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MULTIVARIATE ANALYSIS OF THE BEHAVIORAL RESPONSES OF RODENTS STUDIED BY MEANS OF DIFFERENT ANXIETY-INDUCING TESTS

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“La ricerca della conoscenza non si nutre di certezza: si nutre di una radicale mancanza di certezza”.
Carlo Rovelli

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1. INTRODUCTION

1.1. Anxiety, fear and behavior

"Anxiety", object of this dissertation, has become one of the most frequent topics of discussion both in medical and popular discourse as well. To understand the meaning of the word "Anxiety" we have to come back to 16th century, to the French word "*anxiété"* (from Latin *angere)* which means "to press tightly". Only in the 17th century did anxiety become a descriptive category for feelings of fearfulness accompanied by precordial tightness or discomfort. As a product of humoral or nervous dysfunction, anxiety was physiologically as well as psychologically related to constitutional and bodily imbalances. In the 19th century, anxiety was redefined as a pathological mental state. Psychiatrists produced specific models of anxiety that linked irrational fears to the formation of the psyche. In the early 20th century, this trend was reinforced by the work of Sigmund Freud. In the post-Freudian age, anxiety continues to be defined in terms of mental ill-health. Although causal factors may be individual or social and attached to particular situations, people or places, they are as likely to be understood as products of chemical imbalance or brain dysfunction, most recently linked to amygdala activity [Bound, 2004]. Anxiety is a function of the brain, but it is also something more; it is something that we inherit from past generations and that we can feel in ourselves as in other people. What we commonly call the mind is a set of operations carried out by the brain and the study of behavior represents the science of the mind [Kandel et al, 2012]. Actually, the behavior is the expression of the activity of the nervous system [Hogan, 2015]. Every behavior is mediated by specific sets of interconnected neurons, and every neuron's function in a given behavior is determined by its connections with other neurons [Kandel et al, 2012]. The basic units of behavior are perceptual mechanisms, central mechanisms, and motor mechanisms. These units can be organized into more complex units called behavioral systems such as the ones related to hunger, sex, aggression, fear, etc. [Hogan, 2015]. A kind of behavioral system is represented by anxiety. We can define it like a set of physiological responses that occur more or less unconsciously when the brain detects certain challenging situations [Kandel et al, 2012]. Anxiety belongs to the big family of emotions. Animals as well as human beings can show an anxious behavior, but it's important to specify that emotions and feelings are different. Emotions are automatic, largely unconscious behavioral and cognitive responses triggered when the brain detects a positively or negatively charged significant stimulus. It refers to physiological responses to certain kinds of stimuli. Feelings are the conscious perceptions of emotional responses [Kandel et al, 2012]. For example, in a dangerous situation your muscles tense and your heart pounds; the conscious experience that often, but not always, is associated with these bodily responses let us speak about feelings. Anxiety as a conscious anticipation of danger only appears in great

apes. In the case of lower mammals such as rodents, this anticipation may not be conscious and may not be related to the ability to activate a representation of the situation with its possible consequences [Belzung, 2007]. As described by Joseph Le Doux in his book "Synaptic self: how our brains become who we are" the organization of the emotional brain is simple: it involves the synaptic delivery of information about the outside world to the amygdala and the control of responses that act back on the world by sinaptic outputs of the amygdala. If the amygdala detects something dangerous the result is freezing, changes in blood pressure and heart rate, release of hormones, and lots of other responses that either are preprogrammed ways of dealing with danger or are aspects of body physiology that support defensive behaviors [Le Doux, 2003]. The ability to respond appropriately to threatening stimuli is important for survival. Neural circuit that learn about such threats in the environment appears to be highly conserved across species [Pendyam, 2013]. The medial division of the central nucleus of the amygdala projects to various brain areas involved in the production of several symptoms related to anxiety, fear and panic symptoms observable in people with fear-related disorders [Parsons and Ressler, 2013] (fig. 1).

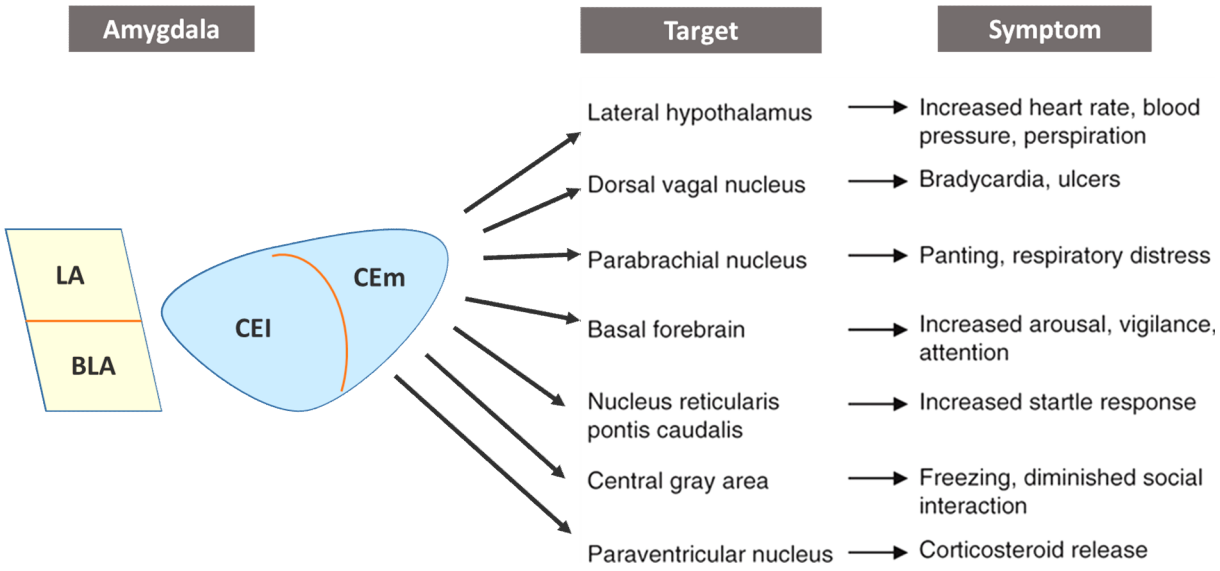


Figure 1 - Connections of Amygdala. Medial division of the central nucleus, CEm, projects to various brain areas that produce several symptoms related to anxiety and fear. LA, lateral nucleus; BLA, basolateral nucleus; Lateral portion of the central nucleus, CEl. Modified from [Parsons and Ressler, 2013]

The amygdala, named for its almond shape by Karl-Friedrich Burdach during the early 19th century, is an evolutionarily conserved structure [Pabba, 2013]. It is a collection of nuclei deep in the temporal lobe that together constitute a tightly knit microcircuit [Likhtik, 2015]. Burdach originally described a group of cells that are now known as the basolateral complex. Subsequently, a large number of structures that surround the basolateral complex have been identified

in many species and constitute what is now known as the amygdaloid complex [Sah et al, 2003]. The lateral nucleus (LA) is typically viewed as the sensory interface of the amygdala and as a key site of plasticity, while the central nucleus is viewed as the output region. LA receives inputs from both thalamic and cortical stations in the auditory system. LA projects to CE both directly and indirectly [Phelps and Le Doux, 2005]. Outputs of the CE then control the expression of fear responses, including freezing behavior and related autonomic nervous system and endocrine responses [Medina et al, 2002; Parsons and Ressler 2013].

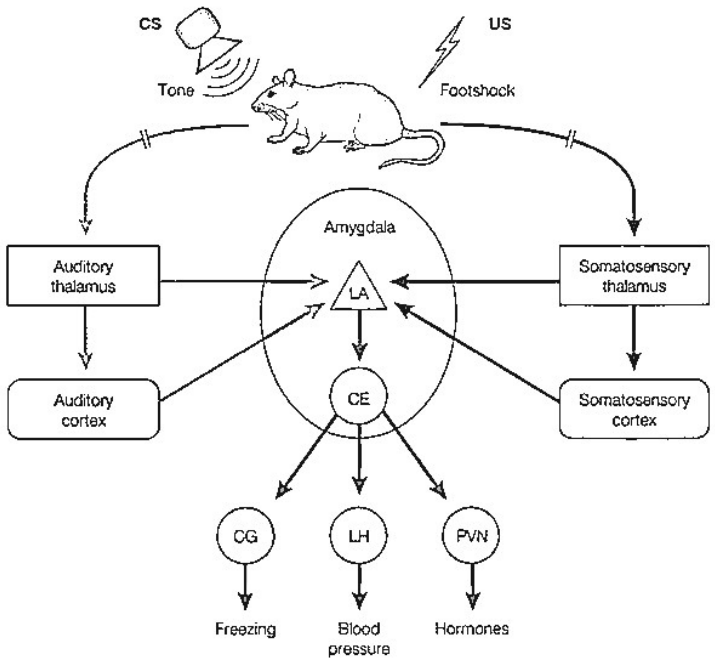


Figure 2 Neural pathways underlying fear conditioning. LA,lateral Amygdala; CE, central Amygdala; CG, central gray; LH, lateral hypothalamus; PVN, paraventricular hypothalamus. Modified from [Medina et al. 2002]

It's easy to understand the concept of anxiety because it's easy to experience it during daily life. During an exam for example, or a competition. But there is a more unusual way to think about it. An anxious experience continuously changes our synapses, our behavior is the manifestation of a never ending process of learning and anxiety, as fear, is a kind of emotional learning. Anxiety is a kind of memory, an aversive memory [Squire and Kandel, 1999]. Fear and anxiety share many common properties but they can also be distinguished [Sylvers et al, 2011]. Fear is usually elicited by specific stimuli and tends to be short-lived, decreasing once a threat has dissipated; on the other hand, anxiety may be experienced in the absence of a direct physical threat and tend to be long-lasting [Hartley and Phelps, 2012]. Anxiety disorders involve abnormal regulation of fear [Kandel et al, 2012]. Neurocircuitry of fear has been investigated using the classical fear conditioning as a model paradigm (anxiety

is commonly conceptualized as a state of sustained fear). During fear conditioning a neutral stimulus such as a tone, is paired with an aversive stimulus such as an electric shock, eliciting a range of automatic, unconditioned fear responses. After one or more pairings, the presentation of the tone alone is sufficient to elicit a fear response, the conditioned one [Hartley and Phelps, 2012]. Research on the neural system underlying fear responses has implicated circuits into and through the amygdala as essential to the acquisition and storage of a memory of the conditioning experience and the expression of fear responses [Hartley and Phelps, 2012]. In addition, the answer to learned fear is suppressed by a double damage of amygdala [Squire and Kandel, 1999]. Lesion and imaging studies have confirmed that the human amygdala is also involved in fear conditioning, but the involvement of amygdala's subregions is still poorly understood in humans [Medina et al, 2002]. Another brain formation which worth to be mentioned is the Lateral Habenula, it is a small epithalamic formation which 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). From an anatomical perspective it receives, through the stria medullaris, inputs mainly from the basal ganglia and from the limbic system [Hikosaka et al., 2008]. 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); also, indirect connections take place through the GABA-ergic rostromedial tegmental nucleus (RMTg) [Hikosaka, 2010; Proulx et al., 2014]

1.2. Aims of the present study

The regulation and alleviation of anxiety is a key factor in the promotion of human well-being [Belzung and Philippot, 2007]. Anxiety disorders are very common in older adults and cause considerable distress and functional impairment; geriatric anxiety disorders will become an increasing human and economic burden because of absent improvements in detection and management [Lenze and Loebach Wetherell, 2011]. As a consequence, the interest of researchers in this field is growing up and the necessity to better characterize anxiety-related behavior is increasing. In such a context our research plays an important role. Our laboratory is committed with the analysis of behaviour by applying advanced analytical tools. Our idea orbits around the following simple point of view: if on the one hand conventional approaches to the analysis of behaviour, e.g. utilization of percent distributions, frequencies or durations of individual behavioural events, are certainly useful from a descriptive point of view since they provide precise information concerning each investigated item, on the other hand they are quite ineffective in depicting behavior's most important features, that is, relationships among the observed behavioral

elements. In other terms, it is our contention that the possibility to characterize each component of a given behavioural repertoire through even thousands of numbers does not imply the possibility to use those numbers to figure out what the behavior is in its wholeness. As a consequence, descriptive approaches to behavioral studies should be partnered, if possible, with more sophisticated techniques of analysis. In such a context multivariate analyses hold an important role. Above all, the study of relationships among events from a temporal point of view allows us to interprete behavior in a more accurate way. By means of a new analytical approach, the T-pattern analysis, repeated sequences of behavior can be appraised and analyzed. The present research project is aimed at the study of the *structural characteristics* of anxiety-related behavior in rodents tested in two different experimental assays classically used in the study of anxiety. The synergic use of descriptive and multivariate analyses has been employed, for the first time, to describe the structural characteristics of the behaviour of two different strains of rodents and to better understand the relationship between anxiety and nicotine.

1.3. Anxiety and animal models

Animal models are potentially relevant to understand human anxiety disorders, indeed many responses to frightening stimuli are conserved across mammalian species [Kandel et al, 2012]. Rodents are usually used as animal models of human behavior. Animal models are defined as experimental preparations developed in one species to study phenomena occurring in another species [McKinney, 1984]. Kaplan added that a model may be valid if it has the same structure as the human behavior or pathology, that is whenever a relation holds between two elements of the animal model, a corresponding relation may hold between the corresponding elements of the human behavior [Belzung, 2001]. We can just start to understand how important is an accurate description of the structure of behavior. Animal models can be artificially created in laboratory like the transgenic mouse model AT-ENPP1-Tg, a C57/Bl6 background with targeted over-expression of human ENPP1 (Ectonucleotide pyro-phosphatase-phosphodiesterase1) in adipocytes [Pan et al., 2012] or they can be spontaneously obtained, as consequence of a mutation for example, like the dystrophic mouse C57BL/10ScSn-Dmdmdx. In the field of anxiety research, animal models can be used for their peculiar characteristics, like the Dark Agouti rat for his high level of anxiety; otherwise normal rats can be used and the stimuli that elicit fear or anxiety can be produced in the laboratory. Usually the goal is the search for compounds with therapeutic potential or the discovery of new mechanisms underlying emotional behavior [Rodgers et al, 1997a,b]. Two different kind of anxiety can be studied: state anxiety and the trait one. ‘State anxiety’ is considered a ‘normal’ anxiety, while ‘trait anxiety’ represents

a ‘pathological’ one [Belzung, 2001]. State anxiety is the anxiety that a subject experiences at a particular moment in time, and is increased by the presence of an anxiogenic stimulus. Trait anxiety, in contrast, does not vary from moment to moment, and is considered to be an enduring feature of an individual [Lister, 1990]. Behavioral models may conveniently be classified as either conditioned or unconditioned responses to stimuli which appear capable of causing anxiety in humans [Rodgers, 1997a,b]. The natural tendencies of rats and mice to avoid predators and open spaces was exploited to study innate or instinctual fear [Kandel et al, 2012] while the study of learned fear requires a more considerable participation of human beings. An ingenious tool to study it is for example food or water deprivation, but usually training of subjects or the use of electric shock as an aversive stimulus is necessary [Rodgers et al, 1997b]. Studies of learned or conditioned fear exploit the ability of rodents and other animals to form powerful associations between previously neutral cues and temporally linked danger [Kandel et al, 2012]. The study of unconditioned responses to various forms of external threat allows for a complete behavioral characterization of the effects of experimental manipulations [Rodgers et al, 1997b]. Examples of models of unconditioned responses are the Elevated Plus Maze, the The Hole-Board, the Open Field, the Social Interaction [Rodgers et al, 1997b]. The Elevated Plus Maze (EPM) test has been in use as a rodent model of anxiety for decades, and is representative of those tests that are based upon the study of spontaneous behavior patterns [Rodgers et al, 1997b].

