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Evidence for a vocal signature in the rat and its reinforcing effects; a key role for the subthalamic nucleus

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Abstract

Although rodents have a well-structured vocal form of communication, like humans and non-human primates, there is, to date, no evidence for a vocal signature in the well-known 50- and 22-kHz ultrasonic vocalizations (USV) emitted by rats. Here, we show that rats can recognize the identity of the USV emitter since they choose to self-administer preferentially playback of 50-kHz USV emitted by a stranger rat over those of their cage-mate. In a second experiment, we show that only stranger, but not familiar, 50-kHz USV reduce cocaine self-administration. Finally, to study the neurobiological substrate of these processes, we have shown that subthalamic nucleus (STN)-lesioned rats did not lever press much for any USV playback, whatever their emotional valence, nor did they seem able to differentiate familiar from stranger peer. Advocating for the existence of a vocal signature in rats, these results highlight the importance of ultrasonic communication in socio-affective influence of behavior, such as the influence of proximal social factors on drug consumption and confirm the role of the STN on this influence.

Introduction

Like humans, rats are highly social animals, living in complex social groups [1,2], requiring a large panel of socio-affective skills (for review: [3]). Rats thus need to assess information on the identity of an encountered conspecific in order to engage in appropriate behavior during a second encounter with the same individual. To do so, like humans, rats process and memorize sensorial signature of individuals, such as their scent [4,5].

Their socio-affective abilities make rodent models relevant to study the socio-affective modulation of behavior. For instance, studies on drug consumption showed that the presence of a peer modulates drug intake [6], in a substance-specific way [7]. In both rats and humans, the presence of a peer decreases cocaine intake [8]. This protective effect of the peer's presence over drug consumption would result from its role as an alternative reinforcer, overcoming the rewarding value of drugs [9]. Interestingly, in both humans and rats, the relationship between the subject and the observer is important since the presence of a stranger peer decreases cocaine consumption more than the presence of a familiar one [8]. However, some questions remain to be addressed regarding the socio-affective modulation of behavior, such as the sensory modalities involved and their neurobiological basis [10].

Among sensory modalities, the ultrasonic vocalizations (USV) have important communicative functions in rats [11–13]. The USV of adult rats are classified into 2 major types relying on their acoustic features and emotional content: positive and negative USV [11]. Reflecting different communication functions, both types of USV elicit distinct biological and behavioral responses in recipient rats [11,12,14].

The negative USV are characterized by a stable sound frequency of ~22-kHz [12], known to be emitted generally in anxiogenic or aversive situations, such social defeat [15] or withdrawal from drug [16–18], and may be considered as an evolutionary equivalent of human crying [19]. These calls serve as signal for fear transmission [20]. They elicit alertness/anxiety [21] and defensive behaviors in the receiver, such as freezing, fight or behavioral inhibition [22]. Playback of 22-kHz USV induces a conditioned place aversion over a background noise, and when associated with cocaine self-administration, it also transiently increases cocaine intake [23].

On the other hand, the positive 50-kHz USV calls are characterized by a broad frequency range (from 35 to 80-kHz), high variable frequency modulations and a very short duration (10-150ms) [12]. These USV are heterogenous and can be divided into several categories from flat calls to trills with a very high level of frequency modulation [24]. Considered as an evolutionary equivalent of human laughter and

joy expression [25], 50-kHz USV are emitted in appetitive situations such as tickling by a human experimenter [26] or self-administration of psychostimulants [27]. They serve as pro-social affiliative calls [12]. Their playback induces approach behavior in recipient rat [28–30], conditioned place preference over a background noise [23] and reduced cocaine self-administration [23].

Interestingly, while there is low intra-subject variability in term of call types, acoustic parameters (i.e., duration, bandwidth, and mean peak frequency) and quantity of USV production in a same experimental context, there is a large inter-subject variability [31,32], depending on several factors, such as social status, sex and strain [33]. This inter-subject variability in USV acoustic parameters and call profile, mirroring human prosody, suggests the existence of a vocal signature in rats allowing them to recognize individual conspecifics. Nevertheless, no study to the best of our knowledge investigated the ability of rats to recognize the identity of the USV emitter. Such a finding would have an important evolutionary value as it would argue in favor of the existence of a vocal signature in rodents, already known as “voice” in non-human and human primates.

Regarding the neurobiological basis of USV perception, replay of 50-kHz USV activates cerebral structures involved in the reward circuit, such as the prefrontal cortex, nucleus accumbens, but also the thalamic parafascicular and paraventricular nuclei [34,35] and decreases the activity in the amygdala [36]. Playback of 22-kHz activates brain areas associated with fear and anxiety processing, such as perirhinal cortex, amygdala, periaqueductal grey and the antero-dorsal nucleus of the bed nucleus of the stria terminalis [14,34,37,38]. Less is known regarding the neurobiological substrate of the behavioral modulation induced by USV playback. The subthalamic nucleus (STN), a deep cerebral structure at the interface of limbic and reward circuits [39] could play a key role. Indeed, STN inactivation differentially affects motivation for cocaine and food [40,41] and modulates affective responses to emotional stimuli [42]. Moreover, STN lesion blunts the effect of 50- and 22kHz USV playback emitted by stranger rats on cocaine consumption [23]. It is thus important to understand to which extent STN is involved in discrimination of the USV emitter and the rewarding properties of these USV.

In this study, we first evaluated the ability of rats to recognize the USV emitter, testing if they could self-administer playback of 50- and 22-kHz USV recorded from a stranger or a familiar (i.e., their cage-mate) congener. Since rats self-administer 50-kHz USV emitted by strangers [43] and lab rats naturally prefer novel over familiar social stimuli [44–47], we hypothesized that they would self-administer more 50-kHz USV from the stranger rat than for familiar one, if they can discriminate them. In a second experiment, we evaluated the influence of the familiarity of the USV emitter on the receiver’s behavior. We therefore expanded upon our previous experiment [23], whereby we investigated the effect of contingent playback of 50- and 22-kHz USV emitted either by a stranger rat or the cage-mate on cocaine self-administration. Finally, to investigate the neurobiological basis of this influence, we tested the effect of STN lesion on these processes.

Materials and Methods

Animals

76 male Lister Hooded rats (Charles River, Saint-Germain-sur-l’Arbresle, France) weighing ~315 g at their arrival were used. Animals were housed in pairs, maintained under a 12-hour inverted light/dark cycle, with all experiments conducted during the dark cycle (7am-7pm). Water and food (Scientific Animal Food and Engineering, Augy, France) were available *ad libitum* in the home cages. Rats were handled every day. All animal care and use conformed to the French regulation (Decree 2010-118),

were approved by the local ethic committee and the French Ministry of Agriculture under the license #3129.01 and followed the 3R European rules.

Surgery

Once rats reached ~400 g, they were anesthetized with ketamine (Imalgene, Merial, 100 mg/kg, i.p.) and medetomidine (Domitor, Janssen 30 mg/kg, i.p.) following a preventive antibiotic treatment with amoxicillin (Duphamox, LA, Pfizer, 100 mg/kg, s.c.) and placed in the stereotaxic frame (David Kopf apparatus) for bilateral injection of either 0.5 μ L of 53 mM ibotenic acid (9.4 mg/mL ; STN-lesioned group, n=42) or vehicle solution (phosphate buffer, 0.1 M ; sham control group, n=34) into the STN (with tooth bar set at -3.3 mm ; at the following coordinates anterior/posterior = -3.7 mm ; lateral = \pm 2.4 mm from bregma ; dorsoventral = -8.35 mm from skull; from Paxinos & Watson, 2007; [48] as previously described [40,42]. At the end of the surgical procedure, anesthesia was reversed with atipamezole (Antisedan, Janssen, 0.15 mg/kg, i.m.). For analgesia, rats received meloxicam (Metacam, Boehringer Ingelheim, 1 mg/kg, s.c.) at the time of surgery.

