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Excessive habit formation in schedule-induced polydipsia: Microstructural analysis of licking among rat strains and involvement of the orbitofrontal cortex

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Schedule-induced polydipsia (SIP) is an animal model of compulsive drinking that selects for individual differences and varies across rat strains. The aim of this study was to investigate excessive habit formation by analyzing the SIP licking microstructure among rat strains, and to compare the brain areas activated by SIP in different populations. Wistar, Long Evans and Roman High- and Low-Avoidance rat strains were compared using a cluster analysis of 2 main variables, that is, frequency of licking (percentage of interpellet intervals with drinking episodes) and intensity of licking (mean number of licks per interpellet interval), and were found to exhibit high intensity and frequent licking (compulsive drinkers, CD), low intensity but frequent licking (habitual drinkers, HD), and low intensity and low-frequency licking (low drinkers, LD). The Wistar strain showed a higher frequency and intensity of licking, and had the largest group of CD rats when compared with the other strains. Regarding the acquisition of SIP, CD rats showed a higher intensity of licking when compared with the HD and LD rats. Moreover, c-Fos quantification revealed that rats in the CD group showed hyperactivity in the lateral orbitofrontal cortex and basolateral amygdala when compared with the LD group. Analyzing the SIP microstructure could be a valuable tool for understanding the role of excessive habit formation in the development of compulsive drinking and its underpinning neurobiological mechanisms.

KEYWORDS
amygdala, compulsivity, habit formation, individual differences, inhibitory control, licking, orbitofrontal cortex, Roman high- and low-avoidance rats, schedule-induced polydipsia, strain comparison

1 | INTRODUCTION

Compulsivity is characterized by the persistence of activities that become disconnected from the prevailing environmental contingencies, and lack an obvious relationship to the overall goal of the activity.[1] This symptom is present in different psychiatric disorders, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder, hoarding disorder, hair-pulling disorder and skin-picking disorder, which comprise the Obsessive-Compulsive and Related Disorders cluster in the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-V[2]). Growing evidence from human and animal research suggests that alterations in neurocognitive mechanisms mediating behavioral inhibition (motor inhibition, cognitive inflexibility), and habit formation (shift from goal-directed to stimulus-driven habits) may contribute to a vulnerability to compulsive behavior in a variety of disorders.[3]

Schedule-induced polydipsia (SIP) is a phenomenon characterized by the development of nonregulated excessive drinking in food-deprived animals exposed to intermittent food-reinforcement schedules.[4,5] Owing to its characteristics of "excessiveness" and "persistence," SIP has been proposed as a useful model to study neuropsychiatric disorders characterized by the presence of compulsive behavior.[6–12] In our laboratory, Wistar (WIST) rats are commonly used for SIP as they show important individual differences between high drinker (HD) and low

drinker (LD) phenotypes, according to a median split. Relative to their LD counterparts, HD WIST rats show behavioral inflexibility in a spatial reversal learning task[13] and a lack of inhibitory control in the 5-choice serial reaction time (5-CSRT) task,[14] which has been associated with changes in monoaminergic systems in the limbic-striatal circuitry.[12,14,15] Compared with WIST rats, Lister hooded (LH) rats do not acquire SIP drinking,[16] and show greater inhibitory control, as measured by the low-anticipatory responses in the 3-CSRT task and lower food hoarding behavior.[17] In addition, the selective breeding of Roman high-avoidance (RHA) and Roman low-avoidance (RLA) rats for rapid vs poor acquisition of active avoidance behavior in the shuttle box test resulted in 2 phenotypes that present with differences in SIP acquisition.[18] RHA rats, which show traits such as higher novelty seeking, susceptibility to addictive drugs, and impulsive behaviors in the delay-discounting task and 5-CSRT task,[18–20] display increased SIP acquisition when compared with RLA rats. Other strains, such as the spontaneously hypertensive rats (SHR) that are characterized as hyperactive and impulsive in terms of their exacerbated sensitivity to delayed reinforcement, display increased SIP drinking when compared with Wistar-Kyoto (WKY) control rats,[11,21] as well as 2 rat lines selectively bred for high-ethanol preference compared with their control counterparts.[8] In addition, different lines of inbred Fisher 344 and Lewis rats also exhibit different levels of SIP.[22] Therefore, high or excessive SIP drinking seems to be sensitive to inhibitory control deficits among different phenotypes and strains of rats.

So far, research on individual differences in SIP has mainly focused on the overall number of licks and water intake in stable sessions of SIP in select phenotypes.[6,12] However, recent studies have pointed toward the importance of analyzing the pattern of licking using microstructural measures to better identify deficits in inhibitory control.[23,24] For instance, SHR rats display similar licking rates in SIP under fixed time 60 seconds (FT-60 seconds) and 30 seconds schedules of reinforcement when compared with WIST rats, even although SHR rats show poorer performance in a delay-discounting task.[21] When comparing microstructural measures in SIP between SHR and WIST rats, SHR rats show an increased proportion of trials with drinking bouts and a higher number of drinking bouts of shorter duration per drinking episode, indicating a hyperactive-like pattern of licking.[24] Similarly, SIP microstructural measures could be useful tools to model aspects of compulsivity. From a theoretical perspective, deficits in goal-directed control and associated overreliance on habits seem to play a key role in compulsivity across different psychiatric disorders.[25] Hence, the study of the frequency and intensity of licking could clarify the nature of compulsive drinking in SIP in terms of excessive habit formation.

The largest body of work investigating the role of habit in human compulsivity has been conducted in OCD.[26] There is broad consensus that OCD is characterized by abnormalities in a particular brain network, the cortico-striato-thalamo-cortical circuitry, which involves areas of the motor, associative and limbic cortices.[27,28] Previous studies on SIP have found hyperactivity in the lateral and ventral lateral orbitofrontal cortex (OFC), and medial prefrontal cortex (mPFC) in compulsive drinking rats, whereas areas of the caudate-putamen do not show significant differences between groups.[15,29] Among the brain structures located in the limbic circuit, the amygdala may be of particular interest because of its role in anxiety and its strong integration within the cortico-striatal system.[30] However, no previous research has evaluated the neuronal activation of the amygdala during SIP compulsive drinking.

We hypothesized that the rat strains showing poorer inhibitory control (as is known from the literature) such as WIST and RHA, would exhibit specific strain-dependent patterns of licking in the microstructural SIP measures recorded, and that the rats classified by the cluster analysis as the most excessive in terms of the frequency and intensity of their licking would have altered neuronal activation in brain areas related to compulsivity, such as regions of the OFC and amygdala. To test these hypotheses, we first explored the microstructural pattern of licking following SIP in different rat strains that have not been compared previously, such as WIST, Long Evans (LE), RHA and RLA rats. The microstructural measures that better characterized the strain-dependent differences in drinking rates, that is, the frequency and intensity of licking, were used to cluster rats into high frequency and high intensity (compulsive drinkers, CD), high frequency and low intensity (habitual drinkers, HD) and low frequency and low intensity (low drinkers, LD) drinking groups. We then calculated the percentages of rats from each strain classified as CD, HD and LD to identify the strain that is most vulnerable to compulsive behavior. Once this first study had been concluded, we analyzed the acquisition of SIP microstructural measures in the WIST strain, which showed the highest percentage of CD rats, to identify vulnerabilities in the development of frequent and intense licking in terms of excessive habit formation, and compared neuronal activity in different brain areas related to compulsivity during SIP in CD, HD and LD rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male rats from 4 strains, weighting 290 to 400 g at the beginning of the experiment, were housed 3 to 4/cage (57 × 35 × 20 cm) at 22°C on an 08:00 to 20:00 dark-light cycle (lights off at 08:00 hours), with food and water available *ad libitum*. The first experiment compared the SIP microstructure in 40 WIST and 15 LE rats obtained from Janvier Labs (France), and 20 RHA and 21 RLA inbred rats from colonies established in 1997[19,31] at the Autonomous University of Barcelona (Spain). The second experiment used WIST rats from 2 companies, 40 rats from Janvier (France) and 48 rats from Charles River (Barcelona, Spain). Before the SIP training, and after 10 days of habituation to the vivarium conditions, the rats were weighed and handled daily. They were gradually reduced to 85% of their free-feeding body-weight by controlled feeding and then maintained at this level of deprivation throughout the experiment. Food was made available by daily feeding with lab chow approximately 30 minutes after each experimental session. Water was always available in the home cages. All testing was performed between 9:00 and 15:00 hours. Two WIST rats were not included in the statistical analysis owing to the inadequacy of their drinking behavior (spilling water from the water bottle during the SIP sessions), as well as 6 WIST rats that were classified by the cluster analysis as outliers. Therefore, the total sample was 91 rats in Experiment 1 and 80 rats in Experiment 2.

