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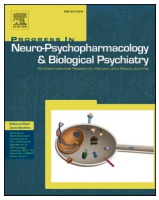

Sex-dependent behavioral effects of chronic nicotine during adolescence evaluated in young adult rats tested in Hole-Board

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ABSTRACT

As one of the leading causes of death and serious illnesses, tobacco smoking remains a significant issue in modern societies. Many individuals smoke during adolescence, a trend that has been exacerbated by the prevalence of vaping among young people. In this context, studying the behavioral effects induced by nicotine administration in male and female rats, during the adolescent period, assumes great importance because it can help to better understand the dynamics underlying tobacco use in the two sexes. For this purpose, we employed 4 groups of rats, 2 male and 2 female groups, chronically treated with saline or nicotine 3 mg/kg i.p. for 30 days, spanning from postnatal day 30 to postnatal day 60. Utilizing quantitative analyses and T-pattern detection and analysis, our findings revealed a complex and multifaceted behavioral reorganization in adolescent rats subjected to chronic nicotine administration. Specifically, we observed an increase of anxiety in males and a reduction in females. The distinctive structural changes, induced by chronic nicotine in both sexes, have significant implications, from a translational perspective, for studies on nicotine dependence disorders.

1. Introduction

As one of the first and most important causes of death worldwide (CDC, 2024), cigarettes and, more generally, tobacco smoke are serious problems in modern societies (Matsumura et al., 2024; Vella and Di Giovanni, 2013). While tobacco use is decreasing overall, the popularity of nicotine-containing vaping products is on the rise among younger populations (Cullen et al., 2018). This has underscored the importance of understanding sex-dependent brain development and its vulnerability to drugs of abuse during adolescence (Ng et al., 2024). Cigarette smoking is very well known to be strictly associated with a long list of diseases and serious health problems such as hypertension, pulmonary diseases, hearth diseases and psychiatric disorders (Matsumura et al., 2024; AHA, 2024; Yuan et al., 2020). In addition, it is present an aspect that should not be underestimated, as it is an insidious opponent when trying to intervene to induce people to quit smoking: nicotine, one of the most important neuroactive components of tobacco, indeed, is highly addictive and after the development of dependence it is very well known to be extremely difficult to quit (Chellian et al., 2021a, 2021b; Casarrubea et al., 2015a). Nicotine affects the central nervous system through specific pentameric receptors (nAChRs) implicated, among other things, in modulating the release of a surprisingly large amount of neurotransmitters such as dopamine, serotonin, glutamate and nitric oxide (Pierucci et al., 2022; Pierucci et al., 2004, 2014; Di Matteo et al., 2007, 2010; Di Giovanni, 2012; Lester, 2014). Thus, given the consistent amount of neurotransmitters involved in central nicotine activity, this alcaloid produces a number of different behavioral effects often extremely difficult to interpret (Picciotto et al., 2002).

Undoubtedly, one of the topics on which nicotine raises numerous questions, perplexities and few unambiguous results concerns anxiety and anxiety-related behaviors (Picciotto et al., 2002). Nicotine has multifaceted and complex effects on anxiety and can provoke a variable degree of responses, ranging from anxiolytic to anxiogenic, depending on the behavioral model utilized, doses, time course and route of administration (Caldarone et al., 2008; Picciotto et al., 2002) and on sex of tested subjects (Rupprecht et al., 2015; Caldarone et al., 2008;

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Stringfield et al., 2018; Cheeta et al., 2001a; Elliott et al., 2004). Several reviews on the effects of nicotine and nicotinic receptors on anxiety and anxiety-related behavior have been conducted (Picciotto et al., 2002; Rupprecht et al., 2015; Chellian et al., 2021b).

In this context, the discovery of possible behavioral differences between males and females, *in terms of behavioral structure*, could lead to new insights into the dynamics underlying nicotine addiction and, from a translational perspective, could lead to the development of more targeted interventions in humans. For this purpose, in this study, we employed a method known as T-pattern detection and analysis (TPA) to conduct an in-depth analysis of the structural and temporal characteristics of the behavior of adolescent male and female rats chronically treated with nicotine intraperitoneally (i.p.) TPA has been shown to be able to reveal behavioral features that are very difficult or impossible to observe with more conventional methods of purely quantitative data analysis. For instance, over the past two decades or so, by means of TPA it has been possible to study rodent behavior in a model of Tourette's syndrome (Santangelo et al., 2018) and in a model of Parkinson's disease (Casarrubea et al., 2019a), route-tracing stereotypies in mice (Bonasera et al., 2008), the interactions between humans and artificial agents (Kerepesi et al., 2006) or between humans and animals (Kerepesi et al., 2005), feeding behavior in rodents (Casarrubea et al., 2019b), neuropsychiatric diseases (Lyon and Kemp, 2004; Kemp et al., 2008; Sandman et al., 2012; Kemp et al., 2016), interactions between hormones and behavior (Hirschenhauser et al., 2002), the behavior of non-human primates (Gunst et al., 2020; Cenni et al., 2020, 2021), behavioral and neurochemical changes in genetic absence epilepsy rats from Strasbourg and non-epileptic controls (De Deurwaerdere et al., 2022), movement and behavioral disorders in human and animals (Aiello et al., 2020). Finally, TPA has been successfully used to study rodent anxiety and anxiety-related behaviors in the Elevated Plus Maze (Casarrubea et al., 2013a, 2013b, 2014, 2016), in the emotional object recognition task (Casarrubea et al., 2021b) and, importantly, in the experimental apparatus utilized in the present study, i.e. the Hole-Board (Casarrubea et al., 2015a, 2020, 2021a).

2. Methods

2.1. Animals and housing

Twenty-eight Black Hooded rats, 14 males and 14 females of the same age, weighing at the post-natal day 60 (P60) 300 ± 30 g (males) and 250 ± 20 g (females), were bred and housed (2 or 3 animals per home-cage, male rats housed with males and female rats with females) at the animal-house facility of the Department of Physiology and Biochemistry, University of Malta, with the room temperature constantly monitored and maintained at 21.5 ± 1 °C with a humidity of 60 ± 5%. A light-dark cycle of 12 h with lights on at 7:00 am and lights off at 7:00 pm was provided. All subjects received "ad libitum" access to food and water.