42 rats were also subjected to intra-jugular implantation of a catheter using standard surgical procedures [40]. The catheters were then flushed daily with a sterile saline solution containing heparin (Heparin Sodium, Sanofi, 3g/L) and enroflorilexine (Baytril, Bayer, 8g/L) during all the experiment to maintain their patency and to reduce infection risk. Catheters were also regularly tested with propofol (Propovet, Abbott, 10 mg/ml) to confirm their patency. All rats were allowed 10 days for recovery. Then they were tested for their auditory capacities, using startled auditory reflex, and their USV have been recorded before experimentation began (see **Supplementary material** [49]).

Apparatus

Standard self-administration chambers controlled by a custom-built interface and associated software (built and written by Y. Pelloux) were used [23] (for details see **Supplementary material** [49]).

Experimental procedures

USV self-administration

After 5 days of habituation (i.e., animals were placed in the chambers for 30-min, with no levers nor house-light), 34 rats were daily exposed to a 30-min session of USV playback self-administration. At the start of the session, the house-light was turned on and the two levers were extended. One press on one lever delivered the playback of a USV file (1.5 sec length of either 50 or 22 kHz USV) while pressing the other lever resulted in the playback of the background-noise file (1.5 sec length). Assignment of USV or background-noise to one of the two levers were counterbalanced between animals. Each lever press switched on the cue-light above the lever for 5-s, followed by a 20-s time-out period, during which the house-light was switched off and any further lever press (perseveration) was recorded, as it reflects a goal-directed behavior, but had no consequence. This time-out period was used to avoid an over-exposure of the rats to the USV and thus minimize a possible habituation effect. The experiment consisted in 3 different blocks of 5 consecutive days in the following order: 50-, 22- and 50-kHz USV, .vs background-noise. The addition of the 2nd 50-kHz USV playback block allowed to evaluate if a decrease in the number of lever presses on the USV-associated lever during the 22-kHz USV could be reversed with the appetitive 50-kHz USV or would reflect a loss of interest for the task. Two different groups of animals were exposed to either USV emitted by a familiar (n=17, familiar playback condition) or a stranger rat (n=17, stranger playback condition). Then, a 4th block was added,

during which both levers delivered 50-kHz USV, one emitted by a stranger peer, the other emitted by the cage-mate. Levers attribution was randomly re-assigned.

Playback display of familiar or stranger USV during cocaine self-administration

42 rats were trained to press a lever to self-administer cocaine for one-hour daily sessions under a FR5 schedule of reinforcement, as previously described in [23] (see **Supplementary material** [49]). Once rats showed less than 25% of variability in the number of cocaine injections for 5 consecutive days – constituting their baseline cocaine consumption - they were exposed to the playback sessions. During these sessions, the first press on the active lever resulted in the playback of the appropriate file (1.5 sec length of either 50 or 22 kHz USV or background-noise) while the 5th press started the cocaine injection. These playback conditions were run in 3 different blocks of 5 consecutive days in the following order: 50-kHz USV, background-noise and 22-kHz USV. The starting order of the USV (50 or 22-kHz) was counterbalanced between rats. Two different groups of animals were exposed to either USV emitted by their cage-mate (n=21; familiar playback condition) or by rat living in a different home cage (n=21; stranger playback condition).

Acoustic Stimuli

Recording and playback of acoustic stimuli for these two experiments are detailed in **Supplemental material** [49] and representative spectrographs of USV and background noise files are illustrated in **Supplementary Figure 1** [49].

In the experiment of USV playback self-administration only, for the “familiar playback condition”, every familiar USV file was collected from the cage-mate of the tested individual. The material used (50- and 22-kHz USV) for the “stranger playback condition” was recorded from a unique stranger rat (from another experiment). Parameters of the files used for the first 3 blocks of USV playback self-administration are illustrated in **Supplementary Figure 1D,E,F**, (for 50-kHz USV files used in 1st and 2nd 50-kHz USV playback blocks) and in **Supplementary Figure 1G** (for 22-kHz USV files). Multiple comparisons Dunnett test was used to detect differences in stranger vs familiar USV file parameters (mean peak frequency, mean bandwidth and total call duration) and USV call subtypes composition (trill, non-trill frequency modulated and trill calls). Detailed results of these tests can be found in **Supplemental material** [49]. Importantly, no significant difference between these USV files were found, only the duration of the 22-kHz USV significantly differed between files used for stranger vs familiar sham-control and STN-lesioned rats.

For the 4th block of USV playback self-administration, to confirm our former results, files from two different rats were used for the “stranger 50-kHz USV”. The first file, described before, was used for the rats previously self-administering familiar USV. However, to avoid an habituation effect, another file was displayed to the rats previously self-administering stranger USV. This new file was recorded from another rat, stranger to the experiment. Parameters of these 50-kHz USV files are illustrated in **Supplementary Figure 1H,I,J**. Once again, multiple comparisons Dunnett test was used to reveal that there was no differences between files used as “stranger 50-kHz USV” (n=2 for both sham and STN-lesioned rats) and files used as “familiar 50-kHz USV” for sham rats (n=14) and for STN-lesioned rats (n=15) (**Supplemental material** [49]).

For the experiment of USV playback during cocaine self-administration, each rat was recorded for positive and negative USV material. Each USV file served twice: first to the cage-mate of the recorded rat in the “familiar playback condition” and second, to a stranger rat in the “stranger playback condition” (see **Supplementary figure 1K,L,M and N** for parameters). A Kruskal-Wallis test was performed to detect differences between the different parameters of files in the 4 experimental

groups (i.e. files for familiar sham n=8; files for stranger sham n=7; files for familiar STN-lesioned rats n=7 and files for stranger STN-lesioned rats n=9). All USV files parameters were similar between groups (**Supplemental material [49]**).

Histology

At the end of the experiments, rats were anesthetized with isoflurane and then euthanized using pentobarbital sodium (Dolethal, Vetoquinol, 200mg/kg, intracardiac). Brains were removed, frozen in isopentane (Sigma-Aldrich) and kept at -80°C before being coronally sectioned at 40 µm thickness, using a cryostat, and stained with thionine (Sigma-Aldrich). The location and extent of STN lesions have been estimated by visualizing neuronal loss and associated gliosis (**Figure 1**).

Statistical analysis

All variables are expressed as mean number ± SEM and the p-value threshold have been set at $\alpha=0.05$.

USV self-administration

First, in each playback block (1st 50-, 22- and 2nd 50-kHz USV, .vs background-noise), we compared the average number of presses (i.e., active presses and perseverations per session) on the USV-associated lever vs. the background noise-paired one, using Wilcoxon matched-pairs signed-ranks tests, separately within subgroups (familiar and stranger USV conditions, in sham-control and STN-lesioned rats). To assess a possible effect of the sessions, we compared the number of presses on the USV-associated lever during the 1st vs the 5th session, separately within subgroups, using Wilcoxon matched-pairs signed-ranks. To assess the effect of the familiarity with the USV emitter and the STN lesion, we first calculated a preference score for the USV over the background noise (mean number of USV lever presses - mean number of background noise lever presses). Then, we compared these preference score between appropriate subgroups (ex. familiar vs stranger conditions, in sham-control or STN-lesioned rats, or familiar condition in sham-control vs STN-lesioned rats) using Wilcoxon sum rank tests. To check the USV-related specificity of all these effects, we compared the number of presses on the background noise during the 1st vs the 5th session within subgroups, using Wilcoxon matched-pairs signed-ranks tests. We also compared the average number of presses between appropriate subgroups using Wilcoxon sum rank tests. Finally, we compared the number of presses on each lever between the 3 playback blocks using Friedman tests, separately within each subgroup.

To interpret these previous effects, we estimated a panel data random-effects (RE) model with the effects of USV familiarity condition, playback blocks and sessions (as dummy variables) effects on the discrimination between the two levers. We adopted a panel data approach because it allows an analysis of cross-sectional time-series data (see **Supplemental (49)**).

For the analysis of the 4th block, we compared the mean number of lever presses on the familiar vs the stranger USV-associated lever, separately for the sham and the STN-lesioned rats, using Wilcoxon matched-pairs signed rank tests.

