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Ultrasonic Rat Vocalizations During the Formalin Test: A Measure of the Affective Dimension of Pain?

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The emission of ultrasonic vocalization (USV) by rats submitted to the formalin test has not yet been demonstrated. We performed two experiments to establish the formalin concentration to induce USV and the relationship of USV emission with motor behaviors and the effects of morphine and naloxone on USV during the formalin test. Male Wistar rats were used. In Experiment 1, 3 different groups of rats were subcutaneously injected with 5%, 10%, or 12.5% formalin in 1 of the anterior paws. Experiment 2 was intended to verify the effect of morphine 1, 2.5, or 5 mg/kg on USV during the 12.5% formalin test, whereas other groups of rats received naloxone 2 mg/kg with each one of the morphine doses to verify the specificity of opioid action. USV and motor behaviors were simultaneously measured in 5-min windows for 40 min, and early (0–5 min), interphase (5–20 min), and late (20–40 min) phases of the test were characterized. Vocalization was detected mostly during the interphase of the formalin test, mainly after formalin 12.5%. Morphine suppressed USV in a naloxone-reversible manner. This is a demonstration of USV during the formalin test, allowing the inclusion of an additional nonreflex behavioral measure to help characterize more clinically relevant integrated behavioral patterns in this rat model of pain.

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Unpleasant emotional feelings are integral components of pain because of their unique sensory qualities and because these qualities often occur within a threatening context. Thus, pain contains both sensory and affective dimensions and is often accompanied by desires to terminate, reduce, or escape it (1). Part of the affective unpleasant dimension of pain is made up of emotional feelings, such as distress or fear, and is linked to the intensity of the painful sensation. Therefore, pain involves both a sensory-discriminative and an affective-emotional component. The sensory component of pain has been extensively studied, whereas data about the psychological and neural mechanisms of the affective dimension of pain are still limited (1). The challenge to understand the occurrence of persistent pain after tissue trauma or inflammation is an important clinical problem, and studies of pathophysiology are being developed for both of these components.

Frequently used animal models of persistent pain are still not ideal for studying the affective-emotional component of pain and are limited because they rely on observing simple reflex limb removal after application of noxious stimuli and do not assess higher cognitive functions and the subjective qualities of pain sensation and pain unpleasantness, the integrative processes that are associated with the perception of pain. Therefore, it is desirable to develop additional behavioral measures that may help characterize a broader range of signals in models of pain. It has been proposed that ultrasonic vocalizations (USV) of rodents may serve as an indicator of affective states (2,3). Rodents produce audible vocalizations (<20 kHz) and ultrasound vocalizations (>20 kHz) (4). USV around 20–35 kHz transmit different types of aversive, unpleasant emotional states (5). These USVs are emitted by rats in distinctive unpleasant contexts, such as maternal separation in pups (6), exposure to a predator (7), startling stimuli (8), drug withdrawal states (9), stressful (3) or noxious stimulation (10), and during social interactions between animals with arthritic pain (11). Therefore, monitoring rodent USV is a potential method of measuring the negative affective components of pain (12)

As reviewed above, to this moment, rat USVs are reported in a number of significant affective situations, including in models of pain. However, USVs are sometimes said not to occur in persistent (13) or chronic models of pain (14). The formalin test is currently one of the most useful chemical models (15);

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however, to mimic acute clinical pain, it is still required to develop the testing of an affective state during the formalin test. In one recent study with negative results (13), only one formalin dose was used in a limited number of individuals, and no USV was correlated to pain. Nevertheless, it is still reasonable to hypothesize that monitoring USV, in association with otherwise established nociceptive variables of the formalin test, might provide additional data connected to the affective dimension of pain.

The effects of opioids are extensively studied in the formalin test (16–18), and although an influence of morphine in the affective aspects of pain has been discussed in the clinical setting (19), there are few studies of opioid effects on the affective dimension of pain using animal models (20–22). Morphine changes pain-induced and nonpain-induced USV (22,23), but correlation of its effect and its reversibility with pain behaviors during formalin tests have not been tested. The present study examined USV emission as an additional variable in rats submitted to the formalin test, and the morphine effect on affective modulation in pain was also explored to investigate the predictive validity of USVs during the pain test.

