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A reassessment of stress-induced "analgesia" in the rat using an unbiased method

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ABSTRACT

An increased tail-flick latency to noxious heat during or after stress in the rodent is usually interpreted as a stress-induced reduction in pain sensitivity and often described as a form of stress-induced "analgesia." However, this measure is an indirect and flawed measure of the change in nociceptive threshold to noxious heat. A major confound of the latency measure is the initial temperature of the tail, which can drop down to room temperature during stress, the consequence of a marked sympathetically mediated vasoconstriction in the skin of the extremities. We addressed this issue with tail-flick tests during contextual fear using infrared thermography to monitor temperature changes and a CO_2 laser to deliver the heat stimulus. The experiment revealed a 4.2°C increase of the nociceptive threshold, confirming a true antinociceptive effect. However, its contribution to the increased withdrawal latency was less than two-thirds (63.2%). Nearly one-third (32.2%) was due to the drop in tail temperature (4.4°C), which also slowed conduction along sensory fibers (2.2%, included in the 32.2%). The remaining 4.6% was due to an increase in decisional/motor latency. This new unbiased method establishes beyond doubt that a conditioned stress response is associated with true antinociception to noxious heat. It also confirms that stress-induced changes in skin temperature can be a major confound in tail-flick tests. The present study shows, for the first time, the exact contribution of these two components of the tail-flick latency for a stress response.

1. Introduction

Stress-induced analgesia is a well-described phenomenon that can be observed and reproduced in laboratory animals [2,11,12,41,43,46,59]. The common view is that this form of reduced reactivity to pain is due to activation of descending inhibitory pain pathways leading to an increase in nociceptive threshold, that is, an increase of the threshold at which the nociceptive stimulus becomes painful and triggers a withdrawal response. However, the actual nociceptive threshold and its changes during stress in the conscious animal have in fact never been measured.

While a number of nociceptive tests have been used to study the effect of stress on pain, the most common ones are thermal tests such as the tail-flick, in which the degree of presumed analgesia is evaluated from changes in the withdrawal reaction time (or latency) [39]. This is a convenient behavioral measure; however, it is an integrated measure that depends only partly on the nociceptive threshold [5]. As has been pointed out already, one critical variable that can affect the withdrawal reaction time is the initial temperature of the skin where the stimulus is applied [5,20,30,38].

The confounding effect of the initial temperature is a major concern when testing thermal nociception during stress because stress can lead to large variations in skin temperature. It is well known that centrally evoked defense reactions or naturally evoked stress responses are associated with sympathetically mediated vasoconstrictions in the skin that lead to cooling of cutaneous territories, especially in the extremities [1,3,8,9,19,22,47]. This is best observed with infrared thermography, as we recently reported, in fear-conditioned rats [56]. In this study, which was conducted at room temperature, marked reductions in skin temperature (4–6°C) were observed in the tail and paws. Such a drop in skin temperature is not trivial. It could significantly increase the withdrawal reaction time of a tail-flick independently of any change in nociceptive threshold. At present, there is no way to determine the contributions of these 2 factors. Methods to minimize or control for the confounding effect of the initial temperature have been proposed, however, they are tedious and not always amenable to some forms of stress, such as conditioned fear, where the animals must be freely moving and preferably untethered [54].

The aim of this study was to reassess the stress-induced "analgesia" evoked by conditioned fear to context (also known as condi-

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tioned analgesia), using a new method for testing and analyzing the tail-flick in conscious unrestrained rats. The method uses infrared thermography to record the changes in temperature at a spot on the tail where noxious heat is delivered by a CO_2 laser beam. This new method allowed us to determine for the first time (1) the actual change in the nociceptive threshold to noxious heat and (2) the exact contributions of this change in nociceptive threshold and of the change in initial skin temperature to the tail withdrawal reaction time during contextual fear.

2. Methods

2.1. Animals

Experiments were performed on adult male Sprague-Dawley rats (400–550 g, n = 36) in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, the European Communities Council Directive 86/609/EEC regulating animal research, and the ethics committee of the International Association for the Study of Pain (IASP) [17,62]. The animals were housed in groups of 8 in a room maintained at 20–25°C with a normal day/night cycle. The experiments were conducted between 11 AM and 7 PM.

2.2. Fear conditioning and testing

Contextual fear conditioning and testing was done in foot-shock chambers (21 cm long × 21 cm wide × 60 cm tall) made of clear Polymethyl Methacrylate (Plexiglas) walls and open at the top with a grid floor composed of 12 stainless steel rods (1 cm in diameter), spaced 1.7 cm apart, and wired to a shock generator (Fig. 1). The chambers were cleaned before and after use with 0.5% acetic acid. Conditioning started with 2 preconditioning sessions lasting 5 minutes each, during which the animal was allowed to explore the shock chamber with no shock being delivered. It was followed by 4 shock sessions done on separate days over a period of 1 week. Each shock session consisted of a 40-minute-long exposure to the foot-shock chamber with 4 unsignaled electric foot-shocks (1 mA, 1 s) delivered at approximately t = 5, 15, 25, and 35 minutes. The conditioned fear response was tested by re-exposing the rats to the same foot-shock chamber for 40 minutes, with no shock delivery. Half of the animals were fear-conditioned with the shocks (Fear group). The other half was treated in exactly the same way but never received shocks (Control group).

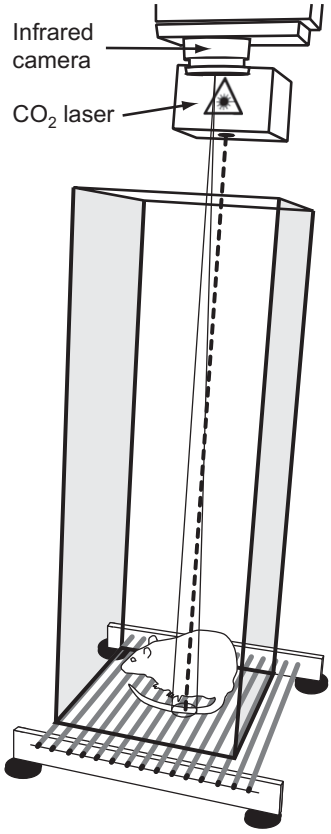

Fig. 1. The experimental setup used for testing the tail-flick. The dotted line shows the path of the CO_2 laser beam aimed at the midsection of the tail. The infrared camera, which was mounted next to the laser unit, was focused on the same area. The tail was brought under the laser beam by gently sliding the whole cage on the smooth surface of the bench.

2.3. Laser heat stimulation

The heat source was a laser beam generated by a CO_2 laser unit (CO_2LSD, SIFEC, Ferrière, Belgium; see Benoist et al. [5]) placed 65 cm above the floor of the foot-shock chamber in which the animal was tested (Fig. 1). The position of the beam, which was fixed, was made visible by a red light beam (He–Ne) invisible to the rat, collimated with the laser beam. To bring the tail of the unrestrained rat under the beam, the chamber was mounted onto 4 feet that could slide smoothly on the hard surface of the bench. Thus, the tail of the rat was positioned under the beam without touching or disturbing the animal, simply by slowly moving the chamber. Stimulation could be performed only while the animal was immobile, which was either while freezing (Fear group) or when at rest (Control group). The surface stimulated was circular and did not exceed the width of the tail. The actual diameter of the stimulus, its intensity and duration depended on the type of experiment (see below, 2.5. Experimental design).

