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Between Scylla and Charybdis: assessing the multidimensional aspects of pain behaviors in rats using a double avoidance place preference paradigm

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Abstract

Although the behavioral response to pain is complex and involves supraspinal processes, assessment of pain symptoms in animal models still mainly relies on reflex-based nociceptive tests, which do not account for the affective-motivational nor cognitive components of pain. We introduce a double avoidance place preference paradigm, an integrated testing procedure in freely moving rats that relies on the conflict between the avoidance of a dark compartment in which a thermal ramp is activated, and the escape towards an aversive brightly lit compartment. We were able to differentiate the first nociceptive threshold from the temperature of definitive escape from the dark compartment, conveying information on the adaptive behavior of animals. Measures were repeated after an hour to evaluate the adaptive learning response upon reexposure. In naive animals, there was a significant decrease in the time spent in the dark compartment at all stages of the testing paradigm upon reexposure, leading to a final escape before the flood had reached nociceptive values. This adaptive behavior was blunted by anxiolytic treatment. In animals exhibiting hyperalgesia following intraplantar complete Freund adjuvant injection, escape thresholds were significantly higher than that of control animals, hinting at a maladaptive affective-motivational response to noxious stimulation. However, in cuff animals, we failed to reveal any hot nociceptive hypersensitivity, but animals exhibited a strong adaptive response to cold simulation upon reexposure. Overall, the proposed paradigm allows for an integrated cortical response leading to a proactive avoidance behavior, while fully complying with ethical standards in animal experimentation.

Keywords: Pain behaviors, Nociception, Escapable place preference, Coping, Adaptive avoidance

1. Introduction

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage, or described in terms of such damage."[21] Several components of pain are commonly distinguished after recruitment of the nociceptive system: (1) the sensoridiscriminative component, the most straightforward, refers to the decoding of the nociceptive message (quality, duration, localization, intensity); (2) the affective-motivational aspect underlies the unpleasantness of the pain experience and mobilizes the organism to adapt; (3) the cognitive component refers to the significance given to the pain experience and its memorization; and finally, (4) the behavioral aspect of pain underlies the motor (including unconscious autonomic regulations) and verbal expression associated with the experience of pain.[14]

The study of pain and its underlying mechanisms is of utmost importance for the elaboration of new antalgic treatments. It is particularly relevant in the case of refractory chronic pain, a growing worldwide burden and the origin of more than half of medical consultations in Europe and the United States.[5,8,22] A plethora of animal models, ranging from inflammatory sensitization to neuropathic pain,[7] have emerged over time and proven efficient in furthering our knowledge on the apparition and maintenance of pain symptoms. However, assessment of pain symptoms in animal models still mainly relies on reflex-based nociceptive tests, which mostly translate the sensoridiscriminative component of pain through the measure of thresholds eliciting an avoidance response to a noxious stimulus but do not enable the evaluation of the other components of pain. Indeed, most of those studied reflexes, such as tail flick or paw withdrawal, are exhibited in decerebrated animals[15] and hardly translate the supraspinal integrated responses associated with pain. It is also of note that nociceptive thresholds vary depending on the test used.[7]

Although some tests have been developed to try to evaluate a more integrated concept of pain in animals, such as the

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conditioning placement preference or other related operant paradigms,[3,10,11,13,16,19] aversiveness is still under investigated in pain laboratories, which mostly focus on nociceptive thresholds without taking into account the multidimensional aspect of pain or its cognitive impact.

The aim of this work was to elaborate an original testing procedure (DAPP: Double Avoidance Place Preference paradigm) capable of providing measures of nociceptive thresholds, as well as assessing affective-motivational aspects and adaptive behaviors (ie, cognitive determinants) in freely moving rats faced with 2 unpleasant stimuli. This procedure stems on rats' natural aversion to brightly lit environments and the avoidance behavior elicited by a potentially painful thermal hot or cold stimulation. This procedure was tested on naive animals, animals treated with anxiolytic compounds, and animals exhibiting inflammatory or neuropathic pain symptoms.

2. Materials and methods

2.1. Animals

Adult Wistar rats (Charles River, Saint Germain Nuelles, France) aged 6 to 8 weeks were used for this study. Animals were housed in a temperature-controlled (22 ± 1°C) and humidity-controlled (50 ± 10%) room under a 12-hour light–dark cycle (lights on at 7:00 AM), with ad libitum access to food and tap water. Both male and female rats were used for this study, housed in collective cages according to sex. All procedures were conducted in accordance with EU regulations and approved by the regional ethical committee (CREMEAS authorization number 2019071018511286 v5).

2.2. Double avoidance place preference paradigm

We developed a DAPP paradigm to simultaneously assess the sensory, affective, and cognitive components of behavior in rats exposed to noxious thermal stimuli. This procedure was performed in a light/dark box arena, well known for the assessment of anxiety-like symptoms (preference for the dark compartment), using a thermal place preference apparatus (Ugo-Basile, Italy) made of 2 Plexiglas cylinders (h: 25.5 cm, diameter: 20 cm) placed on separate temperature-controlled floors. The dark compartment was created using a transparent, red, plastic film: perceived as dark for Wistar rats who cannot see in the red spectrum, the compartment was see-through for humans, allowing both video monitoring and the scoring of rat behaviors at any time of the testing procedure (**Fig. 1A**). The light in the lit compartment was set at 120 lux, an intensity capable of creating a compartment aversive enough to establish a conflict with the dark nociceptive compartment.

At the time of testing, animals were placed in the dark compartment, and their behavior was manually monitored and video recorded. The 10-minute procedure consisted in a 5-minute habituation period during which both floor temperatures were set at 25°C (close to room temperature), followed by 5 minutes of dynamic thermal stimulation in the dark compartment. Both compartments were accessible at all times of the test. An hour after the first exposition to the test, the procedure is repeated in a second session to assess the learning response upon reexposure. It is worth noting that the span of one hour between both sessions was selected following careful consideration. In particular, the learning response could not be assessed when the second session was held 24 hours after the first one, as no learning could be seen in this case (Supplementary Fig. 1, http://links.lww.com/PAIN/C117).