2.2. Groups and drugs

The males and females were divided into 2 subgroups, so obtaining four different groups each encompassing 7 subjects: male saline ($n = 7$), female saline (n = 7), male nicotine (n = 7) and female nicotine (n = 7). Starting from P30 to P59, all the rats were chronically administered i.p. (30 consecutive days, 3 times per day: 08:00 am, 12:00 am and 04:00 pm) with nicotine (−)-nicotine hydrogen tartrate salt (C18H26N2O12) dissolved in physiological saline (0.9% NaCl) and adjusted to a pH of 7.4) at a dose of 1 mg/kg (for a cumulative dose of 3 mg/kg/day) and saline (1 ml/kg) 3 times per day as a control. On the final day (31st day), rats were moved from their housing room to the testing room in their home cages to minimize possible transfer effects, given their final i.p. administration of either saline or nicotine and allowed a 30-min acclimation period away from the experimental apparatus. Finally, 30 min after the administration, each subject was tested in the Hole-Board for 10 min and their behavior recorded by means of a digital videocamera (see below).

2.3. Experimental apparatus and behavioral recordings

The Hole-Board apparatus utilized in this study consisted of a square arena (50 × 50 cm) made of white opaque Plexiglas floor with four equidistant holes (diameter 4 cm). The center of each hole was 10 cm distant from the two adjacent walls so that all the holes were equidistant. The Hole-Board floor was elevated 5 cm above a white opaque Plexiglas sub-floor. The ground floor perimeter was enclosed by three white opaque Plexiglas walls (50 × 50 cm) and a front transparent one (50 × 50 cm). A digital videocamera (Toshiba Corporation, model HDDV P-10), positioned in front of the transparent wall, was used to digitally record the behavior of each rat. All video files, initially recorded on the digital camera's removable SD card, were permanently stored on a personal computer for the following analyses.

2.4. Data analyses

Fig. 1 shows an outline of all the components of the behavioral repertoire and their formal descriptions. This ethogram, based on our previous studies (Casarrubea et al., 2023; Casarrubea et al., 2009b; Casarrubea et al., 2017; Casarrubea et al., 2020), encompasses four different behavioral categories, overall containing eleven behavioral components. *General Exploration*: Walking (Wa), Immobile Sniffing (IS), Climbing (Cl), Rearing (Re); *Focused exploration*: Edge-Sniff (ES), Head-Dip (HD); *Grooming activity*: Front-Paw Licking (FPL), Hind-Paw Licking (HPL), Face Grooming (FG), Body Grooming (BG); *Immobility* (Im). Utilizing this ethogram, video files were annotated by a trained observer, blind to groups (i.e. subjects' sex and treatment), using a professional software coder (The Observer, Noldus Information Technology bv, Netherlands). As a result of coding process, event log files were generated for each subject and represented the starting point for all the analyses presented and discussed in this study, i.e. quantitative and T-pattern analyses (TPA). The propaedeutic assessment of intra-rater reliability was evaluated by using Cohen's Kappa coefficient on five video files, randomly taken from the experimental recordings and each file scored by the observer two times, in two different moments; the following coefficients were achieved for each pair of event-log files respectively: $\kappa = 0.866$, $\kappa = 0.881$, $\kappa = 0.832$, $\kappa = 0.854$, $\kappa = 0.815$.

2.4.1. Univariate analyses

For each group, the following univariate variables were analyzed: mean occurrences, mean durations and percent distributions of each individual component of the behavioral repertoire (Fig. 1).

2.4.2. Multivariate T-pattern detection and analysis

TPA is a technique conceived to reveal the structure of behavior in terms of temporal features. Theories and concepts underlying T-patterns and their detection can be found in various monographies (Magnusson et al., 2016; Casarrubea and Di Giovanni, 2020) and articles (Magnusson, 1996; Magnusson, 2000; Magnusson, 2004; Magnusson et al., 2016; Magnusson, 2020a, 2020b; Casarrubea et al., 2015b, 2022; Maseroli et al., 2020). In brief, by means of Theme software (Theme, PatternVision Ltd., Iceland), i.e. a computer program specifically created and used for this purpose (Magnusson, 1996; Magnusson, 2000) it is possible to unveil hidden sequences of events based on the detection of significant constraints on the time intervals separating them. An advanced algorithm processes event-log files within a T0-Tx observation period, for example events "a"… "b"… "c"… "d"… etc., and their discrete time-points, comparing the distributions of each pair of the behavioral events searching for a time window so that, e.g. "a" is followed by "b" within such time interval. This time interval, called "critical interval relationship" is at the heart of the search algorithm for the detection of T-