2.4. Infrared thermographic recording

The spatial and temporal evolution of the change in skin temperature caused by the laser stimulation was recorded with a thermographic video camera (JADE MWIR camera, 3–5 μm optical bandpass, CEDIP Infrared Systems, Croissy-Beaubourg, France; see Benoist et al. [5]). This camera had a 500 μs integration time, and supplied images of 320 × 240 pixels at 172 Hz, with a sensitivity of 0.02°C at 25°C. The camera was also positioned 65 cm above the animal, next to the laser unit (Fig. 1). It was operated by the Cirrus software (CEDIP Infrared Systems) and calibrated by means of a black body (CI SR80 CI Systems, Migdal Haemek, Israel). Spatial and temporal resolutions were 0.3 mm and 5.8 ms, respectively. The recording started 500 ms before the application of the stimulus and was stopped within a few seconds of the tail-flick (<1 mm displacement of stimulated area) or, if the tail had not moved, 6 s later.

2.5. Experimental design

Two series of experiments were conducted. In both experiments, the entire tail was depilated either on the eve or on the morning of the experiment with a depilatory cream (Hair removal cream Dermo Tolerance, Vichy Laboratories, Cusset, France.). The cream was applied for 10 minutes, followed by thorough rinsing.

2.5.1. Variable intensity experiments

The tail-flick response is defined by 3 parameters (Fig. 2). These are: the true nociceptive threshold (referred to as behavioral threshold, or Tβ, from now on), the behavioral latency (Lβ, the time it takes for the tail to withdraw once the behavioral threshold has

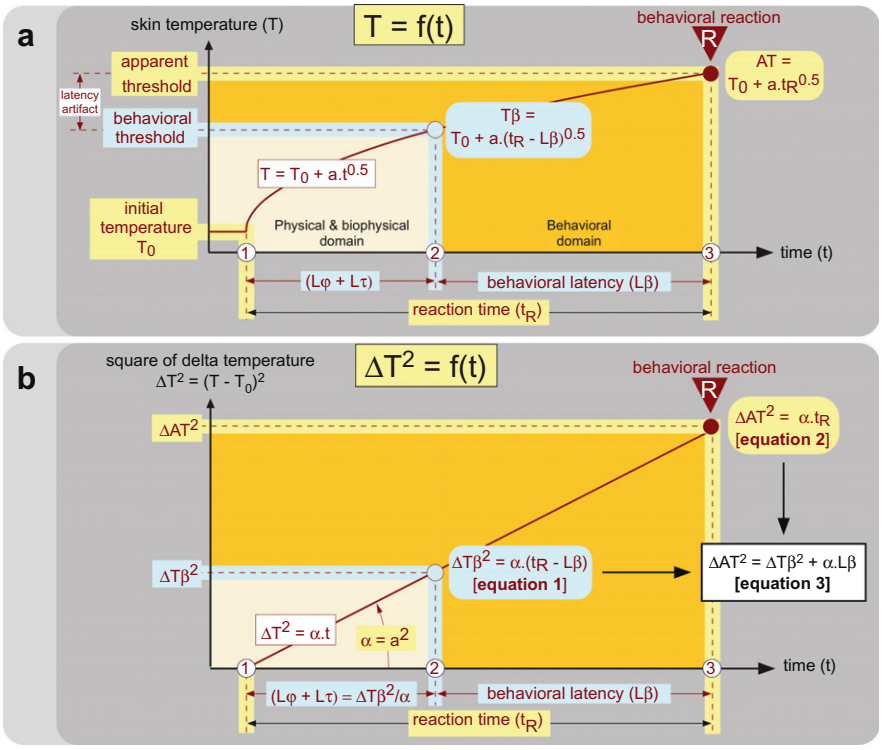

Fig. 2. Modeling of the tail-flick response, critical parameters, and principle of analysis. The measurable variables are indicated with a yellow background, and variables to be determined are indicated with a blue background. Abbreviations are available in Table 1. (a) When the skin is exposed to a constant source of radiant heat, its temperature (T) increases with the square root of time from an initial temperature T_0, according to the law of physics $T = f(t) = T_0 + a * t^{0.5}$. The constant term "a" is proportional to the power density of the heating source. When this density is high enough, a withdrawal response (tail-flick) is triggered within a period, the reaction time t_R. The reaction time t_R is the sum of the time it takes to warm the skin up to the behavioral threshold Tβ (ie, the true nociceptive temperature threshold) plus the time it takes for the withdrawal to occur once this threshold has been reached. The first period belongs to the physical and biophysical domains, including heating (Lφ) and transduction (Lτ) processes. During the second period, the temperature of the skin continues to increase up to the apparent temperature threshold (AT) reached at the time of the withdrawal. This second period, which is the true latency of the reflex, is defined as the behavioral latency (Lβ) and includes the conduction time of the nociceptive fibers carrying the information from the point of stimulation to its entry in the central nervous system. (b) Expressed in terms of squared temperature variations, the relation $T = f(t) = T_0 + a * t^{0.5}$ becomes linear: $\Delta T^2 = [T(t) - T_0)]^2 = a^2 * t = \alpha * t$. Overall, 4 variables are potentially accessible to experimental measurements: T_0, AT, t_R, and α. The heating process can be described by 3 key moments: (1) the beginning of the stimulation, $t = t_0$ and $T = T_0$; (2) the moment of the triggering of the reaction defined by $\Delta T\beta^2 = (T\beta - T_0)^2 = \alpha * (t_R - L\beta)$ [equation 1]; (3) the moment of the reaction defined by $\Delta AT^2 = (AT - T_0)^2 = \alpha * t_R$ [equation 2]. Equation 3 is obtained by substituting $\alpha * t_R$ of equation 2 into equation 1 and is used for extraction of the 2 variables to be determined, Lβ and Tβ (see Fig. 3).

been reached), and the initial temperature of the tail (T_0) (see Table 1 for definitions of all abbreviations and symbols). The aim of the first series of experiments was to extract the behavioral threshold Tβ and the behavioral latency Lβ of the tail-flick responses in the animals of the Fear and Control groups (n = 10 each). This was done during a single re-exposure by applying laser stimuli of variable intensities to a 2-cm long section of the tail, 5 cm from its base. The stimuli, which had a constant diameter of 15 mm (Fig. 3a) were applied in the following recurring sequence: 5 cm, 6 cm, 7 cm, 5.5 cm, and 6.5 cm from the base of the tail, each no less than 2 minutes apart. It follows that the time between 2 stimulations at a given site was always more than 10 minutes. The stimulations were aborted or discarded if any spontaneous movement occurred at the beginning of the stimulation. The stimulations were also automatically cut off 6 s after their onset to avoid burning injuries in case the tail would not react. The intensities of stimulation varied within a power range of 0.25 to 2.5 W, which produces responses within 0.3–6 s without damaging the skin. In these conditions, the slope α (an index of the heat intensity of the stimulus, Fig. 2) was in the 0.03–3°C^2 ms^{-1} range and the maximum temperature reached at the actual moment of the tail reaction was always lower than 70°C.