2.2.1. Hot ramp protocol

By heating the floor from room temperature (25°C) to noxious hot (52°C) in the dark compartment at a heating speed of 7°C/minute starting at the fifth minute, animals have to solve a conflict between their aversion towards the lit compartment and the motivation to escape the noxious hot stimulation in the dark compartment. The temperature of 42°C was reached after 2 minutes of heating (ie, minute 7-8), whereas the final temperature of 52°C was reached at the ninth minute (ie, after 4 minutes of heating). To avoid potential tissue damage, exposure to the 52°C-heated floor was limited with an a priori cutoff set at 30 seconds during the last minute of the test. However, in this study, no animal ever remained in the 52°C-floor dark compartment long enough for the cutoff to be reached.

The protocol can be divided into 3 specific periods: (1) the baseline period (minutes 0-5), when animals freely explore both compartments with the same floor temperature; (2) the anticipation period (minutes 5-7), during which the heating ramp has started but has not yet reached the nociceptive threshold of 42°C; and (3) the coping period (minutes 7-10), in which animals remain in the nociceptive compartment until their definitive escape.

2.2.2. Cold ramp protocol

Because the device could not reach cold temperatures as fast as hot ones, the testing protocol was adapted to a cooling speed of 2°C/minute. After 5 minutes, the cold ramp started, and the floor temperature of 15°C, possibly nociceptive based on the literature, was reached at the 10th minute. The test lasted for 18 minutes, when the final temperature of 0°C was reached.

The cold ramp protocol can also be divided into 3 periods: (1) the baseline period during the first 5 minutes; (2) the anticipation period (minute 5-10), when the temperature is decreasing but still above 15°C; and (3) the coping period from (minute 10-18) when the floor in the dark compartment is at a temperature below 15°C.

2.2.3. Test variables

(1) Time spent in the dark compartment: the percentage of time spent in the dark compartment is measured for each minute of the test and compared between groups and sessions.
(2) Nociceptive threshold: temperature prompting the first nociceptive reflex excluding escape (paw withdrawal, paw licking, flinching), measured in the dark compartment during the first session. Therefore, values can only be measured in animals who were still present in the dark compartment, as in some rare cases, animals left the dark compartment before having exhibited any nociceptive behavior (**Table 1**).
(3) Escape threshold: final temperature at which animals crossed to the lit compartment and did not return to the dark compartment, used to plot survival curves.
(4) Escape-nociception delta: difference of temperature between the nociceptive threshold and the definitive escape threshold.
(5) Half-escape temperature: extracted from survival curves, represents the temperature at which 50% of animals have left the dark compartment.

2.3. Model of painful inflammatory sensitization

Animals were anesthetized with 3% isoflurane (Ventoflurane, Vibrac, France) pushed by compressed air (700 mL/min). 100 μL of complete Freund's adjuvant (CFA, Merck, Darmstadt, France) was then injected into the plantar surface of the right hindpaw.

