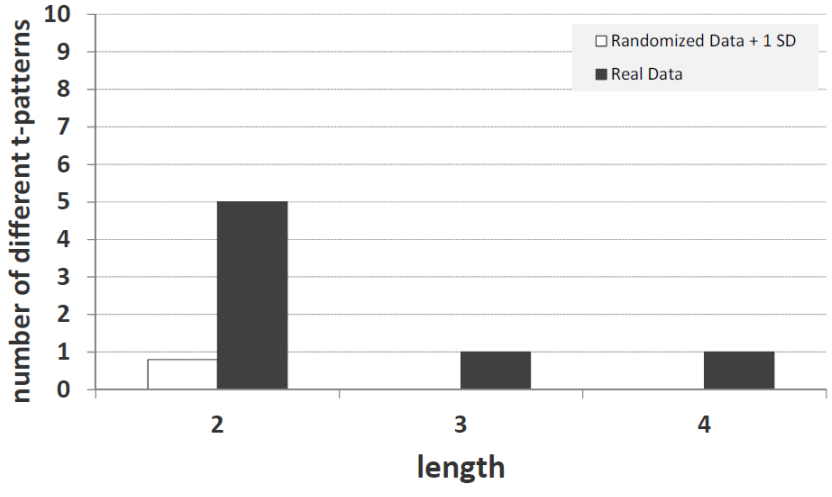
Figure 6.TIF

LHb-Les + Saline

TP#	String	Occs
1	(CI IS)	91
2	(ES HD)	133
3	(FPL FG)	7
4	(HD ES)	102
5	(IS Wa)	493
6	(CI (IS Wa))	61
7	((HD ES)(IS Wa))	40

Overall number of T-patterns detected: 927

Mean number for each subject: 154.5 ± 14.15



LHb-Les + Nicotine 1 mg/kg

TP#	String	Occs
1	(BG FG)	6
2	(CI IS)	29
3	(CI Wa)	36
4	(ES HD)	102
5	(ES IS)	242
6	(FG BG)	10
7	(FPL BG)	9
8	(FPL FG)	10
9	(HD ES)	103
10	(HD IS)	118
11	(Imm IS)	120
12	(Wa ES)	162
13	(FPL (BG FG))	4
14	(FPL (FG BG))	7
15	((Wa ES) HD)	55