2.5.2. Constant intensity experiments

The aim of the second series of experiments was to extract the conduction velocity of the nociceptive fibers that triggered the tail-flick response in the animals of the Fear and Control groups (n = 8 each). The conduction time contributes to the behavioral latency (Lβ). For this experiment, laser stimuli of constant short duration and high intensity (100 ms duration; 150 mJ; 11 mm diameter) were applied from the base to the tip of the tail, each 5 mm apart in a random sequence. Each site of stimulation was stimulated only once. Conduction velocities were calculated on the basis of the linear relationship between the tail withdrawal reaction time (t_R) and the distance that separates the site of stimulation to the spinal entry zone of tail sensory fiber (D). To determine this distance, the animals were autopsied at the end of the experiment after being sacrificed with an overdose of pentobarbital. The L3 vertebra was carefully identified and the distance between the base of the tail and the L3 vertebra was measured. This level was considered as the main entry zone in the cord for afferent signals from the tail on the basis that: (1) the 4 major nerves innervating the tail, namely the dorsolateral and ventrolateral tail nerves, project to dorsal horn superficial laminae of S2–Co2 segments; (2) these segments are located within vertebrae L2–L4; and (3) the maximum N-wave dorsum potential elicited by electrical stimulation of the tail is found in the middle of a laminectomy of vertebrae L2–L4 [5].

2.6. Analysis

The thermographic films were analyzed with the Altair software (CEDIP Infrared Systems) as described in Benoist et al. [5]. For the

Table 1
Symbols, abbreviations and units.

a	Constant of the heating ramp (°C $ms^{-0.5}$)
α	Slope of the squared temperature variation over time (°C^2 ms^{-1}) = a^2
AT	Apparent threshold (°C) = temperature reached when the behavioral reaction occurs
Co2	Second coccygeal level of the spinal cord
control	Related to a variable recorded in the control situation
D	Distance between the stimulation site and the dorsal horn entry zone (mm)
δ_1	Delay in the reaction time t_R due to the lower initial temperature of the skin T_0 in the fear animals (ms)
δ_2	Delay in the reaction time t_R due to the increase in behavioral threshold Tβ in the fear animals (ms)
δ_3	Delay in the reaction time t_R due to slower motoneurons recruitment in the fear animals (ms)
δT_0	Decrease of the initial skin temperature in fear animals relative to control animals (°C) = $T_{0control}$ - T_{0fear}
δTβ	Increase of the behavioral threshold in fear animals relative to control animals (°C) = $T\beta_{fear}$ - $T\beta_{control}$
δt_R	Increase of the reaction time in fear animals relative to control animals (ms)
ΔAT	Temperature variation at the apparent threshold, relative to the initial temperature (°C) = AT - T_0
ΔT	Temperature variation relative to the initial temperature (°C) = T – T_0
ΔTβ	Temperature variation at the behavioral threshold, relative to the initial temperature (°C) = Tβ – T_0
fear	Related to a variable recorded during fear
L2	Second lumbar vertebra
L4	Fourth lumbar vertebra
Lβ	Behavioral latency (ms) = time taken for the behavioral reaction R to occur once the behavioral threshold Tβ has been reached
Lφ	Physical latency (ms) = time taken to reach the temperature at which transduction in nociceptors is triggered, from the initial skin temperature T_0
Lτ	Transduction latency (ms) = time required for noxious heat to be transduced into action potentials by nociceptors
Q_{10}	Rate of change in nerve conduction velocity for a 10°C increase in temperature
R	Behavioral reaction = tail withdrawal reaction or tail-flick
S2	Second sacral level of the spinal cord
t	Time (ms)
t_0	Beginning of the stimulation
t_R	Time at which the behavioral reaction occurs (ms) = reaction time
T	Skin temperature of the tail (°C)
T_0	Initial skin temperature of the tail (°C)
T_a	Ambient temperature (°C)
Tβ	Behavioral threshold (°C) = temperature at which the true nociceptive threshold is reached
V	Conduction velocity of the sensory fibers that trigger the behavioral reaction (m s^{-1})
virtual	Related to a computed virtual variable

variable intensity experiments, this involved the following steps: (1) delineation of the stimulated area; (2) extraction of the initial temperature T_0 in the center of this area; and (3) extraction of the temperature (T) of the warmest pixel in this area from each image captured during stimulation (every 5.8 ms) until the last image preceding the tail withdrawal reaction. Examples of the resulting curve T = f(t) are shown in Fig. 3b. The tail withdrawal reaction time t_R was calculated by counting the number of images captured until the tail reaction where the temperature curve exceeded T_0 + 1°C (~5 SD calculated within a 0.5 s control period) and multiplying this count by 5.8 ms. The data from each curve were then imported into Excel (Microsoft Corporation, Redmond, WA) and further analyzed to extract the relevant variables. The principles of the method are illustrated in Figs. 2 and 3b–e. For a detailed description of the psychophysics of the tail-flick response, see our previous work [5]. For the constant intensity experiments, the only variable extracted from the thermographic films was the reaction time t_R as explained above. t_R was then plotted against the distance of the site of stimulation from the spinal entry zone of sensory fibers (D) to extract the conduction velocity of the fibers.

2.7. Statistical analyses

Least squares linear regressions and one-way analyses of variance were used for statistical purposes. Calculations were performed with the statistical software StatView 5.0 (SAS Institute, Cary, NC). Results were considered significant at $P < 0.05$. Data are expressed as means (±95% confidence interval).

3. Results

3.1. Behavioral changes

Fear-conditioned animals re-exposed to the foot-shock chamber displayed the typical tense freezing immobile posture that characterizes conditioned fear responses. As we have shown previously [56], freezing lasted for most of the re-exposures (40 minutes) except in the last 10 minutes, when it was interrupted by short periods of slow and cautious movements. In contrast, control animals first displayed activity (5–10 minutes), then went to rest, adopting a relaxed, immobile posture. Tail-flick tests were performed during these periods of immobility, that is, during freezing in the Fear group or during rest in the Control group.

3.2. Behavioral thresholds Tβ and behavioral latencies Lβ

The behavioral thresholds and behavioral latencies of the tail-flick response tested during the re-exposures were determined for each rat of the Fear and Control groups by running tail-flick tests at variable intensities of laser heat. Fig. 3 shows how the data were analyzed for 2 single re-exposures, one of a control animal (in blue) and one of a fear-conditioned animal (in red). The 2 images shown in Fig. 3a were captured just before the tail withdrawal during laser stimulations of the same intensity (see also Movie 1 and Movie 2 in Supplementary Material for examples of movies showing the whole sequence). Two important observations can already be made by comparing the 2 images: first, the surface temperature of the tail outside of the stimulated area is colder in the fear-conditioned animal than in the control; second, the surface temperature reached inside the stimulated area just before the tail reaction is warmer in the fear-conditioned animal than in the control.

