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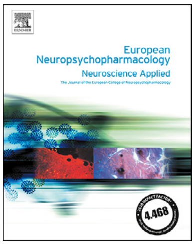

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Characterization of rat ultrasonic vocalization in the orofacial formalin test: Influence of the social context

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Abstract

Rats emit ultrasonic vocalizations (USVs) about 22 kHz and 50 kHz sound frequency to communicate the presence of negative or positive emotional states, respectively. The calling behavior may be influenced by several factors, including environmental factors. Likewise, pain behavior can be modulated according to the social context, and also can be transferred to conspecifics through direct observation and/or social interaction. Herein we investigated if acute pain induction was related to changes in emission of aversive and appetitive calls and how different social contexts affected the nociceptive behavior and USVs. Our results demonstrated that orofacial formalin injection in rats induced aversive calls in addition to the nociceptive behavior, and both are reduced by systemic treatment with morphine (2.5 mg/kg). Exposure of formalin-injected rats to cagemates had no effect on the nociceptive behavior or calls emitted by the demonstrator, but the observer showed emotional contagion of pain. In contrast, exposure of formalin-injected rats to non-cagemates decreased the nociceptive behavior of the demonstrator, without affecting the calls emission. The emotional contagion was not detected in non-cagemates or in cagemates separated by a visual barrier. In conclusion, we suggest that familiarity and the visual contact contributes to emotional contagion of pain. USV analysis may

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represent an additional measure in the evaluation of the emotional aspect of orofacial pain, and for the study of pain modulation.

1. Introduction

Rodents emit ultrasonic vocalizations (USVs) i.e., above the human hearing threshold of about 20 kHz, to express positive or negative emotional states and to coordinate social interactions (for review see Wöhr and Schwarting, 2013; Simola and Granon, 2018). It is well characterized that laboratory rats emit 50-kHz USV in appetitive situations, such as playing behavior in juvenile rats, mating or in response to psychostimulants, whereas 22-kHz USVs may index a state of negative activation, including stressful and painful situations (Jourdan et al., 1995; Knutson et al., 1998; Dinh et al., 1999; Knutson et al., 2002; Brudzynski, 2005, 2009). Thus, aversive 22-kHz USV and appetitive 50-kHz USV serve distinct communicative functions as they reflect distinct patterns of brain activation (Wöhr and Schwarting, 2013). It is also noteworthy that at least three subtypes of appetitive calls have been described, (1) flat, (2) frequency modulated and (3) trill calls, which are believed to be emitted in different social contexts and affective states (Brudzynski, 2015).

Pain research has been largely based on evaluation of behavioral responses to distinct modalities of noxious stimuli. However, pain is a multidimensional experience, which involves affective and motivational features that may be discriminated from its sensory and discriminative qualities (for review, see Fields, 1999; Porreca and Navratilova, 2017). In this context, the identification of USVs in rats submitted to different pain models may represent a new approach for understanding the brain circuitry underlying mammalian affective states, as well as a useful tool in the development of suited pharmacological therapies (Haney and Miczek, 1994; Knutson et al., 2002; Simola and Granon, 2018).

In fact, substantial increase in the emission of 22-kHz USVs in rats has been demonstrated in different pain models, including experimental arthritis, muscle pain, experimental migraine, tail electric stimulation, tail flick and the paw formalin test (Calvino et al., 1996; Jourdan et al., 1998; Dinh et al., 1999; Vivian and Miczec, 1999; Han and Neugebauer, 2005; Han et al., 2005; Oliveira and Barros; 2006; Martino and Perkins, 2008; Bobade et al., 2015). Some of these studies have also shown that the emission of aversive USV is significantly reduced by potentially analgesic drugs, suggesting that USV analysis can be used to predict the effectiveness of new analgesic agents in reducing pain-induced affective discomfort (Calvino et al., 1996; Jourdan et al., 1998; Oliveira and Barros, 2006; Martino and Perkins, 2008; Bobade et al., 2015). In spite of these observations, to the best of our knowledge, there is no study evaluating USV emission in orofacial pain models. Moreover, most pain studies that evaluated USVs limited their analysis to aversive calls, without considering that the pain state may modify the emission of appetitive calls. Changes in the emission of 50-kHz may indicate mood changes and/or impaired social interaction, conditions that have been frequently associated to pain states (Simola, 2015; Karos et al., 2018)

Besides, there is growing evidence that USVs can elicit emotional contagion, a form of empathy, in which an individual acquires the emotional state of another via social cues. For instance, it has been shown that rats subjected to a cognitive task signaled with a positive outcome after hearing appetitive calls from conspecifics, but responded with a negative outcome if previously exposed to aversive calls (Saito et al., 2016). It is widely accepted that empathy may modify the perception of pain in rodents (Langford et al., 2006; Li et al., 2014; Lu et al., 2018), nonetheless, the influence of changes in the social context in the emission of USVs in pain models has not yet been investigated.

Here, we tested the hypothesis that the formalin test applied to the orofacial region induces facial grooming behavior and increases the emission of aversive calls, which are both amenable to reduction by systemic morphine. We also explored the emission of aversive and appetite calls in rats subjected to the orofacial formalin test in a variety of social contexts, including, isolated rat, rat in the presence of a cagemate and a non-cagemate conspecific.

2. Material and methods

2.1. Animals

The experiments were conducted on 536 male Wistar rats (weighing 280–320 g, 8–9 weeks old) provided by Federal University of Parana. All animals were housed together for at least 1 week before testing, in groups of 4, under controlled conditions of light (12:12 h light: dark cycle; lights on 7:00 a.m.) and temperature (22± 1 °C), and with chow and water ad libitum. All experiments were conducted between 9 a.m. and 4 p.m. and animals were randomly assigned to the different groups. All experimental protocols were approved by the Institutional Committee on the Ethical Use of Animals (authorization # 1151) and conducted in accordance with the Brazilian regulations on animal welfare.

2.2. Orofacial formalin test

This test was conducted as previously described (Chichorro et al., 2004). Briefly, each animal was placed in an individual plastic cage (clear acrylic box, 40 × 40 × 40 cm), and left to adapt to the environment for at least 15 min. Subsequently, each animal was gently held and received a subcutaneous 50 μL injection of 2.5% formalin (Alphatec, Brazil) or vehicle (0.9% sterile saline) into the right upper lip and was returned immediately to the observation cage. For the injection, it was used BD (Beckton-Dickinson) ultra-fine syringes (0.5 mL/cc) with short needles (30G: 0,30 × 8 mm). The animals were recorded for 30 min for subsequent analysis of the facial grooming time, which was considered the time (in seconds) each animal spent rubbing the injected area with its forepaws. The first and second phases of the formalin response were considered to be 0–3 min and 12–30 min after the formalin injection, respectively. Concomitantly, the emission of ultrasonic vocalizations was recorded, as described in the below section.

2.3. Analysis of ultrasonic vocalization (USV)

One rat was tested at a time following the methodology previously described by Oliveira and Barros (2006), with minor modifications. The emission of USV by the subject animal was recorded by an ultrasound gate condenser microphone CM16 (Avisoft Bioacoustics, Berlin, Germany), sensitive to frequencies between 15 and 180 kHz. This apparatus was mounted 45 cm above the observation cage and connected to a computer with the recording software Avisoft Recorder (version 2.95, Avisoft Bioacoustics). Experiments were done under 40 lx illumination, with USVs being recorded for 30 min immediately after the injection of 2.5% formalin or vehicle, subcutaneously into the upper lip. In order to decrease the aversivennes of the test situation and facilitate the detection of USV, the observation cage contained fresh bedding (Natusch and Schwarting, 2010). The effect of a conspecific was assessed by the presence or absence of a second animal put into another acrylic box, 60 cm apart from the experimental subject. This protocol was repeated in rats subjected to different social contexts, as described below. The distance used to separate the animals allowed to evaluate the influence of USV emission in the observer, but the recording of the calls was limited only to one rat (i.e. observer or demonstrator).