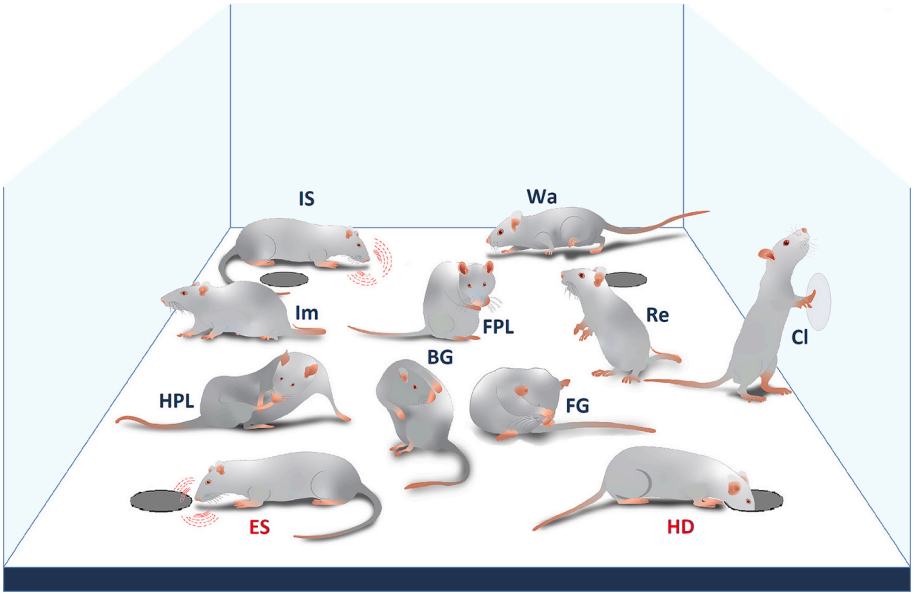

Fig. 1. Behavioral repertoire of male and female rats in the Hole-Board. *General exploration*: Walking (Wa) = rat walks around sniffing the environment; Immobile-Sniffing (IS): rat sniffs the environment while standing on the ground; Rearing (Re) = rat maintains an erect posture without leaning against the Plexiglas box; Climbing (Cl) = rat maintains an erect posture leaning against the Plexiglas wall. *Focused exploration*: Edge-Sniff (ES) = rat sniffs the border of one of the four holes; Head-Dip (HD) = rat puts its head into one of the four holes. *Grooming activity*: Front-Paw Licking (FPL) = rat licks or grooms its forepaws; Hind-Paw Licking (HPL) = rat licks or grooms its hind paws; Face-Grooming (FG) = rat rubs its face (ears, mouth, vibrissae, eyes) with rapid circular movements of its forepaws; Body-Grooming (BG) = rat licks its body combing its fur with fast movements of incisors. *Immobility* (Im) = rat maintains a fixed posture. No movements are produced. Modified from Casarrubea et al., 2023.

patterns (Magnusson, 1996; Magnusson, 2000; Magnusson, 2004; Magnusson et al., 2016; Magnusson, 2020a, 2020b). Briefly, using the words of Prof. Magnusson, who developed T-pattern analysis, "*if A is an earlier and B a later component of the same recurring T-pattern, then, after an occurrence of A at t, there is an interval* $[t + d_1, t + d_2]$ $(d_2 \geq d_1 \geq 0)$ *that tends to contain at least one occurrence of B more often than would be expected by chance. This relation is here called a critical interval relation […]*" (Magnusson, 2000). If this condition between event "a" and event "b" is present, the two-event T-pattern (a b) is detected; in a second phase such a first level (a b) simple sequence is considered to be the "a" or "b" terms for the detection of higher-order patterns, e.g., ((a b) c), ((a b) d), etc. The search algorithm stops when no more patterns are identified. Important features of T-patterns are their *variability* (i.e. the number of T-patterns of different composition detected), their *complexity* (i.e. the length of T-patterns, namely, the number of events in sequence) and their *recursivity* (i.e. the number of times each T-pattern does occur) (Casarrubea et al., 2019b, 2021a). Search parameters used were as follows: significance level = 0.0001, lumping factor = 0.90, and minimum percent of samples = 100. For each group, the following parameters of the behavioral responses were analyzed: terminal strings of all the T-patterns detected (i.e., simply stated, a textual representation indicating the events included in each T-pattern, their order, and parentheses indicating their hierarchical level); length distribution of different T-patterns, both in real and randomly generated data; mean length and mean occurrences of T-patterns; finally, percent distribution of T-patterns encompassing behavioral components of hole-exploration have been assessed as well.

2.4.3. Statistics

As to mean occurrences and mean durations of each component of the behavioral repertoire, two-way ANOVA for independent samples (factors: sex, drug) followed by Newman-Keuls post-hoc analysis for multiple comparisons among groups were utilized; the chi-square test was utilized to evaluate possible significant differences in percent distributions of Head-Dip and Edge-Sniff components. As to TPA, even if each sequence involves the detection of statistical significant constraints among events in sequence, data with thousands of events might rouse a spontaneous question: whether the T-patterns detected are there only by mere chance. Theme software deals with this crucial aspect by repeatedly randomizing and reanalyzing the original data, using exactly the same search parameters utilized in the detection run with the real dataset. By doing so, it is possible to compare the mean number of T-patterns detected in the randomized data with the number of patterns identified in the original data. Mean occurrences and mean length of T-patterns detected in real data were assessed using Kruskal-Wallis test followed by Mann-Whitney post-hoc for multiple comparisons; finally, to evaluate possible significant differences in T-patterns encompassing events of hole-exploration, chi-square test was utilized. $P \leq 0.05$ was considered to indicate statistical significance in all the analyses used in the present study.

2.5. Ethical statement

Procedures involving animals and their care were conducted in accordance with European Law (EU Directive 2010/63/EU) and the Institutional Animal Use and Care Committee (IAUCC) at the University of Malta. All efforts were made to reduce the number of animals used and minimize their suffering.

3. Results

3.1. Univariate results

As to mean occurrences, ES and HD scores are illustrated in Fig. 2A,B for all groups. Nicotine induced a diverging effect in the two sexes, with decreases in males and increases in females. Two-Way ANOVA (drugs, sex) showed: significant differences for ES ($F = 8.93$; $p < 0.01$; $\eta^2 = 0.271$), with a significant interaction of factors ($F = 6.02$; $p < 0.05$; $\eta^2 = 0.201$) and HD ($F = 8.81$; $p < 0.01$; $\eta^2 = 0.268$), with a significant interaction of factors ($F = 4.95$; $p < 0.05$; $\eta^2 = 0.191$. Newman-Keuls post-hoc test, utilized to perform multiple comparisons among all groups, highlighted significant differences ($p < 0.05$) between male