Playback display of familiar or strangers USV during cocaine self-administration

Results were analyzed separately for sham-control and STN-lesioned rats. Because of the inter-individual variability in cocaine consumption during baseline, consumption per session was divided by the average number of cocaine injections per session during the baseline, separately for each rat.

Within each group, we first compared this variation ratio of cocaine consumption between each playback block (50- and 22-kHz USV, and background-noise) with the baseline via a Wilcoxon matched-pairs signed-ranks test, separately for the familiar and stranger USV conditions. Then, variation in consumption was compared between familiar and stranger USV conditions, using a Wilcoxon rank sum test, for each playback block. When appropriate, to assess the effect of the STN lesion, we compared the variation in consumption between sham-control and STN-lesioned rats subjected to the same familiarity condition, in a playback block, using Wilcoxon rank sum test. Finally, we estimated a panel data RE model with the effects of USV familiarity condition, playback blocks and sessions (as dummy variables) on the log-transformed cocaine consumption variation (See **Supplemental (49)**).

The statistical tests were two tailed and performed using R software. The graphs were performed using GraphPad Prism 6 (version 6.07).

Results

In total, 29 rats were included in the analysis of USV self-administration and 31 rats in the analysis of the cocaine self-administration over USV. Animals were excluded from STN-lesioned group because of unsatisfying lesions (partially outside the STN, too restricted or unilateral; n=11), from the sham-control group because of apparent mechanic cerebral lesion (n=1) or lack of startled auditory reflex or catheter occlusions (n=4).

Do rats lever press for playback of USV?

Sham-control rats press more for stranger than familiar 50-kHz USV playback

As illustrated in **Figure 2B**, during the first playback block, sham-control rats subjected to stranger (n=7) and familiar (n=7) USV playback significantly preferred to self-administer 50-kHz USV over background playback (V=28, p=0.016 and V=26, p=0.031, respectively). Interestingly, the number of presses on the USV-associated lever decreased between the 1st and the 5th session (V=25, p=0.047) only for familiar playback, not for stranger playback (V=20, p=0.109). As a result, the preference score for the USV over the background noise was stronger in rats subjected to stranger playback (W=3.5, p=0.005). Both groups pressed equally on the lever delivering background noise (W=27, p=0.805), with no difference between the 1st and the 5th session, neither in rats subjected to stranger or familiar playback (V=20, p=0.109 and V=7, p=0.689). These results suggest that playback of stranger, but not familiar, 50-kHz USV is rewarding.

When the USV-associated lever delivered 22-kHz USV, neither rats subjected to stranger, nor familiar, playback discriminated between the two levers (V=20, p=0.375 and V=17, p=0.688, respectively). Presses for stranger 22-kHz USV playback decreased between the 1st and the 5th session (V=24, p=0.047), but was stable across sessions for familiar ones (V=3, p=0.848). The preference score for the USV vs background noise lever was similar between the two groups (W=17, p=0.383). Finally, both groups pressed equally for the background noise (W=14, p=0.197) and number of presses was stable between the 1st and the 5th session for stranger and familiar playback (V=4, p=0.781 and V=2, p=0.906 respectively). These results suggest that neither stranger, nor familiar 22-kHz USV, have reinforcing properties, nor aversive ones.

When the USV-associated lever delivered again 50-kHz USV, only rats subjected to stranger playback pressed more for USV than for background noise (V=27, p=0.031). The stranger USV self-administration increased between the 1st and the 5th session (V=28, p=0.016), while it remained stable for familiar USV (V=7, p=0.563). As a result, the preference score for the USV over background noise was

significantly stronger for rats subjected to stranger than familiar playback ($W=7$, $p=0.026$). Finally, both groups pressed equally for the background noise ($W=19$, $p=0.535$) in a stable manner ($V=11$, $p=0.313$ and $V=3$, $p=0.844$, for stranger and familiar groups). These results suggest that only stranger, but not familiar, 50-kHz USV present strong and stable rewarding properties.

To check robustness, we compared lever presses on each lever across the three playback blocks. In rats subjected to stranger playback, lever presses for the USV are modulated by the three playback blocks ($\chi^2(2)=10.57$, $p=0.003$). Post-hoc comparisons confirmed that they pressed more for the 50-kHz USV (first ($p<0.010$) and third ($p=0.023$) playback blocks) than for 22-kHz USV (second playback block). The number of presses was equivalent during the first and third playback blocks ($p>0.999$).

Also in rats subjected to familiar playback, lever presses differed across the playback blocks ($\chi^2(2)=10.57$, $p=0.003$). Post-hoc comparisons revealed that lever presses decreased between the 1st and the 3rd playback blocks ($p=0.023$).

Results of the RE panel data model (**Supplementary Table 1** [49]) confirmed that 1) rats subjected to stranger USV playback had a stronger preference for the USV than those subjected to familiar USV during the 1st ($\beta=-17.486$, $p=0.003$) and the 2nd 50-kHz USV playback blocks ($\beta=-21.857$, $p<0.001$),

2) no preference for the 22-kHz USV was observed (stranger: $\beta=-21.857$, $p<0.001$, familiar USV condition: $\beta=-26.114$, $p<0.001$)

3) rats self-administering stranger USV showed a similar preference for the lever delivering 50-kHz USV during the 1st and the 3rd playback block ($p=0.902$).

Finally, during the 4th playback block, sham-control rats preferred stranger over familiar 50-kHz USV ($V=85$, $p=0.042$, $n=14$) (**Figure 2C**).

STN-lesioned rats do not press to obtain playback of USV

In none of the three playback blocks, STN-lesioned rats subjected to stranger ($n=6$) or familiar ($n=9$) playback discriminated between the two levers (stranger: $V=10$, $V=14$ and $V=13$, $p>0.05$, familiar: $V=17$, $V=16.5$ and $V=21$, $p>0.05$) (**Figure 2D**). Their USV self-administration remained stable between the 1st and 5th session in each block (stranger: $V=17$, $V=3$ and $V=15$, $p>0.05$; familiar: $V=20$, $V=2$ and $V=13$, $p>0.05$). Both stranger and familiar playback induced similar preference score ($W=26$, $W=23$ and $W=26.5$, $p>0.05$). During the 3 playback blocks, USV familiarity did not modify presses for the background noise ($W=39$, $W=39.5$ and $W=38.5$, $p>0.05$).

The robustness check confirmed that, in STN-lesioned rats, the lever presses for both stranger and familiar USV playback tended to decrease across the three playback blocks ($\chi^2(2)=6.333$, $p=0.052$ and $\chi^2(2)=1.333$, $p=0.571$). STN-lesioned rats showed no preference between familiar and stranger 50-kHz USV playback ($V=53$, $p=0.720$), confirming that the STN-lesion blunts the rewarding value of stranger 50-kHz USV, (**Figure 2E**).

Direct comparison of sham-control and STN-lesioned rats revealed that sham-control rats showed a stronger preference score for the stranger USV over the background noise than the STN-lesioned animals during the 1st ($W=0$, $p=0.001$) and 3rd ($W=3.5$, $p=0.009$), but not during the 2nd playback block ($W=15$, $p=0.445$). Sham and STN-lesioned groups subjected to stranger playback did not differ in their number of presses for the background noise in any block (1st: $W=12$, 2nd: $W=13$ and 3rd: $W=14$, $p>0.05$). Sham-control and STN-lesioned groups did not differ either in their preference score for the familiar

USV over the background noise in any playback block (1st: W=22, 2nd: W= 27, and 3rd: W=29, p>0.05), nor in their number of presses for the background noise (1st: W=18, 2nd: W=20, and 3rd: W=23, p>0.05).

2) How does the familiarity with the USV emitter modulate the effect of the USV playback on cocaine consumption?

Stranger, but not familiar, 50-kHz USV playback decreases cocaine intake in sham-control rats

As illustrated in **Figure 3B**, sham-control rats subjected to stranger 50-kHz USV playback (n=7) showed a significant reduction from their basal cocaine consumption (mean number of injections: 21.03 ±3.33) (V=28, p=0.016). In contrast, no difference was found with background-noise (V=17, p=0.688), nor 22-kHz USV playback (V=21, p=0.297). A trend towards increased cocaine consumption between the 1st and 5th session of the 22-kHz USV playback was observed (V=1, p=0.059), in line with our former results with stranger USV only [23].