Analysis of USV emitted by the experimental rat was made afterwards, using the AvisoftSASLab Pro software (version 4.34; Avisoft Bioacoustics). For manual counting and classification of USV, spectrograms from the recordings were generated at a frequency resolution of 488 Hz and a time resolution of 0.512 ms (Pereira et al., 2014; Wendler et al., 2019; Natusch and Schwarting, 2010). USV of 22 kHz are calls in the 18–32 kHz range of frequency, emitted either in bouts or individually (Wohr et al., 2015). The separation between one call and another was defined by an inter-call interval of at least 190–320 ms, which is the time for inhalation between two voiced calls. All USVs above 33 kHz were regarded as 50 kHz USV, and their classification into subtypes was based on their spectrographic shapes, being called flat when peak frequency changes within a single call were equal or lower than 5 kHz; step, when a main flat call had also another short flat element was shifted 5 kHz higher in frequency; trill, a single call element with peak frequency changes inopposed directions at least 5 kHz apart forming a "zig-zag"; and mixed, which were calls that did not fit in other categories (Pereira et al., al., 2014; Wöhr et al., 2015; Wendler et al., 2019).

2.4. Experimental procedures

The term "isolated" refers to rats placed alone only for the conduction of experiments; the term "cagemates" refers to rats taken from the same cage; "non-cagemates" refers to rats taken from different cages, "cagemates separated by visual barrier" refers to rats taken from the same cage, and using a white side of the cage as a non-transparent barrier between them during the testing. The term "demonstrator" is used to define the rats that received formalin or saline injection. The term "observer" refers to naive rats in presence of an injected rat with saline or formalin. All rats were tested only once.

Firstly, to assess whether orofacial formalin injection was able to evoke USV and facial grooming behavior in isolated rats, animals were administered 50 μL of formalin (2.5%) or vehicle subcutaneously into the upper lip and, immediately, placed on the observation cage. A separated group of animals received morphine (2.5 mg/kg s.c. diluted in 0.9% sterile saline – Merck S.A-São Paulo-Brazil) or vehicle (0.9% sterile saline, 1 mL/kg, s.c.) 30 min before formalin administration. In the observation box, facial grooming behavior was videotaped concomitantly to the ultrasonic vocalization recording, both for 30 min (Supplementary Fig. 1(A)).

Secondly, to evaluate whether the presence of other animals was able to change USV emission and grooming behavior by the demonstrator, experimental animals were subjected to the same protocol, but while under the effect of formalin, they were also exposed to cagemates, non-cagemates, and cagemates separated by visual barrier (Supplementary Fig. 1(B)–(D)). Both grooming behavior and USV emission were recorded in the presence of another animal, with the boxes of the subject and the other animal separated by 60 cm distance.

Lastly, to analyze whether the presence of a demonstrator that received formalin or saline injection affects USV emission and grooming behavior of the observer rats, the later was placed in the observation cage, in different social contexts: in the presence of cagemates, non-cagemates and cagemates separated by visual barrier (Supplementary Fig. 2). One additional group of cagemates was tested in grid cages, instead of plastic cage walls, to facilitate the transfer of auditory information (Supplementary Fig. 2). Both, grooming behavior and USV emission by the observer were recorded in the presence of another animal, with the boxes of the subject and the other animal separated by 60 cm distance. At the end of the experiments, each animal was placed in a new box to prevent contact with cagemates that were not subjected to the procedure.

2.5. Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M.) of 6–10 animals per group. Two-way ANOVA with Bonferroni post hoc test was used to analyze the time-course of facial grooming behavior and the number of 22-kHz calls in each time point, with treatment and time as independent factors. One way ANOVA with Bonferroni post hoc test was used to analyze the cumulative grooming response for phase I (0–3 min) and phase II (12–30 min) in the isolated group. Phase I and phase II cumulative response in all other experimental groups was analyzed with unpaired *t*-test, which was also applied to analyze the total number of 22-kHz and 50-kHz calls. Two way ANOVA followed by Bonferroni post hoc test was employed in the analysis of subtypes of 50-kHz calls. Data were transformed in $y=\log+1$ function when F test to compare variances demonstrates $P < 0.05$.

3. Results

The main findings of the study are summarized on Supplementary Table 1.

3.1. Analysis of facial grooming behavior and USVs induced by formalin in the isolated rat

Subcutaneous administration of formalin into the upper lip of isolated rats induced an increase in the facial grooming behavior (time factor: $F_{(9,270)} = 3.289$, $P = 0.0008$; treatment factor: $F_{(3,270)} = 55.44$, $P < 0.0001$; interaction factor: $F_{(27,270)} = 2.573$, $P < 0.0001$, Fig. 1(A)). There was a significant increase in the grooming time in phase I (0–3 min, $F_{(3,28)} = 5.678$, $P = 0.0036$) and phase II (12–30 min, $F_{(3,28)} = 26.07$, $P < 0.0001$) after formalin injection (Fig. 1(B)). Previous treatment with morphine was able to inhibit facial grooming behavior in both phases (Fig. 1(A) and (B)). The concomitant USV analysis indicated significant increase of 22-kHz calls during the phase I, which was also prevented by morphine (time factor: $F_{(9,210)} = 3.474$, $P = 0.0005$; treatment factor: $F_{(3,210)} = 11.09$, $P < 0.0001$, interaction factor:

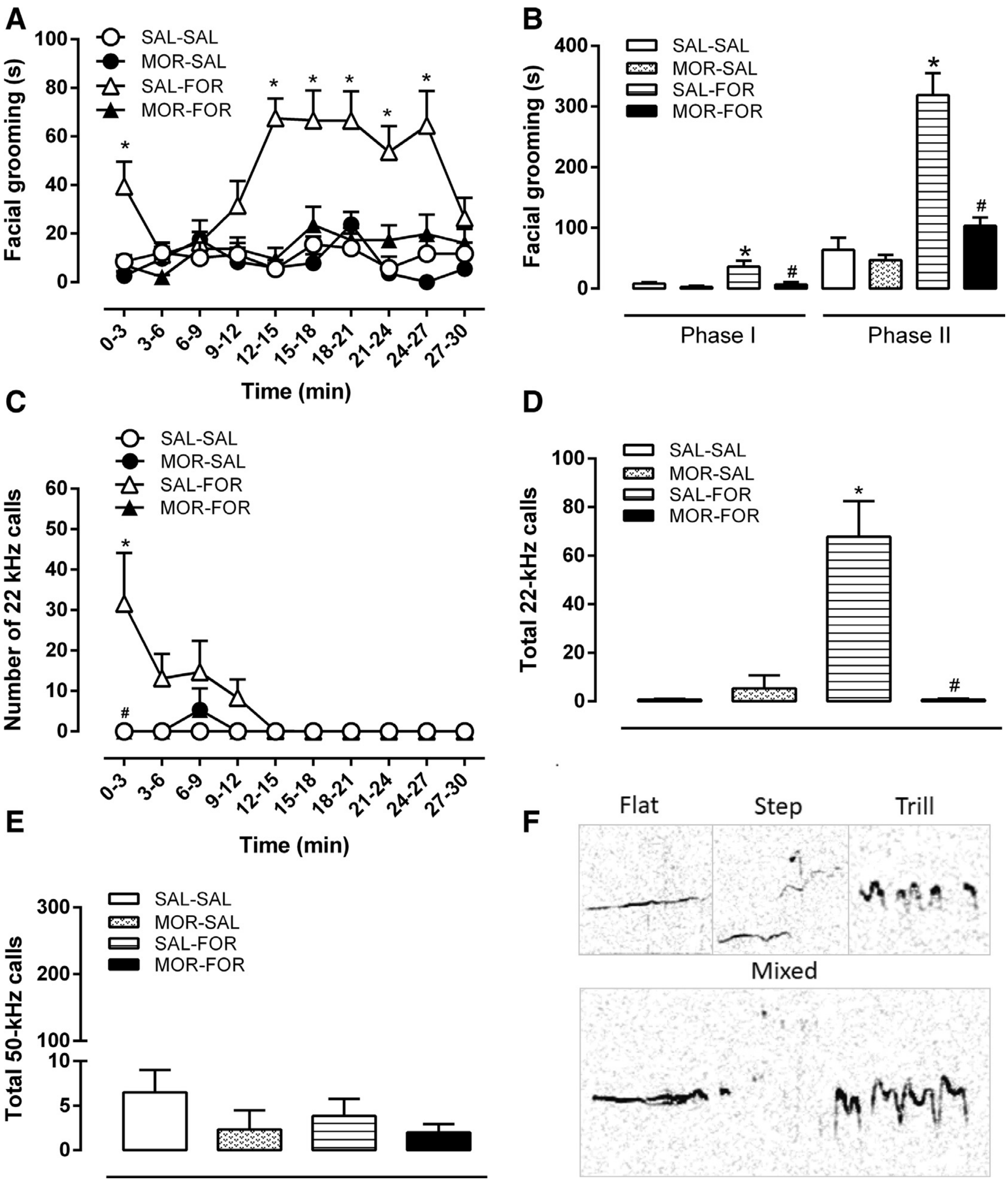

Fig. 1 Facial grooming behavior and USV analysis after upper lip administration of formalin in isolated rats. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response for phase I and phase II. (C) Number of 22-kHz calls in each time point after formalin injection. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Representative examples of 50-kHz calls subtypes. $^{*}P \leq 0.05$ vs SAL-SAL, $^{\#}P \leq 0.05$ vs SAL-FOR ($n = 6$-10/group). Two-way ANOVA (A and C) and One-way ANOVA (B, D and E) followed by Bonferroni multiple comparisons post-hoc test. All graphs show mean ± SEM (*SAL*, saline; *FOR* formalin; *MOR*, morphine).

$F_{(27,210)} = 2.874$, $P < 0.0001$, Fig. 1(C)). The total number of 22-kHz calls was significantly increased in formalin-compared to saline-injected rats. Morphine pre-treatment failed to change the USVs emission in saline-injected rats and abolished the emission of aversive USVs by rats that received formalin ($F_{(3,25)} = 19.68$, $P < 0.0001$, Fig. 1(D)). There was no significant difference in the emission of 50-kHz calls among all the groups ($F_{(3,25)} = 1.277$, $P = 0.3037$, Fig. 1(E)). Representative examples of 50-kHz call subtypes are demonstrated in Fig. 1(F). No significant differences were found in 50-kHz subtypes among all the groups (USV factor: $F_{(3,112)} = 4.634$, $P = 0.004$; treatment factor: $F_{(3,112)} = 3.20$, $P = 0.026$; interaction factor: $F_{(9,112)} = 2.209$, $P = 0.026$, data not shown).

3.2. *Influence of the social context on the facial grooming behavior and USVs induced by formalin:* analysis of cagemates

The time course analysis of facial grooming of the demonstrator in the presence of a cagemate observer revealed

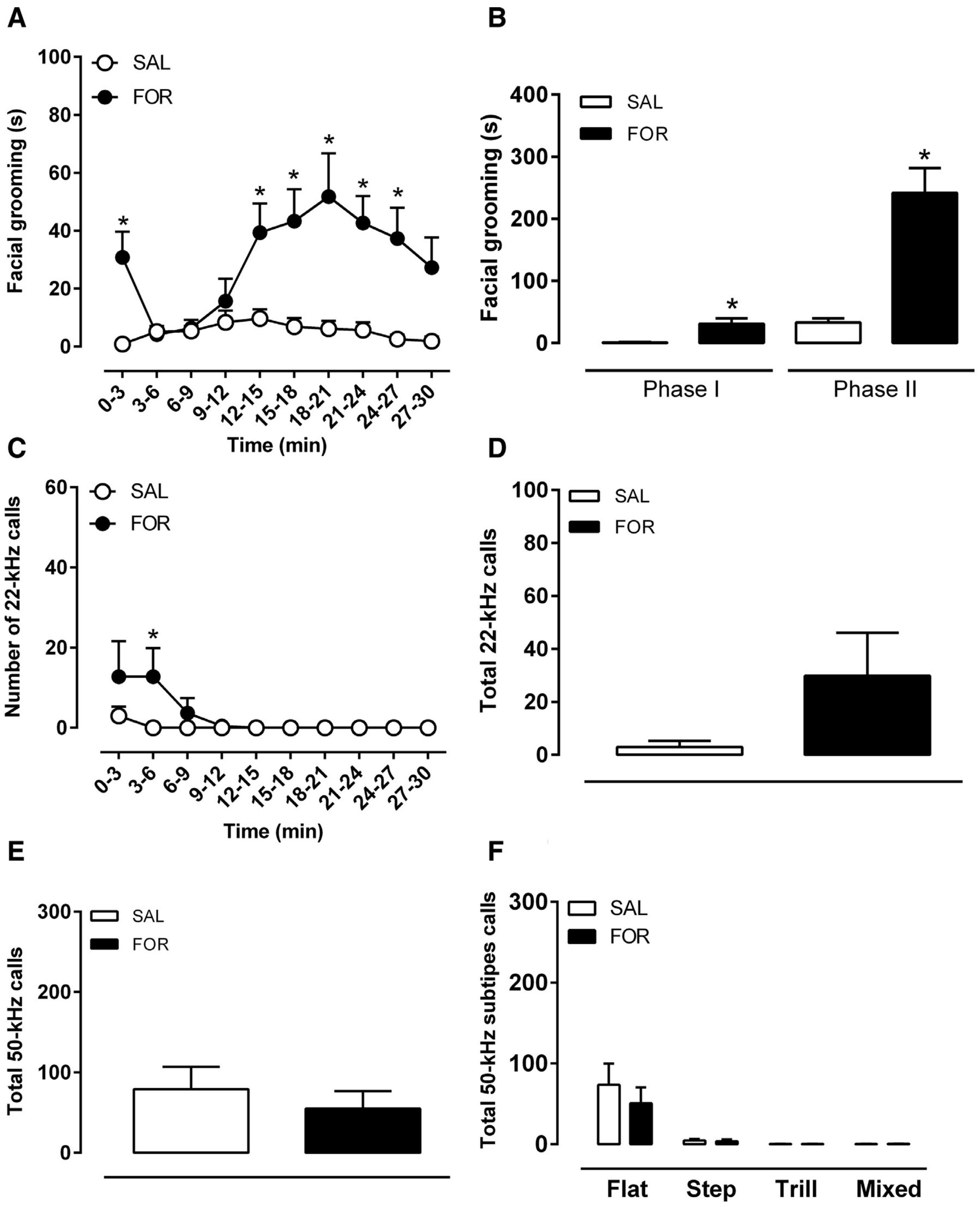

Fig. 2 Facial grooming behavior and USV analysis in the demonstrator after upper lip administration of formalin in the presence of cagemates. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response for phase I and phase II induced by formalin in the demonstrator. (C) Number of 22-kHz calls in each time point after formalin injection in the demonstrator. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: flat, step, trill and mixed emitted by the demonstrator. $^*P \leq 0.05$ vs SAL (n = 8-10/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired t-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

a biphasic response, that seemed not differ from isolated rats (time factor: $F_{(9,180)} = 2.921$, $P = 0.003$; treatment factor: $F_{(1,180)} = 62.97$, $P < 0.001$; interaction factor: $F_{(9,180)} = 2.786$, $P = 0.0044$, Fig. 2(A)). Formalin-injected rats in the presence of a cagemate observer displayed a significant phase I and phase II of response ($t = 2.09$, $P = 0.05$ and $t = 5.169$, $P < 0.0001$, respectively, Fig. 2(B)). A significant increase of 22-kHz calls was found from 3 to 6 min after formalin injection (time factor: $F_{(9,180)} = 2.428$, $P = 0.0126$; treatment factor: $F_{(1,180)} = 4.908$, $P = 0.028$, Fig. 2(C)), but