The analysis was conducted in 4 steps, as shown in Fig. 3b-e. The principles of the method are illustrated on the left of Fig. 3 in reference to the tail-flick model shown in Fig. 2. First, the temperature of the hottest pixel in the stimulated area was extracted from each frame of the video and plotted against time to build the response curves (T = f[t]; Fig. 3b). Each curve corresponds to a single stimulation made during the re-exposure. Each curve starts 500 ms before the laser stimulation and ends at the time

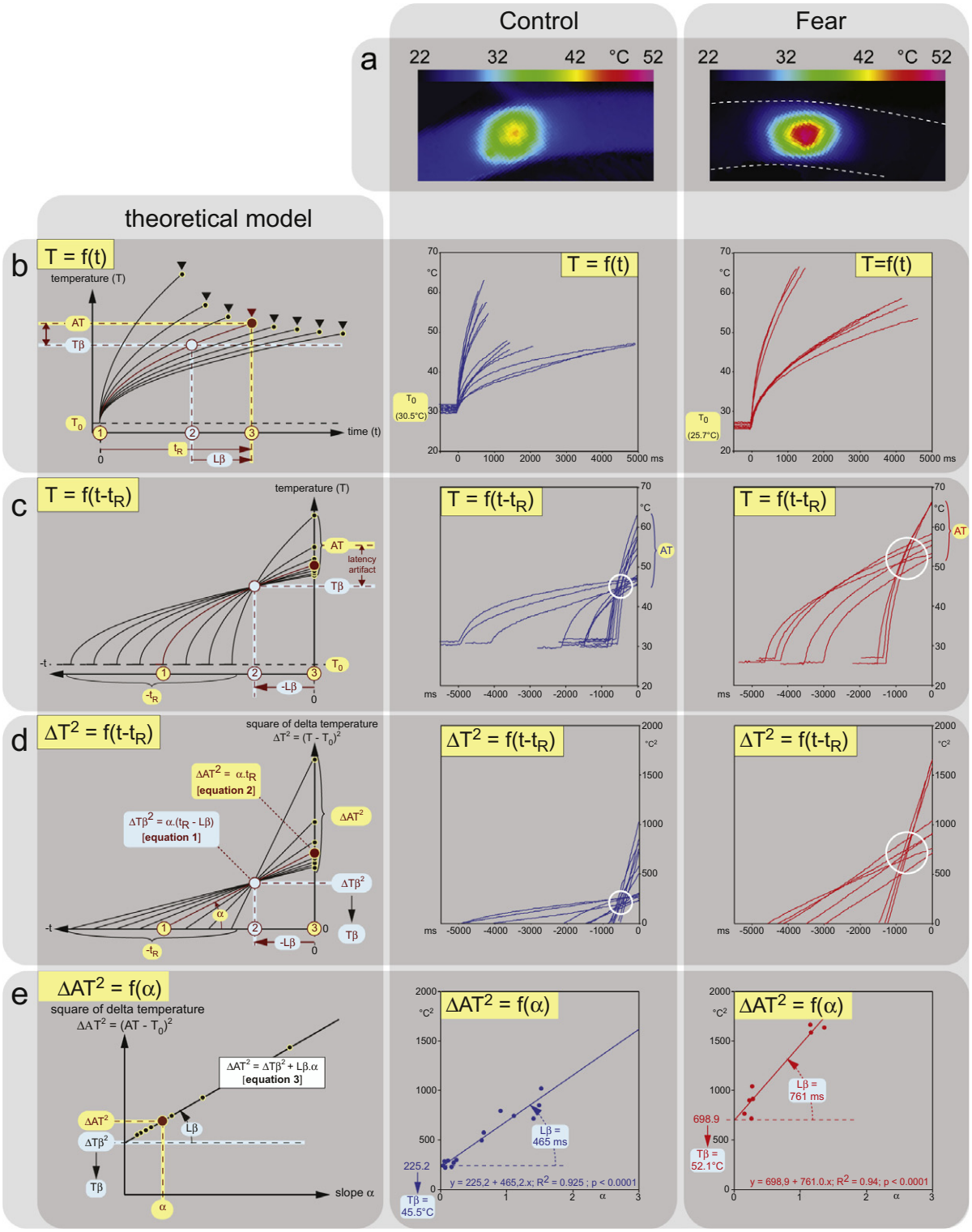

Fig. 3. Extraction of the behavioral threshold (Tβ) and behavioral latency (Lβ) from the tail-flick response. This is an example taken from a control rat (blue) and a fear-conditioned rat (red) using data gathered during a single re-exposure with stimulations done at variable intensities. (a) Thermal images showing the area stimulated by the CO_2 laser (same intensity) just before tail withdrawal. (b) Temporal evolution of the temperature of the skin for each stimulation (14 stimulations in the control rat and 8 in the fear-conditioned rat, done at varying intensities). Note the lower initial temperature (T_0) in the fear-conditioned animal. (c) The same curves realigned at the time of the withdrawal (back timing). The white circles correspond to the privileged zone where the curves cross each other, which is when the behavioral threshold Tβ is reached. The apparent thresholds (AT) (temperature at the time of withdrawal) vary depending on the intensity of the stimulation. (d) The same curves now expressed as the square of the difference in temperature ($\Delta T^2 = [T - T_0]^2$), transforming them into straight lines of variable slopes α that cross the *y* axis at $\Delta AT^2 = (AT - T_0)^2$. (e) Plots of each ΔAT^2 as a function of its respective α. According to Equation (3) (see also Fig. 2), the points should be distributed along a line whose slope and intercept are Lβ and $\Delta T\beta^2$, respectively. These 2 parameters are then determined by regression analysis and Tβ is finally extracted ($\Delta T\beta^2 = [T\beta - T_0]^2$). Note the higher Tβ and Lβ values in the fear-conditioned animal. See also Supplemental Movie 1 and Movie 2 for examples of movies showing the temperature changes of the tail where the laser stimulus is applied.

of the tail reaction (reaction time, t_R). Curves obtained with stimuli of higher intensities appear on the left and are characterized by faster rises in temperature and shorter reaction times. As expected, there was a clear difference between the 2 animals in the initial temperature of the tail (T_0); the fear-conditioned animal being on average approximately 5°C colder than the control animal.

The same curves were then realigned at the time of the tail reaction ($T = f[t - t_R]$; Fig. 3c). Note that all the curves tend to meet in a privileged zone (white open circle). This corresponds to the point at which the true behavioral threshold Tβ of the tail-flick response is reached (Fig. 2). The tail reaction occurs shortly after, with a delay that corresponds to the behavioral latency of the response (Lβ). When the reflex occurs, the temperature reached is the apparent temperature threshold (AT), which increases with the intensity of the stimulus.