Methods

Experimentally naive adult male Wistar rats (210–320 g) from the Animal House of FFFCMPA, Brazil, were housed in groups of 4 with food and water *ad libitum* at a room temperature of 22°C ± 2°C and a 12-hour light cycle (lights on at 7:00 AM). The study was approved by the Ethical Committee of Fundação Faculdade Federal de Ciências Médicas de Porto Alegre and was performed in accordance with the recommendations and policies of the U.S. National Institutes of Health Guidelines for the Use of Animals. The animals were killed immediately after the behavioral testing.

Drugs used were formalin (formaldehyde 37%, wt/wt, diluted to 5%, 10%, or 12.5% in 0.9% saline), morphine (1, 2.5, or 5 mg/mL; Cristalia Produtos Quimicos e Farmaceuticos Ltda, Itapira, Sao Paulo, Brazil; diluted in 0.9% saline), and naloxone (0.4 mg/mL; Cristalia Produtos Quimicos e Farmaceuticos Ltda, Itapira, Sao Paulo, Brazil). The control solution used was saline 0.9%.

Two experiments were performed. Experiment 1 aimed to establish the smallest formalin concentration to induce the whole range of sensory-discriminative (motor) and affective-emotional responses (USV emission). Three groups of rats ($n = 10$ per group) were injected with 5%, 10%, or 12.5% of formalin. A subcutaneous injection of 50 μL of the respective formalin solution was performed in the dorsal surface of the right forepaw of each rat, after 20 min of acclimatization to the testing chamber. After formalin administration, the rats were returned to the containment cage, and a video and audio recording were performed for 40 min, as described below.

The doses of formalin were chosen after preliminary observations in our laboratory when we could replicate the dose-response effects of formalin for pain behaviors but did not observe any USV emission associated with subcutaneous concentrations of formalin smaller than 5% (data not shown). We could then also verify that the behavioral analysis depicted two phases of motor pain behaviors, separated by a quiescent phase of behaviors called the "interphase." Also, no vocalizations where detected from rats injected with saline or not submitted to any external stimuli under our experimental procedures, as described Brudzynski (36).

Experiment 2 was conducted to establish the influence of morphine on USV emission during the 12.5% formalin test. The larger dose was chosen because it was established in Experiment 1 that it causes the most USV at the 10- to 15-min period of observation, the middle period of the interphase. After the adaptation period, morphine doses of 1, 2.5, or 5 mg/kg, combined or not with naloxone 2 mg/kg, were injected intraperitoneally (i.p.) at 30 and 15 min, respectively, before formalin administration. Rats were randomly assigned to one of the following treatment groups ($n = 10$ per group): (a) control; (b) naloxone 2 mg/kg; (c) morphine 1 mg/kg; (d) morphine 2.5 mg/kg; (e) morphine 5 mg/kg; (f) naloxone + morphine 1 mg/kg; (g) naloxone + morphine 2.5 mg/kg; or (h) naloxone + morphine 5 mg/kg. To homogenize the procedures, every rat received two i.p. injections; when only one substance was tested, the other injection was saline. After drug administrations, the rats were returned to the containment cage, and the video and audio recordings were performed for 40 min.

Experiments were conducted in a sound-attenuated chamber (60 × 60 × 60 cm) that was illuminated by a 10-W red light and was fitted with mirrors (30 × 30 cm) to allow behavioral observations from a camera in front of the containment cage and connected to a VCR for posterior behavioral analysis. Inside the sound-attenuated chamber, a clear Perspex testing cage (25 × 25 × 40 cm) was used for animal containment. USV was measured using a high-frequency condenser microphone (Model D940, Pettersson Elektronik AB, Uppsala, Sweden) connected to a personal computer using the LP 900 Signal Analyser System Version 3.10 (serial number 937) and with R 900 Sound Analysis Program (Version 1.0).

