



Prolonged fear incubation leads to generalized avoidance behavior in mice

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ARTICLE INFO

Article history:

Received 13 April 2010

Received in revised form

15 June 2010

Accepted 29 June 2010

Keywords:

Post-traumatic stress disorder

PTSD

Avoidance

Fear incubation

Conditioned fear

ABSTRACT

Long-lasting presence of avoidance and emotional numbing are reliable behavioral markers for PTSD, but little is known about its psychological and biological underpinnings. We employed our recently established mouse model of PTSD (i) to study the emergence of avoidance behavior in the aftermath of a trauma, (ii) to disentangle the impact of context generalization vs. lack of motivation vs. novelty fear and (iii) to assess the therapeutic value of benzodiazepines and selective serotonin reuptake inhibitors (SSRIs). Specific conditioned avoidance to shock-paired odor turned into generalized avoidance after 28 days of fear incubation. Combination of habituation to the novel environment and extinction of contextual fear abolished both generalized and specific avoidance behavior. Chronic fluoxetine treatment partially reversed the phenotype, whereas acute treatment with diazepam did not. Our animal model may help understanding the mechanisms underlying psychological and biological mechanisms of PTSD for the benefit of developing pharmacotherapeutic strategies, which specifically address generalized avoidance.

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1. Introduction

Post-traumatic stress disorder (PTSD) is a severely impairing, long-lasting psychiatric disease that develops in the aftermath of potentially life-threatening events (diagnostic criterion A; American-Psychiatric-Association, 1994). According to DSM-IV (American-Psychiatric-Association, 1994), symptomatology of PTSD is categorized by three clusters that have to persist for more than a month: intrusive re-experiencing of trauma (criterion B), avoidance of trauma-related stimuli/emotional numbing (criterion C) and hyperarousal (criterion D). Symptoms may directly relate to traumatic memory (re-experiencing, avoidance of trauma-related cues) or represent emotional overreactions to unspecific 'neutral' stimuli (hyperarousal, emotional numbing, social withdrawal). Accordingly, both associative fear memories and non-associative fear sensitization seem to play a role in development and maintenance PTSD (Charney et al., 1993).

Among the diagnostic criteria, the long-lasting presence of avoidance and emotional numbing appears to be particularly reliable behavioral markers for PTSD (Breslau et al., 2005; North et al., 2009). For example, 94% of the bombing survivors from the terrorist attack in Oklahoma City that met criterion C fulfilled the PTSD

diagnosis, while criteria B and D by themselves failed to predict PTSD (North et al., 1999).

Despite this apparent importance of avoidance behavior on PTSD diagnosis, little is known about its psychological and biological underpinnings, in particular because preclinical research paid surprisingly little attention to this issue. Mowrer's two-factor learning theory of fear would predict the emergence of avoidance behavior by higher-order conditioning (classical and operant) and stimulus generalization following initial classical conditioning processes (Mowrer, 1960). Alternatively, lack of motivation/emotional numbing may play a significant role. Here we employed our recently established mouse model of PTSD (i) to study the emergence of avoidance behavior in the aftermath of a trauma, (ii) to disentangle the impact of context generalization vs. lack of motivation vs. novelty fear and (iii) to assess the therapeutic value of benzodiazepines and selective serotonin reuptake inhibitors (SSRIs). In our model, mice receive a brief electric foot-shock and subsequently develop exaggerated conditioned and unconditioned fear responses, hyperarousal, increased depression-like behavior and social withdrawal after a period of at least 28 days of fear incubation (Golub et al., 2009; Siegmund and Wotjak, 2007). The main objective of the present study was to design a behavioral task to investigate the development of avoidance to trauma-related (specific) and neutral (unspecific) stimuli following fear incubation. This manuscript describes the establishment of a behavioral paradigm, the conditioned odor avoidance (CODA) task, which relies on

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the ability of mice to associate an odor cue with an aversive experience (foot-shock) and to discriminate between this conditioned odor and a neutral one when both are presented in concurrent compartments at the same time. We could demonstrate generalized avoidance after incubation of the trauma for 28 days that was resistant to acute treatment with diazepam, but partially reversed by chronic fluoxetine. This underscores the predictive validity of the approach and is explained best by the fact that generalized avoidance primarily relates to an unspecific increase in novelty fear. Finally, we provide evidence that exposure therapy, such as the combination of habituation to the novel environment and extinction of contextual fear may abolish both generalized and specific avoidance behavior.

2. Methods

2.1. Animals

A total of 352 adult male C57BL/6NCrl mice were purchased from Charles River (Germany) with 7–8 weeks of age and single housed in the animal facility of the Max Planck Institute of Psychiatry for about 2 weeks before starting with the experiments. Each animal was isolated in standard macrolon cages (type 2) with sawdust bedding, water and food *ad libitum*, at 22 ± 2 °C room temperature and $55 \pm 5\%$ humidity, under an inverse 12 h light/dark cycle (lights off at 09:00 h) and remained under these conditions throughout behavioral testing. Mice were always tested during the dark phase of the period, between 10:00 h and 18:00 h. The number of animals used in each experimental group is mentioned in the figure legends. All experimental procedures were approved by the Committee on Animal Health and care of the State of Upper Bavaria and performed in strict compliance with the European Union recommendations for the care and use of laboratory animals (86/609/CEE).