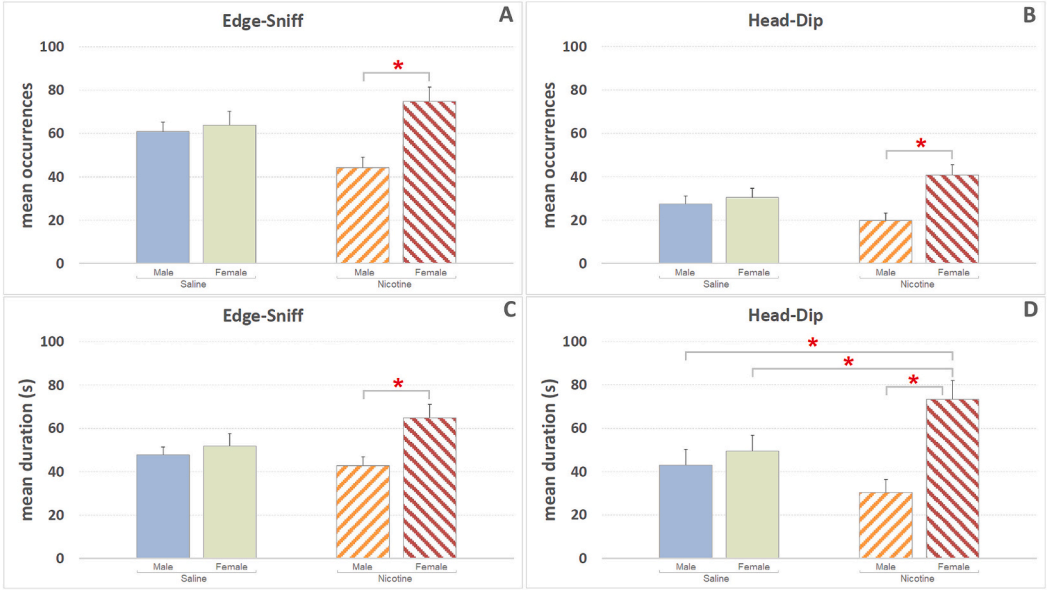

Fig. 2. Mean Occurrences (A,B) and mean durations (C,D) of Edge-Sniff and Head-Dip for all groups. * = significant ($p < 0.05$) differences between groups as assessed by 2-way ANOVA followed by the Newman-Keuls post-hoc test for multiple comparisons. The data were obtained from the analysis of 28 rats.

nicotine Vs female nicotine groups both for ES (Fig. 2 A) and HD (Fig. 2B) values. Supplementary online Fig. S01A presents mean occurrences of all the components of the behavioral repertoire. Except for ES and HD, two-way ANOVA revealed no additional statistical significance for any of the other components of the behavioral repertoire.

As to mean durations, ES and HD values are illustrated in Fig. 2C,D for all groups. Similarly to what was observed regarding average frequencies, also for durations, a divergent effect of chronic nicotine is observed in the two sexes. Two-Way ANOVA showed: significant differences for ES ($F = 6.68$; $p < 0.05$; $\eta^2 = 0.218$), with no significant interaction of factors and HD ($F = 11.26$; $p < 0.005$; $\eta^2 = 0.319$), with a significant interaction of factors ($F = 6.08$; $p < 0.05$; $\eta^2 = 0.202$). Newman-Keuls post-hoc test utilized to perform multiple comparisons among all groups showed significant differences ($p < 0.05$) between male Vs female rats treated with nicotine for ES (Fig. 2C) and nicotine-treated female rats Vs all the remaining groups for HD (Fig. 2D).

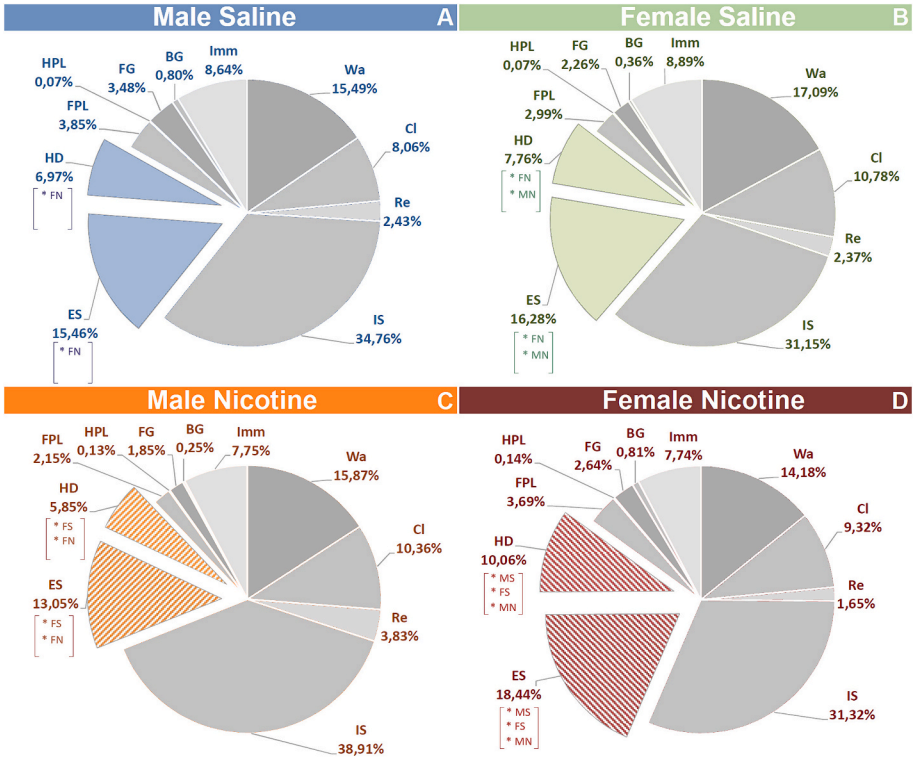

Fig. 3. Percent distributions of all the components of the behavioral repertoire for all groups (A-D). Acronyms within square brackets indicate significant ($p < 0.05$) differences compared to the indicated group, as assessed by means of Chi-square test: MS = male saline, FS = female saline, MN = male nicotine, FN = female nicotine. For abbreviations see Fig. 1. The data were obtained from the analysis of 28 rats.

Supplementary online Fig. S01B presents mean durations of all the components of the behavioral repertoire; except ES and HD (see above), two-way ANOVA revealed significant results only for IS component ($F = 41.97$; $p < 0.0001$; $\eta^2 = 0.636$), with no significant interaction of factors and Newman-Keuls highlighted significant differences between male groups Vs both female groups.

Finally, percent distributions of all the behavioral components of the behavioral repertoire are illustrated in Fig. 3 for all groups. Chi-square test, utilized to perform multiple comparisons among all groups, showed significant differences between female saline Vs female nicotine for both ES ($p < 0.05$) and HD ($p < 0.005$) and male nicotine Vs female nicotine for both ES ($p < 0.0001$) and HD ($p < 0.0001$).

3.2. T-patterns

Fig. 4 illustrates the distribution of all the different T-patterns detected on the basis of their length. Male rats treated with saline showed 51 different T-patterns: Length(L) 2 (i.e., encompassing only 2 events in structure) = 13, L3 = 17, L4 = 10, L5 = 11 (Fig. 4 A); female rats treated with saline presented 122 T-patterns of different composition: L2 = 18, L3 = 19, L4 = 20, L5 = 22, L6 = 18, L7 = 6, L8 = 8, L9 = 5, L10 = 5, L11 = 1 (Fig. 4B); male rats treated with nicotine showed 40 different T-patterns: L2 = 14, L3 = 13, L4 = 7, L5 = 4, L6 = 2 (Fig. 4C); finally, as to female rats treated with nicotine, 39 different T-patterns have been observed: L2 = 16, L3 = 14, L4 = 6, L5 = 2, L6 = 1 (Fig. 4D).