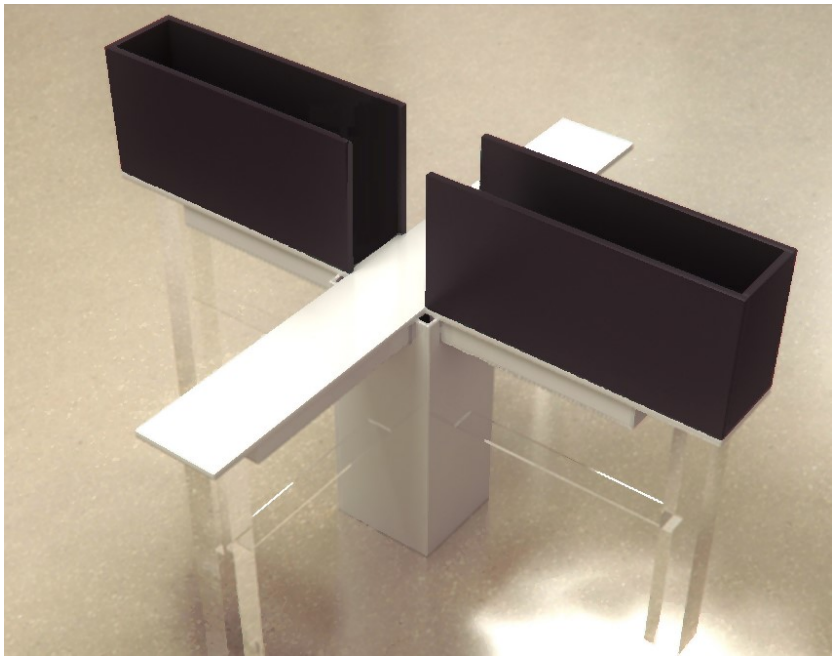


Figure 3. Elevated Plus Maze. The apparatus consists of a plus-shaped platform with two open and two enclosed arms. The test involves placing the rat (or mouse) in the center of the apparatus and allowing it to explore for a short period (usually 5 min).

Montgomery, in 1955, proposed that the behavior of animals exposed to a novel situation results from a competition between an exploratory tendency (motivated by curiosity) and a withdrawal tendency (motivated by fear) [Lister, 1990]. At one level this might be considered a form of approach-avoidance conflict [Lister, 1990]. There is substantial evidence that, during the conventional 5min test, the rodent has a clear preference for the closed arms and only subjects with a reduced anxiety level do increase their activity in the open arms [Carobrez and Bertoglio, 2005].

Another apparatus usually used to study unconditioned responses is the Hole Board (HB). It's peculiar characteristic is the presence of holes (frequently four) in the floor through which an animal can poke its head [Lister, 1990]. An increase in head-dipping is suggestive of an anxiolytic action in animals naive to the test apparatus [Lister, 1990].

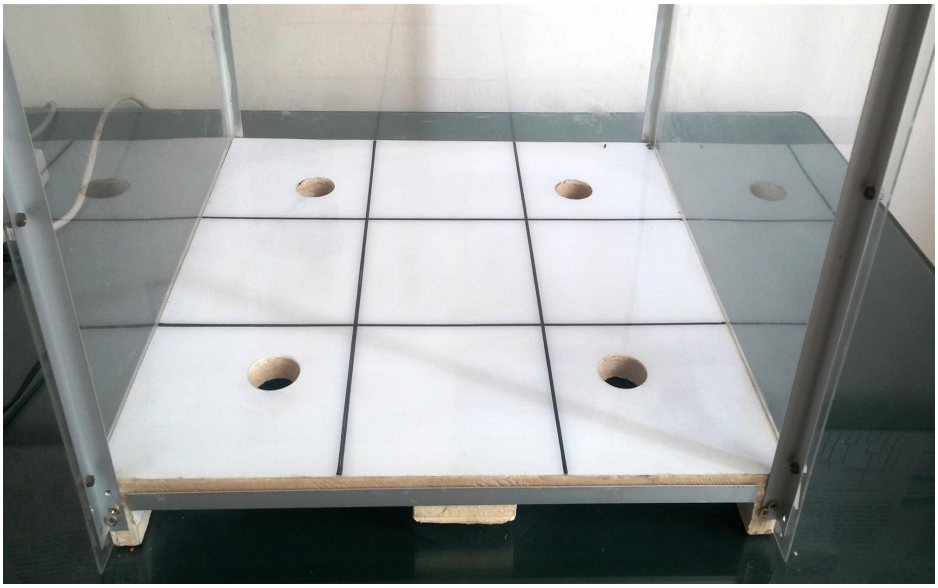


Figure 4. Hole board. The apparatus consists of a 50x50 cm board provided with four equidistant holes 4 cm in diameter. The test involves placing the rat in the center of the apparatus and allowing it to explore for a short period (usually 5 or 10 min).

2. MATERIALS AND METHODS

2.1. Animals and housing

Wistar, Sprague-Dawley and Dark-Agouti (DA/Han) rats were used. The Wistar rat is one of the most popular rats used for laboratory research. Currently about 247000 papers on PubMed deal with Wistar rats. The DA/Han strain has been used because of his higher anxiety level. This peculiar trait could depend on an alteration of the enzymes belonging to the CYP2D- subfamily (member of the cytochrome P450 system) [Mechan et al., 2002] whose activity has been demonstrated to be markedly reduced in the DA/Han rat, in comparison with the Wistar one [Schulz-Utermoehl et al, 1999]. Interestingly, also in humans, a correlation has been shown between the activity of specific enzymes belonging to this family and anxiety disorders [González et al., 2008]. Both Wistar and DA/Han subjects were three months old. Animals were born in the animal facility of the University of Rouen (France) and breeders originated from Janvier (Le Genest-St-Isle, France). Both Wistar and DA/Han rats were housed in groups of three in a room maintained at the constant temperature of 21 ± 2 ◦ C, under the following light/dark cycle: light on = 12 noon; light off=12 midnight. Sprague-Dawley rats (Charles River, Margate, UK) weighing between 250–350g 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 h: 12 h with the lights being turned on at 6 am. Food and water was provided to the animals *ad libitum*.

2.2. Drugs and treatment

As regards the HB study we can distinguish 2 groups. Lesioned and unlesioned subjects. As to unlesioned subjects, the treatment groups were: saline (vehicle), nicotine 0.1 mg/kg, 0.5 mg/kg and 1 mg/kg, all administered intraperitoneally (i.p.). Sham-lesioned and LHb- lesioned rats were treated with saline or nicotine 1 mg/kg, i.p. Nicotine hydrogen tartrate salt was diluted in saline and adjusted to pH 7.4. All drug doses refer to the weight of the salt. Saline or nicotine was given in 1 ml/kg volume, 30 min before the test. Concerning EPM, no drugs have been used since the study involved the comparison of two strains with different emotional reactivity.

2.3. Experimental apparatus

The EPM consists of an elevated plus-shaped platform characterized by the presence of two open and two enclosed arms. The apparatus is elevated at a height of 50 cm above the floor [Roy et al, 2009]. The closed arms are

surrounded by a 50 cm wall while open arms present 0.5 cm edges in order to maximize open-arm entries [Treit et al, 1993]. Rodents exposed to the apparatus will respond to a conflict elicited by the presence of safe parts of the maze that are closed and protected, and aversive parts of the maze that are open, unprotected and more brightly lit [Carobrez and Bertoglio,2005]. The HB apparatus used in the experiment consisted of a square (50 × 50 cm) open-field arena made of white opaque Plexiglas with a raised floor, containing four equidistant holes 4 cm in diameter. HB floor was positioned 5 cm above a white opaque Plexiglas sub-floor. Changes of head-dipping behavior (frequency, latency, duration) reflect the anxiogenic and/or anxiolytic state of animals.

2.4. Procedures

As regards Wistar/Dark-Agouti comparison, rats were transported from housing to testing room inside their home-cages to minimize transfer effect. To avoid possible visual and/or olfactory influences, animals were allowed to acclimate for 30 min far from the observational apparatus. Each subject, experimentally naïve, was placed in the central platform of EPM, facing an open arm, and allowed to freely explore for 5 min. After each observation, EPM was cleaned with ethyl alcohol (10%) to remove scent cues left from the preceding subject. Concerning Sprague-Dawley study, 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 received the drug treatment and was brought into the testing room and left for 30 min to acclimatize. The animals were subsequently placed in the center of the HB and allowed to freely explore for 10 min, whilst being recorded by video camera. After each recording the HB was cleaned with ethanol (70%) to remove all scent traces and faeces. The rodents' behavior was recorded by means of a video camera, and video files were stored for subsequent analyses. As regard the lesioning procedure of the HB experiment, twenty rats received bilateral electrolytic lesions at the LHb level. Two holes were made in the skull, 3.6 mm posterior to bregma and 1.8 mm lateral to the midline (Paxinos and Watson, 2007). Two bipolar electrodes made from two stainless steel bifilar wires (California Fine Wire, Grover Beach, CA, USA) with their ends separated 0.5 mm, were attached to a micromanipulator angled 10 to the coronal plane, and lowered into the right and left LHb (depth of 5.0 mm from the surface of the dura). A 500 µA current was applied for 30 s 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 h. Once surgery was complete, rats were given a subcutaneous injection of saline (1 ml) and a topical application of antibiotic cream (mupirocin), and were left for 7–10 days to recover before testing in the HB. An identical procedure was followed for twenty additional rats, except electrodes were only lowered 3.5 mm and no current was passed so that no

electrolytic lesion was made, producing sham-lesioned animals. 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 LHb were considered acceptable when surrounding regions (i.e., medial habenula, dorsal hippocampus and thalamic nuclei) were spared.

2.5. Data analysis

The first step was the construction of an ethogram, namely, a formal decription of each component of the behavioural repertoire. In the EPM for rats, the central platform is a 10 cm × 10 cm square area in the centre of the apparatus (fig. 5, left).

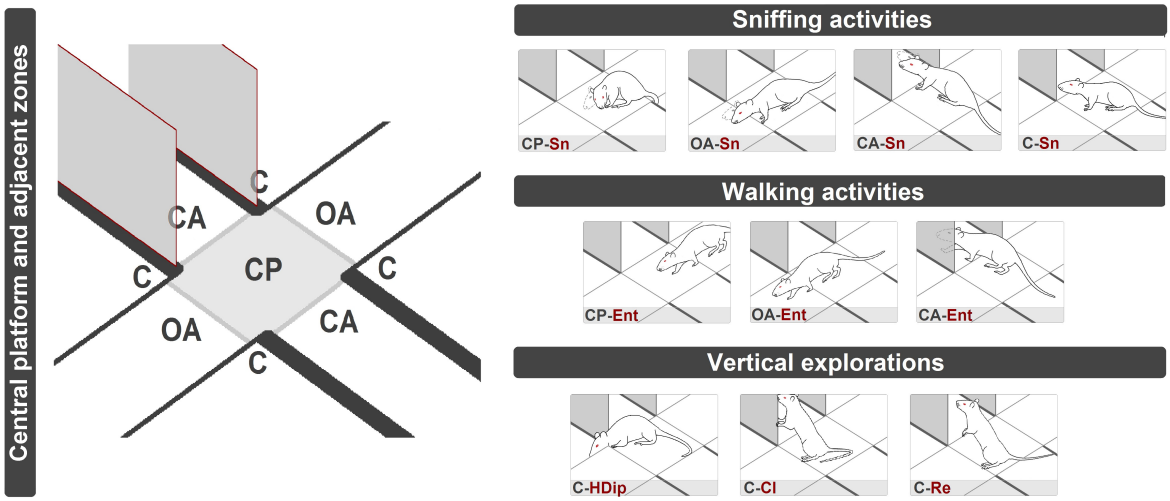


Figure 5. Ethogram of rat behavior in the central plaform of the EPM. ***Left panel***: Only the walls of one closed arm have been represented. ***CP*** = Central Platform Area; ***CA*** = Closed Arm Zones; ***OA*** = Open Arm Zones; ***C*** = Corner Zones (closed-open arm junction and external 90° angle comprised between the two arms). ***Right panel***: ***CP-Sn*** = Central Platform Sniffing: the rat sniffs the ground of the central platform; ***OA-Sn*** = Open Arm Sniffing: the rat sniffs the entrance of one of the two open arms; ***CA-Sn*** = Closed Arm Sniffing: the rat sniffs the entrance of one of the two closed arms; ***C-Sn*** = Corner Sniffing: the rat sniffs the Plexiglas border of one of the four corners; ***CP-Ent*** = Central Platform Entry: the rat moves from an open or from a closed arm to the central platform; ***OA-Ent*** = Open Arm-Entry: the rat moves from the central platform to one of the two open arms; ***CA-Ent*** = Closed Arm Entry: the rat moves from the central platform to one of the two closed arms; ***C-HDip*** = Corner Head Dip: the rat, from one of the four corners, performs scanning head movements in the direction of the floor; ***C-Cl*** = Corner Climbing: the rat maintains an erect posture leaning against the Plexiglas border of one of the four corners; ***C-Re*** = Corner Rearing: the rat, without leaning against the Plexiglas, maintains an erect posture facing one of the four corners. Modified from [Casarrubea et al, 2015a]

On the basis of the adjacent arms and enclosures, the central platform presents nine sub-regions (fig. 5, left): a central area (CP), two borders between the CP and the open arms (OA), two borders between the CP and the closed arms (CA),

and finally, four corners (C). By taking into consideration these regions and borders, it has been possible to arrange an ethogram encompassing 10 behavioural components organized in three main categories:

- *Sniffing activities*, namely, behavioral components characterized by the sniffing of the CP and adjacent zones (all four paws in CP, rapid scanning head movements often associated with movement of vibrissae: CP- Sn, CA-Sn, OA-Sn, C-Sn);
- *Walking activities*, namely, behavioral components where the animal walks from an adjacent arm to the CP or vice versa (head and front-paws across the border line between the two regions: CA-Ent, OA-Ent, CP-Ent);
- *Vertical explorations*, that is, behavioral components where the animal, maintaining a fixed position on the CP, explores above or below the ground (C-Cl, C-HDip, C-Re).

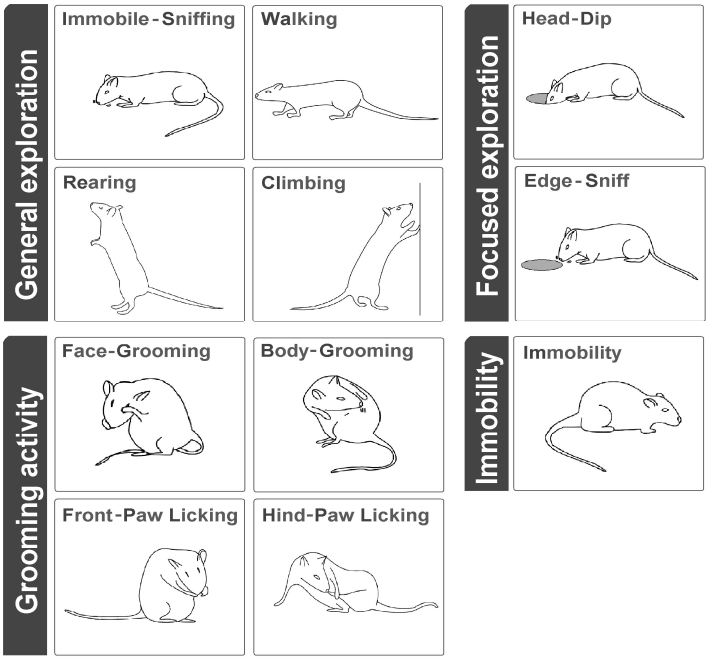


Figure 6. Ethogram of rat behavior in the HB. Immobile sniffing (***IS***): rat sniffs the environment standing on the ground; walking (***Wa***): rat walks around sniffing the environment; rearing (***Re***): rat maintains an erect posture without leaning against the Plexiglas box, usually associated with sniffing; climbing (***Cl***): rat maintains an erect posture leaning against the Plexiglas wall, usually associated with sniffing; head dip (***HD***): rat puts its head into one of the four holes; edge sniff (***ES***): rat sniffs the border of one of the four holes; face grooming (***FG***): rat rubs its face (ears, mouth, vibrissae, eyes) with rapid circular movements of its forepaws; body grooming (***BG***): rat licks its body combing its fur with fast movements of incisors; front-paw licking (***FPL***): rat licks or grooms its forepaws; hind-paw licking (***HPL***): rat licks or grooms its hind paws; immobility (***Im***): rat maintains a fixed posture. Modified from [Casarrubea et al, 2015b]

Concerning the ethogram for the behaviour of the rat in the HB apparatus, it is possible to identify four different categories:

- *General Exploration*: Walking (Wa), the rat walks around sniffing the environment; Immobile Sniffing (IS), the rat sniffs the environment standing on the ground; Rearing (Re), the rat maintains an erect posture without leaning against the Plexiglas box, usually associated with sniffing; Climbing (Cl), the rat maintains an erect posture leaning against the Plexiglas wall, usually associated with sniffing;
- *Focused Exploration*: 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;
- *Grooming Activity*: 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;
- *Immobility* (Imm): the rat maintains a fixed posture, no movements are produced.