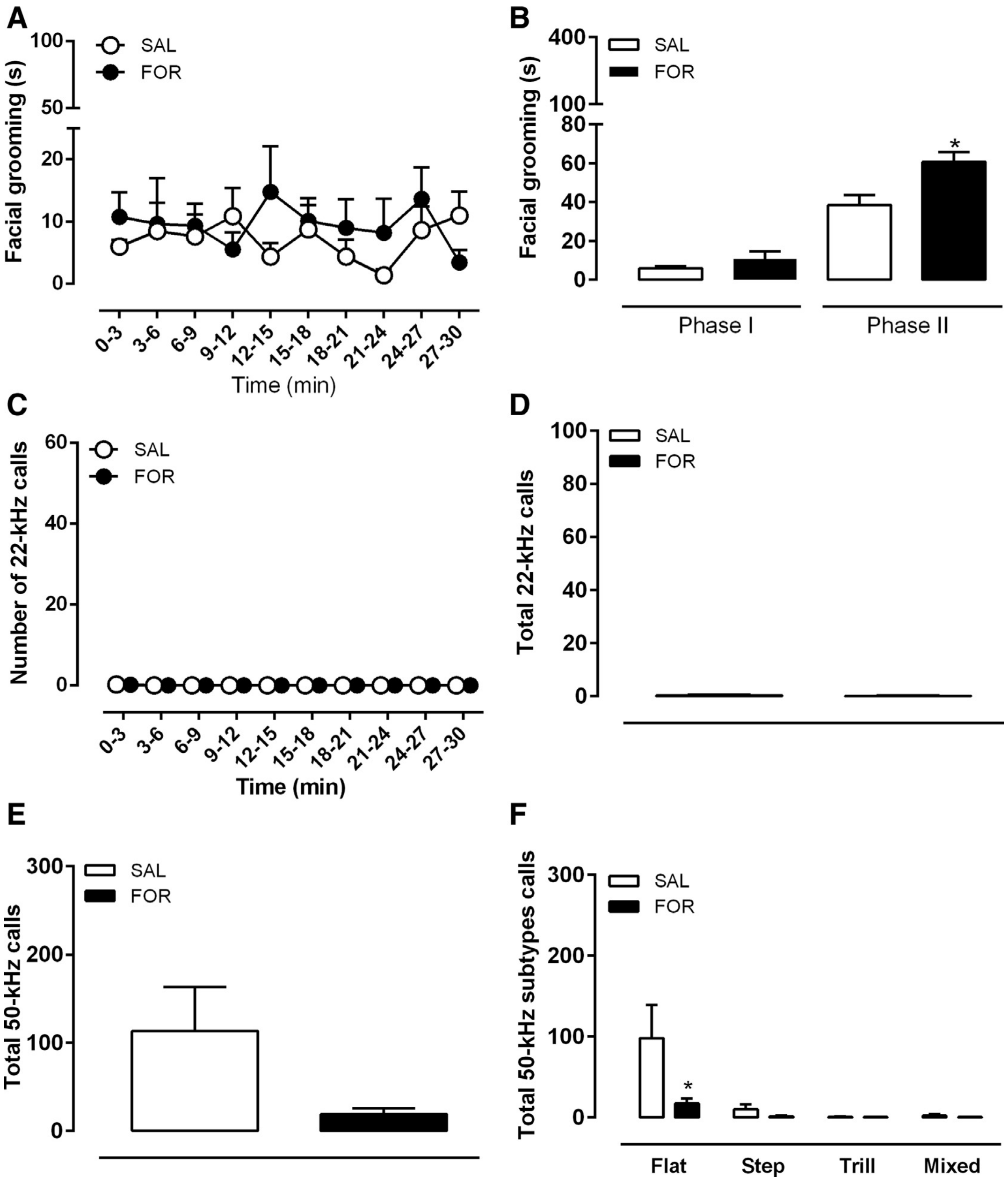

Fig. 3 Facial grooming behavior and USV analysis in the observer after upper lip administration of formalin in the cagemate demonstrator. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response. (C) Number of 22-kHz calls in each time point. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: flat, step, trill and mixed emitted by the observer. $^{*}P \leq 0.05$ vs SAL ($n = 7$-8/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired t-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

the total number of 22-kHz and 50-kHz calls were not statistically different between groups ($t = 1.05$, $P = 0.305$ and $t = 0.688$, $P = 0.499$, respectively, Fig. 2(D) and (E)). No significant differences were found in 50-kHz subtypes (USV factor: $F_{(3,72)} = 13.76$, $P < 0.0001$; treatment factor: $F_{(1,72)} = 0.501$, $P = 0.4814$; interaction factor: $F_{(3, 72)} = 0.4740$, $P = 0.7014$, Fig. 2(F)).

The time course analysis did not demonstrate difference in the grooming time of the cagemate observer in the presence of a saline- or a formalin-injected rat (time factor: $F_{(9,150)} = 20.3359$, $P = 0.9618$; treatment factor: $F_{(1,150)} = 1.451$, $P < 0.2303$; interaction factor: $F_{(9,150)} = 0.7979$, $P = 0.6187$, Fig. 3(A)). However, when both phases were evaluated separately, it was detected an increase in the facial grooming time in the period that corresponds to the phase II of the formalin test ($t = 3.074$, $P = 0.0073$), but no difference in the phase I ($t = 0.6093$, $P = 0.5514$, Fig. 3(B)).

The cagemate observer did not emit 22-kHz calls (Fig. 3(C) and (D)). There was no difference in the emission of 50-kHz calls by the cagemate observer in the presence of a saline- or a formalin-injected rat ($t = 1.923$, $P = 0.075$, respectively, Fig. 3(E)). When the subtypes of 50-kHz calls were evaluated, there was a decrease of flat calls emitted by cagemate observers in the presence of formalin-demonstrator rats (USV factor: $F_{(3,56)} = 9.08$, $P = 0.0001$; treatment factor: $F_{(1,56)} = 6.196$, $P = 0.015$; interaction factor: $F_{(3,56)} = 4{,}45$, $P = 0.007$, Fig. 3(F)).

When tested in a grid box, the cagemate observer in the presence of a formalin-injected rat showed similar results. It was detected an increase in the facial grooming during the time course evaluation of the formalin-induced response (time factor: $F_{(9,130)} = 4.474$, $P < 0.0001$; treatment factor: $F_{(1,130)} = 3.113$, $P < 0.08$; interaction factor: $F_{(9,130)} = 5.8$, $P < 0.0001$, Fig. 4(A)). Likewise, the total grooming time was increase in the period corresponding to the phase II of the formalin response ($t = 2.844$, $P = 0.0138$, Fig. 4(B)), but not in the period corresponding to phase I ($t = 0.5870$, $P = 0.5673$). However, no emission of 22-kHz calls was detected (Fig. 4(C) and (D)). In the grid box, the cagemate observer showed marked emission of 50-kHz calls in the presence of a saline-injected rat, which was significantly reduced by the presence of a formalin-demonstrator rat ($t = 2.135$, $P = 0.05$, respectively, Fig. 4(E)). The analysis of 50-kHz calls subtypes revealed a decrease of flat calls emitted by cagemate observers in the presence of formalin-demonstrator rats compared to saline-injected rats (USV factor: $F_{(3,52)} = 26.25$, $P = 0.0001$; treatment factor: $F_{(1,\ 52)} = 5.34$, $P = 0.024$; interaction factor: $F_{(3,\ 52)} = 4.5$, $P = 0.007$, Fig. 4(F)).