Overall number of T-patterns detected: 1013

Mean number for each subject: 168.83 ± 33.94

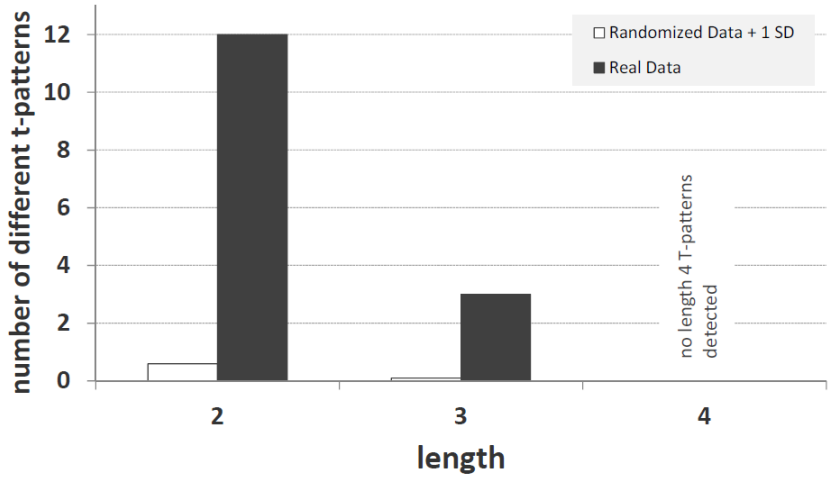


Figure 7.TIF

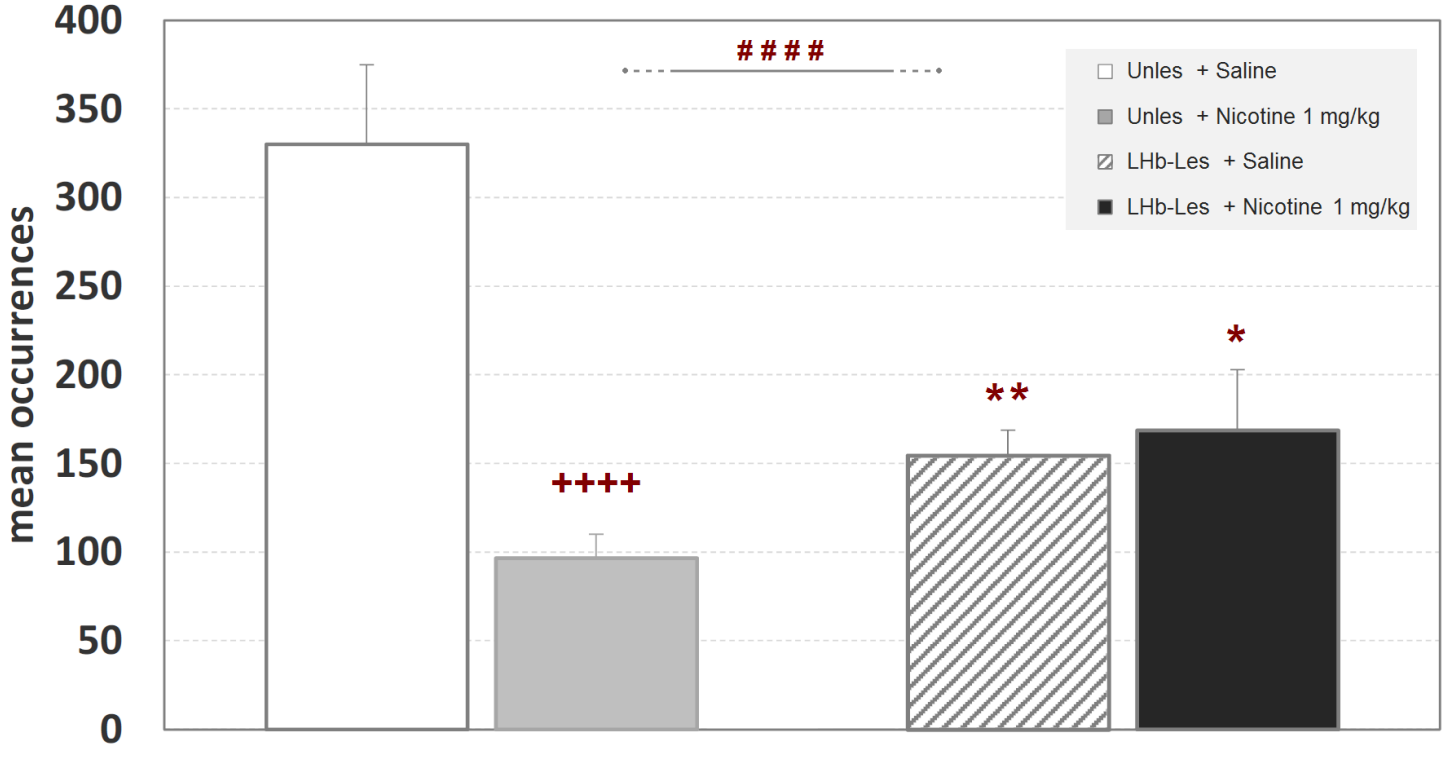
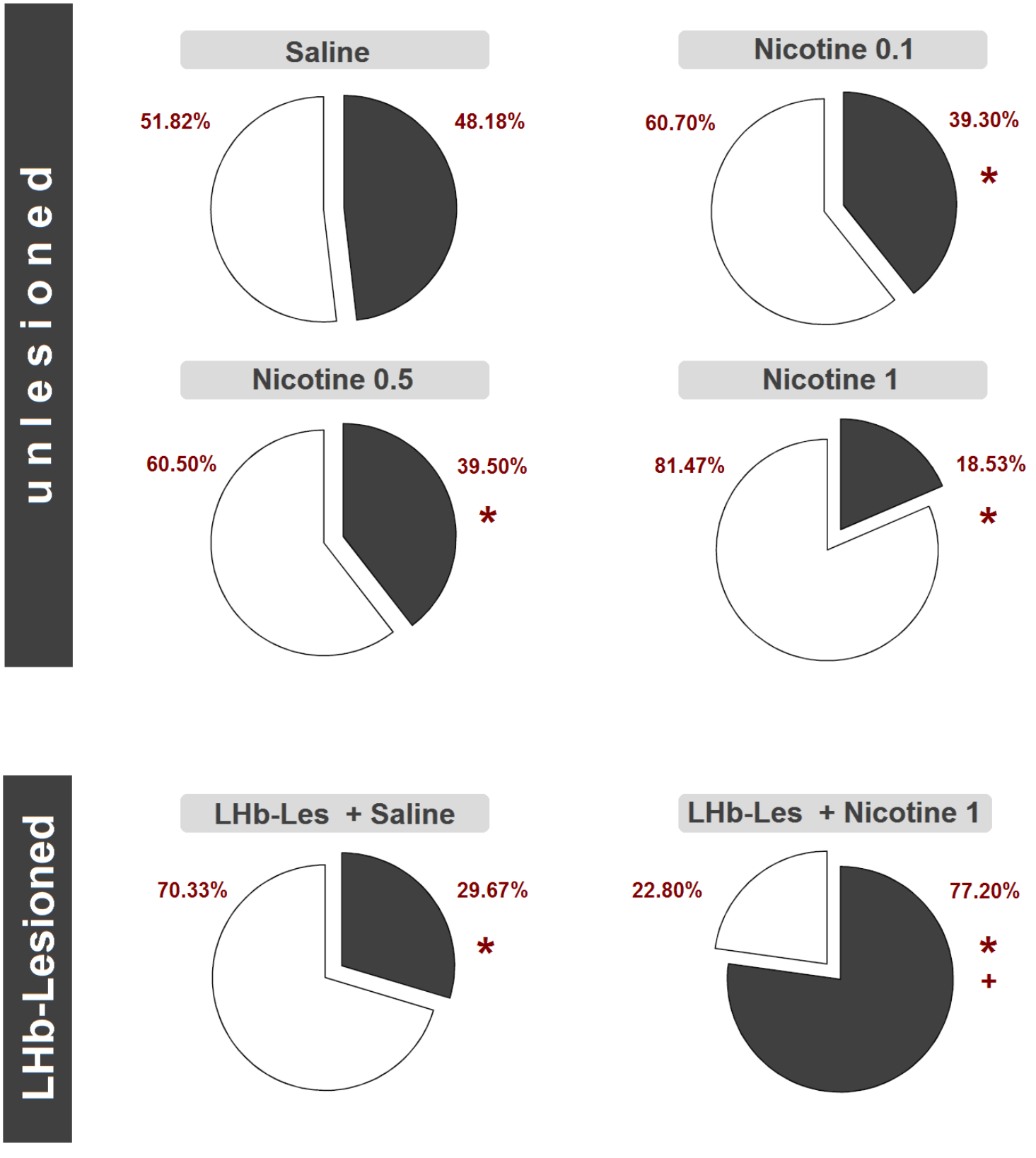

400
350
300
250
200
150
100
50
0
mean occurrences
####
++++
**
*
Unles + Saline
Unles + Nicotine 1 mg/kg
LHb-Les + Saline
LHb-Les + Nicotine 1 mg/kg

Figure 8.TIF


unlesioned
Saline
51.82%
48.18%
Nicotine 0.1
60.70%
39.30%
*
Nicotine 0.5
60.50%
39.50%
*
Nicotine 1
81.47%
18.53%
*
LHb-Lesioned
LHb-Les + Saline
70.33%
29.67%
*
LHb-Les + Nicotine 1
22.80%
77.20%
*
+
Percent of T-patterns containing edge-sniff and/or head-dip
Percent of T-pattern without edge-sniff and/or head-dip