One rat was tested at a time, and the ultrasound detector was positioned above the containing cage. The ultrasound detector was set to detect frequencies of 30 ± 10 kHz with an amplitude filter setting of 4 to minimize background noise. The total number and duration of USVs for each rat were recorded during 5-min periods for 40 min. Environment noise levels

were standardized to minimize their influence in ultrasound recording (24).

A trained observer conducted the behavioral analysis of the video recordings to determine the motor behaviors induced by formalin. The observer was trained to provide a similar rating performance (at the 95% confidence limit) for each behavior during the tests of different animals. The videotapes were analyzed through direct computer keyboard input into a BASIC written software. The observer depressed the key encoding the motor behavior observed according to what was displayed in the video. The observer recorded the amount of time spent in each one of 4 behavioral categories, which were further scored as: 0 = the injected paw is not favored, 1 = the injected paw has little or no weight on it, 2 = the injected paw is elevated and is not in contact with any other surface, and 3 = the injected paw is licked, bitten, or shaken. An average pain intensity score was calculated, according to the weighed-scores technique (16), by multiplying the amount of time spent in each category by its assigned category score, adding these products and dividing by the total time of observation (i.e., 300 s). Nonpainful behaviors were also measured and were defined as "active behaviors" (walking, exploring, or grooming) and "inactive behaviors" (sitting, freezing, lying down, or sleeping) to improve the efficiency of formalin test observations (10).

The early (0–5 min), interphase (5–20 min), and late (20–40 min) phases of the formalin-induced pain behaviors were used to set the limits for analysis of USV emission, pain behaviors, and active/inactive behaviors (25).

Data were analyzed for statistical significance with Sigma Stat 2.0 for Windows (SPSS Inc., Chicago, IL) by repeated-measures two-way analysis of variance test using treatments and intervals of observations as independent factors for pain scores and USV behavioral analysis. For pair-wise multiple comparisons, the *post hoc* HSD Tukey test was used when appropriate. Pearson test was performed to correlate pain behaviors, USV emission, and nonpainful behaviors. The accepted level of significance was $P < 0.05$. Data are shown as mean ± SEM.

Results

The pain scores and number of USV in Experiment 1 are represented in Figure 1. Two-phases of painful behaviors, early and late phases, separated by an interphase were observed. There was a significant difference in pain scores according to the formalin concentrations ($F_{(2,239)} = 36.169$; $P < 0.001$) and according to the phases ($F_{(7,239)} = 14.04$; $P < 0.001$) during the total test time. There was an interaction between formalin concentration and phases ($F_{(14,239)} = 1.978$; $P < 0.05$). The formalin concentration of 5% determined higher pain scores than other concentrations during the late phase. The comparison between phases showed lower pain scores during the interphase. The number of USVs was not influenced by formalin dosing, but more USV were detected during the interphase ($F_{(7,239)} = 15.219$; $P < 0.001$). Length and frequency of USV are represented in Table 1. The time of USV was significantly longer in the 12.5% formalin group than in the 10% and 5% groups ($F_{(2,29)} = 5.539$; $P < 0.01$). Frequency was not different among the formalin concentrations.

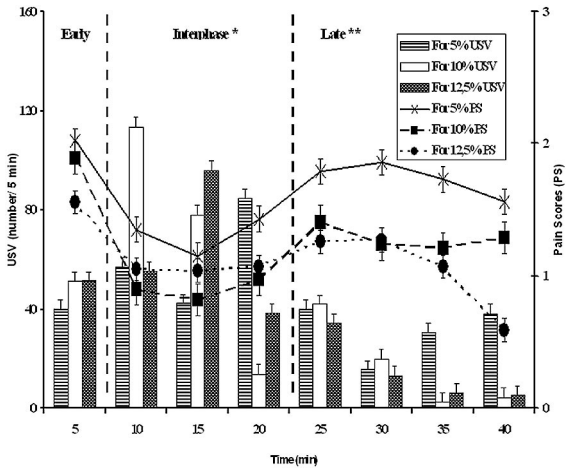

Figure 1. Pain scores (PS) and ultrasonic vocalizations (USV) during the total time of the formalin test. *More USV were seen in the interphase than in the early and late phases ($P < 0.001$). **The formalin 5% group had higher pain scores than the other groups in the late phase ($P < 0.001$) ($n = 10$ per group). Mean ± SEM.