In contrast, in sham-control rats subjected to familiar playback (n=8), cocaine consumption remained equivalent to baseline (mean number of injections: 15.18 ±2.02) with 50-kHz USV (V=20, p=0.844), background-noise (V=22, p=0.641) and 22-kHz USV (V=25, p=0.383). Cocaine intake was thus significantly different during stranger vs familiar 50-kHz USV playback (W=9, p=0.029), while not during background-noise (W=31, p=0.779) nor 22-kHz USV (W=21, p=0.463) playbacks.

To check robustness, results from the RE panel data model (**Supplementary Table 2** [49]) confirmed that stranger ($\beta=-25\%$, p=0.003), but not familiar (p=0.452) 50-kHz USV playback strongly decreased cocaine consumption, but familiarity with the USV emitter had no general effect (p=0.922). No effect of the sessions was found, suggesting that there was no habituation within the playback blocks.

Cocaine consumption of STN-lesioned rats is not affected by playback of USV

In contrast with sham-controls, STN-lesioned rats subjected to stranger playback (n=9) showed no variation from their basal cocaine consumption (mean number of injections: 17.87, ±3.35) with 50-kHz USV (V=20, p=0.353), background-noise (V=22, p>0.999), 22-kHz USV (V=27, p=0.652)(**Figure 3C**). As a results, the decreased cocaine intake induced by stranger 50-kHz USV playback was stronger in sham-control than in STN-lesioned rats (W=12, p=0.042; **Figure 3D**), in line with [23]. STN-lesioned rats subjected to familiar USV playback (n=7) showed no variation from their basal consumption (mean number of injections: 16.63, ±4.62) (50-kHz USV: V=22, background-noise: V=23, 22-kHz USV: V=18, p>0.05).

Discussion

We showed that in sham-control rats, 50-kHz USV playback was more rewarding if emitted by a stranger than a familiar rat. This discrimination between stranger and familiar USV advocates for the existence of a vocal signature in rats. Consequently, only stranger 50-kHz USV playback decreased cocaine intake. Independently of the USV emitter, rats did not press for 22-kHz USV and their playback had no effect on cocaine consumption. In contrast, neither the familiarity nor the emotional valence of the USV affected STN-lesioned rats, which did not self-administer their playback and maintained stable cocaine intake in playback conditions.

Positive USV emitted by a stranger are more rewarding than those emitted by a familiar peer.

We showed that rats actively self-administer stranger 50-kHz USV playback, in line with former work [43], confirming the rewarding properties of these USV [23,43]. Moreover, the rewarding properties of stranger 50-kHz USV playback remained stable over the sessions. On the other hand, familiar USV induced less preference than stranger USV over background noise during both 50-kHz USV playback blocks and this preference even faded over time and was no longer significant during the 2nd 50-kHz USV playback block. This diminished preference could result from habituation to the 50-kHz calls emitted by the cage-mates. Finally, control rats chose to press more for stranger 50-kHz USV than for familiar ones. Taken together, these results show that listening to stranger 50-kHz USV is more rewarding than familiar ones, which is in line with rats' social novelty preference [44–47].

In this experiment only, it is noteworthy that we used a unique file as stranger 50-kHz USV stimulus (yet two when rats faced the choice between stranger and familiar 50-kHz USV), while familiar 50-kHz USV stimuli were as numerous as subjects. Could this unique stranger 50-kHz stimulus convey something distinctive that justify the preference of rats for it? Firstly, USV files parameters (total duration and number of calls, mean peak frequency and bandwidth) and call subtypes composition were found similar between stranger and familiar 50-kHz USV files. Secondly, results are consistent between conditions and experiments (USV self-administration and cocaine self-administration with USV playback), while stranger USV files used were different. However, in line with the literature [31,33], we observed an inter-subject variability in these USV files parameters within groups. Then, this differential preference for stranger over familiar USV indicates that rats are able to recognize the vocal signature of their cage-mate. The ability to discriminate the emitter of USV refers to the concept of “voice recognition” in other species such as non-human and human primates, that was not demonstrated in rodents and is therefore critical for translational approaches.

The fact that stranger 50-, but not 22-kHz, USV playback diminished cocaine consumption in our experiment is in line with previous results [23] and could rely on the hypothesis that social reward – here 50-kHz USV playback - can counterbalance the drug reinforcing properties [9]. Indeed rats consume less drug in the presence of a stranger peer than when alone [8]. Also, rats choose social interaction over drug, confirming the beneficial effect of proximal social stimuli to reduce drug intake [50]. Besides, both cocaine intake and listening to 50-kHz USV activate the reward system, highlighting their competing reinforcing effects. However, the influence of the familiarity of USV on this activation of the reward system has not yet been assessed.

In contrast to stranger, familiar 50-kHz USV playback did not affect cocaine consumption, suggesting that the rewarding value of familiar 50-kHz USV playback is not strong enough to counterbalance the reinforcing effect of cocaine. The lower impact of familiar USV playback (compared with unfamiliar) on cocaine self-administration is also in line with the lower influence of the presence of the cage-mate [8]. One could hypothesize that the poor rewarding properties of familiar presence and USV playback and their consequences on drug consumption cannot be translated to human because rats do not choose their cage-mate, contrary to human beings. This difference could be critical for the rewarding value of familiar stimuli. However, in Giorla et al.[8], human stimulant users also reported to take less drug in presence of a stranger over a familiar peer. Taken altogether, our results suggest that positive communication and familiarity constitute key factors in the influence of social contact on cocaine consumption.

The results of self-administration of stranger or familiar 22-kHz USV or their influence on cocaine intake did not show aversive effect. Nevertheless, it should be noted that to compare the effects between 50- and 22-kHz USV and between individuals, we used USV files of a similar short duration (only 1.5s), which lead to a trunked 22-kHz call. It is possible that the apparent lack of aversive effect of their playback could reflect a sub-optimal duration of these calls, compared to ecological situations. In

contrast, in our former experiment [23], stranger 22-kHz USV playback induced a conditioned place aversion and a transient increase in cocaine intake, confirming its aversive property.

Discrepancies between behavioral responses to 22-kHz calls across studies are common [22]. For instance, Burman et al. [51] found that 22-kHz USV playbacks emitted by two different rats induced different behavioral responses. Different factors may interfere on the behavioral response to 22-kHz USV like rats' environment and anxiety history [52,53]. Kim et al. [20] showed that 22-kHz USV induces a fear-related response only in rats with prior exposure to stress, not in naïve animals. Further studies are then needed to better understand this variability in behavioral responses to the 22-kHz calls.

It is important to note that this study was exclusively conducted on male rats, as a first step before testing female rats, although they do not show social novelty preference [47] and emit less USV than males [33]. There is, to our knowledge, no study investigating the female rat behavioral response to USV emitted by female. We will try to fill this gap in the future.

The STN is involved in USV rewarding properties and their consequences on cocaine intake

Our results showed that STN-lesioned animals never discriminated between USV and background-noise, whatever the USV emotional valence and the familiarity of the emitter. The lack of rewarding properties of the 50-kHz USV emitted by a stranger rat in STN-lesioned animals is in line with former results [23]. Because STN-lesioned rats did never show a preference for one lever, we cannot conclude regarding the effect of the STN lesion on the aversive properties of 22-kHz USV emitted by a stranger or a familiar rat. However, we showed [23] that STN lesion blunts the conditioned place aversion induced by stranger 22-kHz USV. STN-lesioned rats remained insensitive to USV playback in their cocaine consumption, whatever their emotional value or the familiarity with their emitter. These results question the role of STN in social emotional processes.