The next 2 rows in Fig. 3 show how Tβ and Lβ were extracted. First, a linear transformation was done by expressing the square of the temperature change as a function of time ($\Delta T^2 = [T - T_0]^2 = f[t - t_R]$; Fig. 3d). The resulting lines confirm that the temperatures increased with the square root of time, with a slope α proportional to the squared power density of the laser beam. As with the temperature curves, the privileged zone where the lines cross should correspond to the point when Tβ is reached. By definition, the coordinates of this virtual point on each line are $x = -L\beta$ and $y = (T\beta - T_0)^2 = \Delta T\beta^2$ (see also Fig. 2b). By definition also, these 2 values are bound in a linear relationship between the square of the apparent threshold ΔAT^2 and the slope α of each line, since substitution of $\alpha.t_R$ of equation (2) in equation (1) (Figs. 2b and 3d), gives after rearrangement: $\Delta AT^2 = \Delta T\beta^2 + L\beta.\alpha$ [equation (3)] (Figs. 2b and 3e). From this linear relationship, one can extract the slope Lβ and intercept with the ordinate $\Delta T\beta^2$ by plotting each ΔAT^2 value against its respective α value. Indeed, clear linear relationships were observed when these plots were constructed for each animal, which allowed extraction of Lβ and $\Delta T\beta^2$ by regression analysis (Fig. 3e). Tβ was finally calculated by subtracting the average T_0 from the square root of $\Delta T\beta^2$. The results in these 2 animals gave a Tβ of 52.1°C for the fear-conditioned animal, which is 6.6°C higher than in the control animal (45.5°C), and an Lβ of 761 ms, which is 296 ms longer than in the control animal (465 ms).

The group results (n = 10 in each group) confirmed the trend revealed in the 2 examples (Fig. 4). First, T_0 was ~4.4°C lower in the Fear group compared to the Control group (26.1 [25.7–26.5] vs 30.5 [29.6–31.4]°C, $P < 0.001$), while there was no difference in ambient temperature (T_a) (24.7 [24.5–24.9] vs 25.0 [24.7–25.3]°C, respectively). Second and most importantly, Tβ in the Fear group was ~4.2°C higher (50.7 [48.0–53.3] vs 46.5 [45.1-47.9]°C, $P < 0.02$) and Lβ ~127 ms longer (548 [474–622] vs 421 [358–484] ms, $P < 0.02$) than in the control group. Thus, there was a significant increase of the nociceptive threshold Tβ during fear.

3.3. Conduction velocity of afferent fibers

A second experiment was carried out to determine the conduction velocity of the sensory fibers carrying the afferent signal from the periphery to the spinal cord (Fig. 5). This conduction speed, which is slow because it is mediated by unmyelinated nociceptive fibers, makes a significant contribution to the behavioral latency Lβ [5]. Because Lβ was 127 ms longer in the fear-conditioned animals, we sought to find out if the conduction velocity of the afferent signal in these animals had changed. The constant stimulus (see insert in Fig. 5a) was applied at varying distances on the tail between its base and tip during single re-exposures, and the reaction time t_R was plotted against the distance (D) between the point being stimulated and the level of entry of the sensory fibers in the spinal cord (Fig. 5a). In all cases (8 rats in each group), there was a highly significant linear relationship between D and t_R. From these we extracted regression lines for each re-exposure (Fig. 5a). The figure shows that the lines were roughly parallel within each group and that those of the Fear group were steeper, indicating a slower conduction velocity (inverse of the slope). Note that all the lines tended to cross in a privilege zone (white circle, ~98 mm from the level of entry, see Methods). This zone corresponds approximately to the transition between tail and core, which is where temperature differences between the 2 groups should disappear.

The averages of the conduction velocities (V) and initial tail temperatures (T_0) were then calculated for each group (Fig. 5b). As can be seen, there was a significant reduction (39%) of the speed of conduction in the animals of the Fear group compared to those of the Control group (0.57 [0.54–0.61] vs 0.93 [0.80–1.06] m s^{-1}, $P < 0.001$). The tail temperature in the animals of the Fear group was also significantly reduced, as observed in the first experiment (−4.3°C, $P < 0.001$). In fact, a clear relationship is apparent between the changes in conduction velocity and the changes in tail temperature (Fig. 5c), which is entirely consistent with similar data reported in a parallel study [5] (white dots in Fig. 5c). A regression analysis done on all these points combined reveals a tight and significant correlation ($y = -0.455 + 0.041x$; $R^2 = 0.836$, $P < 0.001$), indicating a clear effect of the temperature of the tail on the conduction velocity of the sensory fibers that trigger the tail-flick response (Q_{10} ~200% between 20°C and 30°C). Thus, there is a

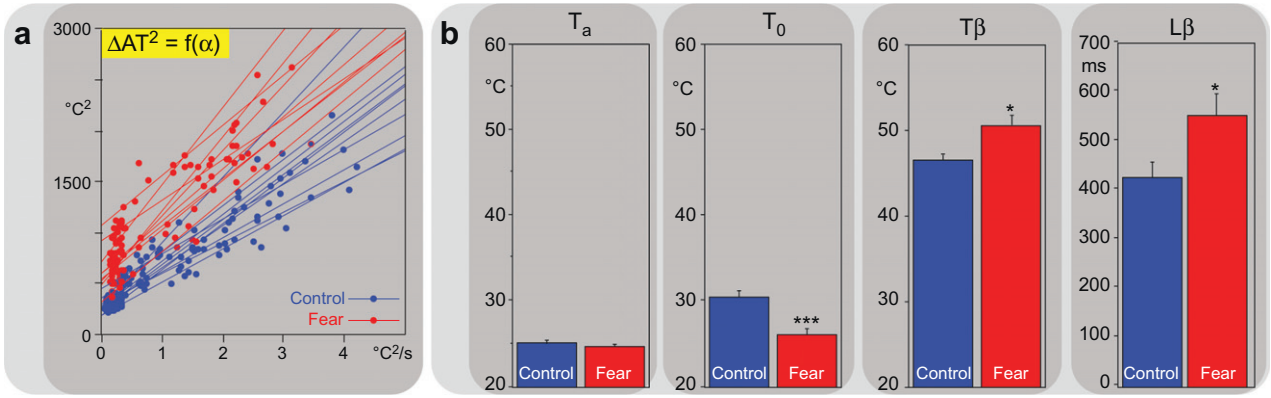

Fig. 4. Group results from the variable intensity experiments showing the effect of conditioned fear on the behavioral threshold (Tβ) and behavioral latency (Lβ) of the tail-flick response. The mean values are based on 10 control rats (blue) and 10 fear-conditioned rats (red). (a) Regression lines ($\Delta AT^2 = f[\alpha]$) (as in Fig. 3e) for each animal. Note that both the slopes and intercepts of the lines tend to be greater in the fear-conditioned animals. (b) Summary of the results for the ambient temperature (T_a), initial temperature of the tail (T_0), behavioral threshold (Tβ), and behavioral latency (Lβ). Mean ± 95% confident interval. $^{*}P < 0.02$; $^{***}P < 0.001$.