Correlation between pain scores in early and late phases and USV in the interphase did not occur with formalin 5% and 10% but was present with formalin 12.5%. This latter concentration determined a significant inverse correlation during the interphase ($r = -0.64$; $P < 0.05$); rats with less pain scores during the interphase had more USV. Correlation between pain scores and nonpainful activity showed that during both early and late phases, as the rats pain behaviors increased, nonpainful activities at all three concentrations of formalin also increased (Table 2). Thus, more time was spent in inactive behaviors when rats presented higher pain scores. Correlation between USV and nonpainful activity did not show statistical significance.

The influence of morphine on pain scores in the formalin test and the reversion by naloxone can be seen in Figure 2 and Table 3. There was a significant difference of pain scores in respect to time ($F_{(7,639)} = 14.150$; $P < 0.001$), with lower pain scores during the interphase, as already seen in Experiment 1. The rats treated with the larger morphine dose (5 mg/kg) showed lower pain scores ($F_{(7,639)} = 15.972$; $P < 0.001$). Morphine produces a dose-dependent decrease in

Table 1. Frequency and Length of USV During the Formalin Test

Experiment	Group	Frequency (kHz)	Length (ms)
Formalin only	5%	29.7 ± 0.91	2.91 ± 1.67
	10%	31.16 ± 0.89	4.57 ± 1.73
	12.5%	31.30 ± 0.94	10.56 ± 1.71[a]
	Overall	30.72 ± 0.56	6.01 ± 0.98

Data are represented as mean + SEM.
[a]Formalin 12.5%-treated rats produced longer USV than the other groups (Tukey test; $P < 0.05$).

pain scores, as seen in Figure 3A. When the rats were treated with morphine plus naloxone, the pain scores were similar to the ones seen in control animals, demonstrating the reversibility of the antinociceptive effect of morphine.

In Figure 4, the USV of rats treated with different doses of morphine and of the control group are represented at different times of the formalin test. All morphine doses decreased the number of USV in the formalin test during the initial periods of the interphase, i.e., by 10 and 20 min of the test. However, no dose-response relationship was detected. As already seen in Experiment 1, rats in the interphase of the formalin test had more USV than in the early and late phases ($F_{(3,319)} = 7.281$; $P < 0.001$) of the 12.5% formalin test. The number of USV showed a significant difference among treatments ($F_{(7,319)} = 2.168$; $P < 0.05$), when all doses of morphine reduced the number of USV during the formalin test, which are antagonized by naloxone (Fig. 3B). Whereas 2 mg/kg of naloxone reversed the morphine 1 mg/kg effect on USV, rats treated with naloxone plus morphine 2.5 mg/kg and naloxone plus morphine 5 mg/kg had more USV emission than controls.

There were no differences in length and frequency of USV among all 8 groups of the second experiment, with overall means of 13.8 ± 1.56 ms and 27.59 ± 1.23 kHz, respectively.

Discussion

The main findings in our study were that rats submitted to a persistent somatic pain model, the formalin test, emitted USV, especially during the interphase, a period of quiescent motor demonstrations of pain, and morphine decreased these vocal emissions in a naloxone-reversible manner.