Interestingly, in human, the involvement of STN in social emotions was documented in Parkinson's disease (PD) patients with STN deep brain stimulation (DBS). STN-DBS induces alterations of social emotion decoding [54], notably vocal emotion [55,56]. Besides, in healthy subjects, the STN presents a functional connectivity with structures involved in emotional prosody decoding [57]. In agreement with these human studies, our results could suggest that STN lesion affects USV emotional decoding, thereby blunting the emotion contagion induced by USV listening, and *in fine*, the behavioral response to those USV. In PD patients, STN-DBS can also induce apathy [58,59], while in rats, STN lesion blunts emotional response to hedonic and aversive stimuli [42]. This general diminished affective state could account for the dampened response to USV.

Importantly, STN-DBS also affects vocal expression by improving the oral motor control of PD patients' speech [60]. Although in parallel, it could negatively impact their expression, and decoding of emotions [61,62]. These effects would depend on the exact location of the electrode's implantation in the STN (e.g., limbic vs sensorimotor part) [63]. Besides, previous work has shown that STN-lesioned rats emit less 22-kHz USV in response to an aversive stimulus [42]. It would be interesting to further investigate STN's involvement in emotional expression.

In every case, our results confirm the involvement of the STN in vocal emotion decoding and more broadly, its role on the influence of proximal social factors on drug consumption.

Conclusions

Taken as a whole, our study advocates for the existence of an identifiable vocal signature in rats that can be differentiated between cage-mate and stranger rats, like primates can recognize a type of "voice". It shows the importance of ultrasonic communication in socio-affective influence of behavior

since the playback of stranger 50-kHz USV has stronger rewarding properties that might counterbalance cocaine effects, leading to a reduced consumption, when paired with drug self-administration. The STN seems to be a key actor in the influence of social contact on drug consumption.

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Author Contributions

CV: designed the research, performed research, analyzed data and wrote the paper;

CM: performed the research and contributed to experimental material;

YP: designed the research and contributed to analytical tools;

CB: designed and supervised the research, performed surgery, wrote the paper and obtained the funding.

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Data accessibility statements

Data presented in this article are available to the public via: <https://figshare.com/s/d81f9e3b541902a0af4c> ; DOI: 10.6084/m9.figshare.16789264.

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Figure and Figure captions

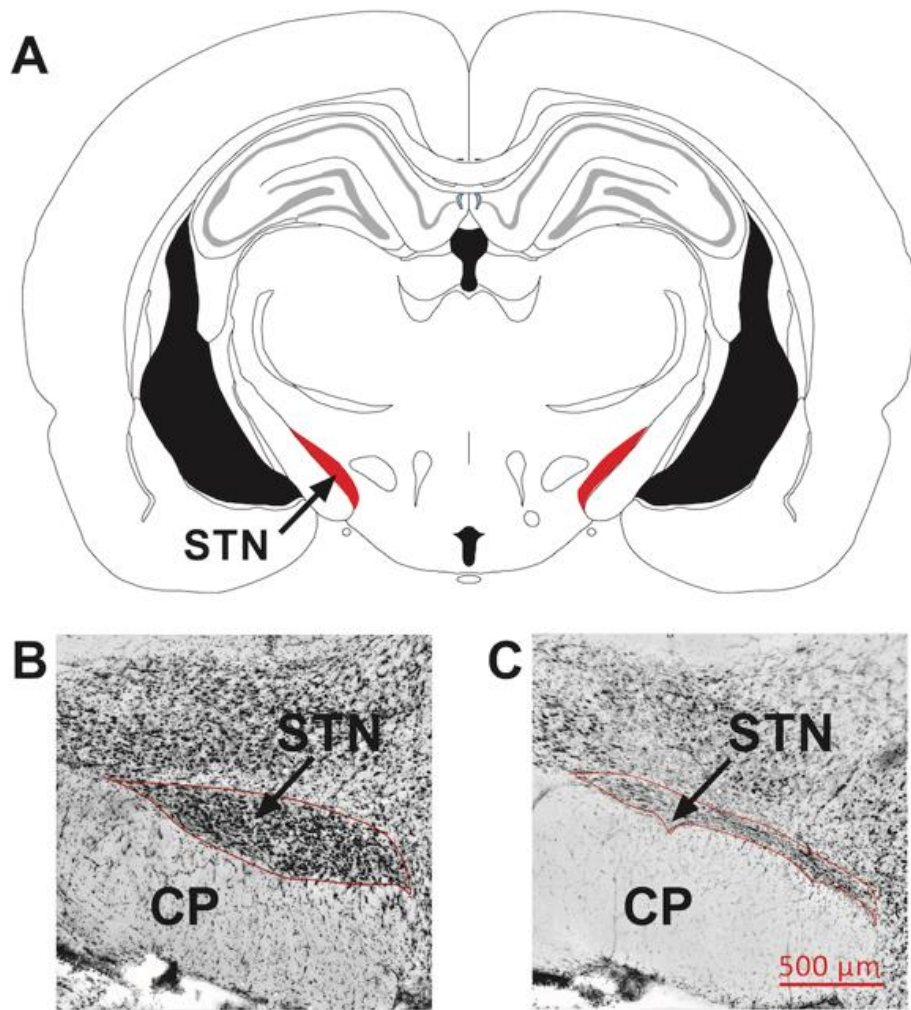


Figure 1: Frontal sections of the subthalamic nucleus stained with thionine

A: Schematic coronal section of the rat brain at the level of the STN targeted (AP: -3.7 mm from bregma) (left STN filled in red indicated by the arrow), from Paxinos & Watson, 2007).

B-C: Representative STN delineated by the red lines in a sham-operated rat (B) and a representative STN-lesioned rat (C). The lesions were characterized by neuronal loss, shrinkage of the structure and gliosis. They were considered as satisfying if restricted to the STN, bilateral and covering at least the medial two-third of the STN. The red bar indicates the scale.

CP: cerebral peduncle; STN: subthalamic nucleus.