After the description of the activity of the rat through an ethogram, video files were frame-by-frame analyzed using a personal computer equipped with a software coder (The Observer, Noldus IT, The Netherlands). Event log files, obtained from the software coder, were analyzed by means of quantitative approaches, multivariate approaches based on the elaboration of transition matrices and by means of T-pattern analysis. To detect temporal relationships among behavioral elements, event log files were processed with *Theme software* (PatternVision Ltd, Iceland; Noldus Information Technology, The Netherlands).

2.6. Quantitative analyses

All the quantitative analyses have been carried out on the basis of the event log files obtained from coding process. Concerning the EPM study, a min-by-min and overall average time spent in the CP, the mean number of all behavioral components, the mean number of each behavioral component and their per cent distribution were calculated for both strains. Moreover, the ratios between the entrances in the arms and the preceding sniffings, i.e., open arm entries/open arm sniffings and closed arm entries/closed arms sniffings, were also analyzed. A value of ratio >1 indicates that entries are more represented than preceding sniffings; a value <1 indicates that not all the sniffings are followed by arm

entries. To evaluate the amount of time each subject spent in the CP, in the OA and in the CA, mean durations have been measured. To evaluate the number of entries in the CP, in the OA and in the CA mean occurrences have been calculated. To calculate the amount of time each subject spent in walking activities, sniffing activities and vertical explorations, mean durations have been measured. Finally, to evaluate the onset of the behavioural categories, mean latencies of the first occurrence of walking activities, sniffing activities and vertical explorations have been calculated. Concerning the HB experiments, the following parameters of the behavioral response were analyzed: mean duration of each behavioral element, for each subject; mean occurrence of each behavioral element, for each subject.

2.7. Transition matrices

Behavioral elements were coded from the collected video files. Through the relevant option available within the Observer, transitions from an element to another one were traced in a transition matrix (TM). TMs for each subject were calculated and summed together, obtaining a total TM. It represented the starting point for Cluster Analysis.

2.8. T-Patterns analyses

"*Behavior consists of patterns in time. Investigations of behavior deal with sequences that, in contrast to bodily characteristics, are not always visible*" [Eibl-Eibesfeldt, 1970]. The ability to recognize patterns in the environment is critical for an organism's survival. Human environments consist to a large extent of repeated spatiotemporal patterns which are typically composed of simpler patterns. [Magnusson, 2004]. Patterns are often hidden and very difficult to be perceived. *Theme software* (PatternVision Ltd, Iceland; Noldus Information Technology, The Netherlands) 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]. 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 first 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. Each critical interval underlines the detection of a statistically significant constraint. Notwithstanding, the extremely high number of possibilities of such relationships in data with hundreds or even thousands occurrences of behavioural events might raise the question whether the observed T-patterns are

detected only by chance. Theme deals with such an issue by repeatedly randomizing and re-analysing the original data, using exactly the same search parameters used with the real behavioural data. The average number of patterns of each length detected in the randomized data is then compared with that obtained from the original data.

2.9. Statistics

Possible significant differences of mean values between Wistar and DA/Han rats were assessed using Student's t-test for independent samples. Possible significant time-related changes and differences in comparison with minute 1 were calculated using one-way ANOVA followed by Newman–Keuls post-hoc test for multiple comparisons. Finally, possible differences between the per cent distributions were evaluated by means of the chi-square test.As to HB study, 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 unlesioned groups. Two-way ANOVA (treatment x lesion) was used to analyze differences among saline in sham-lesioned rats, saline in LHb- lesioned rats, nicotine 1 mg/kg in sham-lesioned rats and nicotine 1 mg/kg 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 x treatment interaction, the data of the sham-lesioned and LHb-lesioned groups, comparisons of nicotine to the vehicle control condition were made by paired *t*-tests. Differences were considered significant at $p < 0.05$. Two-way ANOVA (lesion x treatment) was used to analyze differences among saline in sham-lesioned rats, saline in LHb-lesioned rats, nicotine 1 mg/kg i.p. in sham-lesioned 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. 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 of whether the number of different detected T-patterns is different by chance. The software used for T-pattern 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.

2.10. Ethical Statement

All efforts were made to minimize the number of animals used and their suffering. The experiments were conducted in accordance with the European Communities Council Directive 86/609/EEC concerning the protection of animals used for experimental scientific purposes.

◊

Results and illustrations presented in following sections are partially originating from our recent studies:

- Casarrubea M., **Faulisi F.**, Sorbera F., Crescimanno G. The effects of different basal levels of anxiety on the behavioral shift analyzed in the central platform of the elevated plus maze. Behavioural Brain Research. 2015; Vol. 281, pp 55-61

- Casarrubea M., Davies C., **Faulisi F.**, Pierucci M., Colangeli R., Partridge L., Chambers S., Cassar D., Valentino M., Muscat R., Benigno A., Crescimanno G. and Di Giovanni G. Acute nicotine induces anxiety and disrupts temporal pattern organization of rat exploratory behavior in hole-board: a potential role for the lateral habenula. Frontiers in Cellular Neuroscience. 2015; 9:197

- Casarrubea M., **Faulisi F.**, Caternicchia F., Santangelo A., Di Giovanni G., Benigno A., Magnusson M.S., Crescimanno G. Temporal patterns of rat behaviour in the central platform of the elevated plus maze. Comparative analysis between male subjects of strains with different basal levels of emotionality. Journal of Neuroscience Methods. 2016; Vol. 268, pp 155-162

3. RESULTS OF ELEVATED PLUS MAZE STUDY

3.1. Quantitative Analysis

Overall, DA/Han rats stayed significantly ($p < 0.0001$) longer in the CP in comparison with Wistar ones: 127.08 ± 9.87 s the former, 74.11 ± 5.11 s the latter (fig. 7). Min-by-min assessment confirmed significant differences ($p < 0.05$) between the two strains in the average time spent in the CP.

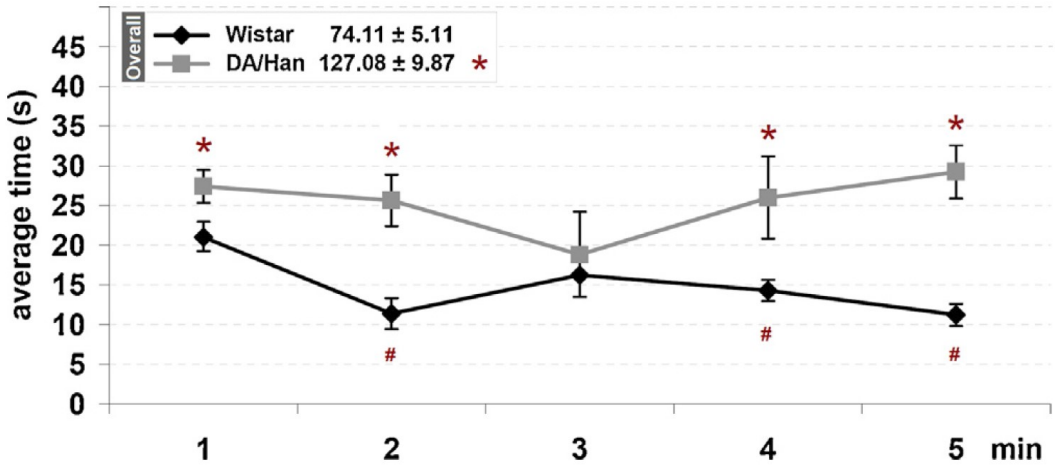


Figure 7. Average time spent by both strains in the CP. Modified from [Casarrubea et al, 2015a]

In addition, a significant variation ($F_{4,49} = 4.12$; $p = 0.006$) over time was detected for Wistar rats; post-hoc analysis revealed a significant ($p < 0.05$) reduction of the time spent in CP during the observation. On the other hand, no significant variations ($F_{4,49} = 0.92$; $p = 0.459$) of the time spent in CP over time were detected for DA/Han subjects.

Fig. 8 shows that in Wistar rats walking activities (central-platform entry, open-arm entry and closed-arm entry) represent 46.25%, sniffing activities (central-platform sniffing, open-arm sniffing, closed-arm sniffing and corner-sniffing) 45.82% and vertical explorations (corner-head dip, corner-climbing and corner-rearing) 7.93%.

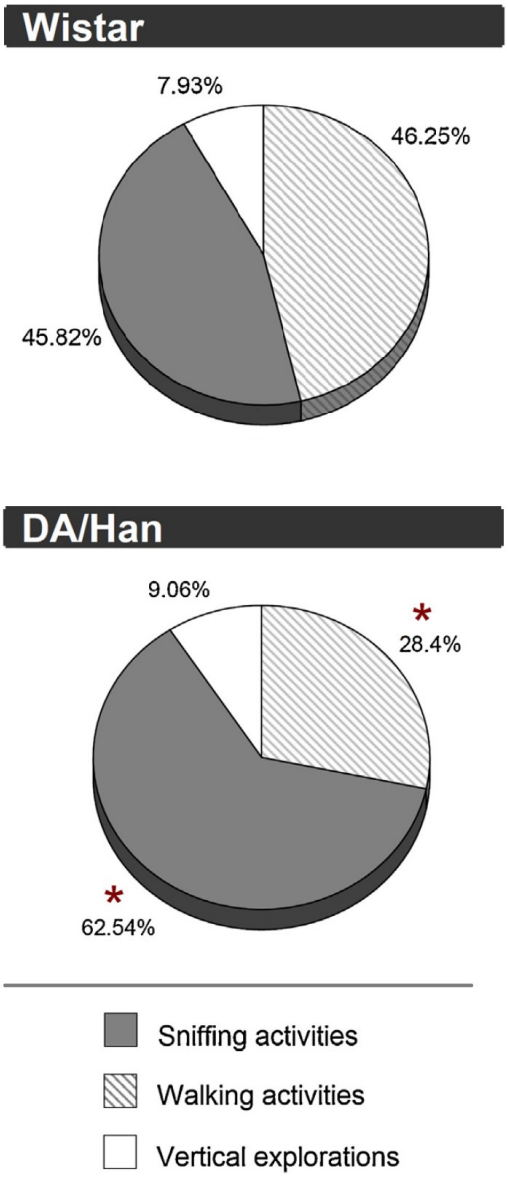


Figure 8. Per cent distribution of walking activities, sniffing activities and vertical explorations in both strains. Modified from [Casarrubea et al, 2015a]

When compared to Wistar strain, DA/Han is characterized by a significantly lower value ($p < 0.0001$) of walking activities (28.4%), counterbalanced by a significantly higher value ($p < 0.0001$) of sniffing activities (62.54%). vertical explorations, with a value 9.06%, do not show any statistically significant differences.

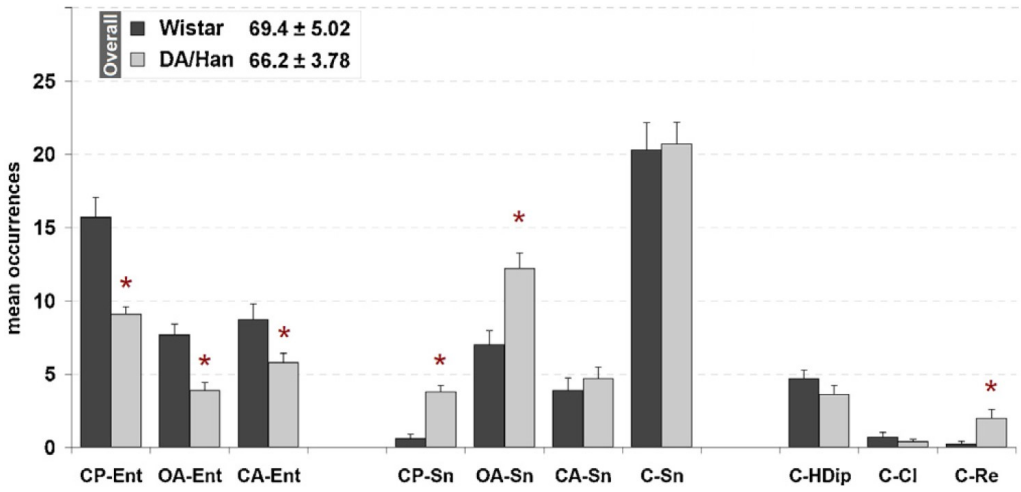


Figure 9. Mean occurrences of behavioral elements in both strains. See fig. 5 for abbreviations. Modified from [Casarrubea et al, 2015a]

Mean occurrences of the behavioral elements (fig. 9) show that Wistar rats performed an average of 69.4 ± 5.02 behavioral elements for each subject. Values not significantly different ($p = 0.617$) were detected in DA/Han rats (66.2 ± 3.78). As to the mean occurrences of each behavioral element, in comparison with Wistar rats, DA/Han rats showed a significantly lower value of central-platform entry ($p < 0.0001$), open-arm entry ($p < 0.0001$) and closed-arm entry ($p < 0.033$); significantly higher values were detected for central-platform sniffing ($p < 0.0001$), open-arm sniffing ($p < 0.002$) and corner-rearing ($p < 0.006$).

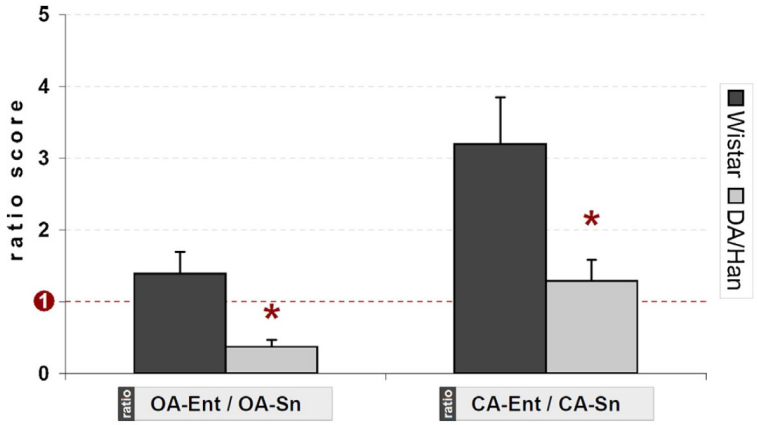


Figure 10. Ratio between entries (-Ent) and sniffings (-Sn) in open arms (OA) and in closed arms (CA) of the EPM. Modified from [Casarrubea et al, 2015a]

The ratio between open-arm entry/open-arm sniffing and closed-arm entry/closed-arm sniffing is presented in Fig. 10. A value of ratio >1 indicates that entries are more represented than preceding sniffings; a value <1 indicates that not all the sniffings are followed by arm entries. Wistar presented a value of ratio of 1.39 ± 0.30 for the open arms and 3.18 ± 0.75 for the closed ones. Significantly different values were observed for DA/Han rats where the ratio was 0.37 ± 0.10 ($p < 0.005$) (Fig. 10, left) for the open arms and 1.29 ± 0.30 ($p < 0.03$) (Fig. 10, right) for the closed ones.