All procedures were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, with the European Community Council Directives (2010/63/EU), and with the University of Almería Animal Research Committee approval.

2.2 | Schedule-induced polydipsia

2.2.1 | Apparatus

We conducted the tests in 10 standard operant conditioning chambers (MED Associates, Inc., Cibertec, Madrid, Spain) that were 32 cm long × 25 cm wide × 34 cm high, with stainless steel grid floors. A detailed description of the apparatus for SIP has been provided previously.[18,32] The scheduling and recording of experimental events were controlled by a Med PC IV computer and commercial software (Cibertec SA, Madrid, Spain).

2.2.2 | Baseline water consumption

All rats were individually housed in plastic cages containing a dish with the same amount of food as that delivered in the experimental sessions and the same water bottle used in the operant chambers. Over 2 successive days, 60 food pellets were placed together in a dish and the amount of water consumed by each rat in 60 minutes was measured.

2.2.3 | SIP procedure

First, the animals were habituated on day 1 to the test chambers for 60 minutes, and were given 30 food pellets placed in the food magazine. After magazine habituation, the rats were exposed to an FT-60 seconds schedule of food pellet presentation during the 60 minutes sessions. Water bottles containing 100 mL of fresh water were provided immediately before each session. The SIP procedure lasted until water intake and licks were stable across 5 consecutive sessions. RHA, RLA and LE rats underwent 14 sessions of SIP, whereas WIST rats completed 20 sessions of SIP.

2.2.4 | SIP measures

The measures registered for each rat could be divided into Traditional Measures, which are those previously used in our laboratory ([13,16,33,34]; for review see[6,14]) and Microstructural Measures, which are those obtained from the exact temporal time that each lick was performed for during the session. Traditional Measures incorporated the following: (1) total number of licks, (2) water intake (total volume of water removed from the bottle) and (3) total number of magazine entries.

To analyze the SIP microstructure we counted *drinking episodes*, defined as the number of licks (with a minimum of 5 licks) in which the time between licks was no longer than 500 milliseconds. The criterion of 500 milliseconds has been previously used for clustering licks.[35] The microstructural measures were as follows:

1. Frequency of licking: percentage of interpellet intervals with drinking episodes.
2. Intensity of licking: mean number of licks per interpellet interval.
3. Number of episodes: mean number of drinking episodes per interpellet interval.
4. Latency to licking: mean time spent from the delivery of the pellet to the first drinking episode.

2.2.5 | Classification

Previous studies from our laboratory classified rats as high drinkers and low drinkers if their average water intake was above or below the group median, respectively.[6,14] However, dichotomizing a continuous variable via the median split might lead to type II error and a reduction in effect size and power.[36] For this reason, in this paper we have classified rats according to the frequency and intensity of their licking from the average of the 3 last sessions of SIP using hierarchical clustering (Ward's method) and K-means clustering approaches.

2.3 | Brain analyses: c-Fos immunohistochemistry

The animals were deeply anesthetized 60 minutes after a 60-minute SIP session using intraperitoneal injection of sodium pentobarbital (100 mg/kg), and they were then perfused transcardially with 0.1 M phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were rapidly removed and maintained in the same fixative at 4°C. Coronal free-floating sections were cut at 50 μm on a sliding microtome and stored in cryoprotectant (ethylene glycol and glycerol) at −20°C. The slices were rinsed 3 times in PBS, incubated for 30 minutes in 0.3% H_2O_2 in absolute methanol to quench endogenous peroxidase, rinsed 3 times in PBS, and incubated for 1 hour in 3% goat serum in PBS. The slices were then transferred, without rinsing, to the primary antibody solution, which consisted of a 1:10 000 dilution of c-Fos polyclonal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes residues 3 to 16 of the c-Fos protein. After a 36-hour incubation at 4°C, the slices were rinsed 10 times in PBS and processed using the avidin-biotin complex (Elite ABC kit) method (Vectastain, Vector Labs, PALEX MEDICAL, S.A., Barcelona, Spain). Briefly, the slices were transferred to a solution containing biotinylated anti-rabbit IgG for 1 hour, rinsed 3 times in PBS, transferred to avidin-biotin peroxidase for 1 hour, rinsed 3 times in PBS for 30 minutes, then rinsed 3 times in PB for 30 minutes, and developed with nickel-intensified diaminobenzidine substrate (6 minutes) (DAB Peroxidase substrate kit, Vector labs, PALEX MEDICAL, S.A.). The sections were mounted on gelatin-coated slides, air dried, dehydrated with ethanol, cleared in xylene and coverslipped with DPX mounting medium. Prefrontal sections were examined with a light microscope (Nikon E400) equipped with a digital camera (Nikon Coolpix 4500). Amygdalar sections were examined under a Nikon Multizoom AZ-100 microscope and images were acquired with a Nikon DS-5 M digital camera using the Nis-Elements imaging software (Nikon Corporation, Minato, Tokyo, Japan), both at ×10 magnification. c-Fos positive nuclei were measured in the following brain areas: ventral orbitofrontal cortex (VOFC: bregma +4.2 and + 3.2 mm), lateral orbitofrontal cortex (LOFC: bregma +4.2 and + 3.2 mm), prelimbic cortex (PrL: bregma +4.2, +3.2 and + 2.2 mm), infralimbic cortex (IL: bregma +3.2 and + 2.2 mm), basolateral amygdala (BLA: bregma −2.3 and − 3.14 mm) and central amygdala (CA: bregma −2.3 and − 3.14 mm). Counting was performed by an individual who was blind to the experimental condition of the animals. The nuclei were quantified in both brain hemispheres using the image analysis computerized system ImageJ 1.34 seconds (National Institutes of Health (NIH), Bethesda, Maryland). Before counting, images were set to a threshold on a standardized gray-values scale level empirically determined to

allow for the detection of stained cells from low to high density, with suppression of very lightly stained nuclei. Since the immunochemistry procedure was performed on different days, differences in signals were observed. Therefore, the number of c-Fos-positive cells divided by area (mm^2) was transformed into *z*-scores to take into account the fact that each day was counterbalanced, and we compared differences among clusters.