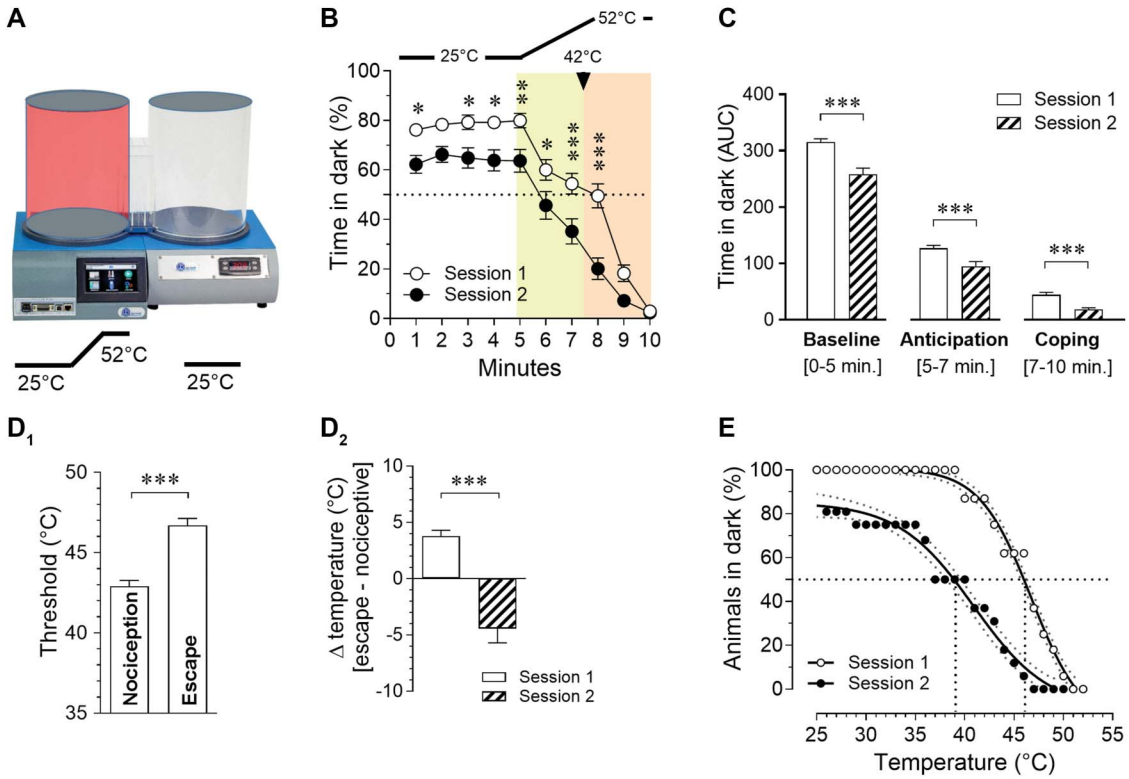

Figure 1. General behavior of naive rats exposed to the double-avoidance place preference (DAPP) paradigm during 2 10-minute sessions set 1 hour apart. (A) DAPP paradigm light/dark box arena with 2 separate temperature-controlled floors. (B) The progressive escape from the dark compartment observed after the start of the heat ramp is significantly anticipated in session 2 in control animals (n = 31). Statistical significance was assessed with Sidak multiple comparison test. (C) Areas under the curves (AUC) show a significant reduction in the time spent in the dark compartment during the second session for all 3 phases of the testing paradigm in control animals (n = 31). Statistical significance was assessed with a paired *t* test. (D_1) During the first session of the DAPP paradigm, the escape threshold is significantly higher than the nociceptive threshold (n = 32). Statistical significance was assessed with a paired *t* test. (D_2) Delta between the escape and the nociceptive threshold temperatures for both sessions (n = 32). Statistical significance was assessed with a paired *t* test. (E) Survival curve representing the percentage of control animals present in the dark compartment following increase in the floor temperature. Circles indicate the percentage of animals present in the dark compartment for a given temperature range during the first (white circles) and second (black circles) sessions. Half-escape temperatures are represented in dotted lines. For the whole panel, statistical significance was illustrated as follows: $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

Animals were exposed to the DAPP paradigm 24 hours after CFA injection, to be outside of the acute inflammatory phase, which peaks 7 hours after injection. Control animals received an intraplantar injection of saline.

2.4. Model of neuropathic pain

Neuropathic pain was induced by chronic constriction of the right sciatic nerve using a procedure previously validated in our laboratory.[4] Animals were anesthetized with 4% isoflurane (Ventoflurane, Vibrac, France) pushed by compressed air (700 mL/min). An incision was made to expose the right sciatic nerve of the animals, and a polyethylene cuff (1-mm-long split section; ID = 0.86 mm, OD = 1.27 mm; PE-90, Harvard Apparatus, Les Ulis, France) was placed around it. The skin was then closed with a nylon suture (Ethilon 4-0, Ethicon Plymouth, MA). Control rats underwent the same procedure without the cuff implantation (sham group). Animals were exposed to the DAPP paradigm 2 weeks after surgery to be within the nociceptive hypersensitive phase, which lasts 40 days in this model.

2.5. Pharmacological treatments

Diazepam (TVM, Centravet, Nancy, France) was diluted in NaCl 0.9% to reach a dose of 1 mg/kg and injected subcutaneously (s.c.) at a final volume of 20 μL with a Hamilton syringe 20 minutes before the DAPP paradigm.

Etifoxine (EFX; 2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) was kindly provided by Biocodex laboratories (Biocodex, Gentilly, France, batch n°562). Etifoxine

Table 1

Half escape temperatures for both sessions obtained after plotting the whole distribution for each animal group.

	CTRL	Saline	DZP	EFX	Saline	CFA	Sham (hot)	Cuff (hot)	Sham (cold)	Cuff (cold)
Half escape temperature S1 (°C)	46.8	49.1	48.0	49.1	47.6	49.8	47.7	48.9	8.7	7.5
% of escape during baseline	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Half escape temperature S2 (°C)	40.9	43.7	45.6	45.7	42.9	48.3	38.4	45.7	15.1	18.9
% of escape during baseline	*13*	*0*	*6.6*	*0*	*18.7*	*0*	*12.5*	*25*	*14.2*	*37.5*

The percentage of animals who never entered the dark heated compartment after the heat ramp started is indicated in italic.

was prepared in NaCl 0.9% containing 1% Tween 80 (vol/vol; Merck, France) after dissolution in 1.5% ethanol. Etifoxine solution was administered intraperitoneally (i.p.; volume 0.5 mL/100 g) at a dose of 50 mg/kg once per day, for 2 days before the day of testing. The third and last injection was done 20 minutes before the DAPP paradigm. Control animals received an equivalent i.p. volume of NaCl 0.9%.

Lidocaine/prilocaine cream 5% (Zentiva, Paris, France) was applied topically on the right hindpaw 20 minutes before exposure to the DAPP paradigm.

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was performed using the GraphPad Prism software (La Jolla, CA). After checking the linear distribution of the data (Shapiro–Wilk normality test), parametric statistical tests (Student *t* test) were performed to compare data obtained from 2 paired measures in a single group. Two-way (time × group) ANOVA analysis, with repeated measures for the time variable (2w RM ANOVA) were used to compare the time spent in the dark compartment, as well as the delta between escape and nociceptive threshold temperatures. This was followed by Sidak post hoc multiple comparison test. To compare temperature thresholds, a 2w ANOVA (threshold × group) was used, followed by Sidak multiple comparison test. When data were not linear, the nonparametric Kruskal–Wallis test was used to compare thresholds between the groups, followed by Dunn multiple comparison test. Kolmogorov–Smirnov statistics were used to compare the survival curves (definitive escape distributions) between sessions 1 and 2. Differences were considered statistically significant for $P < 0.05$. Because no statistical difference was found between male and female rats (Supplementary Fig. 2, http://links.lww.com/PAIN/C117), data were pooled and analyzed together.

3. Results

3.1. General behavior of adult Wistar rats in the double avoidance place preference paradigm

Figure 1B illustrates the time spent by control Wistar rats in the dark compartment of the apparatus during two 10-minute sessions separated by an hour. As expected, during the 5-minute baseline of the first session, animals spent most of their time (79%) in the dark compartment. As soon as the heat ramp started (after the fifth minute), they progressively left this dark compartment, until floor temperature reached 52°C. During the second session, the progressive escape from the dark compartment was significantly anticipated (2w RM ANOVA, time x session, $F_{(9,270)} = 2.351$, $P = 0.0144$). For instance, animals spent significantly less time in the dark compartment during most of the baseline, as well as during the beginning of the heating ramp (eg, minute 7: 54.4 ± 4.2% in S1 vs 35.2 ± 5.1% in S2) and when floor temperature was above the nociceptive threshold of 42°C (Minute 8: 49.6 ± 4.8% in S1 vs 20.1 ± 4.4% in S2).