Classically, in the formalin test, only the motor painful behaviors are generally analyzed (25). We have also observed two phases of painful behaviors during the formalin tests performed, with high pain behavior scores intercalated by an interphase with low pain scores, as already described (26). In a previous experiment in our laboratory, we verified that the pain scores dose-dependently increase with formalin in concentrations up to 5%, and in this study, further increments in formalin concentrations did not increase pain scores, which were shown to be at a similar level or even declining, because by the end of the observation period with the largest dose used (12.5%), there was a significantly lower pain score. Other researchers described a similar pattern of effects with concentration-dependent pain scores between formalin concentrations of 0.25% and 2.5%, a plateau at concentrations between 2.5% and 5%, and declining pain behaviors at larger formalin concentrations (37). Although there is no correlation between pain scores and USV with smaller formalin concentrations, there is an inverse correlation between pain scores and USV with 12.5% formalin during the interphase. One may suppose that a dual facilitatory and inhibitory influence on spinal nociception by the concomitant activation of the off-cells in the rostroventromedial medulla (RVM) is produced when larger concentrations of formalin are injected to produce a high intensity stimuli, and therefore, pain scores may appear decreased (38). Interestingly, according to our observation, these larger concentrations of formalin also induce USV, which seem to belong in the behavioral complex of the formalin test. The USVs occur only after formalin 5% or larger concentrations are injected in the rat's paw. They become longer with larger concentrations of formalin, such as 12.5%, and therefore, may be denoting increasing intensity of pain. In fact, one could expect that since the motor manifestations of pain are plateaued or decreased after this formalin dose, additional behaviors might be included in the repertoire of behaviors to denote increased intensity of pain in the formalin test.

The observation of USVs during the formalin test occurs mainly during the interphase in all three concentrations of formalin tested. The interphase takes place 10–20 minutes after the formalin injection and is associated with less discharge firing of C fibers (28), seeming to be the result of spinal or supraspinal inhibition through γ-aminobutyric acid (GABA) neurotransmission with central involvement of $GABA_A$ receptors in the RVM (29–31). The release of GABA into the RVM medulla could elicit USV by the influence of other components of the descending pain modulation system, including the periaqueductal gray (22) and ventral tegmental area (32), which are regions also involved in the emission of USV in anxiety or stressful situations.

Different from the observation of morphine dose-dependent decrease on startle-induced USV in socially defeated rats (23), in the dose range we used, all morphine-treated rats decreased vocalizations during the formalin pain test. Our observation is in accordance with the verification that the intensity of USV after brief electrical pulses in rats tails was also

Table 2. Correlation Values (*r*) Between Inactivity and Pain Scores and Inactivity and USV During the Formalin Test

	Inactivity and pain scores			Inactivity and USV		
Group	Early phase	Interphase	Late phase	Early phase	Interphase	Late phase
5%	0.76[a]	−0.30	0.77[a]	−0.15	−0.35	−0.33
10%	0.83[a]	−0.94[a]	0.98[a]	0.01	−0.41	−0.26
12.5%	0.89[a]	−0.14	0.97[a]	−0.09	−0.35	−0.19

[a]Significant *r* values.