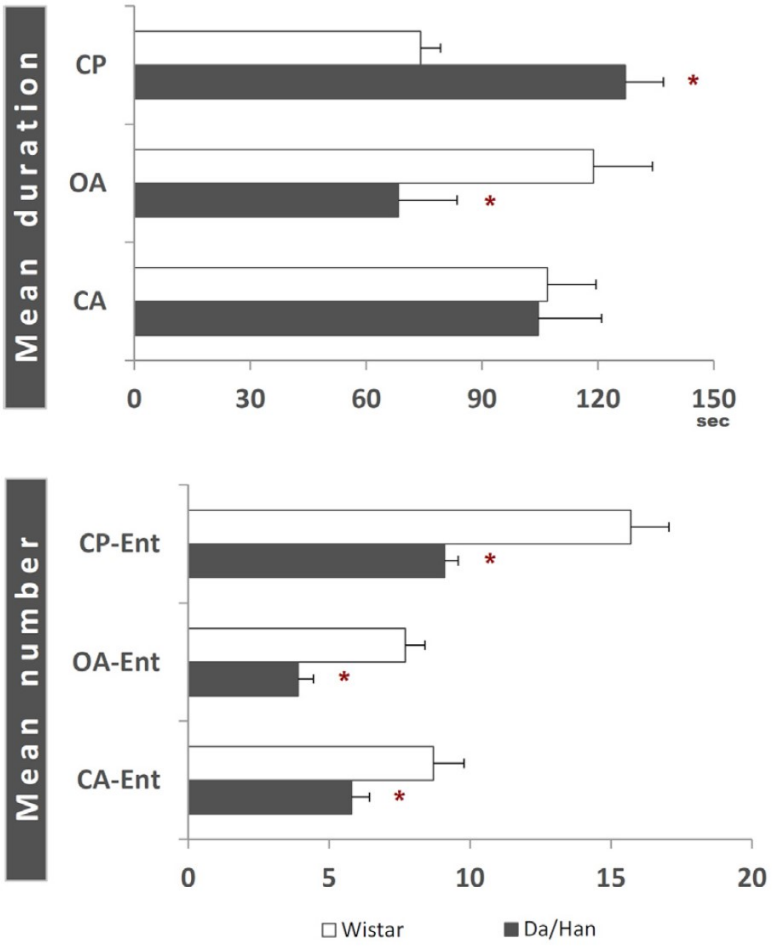


Figure 11. Mean time ± SE (s) that each subject spends in the CP, in the OA and in the CA (upper panel) and mean number of entries in the three EPM zones (bottom panel). See fig. 5 for abbreviations. Modified from [Casarrubea et al, 2016]

Student's t-test revealed significant differences between the two strains for the time spent in CP ($p < 0.0001$) and for the time spent in OA ($p < 0.05$); no significant difference has been detected for the time spent in the CA ($p = 0.908$). In addition, Student's *t*-test revealed significant differences between the two strains for the number of entries in CP ($p < 0.0001$), in OA ($p < 0.0001$) and in CA ($p < 0.05$).

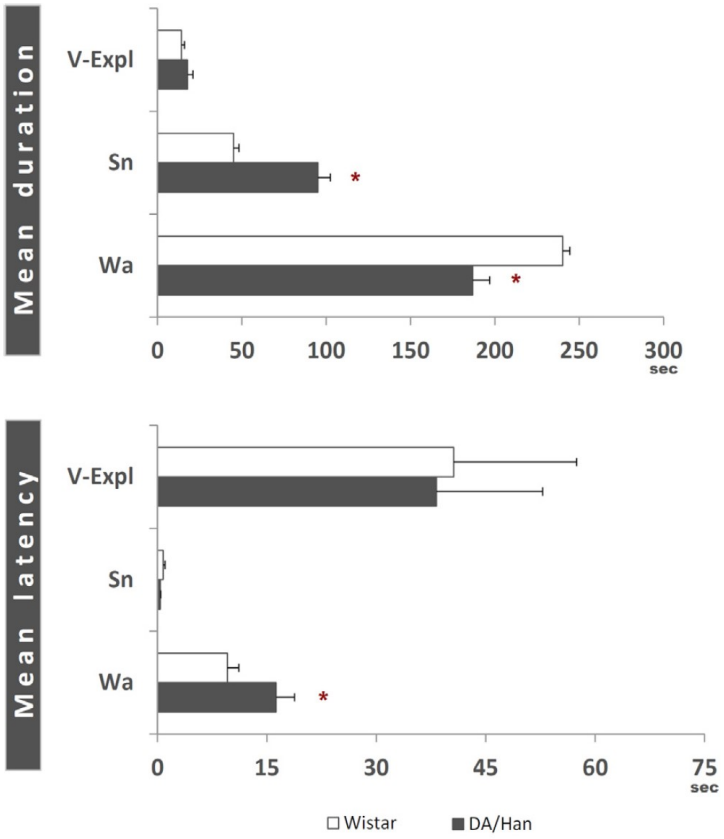


Figure 12. Mean time ± SE (s) that each subject spends in walking activities (Wa), sniffing activities (Sn) and vertical explorations (V-Expl) (upper panel) and mean latency of the first occurrence of each activity (bottom panel). Modified from [Casarrubea et al, 2016]

Student's t-test revealed significant differences between the two strains for walking ($p < 0.0001$) and for sniffing activities ($p < 0.0001$); no significant difference has been detected for vertical explorations ($p = 0.350$). Student's t-test revealed significant differences between the two strains for walking activities ($p<0.05$); no significant differences have been detected for sniffing activities ($p = 0.179$) and for vertical explorations ($p = 0.917$).

3.2. Cluster analysis

Similarity matrices were exemplified by means of dendrograms (fig. 13) where the similarity values among all the components of the comprehensive behavior are illustrated.

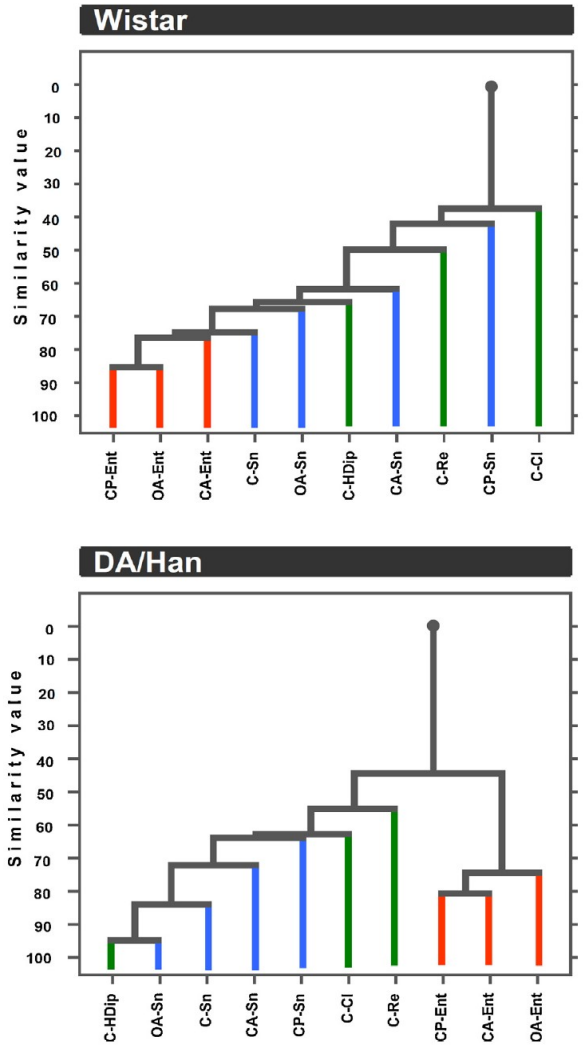
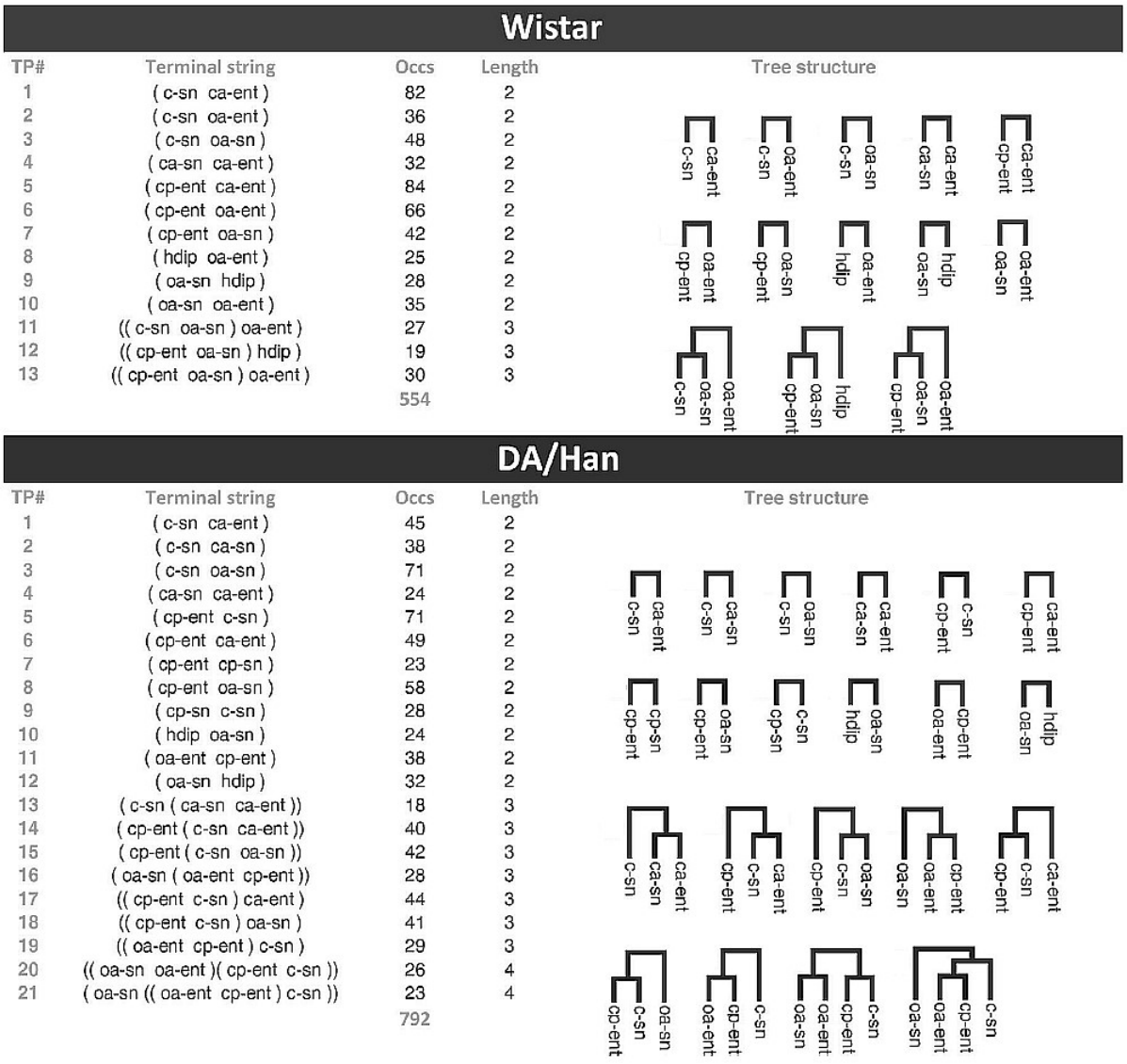


Figure 13. Dendrograms of Wistar and DA/Han behavior in the EPM emphasize evident structral differences in the behavior of the two strains. See fig. 5 for abbreviations. Modified from [Casarrubea et al, 2015a]

As regards Wistar rats, walking activities are arranged together, closely followed by corner-sniffing, open-arm sniffing, corner-head dip and closed-arm sniffing; central-platform sniffing, corner-rearing and corner-climbing represent the peripheral zone of the hierarchical structure. A different organization emerges from the dendrograms of DA/Han rats where walking activities remain clusterized together. Interestingly, all the sniffing activities (open-arm sniffing, corner-sniffing, closed-arm sniffing and central-platform sniffing) are linked with the corner-head dip. Corner-climbing and corner-rearing conclude such a pattern with the more peripheral position.

3.3. T-pattern analysis

Wistar rats performed 13 different T-patterns distributed and composed as follows: 10 encompassing two events and 3 with three events in their structure.



Wistar

TP#	Terminal string	Occs	Length
1	(c-sn ca-ent)	82	2
2	(c-sn oa-ent)	36	2
3	(c-sn oa-sn)	48	2
4	(ca-sn ca-ent)	32	2
5	(cp-ent ca-ent)	84	2
6	(cp-ent oa-ent)	66	2
7	(cp-ent oa-sn)	42	2
8	(hdip oa-ent)	25	2
9	(oa-sn hdip)	28	2
10	(oa-sn oa-ent)	35	2
11	((c-sn oa-sn) oa-ent)	27	3
12	((cp-ent oa-sn) hdip)	19	3
13	((cp-ent oa-sn) oa-ent)	30	3
		554	

Tree structure

DA/Han

TP#	Terminal string	Occs	Length
1	(c-sn ca-ent)	45	2
2	(c-sn ca-sn)	38	2
3	(c-sn oa-sn)	71	2
4	(ca-sn ca-ent)	24	2
5	(cp-ent c-sn)	71	2
6	(cp-ent ca-ent)	49	2
7	(cp-ent cp-sn)	23	2
8	(cp-ent oa-sn)	58	2
9	(cp-sn c-sn)	28	2
10	(hdip oa-sn)	24	2
11	(oa-ent cp-ent)	38	2
12	(oa-sn hdip)	32	2
13	(c-sn (ca-sn ca-ent))	18	3
14	(cp-ent (c-sn ca-ent))	40	3
15	(cp-ent (c-sn oa-sn))	42	3
16	(oa-sn (oa-ent cp-ent))	28	3
17	((cp-ent c-sn) ca-ent)	44	3
18	((cp-ent c-sn) oa-sn)	41	3
19	((oa-ent cp-ent) c-sn)	29	3
20	((oa-sn oa-ent)(cp-ent c-sn))	26	4
21	(oa-sn ((oa-ent cp-ent) c-sn))	23	4
		792	

Tree structure

Figure 14. Results of T-pattern analysis in the two strains. TP# = T-pattern idintificative number; Terminal String = text rapresentation of events in pattern; Occs = number of occurrences of the given pattern; Length = number of events in pattern. Tree structures of detected patterns are illustrated on the right side of the figure. The analysis revealed a very different behavioral structure of Wistar and DA/Han. See fig. 5 for abbreviations. Modified from [Casarrubea et al, 2016]

No higher order patterns have been detected. DA/Han rats presented 21 different patterns: 12 encompassing two events, 7 with three events and 2 with four events. Overall, 554 T-patterns occurred in Wistar and 792 in DA/Han rats (fig 14). First column means the number of T-pattern, the second one shows

different events which create a T-pattern, the third column how many time a peculiar pattern occurs and the following how many events perform each T-pattern. On the right, the graphical rapresentation of each T-pattern can help us to better understand what a T-pattern is.