3.3. *Influence of the social context on the facial grooming behavior and USVs induced by formalin:* analysis of cagemates separated by a visual barrier

The visual barrier did not influence the time course of formalin response in formalin-injected-rats in the presence of cagemates when compared with isolated rats (time factor: $F_{(9,160)} = 4.665$, $P < 0.0001$; treatment factor: $F_{(1,160)} = 127.4$, $P < 0.0001$; interaction factor: $F_{(9,160)} = 3.94$, $P = 0.0001$, Fig. 5(A)). Demonstrator rats showed significant phase I and phase II responses ($t = 3.845$, $P = 0.0014$ and $t = 8.298$, $P < 0.0001$, respectively, Fig. 5(B)). However, there was no emission of 22-kHz calls by formalin-demonstrator rats or control rats (Fig. 5(C) and (D)). Moreover, there was no significant difference in the total number of 50-kHz calls between groups ($t = 2.01$, $P = 0.061$, Fig. 5(E)), but formalin-injected rats showed a significant reduction in the emission of flat calls (USV factor: $F_{(3,\ 72)} = 9.909$, $P < 0.0001$; treatment factor: $F_{(1,\ 72)} = 3.56$, $P = 0.063$; interaction factor: $F_{(3,\ 72)} = 3.037$, $P = 0.034$, Fig. 5(F)).

Cagemate observers separated from formalin or saline-demonstrator rats by a visual barrier did not show changes in the time course of the grooming behavior (time factor: $F_{(9,150)} = 1.542$, $P = 0.1380$; treatment factor: $F_{(1,150)} = 0.2680$, $P = 0.6054$; interaction factor: $F_{(9,150)} = 1.285$, $P = 0.2492$, Fig. 6(A)), as well as in the period that corresponded to phase I and phase II of formalin test ($t = 1.044$, $P = 0.312$ and $t = 0.129$, $P = 0.898$, respectively Fig. 6(B)). Emission of 22-kHz calls in the presence of formalin- or vehicle-demonstrator rats was not detected (Fig. 6(C) and (D)). They also did not show differences in the emission of total 50 kHz calls ($t = 0.118$, $P = 0.9069$, Fig. 6(E)), as well as in the emission of 50-kHz subtypes (USV factor: $F_{(3,60)} = 17.44$, $P < 0.0001$; treatment factor: $F_{(1,60)} = 0.017$, $P = 0.8938$; interaction factor: $F_{(3,60)} = 0.025$, $P = 0.9945$, and Fig. 6(F)).

3.4. *Influence of the social context on the facial grooming behavior and USVs induced by formalin:* analysis of non-cagemates

The time course analysis of facial grooming of the demonstrator in the presence of a non-cagemate observer revealed a significant increase in the facial grooming time of formalin injected rats compared to saline only at 24-27 min (time factor: $F_{(9,162)} = 2.189$, $P < 0.0253$; treatment factor: $F_{(1,18)} = 6.78$, $P < 0.017$; interaction factor: $F_{(9,162)} = 1.58$, $P < 0.0001$, Fig. 7(A)). In spite of that, there was still a significant increase in the amount of grooming in the second phase ($t = 3.555$, $P = 0.0026$, Fig. 7(B)), but not in the phase I of the response ($t = 1.001$, $P = 0.3302$).

Formalin-demonstrator rats showed an increase of 22-kHz calls from 3 to 6 min in the presence of non-cagemates rats (time factor: $F_{(9,117)} = 3.68$, $P < 0.0004$; treatment factor: $F_{(1,\ 13)} = 3.32$, $P = 0.091$; interaction factor: $F_{(9,\ 117)} = 3.96$, $P < 0.0002$, Fig. 7(C)). The total number of 22-kHz calls was not significantly increased compared to control rats ($t = 1.126$, $P = 0.2821$, Fig. 7(D)), and there was no difference in the total number of 50-kHz calls ($t = 0.62$, $P = 0.546$, Fig. 7(E)) or in the number of each 50-kHz call subtypes (USV factor: $F_{(3,33)} = 18.77$, $P < 0.0001$; treatment factor: $F_{(1,11)} = 0.2773$, $P = 0.608$; interaction factor: $F_{(3,33)} = 0.2288$, $P = 0.8757$, Fig. 7(F)).

The time course analysis of facial grooming of the observer in the presence of a non-cagemate demonstrator did not show a difference in the facial grooming time between saline- and formalin-injected rats (time factor: $F_{(9,170)} = 1.07$, $P = 0.3873$; treatment factor: $F_{(1,17)} = 1.436$, $P = 0.2324$; interaction factor: $F_{(9,170)} = 0.9594$, $P = 0.4755$, Supplementary Fig. 3(A)). The analysis of non-cagemate observers demonstrated no difference in the facial grooming behavior in the periods corresponding to the phase I and phase II of the test ($t = 1.193$, $P = 0.07$ and $t = 1.263$, $P = 0.223$, Supplementary Fig. 3(B)). There was no emission of 22-kHz calls (Supplementary Fig. 3(C) and (D)). Moreover, there was no difference in the emission of total 50-kHz calls between groups ($t = 1.402$, $P = 0.1788$; Supplementary Fig. 3(E)), as well in the 50-kHz calls subtypes (USV factor: $F_{(3,68)} = 5.491$, $P = 0.0019$; treatment factor: $F_{(1,68)} = 0.0523$, $P = 0.8197$; interaction factor: $F_{(3,68)} = 0.028$, $P = 0.9934$, Supplementary Fig. 3(F)).

4. Discussion

Formalin typically produces a biphasic pain response, with the first phase thought to be mediated by direct activation of nociceptors and the second phase mediated by the development of central sensitization due to the ongoing afferent fiber activity and localized inflammation (Tjolsen et al., 1992). Both phases of the formalin response were reduced by systemic morphine (Clavelou et al., 1989). In line with these observations, our data demonstrated that formalin injection to a single rat resulted in a biphasic nociceptive