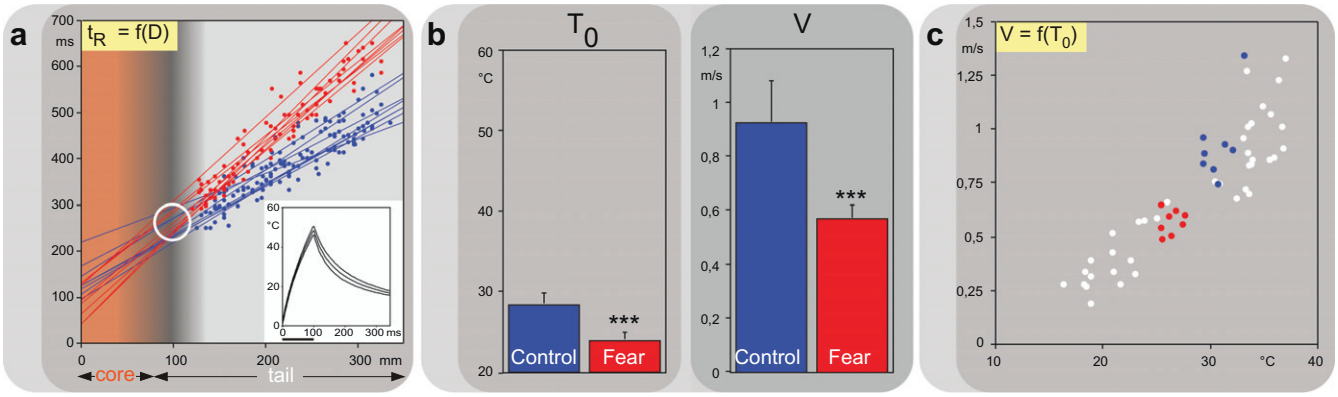

Fig. 5. Group results from the constant intensity experiments showing the effect of conditioned fear on the conduction velocity of afferent fibers. (a) Plots showing the relationship between the level of stimulation along the tail (distance [D] calculated from the spinal entry zone of afferent fibers [see Methods]) and the reaction time (t_R) of the tail-flick response in each of 8 controls (blue) and 8 fear-conditioned animals (red). The insert shows the temperature response (mean ± 95% confidence interval) to the brief laser pulse of constant intensity (100 ms, 150 mJ) (horizontal bar) in an individual rat. In each animal, highly significant linear relationships were found between D and t_R, as shown by the regression lines. Note that the regression lines are steeper in the fear-conditioned animals, indicating a slower conduction velocity. The white open circle corresponds to a zone where the temperature changes between that of the tail and that of the core (98 mm, determined statistically from the cluster of intersection points of the straight lines Lβ = f[D] in the positive plane, obtained from the 2 groups of rats). (b) Summary of the group results for the initial tail temperatures (T_0) and conduction velocity (V) in the 2 groups. (c) Relationship between the initial tail temperature (T_0) and the conduction velocity (V) in each of the 16 animals (blue and red dots). The white dots are from Benoist et al. [5] and are shown here to demonstrate the tight relationship between the 2 parameters. $^{***}P < 0.001$.

decrease in the conduction speed of the tail portion of the afferent fibers in the fear-conditioned animals, and this decrease is likely to be related to the drop in tail-skin temperature. For stimulations done 6 cm distal to the base of the tail (as was done in the first experiment), this decrease in conduction velocity would have resulted in a 41 ms delay (60/0.57–60/0.93). This is a significant contribution (about one-third) to the 127 ms increase in Lβ between the fear-conditioned and control animals. The remaining two-thirds (127 − 41 = 86 ms) will be considered in the next section.

3.4. Reconstruction of the tail-flick responses

Finally, we performed calculations to evaluate the contributions of the drop in skin temperature and increase in nociceptive threshold to the increase in reaction time of the tail-flick response (ie, the conventional tail-flick latency). Using the equations of the tail-flick model (Fig. 2) and the 3 variables (T_0, Tβ, Lβ) we have determined, we reconstructed the tail-flick response for an average control and fear-conditioned animal at a stimulation intensity of $\alpha = 0.2°C^2\ ms^{-1}$ (Fig. 6a; these calculations apply only to constant power sources of infrared radiation). The reconstructed responses at this intensity show that the reaction time t_R would be more than doubled in the fear-conditioned animal compared to the control (t_{Rfear} = 3574 vs $t_{Rcontrol}$ = 1701 ms, a difference $\delta t_R = t_{Rfear} - t_{Rcontrol}$ = 1873 ms or a 110% increase). A similar figure is obtained if we select the stimulations that were done at $\alpha \sim 0.2°C^2\ ms^{-1}$ in the first experiment (3419 [3070–3767] ms for the Fear group vs 1683 [1521–1845] ms for the Control group, a difference δt_R = 1736 ms or a 103% increase, calculated from a total of 18 stimulations in 8 fear-conditioned animals and 18 stimulations in 8 control animals done at $\alpha = 0.19°C^2$ [0.18–0.21]$°C^2\ ms^{-1}$ and 0.20 [0.19–0.21]$°C^2\ ms^{-1}$, respectively [see Supplementary Fig. S1]).

To evaluate the contribution of the drop in initial temperature of the tail δT_0 ($T_{0control} - T_{0fear}$) to the doubling of the reaction time (or $\delta t_R = t_{Rfear} - t_{Rcontrol}$ = 1873 ms increase), one must first consider a direct effect of the initial temperature on the behavioral threshold Tβ. Previous work from this laboratory [5] has shown that for each °C drop of the initial tail temperature in the 20–30°C range, there is, in fact, a corresponding reduction of Tβ of 0.27°C (see Fig. 7C in Benoist et al. [5]). Accordingly, for δT_0 = 4.4°C, the reduction would be 1.2°C, bringing the control Tβ of 46.5°C to a "virtual" control Tβ ($T\beta_{virtual}$) of 45.3°C (Fig. 6b). As shown in Fig. 6b, the time required to reach this virtual threshold from the lower T_0 is $(T\beta_{virtual} - T_{0fear})^2/\alpha$ = 1843 ms, which represents an increase of 1843 − 1280 = 563 ms compared to the experimental control condition. The other direct effect of the drop δT_0 is the decrease in conduction velocity of the peripheral sensory fibers, which, as calculated above, adds a 41 ms delay to the behavioral latency Lβ. Thus, independently of any other factor, the direct contribution of the drop in initial temperature to the increase in reaction time δt_R in the fear-conditioned animals is 563 + 41 = 604 ms (shown as δ_1 in Fig. 6b). This represents 604/1873 = 32.2% of δt_R.

The remaining portion of δt_R (1269 ms or 67.8% of δt_R, shown as δ_2 [1183 ms] plus δ_3 [86 ms] in Fig. 6c) corresponds to the real effect of the fear condition on the processing of the nociceptive stimulus leading to the tail reaction. The main component, δ_2 (1183 ms) is the delay due to the increase in Tβ, which is, in fact, 4.2 + 1.2 = 5.4°C because of the direct effect of the temperature drop on Tβ, as mentioned above (Fig. 6b). The other one, δ_3, much smaller (86 ms), is the difference between $L\beta_{fear}$ and $L\beta_{virtual}$ (548 − 462 ms; Fig. 6c) or the difference that remains between $L\beta_{fear}$ and $L\beta_{control}$ after the delay due to slower conduction speed of peripheral fibers (41 ms) has been subtracted (548 − 421 − 41 ms; Fig. 6c). It is also the remaining factor mentioned at the end of the previous section of the Results. This additional delay, which is independent of Tβ and T_0 and represents 4.6% of δt_R, must occur either centrally or on the motor side of the reflex or both, and most likely represents an effect of the fear response on the recruitment of the motoneurons mediating the reflex. It corresponds to the 2 components of Lβ that Benoist et al. [5] called decisional latency and motor latency.

