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Research report

Generalization of contextual fear depends on associative rather than non-associative memory components

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HIGHLIGHTS

- Immediate shock presentation induces lower contextual fear expression and prevents the development of fear generalization.
- Hyperarousal and associative fear can develop independently.
- Changes in fear generalization can be related to a time-dependent reduction in context discrimination.
- Our data supports the hypothesis that generalized fear results from forgetting specific stimulus attributes.
- Basolateral amygdala and dentate gyrus appear to play a role in encoding multimodal contextual information.

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ABSTRACT

Posttraumatic stress disorder (PTSD) is characterized by the presence of three major symptom clusters: persistent fear memories, hyperarousal, and avoidance. With a passage of time after the trauma, PTSD patients show an increase in unspecific fear and avoidance, a phenomenon termed "fear generalization". It is not clear whether fear generalization arises from the time-dependent growth of hyperarousal or changes in associative fear. The present study investigated behavioral and neuroanatomical correlates of non-associative and associative fear memory one week vs. one month after a trauma in a mouse model of PTSD with immediate vs. delayed foot shock application. The immediate shock procedure led to a lower contextual fear, but did not influence the hyperarousal (i.e. increased acoustic startle responses) assessed within the first week after the trauma. Only delayed shocked mice demonstrated generalization of contextual fear and an increase in generalized avoidance behavior, with no changes in hyperarousal one month after trauma. We observed the same increase in c-Fos expression following delayed and immediate shock presentation within the lateral, basolateral, central amygdala and CA1, CA3 and dentate gyrus of hippocampus, suggesting that all of these structures contribute to the development of hyperarousal. Only basolateral amygdala and dentate gyrus appeared to be additionally involved in encoding of contextual information. In summary, our results demonstrate the independence of associative and non-associative trauma-related fear. They support the hypothesis that generalized fear emerges in consequence of forgetting specific stimulus attributes associated with the shock context.

1. Introduction

Posttraumatic stress disorder (PTSD) may develop in the aftermath of a traumatic event. It is characterized by three major clusters of symptoms: (i) persistent re-experience of the traumatic event, (ii) persistent symptoms of increased arousal and (iii) persistent avoidance of stimuli associated with the trauma (DSM-IV). These core symptoms of PTSD are thought to arise from extraordinarily strong associative fear memories of the trauma and from fear sensitization of the individual in a non-associative and cross-modality manner [1]. Classical conditioning is thought to underlie the development of the first cluster of symptoms, whereas fear sensitization and second order operant conditioning may contribute to the second and third [1–5]. With the passage of time after trauma, PTSD patients tend to show enhanced acoustic startle responses [6] and an increase in unspecific fear and generalized avoidance [7].

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Two phenomena, relevant to these time-dependent changes in the fear response of PTSD patients, have been depicted in so far. More than 40 years ago McAllister and McAllister have described a "response increase to fear cues over time in the absence of further stress exposure", a phenomenon that they named as "fear incubation" [8]. This time-dependent response increase to fear cues without any interjacent exposure to conditioned stimuli has been later repeatedly demonstrated in humans and laboratory animals [8–16].

Another phenomenon: a time-dependent extend of fear response to a range of stimuli resembling the original conditioned stimulus was first reported by Ivan Pavlov (Pavlov, 1927) and later replicated in a number of animal studies [17–21]. Animals were shown to easily discriminate between the actual stressful training context and an altered context that contains only a subset of the original cues shortly after training. However, with a longer time interval, animals have difficulties discriminating between the training context and a different one, a phenomenon termed "fear generalization" or "broadening of generalization gradient" [17–21].

Animal models may help dissecting the neuroanatomical and molecular bases of PTSD. We have recently established a foot shock paradigm, which allowed us to study the contribution of associative and non-associative memory components to the formation of PTSD-like symptoms in mice [22]. Despite first evidence for a dissociation of the two components [23–25] it remained unclear whether generalized fear in PTSD result from the time-dependent growth of hyperarousal or is a consequence of changes in associative fear.

In order to delineate these possibilities, our study compared the consequences of delayed vs. immediate shock presentation on the development and/or maintenance of PTSD-like symptoms. This experimental approach was chosen on the basis of observations showing that immediate shock delivery in a specific context precludes the formation of an adequate context representation and, as a consequence, the formation of multimodal contextual fear memories [26]. By comparing specific patterns of c-Fos expression following delayed vs. immediate shock presentation we aimed at identifying those brain regions, which were specifically involved in acquisition of the context-shock association vs. non-associative fear.

2. Material and methods

2.1. Animals

A total of 118 male C57BL/6NCrl mice were purchased from Charles River Germany GmbH (Sulzfeld, Germany) at an age of 6 weeks. Animals were single housed under an inverse 12 h:12 h light-dark cycle (lights off: 09:00 h) with food and water ad libitum for at least 14 days before starting the experiments. All experimental procedures were approved by the Committee on Animal Health and Care of the State of Upper Bavaria (Regierung Oberbayern, Germany; Az. 55.2-1-54-2531-41-09) and performed in strict compliance with the European Union Directive for the care and use of laboratory animals (86/609/EEC).

2.2. Experimental procedures

All experiments were performed during the activity phase of the animals between 09:30 h and 18:00 h. The setup has been described and displayed in detail before [27]. Briefly, experiments were performed in two contexts: (i) the shock context, a cubic-shaped box with a metal grid for shock application, and (ii) the grid-context, a hexagonal shaped prism made of non-transparent Plexiglas with a metal grid floor as a dominant reminder of the shock context. The contexts were cleaned thoroughly after each trial with 70% EtOH (shock context) or 0.05% isoamylacetate (grid context).