2.4 | Statistical analysis

The classification of rats was performed through a combination of hierarchical and nonhierarchical methods, as recommended by current theoretical trends.[37] First, a hierarchical analysis was performed using Ward's method (squared Euclidean distance measure); second, cluster membership was determined through a nonhierarchical K-means analysis. One potential way to decide the number of clusters is to plot the number of clusters on the *x*-axis against the distance at which objects or clusters are combined on the y-axis. Using this plot, we then searched for a distinctive break (elbow) where the squared Euclidean distance among clusters remained smaller, indicating the appropriate number of clusters. Alternatively, the dendrogram plots were used to visually identify the same information.[38]

SIP measures were analyzed using a one-way ANOVA for Experiment 1, with Group (WIST, LE, RHA and RLA) as the between-subject factor. A repeated measures ANOVA was used for the data collected in Experiment 2, with Group (CD, HD and LD) as the between-subject factor, and Sessions as the within-subject factor. We performed a one-way ANOVA to evaluate the *z*-scores for c-Fos positive cells/area (mm^2) with Group (CD, HD and LD) as the between-subject factor. A Pearson's correlation analysis was used to assess the possible relationship between microstructural SIP measures and the *z*-scores for c-Fos positive cells/area (mm^2) for different brain areas. Where appropriate, post hoc comparisons were made using Tukey's test. All analyses were computed using the Statistical Package for the Social Sciences (SPSS) 22.0 software package. Differences with $P<.05$ were deemed statistically significant, and trending toward significance was defined as $P < .08$.

3 | RESULTS

3.1 | Experiment 1: strain-dependent SIP and phenotype differences determined by cluster analysis

First, we analyzed differences among WIST, LE, RHA and RLA rats using traditional SIP measures and microstructural SIP measures. The average (mean ± SEM) water intake for each of the groups at baseline was as follows: 2.95 ± 0.25 mL, 2.73 ± 0.28 mL, 2.73 ± 0.23 mL and 3.68 ±0.26 mL for the WIST, LE, RHA and RLA rats, respectively. A one-way ANOVA of baseline water consumption indicated a trend toward significance among strains ($F_{3,87} = 2.523$; $P = 0.063$), but post hoc comparisons did not indicated statistically significant differences between the strains. Strain effects were found for the total number of licks ($F_{3,87} = 7.119$; $P < .001$) and water intake ($F_{3,87} = 7.199$; $P < .001$), but not for magazine entries ($F_{3,87} = 1.210$; $P = .311$) (Table 1). The post hoc analysis showed that WIST rats had a greater total number of licks and water intake than RHA ($P < .05$ and $P < .01$, respectively) or RLA ($P < .001$ and $P < .001$, respectively) rats. WIST rats were not significantly different with regard to their total number of licks ($P = .520$) when compared with LE rats, despite showing more water intake than LE rats ($P < .05$). RHA and RLA rats did not differ with regard to their total number of licks ($P = .478$) or water intake ($P = .972$). LE rats had a similar total number of licks and water intake to RHA ($P = .733$ and $P = .999$, respectively) and RLA ($P = .090$ and $P = .953$, respectively) rats.

Regarding the microstructural measures, a one-way ANOVA revealed strain differences in the frequency of licking (Figure 1A; $F_{3,87} = 7.029$; $P < .001$), intensity of licking (Figure 1B; $F_{3,87} = 15.503$; $P < .001$), and mean number of drinking episodes (Figure 1D; $F_{3,87} = 3.455$; $P < .05$). The latency to licking was not significantly different among strains (Figure 1C; $F_{3,87} = 1.117$; $P = .323$). The post hoc analysis revealed that the frequency of licking was greater in WIST and RHA rats compared with LE and RLA rats. WIST rats showed an increased frequency of licking when compared with LE ($P < .05$) and RLA ($P < .01$) rats. In addition, RHA rats showed an increased frequency of licking when compared with LE ($P < .05$) and RLA ($P < .01$) rats. WIST and RHA rats did not differ regarding the frequency of licking ($P = 1.000$). On the other hand, the WIST and LE groups had a higher intensity of licking compared with the RHA ($P < .001$ for both) and RLA ($P < .001$ for both) groups. The WIST and LE groups showed similar intensity of licking ($P = .975$), as well as RHA and RLA ($P = .997$). A one-way ANOVA revealed a strain effect with regard to the mean number of drinking episodes; however, Tukey's post hoc comparisons did not show statistically significant differences between the strains.

We clustered rats according to the frequency and intensity of their licking, which represented the best SIP microstructural measures characterizing drinking among rat strains. Figure 2A,B show the rescale squared Euclidean distance plotted against the number of clusters and the difference among the distances to identify the appropriate number of clusters for the mixed population of rat strains. The elbow, where the squared Euclidean distance among clusters remained smaller, is observed from cluster 3 onward (see Figure 2B), showing that the appropriate number of clusters was 3. In addition, the representation of the 3 clusters can be seen in a dendrogram in Figure 2C.

TABLE 1 Water intake (ml), total number of licks, and magazine entries for the average of the last 3 SIP sessions in WIST ($n = 40$), LE ($n = 15$), RHA ($n = 20$), and RLA ($n = 21$) rats

	WIST	LE	RHA	RLA
Water intake (ml)	14.2 ± 1.7*a*	7.8 ± 1.3*b*	7.6 ± 0.9*b*	6.6 ± 0.4*b*
Total licks	2561.5 ± 346.0*a*	1916.4 ± 396.8*ab*	1380.9 ± 250.9*b*	695.8 ± 73.4*b*
Magazine entries	2143.2 ± 195.1	1777.5 ± 169.9	2369.0 ± 233.6	2473.7 ± 326.1

Mean ± SEM values with different letters (a, b) are significantly different ($P < .05$).

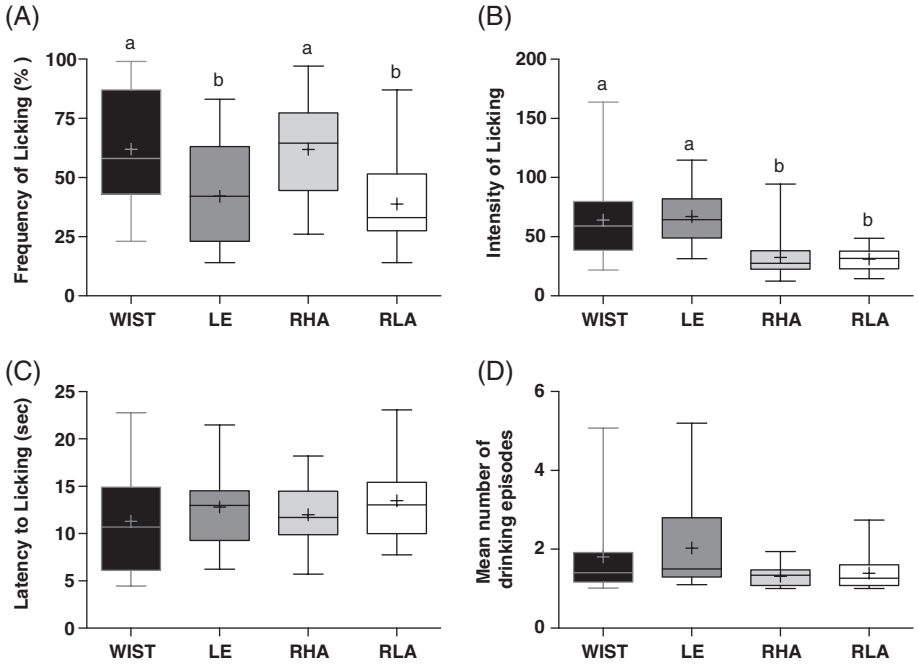

FIGURE 1 SIP microstructure among rat strains. Boxplots of (A) frequency of licking, (B) intensity of licking, (C) latency to licking, and (D) mean number of drinking episodes from the average of the last 3 SIP sessions in: WIST (n = 40), LE (n = 15), RHA (n = 20), and RLA (n = 21) rats. For each boxplot, the central line indicates the median; box and whiskers represent the interquartile range and range, respectively; + symbol indicates the mean values. Boxplots with different letters (a, b) are significantly different (P < .05)