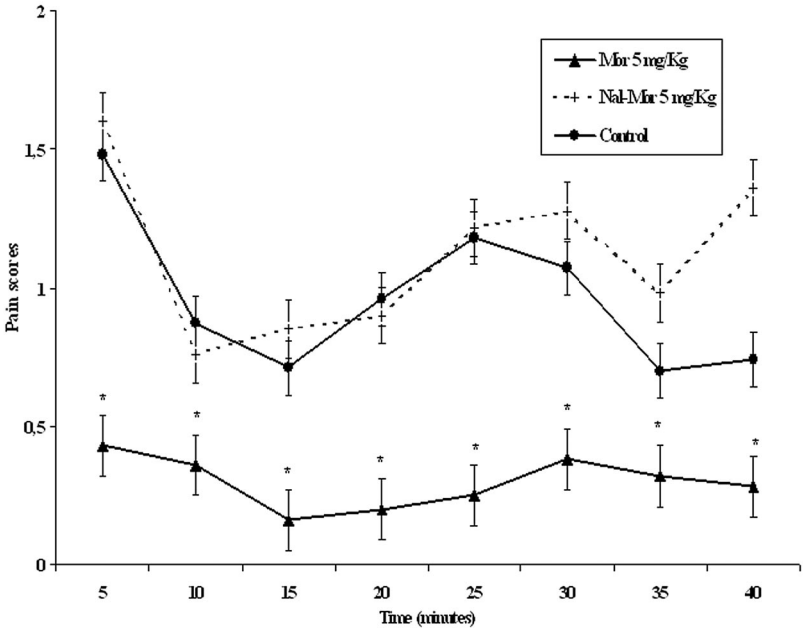

Figure 2. Pain scores (PS) during the formalin test after treatment with morphine 5 mg/kg (Mor 5 mg/kg) and reversion by naloxone (Nal –Mor 5 mg/kg) to control level. *There were significantly lower pain scores with morphine 5 mg/kg ($P < 0.001$; $n = 10$ per group). Mor = morphine; Nal-Mor = naloxone plus morphine. Mean ± SEM.

Table 3. Pain Scores During the Formalin Test

	Time (min)							
Group	0–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40
Control	1.48 ± 0.06	0.86 ± 0.12	0.71 ± 0.05	0.96 ± 0.10	1.18 ± 0.08	1.07 ± 0.11	0.69 ± 0.12	0.74 ± 0.10
Naloxone	1.28 ± 0.12	0.70 ± 0.13	0.65 ± 0.16	0.87 ± 0.17	1.07 ± 0.18	1.28 ± 0.18	0.94 ± 0.17	1.12 ± 0.20
Mor 1 mg/kg	0.60 ± 0.08	0.68 ± 0.08	0.61 ± 0.12	0.37 ± 0.08	0.63 ± 0.06	0.78 ± 0.14	0.77 ± 0.14	0.71 ± 0.18
Mor 2.5 mg/kg	0.61 ± 0.06	0.49 ± 0.06	0.46 ± 0.08	0.39 ± 0.07	0.37 ± 0.04	0.50 ± 0.07	0.63 ± 0.10	0.65 ± 0.08
Nal-mor 1 mg/kg	1.08 ± 0.18	0.77 ± 0.16	0.88 ± 0.16	0.59 ± 0.11	0.84 ± 0.18	1.16 ± 0.17	1.19 ± 0.16	0.99 ± 0.24
Nal-mor 2.5 mg/kg	1.53 ± 0.08	0.84 ± 0.10	0.86 ± 0.07	0.91 ± 0.11	1.38 ± 0.10	1.39 ± 0.08	1.24 ± 0.14	1.07 ± 0.13

Point-by-point analysis show higher effect of morphine treatment during early phase (0–5 min). Data are represented as mean ± SEM. The groups treated with morphine 5 mg/kg and morphine 5 mg/kg plus naloxone are represented in Figure 2. Nal = naloxone; Mor = morphine.

affected by morphine, although no variation among doses in the range of 0.1–3 mg/kg could be detected (20). One explanation for the lack of a dose-response effect of morphine on pain-associated USV might be that larger formaldehyde concentrations increase the ability of morphine to suppress some, but not all, formalin-induced behaviors (18) so that the doses used in our study were within the therapeutic range for motor pain scores but seem to be at the peak of the dose-response curve for USV. This would speak in favor of opioids having higher potency for the affect component of pain (19). Therefore, in future studies, smaller doses of morphine need to be