For application of the inescapable foot shock, animals were placed into the shock chamber, and a single scrambled electric foot shock (2 s, 1.5 mA) was delivered via the metal grid 198 s later (delayed shock; standard protocol). Animals remained in the shock chamber for another 60 s before being returned to their home cages. For the immediate shock protocol, animals received the same electric foot shock 5 s after being placed into a chamber and were removed immediately thereafter to prevent formation of contextual representation of the conditioning environment.

To test for contextual fear, mice were exposed to the conditioning chamber for 3 min. To assess fear generalization, mice were exposed to the grid context for 3 min (see Figs. 1A and 2A for temporal order of the exposure). Contextual fear and fear generalization tests were videotaped by small CCD cameras (Conrad Electronics, Hirschau, Germany). Animals' behavior was rated off-line by a trained observer who was blind to the experimental condition (EVENTLOG, Robert Henderson, 1986). Freezing behavior was defined as immobility except for respiration movements.

To test for hyperarousal, acoustic startle responses were measured as described before [24,28]. In brief, mice were placed into one out of eight identical startle set-ups, consisting of a non-restrictive Plexiglas cylinder (inner diameter 4 cm, length 8 cm) mounted onto a plastic platform, each housed in a sound attenuated chamber (SR-LAB, San Diego Instruments SDI, San Diego, CA, USA). The cylinder movement was detected by a piezoelectric element mounted under each platform and the voltage output of the piezo was amplified and then digitized (sampling rate 1 kHz) by a computer interface (I/O-board provided by SDI). The startle amplitude was defined as the peak voltage output within the first 50 ms after stimulus onset and quantified with SR-LAB software. The response sensitivity of each chamber was calibrated in order to assure identical output levels. Startle stimuli and background noise were delivered through high-frequency speakers placed 20 cm above each cage. Four different startle stimuli consisting of white noise bursts of 20 ms duration and 75, 90, 105 or 115 dB(A) intensity (INT) were presented in a constant background noise of 50 dB(A). Intensity was measured using an audiometer (Radio Shack, 33-2055, RadioShack, Fort Worth, TX, USA). Each session consisted in an acclimation period of 5 min, 10 control trials (background noise only) and 20 startle stimuli of each intensity presented in a pseudorandomized order. The interstimulus interval was 15 s averaged (13–17 s, pseudorandomized). To avoid context reminders, the startle set-ups were localized in a different building and startle measurements were performed by a scientist unfamiliar to the animals, thereby minimizing confounding influences of context generalization. Plexiglas cylinders were cleaned thoroughly with soap water after each trial.

To test for avoidance behavior, a Conditioned Odor Avoidance (CODA) task was performed in a rectangular box made of white PVC, comprising 3 identical compartments (30 cm × 30 cm × 30 cm) that were accessible from the center compartment through small openings (6 cm × 5 cm), which could be closed by guillotine doors (for details see Pamplona et al., 2011 [25]). A filter paper-lined Petri dish (10 cm diameter), containing own home-cage bedding (nest compartment), ethanol 70% (conditioned odor) or acetate 1% (novel odor) vapor was placed in the respective compartment. Ethanol and acetate compartments were placed left or right (counterbalanced) from the nest compartment in the center and cleaned with the respective solution, whereas the center (nest) compartment was cleaned with a damp cloth and dried with paper towels. For CODA testing, mice were enclosed in the nest compartment for 5 min (habituation) followed by free apparatus exploration (test), when the latency to the first exit from the nest compartment and the time spent in each compartment were recorded for 5 min. The animals' behavior was observed and rated online by means of a CCD camera positioned above the CODA apparatus.

2.3. Immunohistochemistry

Mice were anesthetized with isoflurane 70 min following fear conditioning or chamber exposure and transcardially perfused with ice-cold phosphate buffer (0.1 M), pH 7.4 followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Animals from the handling control group were randomly taken out of their home cages and processed similarly. Brains were removed, postfixed in 4% formaldehyde, diluted in phosphate buffered saline (PBS) for 24 h and transferred into 30% sucrose solution at 4 °C for 48 h. The brains were shock frozen in isobutanol and stored at −80 °C until the whole brain was cut into 30 μm coronal sections by cryosectioning. Sections starting at Bregma level −1.22 mm to −2.54 mm [29] were collected and then stored at −20 °C in an anti-freezing solution until processed for immunohistochemical staining. Every third section (90 μm interval) was selected and processed for immunohistochemical staining. The floating coronal sections were incubated with an anti-c-Fos rabbit polyclonal antibody (1:30.000, Calbiochem, Germany) for 20 h. c-Fos immunoreactive cells were visualized using a biotinylated donkey anti-rabbit secondary antibody (1:500, Santa Cruz, Germany) and the avidin-biotin complex (ABC-Elite kit rabbit, Vector Laboratories, Germany) [30].

2.4. Stereological quantification

The number of c-Fos-immunoreactive cells was determined using stereological quantification. The following brain areas were analyzed: dentate gyrus (DG), CA1-and CA3-region of the dorsal hippocampus (CA1, CA3), basolateral amygdala (BLA), lateral amygdala (LA), central amygdala (CeA) devided into CeM (medial devision), CeL (lateral devision) and CeC (capsular part). The examined regions of the hippocampus and the amygdala are depicted in Fig. 6A.

Stereological quantification of the c-Fos positive cells was carried out strictly blind to the experimental conditions with the optical fractionator estimating total numbers of c-Fos positive cells [31–33]. After histological processing the sections had a mounted section thickness of 20 μm, a fixed distance of 2 μm and an optical