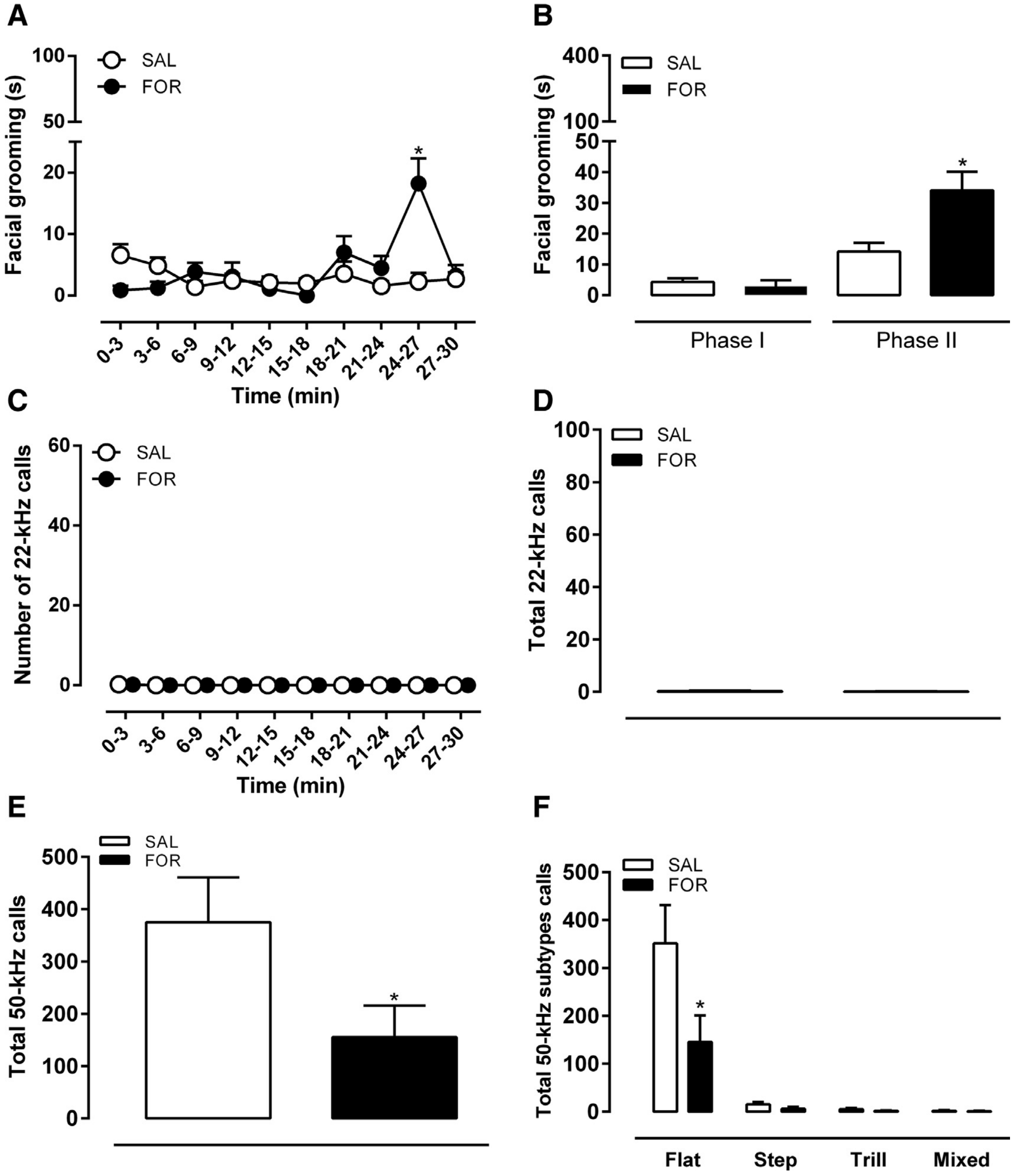

Fig. 4 Facial grooming behavior and USV analysis in the observer after upper lip administration of formalin in the cagemate demonstrator in a grid box. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response. (C) Number of 22-kHz calls in each time point. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: flat, step, trill and mixed emitted by the observer. $^{*}P \leq 0.05$ vs SAL ($n = 8$–10/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired *t*-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

response, which was sensitive to morphine. In addition, our results showed that in parallel to the first phase of grooming behavior, formalin-injected rats showed an increase in the emission of 22 kHz-calls, which were also abolished by morphine pre-treatment (Fig. 1). Oliveira and Barros (2006) had previously shown that paw injection of formalin resulted in a significant increase in the number of aversive calls, which were significantly reduced by morphine treatment. The 22-kHz calls serve as warning and alarm calls and express negative and/or aversive state (Brudzynski, 2013). Considering that in both formalin studies the increase in the number of USVs was detected in the first minutes after formalin injection, it is plausible to suggest that USVs may serve to warn the conspecifics about the danger and reflect the aversiveness of the stimuli. Morphine treatment reduced aversive USV, indicating that it may represent a measure of the emotional aspect of pain, but failed to affect the number of appetitive USV.

When the same analyses were performed in the presence of a cagemate, there was no change in the nociceptive

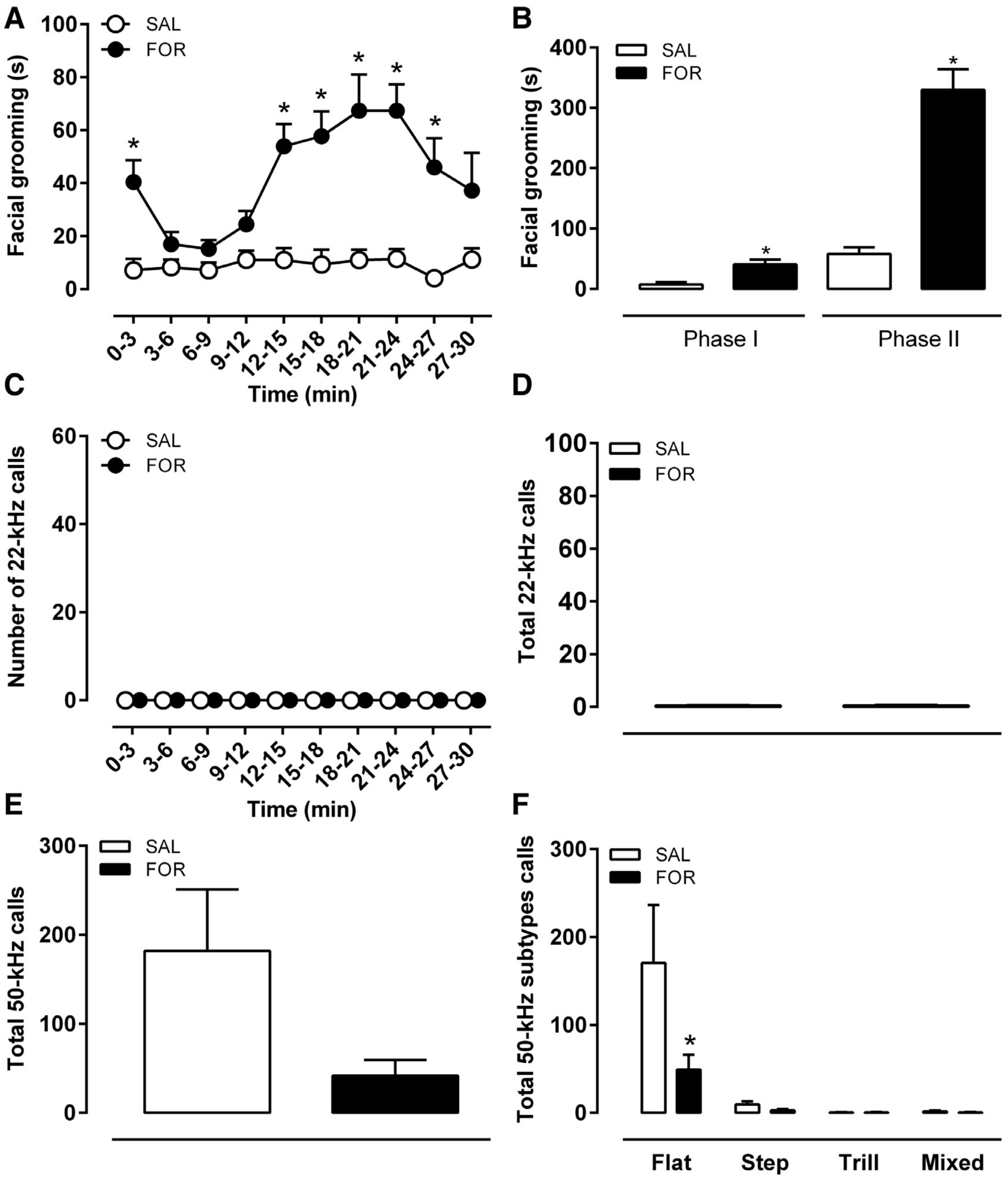

Fig. 5 Facial grooming behavior and USV analysis in the demonstrator after upper lip administration of formalin in the presence of a cagemate separated by visual barrier. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response for phase I and phase II induced by formalin. (C) Number of 22-kHz calls in each time point. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: flat, step, trill and mixed emitted by the demonstrator. $^*P \leq 0.05$ vs SAL ($n = 8$-10/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired *t*-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

behavior displayed by the demonstrator rat. In addition, the emission of aversive calls was also detected in the first minutes after formalin injection (Fig. 2). These data indicate that the presence of a cagemate observer does not influence the nociceptive behavior and the emission of aversive calls by the demonstrator. In fact, it has been reported that the intensity of the alarm calls is dependent on the danger, but independent of the proximity of other conspecifics (Litvin et al., 2007). However, it is important to point out, that in the presence of the observer; demonstrator rats emitted a significant number of 50 kHz-calls. It is widely accepted that these calls are emitted by rats in rewarding situations or in expectation of them, including social encounters with conspecifics (Burgdorf et al., 2008; Brudzynski, 2013; Rippberger et al., 2015).