Mean occurrences and mean length of T-patterns are illustrated in Fig. 5 for all groups. Kruskal-Wallis test showed highly significant differences for both mean occurrences ($H_{(df3)} = 33.16$; $p < 0.0001$) and mean length ($H_{(df3)} = 46.35$; $p < 0.0001$); Mann-Whitney post-hoc test for multiple comparisons highlighted significant differences ($p < 0.05$) of female rats treated with nicotine Vs all the remaining groups for mean occurrences (Fig. 5 A) and female saline Vs all the remaining groups for mean length (Fig. 5B). Supplementary online Fig. S02 presents the results of the comprehensive T-pattern detection process.

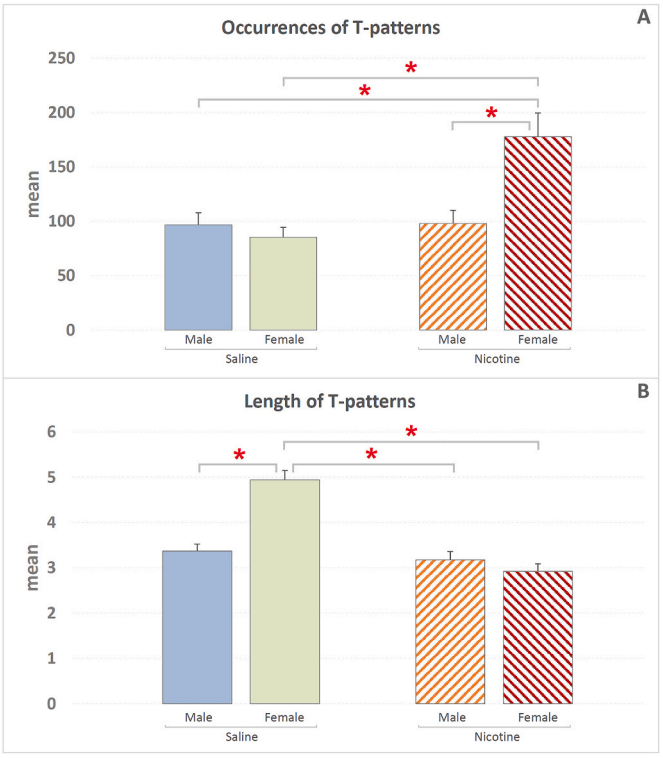

Fig. 5. Mean occurrences (A) and mean length (B) of T-patterns detected in all groups. * = significant ($p < 0.05$) differences between groups as assessed by 2-way ANOVA followed by the Newman-Keuls post-hoc test for multiple comparisons. The data were obtained from the analysis of 28 rats.

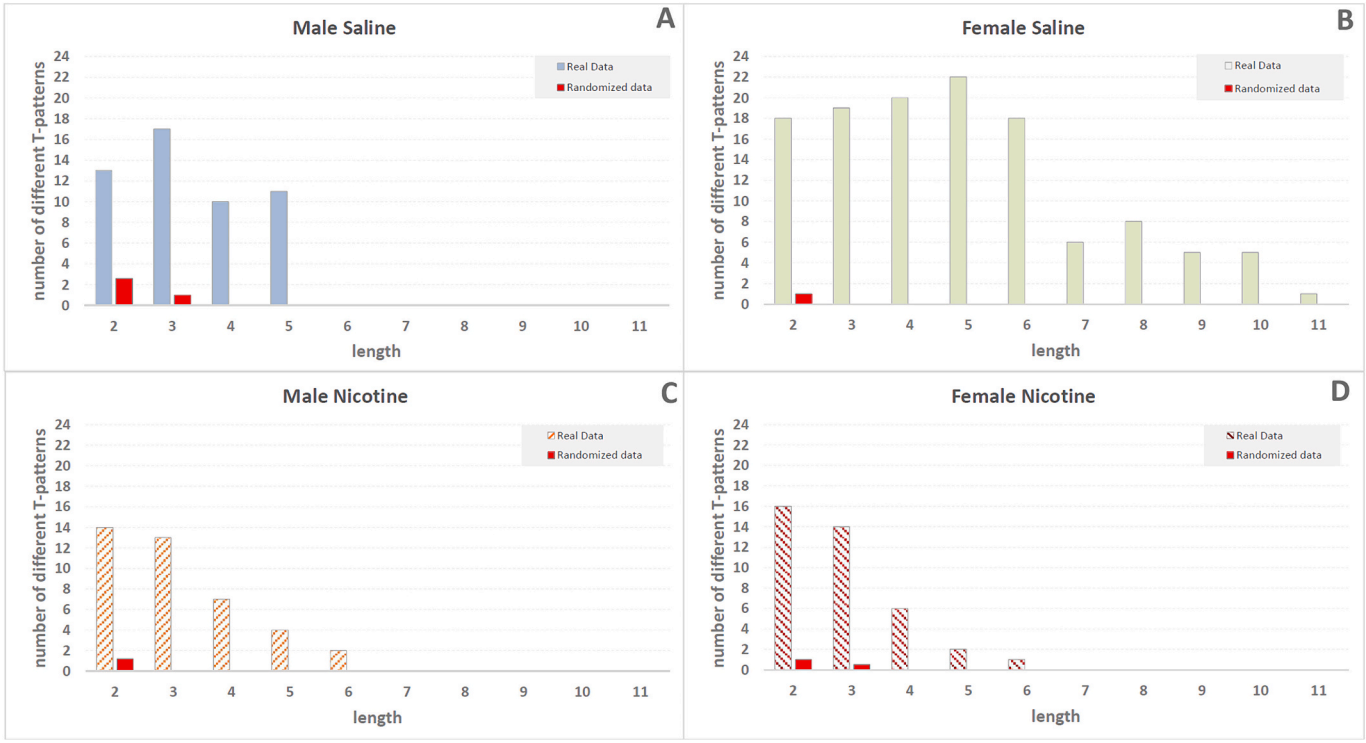

Fig. 4. Distribution of T-patterns based on their length for all groups (A-D). X-Axis = number of events in sequence, Y-axis = number of different T-patterns detected. For each group the numbers of T-patterns detected in real data and in randomly generated data are indicated. The data were data obtained from the analysis of 28 rats.