A 2-dimensional representation of the frequency and intensity of licking for a mixed population of rat strains is shown in Figure 3A. The cluster with a low frequency and low intensity of licking were termed LD rats, and represented 45 out of 91 rats (49% of the total sample). The centroid of LD was 32.4 ± 1.5 for frequency and 45.8 ± 2.9 for the intensity of licking. The cluster with a high frequency and low intensity of licking was termed HD, and represented 33 out of 91 rats (36% of the total sample). The centroid for the HD group was 68.6 ± 2.3 for frequency and 36.0 ± 2.4 for the intensity of licking. The cluster with a high frequency and high intensity of licking was termed CD, and

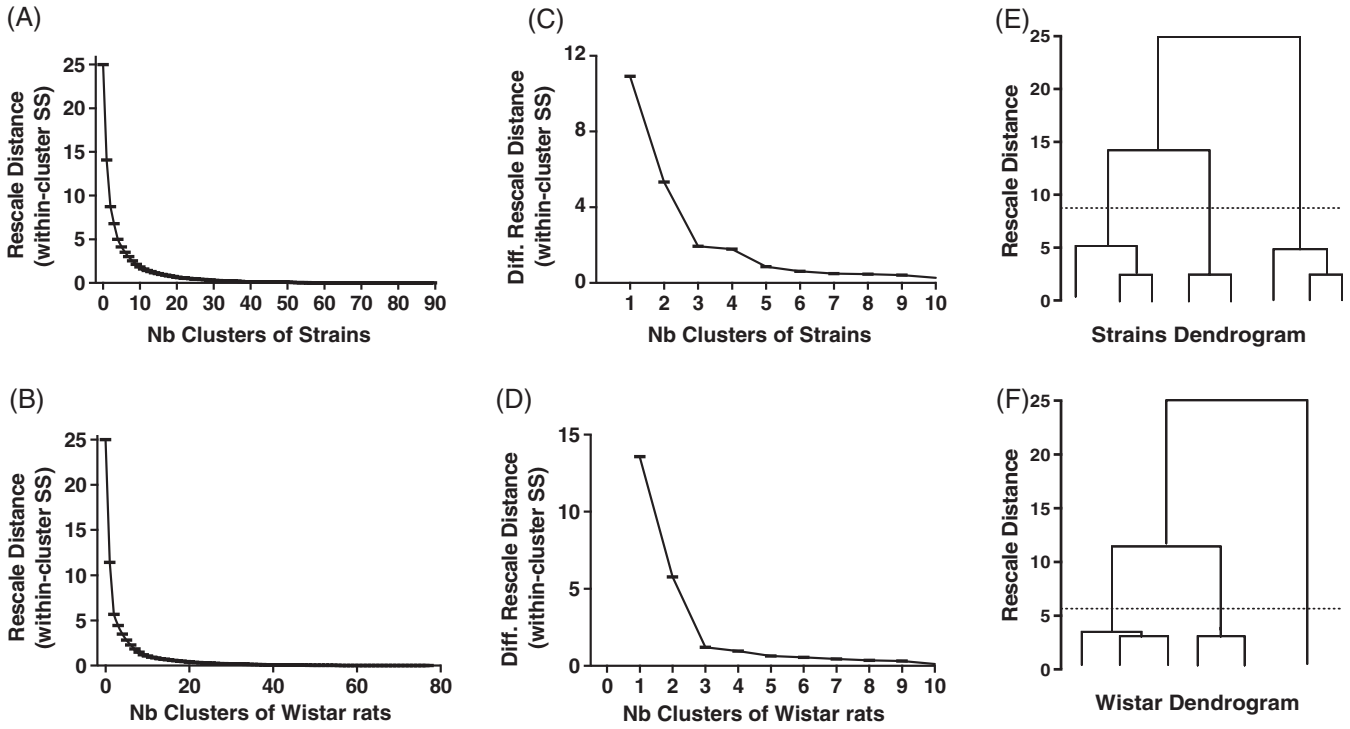

FIGURE 2 Hierarchical clustering approach to determine the appropriate number of clusters according to the frequency and intensity of licking using Ward's criterion. Squared Euclidean distance plotted against the number of clusters (A) for a mixed population of strains (WIST, LE, RHA, and RLA, n = 91), and (B) for Wistar rats only (n = 80), differential distance between the interesting range of number of clusters (C) for a mixed population of strains, and (D) for Wistar rats only. Dendrogram for the application of agglomerative hierarchical clustering using Ward's criterion (E) for a mixed population of strains, and (F) for Wistar rats only

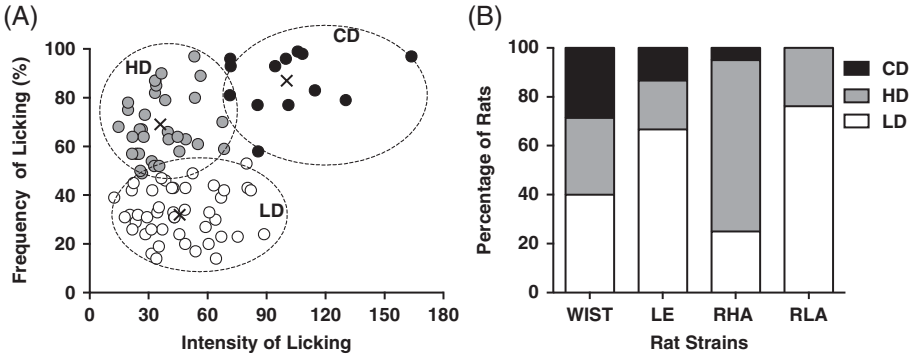

FIGURE 3 Clusters of a mixed population of strains and their percentages from each strain. (A) Two-dimensional representation of the frequency and intensity of licking shows the classification of each individual as CD (n = 13), HD (n = 33), and LD (n = 45) using a K-means clustering approach, and (B) the percentage of rats classified as CD, HD, and LD from the WIST (n = 40), LE (n = 15), RHA (n = 20), and RLA (n = 21) strains (b)

represented 13 of 91 rats (14% of the total sample). The centroid for the CD group was 86.7 ± 3.3 for frequency and 100.3 ± 7.2 for intensity.

One-way ANOVA analyses showed statistically significant differences in the total number of licks ($F_{2,88}$ = 120.39; P < .001) and water intake ($F_{2,88}$ = 82.62; P < .001), but not in magazine entries ($F_{2,88}$ = 0.18; P = .982) among clusters, in a mixed population of rat strains (data not shown). A post hoc analysis revealed that CD rats had a greater total number of licks and water intake than HD (P < .001 for both) and LD (P < .001 for both) rats, and HD rats had a greater total number of licks (P = .010) and higher water intake (P < .05) than LD rats. For the microstructural measures, cluster effects were found for the frequency of licking ($F_{2,88}$ = 155.97; P < .001), intensity of licking ($F_{2,88}$ = 57.19; P < .001), latency to licking ($F_{2,88}$ = 5.87; P < .01) and the mean number of drinking episodes ($F_{2,88}$ = 27.26; P < .001) (data not shown). Post hoc analyses revealed that, when compared with HD and LD rats, CD rats showed more frequency of licking (P < .001) and intensity of licking (P < .001), had a higher mean number of drinking episodes (P < .001), and they also showed a shorter latency to licking than HD (P < .05) and LD (P < .01) rats. HD rats showed a higher frequency of licking (P < .001) and a trend toward a significant higher intensity of licking (P = .066) than LD rats, whereas both groups were similar in terms of mean number of drinking episodes (P = .250), and the latency to licking (P = .430).