The influence of the context was detected on the observer's behavior, which showed a significant increase in the time spent performing the facial grooming in the period that corresponds to the second phase of the formalin response (Fig. 3). This data corroborates previous

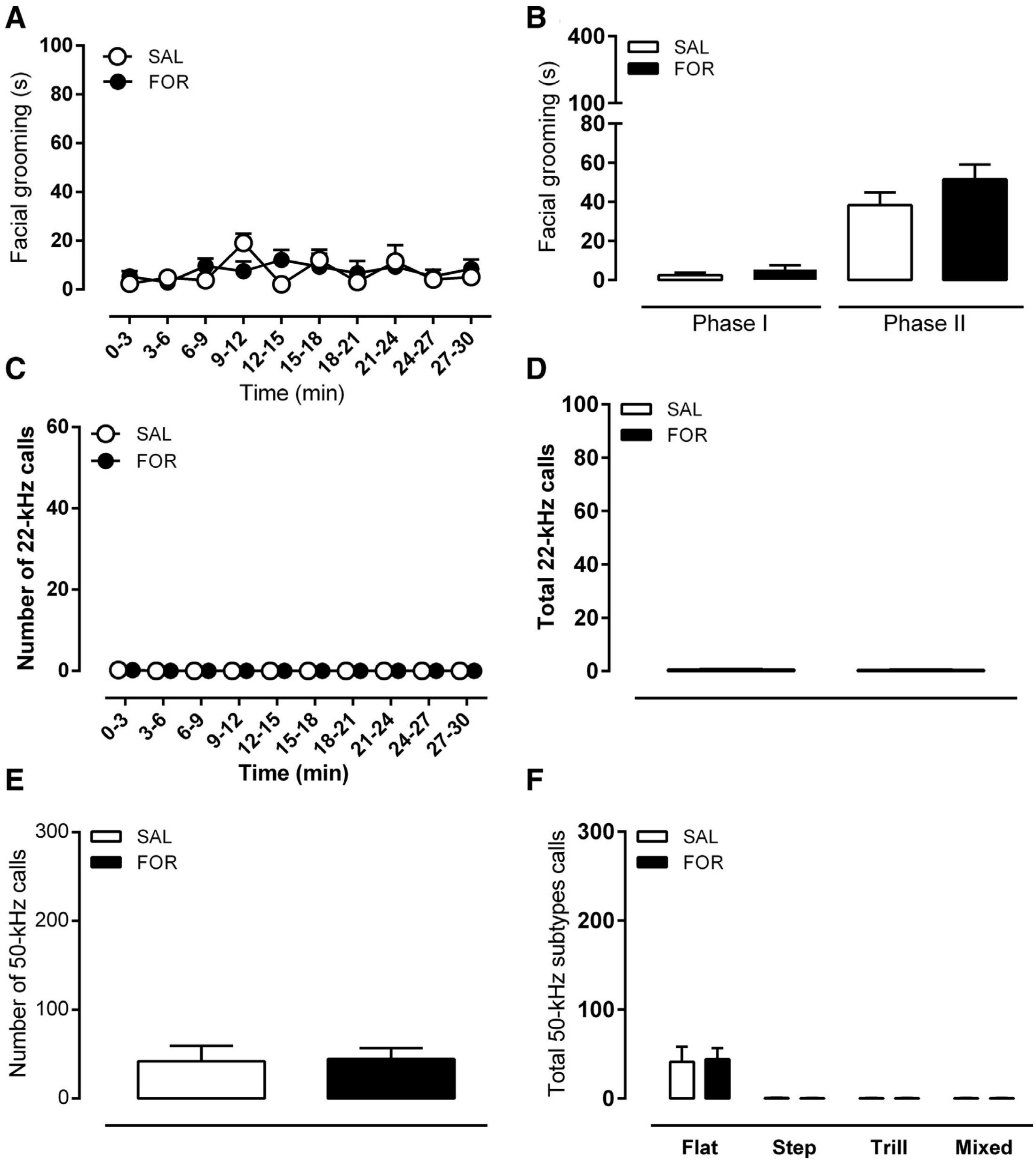

Fig. 6 Facial grooming behavior and USV analysis in the observer after upper lip administration of formalin in the cagemate demonstrator separated by a visual barrier. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response. (C) Number of 22-kHz calls in each time point. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: Flat, step, trill and mixed emitted by the observer. *$P \leq 0.05$ vs SAL ($n = 8$–10/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired *t*-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

observations that rats are able to transfer emotional and/or behavioral states to conspecifics through direct observation and/or through social interaction. In line with this idea, it has been demonstrated that following fear-conditioning, a brief social interaction between a shocked demonstrator and a naïve cagemate observer increased the freezing behavior in the latter (Knapska et al., 2006). Pain has been also demonstrated to affect the observer's behavior. Previous studies have demonstrated that both rats and mice displaying pain behavior when exposed to a cagemate, but not a stranger, showed an increase in the sensitivity to noxious stimuli in the observer, indicating emotional contagion between the members of the dyad (Langford et al., 2006; Li et al., 2014). This idea is reinforced by the fact that non-cagemate observers do not show changes in the grooming behavior in the presence of formalin- or vehicle-injected rat (Supplementary Fig. 3).

Regarding the USVs emission, observers do not emit 22-kHz calls, which is in line with the idea that they function as alarm calls to warn conspecifics about the danger (Brudzynski, 2013). On the other hand, observers exposed to saline injected rats emitted flat 50-kHz calls,