Percent distributions of T-patterns encompassing sequences of ES ➔ HD, HD ➔ ES or HD only, ES only and T-patterns containing all the remaining events (i.e. except ES and HD) are illustrated in Fig. 6. Chi-square test showed significant ($p < 0.001$) differences between all groups, except ES between male nicotine and female saline, and HD ➔ ES between female saline and female nicotine.

4. Discussion

The behavioral effects induced in adolescent rats by chronic nicotine were notably sex-dependent when assessed in male and female rats at P60, considered young/adult animals (Sudakov et al., 2021). Specifically, we observed an increase of anxiety in males and a reduction in females. This aligns with the evidence suggesting that female rats are more sensitive to the rewarding effects of nicotine (Xue et al., 2020) and long-term cognitive impairment (Abela et al., 2023). These aspects place adolescent females at a higher risk for nicotine dependence and negative consequences in comparison with adolescent males. On the other hand, the decrease in anxiety that we observed in females could be interpreted as indicative of the female brain's resilience to nicotine exposure during vulnerable periods of adolescent neurodevelopment, as recently demonstrated by Laviolette and colleagues (Ng et al., 2024).

4.1. Univariate results

Compelling evidence has highlighted the usefulness of the Hole-Board as a suitable and reliable tool to study anxiety and anxiety-related behavior in rodents (Casarrubea et al., 2009a, 2009b, 2015a, 2017, 2020, 2021a, 2023). By evaluating Edge-Sniff (ES) and Head-Dip (HD), it is possible to assess the subject's anxiety-related behavior and, importantly, the effects that anxiolytic substances such as Diazepam (Casarrubea et al., 2009b) or anxiogenic substances such as FG7142 (Casarrubea et al., 2017) produce. ES and HD can be considered the "*raison d'être*" of a Hole-Board that, conversely, would be identical to an Open-Field. The evaluation of ES and HD is therefore of considerable importance when analyzing rodents' behavior in Hole-Board. All results show definite and highly significant differences between male and female and, importantly, a clear interaction between the factors. These differences mainly occurred in the nicotine-treated groups (Fig. 2 and additional online Fig. S01). It is easy to notice that nicotine induces opposite effects: in male rats, both in occurrences and durations, provoking ES and HD decrease; on the other hand, in female rats, nicotine induces a clear-cut and significant increase in ES and HD. Such a different effect in nicotine-treated male rats and female rats compared with the respective saline-treated groups is very easily captured when looking directly at the bars of the two nicotine-treated groups. Notably, with regard to the duration of HD, the effect in female rats is so pronounced as to place the entire group in a significantly different position not only toward nicotine-treated males, but also toward all the others (Fig. 2D). Therefore, HD and ES, in female rats under nicotine but not in male rats, suggest an anxiolytic-related behavior. Regarding all the remaining components of the behavior, only the duration of IS shows noteworthy variations (additional online Fig. S01). IS, i.e. the behavior of sniffing the environment from a fixed position, is an important element of generalized exploration. Indeed, analyses conducted through probabilistic evaluations of transitions between different components of the behavioral repertoire, in both Open-Field and Hole-Board, have revealed that IS receives and sends transitions to and from all the remaining components of the behavioral repertoire (Casarrubea et al., 2008, 2009a). Consistently, it has been proposed that this behavioral component "*could be considered the behavioral expression of an ongoing selection process that, on the basis of incoming inputs, carries out a switching activity toward motor outputs*" (Casarrubea et al., 2008). In short, IS has literally a "central" role in the structure of behavior of rats in Open-Field

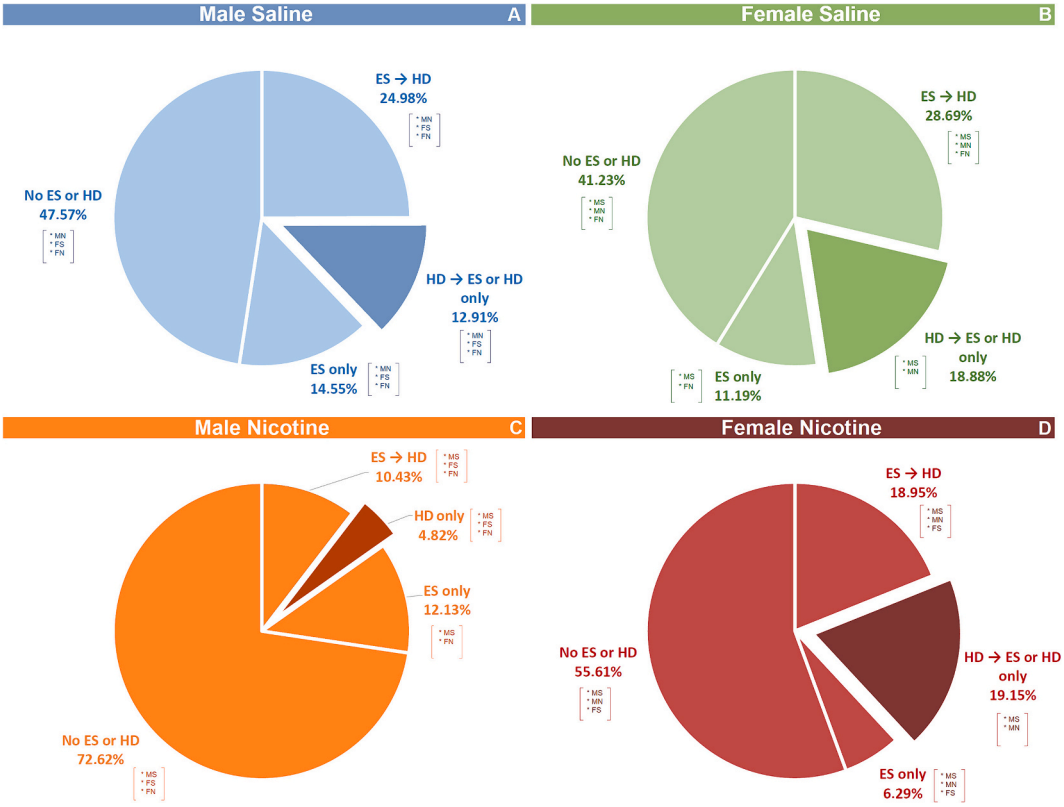

Fig. 6. Percent distributions of T-patterns containing ES ➔ HD events, HD ➔ ES or HD only, ES events only and all the remaining events except HD or ES, in all groups (A-D). Acronyms within square brackets indicate significant ($p < 0.05$) differences with the indicated group, as assessed by means of Chi-square test: MS = male saline, FS = female saline, MN = male nicotine, FN = female nicotine. For abbreviations see Fig. 1. The data were obtained from the analysis of 28 rats.