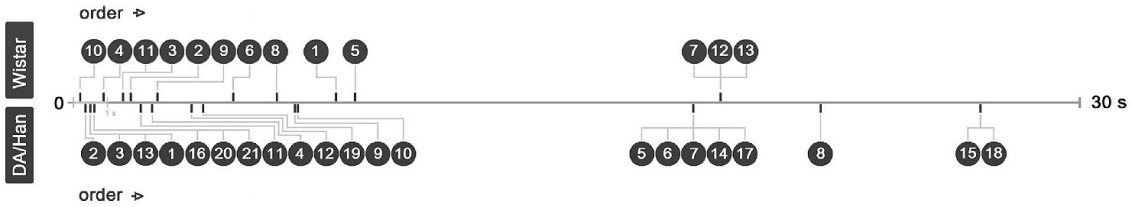


Figure 15. Results of T-pattern analysis in the two strains. Detail of the first 30 seconds. Numbers refer to the corresponding pattern indicated in fig. 14. Modified from [Casarrubea et al, 2016]

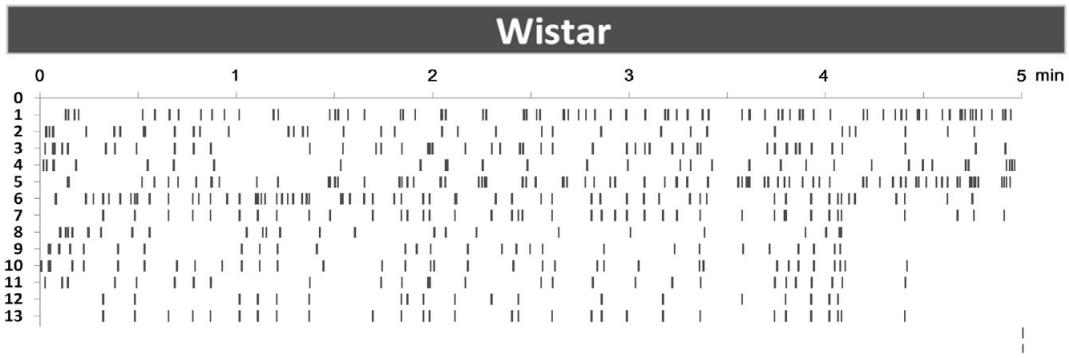


Figure 16. Rasterplot indicating the onset of all detected patterns in the Wistar Strain during the 5 min of observation. Numbers refer to the corresponding pattern indicated in fig. 14. Modified from [Casarrubea et al, 2016]

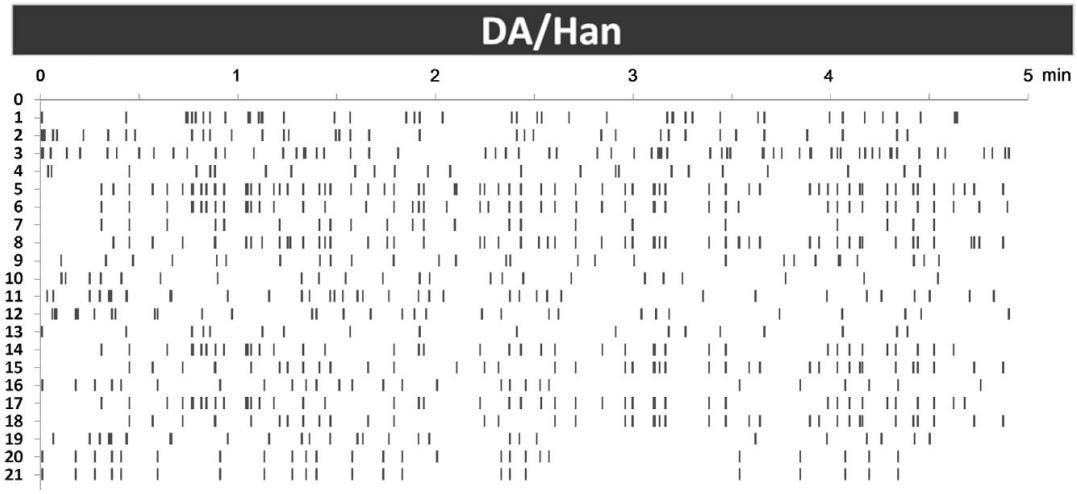


Figure 17. Rasterplot indicating the onset of all detected patterns in the DA/Han Strain during the 5 min of observation. Numbers refer to the corresponding pattern indicated in fig. 14. Modified from [Casarrubea et al, 2016]

Fig. 18 does represent the mean number of T-patterns in each group revealed a significantly ($p<0.0001$) higher number of occurrences in DA/Han subjects.

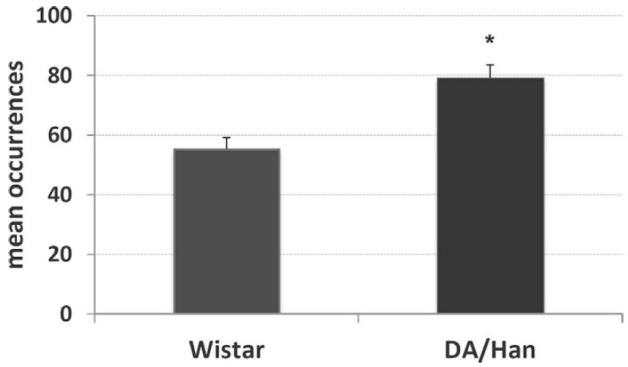


Figure 18. Histogram indicating the mean number of detected patterns in the two strains. Modified from [Casarrubea et al, 2016]

4. RESULTS OF HOLE-BOARD STUDY

4.1 Quantitative Analysis

Mean durations ± SEM of each behavioral component in saline and nicotine (0.1, 0.5, and 1 mg/kg, i.p.) treated unlesioned groups are presented in fig. 19.

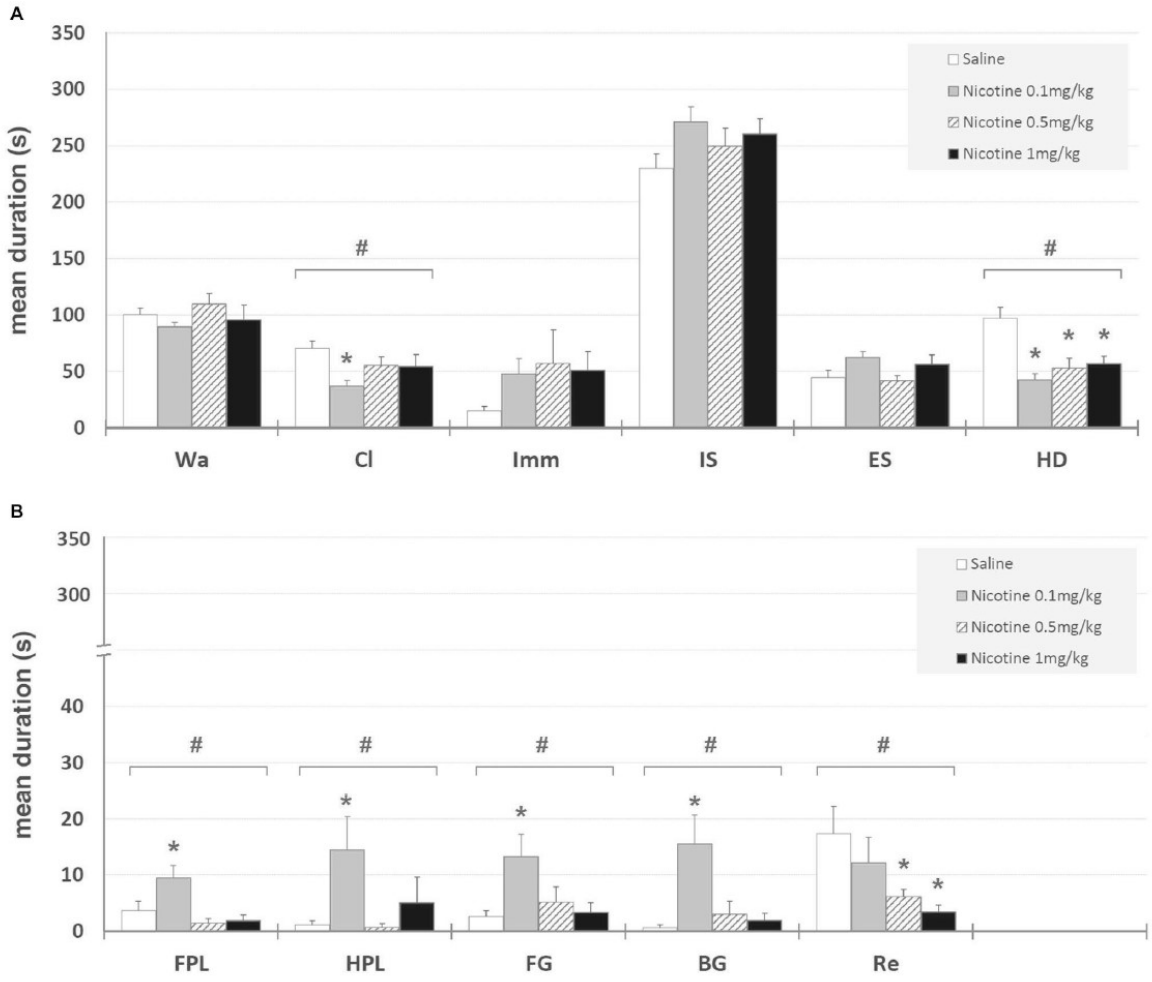


Figure 19. Mean durations of behavioral components in the HB apparatus, following saline and nicotine 0.1, 0.5 and 1mg/kg injection. For abbreviations see fig. 6. Modified from [Casarrubea et al, 2015b]

One-way ANOVA revealed significant nicotine-related changes for climbing ($F3,39 = 3.19$, $p < 0.035$), head-dipping ($F3,39 = 9.58$, $p < 0.0001$), front paw licking ($F3,39 = 6.07$, $p < 0.002$), hind paw licking ($F3,39 = 2.87$, $p < 0.05$), face grooming ($F3,39 = 3.58$, $p < 0.023$), body grooming ($F3,39 = 5.69$, $p < 0.003$) and rearing ($F3,39 = 3.28$, $p < 0.032$). Newman-Keuls *post hoc* test showed significant ($p < 0.05$) nicotine-induced decreases, in comparison with

saline, for head- dipping at all nicotine doses, for rearing at 0.5 and 1 mg/kg and for climbing at 0.1 mg/kg, while a significant increase was observed for front paw licking, hind paw licking, face grooming and body grooming at 0.1 mg/kg.

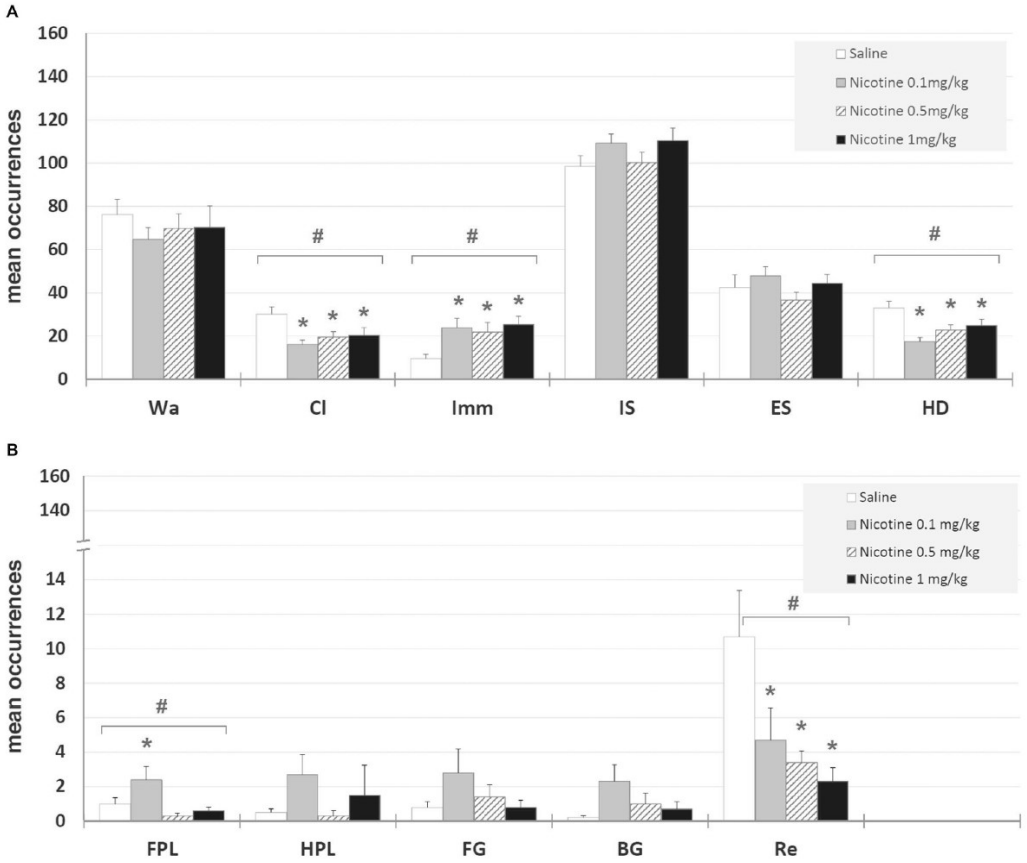


Figure 20. Mean occurrences of behavioral components in the HB apparatus, following saline and nicotine 01, 05 and 1mg/kg injection. For abbreviations see fig. 6. Modified from [Casarrubea et al, 2015b]

Mean occurrences ± SEM of each behavioral component in saline and nicotine (0.1, 0.5, and 1 mg/kg) injected groups are illustrated in fig. 20. One-way ANOVA showed significant drug-related changes for climbing ($F3,39 = 4.23$, $p < 0.012$), immobility ($F3,39 = 3.72$, $p < 0.020$), head-dipping ($F3,39 = 6.53$, $p < 0.001$), front paw licking ($F3,39 = 4.23$, $p < 0.012$) and rearing ($F3,39 = 3.61$, $p < 0.022$). Newman-Keuls *post hoc* test highlighted significant ($p < 0.05$) decreases, in comparison with saline, for climbing, rearing and head-dipping at all doses, and an increase of immobility and front paw licking at all nicotine doses and 0.1 mg/kg, respectively.

