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Research report

# Behavioral, immunocytochemical and biochemical studies in rats differing in their sensitivity to pain

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**Abstract**

The aim of the study was to further explore the anatomical and neurochemical background of differences in response to the conditioned aversive stimuli. The different patterns of behavioral coping strategies (a conditioned freezing response and ultrasonic vocalization) were analyzed in animals differing in their response to the acute painful stimulation, a foot-shock (HS: high sensitivity rats, LS: low sensitivity rats, and MS: medium sensitivity rats, according to their behavior in the flinch-jump pre-test), and correlated with plasma corticosterone levels, expression of *c*-Fos protein, and distribution of 5-HT innervation, in different brain structures. It was found that HS rats showed significantly more freezing behavior, whereas LS animals vocalized much more intensively. The behavior of LS group (less freezing response and stronger vocalization) was related to activation of prefrontal cortex (PFCX), increased activity of adrenal glands and stronger serotonin immunostaining in the PFCX, in comparison with HS animals. The more passive strategy of coping with the aversive event of HS group was related to increased activity of amygdalar nuclei and some areas of the hippocampus, and stronger 5-HT immunostaining in the baso-lateral nucleus of the amygdala, in comparison with LS rats. The present findings suggest that animals more vulnerable to stress might have innate deficits in the activity of brain systems controlling the hypothalamic-pituitary-adrenal axis that would normally allow them to cope with stressful situations. It appears also that response to pain may determine other patterns of emotional behavior, probably reflecting different activation thresholds of some brain structures controlling anxiety, e.g. prefrontal and secondary motor cortex.


*Keywords:* Individual response to foot-shock pain; Freezing response; Ultrasonic vocalization; *c*-Fos protein; Corticosterone; 5-HT immunostaining; Rats

## 1. Introduction

The issue of the anatomical and neurochemical basis of differences in the individual sensitivity to aversive stimuli is still unresolved [11,17,23,25,39,49]. The mechanisms regulating individual sensitivity and reactivity to emotional stimuli are important for a variety of physiological and pathological processes, ranging from pain perception to mood and emotions [7,22,23,25,39,45,49,51,52,55]. It was found previously by us that rats subjected to the flinch-jump test, and divided into two groups according to their response to the acute painful stimulation (high sensitivity and low sensitivity; HS, LS), showed a strong stimulation of brain activity on re-exposure to the shock cage and aversive stimulation (5 foot-shocks, 0.5 mA, 1 s long, repeated every 1 min), on retest 10 days later [32]. A detailed analysis of data revealed a potent enhancement of *c*-Fos expression in a majority of examined brain structures, including cortical areas, indicating their sensitivity to the direct and indirect (conditioned) aversive stimuli. The only significant difference in *c*-Fos expression between LS and HS rats was found in the lateral habenular nucleus (LHAB), indicating this brain structure as selectively engaged in processing of the painful stimulation. It was concluded that the reactivity of LHAB may be responsible for the differences in sensitivity to acute pain.

The aim of the present study was to further explore the phenomenon of differences in animal response to conditioned

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aversive stimuli using the conditioned fear test (a contextual fear). The rationale for this study was the assumption that reactivity to a simple sensory stimulation may determine animal behavior in response to more complex, e.g. conditioned, anxious stimuli. In other words, it has been assumed that rats with different pain thresholds are characterized also by different sensitivity to emotional stimuli. To elucidate this problem, we have analyzed the behavioral and neurochemical changes in animals divided into groups clearly differing in their sensitivity to pain. To this end, the first threshold of pain, flinch response in the flinch-jump test, was selected to avoid too strong response of animals, and the ceiling-like effects. A conditioned freezing response and aversive ultrasonic vocalization were studied in laboratory rats, divided into groups according to their reactivity to the acute painful stimulation. The different patterns of behavioral coping strategies were analyzed in low sensitivity and high sensitivity rats, and correlated with changes in plasma corticosterone levels, expression of *c*-Fos protein and distribution of 5-HT immunoreactivity, in different brain structures. It was assumed that these groups of rats should be characterized also by different sensitivity to emotional stimuli, and reactivity of brain structures and system which are involved in processing of emotional input to the central nervous system.

## 2. Materials and methods

### 2.1. Animals

The experiment was performed in a cohort of 55 male Wistar rats. The rats (180–200 g), bought from a licensed breeder, were housed in standard laboratory conditions under a 12 h light/dark cycle (lights on at 7 a.m.), in a constant temperature (21 ± 2 °C) and 70% humidity. The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). All experimental procedures using animal subjects were approved by the Local Committee for Animal Care and Use at Warsaw Medical University, Poland.