2.2. Drugs

Diazepam (Diazepam-Lipuro®, Braun Melsungen, Germany) was freshly dissolved in saline and injected systemically (1 mg/kg, i.p.). Fluoxetine-ratiopharm solution (Ratiopharm GmbH, Germany) was dissolved in tap water resulting in a daily dose of 20 mg/kg and provided in light-proof drinking bottles (home-cage). The control solution consisted of the respective drug's vehicle. Effective doses of fluoxetine and diazepam were selected based on the literature (Siegmund and Wotjak, 2007) and pilot experiments using the elevated plus maze (see Fig. 5B).

2.3. Conditioning procedure

Setups and procedures were essentially the same as described (Kamprath and Wotjak, 2004; Siegmund and Wotjak, 2007). In brief, mice were placed in a conditioning chamber (MED Associates, U.S.A.) and received a scrambled electric foot-shock (1.5 mA, 2 s) 3 min later. They remained in the conditioning chamber for another 60 s, before being returned to the home-cage. The conditioning chamber was cleaned thoroughly and scented with ethanol (70%) solution between two exposures.

2.4. Conditioned odor avoidance task

The conditioned odor avoidance (CODA) task was conducted in a rectangular box made of white PVC walls and a dark grey PVC floor. The box was divided into three compartments ($30 \times 30 \times 30$ cm³ each) that were interconnected by small opening (6×5 cm²) with guillotine doors. A filter paper-lined Petri dish

(10 cm diameter), containing own home-cage bedding (nest compartment, center), ethanol 70% or acetate 1% (left or right compartment, counterbalanced) was placed in each compartment. Ethanol and acetate compartments were also cleaned with the respective solution, whereas the center (nest) compartment was cleaned with a damp cloth and soapy water and dried with paper towels. For CODA testing, mice were enclosed in the nest compartment for 5 min (habituation phase) followed by 5 min of free apparatus exploration (test phase). During testing, the latency to the first exit from the nest compartment and the time spent in each of the compartments were recorded. The animals' behavior was observed and rated online by means of a CCD camera positioned above the CODA apparatus and a stop-watch.

2.5. Experiments

Experiment 1: Conditioned odor avoidance in PTSD mice – consequences of fear incubation. In a two-factorial design (shock, time), mice were randomly assigned to one out of four groups. Two groups received the electric foot-shock at day 0, the other two groups were placed in the conditioning chamber for the same amount of time, but without shock application. One shocked and one non-shocked group were tested for CODA responses 2 days, the other two groups 28 days after shock.

Experiment 2: Early contextual fear extinction. Mice received the electric shock and were randomly assigned to one out of two groups. One group underwent early extinction training of contextual fear by re-exposing the animals to the shock context for 30 min on three consecutive days, from day 2 to day 4 (Golub et al., 2009). The extinction context was exactly the same used for conditioning, including the ethanol odor. The other group remained undisturbed in the home-cage as retention control. All mice were tested in CODA 28 days after foot shock and one day later for contextual fear (day 29; 3 min exposure to the shock context).

Experiment 3: Prolonged habituation to CODA apparatus. Mice received the electric shock and were randomly assigned to one out of two groups. One group was extensively habituated to the empty, unscented CODA apparatus for 20 min on two consecutive days, starting at day 25 after foot-shock. The other group remained undisturbed in the home-cage. All mice were tested in CODA 28 days after foot shock. An independent group of non-shocked mice was also tested during the habituation sessions for comparison with the PTSD mice. The number of door crosses served as a measure of apparatus exploration during prolonged habituation training.

Experiment 4: Habituation training and late extinction of contextual fear. Mice received the foot-shock and were randomly assigned to one out of four groups. The first group underwent habituation training to the test apparatus as described before (days 25/26 after shock), followed by late extinction training (days 28–30 after shock; as described before). The second and third groups underwent either habituation or extinction training. The fourth group served as retention control. All mice were tested in CODA 31 days after shock and one day later for contextual fear during a 3 min exposure to the shock context (day 32).

Experiment 5: Pharmacological validation of CODA. In a first set of experiments, shocked mice were treated either with diazepam (1 mg/kg, i.p.) or with vehicle 30 min before CODA at post-shock day 28. Dose and treatment procedure were shown to exert anxiolytic-like effects in naïve mice in the elevated plus maze test at similar light conditions (see Fig. 5B).

In a second set of experiments, fluoxetine (20 mg/kg/day, p.o.) was provided to shocked mice via drinking water for 25 days, starting from post-shock day 29 (Siegmund and Wotjak, 2007).

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