Figure 3B depicts the percentage of rats from each strain classified as CD, HD and LD. The strain with the highest representation of CD rats was the WIST group (29%), followed by the LE group (13%), whereas the RHA and RLA groups had the lowest percentage or even no CD rats (5% and 0%, respectively). HD rats were widely present in the RHA group (70%) in comparison to the WIST (31%), LE (20%) and RLA (24%) groups. LD rats were predominantly found in the RLA group (76%), followed by the LE (67%) and WIST (40%) groups, with the lowest percentage found in the RHA group (25%).

3.2 | Experiment 2: microstructural measures and c-Fos activity in clusters of WIST rats

The WIST populations used in this experiment were obtained from 2 different companies (see Section 2). A repeated measures ANOVA did not show significant differences between the 2 groups in the following SIP measures: water intake ($F_{4,213}$ = 0.112; P = .978), total number of licks ($F_{4,213}$ = 0.342; P = .850), number of magazine entries ($F_{4,213}$ = 0.324; P = .862), intensity of licking ($F_{4,213}$ = 1.578; P = .180), latency to licking ($F_{4,213}$ = 1.879; P = .114), and number of drinking episodes ($F_{4,213}$ = 0.656; P = .594). A trend toward significance was obtained in frequency of licking ($F_{4,213}$ = 2.270; P = .062). However, post hoc analysis did not show significant differences between rats from both companies from session 13 onward (data not shown).

Similar to the results of the cluster analyses in Experiment 1, the elbow where the squared Euclidean distance among clusters remained smaller was observed from cluster 3 onward in the WIST rats (see Figure 2B,D). A cross-validation of the 3 SIP clusters can be observed in Experiment 2 because performing the same cluster analyses with a different population of rats showed a similar optimal number of clusters. Figure 2F shows the dendrogram for Experiment 2, with a population of only WIST rats. A 2-dimensional representation of the frequency and intensity of licking for the WIST rats is shown in Figure 4. The LD cluster represented 36% of the population (29 of 80 rats), and the centroid was 35.0 ± 2.4 for the frequency and 38.6 ± 3.1 for the intensity of licking. The HD cluster represented 38% of the population (30 of 80 rats), and the centroid was

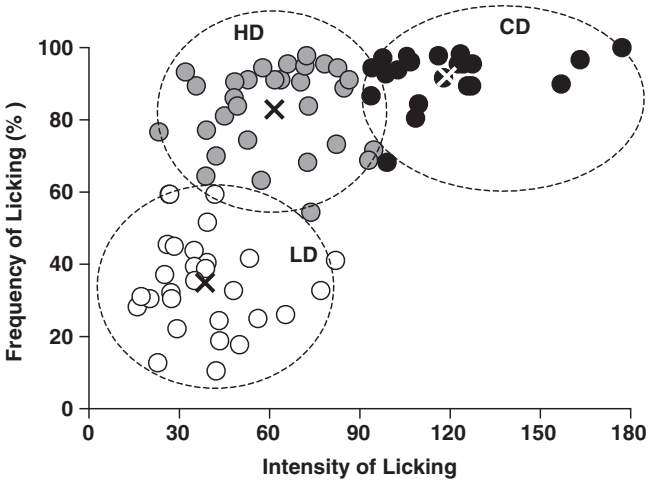

FIGURE 4 Clusters of Wistar rats. Two-dimensional representation of the frequency and intensity of licking shows the classification of each individual from the Wistar strain as CD (n = 29), HD (n = 30), and LD (n = 29) using a K-means clustering approach

TABLE 2 Water intake (ml), total number of licks, and number of magazine entries for the average 1-4, 5-8, 9-12, 13-16, and 17-20 SIP sessions in CD (n = 21), HD (n = 30), and LD (n = 29) Wistar rats

	Water intake (ml)			Total licks			Magazine entries		
Session	LD	HD	CD	LD	HD	CD	LD	HD	CD
1-4	3.5 ± 0.6	3.5 ±0.4	7.2 ± 1.1	414 ± 95	565 ± 87	1357 ± 283	1632 ± 136	1753 ± 142	1548 ± 217
5-8	5.7 ± 1.1	9.2 ± 0.9	17.1 ± 2*	837 ± 190	1847 ± 201#	3866 ± 392*	2016 ± 164	2111 ± 151	1982 ± 177
9-12	7.4 ± 1.1	12.8 ± 1#	23.1 ± 1.9*	1047 ± 172	2557 ± 218#	5410 ± 407*	2171 ± 171	2151 ± 188	2090 ± 191
13-16	8.3 ± 1.2	14.6 ± 1#	26.6 ± 1.7*	1181 ± 147	2914 ± 222#	5909 ± 306*	2190 ± 185	2072 ± 195	2209 ± 238
17-20	7.8 ± 1.1	16.1 ± 0.9#	27.9 ± 1.7*	989 ± 112	3061 ± 185#	6469 ± 256*	2283 ±220	1996 ± 193	2122 ± 233

Data are shown as mean ± SEM. *Statistically significant differences between the CD and HD groups (P < .001). #Statistically significant differences between the HD and LD groups (P < .05).

82.9 ± 2.1 for the frequency and 61.7 ± 3.4 for the intensity of licking. The CD cluster represented 26% of the population (21 of 80 rats), and the centroid was 92.0 ± 1.6 for the frequency and 119.0 ± 5.0 for the intensity of licking.

With regard to water baseline consumption, CD, HD and LD rats drank 2.90 ± 0.24 mL, 2.72 ± 0.21 mL and 2.81 ± 0.27 mL, respectively. No statistically significant differences were found among clusters ($F_{2,77}$ = 0.129; P = .880) for water baseline consumption. When analyzing the total number of licks, a repeated measures ANOVA revealed an interaction of cluster × session ($F_{8,308}$ = 41.031; P < .001), a main effect of session ($F_{4,308}$ = 206.794; P < .001) and a main effect of cluster ($F_{2,77}$ = 97.689; P < .001) (Table 2). A post hoc analysis indicated that CD rats had the highest total number of licks compared with HD and LD rats from sessions 5 to 8 onward (P < .001 for both), followed by HD compared with LD rats (P < .05). LD rats did not show any increase in the total number of licks, whereas the total number of licks performed by the CD and HD rats stabilized in sessions 13 to 16 (P = .344) and 9 to 12 (P = .225), respectively. With regard to water intake, an interaction of cluster × session ($F_{8,308}$ = 25.562; P < .001), a main effect of session ($F_{4,308}$ = 199.385; P < .001) and a main effect of cluster ($F_{2,77}$ = 42.950; P < .001) were also found (Table 2). Similarly, a post hoc analysis revealed that CD rats drank the highest volume compared with HD and LD rats from sessions 5 to 8 onward (P < .001 for both), whereas HD rats differed from LD rats and showed an increase in water intake during sessions 9 to 12 onward (P < .05). Water intake in the CD and HD rats stabilized during the acquisition phase in sessions 13 to 16 (P = .992 and P = .902, respectively), while water intake in the LD rats stabilized in sessions 5 to 8 (P = .446) following a slight increase in water intake. There were no statistically significant differences in magazine entries among the clusters (Table 2; session × cluster, $F_{8.308}$ = 1.137; P = .338).

Concerning the frequency of licking, a repeated measures ANOVA revealed a cluster × session interaction ($F_{8,308}$ = 19.277; P < .001), a main effect of session ($F_{4,308}$ = 245.101; P < .001), and a main effect of cluster ($F_{2,77}$ = 73.613; P < .001) (Figure 5A). A post hoc analysis showed that LD rats had a lower frequency of licking than CD (from sessions 1-4; P < .01) and HD (from sessions 5-8; P < .001) rats. Despite the fact that CD rats generally showed a higher frequency of licking compared with HD rats based on the post hoc comparisons among clusters (P < .01), we only found trends toward significance in sessions 1 to 4 (P = .062) and sessions 5 to 8 (P = .070), while the rest of the sessions did not show significantly different results. In the comparison among different sessions, the frequency of licking stabilized in sessions 9 to 12 for both the CD and HD rats (P = .963 and P = .172, respectively), while the LD rats showed earlier stabilization in sessions 5 to 8 (P = .129).