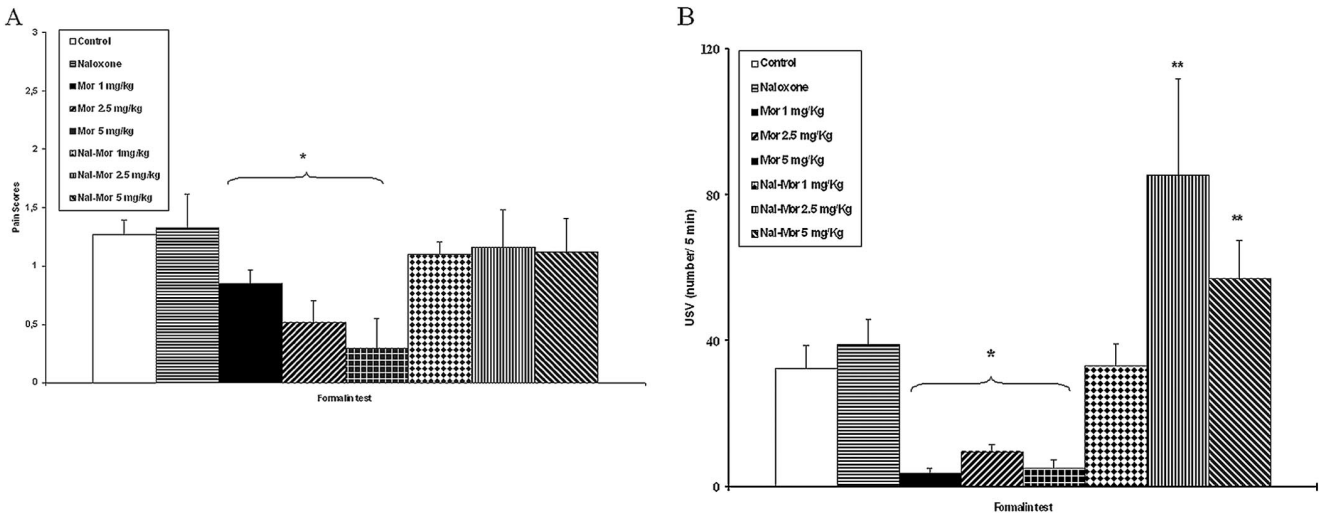

Figure 3. Morphine (Mor) and naloxone (Nal) effects on (A) pain scores (PS) and (B) ultrasonic vocalization (USV) of rats submitted to the formalin test. *Morphine-treated rats had lower PS and USV than control and naloxone groups. **Naloxone plus morphine 2.5-mg/kg– and naloxone plus morphine 5-mg/kg–treated groups had significant higher USV than controls ($P < 0.05$). Cumulative data are represented ($n = 10$ per group).

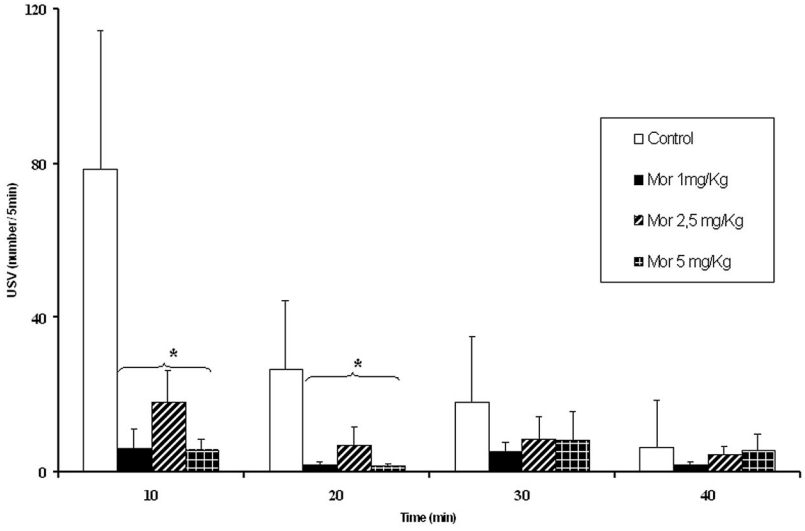

Figure 4. Ultrasonic vocalizations (USV) in morphine-treated (Mor) and control rats. *Morphine-treated rats were different from control at 10 and 20 min of observation ($P < 0.05$; $n = 10$ per group). Mean ± SEM.

used to test this explanation and to allow for estimates of the drug potency to change the motor and the affective component of formalin-induced pain. Another possibility would be that morphine could also be acting indirectly on the USV by interfering with other neurotransmitter systems in the circuitry of pain and affect.