Values from **Figure 1B** can be expressed as areas under the curve (AUC) for 3 distinct periods (baseline, anticipation, coping), depending on the floor temperature in the dark compartment (see Methods for further details), as illustrated in **Figure 1C**. For the sake of clarity, this representation was favored over the detailed time course for the rest of the article. As shown here, a significant reduction in the time spent in the dark compartment was observed during the second session compared with session 1 before the start of the ramp (baseline: paired *t* test: t = 6.384, df = 30, $P < 0.0001$), before the ramp reached noxious values (anticipation: paired *t* test: t = 4.095, df = 30, $P = 0.0003$), and during the coping period (paired *t* test: t = 5.117, df = 30, $P < 0.0001$), confirming the efficacy of the reexposure to elicit an adaptive behavior.

As mentioned above, during the first session, most animals remained in the dark heated compartment until it reached a thermal hot nociceptive value. This permitted to measure the temperature corresponding to the apparition of the first nociceptive response (defined as the nociceptive threshold) before any adaptive decision to escape the thermal stimulus (escape threshold). As seen in **Figure 1D$_1$**, the thermal nociceptive threshold was of 42.9 ± 0.4°C during the first session, in accordance with values found in the literature, whereas the definitive escape only happened when floor temperature reached the significantly higher value of 46.7 ± 0.4°C (paired *t* test: t = 7.364, df = 31, $P < 0.0001$). We then measured the difference between escape and nociceptive threshold temperatures in both sessions. This delay was significantly reduced in the second session, dropping from 3.8 ± 0.5°C to −4.4 ± 1.3°C (**Fig. 1D$_2$**; paired *t* test: t = 6.355, df = 31, $P < 0.0001$). This drop in the negative range demonstrates the anticipated adaptive escape response upon reexposure, with animals leaving the dark compartment before it reached nociceptive levels. In addition, using the temperature of definitive escape, we also plotted the distribution of the rat population present in the dark compartment throughout the duration of the test (**Fig. 1E**). In agreement with previous observations, less animals were present in the dark compartment in session 2 during the baseline period (about 20% less). Animals also escaped from the dark compartment significantly sooner during session 2, as demonstrated by the temperature of half-escape, which diminished from 46.8°C to 40.9°C (**Table 1**), for sessions 1 and 2, respectively (Kolmogorov–Smirnov, KS = 0.6071, $P < 0.0001$).

3.2. Sensitivity of the rat behaviors to anxiolytics

In the next set of experiments, animals received 1 of 2 anxiolytics with different pharmacological profiles and side effects on affective/cognitive functions. **Figure 2A** shows the time spent in the dark compartment during the periods of interest after injection of the classical benzodiazepine diazepam (DZP). During the first session, DZP-treated animals remained significantly longer in the dark compartment during the anticipation period (2w RM ANOVA, group × session, group factor: $F_{(1,28)} = 24.74$, $P < 0.0001$), although no difference was seen compared with saline-injected control animals for both the baseline (2w RM ANOVA, group × session: $F_{(1,28)} = 9.592$, $P = 0.0044$) and coping periods (2w RM ANOVA, group × session: $F_{(1,28)} = 0.8391$, $P = 0.3675$). Moreover, DZP-treated animals showed no difference in behavior between both sessions, contrary to the learned escape behavior observed in control animals. In line, half escape temperatures were similar between the sessions (**Table 1**). As seen in **Figure 2B**, animals that received the nonbenzodiazepine anxiolytic EFX also showed a progressive decrease in the time spent in the dark compartment throughout the 10-minute test, although they stayed longer than control animals during the heating ramp of the first session, both before and after the temperature threshold of 42°C. During reexposure, if EFX-treated animals still remained significantly in the dark compartment longer than control animals during all periods (baseline: 2w RM ANOVA, group x session: $F_{(1,29)} = 11.46$, $P = 0.0021$; anticipation: 2w RM ANOVA, group × session: $F_{(1,29)} = 5.848$, $P = 0.0221$; coping: 2w RM ANOVA, group x session:

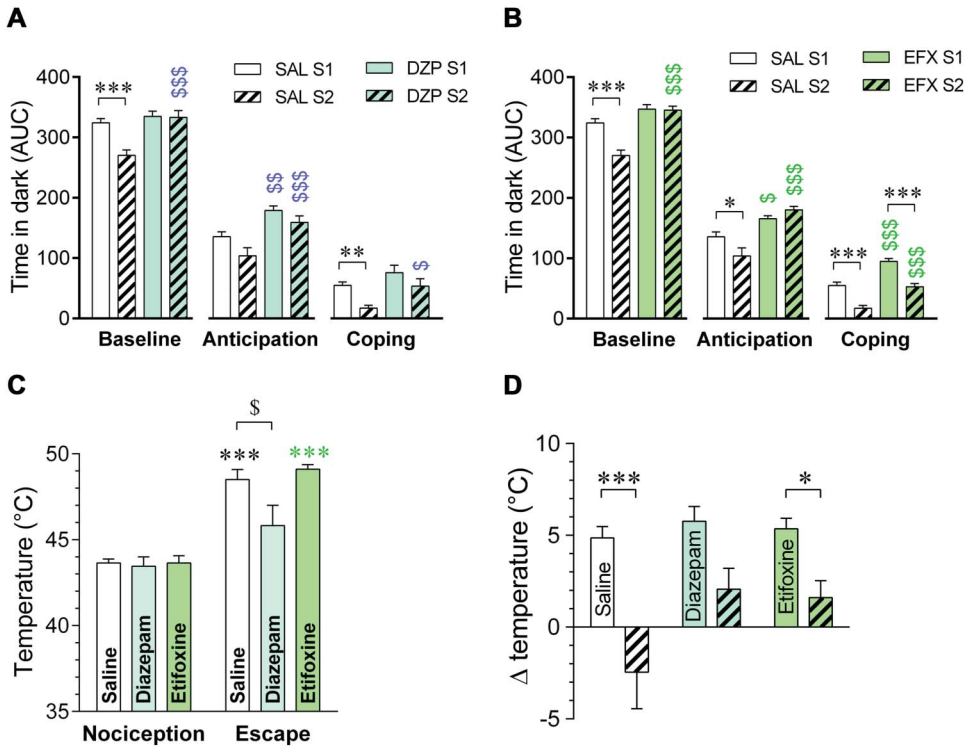

Figure 2. Sensitivity of the DAPP parameters to anxiolytic treatment. (A) Areas under the curves (AUC) of the time spent in the dark compartment during the 3 phases of the testing paradigm for saline-injected (SAL; n = 16) and diazepam-injected (DZP; n = 14) animals during the first (S1) and second (S2) sessions. (B) Areas under the curves (AUC) of the time spent in the dark compartment during the 3 phases of the testing paradigm for saline-injected (SAL; n = 16) and etifoxine-injected (EFX; n = 15) animals during the first (S1) and second (S2) sessions. (C) Nociceptive and escape thresholds during the first session of the DAPP paradigm for animals injected with a saline solution (n = 8), diazepam (n = 15), or etifoxine (n = 15). (D) The delta between the escape and nociceptive threshold temperatures is reduced in the second session (black stripes) for saline-treated (n = 8) and EFX-treated (n = 15) animals but not in the diazepam group (n = 9). For the whole panel, statistical significance was assessed with Sidak multiple comparison test and illustrated as follows: $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) for intragroup comparisons, and $P < 0.05$ ($), $P < 0.01$ ($$), or $P < 0.001$ ($$$) for intergroup comparisons.

$F_{(1,29)} = 0.2278$, $P = 0.6367$), a significant decrease in the time spent in the dark compartment was seen between both sessions of the coping period, with animals escaping from the dark heated compartment sooner than in session 1. This observation fits well with the half escape temperature values for session 2, which were similar to that of control or saline-treated animals (**Table 1**).

As illustrated in **Figure 2C**, the mean nociceptive threshold was not affected by either anxiolytic treatment, with thresholds ranging from 43.7 ± 0.2°C for saline-injected control animals, to 43.5 ± 0.5°C and 43.6 ± 0.4°C for DZP- and EFX-treated animals, respectively. In both saline-treated and EFX-treated animals, the escape temperature threshold in the first session was significantly higher than the nociceptive threshold, with temperatures of 48.5 ± 0.6°C and 49.1 ± 0.3°C, respectively (2w ANOVA, group × threshold, threshold factor: $F_{(1,64)} = 46.62$, $P < 0.0001$). Conversely, DZP-treated animals showed no difference between the first nociceptive reflex and the definitive escape threshold, hinting at a possible reduction in conflict aversion, leading to the prompt avoidance of the nociceptive environment. Similarly, in the second session, DZP-treated animals failed to show any anticipated escape, as demonstrated by the lack of significant difference between delta temperatures for both sessions (**Fig. 2D**; 2w RM ANOVA, group × session, session factor: $F_{(1,27)} = 35.08$, $P < 0.0001$). In EFX-treated animals however, a significant decrease in the delta between escape and nociceptive temperatures can be seen, albeit not as strong as in control animals, confirming the delay in the escape behavior observed in **Figure 2B** for EFX-treated animals.

3.3. Influence of inflammatory or neuropathic sensitization

We then measured the effect of a painful inflammation induced by an intraplantar CFA injection on the parameters evaluated in the DAPP test. As seen in panel A of **Figure 3**, animals that received an intraplantar saline injection behave similarly to noninjected controls (**Fig. 1**) and other saline-injected animals (**Fig. 2**), with a progressive decrease in the time spent in the dark compartment as the heat ramp begins. During the first session, CFA animals display an escape behavior similar to that of controls, except during the coping period during which they remain significantly longer in the dark heated compartment (**Fig. 3A**$_1$; coping: 2w RM ANOVA; group x session, $F_{(1,27)} = 9.192$; $P = 0.0053$), suggesting an increased aversion conflict. Furthermore, adaptive behavior seems to be only partial in CFA animals upon reexposure, as they only show a significant reduction in the AUC of time spent in the dark compartment during the coping period (baseline: 2w RM ANOVA; group × session, $F_{(1,27)} = 2.323$; $P = 0.1391$, anticipation: 2w RM ANOVA; group × session, $F_{(1,27)} = 0.8086$; $P = 0.3765$).