With regard to the intensity of licking, we found a cluster × session interaction ($F_{8,308}$ = 21.437; P < .001), a main effect of session ($F_{4,308}$ = 111.229; P < .001), and a main effect of cluster ($F_{2,77}$ = 69.663; P < .001) (Figure 5B). A post hoc analysis revealed that CD rats showed a greater licking intensity compared with HD (from sessions 5-8; P < .001) and LD (from sessions 1-4; P < .05) rats. HD rats showed a greater intensity of licking when compared with LD rats in sessions 17 to 20 (P < .01). In the comparison among different sessions, the intensity of licking increased slightly in the HD and LD rats, and this stabilized in sessions 9 to 12 (P = .986) and 5 to 8 (P = 1.000), respectively. The intensity of licking increased in the CD rats from 46.64 ± 3.84 to 118.77 ± 4.0 (average licks per interpellet interval) from sessions 1-4 to 17-20, respectively, reaching stable levels in sessions 13 to 16 (P = .191).

A statistically significant cluster × session interaction ($F_{8,308}$ = 5.033; P < .001), a main effect of session ($F_{4,308}$ = 252.673; P < .001), and a main effect of cluster ($F_{2,77}$ = 19.317; P < .001) were found for the latency to licking (Figure 5C). A post hoc analysis revealed that LD rats showed a longer latency to licking after obtaining the pellet than the CD (from session 5 to 8; P < .001) and HD (from sessions 9 to 12; P < .05) rats. Comparisons among the clusters indicated that CD rats had a generally lower latency to licking than HD rats (P < .001). However, there were no statistically significant differences in any of the sessions between the CD and HD rats according to a Tukey's post hoc analysis. CD, HD and LD rat groups showed a similar stabilization of their latency to licking in sessions 9 to 12 (P = .982, P = .175 and P = .662, respectively).

With regard to the mean number of drinking episodes, we found a cluster × session interaction ($F_{8,308}$ = 8.308; P < .001), a main effect of session ($F_{4,308}$ = 12.318; P < .001), and a main effect of cluster ($F_{2,77}$ = 9.207; P < .001) (Figure 5D). CD rats showed a greater mean number of drinking episodes per interpellet interval compared with HD and LD rats from sessions 13 to 16 onward (P < .001 for both), and this increment remained stable (P = .731). HD and LD rats did not differ in any session, nor did they show an increase in the number of drinking episodes per session.

Figure 6 shows the z-scores for c-Fos positive cells/area (mm^2) in different brain areas in CD, HD and LD rats. One-way ANOVA analyses identified cluster effects in the LOFC (Figure 6A; $F_{2,11}$ = 4.559; P < .05), and a trend toward significance in the BLA (Figure 6E; $F_{2,10}$ = 3.424; P = .074), whereas no statistically significant effects

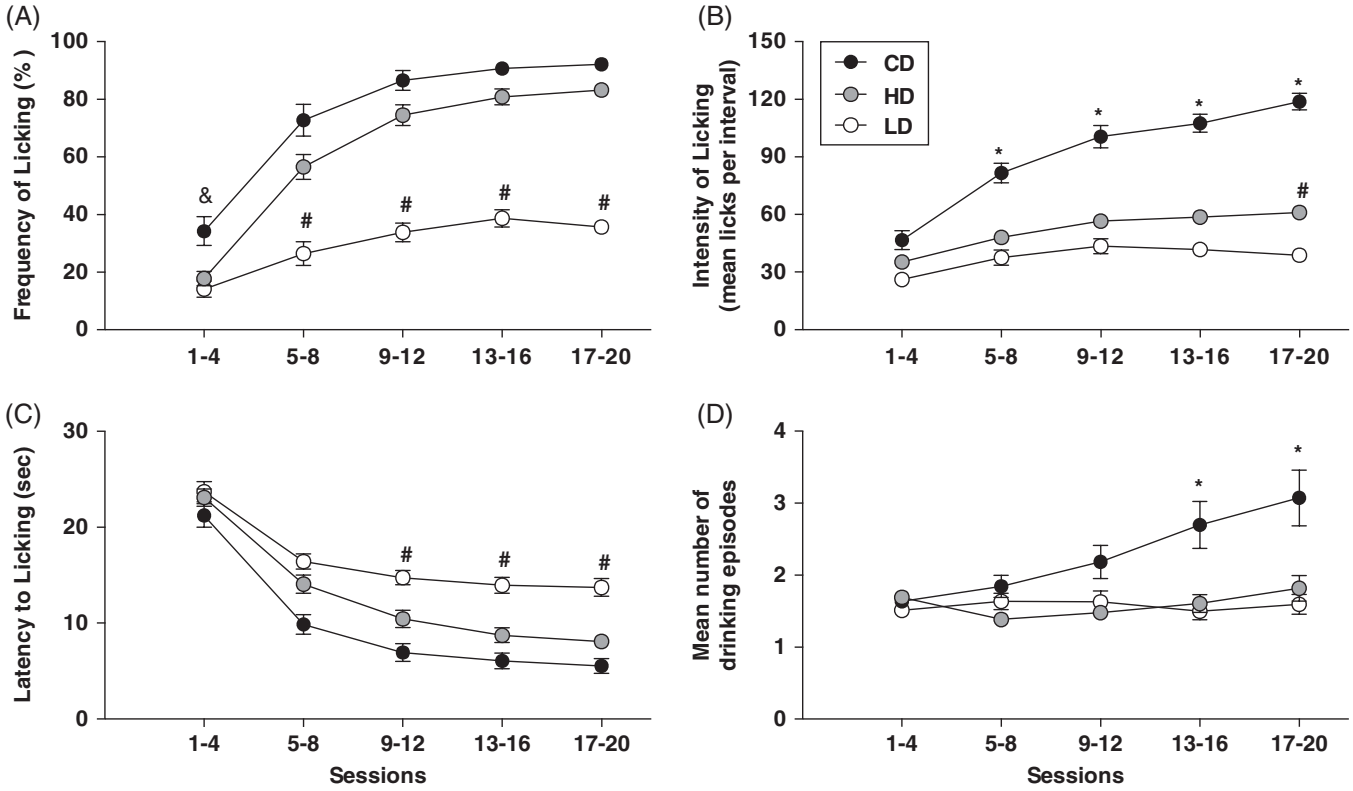

FIGURE 5 SIP microstructure in clusters of Wistar rats. (A) Frequency of licking, (B) intensity of licking, (C) latency to licking, and (D) mean number of drinking episodes of the average 1-4, 5-8, 9-12, 13-16, and 17-20 SIP sessions in CD (n = 29), HD (n = 30), and LD (n = 29). Data are shown as mean ±SEM. *Statistically significant differences between the CD and HD groups (P < .05). #statistically significant differences between the HD and LD groups (P < .05). &Trend toward a significant difference in the CD compared with the HD group (P = .062)

were found in the VOFC (Figure 6B; $F_{2,11}$ = 2.280; P = .149), PrL (Figure 6C; $F_{2,11}$ = 2.956; P = .094), IL (Figure 6D; $F_{2,11}$ = 0.198; P = .823), or CA (Figure 6F; $F_{2,10}$ = 0.357; P = .715). A post hoc analysis revealed that CD rats had more activity in the LOFC (P < .05) and higher activity in the BLA that showed a trend toward significance (P = .064) compared with LD rats. No statistically significant differences were found between the CD and HD rats for the LOFC (P = .975) or BLA (P = .680), or between the HD and LD rats for the LOFC (P = .099) or BLA (P = .341).