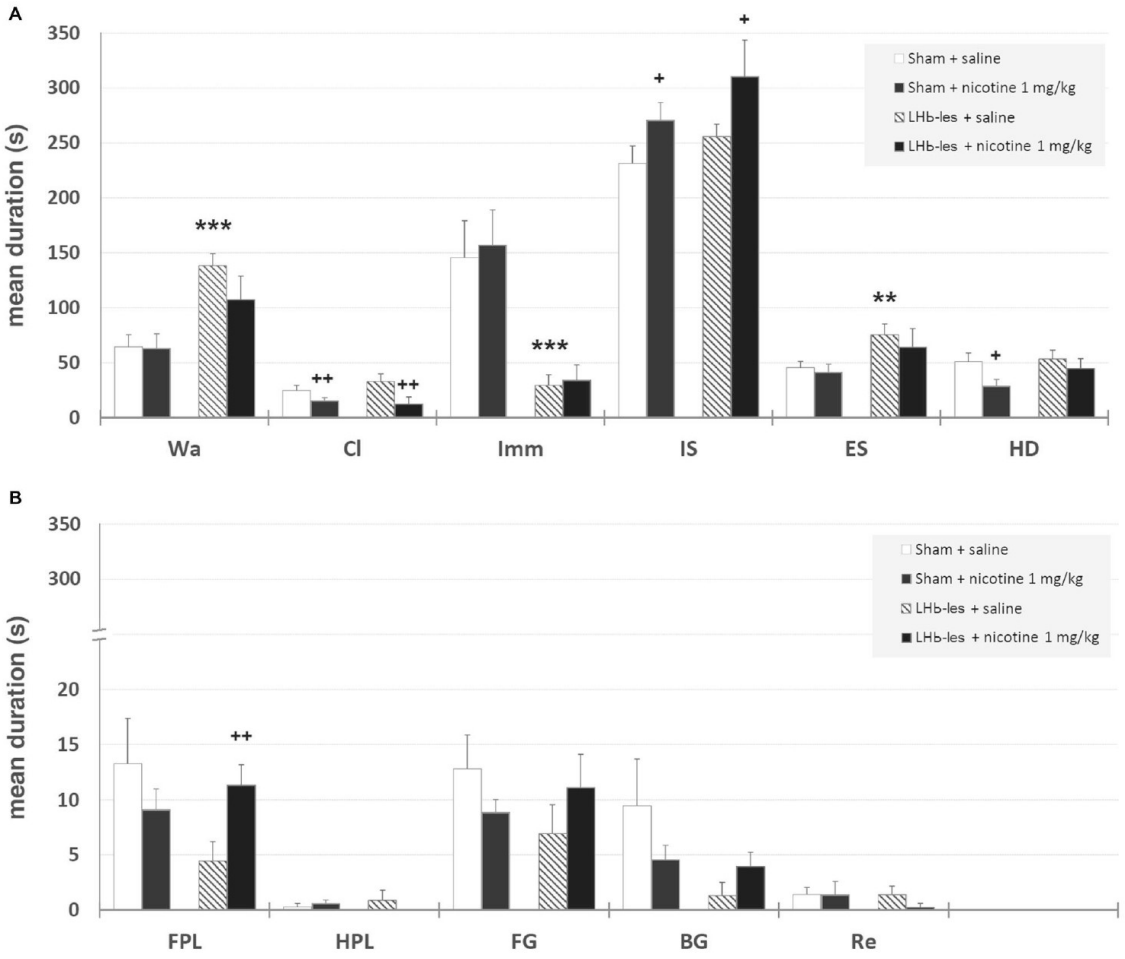


Figure 21. Mean durations of behavioral components in the HB apparatus, following sham lesion+saline, sham lesion+nicotine 1mg/kg, LHb lesion+saline and Lhb lesion+nicotine 1mg/kg. For abbreviations see fig. 6. Modified from [Casarrubea et al, 2015b]

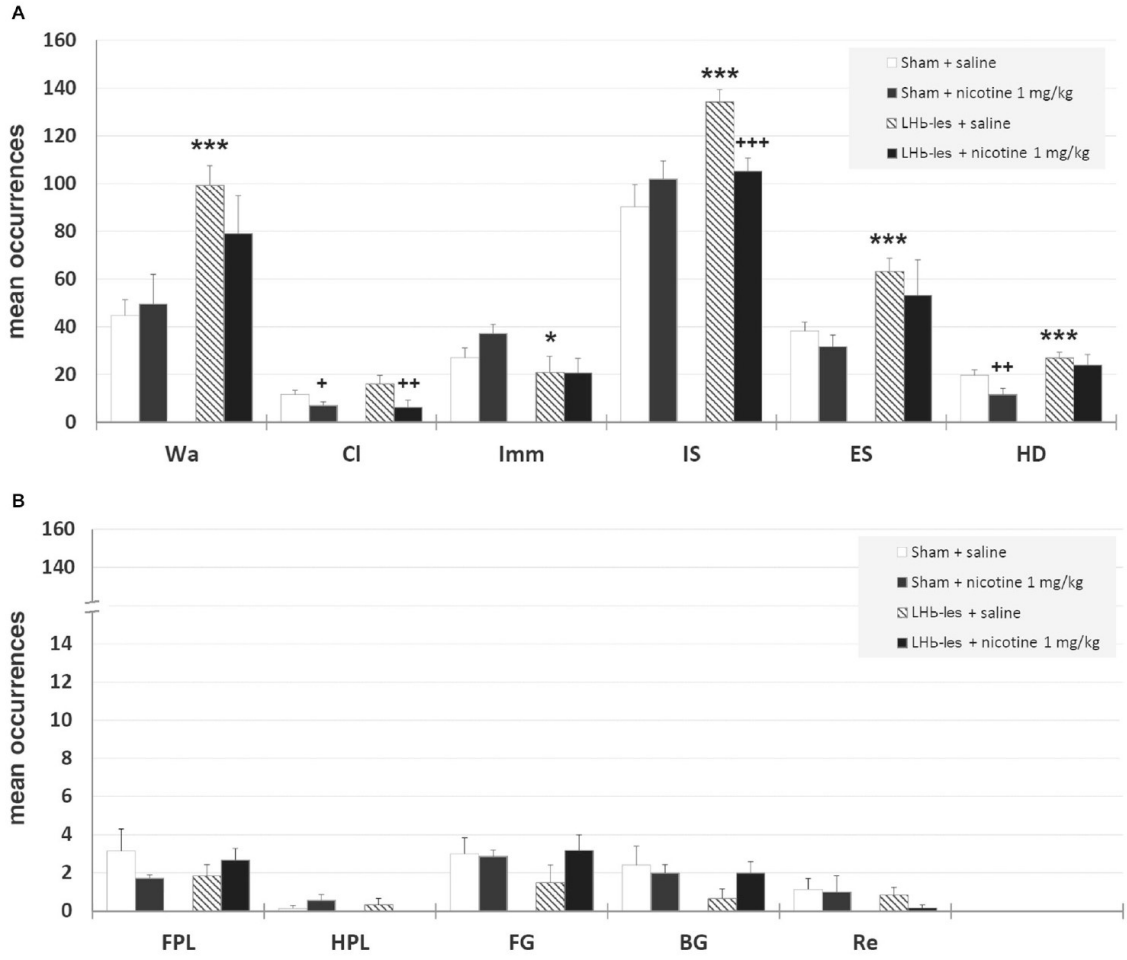


Figure 22. Mean occurrences of behavioral components in the HB apparatus, following sham lesion+saline, sham lesion+nicotine 1mg/kg, LHb lesion+saline and Lhb lesion+nicotine 1mg/kg. For abbreviations see fig. 6. Modified from [Casarrubea et al, 2015b]

Saline-treated sham-lesioned animals, in comparison to unlesioned animals, exhibit significant changes in different behavioral components for both durations and occurrences (Wa, Cl, HD, FG, BG, and Re; $p < 0.05$) as measured in HB, reflecting an enhanced anxiety-like state (fig. 21 and fig. 22)

Walking: Two-way ANOVA showed significant differences between sham-lesioned and LHb-lesioned groups ($F1,22 = 16.8$; $p = 0.0005$), no significant effect of nicotine treatment $F1,22 = 1.3$; $p = 0.28$), and a lack of interaction between the two factors (lesion x treatment; $F1,22 = 1.01$; $p = 0.33$) on walking mean duration. Similar results were observed for the mean occurrences of walking behavior (lesion $F1,22 = 14.2$; $p = 0.001$; treatment $F1,22 = 0.5$; $p = 0.5$; lesion x treatment $F1,22 = 1.3$; $p = 0.27$)

Climbing: Two-way ANOVA revealed a non-significant effect of LHb- lesion ($F1,22 = 0.31$; $p = 0.59$) and a significant main effect of treatment ($F1,22 = 8.8$; $p = 0.007$) on climbing mean duration. However, no significant interaction of

the two factors was observed ($F1,22 = 0.1$; $p = 0.3$). Similarly, there was a no significant effect of lesion ($F1,22 = 0.6$; $p = 0.5$) and a significant effect of drug treatment ($F1,22 = 8.9$; $p = 0.007$) on the occurrence of climbing behavior. In addition, no significant interaction of lesion group x drug treatment ($F1,22 = 1.2$; $p = 0.3$) was observed on the occurrence of climbing behavior.

Immobility: There was a significant effect of lesion on the duration ($F1,22 = 20.7$; $p = 0.0002$) and mean occurrence ($F1,22 = 4.7$; $p = 0.05$) of immobility. No significant effect of drug treatment on the duration ($F1,22 = 0.1$; $p = 0.8$) and mean occurrence ($F1,22 = 0.9$; $p = 0.4$) was observed, whilst there was also no significant interaction of lesion x treatment on the duration ($F1,22 = 0.02$; $p = 0.9$) and mean occurrence ($F1,22 = 1.0$; $p = 0.3$).

Immobile-Sniffing: There was no significant effect of lesion on the duration of immobile sniffing ($F1,22 = 2.2$; $p = 0.1$), and a significant effect of drug treatment ($F1,22 = 4.8$; $p = 0.04$), but no significant interaction of lesion x drug treatment ($F1,22 = 0.1$; $p = 0.7$). As for occurrence, there was a significant effect of lesion ($F1,22 = 10.3$; $p = 0.004$), no significant effect of drug treatment ($F1,22 = 1.4$; $p = 0.2$), but a significant interaction of these factors ($F1,22 = 7.6$; $p < 0.05$) as revealed by two-way ANOVA. *Post hoc* analysis revealed that LHb lesion induced a significant increase in the occurrence ($p = 0.002$) of immobile sniffing in the saline group. Nicotine reduced the mean occurrence ($p = 0.003$) in LHb- lesioned animals and was ineffective in sham-lesioned animals.

Edge Sniff: There was a significant effect of lesion on the duration ($F1,22 = 3.8$; $p = 0.01$) and on mean occurrence ($F1,22 = 3.6$; $p = 0.008$) of edge sniff. Conversely, there was no significant effect of drug treatment on duration ($F1,22 = 0.6$; $p = 0.4$) and mean occurrence ($F1,22 = 1.1$; $p = 0.3$), nor any significant interaction of lesion x treatment on duration ($F1,22 = 0.1$; $p = 0.7$) or frequencies ($F1,22 = 0.05$; $p = 0.8$).

Head-Dipping: There was no significant effect of lesion on the duration of head-dipping behavior ($F1,22 = 1.5$; $p = 0.2$), while a significant effect of drug treatment ($F1,22 = 4.1$; $p = 0.05$), but no significant interaction of lesion x treatment ($F1,22 = 0.8$; $p = 0.4$) were observed. As for occurrence, there was a strong significant effect of lesion ($F1,22 = 10.8$; $p = 0.003$), but no effect of drug treatment ($F1,22 = 3.4$; $p = 0.08$) or interaction of these factors ($F1,22 = 0.7$; $p = 0.4$)

Front Paw Licking: There was no significant effect of lesion on the duration of front paw licking behavior ($F1,22 = 1.5$; $p = 0.2$), no significant effect of drug treatment ($F1,22 = 0.2$; $p = 0.6$), but significant interaction of lesion x treatment ($F1,22 = 4.1$; $p = 0.05$). As for occurrence, there was no significant effect of lesion group ($F1,22 = 0.05$; $p = 0.8$), no effect of drug treatment ($F1,22 = 0.5$; $p = 0.7$) and no interaction of these factors ($F1,22 = 2.2$; $p = 0.1$). *Post hoc* analysis revealed that LHb lesion induced a significant decrease in the duration

($p = 0.005$) of front paw licking. Nicotine did change duration and occurrence in sham- lesioned 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 the LHb lesion on the duration ($F1,22 = 0.1$; $p = 0.9$) nor on mean occurrence ($F1,22 = 0.6$; $p = 0.4$) of hind paw licking. Neither was there a significant effect of drug treatment on duration ($F1,22 = 0.4$; $p = 0.5$) and mean occurrence ($F1,22 = 0.1$; $p = 0.8$) nor any significant interaction of lesion group x drug treatment for duration ($F1,22 = 1.6$; $p = 0.2$) and occurrences ($F1,22 = 2.6$; $p = 0.1$)

Face Grooming: There was no significant effect of lesion on the duration ($F1,22 = 0.5$; $p = 0.5$) or on mean occurrence ($F1,22 = 0.6$; $p = 0.5$) of face grooming. Moreover, there was no significant effect of drug treatment on duration ($F1,22 = 0.01$; $p = 0.9$) and mean occurrence ($F1,22 = 1.1$; $p = 0.3$) nor any significant interaction of lesion group by drug treatment for duration ($F1,22 = 2.5$; $p = 0.1$) and occurrences ($F1,22 = 1.4$; $p = 0.2$)

Body Grooming: There was no significant effect of lesion group on the duration of body grooming behavior ($F1,22 = 3.0$; $p = 0.09$), nor significant effect of drug treatment ($F1,22 = 0.2$; $p = 0.7$), and neither was there an interaction of lesion x treatment ($F1,22 = 2.2$; $p = 0.1$). As for occurrence, there was no significant effect of lesion group ($F1,22 = 1.7$; $p = 0.2$), nor significant effect of drug treatment ($F1,22 = 1.7$; $p = 0.2$) nor any significant interaction of these factors ($F1,22 = 1.7$; $p = 0.2$)

Rearing: There was no effect of lesion on the duration ($F1,22 = 0.5$; $p = 0.5$) nor on mean occurrence ($F1,22 = 1.0$; $p = 0.34$) of rearing. Neither was there a significant effect of drug treatment on duration ($F1,22 = 0.5$; $p = 0.5$) and mean occurrence ($F1,22 = 0.2$; $p = 0.7$), nor any significant interaction of lesion x drug treatment for duration ($F1,22 = 0.5$; $p = 0.5$) and frequency ($F1,22 = 0.2$; $p = 0.7$)

4.2. T-Pattern Analysis

The left part of fig. 23 shows, for each T-pattern, the terminal string and the respective occurrences. 17 different T-patterns were detected in the saline - administered group.

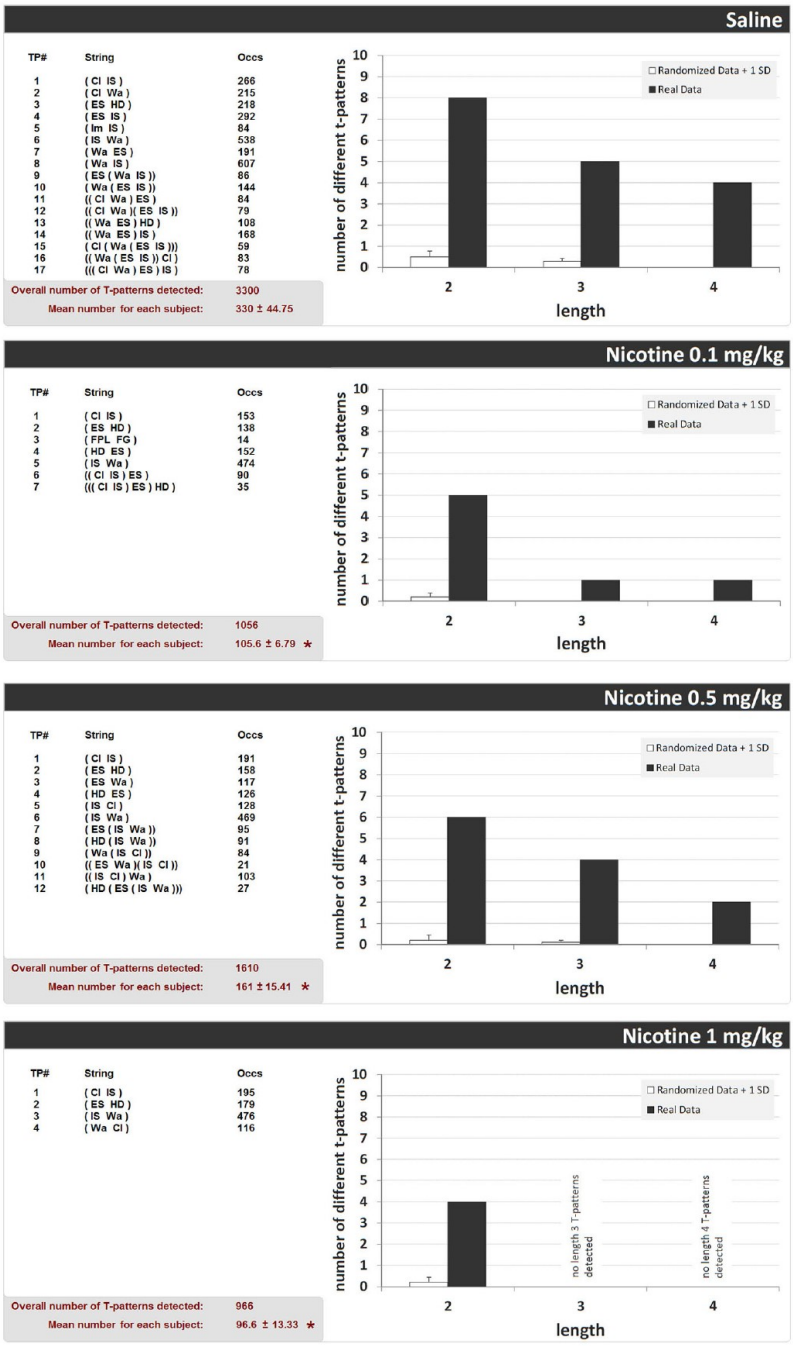


Figure 23. Structure of all T-patterns detected in unlesioned rats treated with saline or nicotine (0.1, 0.5, and 1 mg/kg, i.p.). Modified from [Casarrubea et al, 2015b]

Nicotine 0.1, 0.5, and 1 mg/kg groups revealed 7, 12 and 4 different T-patterns, respectively. Histograms on the right show for each group, T-pattern length distribution in real data and in randomly generated data ± 1 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 rather than in randomly generated data. The mean number of T-patterns shows a clear-cut reduction in all nicotine-administered normal groups. ANOVA ($F3,39 = 19.03$, $p < 0.0001$), 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.