As expected, CFA animals exhibited significantly lower nociceptive thresholds compared with saline animals, with thresholds of 43.0 ± 0.7°C and 34.4 ± 1.3°C, respectively (**Fig. 3A**$_2$; 2w ANOVA, group ×threshold, $F_{(1,55)} = 48.69$, $P <$

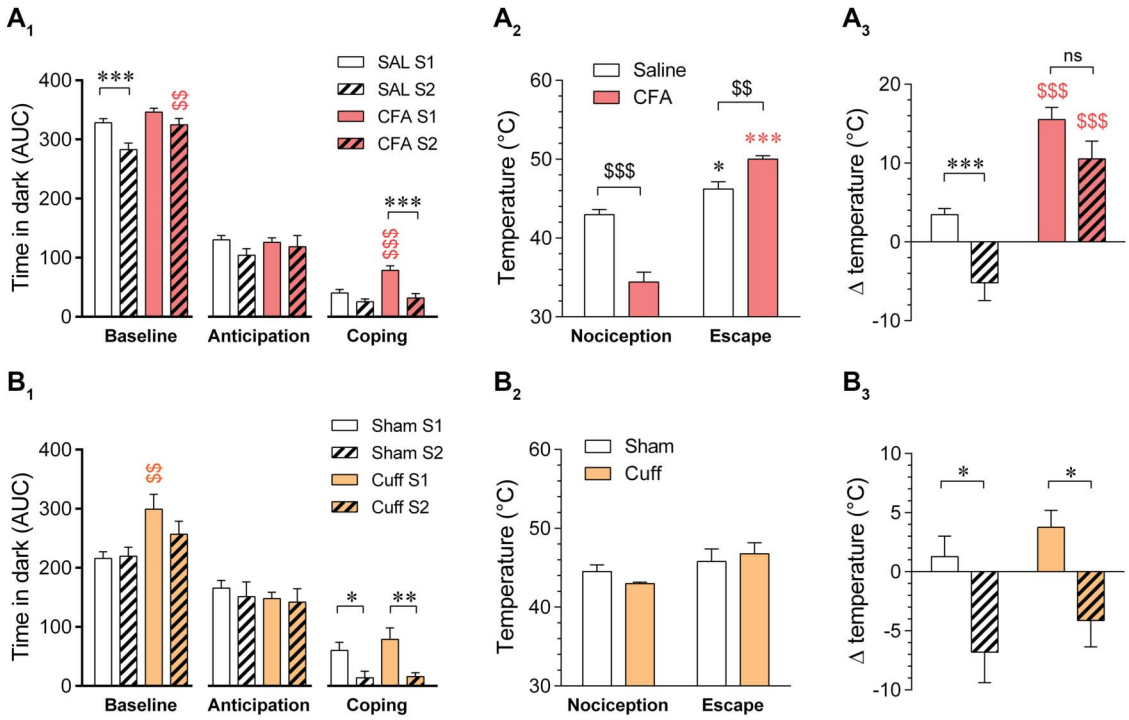

Figure 3. Influence of inflammatory or neuropathic painful sensitization on the DAPP parameters. (A_1) Areas under the curves (AUC) of the time spent in the dark compartment during the 3 phases of the testing paradigm during the first (S1) and second (S2) sessions in animals having received an intraplantar injection of saline (SAL; n = 16) or CFA (n = 13). Statistical significance was assessed with Sidak multiple comparison test. (A_2) Nociceptive and escape thresholds during the first session of the DAPP paradigm for saline (SAL; n = 16) and CFA animals (n = 14). Statistical significance was assessed with Sidak multiple comparison test. (A_3) The delta between the escape and nociceptive threshold temperatures is reduced in the second session (black stripes) for saline animals (n = 15) but not in the CFA group (n = 13). Statistical significance was assessed with Sidak multiple comparison test. (B_1) Areas under the curves (AUC) of the time spent in the dark compartment during the 3 phases of the testing paradigm during the first (S1) and second (S2) sessions in sham (n = 8) and cuff (n = 8) animals. Statistical significance was assessed with Sidak multiple comparison test. (B_2) The nociceptive and escape thresholds during the first session are similar in the sham (n = 8) and cuff (n = 8) groups. Statistical significance was assessed with Dunn multiple comparison test. (B_3) The delta between the escape and nociceptive threshold temperatures is reduced in the second session (black stripes) for sham (n = 8) and cuff (n = 8) animals. Statistical significance was assessed with Sidak multiple comparison test. For the whole panel, statistical significance was illustrated as follows: $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) for intragroup comparisons, and $P < 0.01$ ($$), or $P < 0.001$ ($$$) for intergroup comparisons.

0.0001), highlighting the fact that CFA animals are already in the nociceptive range during the anticipation period (ranging from 25°C to 42°C). This is further supported by the results obtained with a group of CFA rats, which received a topical lidocaine treatment that restored nociceptive thresholds (Supplementary Fig. 3, http://links.lww.com/PAIN/C117). If the escape threshold is higher than the nociceptive threshold in both saline and CFA groups, showing the conflict to enter the anxiogenic lit compartment, the escape threshold in CFA animals is significantly higher than that of saline animals (and that of CFA animals treated with topical lidocaine, see Supplementary Fig. 3B, http://links.lww.com/PAIN/C117), suggesting a maladaptive response. The increased aversion conflict can also be observed in the delta between escape and nociceptive threshold temperatures illustrated in **Figure 3A**$_3$, significantly higher in CFA animals compared with saline (2w RM ANOVA, group × session, group factor: $F_{(1,26)} = 45.10$, $P < 0.0001$). No difference between delta temperatures could be seen in CFA animals between the sessions (see also **Table 1**), possibly translating an impairment of the learning response during the anticipation phase, during which hypersensitive CFA rats stay in the dark compartment during the heating ramp despite already exhibiting nociceptive reflexes. Following lidocaine application, however, the adaptive behavior and learning response are restored, as highlighted by the lower escape temperature and the negative delta between escape and nociceptive temperatures during the second session (Supplementary Fig. 3, http://links.lww.com/PAIN/C117).

We next tested rats expressing neuropathic pain symptoms following constriction of the right sciatic nerve. As seen in **Figure 3B**$_1$, the control group, who underwent sham surgery, generally behaved in a similar fashion as control-naive animals, although a quite drastic reduction in the time spent exploring the dark compartment during the baseline could be seen, which blunted the comparison between both sessions. No difference could be seen between the sham and cuff groups in the time spent in the dark compartment, although the exploration period during the first session is restored in cuff animals. For instance, during the second session, both groups only spent significantly less time in the dark compartment once the temperature had reached 42°C, ie, during the coping period, whereas the AUC of time spent in the dark compartment did not differ between sessions during the baseline or anticipation period (baseline: 2w RM ANOVA, group × session: $F_{(1,14)} = 3.063$, $P = 0.1020$; anticipation: 2w RM ANOVA, group × session: $F_{(1,14)} = 0.0547$, $P = 0.8184$; coping: 2w RM ANOVA, group × session: $F_{(1,14)} = 0.4174$, $P = 0.5287$). The same conclusion can be drawn when looking at the half escape temperatures (**Table 1**).