A Pearson's correlation analysis identified a strong positive correlation between the LOFC z-scores and the frequency of licking (r = 0.648, P < .05, n = 14), and a moderate positive correlation was found between the PrL z-scores and the frequency of licking (r = 543, P < .05, n = 14). The correlation between the z-scores for the BLA and frequency of licking did not reach statistical significance (r = 0.495, P = .085, n = 13). Similarly, we found moderate negative correlations between latency to licking and the LOFC z-scores (r = −0.573, P < .05, n = 14), and the PrL z-scores (r = −582, P < .05, n = 14). The intensity of licking and mean number of drinking episodes were not significantly correlated with any of the brain areas.

4 | DISCUSSION

The results of the present study show that rat strains with phenotypic differences display specific patterns of licking, as indicated by SIP microstructural measures. The microstructural measures allowed us to differentiate the rat populations more precisely according to their SIP habitual and excessive drinking, which were modeled by the frequency and intensity of licking, respectively. The populations obtained by cluster analyses showed high intensity and frequent licking (CD), low intensity but frequent licking (HD), and low intensity and low-frequency licking (LD). The WIST strain showed a higher frequency and intensity of licking and had the most CD rats when compared with the LE, RHA and RLA groups, indicating that this strain is the most suitable for studying compulsive behavior. The high intensity of licking was the behavior that best distinguished CD rats among clusters that displayed similar habitual or frequent licking, such as the HD and CD groups. Furthermore, CD rats presented hyperactivity in the lateral OFC, and a trend toward significance was found for higher activity in the BLA.

This is the first experiment to compare SIP in different strains of rats, pigmented LE, albino WIST, RHA and RLA rats, which are commonly used in experimental psychology studies. Pigmented strains, such as LE and LH rats, are widely used for cognitive tasks, whereas albino rats, such Sprague-Dawley and WIST rats, are most frequently used for behavioral pharmacology experiments and as models of psychopathology.[39,40] In general, pigmented rats show increased ambulatory activity and reduced habituation to a novel environment in the open field test when compared with albino rats.[17,40–42] However, some studies have specifically evaluated differences between LE and WIST rats in different paradigms, and showed that LE rats display increased locomotor activity in the open-field test, less anxiety, as

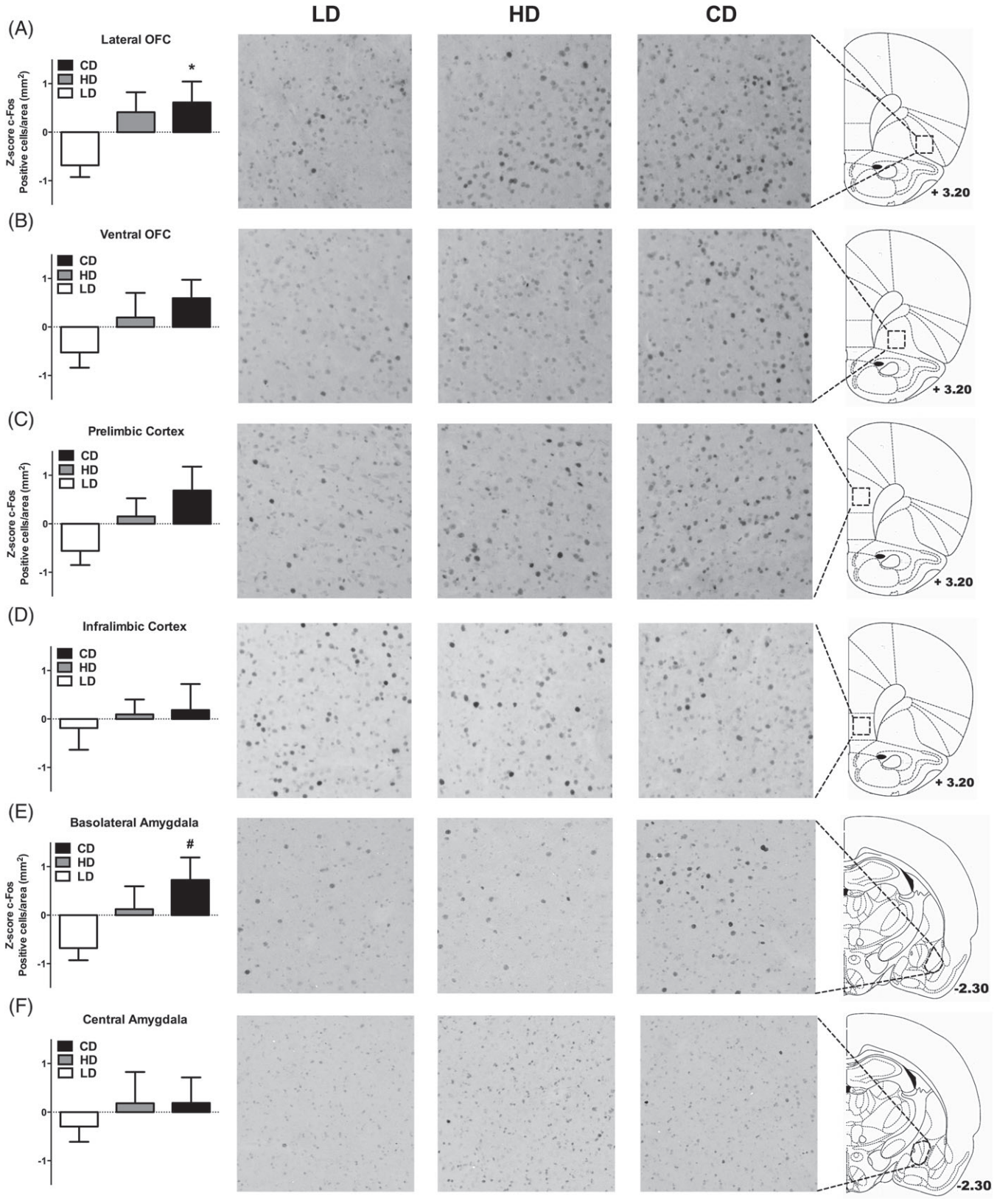

FIGURE 6 Neuronal activity in clusters of Wistar rats. C-Fos positive cells/area (mm^2) in CD (n = 4), HD (n = 4) and LD (n = 5-6) in the following brain areas: (A) lateral orbitofrontal cortex, (B) ventral orbitofrontal cortex, (C) prelimbic cortex, (D) infralimbic cortex, (E) basolateral amygdala and (F) central amygdala. The drawings were adapted from the rat brain atlas of Paxinos and Watson (1998)[79]. The rostral distances (in mm) to bregma were indicated by numbers. Data were transformed into z-scores. *Statistically significant difference between the CD and LD groups ($P < .05$). #Trend toward a significant difference in the CD compared with the LD group ($P = .064$)

revealed through an increase in the amount of time spent in the open arms of the plus maze,[42] and a lower acoustic startle response amplitude[43] than WIST rats. Regarding learning and memory, LE and WIST rats show similar performances with regard to lever pressing in

an auto-shaping task, in the Morris Water Maze,[44] and in a visual discrimination touch-screen task,[45] even although LE rats are faster learners. In the 5-CSRT task, the accuracy and number of premature responses does not differ among LE, WIST,[46] and Sprague-Dawley rats.[39] Even although the 5-CSRT task does not detect inhibitory control deficits between LE and WIST rats with regard to premature and perseverative responses, more recent studies have found poorer cognitive flexibility in a visual discrimination reversal task in WIST compared with LE rats,[45] and increased perseverative swimming in a probe trial of the Morris Water Maze in which the platform is removed and the animals can display a preference for quadrants over 60 seconds.[47] Taken together, these findings suggest that WIST rats are less active and more anxious and inflexible when compared with LE rats, indicating a possible compulsive phenotype.