In summary, the overall effect of the drop in tail-skin temperature in our fear-conditioned animals accounts for 32.2% of the increase in tail-flick latency. The real contribution of the increase in nociceptive threshold is not more than 63.2% of the increase in tail-flick latency. The remaining 4.6% is due to an increase in decisional/motor latency.

Using a similar approach, we also ran simulations of the tail-flick in control and fear-conditioned animals for varying intensity of stimulation α (see Supplementary Fig. S2). As expected, the tail-flick latency decreases as the stimulation intensity increases, however, the relative contributions of the drop in initial tail temperature (ΔT_0) and of the increase in threshold ($\Delta T\beta$) to the in-

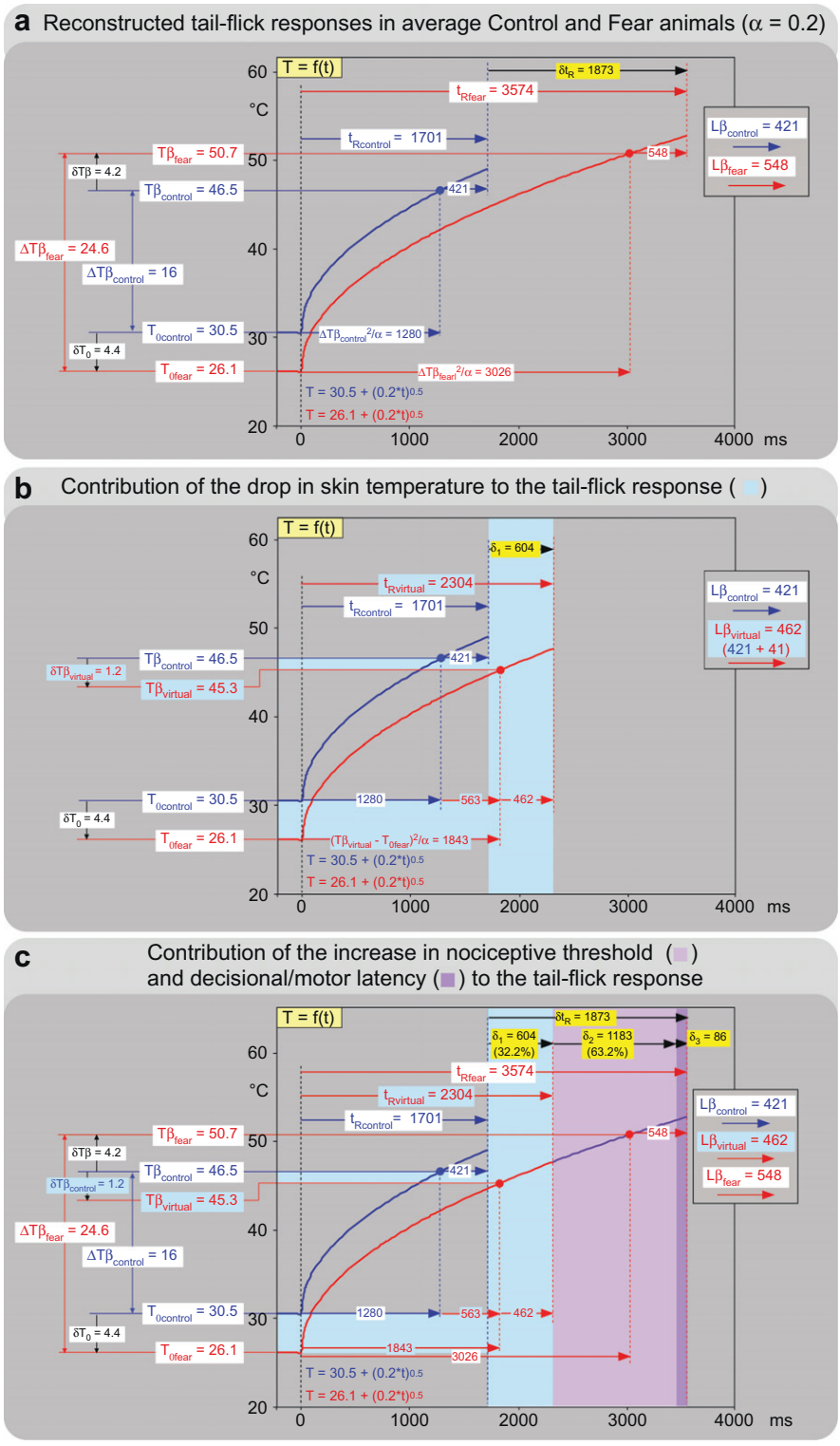

Fig. 6. Reconstruction of the tail-flick response showing its different components in control and fear-conditioned animals. (a) Reconstruction of the tail-flick response for an average fear-conditioned and control animal at a stimulation intensity of $\alpha = 0.2°C^2\ ms^{-1}$. The reconstructions were made using the behavioral threshold (Tβ) and behavioral latency (Lβ) determined in Fig. 4, according to equations of Fig. 2. The increase in reaction time is indicated as Δt_R (1873 ms). (b) Contribution of the drop in temperature of the tail skin ΔT_0 (blue area). The reconstruction is shown as a virtual response (ie, a control rat starting from a lower T_0). According to our previous work [5], and knowing that ΔT_0 is a 4.4°C drop, a 1.2°C reduction of the behavioral threshold is expected ($T\beta_{virtual}$). The time it takes to reach this new threshold from T_{0fear} is 1843 ms. The lower temperature also reduces the conduction velocity of the afferent fibers, adding an additional 41 ms to Lβ ($L\beta_{virtual}$). Overall, the net effect of the tail cooling is a Δ_1 = 604-ms increase of t_R. (c) Contributions of the increased threshold ΔTβ (pink area) and increased decisional/motor latency (purple). The stress-induced increases in ΔTβ and decisional/motor latency add a delay of δ_2 = 1183 ms and δ_3 = 86 ms, respectively. The latter is added to the latency $L\beta_{virtual}$ to result in a $L\beta_{fear}$ of 548 ms. To summarize, the 3 components δ_1, δ_2, and δ_3 account for 32.2%, 63.2%, and 4.6% of δt_R, respectively. A similar reconstruction is shown on Supplementary Fig. S2, to demonstrate that the relative contributions of δ_1 and δ_2 to δt_R remain approximately the same with varying intensities of stimulation α.

crease in tail-flick latency (δt_R) remains stable, roughly one-third to two-thirds, respectively.

4. Discussion

To the best of our knowledge, this study is the first to show the actual change in thermal nociceptive threshold for the tail-flick during stress in the conscious rat. Using contextual fear in unrestrained rats, we observed an increase of the behavioral threshold to noxious heat in the 4–5°C range, which confirms beyond doubt the existence of a stress-induced reduction of nociception. This observation is very much in keeping with the report in humans of a slight but consistent and reproducible increase of the threshold of the R_{III} nociceptive flexion reflex recorded from the lower limb, elicited by stress anticipation of pain [60].