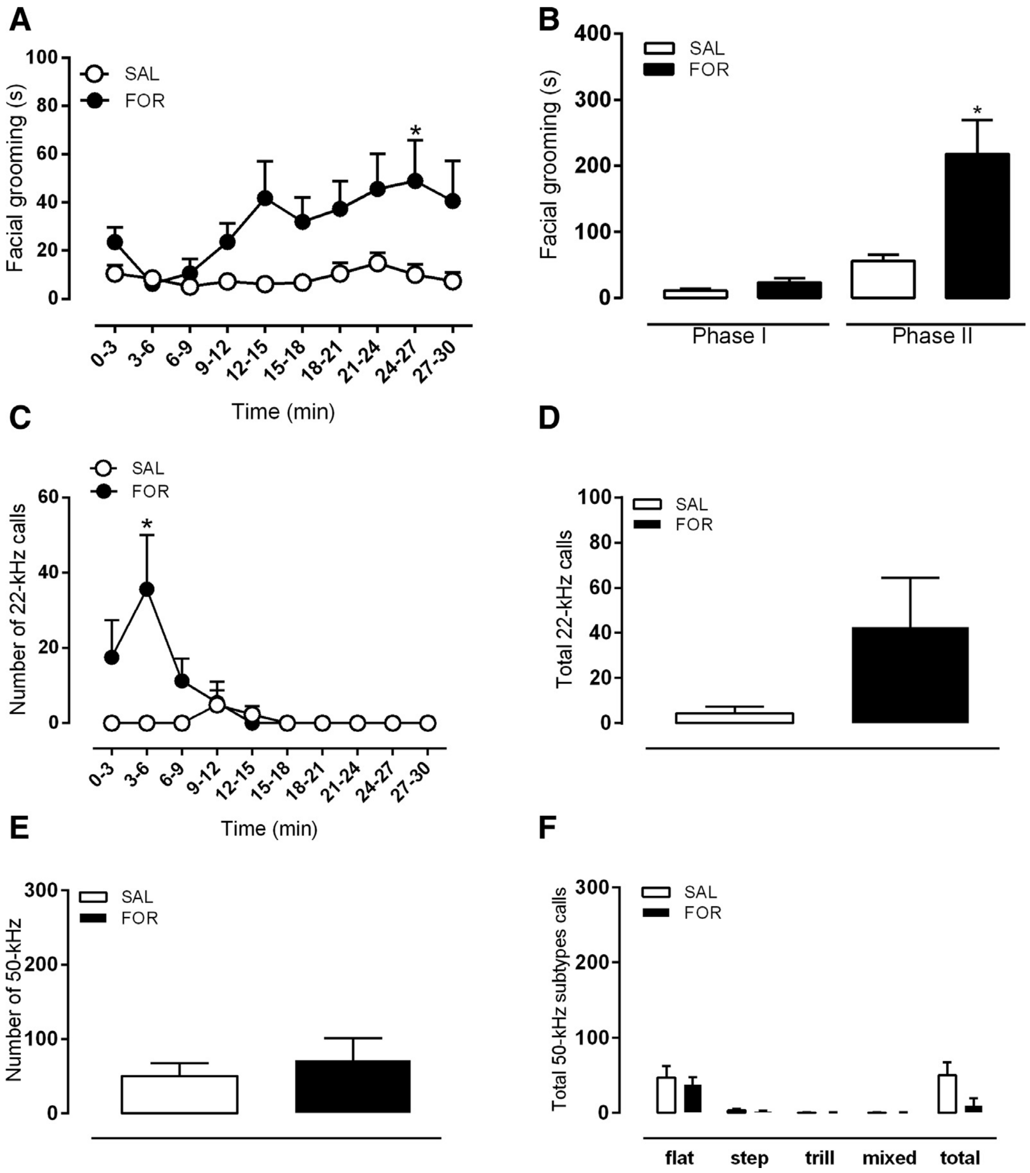

Fig 7 Facial grooming behavior and USV analysis in the demonstrator after upper lip administration of formalin in the presence of a non-cagemates. (A) Time-course of facial grooming behavior. (B) Cumulative grooming response for phase I and phase II induced by formalin. (C) Number of 22-kHz calls in each time point. (D) Total number of 22-kHz calls. (E) Total number of 50-kHz calls. (F) Total number of 50-kHz calls subtypes: Flat, step, trill and mixed mitted by the demonstrator. $^*P \leq 0.05$ vs SAL ($n = 9$–11/group). Two-way ANOVA (A, C and F) followed by Bonferroni multiple comparisons post-hoc test and unpaired *t*-test (B, D and E). All graphs show mean ± SEM (*SAL*, saline; *FOR*, formalin).

which were markedly reduced when they were exposed to formalin-injected rats (Fig. 3). The full implication of this finding remains to be clarified, but some hypotheses may be discussed. The flat subtype may serve as contact calls during social interaction and are considered to be dependent of mesolimbic dopamine system (Brudzysnki, 2015; Mulvihill and Brudzysnki, 2019). Thus, the emission of 50-kHz calls by the observer may reflect social interaction with a familiar conspecific; but when the conspecific is in pain the observer may be receiving some cues from the demonstrator that inhibit the emission of flat calls. It has been suggested that the pain state caused a significant reduction in the emission of 50 kHz calls, which may be related to decrease in dopamine release (Faramarzi et al., 2016; Siahposht-Khachaki et al., 2017; Shafiei et al., 2018). In line with this idea, a recent study demonstrated that footshocks directed to the conspecific modified the behavior and increased dopamine release, suggesting that rats may have been using cues from conspecific (e.g., vocalizations, odors, visual, etc.) to determine that the shock had been delivered to the conspecific (Lichtenberg et al., 2018).

Herein, we have tested the influence two different cues on pain behavior and USVs emission. The first was the influence of the vocalization emitted by the demonstrator on the behavior of the familiar cagemate. Vocalizations are characterized by high frequencies, meaning that they are easily reflected by objects, such as transparent and non-transparent walls. Thus, the acrylic box may restrict the transfer of the auditory information (Lopuch and Popik, 2011). Therefore, we performed the same experiments represented in Fig. 3 in grid boxes, which allow more easily the transfer of auditory information. The results showed in Fig. 4 demonstrated that auditory input from the demonstrator did not change grooming behavior or the emission of 22-kHz calls emitted by the observer, compared to the experiments performed in the acrylic boxes. Likewise, in this setting cagemate observers emitted a marked number of 50-kHz calls which were significantly reduced in the presence of a demonstrator in pain. It is important to mention that the number of 50-kHz calls emitted by observers placed in grid boxes were much higher than those emitted by observers in acrylic boxes, indicating that contact calls are influenced by the auditory stimuli. However, when placed in grid boxes, rats showed pronounced exploratory behavior, which interfere with the other analysis, mainly the pain behavior. Thus, we have opted for performing the next set of experiments in acrylic boxes.

The next cue we have addressed in the present study was visual contact, by the analysis of cagemate dyads separated by a visual barrier (Fig. 5). In this context, demonstrator rats injected with formalin continue to present a biphasic nociceptive response, but failed to emit 22-kHz calls, suggesting that emission of aversive calls in a social context depends, at least partially, of visual contact. In fact, it has been reported that social communication of pain seems to be mediated visually (Levenson and Ruef, 1992; Langford et al., 2006; Ueno et al., 2018), and to support this notion, cagemate observers separated with a visual barrier did not show changes in the grooming behavior or USV emission in the presence of a formalin-injected rat (Fig. 6). Other studies have shown that odor cues released by animals in pain are perceived by their conspecifics and modulate their behavior (Alves and Palermo-Neto, 2015; Smith et al., 2016), but this hypothesis remains to be investigated in our experimental conditions. Interestingly, the emission of 50-kHz flat calls by the demonstrator was not affected by the blockade of visual contact, suggesting that the social interaction with the conspecific does not require the visual contact.

There is evidence that pain sensitivity in mice is observed only by exposure to cagemates, but not to non-familiar ones in pain (Langford et al., 2011). Corroborating this observation, we demonstrated that rats injected with formalin in the presence of a non-familiar conspecific did not present significant grooming behavior in the period corresponding to the first phase of the test, and a less consistent second phase of response (Fig. 7). It has been suggested that the social interaction with a non-familiar conspecific induces stress response which results in an analgesic effect (Langford et al., 2011; Martin et al., 2015). Likewise, the observer exposed to a non-familiar demonstrator did not show increase in the facial grooming, indicating failure in the emotional contagion of pain. Additionally, the emission of 50-kHz calls was not significantly changed in the analysis of non-cagemate observers, suggesting that if 50-kHz flat call serves as a form of social communication, its expression may depend on whether one is in the presence of a cagemate or non-cagemate conspecific (Supplementary Fig. 3).

In conclusion, our data corroborate previous evidence that USV analysis may represent a useful and additional measure to be applied in different pain models with the potential to enhance the translational meaning of the findings, since it may reflect that affective component of pain. The results also contribute to the current understanding of how the social context may modulate pain, showing that emotional contagion of pain is related to familiarity and visual contact and that exposure to a stranger may produce analgesia, due to reasons that remain to be explored.

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Contributors

All authors designed the study and established the experimental protocols. Barroso and Araya performed the experiments. Pasquini, Araya and Barroso undertook the statistical analysis. Chichorro and Andreatini wrote the manuscript and all authors approved its submission.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgment

None.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.08.298.

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