and Hole-Board (Casarrubea et al., 2008, 2009a). This peculiar difference between males and females in IS duration might be interpreted as the expression, in the two sexes, of a different exploratory strategy completely independent from the drug treatment. The percent distribution of the different behaviors (Fig. 3) poses further topic of discussion because it underscores divergent effects of chronic nicotine: reduction for HD and ES in males and a significant increase in females. Although these data do not allow a clear conclusion in this sense, following chronic nicotine it is possible to hypothesize, in females but not in males, a behavior indicative of a reduction of the anxiety level.

4.2. T-patterns and the structure of behavior in the Hole-Board

As mentioned in methods section, *variability* refers to the number of T-patterns of different composition detected, *complexity* to the length of T-patterns, namely, the number of events in sequence; finally, the *recursivity* indicates the number of times each T-pattern does occur (Casarrubea et al., 2019a, 2019b, 2021a). These features are strongly different between the two sexes, not only in the saline-treated groups but also as a result of nicotine. Evaluation of T-pattern composition sheds light on these variations by showing a clear effect of chronic nicotine in the two sexes, and in female rats a conspicuous increase of anxiolytic effect, not present in male rats.

4.2.1. Variability, complexity and recursivity of detected patterns

The variability, i.e., the number of different T-patterns according to their length highlights that the group that differs from the others is the saline-treated females (Fig. 4). Thus, the behavior of females, chronically treated with saline, is far more variable and articulated than that of males. This finding calls for a twofold reflection motivated, on the one hand, by the "horizontal" comparison with saline males and, on the other hand, by the "vertical" comparison with the effects that nicotine carries out in females. As to the comparison between male saline and female saline (Fig. 4 A,B), well over four decades ago different works highlighted considerable differences between male and female rats in exploration and anxiety-related behavior (e.g. see Archer, 1975; Beatty, 1979). In an interesting study Fernandes et al. (1999), by using factor analysis, clearly showed in the Hole-Board and Elevated Plus Maze, that the behavior of female rats was primarily characterized by activity, while male rats were more driven by anxiety. Consistently, the Authors concluded that detected outcomes much probably "*reflect sex differences in behavioral strategies and response priorities*" (Fernandes et al., 1999). Regarding the comparison between nicotine-treated female groups (Fig. 4B,D), it is readily observed that the alkaloid brings the variability back to the levels of the other groups. The effect of nicotine, therefore, seems to bring the behavior of female subjects back to the levels of their male counterparts. This is also quite evident when looking at the length of the T-patterns (Fig. 5B): *the complexity*, i.e., the number of sequential events in the T-patterns, shows that under saline consistent differences in the basic behavior of the two sexes in the Hole-Board are present; on the other hand, nicotine produces an important response only in females, where the complexity of the T-patterns is greatly reduced. Finally, further evaluation of the results points out that, in females, nicotine possesses a surprising effect with regard to the repetitiveness of T-patterns, which is greatly increased (Fig. 5A): female rats, following chronic nicotine, show a reduction in the length of T-patterns but characterized by much higher occurrences. The behavior of females under chronic nicotine is, therefore, centered on greater *recursiveness*, i.e. a greater repetitiveness of T-patterns detected.

In summary, our results suggest that both in terms of the quantitative data and the general characteristics of the T-patterns, chronic administration of nicotine at high dose has relatively few effects on adolescent male subjects and, conversely, appreciable effects only when administered to adolescent female subjects. Evaluating the composition of the T-patterns in terms of focused exploration events (i.e., HD and ES) sheds further light on the behavioral changes observable following chronic nicotine in the two sexes.

4.2.2. HD and ES in the T-patterns' structure

The focused exploratory activity of the rat in the Hole-Board, namely the exploration of the holes is a surprisingly complex activity even characterized by a microstructure encompassing behavior aimed at the exploration of the edge of the holes and of the hole inside (Casarrubea et al., 2010). On this subject, ES and HD are not just two behavioral components unconnected from the remaining behavioral context. As Fig. 1 clearly suggests, ES and HD, which characterize the rat's behavior in the Hole-Board, are part of a much larger behavioral repertoire and are reciprocally linked by motivational constraints of considerable magnitude. In other words, the ground holes of an Hole-Board do represent "objects" unknown to the naïve rodent: according to the well known principle of approach-avoidance conflict, rodents first do sniff the edge of the ground holes (i.e., Edge-Sniff, ES) and in a second step they insert the head inside the hole (i.e., Head-Dip, HD). It goes without saying, if a rodent does so, it depends exclusively on the motivation, in turn hardwired in subject's emotional assets and anxiety level (Casarrubea et al., 2023). Consistently, ES ➔ HD and HD ➔ ES sequences have profoundly different ethological implication since they are fueled by different motivational and emotional processes. In male rats, all sequences containing events of hole exploration are significantly reduced following chronic nicotine: it is present an evident reduction in all T-patterns containing sequences ES➔HD (indicative of cautious exploration) and HD➔ES (indicative of exploration less bound by the constraints of anxiety); notably, in male rats under nicotine a clear cut reduction of T-patterns containing HD alone is also present; it is therefore possible to conclude that young adult males, treated during adolescence with nicotine, develop significantly more anxious behavior than males treated with saline; in contrast, in females there is an opposite situation since the rates of ES➔HD are reduced but the rates of HD➔ES remain unchanged; it indicates that female young adults, treated during adolescence with nicotine, develop far less anxious behavior than female subjects treated with saline. These results are consistent with previous observations highlighting significant differences in exploration behavior and anxiety-related behavior of male and female rats evaluated in both the Hole-Board and the Elevated Plus Maze (Fernandes et al., 1999); interestingly, female rats during adolescence are far more sensitive to the anxiolytic effects of nicotine than male rats, as clearly indicated by Cheeta and Colleagues utilizing the Social Interaction Test (Cheeta et al., 2001a).