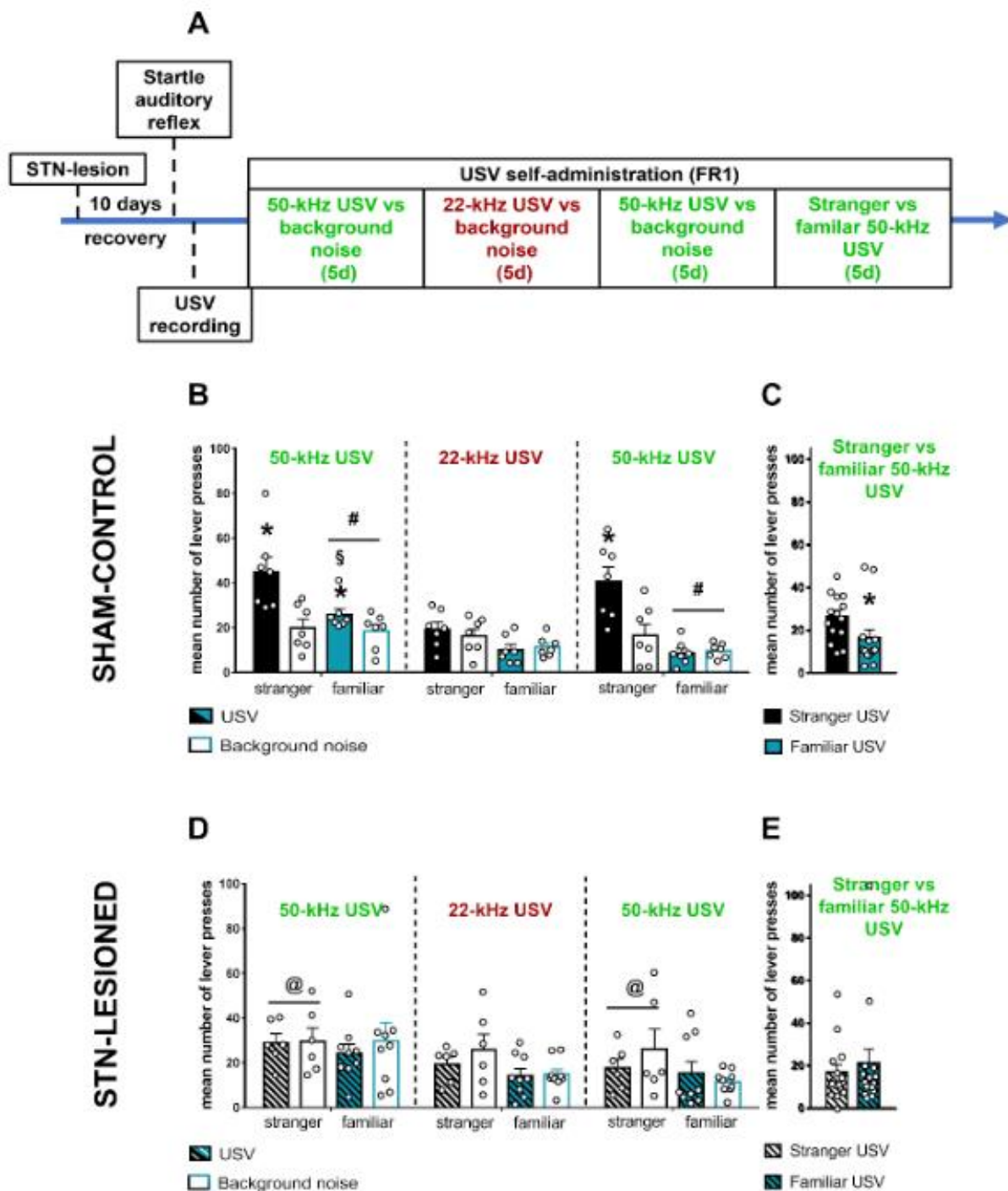


Figure 2: Familiar and stranger USV playback self-administration in sham-control (B-C) and STN-lesioned (D-E) rats

A: Timeline of the experiment.

B-E: Mean number of presses per session on the lever associated with each acoustic stimulus during the 3 first playback blocks (1st: 50-kHz USV (plain bars) vs. background noise (empty bars), 2nd: 22-kHz USV vs. background noise and 3rd: 50-kHz USV vs. background noise; B (sham; stranger USV: black bars n=7; familiar USV: blue bars n=7) and D (STN-lesioned; stranger USV: black striped bars n=6; familiar USV: blue striped bars n=9)) and the 4th playback block (familiar vs stranger 50-kHz USV; C (sham, n=14) and E (STN-lesioned, n=15)) of the USV playback self-administration experiment.

**,#,@, §: $p \leq 0.05$ for respectively: *: vs background noise or vs stranger USV lever, accordingly; #: preference score familiar vs stranger USV; @: preference score sham vs STN-lesioned groups; §: 1st vs 2nd 50-kHz USV playback block.*

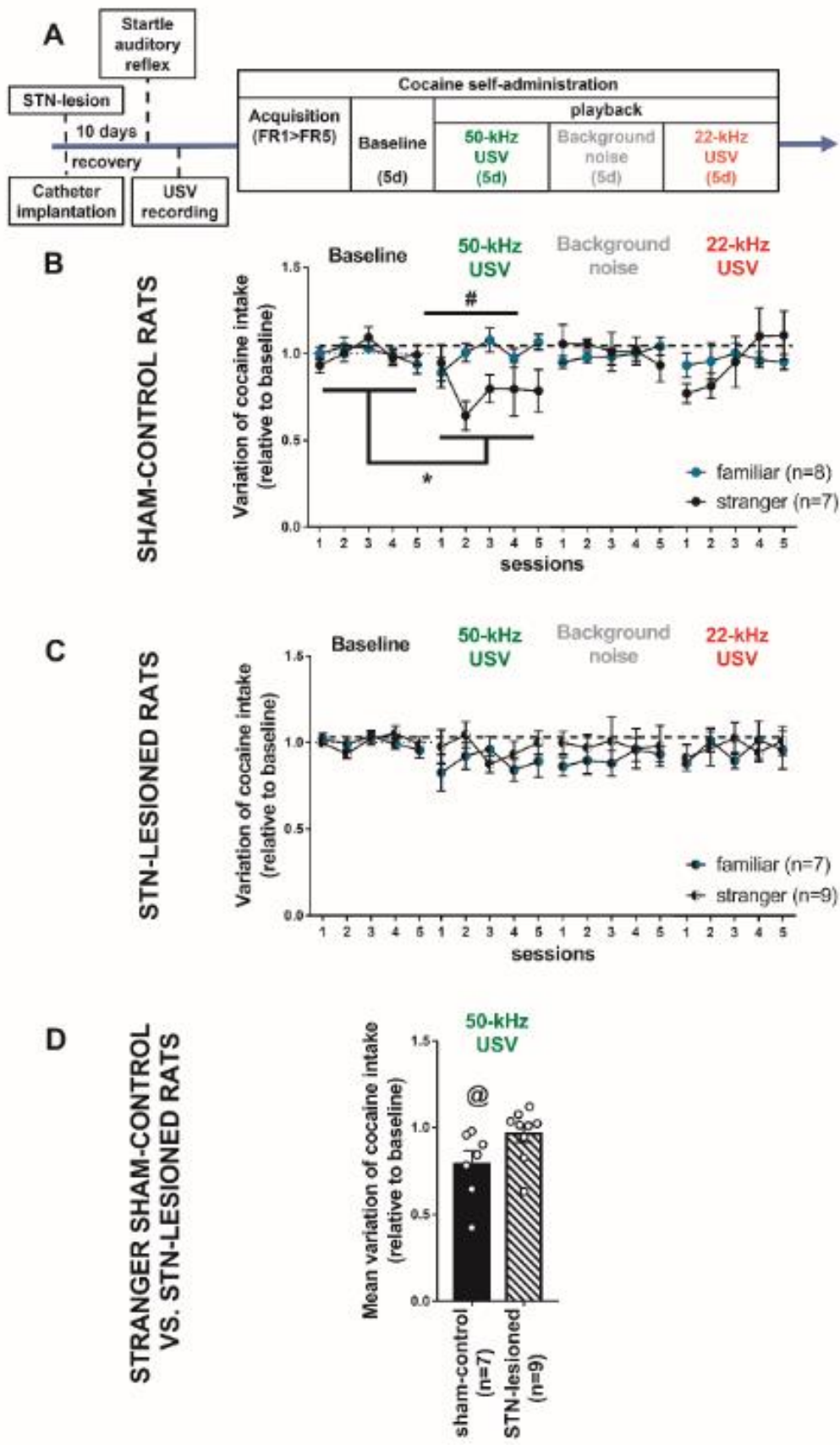


Figure 3: Variation of cocaine consumption under familiar and stranger 50- and 22-kHz USV and background noise playback in sham-control and STN-lesioned rats.

A: timeline of the experiment.

B-C: Mean variation of cocaine intake relative to baseline (\pm SEM) for sham-control (B; n=15; subjected to stranger USV playback, n=7, black dots ; or familiar USV playback, n=8, blue dots) and STN-lesioned

rats (C; n=16; subjected to stranger USV playback, n=9, black and white dots; or to familiar USV playback, n=7, blue and black dots) during the 5 sessions of each playback block (50- and 22-kHz USVs and background noise). The dotted line represents no change from baseline.

D: Mean variation in cocaine intake from baseline averaged for a block of 5 sessions in sham-control (n=7; full bar) and STN-lesioned (n=9; striped bar) rats subjected to stranger 50-kHz USV playback.

**,#,@: p ≤ 0.05 vs baseline; vs familiar USVs condition ; vs STN-lesioned rats*

Supplementary material:

Apparatus

Standard self-administration chambers (Camden Instruments, Loughborough, England; length: 24 cm, width: 25 cm, height: 26 cm) placed in sound and light attenuating cubicles were used. Each chamber was equipped with a white house-light in the ceiling, a cue-light above each of the two retractable levers on the right-hand wall and an ultrasonic loudspeaker (Ultrasonic Dynamic Speaker Vifa, Avisoft Bioacoustics, Glienicke, Germany), connected to a portable ultrasonic power amplifier (Avisoft Bioacoustics), linked to a computer audio interface (Quad-Capture USB 2.0 Audio Capture, Roland Corporation, Los Angeles, CA, U.S) in an opening in the middle of the chamber ceiling. All the chambers described above were controlled by a custom-built interface and associated software (built and written by Y. Pelloux).