This is the first demonstration that rats submitted to the formalin test show increased USVs. One study concerning USV during the formalin test did not find USV associated with the formalin test (13). The difference with our study might be explained by different experimental procedures in the cited study, such as the use of only one, small concentration of formalin, the small number of animals used, and the observation of frequent vocalization rates before starting the experiment, during the acclimatization phase, that could have lead to habituation and decreased vocal emissions during the test (36). Further experiments need to be planned to explain the differences between the two studies. In fact, USV emission in other pain models are described, as in the tail flick (22), the tail electric stimulation (10), the IM-injected antimicrobial pain tests (33), and the inescapable electric foot-shock (34). All of these models may be useful to finally clarify the relationship of the affective nature of USV emitted during pain. Different from these acute pain tests, tests using chemical stimuli can be distinguished

clearly from the others because of their physical nature and duration and by the fact that they are inescapable suprathreshold stimulus. These chemical experimental models are considered the closest in nature to clinical pain (15), and now it is clear that they also evoke USVs. Interestingly, the present study shows that the quiescent phase of the formalin test is only quiescent for motor behaviors. In the future, it would be interesting to elucidate the reasons for this temporal alternation of motor, vocal, and, again, motor pain behaviors.

In addition to verifying the existence of vocalizations, we also tested to discern if opioid drugs intervene with the vocal behaviors during the formalin test to test the hypothesis that USV may be the behavioral manifestation of increased pain intensity with larger concentrations of formalin. There was inhibition of pain behavior by morphine, in a dose-response manner, and reversion of its analgesic effect by naloxone, whereas the treatment with naloxone alone did not alter pain scores during the formalin test, in agreement with other studies (18,27). Also, morphine decreased USVs of rats submitted to the formalin test, and this effect was reversed or even increased by naloxone, denoting pharmacological specificity of this effect. Opioids act in the same brain areas involved in affective and pain modulation such as the RVM (35), periaqueductal gray (22), and ventral tegmental area (32), and its effects are reversed by naloxone, one of its antagonists, as already described. Furthermore, GABA modulation in the analgesic effect of opioids (35) could be the neurochemical mechanism of the inhibition of USV with morphine in these brain areas. Future studies must elucidate the opioids intrinsic mechanism on USV emission in the formalin test, including the role of GABA.

USVs were almost doubled compared with the control group, when naloxone was concomitantly administered with one of the two larger doses of morphine. A possible explanation is that the rats experience hyperalgesia after naloxone treatment (40) and vocalize more. However, we should have also seen more vocalizations from the naloxone and naloxone plus morphine 1 mg/kg groups. Another explanation might relate to acute withdrawal from large morphine doses when naloxone is administered, denoting aversive effects of naloxone, as seen in a conditioned place aversion paradigm (20,39).

Are the vocalizations a measure of affect to pain in the formalin test? It has been proposed that this behavior is related to aversive, unpleasant emotional states, including pain in other models. With the additional demonstration that morphine decreases the affective dimension of pain in humans (19) and also decreases USV in the formalin test, it is tempting to assert that this is the case. However, more studies will be required because some inconsistencies were found in this study. One would expect to see negative affect connected to pain in this model. The characteristics of USV frequency in adult rats submitted to other affective experimental settings show 2 distinct types: long 22-kHz calls related to negative affect and 50-kHz short calls related to positive affect (5). Interestingly, the length of USV bouts during the formalin test interphase was short. Our data display a similarity with negative affective activation when the frequency is analyzed (around 22 kHz), although the duration was similar to a positive affective activation (ranging from 2 to 10 ms). The shorter length of USV could be considered a byproduct of locomotor and exploratory activity of animals. To eliminate this potential confounding factor, we demonstrated that there is no correlation between nonpainful activity and USVs. The presence of both short calls and high frequency could be a different pattern of USV, characteristic of the test, but that requires confirmation.

In conclusion, USV seems to be an additional useful behavioral measure during the formalin test. It may reflect the affective state of rats and occurs during the interphase, a period that is now demonstrated not to be quiescent and, therefore, becomes relevant to a better understanding of the components of pain. A further challenge is to establish the external face, construct and predictive validity of the test, and the neuroanatomical sites and neurotransmitters involved in this pain behavior.

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