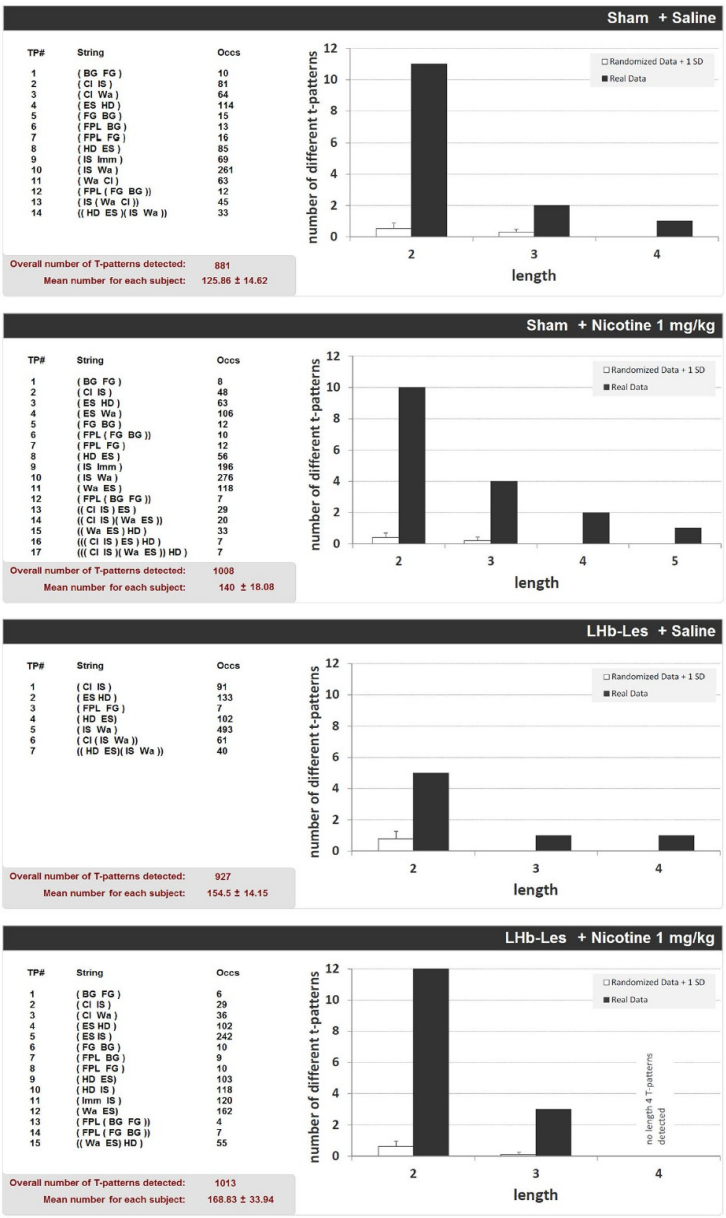


Figure 24. Structure of all T-patterns detected in sham- lesioned and LHb-lesioned subjects injected with saline or 1 mg/kg nicotine. Modified from [Casarrubea et al, 2015b]

The second column from the left indicates the terminal string of each T-pattern and the third one the occurrences. 14 different T-patterns have been detected in sham-lesioned + saline group; 17 in sham-lesioned + nicotine 1 mg/kg; 7 different T-patterns have been detected in LHb-lesioned + saline administered group; 15 different T-patterns have been found in nicotine 1 mg/kg administered group.

Both for sham and LHb-lesioned 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 rather than that which is randomly generated. There was no significant effect of the lesion group on the T- pattern mean occurrence ($F1,22 = 1.6$; $p = 0.2$) nor significant effect of drug treatment ($F1,22 = 0.6$; $p = 0.5$), or interaction of lesion x treatment ($F1,22 = 0.6$; $p = 0.9$).

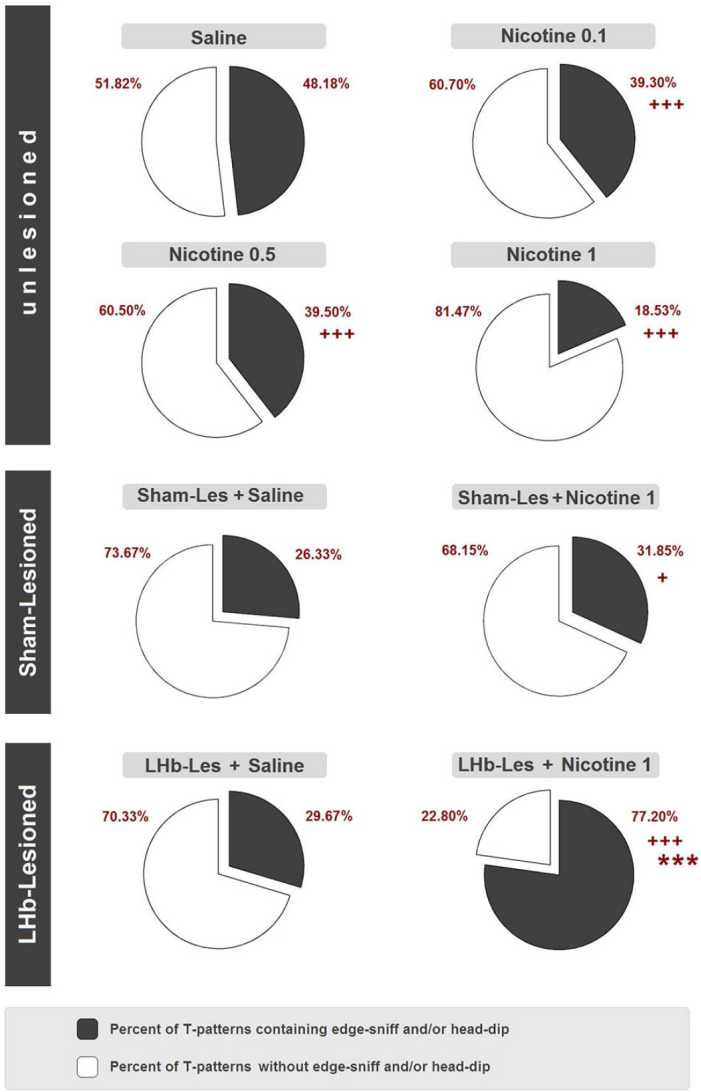


Figure 25. Pie charts illustrating percent distributions of T-patterns containing hole-exploratory behavioral components (i.e., edge sniff and/or head dip) in unlesioned, sham-lesioned and LHb-lesioned groups. Modified from [Casarrubea et al, 2015b]

Concerning unlesioned animals, 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 1 mg/kg. With regard to lesioned subjects, there was no significant difference between sham lesion + saline (26.3%) and LHb-lesion + saline (29.7%). On the contrary, the LHb-lesioned + nicotine 1 mg/kg group showed a significant clear-cut increase of T-patterns containing edge sniff and/or head-dip (77.2%), in comparison with the LHb-lesioned saline group (29.7%) ($p < 0.0001$). Concerning sham-lesioned animals, the administration of nicotine induced a lesser but still significant ($p < 0.05$) increase of T-patterns containing edge sniff and/or head-dip, from 26.3% to 31.9%. Finally, highly significant differences ($p < 0.0001$) were also detected between sham-lesioned vs. LHb-lesioned, nicotine 1 mg/kg groups.

5. DISCUSSION

This research describes general characteristics and temporal profile of anxiety-related behavior of 3 different strains of rats, the Wistar rat, the Dark Agouti rat and the Sprague-Dawley one. The first 2 strains are studied in the central platform of the EPM and a comparison between them has been carried out to fill up the gap of knowledge about their behavior. Wistar and DA/Han strains are characterized by different reactivity to the anxiogenic stimuli. The Sprague-Dawley strain has been studied in the HB apparatus in order to clarify the relationship between nicotine and anxiety. The first aim was to resolve the seemingly conflicting observations in the literature regarding the link between nicotine and anxiety, by comparing the effects of different doses of nicotine on anxiety-like animal behavior.

5.1. Elevated plus-maze study

The DA/Han rat is known for its peculiar emotional reactivity in comparison with other strains [King, 1999; Mechan et al., 2002]. In our laboratory the DA/Han rat, on the basis of observations with the EPM, has been demonstrated to possess a behavioural profile compatible with a higher anxiety level in comparison with Wistar rats [Casarrubea et al., 2013, 2015a]. In detail, we have demonstrated strong differences between Wistar and DA/Han rats in terms of durations and percent distributions of specific behavioral components in the CP (e.g. a clear prevalence of sniffing activities) [Casarrubea et al., 2015a].This study demonstrates the existence of a microstructure of rat behavior in the central platform of the EPM. The synergistic use of both quantitative and multivariate analyses has proved that different basal levels of anxiety deeply modify behavioral shift activity likely by affecting underlying decision making processes.

5.1.1. Quantitative assessments

The analysis of min-by-min durations demonstrates that the two strains spend a very different amount of time in the CP and that DA/Han rats, differently from Wistar ones, do not statistically reduce their permanence in CP over time. The main difference between the two strains is not a different number of behavioral elements but a different distribution of walking and sniffing activities: in the DA/Han rat there is a significant lower walking activity counterbalanced by a significant higher sniffing one. This evidence is confirmed by per cent distributions' pie charts. The walking activities are represented by quick displacements from the CP toward the arms and vice versa (central- platform entry, open-arm entry and closed-arm entry), whereas the sniffing ones are

represented by behavioral activities carried out by an animal standing in the CP. These evidences could explain why the DA/Han rats stay longer in the CP. Since the mean number of behavioral elements carried out by the two strains is not significantly different, we can not explain the increased time spent in CP by DA/Han with a reduction of their motor activity. Interestingly, in DA/Han rats walking activities are all significantly lower than in Wistar; on the contrary, all the sniffing activities are higher and statistically significant only for central-platform sniffing and open- arm sniffing. Therefore, in the CP, the behavior of the DA/Han rat is characterized by sniffing-oriented activities, whereas the Wistar rat by walking-oriented ones. Since it has been highlighted that rodent's behavior in the CP is linked with decision making triggered by the approach-avoidance conflict, and given the strong prevalence that sniffing activities have in the DA/Han rats, a relationship among sniffing activity, decision making processes and anxiety level can be proposed. From the phenomenological point of view, the sniffing of the first portion of the arm (that is, the adjacent part to the CP) usually precedes the entrance in an arm. Therefore, entering or not an arm would trigger a decision making process. The relationships between entering the arms and the preceding sniffing are illustrated through the value of ratio (fig. 10) calculated in the two strains: Wistar rats presented a value higher than 1, that is, entries in the arms are always numerically more represented than sniffings. Very different values were observed in DA/Han rats: below 1 for the open arms and with a value slightly higher than 1 for the closed ones. Wistar rats display a more direct approach to the arms during the second part of the test: while Wistar rats go into the safer closed arm also without sniffing the entries, DA/Han rats go into the safe arms after sniffing the entries; while Wistar rats go into the less safe open arms after sniffing the entry, DA/Han rats do not always go into the open arms after the sniffing. Such evidences suggest that the higher anxiety level drives the DA/Han toward a more cautious exploratory approach in the new environment. Therefore, the value of ratio may be proposed as the expression of the link between anxiety level and decision making. As to rearing, it is a common behavioral element in closed-arm and central-platform but extremely unusual in open-arm [Rodgers et al, 1995, 1997a,b]. Moreover, it has been demonstrated that rearing is a behavioral element indicative of motor activity [Cruz, 1994; Espejo, 1997]. Our data show that a higher frequency of rearing in the CP occurs in the DA/Han rat. Such result confirms our hypothesis that the longer permanence of DA/Han rats in CP is due to the effects of a different reactivity to anxiogenic stimuli and not to a decrease of motor activity of this strain.

5.1.2. Multivariate cluster analysis

The behavioral profiles of Wistar and DA/Han rats, other than by means of quantitative analysis, can be characterized by means of a multivariate approach.

In particular, the multivariate cluster analysis, based on the elaboration of transition matrices, is able to evaluate the behavioral shift, hence the possible relationships between different levels of anxiety and decision making processes. The tree structure of the dendrogram (fig. 13), is arranged on the basis of an aggregative procedure, where a transition matrix is transformed in a half similarity matrix. In such a matrix, each cell is representative of a behavioral transition which, in turn, reflects a behavioral shift driven by a decisional process. Moreover, since it has been reported that the time spent in the central platform is linked to decision making and since the phenomenological expression of decision making processes is the behavioral shift activity, the different transitional arrangement of the two strains of rats suggests that different levels of anxiety influence decision making processes. In Wistar strain a cascade-shaped dendrogram, branching from walking activities is present. This means that the behavior of the Wistar rat is prevalently oriented to cross the CP in direction of one of the two arms. On the contrary, DA/Han rats' dendrogram displays sniffing activities closely linked among them, with walking activities detached from the comprehensive behavior. Both quantitative and multivariate analyses show that the more anxious DA/Han presents a behavior oriented toward the permanence in the CP rather than in other parts of the maze.

5.1.3. T-pattern analysis

T-pattern analysis let us support the idea that both Wistar and DA/Han rats present, in the central platform, a complex behavior organized on the basis of several sequences of events with highly significant constraints on the interval length separating them. DA/Han rats have a behavior more complex and more structured in terms of T-patterns. This feature appears by observing the terminal strings and/or the tree structures, the mean number of detected T-patterns or the rasterplots. Actually, DA/Han strain performs more different patterns in comparison with Wistar (that is, 21 vs. 13). This result demonstrates that the behavior of the DA/Han, in the CP, is more variable. Such a variability is amplified by the length of detected patterns. Indeed DA/Han rats show longer sequences in comparison with Wistar rats (4- length vs. 3-length). These outcomes gain even more emphasis if assessed together with quantitative results. Actually, the amount of time spent in walking and sniffing activities by the two strains is very different. Wistar rats do spend much more time in walking activities and noticeably less in sniffing ones: with significantly higher frequency, indeed, they do cross the central platform to explore the arms; consequently, their permanence in the CP is significantly lower; on the contrary, DA/Han rats do walk less and sniff much more: crossing of CP is significantly reduced both towards the open and the closed arms as well; therefore they do present a significantly higher permanence in CP where they shape the largest

extent of their behaviour. All the results highlight that the different emotional profiles characterizing Wistar and DA/Han rats determine different coping strategies, namely, the behavioral efforts each subject carries out to master an aversive situation [Koolhaas, 1999]: DA/Han coping strategy is aimed at avoiding the aversive situation by maintaining the animal prevalently in the central platform. On the contrary, Wistar coping strategy is aimed at searching for an escape route. The different basal level of anxiety, characterizing the two strains of rats, provokes a different behavioral response to the environment: the closed zones are perceived as more secure by Wistar but not by the DA/Han. On the basis of the different reactivity to the anxiogenic stimuli, DA/Han rat may be proposed as a strain offering a more defensive related behavior in the EPM task. T-pattern analysis supports such an interpretation. Several T-patterns in Wistar rats end with the entry in an open or in a closed arm; hence, the Wistar rat, in the novel and potentially dangerous environment, shows a behaviour highly oriented to cross the CP towards both the open and the closed arm. On the other hand, DA/Han strain shows few patterns concluding with a closed arm entry and never with an open arm entry. On this subject, further details emerge by the evaluation ~~both~~ of latencies, terminal strings and T-patterns' first onset: latencies show that Wistar rats, once in the CP, start walking activities significantly earlier than DA/Han; onsets diagram illustrates that such an early walking activity is characterized by T-patterns #10 and #4, namely, as indicated in fig. 14 (OA-Sn → OA-Ent) and (CA-Sn → CA-Ent). Thus, Wistar rats, during the first second of EPM exploration, promptly enter both open and closed arms. On the other hand DA/Han rats, with T-patterns #2, #3, #13 and #1, namely, (C-Sn → CA-Sn), (C-Sn → OA-Sn), (C-Sn → CA-Sn → CA-Ent) and (C- Sn → CA-Ent) show, during the first second of interaction with the environment, a behavior prevalently organized on the basis of sniffing activities and closed arm entries. Such evidences, highly suggestive of a very cautious approach to the environmental exploration, further highlight the emotional reactivity and the higher anxiety level of the DA/Han supporting the idea of the existance of two different coping strategies linked to a different basal level of emotionality of the two strains. Quantitative analysis allows us to interprete data on the basis of single elements, Cluster analysis allows an immediate understanding of relationships between different groups of behavioural elements; it is a graphical representation of transition matrices. The results provided by this kind of approach could represent a great advantage in terms of synopsis and straightforwardness. A transition matrix and all the related elaborations describe the comprehensive period of observation, without considering the temporal dimension and possible temporal relationships among the components of the behavioural repertoire. A transition matrix is like a "snapshot" of the comprehensive period of observation, it doesn't give information about how one element is followed by another one and so on. There were no information about the development during the time of the behavior of the rat in the central platform

of the EPM until now. By using multivariate T-pattern analysis, we fill such a gap on. For the first time, indeed, information on the fine temporal structure of behavior in this zone of the maze is provided.The great advantage of multivariate T-pattern analysis lies in its ability to provide information concerning the relationships among behavioral events occurring during the observation.