Experimental procedure

1) Playback display of USV during cocaine self-administration

Before the start of the session, catheters of rats were connected to cocaine syringes positioned on motorized pumps (Razel Scientific Instruments, St-Albans, VT, USA), via infusion lines and liquid swivels. At the start of the session, the house-light was turned on and the two levers were extended. Cocaine was assigned to one of the two levers (“active lever”) and counterbalanced between rats. Pressing on the active lever delivered a 5s intravenous infusion of cocaine (250µg/90µl) and switched on the cue-light above the active lever during the cocaine delivery. Each injection was followed by a 20-s time-out period, during which the house-light was switched off and any further lever press was recorded as perseveration but had no consequence. Pressing on the other lever (“inactive lever”) was recorded but had no consequence. After a few sessions ran under a continuous schedule of reinforcement (FR1; i.e. every lever press on the active lever was reinforced), the number of lever presses required to obtain a cocaine injection was increased progressively over days to FR5 (the 5th lever press delivered the infusion of cocaine).

Acoustic stimuli

USV displayed during the experiments were recorded from the tested rats at a sample rate of 192 kHz (16 bits format), using a condenser ultrasound microphone (CM16/CMPA-48, Avisoft Bioacoustics), connected to a computer audio interface (Quad-Capture USB 2.0 Audio Capture, Roland Corporation, Los Angeles, CA, U.S). The software Avisoft-SASLab Pro (Version 4.2, Avisoft Bioacoustics) was used to record and mount USV files.

Long 1.5 s 22-kHz USV calls were recorded from each rat after received a mild electric foot shock (1 mA for 2 s) in a different self-administration chamber than these used for the self-administration experiments described here and located in another room to prevent negative association. For the playback of the 50-kHz USV, a series of 50-kHz calls was recorded from each rat while being tickled alone in its home cage. Background noise was extracted from one of these recordings and was always the same for all the experiments. Representative spectrograms of 50- and 22-kHz USV and background noise files used are illustrated in **Supplementary Figure 1A,B,C**.

In the experiment of USV playback self-administration, for the “familiar USV condition”, every familiar USV file was collected from the cage-mate of the tested individual. The material used (50- and 22-kHz USV) for the “stranger USV condition” was recorded from a stranger rat of another experiment. Parameters of the files used for the 3 first blocks of USV playback self-administration are illustrated in **Supplementary Figure 1D,E,F**, (for 50-kHz USV files used in 1st and 2nd 50-kHz USV playback blocks) and in **Supplementary Figure 1G** (for 22-kHz USV files). Multiple comparisons Dunnett test was used to detect differences in stranger (n=1) vs familiar (n=16; corresponding to: n=7 sham-control and n=9 STN-lesioned rats) USV files parameters (mean peak frequency, mean bandwidth and total call duration) and USV call subtypes composition (trill, non-trill frequency modulated and trill calls).

For the 50-kHz USV files, the analysis revealed no difference in files used for stranger (sham and STN-lesioned rats) vs for familiar sham, neither for stranger (sham and STN-lesioned rats) vs for familiar STN-lesioned rats, regarding the total number (p=0.2459 and p=0.4759, respectively) and duration (p=0.0645 and p=0.1363) of calls per file, nor on their mean peak frequency (p=0.6615 and p=0.8564) and bandwidth (p=0.0704 and p=0.1176). Regarding the USV calls subtypes, the Dunnett multiple comparisons test revealed no difference neither (for flats: p=0.8149 and p=0.9989, non-trill FM: p=0.8848 and p=0.8150 and trill: p=0.9445 p=0.9143 calls proportions).

For the 22-kHz USV files, the analysis revealed a difference in files used for stranger (sham and STN-lesioned rats) vs for familiar sham, neither for stranger (sham and STN-lesioned rats) vs for familiar STN-lesioned rats, regarding the duration of the call (p<0.0001 and p<0.1363) of call per file, but not on the peak frequency (p=0.9530 and p=0.9422), nor the bandwidth (p=0.3651 and p=0.1137).

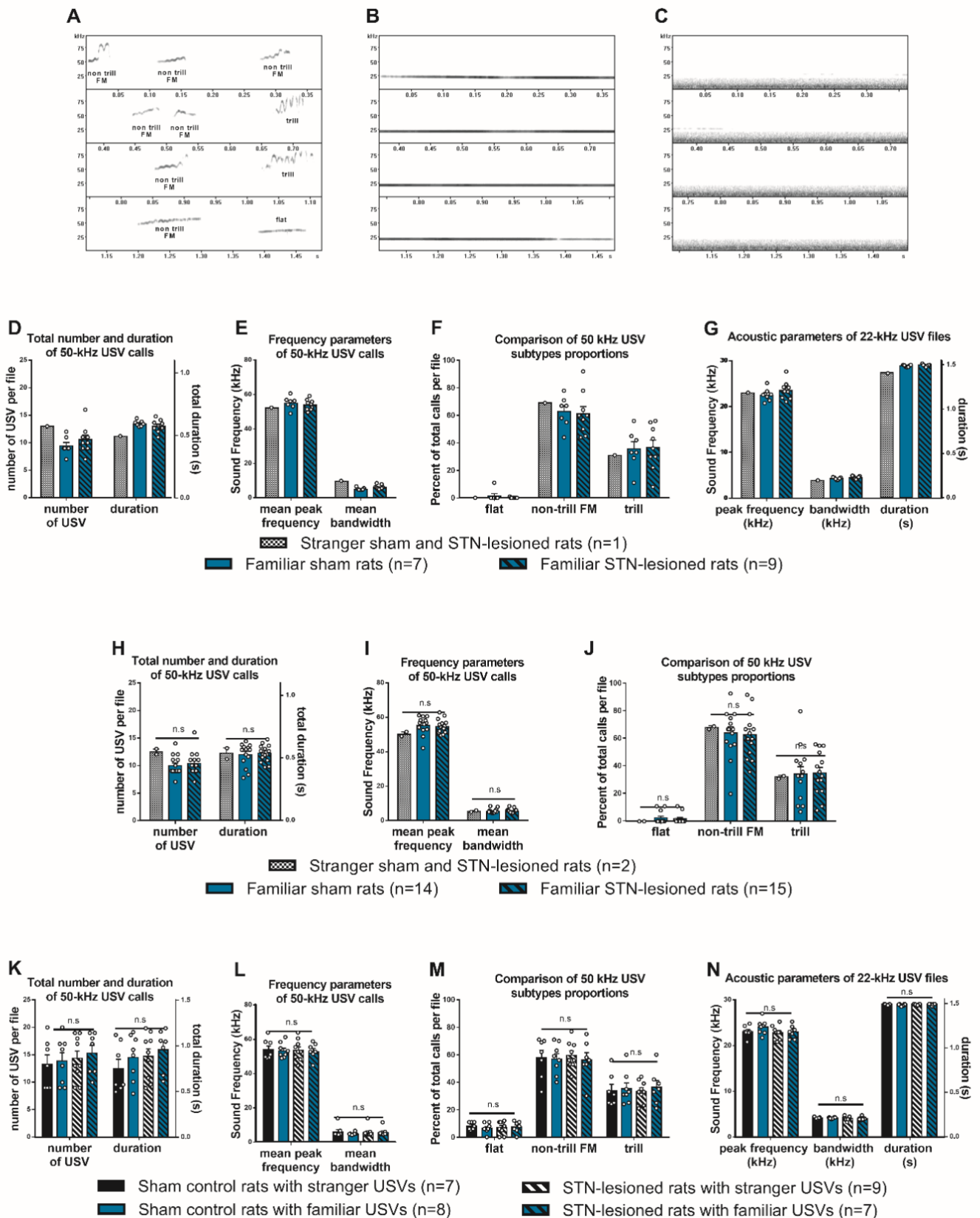
For the 4th block of USV playback self-administration, to confirm our former results, 2 files, from different rats, were played back to rats for the “stranger 50-kHz USV”. The first file, described before, was used for the rats previously self-administering familiar USV. However, to avoid an habituation effect, we played back another file to the rats previously lever pressing for stranger USV. This new file was recorded from another rat stranger to the experiment. Parameters of these 50-kHz USV files used in the 4th block are illustrated in **Supplementary Figure 1H,I,J**. Once again, multiple comparisons Dunnett test was used to detect differences in files used as “stranger 50-kHz USV” (n=2 for both sham and STN-lesioned rats) vs files used as “familiar 50-kHz USV” for sham rats (n=14) and for STN-lesioned rats (n=15). The analysis revealed no difference in files, regarding the total number (p=0.1594 and p=0.2553, respectively) and duration (p=0.9795 and p=0.9998) of calls per file, nor on their mean peak frequency (p=0.2480 and p=0.3293) and bandwidth (p=0.9878 and p=0.9873). Regarding the USV calls subtypes, the Dunnett multiple comparisons test revealed no difference neither (for flats: p=0.6864 and p=0.8459, non-trill FM: p=0.9183 and p=0.8694 and trill: p=0.9816 p=0.9679 calls proportions).