The main technological breakthrough that made this analysis possible was the use of infrared thermography. By monitoring the changes in temperature at the site of stimulation throughout the response, we were able to reconstruct the response and extract its defining variables, which was not possible previously [54]. The other advance was the CO_2 laser, which allowed us to control the intensity of the stimulation and stimulate the tail remotely during the response without interfering with the animal's behavior. The animal would also have been unaware of the laser beam because it was red and therefore invisible to the albino rat.

As previously reported [15,24,27,58], we found that conditioned fear to context markedly increased the tail-flick latency (approximately twice that of controls). It was assumed that this was due to a reduction in thermal nociception, but this assumption may have been challenged if skin temperature had been recorded in these studies. We can now say confidently that the increase in tail-flick latency observed with conditioned fear (or conditioned analgesia) is associated with a significant increase in the true nociceptive threshold to noxious heat. However, the contribution of the increased threshold to the increased latency in our experimental conditions was less than two-thirds, indicating that other factors contributed to the delayed response. The other main factor, which contributed to one-third of the increased latency, was the drop in tail-skin temperature, also in the 4–5°C range. Note that this drop was for tests conducted at room temperature (24.5–25.8°C). It would have been greater at a colder ambient temperature. The drop in skin temperature is the consequence of a sympathetically mediated vasoconstriction of the skin [19]. It is part of the autonomic readjustments to the stressor [13,14], and is therefore independent of central pain modulation. Although the lower temperature of the skin decreased the behavioral threshold [5], its main effect was to increase the time it took to warm up the skin past the threshold. It also increased the conduction time of the peripheral fibers, however, this was a comparatively much smaller effect. Finally, the rest of the increased latency can be attributed to a central effect of the stressor on the motor control of the reflex. It is most probably due to a modulation of propriospinal or supraspinal pathways, which acts to increase tone in postural muscles and produce the immobile freezing posture. Thus, 3 centrally controlled factors contributing to the increased latency of the tail-flick reflex during contextual fear have been identified: one in the dorsal horn that increases the nociceptive threshold (the main one, 63.2%), one in the lateral horn that increases sympathetic vasoconstrictor tone to the skin (32.2% but dependent on ambient temperature), and one in the ventral horn that slows down the somatomotor response (4.6%).

Our study confirms the importance of the initial temperature of the tail when interpreting data from tail-flick tests. The temperature of the skin of the tail can vary between two absolute limits: the ambient temperature if it is fully vasoconstricted and the temperature of the blood if it is fully vasodilated. The regulation of tail-skin blood flow by the sympathetic nervous system is such that variations in temperature can be large and relatively fast. Thus, in our previous work in fear-conditioned animals [56], the tail temperature dropped by 4°C (from 31°C to 27°C) in 8 minutes at the beginning of the fear test and then rebound by 8°C (from 27°C to 35°C) in 6 minutes at the end of the test. It is clear that the tail-flick would return very different values if it was tested during fear or a few minutes after. Tail-skin blood flow is not only extremely sensitive to alerting stimuli [19,22], it is also a main effector for thermoregulation in the rat [18,23,49,61]. Consequently, tail blood flow is constantly fluctuating as the animal adjusts its core temperature (see Supporting Video S1 of Benoist et al. [5] at http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0003125). Furthermore, any surgical or pharmacological intervention that may affect the sympathetic outflow to the tail is likely to affect the temperature of the tail. In fact, Tjølsen and Hole [53] attributed the entire reduction in tail-flick reaction time after section of the spinal cord, lesions of the raphé-spinal serotoninergic system or systemic or intrathecal administration of serotoninergic blocking agents, to an increase in skin temperature. Finally, the tail is not the only organ to behave that way. Although less well studied, the paws also contribute to thermoregulation and show the same fluctuations as the tail in response to fear, as shown in Fig. 2 of Vianna and Carrive [56]. This is likely to affect pain tests that use paw-directed behavioral measures such as the hot plate or the formalin test, which has been shown to be sensitive to temperature [50,52].

The problem of interactions between nociception and peripheral blood flow was identified long ago [4,26] and has been well studied during recordings of lumbar spinal dorsal horn neurons in the cat [20]. Positive correlations between the temperatures of the room and tail [7,25,51] and negative correlations of each of these with the tail withdrawal reaction time [6,36,45,54] have also been reported. Hole and Tjølsen [30] recommended recording skin temperature near the stimulated area. For the tail-flick test, they suggested a regression analysis to remove the confounding effect of tail temperature changes, but the animals had to be restrained [54]. For the hot-plate test, they proposed a gradual warming of the paws [55]. In some cases, the stress paradigm was claimed not to cause any significant change in tail temperature between the experimental and control groups (eg, [36]). However, such studies are not exempt from biases due to the problem of recording the temperature of an interface and the impossibility of measuring the temperature at the very same place of heating. Infrared thermography did not have these constraints and allowed us to record basal tail temperature at the very same spot where the noxious heat was applied.

In our experimental conditions, one-third of the increased latency was due to the change in tail temperature. This may vary depending on the type and conditions of experiments (including ambient temperature), but it confirms the limitations of using the reaction time of a behavioral response to an increasing heat stimulus as a pain index. This method satisfies the criteria of face validity, that is, the extent to which the measurement looks like what it is supposed to measure; however, it does not reach the criterion of construct validity because it does not effectively measure the targeted construct, that is, a quantitative nociceptive response, presupposed to reflect the animal perception of pain [40]. In other words, the withdrawal latency does not measure what it claims to measure. Many acute stressors can increase tail withdrawal latency [2,10,12,15,24,27,41,42,46,48,59] (see, however, [27,34]) or reduce the formalin response [16,28,29], however, the situation is less well documented or not as clear with other tests. Thus, both "analgesia" and "hyperalgesia" [33,44] are reported in the animal literature, with the direction of the effect depending on many factors such as individual, gender, type of stressor (including nature,

magnitude, intensity and duration), and acute/chronic. Interestingly, the threshold for vocalization elicited by electrical shocks, measured by level methods, has repeatedly been reported as decreased by stressors that increase the tail-flick latency [31,32,35–37,57]. It is important to note that electrical stimuli bypass the transduction processes, minimizing the peripheral vasomotor effects on the response.

Our results also highlight the problem of symptom/sign interpretation in animals and the confusions introduced by the terminology. Terms such as pain, analgesia, allodynia, and hyperalgesia are symptoms that presuppose a verbal communication between examiner and subject/patient, offsetting their use in animal studies [21]. Using such a nomenclature in the scientific literature on animals not only installs confusion between semiology (clinical signs and laboratory tests) and symptoms, but may give the false impression of a secure and unrestricted parallel between species in all aspects [40]. As illustrated here, the term ''stress-induced analgesia'' is a relevant example because the term ''analgesia'' is both excessive, for a 4–5°C increase in threshold, and inappropriate, as we are dealing with semiology, not with symptomatology. If an increase in nociceptive threshold can be demonstrated, as we have done here, it would perhaps be more appropriate to refer to it as ''stress-induced antinociception,'' at least in the animal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2010.12.019.

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