Therefore, by evaluating the composition of T-patterns in terms of HD and ES, it has been possible to conclude that nicotine has a very complex effect on behavior and that this effect is realized through a multifaceted behavioral reorganization clearly marked by an anxiety increase in males and anxiety reduction in females. On the one hand, this evidence reinforces the univariate data since it shows that nicotine has an effect not only on individual components of behavior such as HD and ES, but also on entire sequences, i.e., T-patterns containing these focused exploration events; on the other hand, the "difficulties" our data highlight in observing a clear effect in males but not in females, may be expression of the much greater sensitivity of only adolescent females to the anxiolytic effects of nicotine. This hypothesis is supported by the evidence that in female rats is present a much greater sensitivity to anxiolytic effects of nicotine and that even 5 times smaller doses of nicotine in females are enough to induce effects similar to those observed in males with much higher doses (Cheeta et al., 2001a).

4.3. A synoptic view

On the basis of our results, obtained from quantitative analyses and TPA, nicotine chronically administered during adolescence produces opposite anxiety-related behavior, i.e., less anxiety-prone in females, but not in males. The effect, such as the one we observed in males (completely undetectable with common quantitative univariate

methods), *well documented only using an approach such as TPA,* i.e., *a multivariate approach able to describe even subtle variations involving the structure of behavior rather than variations in individual components*, may be one of the reasons why there are so many discrepancies in experimental observations with nicotine. A further analysis of the results gives raise to two questions: first, what could be the explanation behind these results? Second, but not less important, what might be a translational implication of this?

As to the first question, it has been suggested that it is unlikely that the sex differences in response to nicotine in adolescent rats may orbit directly around hormonal differences (Cheeta et al., 2001a); it is much more likely that the differences come from neural changes occurring in different brain structures during the adolescence (Cheeta et al., 2001a). In this context, the complex dynamics of nicotine in modulating the release of numerous neurotransmitters such as dopamine, serotonin or glutamate, at various CNS sites (Pierucci et al., 2004, 2014; Di Matteo et al., 2007, 2010; Di Giovanni, 2012; Lester, 2014), could underlie the observed effects. In particular, the anxiety-related behavioral effects of nicotine may be due to the release of serotonin, which in turn acts on 5-HT1a receptors (Cheeta et al., 2000) at the level of the raphe nuclei and limbic system (Kenny et al., 2000; Cheeta et al., 2001b); moreover, there is also a clear involvement of a cholinergic receptor subtype namely the β2*nAChR. Indeed, it has been demonstrated that 5I-A85380, a selective agonist of this receptor, induces effects very similar to those of nicotine (Anderson and Brunzell, 2015).

As clearly indicated by the National Institute on Drug Abuse (NIDA, 2021), in the USA, male subjects do present a higher prevalence of tobacco usage compared to females (Higgins et al., 2015). Importantly, neuroimaging research findings indicate that smoking triggers stronger activation in the reward circuits of male's brains compared to female's, suggesting that male subjects may smoke for the gratifying effects of nicotine, while women may do so to manage mood (NIDA, 2021). Unfortunately, the situation is similarly serious also during the adolescence (NYTS, 2023). Our results and others (Xue et al., 2020; Ng et al., 2024) are in agreement with what has been described in humans. Indeed, in humans, if on the one hand males often highlight the rewarding effects of cigarettes when they try to explain reasons for smoking, on the other hand females mention the anxiety-reducing effects (Perkins et al., 1999; Crisp et al., 1999); consistently, it is very well known that human females do present a noticeably higher prevalence of neuro-psychiatric affective conditions such depression and anxiety-related disorders (Altemus et al., 2014; Holden, 2005; Kessler et al., 1994; Seeman, 1997). Of course, this does not imply that the hedonistic and/or social aspect related to tobacco smoking is not present in females, but that the reduction of anxiety state is the major component that induces females to initiate and continue nicotine intake through tobacco smoking or other means. If this is true, interventions aimed at strongly discourage the use of cigarette and tobacco, should at the very least be diversified between males and females and aimed at eliminating the different underlying factors. Of course, taking into consideration the complex and multifaceted effects of nicotine in terms of anxiety and anxiety-related behavior, extreme cautiousness is advisable.

4.4. Limitations of the study

Our study has some limitations. For instance, nicotine was not self-infused by the rats but instead administered by experimenters. In addition, we only utilized a single dose of nicotine, despite the well-known dose-dependent nature of nicotine's effects (Casarrubea et al., 2015a, 2020; Picciotto et al., 2002). Concerning strain, the present study employed Black-Hooded rats; while it is conceivable that similar results may be observed in other strains, given the significant variability in nicotine's behavioral effects, it would be highly beneficial to assess the effects of the alkaloid in male and female subjects belonging to other strains as well. Furthermore, within the context of different strains, it would be particularly intriguing to examine the effects of nicotine in both sexes of a rat strain known for its heightened anxious temperament, namely the Dark-Agouti, a strain previously utilized in the study of anxiety-related behavior in the Elevated Plus Maze (Casarrubea et al., 2013b). Regarding this matter, potential behavioral differences in adolescent rats of both sexes under chronic nicotine could have been evaluated also in additional anxiety tests, such as the Social Interaction Test and/or the Elevated Plus Maze.

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Declaration of generative AI in scientific writing

Generative artificial intelligence (AI) and AI-assisted technologies were not used in the writing process.

Ethical statement

Procedures involving animals and their care were conducted in accordance with European Law (EU Directive 2010/63/EU) and the Institutional Animal Use and Care Committee (IAUCC) at the University of Malta. All efforts were made to reduce the number of animals used and minimize their suffering.

CRediT authorship contribution statement

Maurizio Casarrubea: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization, Supervision. **Giuseppe Crescimanno**: Writing – review & editing, Conceptualization, Supervision. **Stefania Aiello**: Writing – review & editing, Investigation. **Daniel Cassar**: Investigation. **Zachary Busuttil**: Investigation. **Fabiana Faulisi**: Investigation. **Antonio Iacono**: Investigation. **Giuseppe Di Giovanni**: Writing – review & editing, Conceptualization, Supervision.

Declaration of competing interest

All the Authors of the above-mentioned manuscript, submitted to Progress in Neuropsychopharmacology & Biological Psychiatry on February 6th 2024, declare no conflict of interest.

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