5.2 Hole Board Study

Trought this experiment our lab demonstrated that acute administration of medium-high doses of nicotine (0.1–1 mg/kg, i.p.) induced clear anxiogenic-like behavior in normal rats. 30 min after injection, the HB findings showed an anxiogenic-like profile of all doses of nicotine when compared to control.

5.2.1. Quantitative assessments

Both mean duration and mean occurrence (fig. 19 e fig. 20) show that head-dipping activity, the main index of anxiogenic-like activity, was statistically decreased by nicotine. This reduction has been shown with all the doses of nicotine (0.1, 0.5 and 1 mg/kg, i.p.). The duration of rearing was significantly reduced following doses of 0.5 and 1 mg/kg, while climbing was reduced only at 0.1 mg/kg; however, their occurrences were reduced by all the doses compared to control. This anxiogenic - like effect following acute nicotine treatment is coherent with previous studies, in which the acute administration of nicotine is used in the EPM platform by Zarrindast et al., 2000, 2010; Ouagazzal et al., 1999a; Hayase, 2007 in rats and mice, and in the HB platform by Nasehi et al., 2011. In agreement with such studies, the mean walking duration and occurrence were not significantly different between treatment groups, indicating that the nicotine- induced reductions in head-dips and rearing were not due to changes in locomotory activity. Another behavioral parameter useful to assess anxiety levels is the grooming activity. As supported by Spruijt et al., 1992 and Kalueff and Tuohimaa, 2005 it is thought to be initiated in response to changes occurring in the animal as a result of anxiogenic stimuli. Consistent with the changes observed on head-dipping and rearing, grooming duration also appeared to be significantly increased by nicotine treatments. It is valid (fig. 19) for all the different grooming's components (FPL, HPL, FG, and BG).

5.2.2. T-pattern analysis

Multivariate T-pattern analysis (fig. 23) revealed that the number of different T-

patterns, their overall occurrences and their mean number are significantly reduced in all nicotine-administered groups, with a maximum effect observed at the higher dose (1 mg/kg), showing that nicotine strongly affects the complex behavior structure in normal rats, drastically simplifying it. It is possible to assert that acute nicotine administration has a negative impact in terms of behavioral variability and organization. On the basis of our data, we can say that the acute administration of nicotine induces an increase in the anxiety- like level in the normal animal (as indicated by the consistent reduction of head-dipping duration) [Takeda et al., 1998]. The simplification of temporal characteristics of behavior could be linked to an increased anxiety condition. However, the simple assessment of T-patterns quantitative features, such as duration and occurrence, is not sufficient to assess whether the animal behavior modifications are coherent with anxiety. To clarify this, we conducted a subsequent evaluation of the sequential structure of T-patterns detected containing edge-sniffing and head-dip following our previous studies [Casarrubea et al., 2009a,b] and we found that nicotine administration reduced them in a significant and almost dose-dependent fashion. Thus, behavior structure is significantly reorganized in terms of a reduced exploratory approach, consistent with an increased anxiety-like level. Our findings support some epidemiological studies suggesting that nicotine dependence increases the risk of anxiety disorder and panic attacks [Bruijnzeel, 2012]. Indeed, first- time smokers report aversion to nicotine and increased anxiety [Newhouse et al., 1990], while long-term smokers show higher levels of anxiety and stress compared to non-smokers [Parrott and Murphy, 2012]. In line with this, a moderate reduction in anxiety levels has been observed 6 months after quitting smoking [McDermott et al., 2013].The contradictory evidence surrounding nicotine and anxiety might be explained by regional nAChR subunit configuration [File et al., 2000]. Indeed, a4-nAChR knock out (KO) mice have decreased anxiety-like behavior [Ross et al., 2000; McGranahan et al., 2011], while a7- [Paylor et al., 1998], b3- [Booker et al., 2007] and b4-nAChR KO mice [Salas et al., 2003] seem to present an increase in anxiety-related behavior. Interestingly, elimination of a4b2-nAChRs specifically from DAergic neurons decreases sensitivity to the anxiolytic effects of nicotine [McGranahan et al., 2011]. Recently, it has been suggested that low nicotine's dose inhibits b2* nAChRs inducing the anxiolytic-like effects, while high doses stimulate them leading to the anxiogenic-like effects of nicotine [Anderson and Brunzell, 2015]. The complex behavioral output following nicotine administration depends on the different brain areas involved in anxiety as a whole; and the neurotransmitter systems regulated by nAChRs all taken together. 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 into the central amygdala [Zarrindast et al., 2008, 2013], the dorsal raphe nucleus (DRN) [Cheeta et al., 2001], lateral septal nucleus [Ouagazzal et al., 1999b] and hippocampus

[Ouagazzal et al., 1999a; Kenny et al., 2000], or applied to different areas of the mesolimbic DA system [Picciotto et al., 2002; Zarrindast et al., 2013] has been shown to induce an anxiogenic-like effect. Of note, nicotine injection into the DRN has differential effects on behavior in the social interaction test depending on the dose used. Low doses of nicotine are anxiolytic, intermediate doses have no effect, and high doses are anxiogenic [Cheeta et al., 2001]. The Lateral Habenula lesion (bilateral) is another variable of the HB study. We dimonstrated that a bilateral lesion lead to significant change in the locomotor activity, when compared to sham-lesioned rats. Our results confirm and validate other studies like that one of Nielson and McIver, 1966; Lecourtier et al., 2008; Gifuni et al., 2012; Wang et al., 2013; Jean-Richard Dit Bressel and McNally, 2014. This locomotor effect is likely due to the strong inhibitory control over midbrain DA neurons exerted by the LHb [Matsumoto and Hikosaka, 2007]. As shown in fig 21 and fig. 22 the occurrence, but not the total time of immobile sniffing and head-dipping, were significantly increased in the LHb-lesioned animals, while no changes in the grooming were revealed. High head-dipping activity is linked to low anxiety while higher grooming activity is a measure of higher anxiety; quantitative data suggest a direct relatioship between the absence of Lateral Habenula and the anxiolytic effect. The somministration of nicotine (1.0 mg/kg) in LHb-lesioned animals has a different effect in comparison with sham-lesioned animals, it was unable to produce the same cheange of head-dipping. While climbing was further inhibited, grooming was increased by nicotine in LHb-lesioned animals (although not significantly). Interestingly, the effect of nicotine on LHb-lesioned animals was the reduction of immobile sniffing in comparison to the LHb-lesioned animals that receive saline. As we suggested in the EPM study a natural more anxious rat (the Dark Agouti) shows a sniffing-oriented behavior. Since both groups are without Lateral Habenula and since nicotine in this case create a profile of behavior not oriented toward Immobile Sniffing but with a reduction of this activity; it seems to be less anxious. Concerning T-pattern analysis, sham-lesioned and LHb- lesioned rats treated with saline are characterized by a modification of anxiety-related behavior compared to unlesioned animals. Indeed, strings and percentage of T-patterns containing edge-sniff and/or head-dipping describe, in both sham and LHb-lesioned animals, a situation essentially consistent with an increased anxiety level, although the influence of the hypolocomotion induced by surgery cannot be excluded. The above discussed condition of increased anxiety, in rats with lesion of the LHb, radically changes if nicotine is acutely administered. Following nicotine administration in LHb-lesioned rats, the number of T-patterns containing head-dip and edge sniff is strongly increased. Interestingly, although less evident, nicotine induced an increase in T-patterns containing head-dip and edge sniff in Sham-lesioned animals, about 32% compared to the 26% of the saline. LHb-lesioned rats treated with nicotine presented the largest extent of patterns, about 77%, containing edge sniff and head dip. Acute

nicotine injected animals with lesion in the LHb do explore the holes significantly more. Such an outcome demonstrates that the lesioning itself had an evident impact in terms of behavioral organization, as indicated by a decrease in locomotion, rearing and head-dipping and increases in immobility and T-patterns containing head dip and edge sniff; typical of an anxiogenic- like phenotype. Some aspects of the surgical procedure used in this study may have been stressful and it is well known that stress induces anxiogenic-like behavior [Bondi et al., 2008]. Thus, some of the nicotine's anxiolytic activity in Sham and LHb-lesioned animals may be related to the drug's known anxiolytic properties under conditions of stress [Hsu et al., 2007]. Strikingly, the LHb lesion strongly amplified the anxiolytic nicotine effect. Such evidence 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) and in behavioral response to stress. One of the most important findings of our study is the evidence that standard quantitative analyses (such as duration and occurrence) provide a reductionist portrait of animal behavior. It allows the description of behavior in terms of individual components, separate from the comprehensive behavioral architecture. Using a multivariate approach, we are able to provide information concerning the structural relationships among each component of the rat behavioral repertoire: T-pattern analys is capable of validating effects that otherwise would be just hypotisized, i.e., anxiolytic nicotine activity in LHb- lesioned rats. It allows us to propone new parameter to measure anxiety: can immobile-sniffing be considered a valid measure of anxiety? Both EPM and HB study share a concept: the activity of sniffing is directly linked to a more anxious behavior. The advantageous use of a multivariate approach like T-pattern analysis can be also well rapresented by head-dip activity; nicotine in LHb-lesioned rats does not affect the duration or occurrences of head-dip compared to its vehicle. As we have discussed in the preceding section, this would have been a wrong conclusion. Actually, when the relationships of head-dip with the other components of the behavior are analyzed, a completely different scenario emerges. The number of head-dips and edge sniffs become components of the largest amount of behavioral sequences performed by the LHb-lesioned animals following nicotine administration. In these animals, the environmental exploration becomes significantly more organized in comparison with the saline administered groups. Our observations are consistent with evidence that chemical inactivation of the LHb limits and abolishes certain behaviors shown under highlighted anxiety states, such as increasing the time spent in the open arms of the EPM, decreasing the time spent burying in the defensive burying task following yohimbine administration and blunting cocaine seeking that is exacerbated by yohimbine [Gill et al., 2013]. Consistently, bilateral electrolytic lesion of the LHb impairs inhibitory avoidance acquisition in the EPM, indicating an anxiolytic-like effect [Pobbe and Zangrossi, 2008]. Our data are in

agreement with previous findings, which show that lesioning of the fasciculus retroflexus improves the behavioral response of depressed rats by increasing the 5-HT level in the DRN [Yang et al., 2008]. Our current findings support and extend these prior studies by showing that the inactivation of the LHb *per se* decreases anxiety-like traits in rats (i.e., increase in head-dipping), an effect never observed before. However, our data do not allow us to be conclusive about the role of the LHb in general and nicotine-induced anxiety-like behavior. Further studies utilizing larger sample size, multiple behavioral tests and anxiolytic drugs should be conducted to validate our results. Concurrently, different types of LHb inactivation/lesion, which might potentially produce control animals with lower levels of basal anxiety compared to those used in our current study, should be considered. Our study therefore highlights an important methodological issue when evaluating behavioral studies that are based on comparisons of only lesioned animals with sham-lesioned with no inclusion of unlesioned controls, which form the majority of the available data. It still remains to be explained how a lesion in Lateral Habenula reverts acute nicotine-induced anxiety- like behavior. LHb-lesioned rats show 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 decreased anxiety observed in animals with lesion in the LHb, and the strong anxiolytic-like effects 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 of which are essential in the homeostasis of anxiety/stress levels [Zweifel et al., 2011; Zangrossi and Graeff, 2014]. Specifically, one possible explanation for the present findings is that nicotine, activating the nAChRs 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 lateral shell of the nucleus accumbens [Hong et al., 2011; Lecca et al., 2011; Lammel et al., 2014], decreasing the rewarding effects of nicotine. 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., 2014]. The mPFC forms part of the anxiety network and has been shown to modulate the amygdala, bed nucleus of the stria terminalis and ventral hippocampal neuronal activity, synchronizing them on the theta band during high state of anxiety [Adhikari, 2014]. Evidence that the LHb spontaneously generates theta oscillations in phase with hippocampus [Goutagny et al., 2013] further suggests that the LHb might also be considered part of the anxiety brain network. The LHb couples the DA and 5-HT systems, and nicotinic activation of 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]. The 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 amygdala leading to the development of an anxiety state. Strikingly, in our conditions the LHb lesion reverses the anxiogenic-like effect mediated by 1 mg/kg of nicotine into an anxiolytic-like 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. Further investigations with habenular lesion/activation, 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 general and nicotine-induced anxiety phenotype.

6. CONCLUSIONS

EPM research shows that Wistar and DA/Han rats present a different behavior in the central platform, that is the zone of the apparatus where the animal selects the arm to explore. It is suggested that the difference between the two strains might represent the behavioral expression of anxiety-induced modifications of decision making process occurring in the central platform and underlying behavioral shift activities. A peculiar defensive-related behavior of the DA/Han strain has been evidenced. Through the T-pattern analysis, present research demonstrates, for the first time, that two strains with different emotional reactivity to the environmental stimuli present important differences in terms of the underlying temporal sequences of activities performed in the central platform of the EPM. In addition, a new useful index able to reveal the effects of pharmacological and/or environmental anxiety manipulation is proposed: the value of ratio between the entrances in the arms from the central platform and the preceding sniffings. HB research demonstrates that nicotine itself leads to anxiety-like behavior under normal conditions and acts as an anxiolytic under some circumstances (i.e., stressful conditions). The Lateral Habenula greatly potentiates the anxiolytic-like properties of nicotine, further supporting the role of the LHb in the neuronal circuits that mediates nicotine's aversive effects [Fowler and Kenny, 2014]. 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 achieve a precise description of the

effects induced by one or more independent variables in animal behavior analysis. The utilization of multivariate analysis lead to an innovative and more complete interpretation. Cluster analysis but above all T-pattern analysis allowed to study the behavior of rats as a whole. As this dissertation shows, the use of both quantitative and multivariate analysis leads the researcher to deeply understand the behavior, to show connections among different elements, to propose new models of interpretation and new measures to study emotions like anxiety. It allows to study anxiety modulators and to give new insights in animal research from a structural point of view but also from a neuroanatomical standpoint.

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