On the other hand, the RHA and RLA strains are well-characterized phenotypes that show rapid vs extremely poor acquisition of active avoidance behavior in a shuttle-box test with different emotional and motivational profiles (for reviews see[19,48–51]). RLA rats show increased anxiety or fear in different paradigms, a passive coping style, and increased stress responses, as indicated by hypothalamus-pituitary-adrenal (HPA)-axis activation.[52,53] RHA rats tend to be novelty/sensation seekers and show relatively low HPA-axis activation,[31,48] while they do show differences in their vulnerability to drug addiction.[20,50] Moreover, RHA rats display higher impulsive choice in a delay-discounting task and increased premature responses in the 5-CSRT task when compared with RLA rats,[18,54] indicating less inhibitory control and an impulsive phenotype.

Previous studies have shown that pigmented LH rats have lower or no acquisition of SIP drinking compared with WIST rats,[16,55] although LE rats have never been compared with WIST rats with regard to SIP before. In this study, we have seen that the WIST strain drank a greater volume than did the other strains, despite showing a similar total number of licks as compared with the LE rats. This discrepancy between the number of licks and water intake has been documented previously and analyzed in terms of drinking efficiency.[16] In this case, it seems that LE rats have a less efficient pattern of drinking compared with WIST rats, because they require more licks to obtain the same volume, which could be another strain-dependent difference in the pattern of licking. The greater total number of licks and water intake observed in WIST rats can be explained by the fact that this strain was the only one to show both a high frequency and high intensity of licking, whereas LE, RHA and RLA rats did not show differences in traditional variables, instead showing different specific patterns of licking. RHA rats have a more frequent but equal intensity of licking when compared with RLA rats. Additionally, LE rats displayed less drinking in traditional measures than WIST rats because of a lower frequency of licking rather than a lower intensity of licking. Surprisingly, a higher frequency of licking, which we related to the concept of habit formation, is common in the WIST and RHA strains that show phenotypes with deficits in inhibitory control.[18,20,45,47,50,54] Indeed, RHA rats had a similar pattern of licking to the impulsive and hyperactive SHR rats. SHR rats were shown by Íbias et al[23] to have an increased frequency of licking, without differences in the duration of the episodes or intensity of licking, compared with WIST and WKY rats. While the role of impulsivity in the acquisition of SIP has been previously disputed,[11] a common feature of the lack of executive control over actions is present in both impulsivity and compulsivity.[56] Thus, we can deduce that the frequency of licking in SIP predicts deficits in inhibitory control, and when accompanied by a high intensity of licking, reveals compulsivity and possible tendencies for engaging in excessive habitual behavior. In fact, the WIST strain was the only one that showed both a high intensity and frequency of licking, and these results are in accordance with previous studies showing behavioral inflexibility in other paradigms.[45,47] This strain also had the highest number of CD rats according to the cluster analysis, indicating that this strain is the most suitable for studying compulsive behavior.

In a second experiment, we used a larger number of WIST rats for the acquisition of traditional and microstructural SIP variables across CD, HD and LD rats. We also analyzed c-Fos neuronal activity in different brain areas related to compulsive behavior among these clusters. In the traditional measures, we observed that CD rats displayed greater water intake and a higher total number of licks compared with the HD and LD rats, whereas HD rats showed increased SIP compared with LD rats for both variables. When the microstructural SIP measures were examined, we found that CD and HD rats did not differ significantly in the acquisition of the frequency of licking, even although HD rats generally showed lower levels in the cluster effects analysis for both variables. The main differences between CD and HD rats can be observed in the intensity of licking, which is superior in the CD rats from sessions 5 to 8, and in the number of drinking episodes from sessions 13 to 16 onward. A previous study has shown a higher number of drinking episodes per interpellet interval in hyperactive SHR rats compared with WIST and WKY control rats, and this increment was not accompanied by the increase of licks/min or duration of licking.[23] However, CD rats presented a higher number of drinking episodes that could be due to the increase of intensity of licking. Moreover, the mean number of drinking episodes of SHR rats was superior compared with CD rats from this study.[23] Therefore, we cannot deduce that CD rats have an impulsive-hyperactive licking behavior like that seen in SHR rats.

From all this we could conclude that the high intensity of licking is what probably best distinguished the SIP compulsive drinking, and points toward the relevance of introducing microstructural measures in studies of individual differences in SIP. We hypothesize that this excessive licking may be due to a lack of inhibition to exert control over habitual processes. In fact, previous studies that classified rats by their median water intake have shown that high-drinker rats displayed resistance to extinction in the 5-CSRT task[14] and had increased lever pressing under a variable-interval 60-second schedule of reinforcement.[13] These findings might indicate an increased habit formation in rats developing high-drinking rates. However, further research should compare the performance of CD, HD and LD rats in outcome devaluation, a traditional procedure that assesses sensitivity of the response to motivational change,[25] in order to determine habitual control deficits in vulnerable populations in SIP.

With respect to neuronal activity, we found increased c-Fos expression in the LOFC of CD compared with LD rats, and a strong positive correlation between this brain area and the frequency of licking. In support of our findings, a previous study observed hyperactivity in the lateral and ventral lateral OFC in rats acquiring SIP.[29] The OFC is involved in goal-directed control over actions in healthy people.[57,58] In patients with OCD, functional imaging studies

have revealed relative metabolic hyperactivity in the OFC when performing an aversive shock avoidance task,[59] and during symptom provocation.[60–64] In the literature, the OFC, in addition to the anterior cingulate/caudal mPFC and caudate region, shows hyperactivity in patients with OCD.[25,65] Regarding SIP, the mPFC shows hyperactivity in compulsive rats as well, whereas brain areas such as the nucleus accumbens, ventral tegmental area, dorsomedial striatum, dorsolateral striatum (DLS), and CA1 region of the hippocampus do not show significant differences.[15,29] However, the involvement of the DLS in SIP should not be dismissed, because neuroadaptation induced by an increase in the spine density in DLS neurons has been observed in rats exposed to SIP compared with a control group.[66] In fact, the DLS has been implicated in actions based on the development of habits.[67,68]

Regarding the amygdalar region, a trend toward significance was found in the BLA, indicating hyperactivity in this region, in CD compared with LD rats. In support of this, previous SIP studies found hyperactivity in serotonergic and noradrenergic neurotransmission,[14] higher binding for D2 receptors[15] in the amygdala, and less myelination of the BLA[13] in HD compared with LD rats selected by the median split. Some studies elucidating the role of the amygdala in habit formation have found that intra-amygdalar infusions of dopamine or noradrenaline agonists are sufficient to bias behavior toward the use of habit-like strategies in a water-maze task.[69,70] The BLA seems to be important for the acquisition of goal-directed actions,[71] whereas the central amygdala seems to be critical for the formation of habits by its interaction with the DLS.[72] In addition, hyperactivity in the amygdala during experimental symptom provocation has been also documented in human OCD studies.[60,73–76] The aberrant LOFC and BLA hyperactivity in CD rats might support the hypothesis that compulsivity in SIP could be due to deficits in the goal-directed system rather than an excessive build-up of stimulus-response habits. Extensive anatomical connectivity exists between the OFC and BLA,[77] which work closely in the encoding and retrieval of valuable information during goal-directed actions.[78] Impaired functioning of this circuitry may induce an inability to use outcome expectancies in adapting future behavior, and therefore a lack of flexibility and perseveration.[3] Future studies on the compulsive phenotype of CD rats with regard to SIP could further identify the nature of excessive habit formation and compulsivity.

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Conflict of interest

The authors declare no conflict of interest.

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