As illustrated in **Figure 3B**$_2$, the nociceptive threshold is similar between the groups, of 44.5 ± 0.8°C and 43.0 ± 0.2°C for sham and cuff animals, respectively (Kruskal–Wallis, KW = 7.556, $P = 0.0561$). Conversely, the escape threshold is also similar between the groups, and we failed to reveal any significant difference between nociceptive and escape threshold during the first session in both groups. When looking at the delta between

escape and nociceptive temperatures illustrated in **Figure 3B**$_3$, a significant decrease can be seen for both groups between the sessions (2w RM ANOVA, group × session, session factor: $F_{(1,14)} = 17.40$, $P = 0.0009$). The negative values in the second session, of −6.8 ± 2.6°C and −4.1 ± 2.2°C from sham and cuff animals, respectively, suggest that despite a blunted overall behavior, both groups still showed an adaptive learning response upon reexposure, leaving the dark compartment before it reached nociceptive values.

3.4. Behavioral responses of neuropathic rats to double avoidance place preference using cold temperatures

Considering the abovementioned results in cuff animals with the hot ramp combined with the well-known sensitivity to cold in this model, we adapted the DAPP test to test this group with a cooling ramp instead of a heating one. As illustrated in **Figure 4A**, during the first session, both groups remained in the dark compartment during most of the testing period, although no animal remained in the dark compartment when it reached 0°C at the 18th minute (data not shown). During the second session, however, a consistent escape from the dark compartment was observed for both groups when the cooling began, demonstrating that the learning response is efficient upon reexposure to the cold environment (baseline: 2w RM ANOVA, group × session: $F_{(1,12)} = 3.351$, $P = 0.0921$; anticipation: 2w RM ANOVA, group × session: $F_{(1,12)} = 0.2587$, $P = 0.6202$; coping: 2w RM ANOVA, group x session: $F_{(1,12)} = 1.194$, $P = 0.2960$). It is worth noting that cuff animals spent less time than controls in the dark compartment during the baseline of the second session, suggesting an increased aversion towards the cold compartment.

As illustrated in **Figure 4B**, we failed to reveal any significative difference in nociceptive thresholds between groups (sham: 12.6 ± 0.9°C, cuff: 15.4 ± 2.4°C; Kruskal–Wallis, KW = 6.923, $P = 0.0744$). However, it should be noted that this could be partially due to the limited number of values for this parameter, part of the animals leaving the dark compartment before having elicited any nociceptive reflex. Once again, the escape threshold was also similar between the groups (see also **Table 1**), and we failed to reveal any significant difference between nociceptive and escape threshold during the first session in both groups. Finally, as hinted at with the AUC of time spent in the dark, **Figure 4C** reveals that both groups showed a significant increase in the delta between escape and nociceptive threshold temperatures between the sessions, translating a proper learning response after reexposure to the cold environment, with animals leaving the dark compartment before it reaches the nociceptive threshold during the second session (2w RM ANOVA, group × session, session factor: $F_{(1,13)} = 19.50$, $P = 0.0007$).

4. Discussion

Although pain is a complex multidimensional integrated process, most preclinical studies only focus on its sensoridiscriminative aspect (ie, using nociceptive tests). It is indeed a challenge to take into account and measure the affective-motivational and cognitive components of pain in nonverbal animals, especially in a single procedure. As such, researchers have tried to come up with paradigms going beyond the mere sensory element of nociception.[19] Although many of those new approaches are based on operant behavior, wherein animals have to be trained to complete a task before being tested, we propose here a test that does not require previous training or habituation and can be used in naive freely moving animals. This test, which uses a thermal aversive environment, is complementary to others that use mechanical aversive stimuli, and which do not require training either.[11,13]

Therefore, we were able to distinguish 2 thresholds: the temperature that elicits the first nociceptive reflex, similar to that found in the literature after a classic hot or cold plate test, and the temperature prompting the animal to leave the dark compartment for good after a period of coping. This highlights the duality between a nociceptive reflex and the conscious decision to escape an aversive situation, as previously hinted at in the literature.[13] Upon reexposure to the procedure, the adaptive behavior in control rats can be attested by the decrease in the time spent in the dark compartment at all stages of the testing paradigm. This led to a final escape before the floor had reached nociceptive temperature values, as illustrated by the negative delta thresholds or by the half-escape temperatures. The use of anxiolytics blunts this adaptive behavior. Although both anxiolytics induced a delay in the escape from the heated dark compartment, the learned escape behavior was partially preserved in EFX-injected animals. However, diazepam animals showed no modification in behavior upon reexposure, nor any