For the experiment of USV playback during cocaine self-administration, each USV file served twice: first to the cage-mate of the recorded rat in the “familiar USV condition” and second, to a stranger rat in the “stranger USV condition”. Parameters of the 50-kHz USV files used in the cocaine self-administration in **Supplementary figure 1K,L,M**. A Kruskal-Wallis test was performed to detect differences between the different parameters of files in the 4 experimental groups (i.e: files for familiar sham; n=8, files for stranger sham; n=7, files for familiar STN-lesioned rats; n=7 and files for stranger STN-lesioned rats; n=9). The analysis revealed no difference in files between groups, regarding the total number ($\chi^2=1.221$, p=0.748) and duration ($\chi^2=2.922$, p=0.404) of calls per file, nor on their mean peak frequency ($\chi^2=0.055$, p=0.997) and bandwidth ($\chi^2=0.424$, p=0.935). The Kruskal-Wallis test also showed no difference between groups in files regarding calls subtypes composition (for flats: $\chi^2=0.613$,

p=0.893, non-trill FM: $\chi^2=0.284$, p=0.963 and trill: $\chi^2=0.340$, p=0.952 calls proportions). Finally, parameters of the 22-kHz USV files used in the cocaine self-administration are illustrated in **Figure 1N**. Again, a Kruskal-Wallis revealed no difference in files between groups regarding the total duration of the call ($\chi^2=2.540$, p=0.468), nor its mean peak frequency ($\chi^2=2.577$, p=0.462) and bandwidth ($\chi^2=2.479$, p=0.479).

It is important to note that in both stranger and familiar USV playback conditions, rats were exposed to USV emitted by a sham-control or a STN-lesioned rat (in a counterbalanced way). Then, if USV playback differently affects sham-control and STN-lesioned rats, it cannot be due to a possible effect of the STN lesion on the USV emission (e.g. frequency, fluency or else), but only an effect of this STN lesion on the listening of USV playback.

As previously described (Montanari et al., 2020), we played back these acoustic stimuli at a sampling rate of 192 kHz (16 bits format). Loudspeakers had a frequency range of 1 to 120 kHz. The dB SPL was calibrated using a Calibrated Reference signal Generator (Avisoft Bioacoustics) and we used an intensity of ~70 dB SPL for the USV, corresponding to the intensity of a USV measured at 20 cm of a rat (Brudzynski et al., 1993) and ~50 dB SPL for the background noise, corresponding to its moderate intensity in the USV files.



Supplementary figure S1: Description of the USV files

Upper graphs: Representative spectrograms with frequency (kHz) and duration (s) of the various audio stimuli used: 50-kHz USV calls (A), 22-kHz USV (B) and background noise (C).

Graphs (D), (E), (F) and (G) illustrate the total number (when appropriate) and duration of the calls per file, their frequency parameters and the 50-kHz subtypes proportion of the USV files used for the 3 first blocks of the USV self-administration experiment.

Graphs (H), (I) and (J) illustrate the total number and duration of the USV calls per file, their frequency parameters and subtypes proportions of the 50-kHz USV files used for the 4th block of the USV self-administration experiment.

Graphs (K), (L), (M) and (N) illustrate the total number (when appropriate) and duration of the calls per file, their frequency parameters and the 50-kHz subtypes proportion of the USV files used for the cocaine self-administration experiment.

FM: frequency-modulated, n.s: non-significative

2) Statistics:

The use of a Random-Effect model:

In the USV self-administration:

RE model controls for unobserved heterogeneity among the individuals and allows to include panel-invariant variables, such as the USV familiarity condition, among regressors. In this model, a constant contains the “reference individual”, corresponding to the discrimination of a rat subjected to stranger USV playback, on the 1st session of the 1st 50-kHz USV playback block. The parameters of the dependent variable (USV familiarity condition, playback block and session) were therefore compared to the reference individual’s parameters. In this model, a significant parameter means that the behavior diverged in a significant manner with regards to this parameter.

Number of observations : n=210		Number of rounds : 5x3		Number of subjects : n= 14	
Variables	Coefficients	s.e	p-value		
Playback blocks x Familiarity of the USV emitter :					
1st familiar 50-kHz USV playback block	-17.486	5.898	0.003**		
familiar 22-kHz USV playback block	-26.114	5.899	< 0.001***		
2nd familiar 50-kHz USV playback block	-25.857	5.900	< 0.001***		
Stranger 22-kHz USV playback block	-21.858	5.315	< 0.001***		
Stranger 2nd familiar 50-kHz USV playback block	- 0.657	5.316	0.902		
Sessions :					
session 2	2.286	4.853	0.638		
session 3	- 2.762	4.854	0.569		
session 4	- 0.595	4.855	0.902		
session 5	- 2.119	4.856	0.662		
Intercept	25.495	5.179	< 0.001***		
2{ Godness of fit :					
Hausman test	$\chi^2 < 0.001$	df=8	p>0.999		
Wald test	$\chi^2 = 41.968$	df=9	p<0.001		

Supplementary table 1: Parameters estimate for the random-effect model of sessions, playback blocks and familiarity of the USVs on the discrimination between the levers for sham-control rats.

Discrimination between the two levers has been calculated as follow: number of lever presses on the lever delivering USV playback minus number of lever presses on the lever delivering background noise playback per session. In this RE model, the reference is the discrimination of a stranger rat during the 1st session of the 1st 50-kHz USV playback block.

****:** $p \leq 0.005$

*****:** $p \leq 0.001$

In the cocaine self-administration experiment:

Log-transformation of drug consumption with dummy variables for treatments allows us to interpret the effects as a percentage variation of consumption. In this model, the reference is the variation of cocaine consumption of a rat subjected to familiar USV playback, on the 1st session of its baseline.

Number of observations : n=300

Number of rounds : 5x4

Number of subjects : n= 15

Variables	Coefficients	s.e	p-value
Playback blocks :			
50-kHz USV	-0.044	0.059	0.0645
background noise	0.016	0.058	0.787
22-kHz USV	-0.095	0.058	0.102
Familiarity of the USV emitter	-0.007	0.075	0.922
Stranger 50-kHz USV playback	-0.250	0.085	0.003**
Stranger background noise playback	-0.034	0.085	0.684
Stranger 22-kHz USV playback	0.050	0.085	0.557
Sessions :			
session 2	-0.005	0.047	0.912
session 3	0.061	0.047	0.195
session 4	0.300	0.047	0.530
session 5	-0.026	0.047	0.576
Intercept	-0.027	0.059	0.645
Godness of fit :			
Hausman test	$\chi^2=1.280$	df=10	p=0.257
Wald test	$\chi^2=38.071$	df=11	p<0.001

Supplementary table 2: Parameters estimate for the random-effect model of sessions, playback blocks and familiarity of the USVs on the log-transformed cocaine consumption of sham-control rats.

In this RE model, the reference is the consumption of a rat subjected to familiar USV playback during the 1st session of the baseline.

** : $p \leq 0.005$