### 2.2. Flinch-jump test

After 4 days of acclimatization to the vivarium, all rats ($n = 49$), with the exception of the control group C ($n = 6$), were subjected to the flinch-jump test (Table 1). The test was performed in a box made of Plexiglas (30 cm × 30 cm × 60 cm, *w*/*l*/*h*), with a grid floor made of stainless steel bars wired to a shock generator. The floor of the box was cleaned after each trial with 95% ethanol. The rats were placed individually into the box. Shocks were delivered to the grid floor of the test box through a shock generator. After a 3 min period of habituation to the test box, shocks titrations were continued upwards in a stepwise manner (0.05 mA, 0.05–1.2 mA range) depending upon responsiveness of the rat. The flinch threshold was defined as the lowest shock intensity that elicited any detectable response. The jump threshold was defined as the lowest shock intensity that elicited simultaneous removal of at least three paws (both hindpaws) from the grid. To avoid foot damage, the cut-off = 1.2 mA was established. In this way, the flinch and jump thresholds in mA were defined for each rat. The time gap between shocks was 10 s, and each animal was tested only once [57].

Next, all animals were divided into three experimental groups according to the following criterion: low sensitivity animals (LS, flinch threshold above 0.65 mA, $n = 15$); high sensitivity animals (HS, flinch threshold below 0.45 mA, $n = 17$); and the medium sensitivity (MS) group with flinch threshold between 0.45 and 0.65 mA ($n = 12$). The criterion was established in the following way: the mean intensity of a stimulus inducing flinch response ± S.D. (0.55 ± 0.1), i.e. the animals with behavioral response above 0.65 mA, or below 0.45 mA stimulus, were allocated to the appropriate experimental groups. Five animals were eliminated from the study because of their inadequate responses to the painful stimulus (i.e. jumping or immobilization as a first reaction). After 7 days, HS and LS rats were subjected to the conditioned fear test, medium sensitivity rats were subjected to the conditioning box only. Another control group was not subjected to any behavioral testing (C, $n = 6$) [32].

The procedure has been applied to group animals in two not overlapping populations, in respect of their response to the painful stimulation. It was assumed that these groups of rats should be characterized also by different sensitivity to emotional stimuli, and reactivity of brain structures and system which are involved in processing of emotional input to the central nervous system. The schemes of experimental protocol are shown in Tables 1 and 2.

### 2.3. Contextual fear conditioning test and ultrasonic vocalization

The fear conditioning experiment was performed using a computerized fear conditioning system (TSE, Bad Homburg Germany), as described previously [33]. Fear conditioning was performed in the experimental cage (36 cm × 21 cm × 20 cm, *w*/*l*/*h*) under constant white noise condition (65 dB). The experiment was performed during three consecutive days in the same testing box and experimental chamber. On the first day, the animals were placed separately for 2 min in a training box, for adaptation to the experimental conditions. The following day, during a 10 min long session, after 2 min of habituation, the animal received three foot-shocks (stimulus: 0.7 mA, 1 s, repeated every 60 s). On the third day, the freezing response of rats was examined for 10 min-long period, in the testing box without any further stimulation. The conditioned response, a freezing response, was recorded and analyzed by the fear conditioning system. The freezing behavior was measured by a photo beam system (10 Hz detection rate) controlled by the fear conditioning system. Photo beams were spaced 1.3 and 2.5 cm in the direction of the *x*-axis and the *y*-axis, respectively. The absolute duration of inactivity was calculated by the fear conditioning system, defined as no interruption of any photo beam over 5 s long periods, and then summarized for the whole 10 min long experimental session (total time of freezing). The fear conditioning system has been validated previously in our laboratory [33,57]. Ultrasonic vocalizations were recorded simultaneously by a microphone, Mini-3 Bat Dector (Noldus Information Technology), attached to the ceiling of the chamber and processed by an interface, Ultravox, Noldus Information Technology, to select the 22 kHz frequency band for aversive vocalization calls (the range: 22 ± 5 kHz, minimum duration of an individual acoustic

Table 1
Treatment scheme for *c*-Fos and serotonin immunocytochemistry (groups: C, MS, HS, LS)

| Days 1–4 | Day 5 | Days 6–11 | Days 12–14 |
|---|---|---|---|
| Habituation to the vivarium | Flinch-jump test<br>Animals divided into groups according to the criterion (flinch response)<br>LS (low sensitivity rats) ↑ 0.65 mA ($n = 8$)<br>HS (high sensitivity rats) ↓ 0.45 mA ($n = 9$)<br>MS (medium sensitivity rats) 0.45–0.65 mA ($n = 6$) | Resting time | Contextual fear conditioning test (LS, HS animals)<br>Exposure to the box only (MS animals) (decapitation for *c*-Fos and serotonin 1.5 h later) |
| | C (control, naive, not disturbed animals) ($n = 6$) | | No exposure to the conditioning box (C animals) (decapitation for *c*-Fos) |