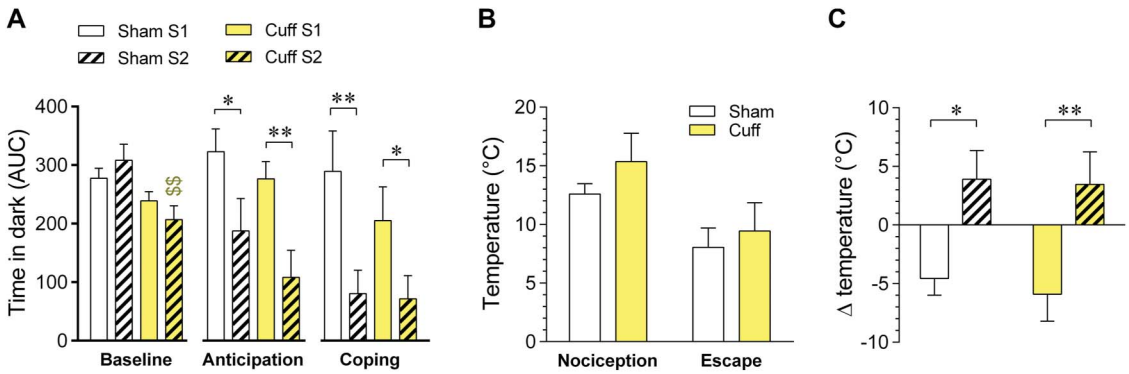

Figure 4. Behavioral responses of neuropathic rats to the DAPP paradigm using cold temperatures. (A) Areas under the curves (AUC) of the time spent in the dark compartment during the 3 phases of the cold-ramped DAPP paradigm during the first (S1) and second (S2) sessions in sham (n = 7) and cuff (n = 7) animals. Statistical significance was assessed with Sidak multiple comparison test. (B) The cold nociceptive and escape thresholds during the first session are similar in the sham (n = 7) and cuff (n = 8) groups. Statistical significance was assessed with Dunn multiple comparison test. (C) The delta between the escape and nociceptive threshold temperatures is reduced in the second session (black stripes) for sham (n = 7) and cuff animals (n = 8). Statistical significance was assessed with Sidak multiple comparison test. For the whole panel, statistical significance was illustrated as follows: $P < 0.05$ (*) or $P < 0.01$ (**) for intragroup comparisons, and $P < 0.01$ ($$) for intergroup comparisons.

difference between the nociceptive and escape thresholds, suggesting a possible effect of the anxiolytic in the reduction of the conflict aversion. This result is consistent with a number of studies reporting the effect of anxiolytics and notably drugs used in the treatment of chronic pain, on cognitive functions.[24] Our results are in good agreement with the pharmacological profile of the 2 anxiolytics used, which induce different consequences on cognitive function. Indeed, etifoxine is a nonbenzodiazepine anxiolytic devoid of mnesic adverse effects at this dosage, as shown in human studies.[20]

In humans, pain and persistent pain are linked to emotional comorbidities, such as anxiety, a vulnerability to depression, or catastrophization. These negative psychosocial factors strongly influence the anticipation and experience of pain and contribute to its long-term outcomes, including work disability and treatment effectiveness.[17] As such, it is of particular interest to see the impact of inflammatory or neuropathic pain on the adaptive response of rats faced with 2 aversive but escapable environments. Indeed, even if CFA rats exhibit hyperalgesia, they tend to remain in the dark heated compartment long after it has become nociceptive, which seems counterintuitive. As pain impacts cognitive capacities,[9] it appears that in this test, inflammatory pain induces a delay in the decision-making processes, leading to the definitive escape from the noxious stimulus, which persists upon reexposure. The review by Moriarty et al. highlights 3 main mechanisms underlying pain-related cognitive impairment, notably limited resources, altered neuroplasticity and dysregulated neurochemistry, which could all interfere with proper cognitive functioning.[18] This hypothesis is in line with the restoration of the learning and adaptive responses of CFA animals following topical lidocaine, which decreases, pain intensity. Conjointly, the anxiety-like phenotype in CFA animals[6] could also be such that animals are more inclined to stay in the lit compartment, although painful. The anxiety specificity of this response could be assessed by exposing CFA animals to the DAPP paradigm following anxiolytic treatment. Overall, CFA animals present a maladaptive response to noxious stimulation beyond the mere nociceptive threshold, translating the major impact of the affective-motivational and/or cognitive response in this group. Conversely, the response to the aversiveness associated with noxious hot appeared drastically different in cuff animals, who did not exhibit nociceptive hypersensitivity.

Although hot plates are widely used in nociceptive tests, cold plates are less common as they provoke less of an obvious reaction in animals, and the scoring relies on the amount of behaviors exhibited in a certain amount of time or the amount of time spent presenting this behavior.[12] The use of a cold ramp in the DAPP test provided new insights on the spontaneous behavior relating to cold of both sham and cuffed rats. Both sham and cuffed animals had a nociceptive threshold around the 15°C cold threshold commonly described in the literature[1] and a strong adaptive response to cooling upon reexposure. It may seem surprising that the nociceptive threshold would not be significantly different between sham and cuffed rats, given that cuff rats usually show very obvious allodynia to cold when the acetone test is used[2]; however, the number of animals exhibiting a nociceptive reflex was probably too low in this study to allow for statistical significance following a very progressive cold ramp. Based on the observation of spontaneous behavior, hypersensitivity to cold does not seem to have an impact on the anticipatory behavior of leaving the noxious cold compartment. This clearly shows that the cold pain in the test remains bearable, allowing the animal to analyze the potential danger of its environment and adapt its escape response. The same behavior was observed in the hot paradigm, but rats did not show hyperalgesia to hot stimuli in this model. Altogether, it is not possible today to exclude the presence of a learning difference in neuropathic animals in models more severe than the cuff model.

In summary, this original paradigm allows not only the measure of classic nociceptive thresholds but also speaks for an integrated cortical and subcortical response leading to a proactive avoidance behavior in freely moving animals without prior training, also described in human studies.[23] In this sense, we believe that the implementation of the DAPP paradigm in preclinical studies could improve the scope of pain-related research, thereby improving the translatability of fundamental research on pain. Beside the all-in-one testing procedure of sensory, affective, and cognitive pain expressions, it is worth mentioning that the DAPP paradigm fully complies with the ethical rule in animal experimentation and promotes the 3R approach (replacement, reduction, and refinement).

Conflict of interest statement

The authors have no conflict of interest to declare.

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All data will be made available to other investigators upon request to the corresponding author.

Data are available upon request.

Supplemental digital content

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