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# Increased levels of conditioned fear and avoidance behavior coincide with changes in phosphorylation of the protein kinase B (AKT) within the amygdala in a mouse model of extremes in trait anxiety

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ABSTRACT

Patients diagnosed for anxiety disorders often display faster acquisition and slower extinction of learned fear. To gain further insights into the mechanisms underlying these phenomenona, we studied conditioned fear in mice originating form a bi-directional selective breeding approach, which is based on elevated plus-maze behavior and results in CD1-derived high (HAB), normal (NAB), and low (LAB) anxiety-related behavior mice. HAB mice displayed pronounced cued-conditioned fear compared to NAB/CD1 and LAB mice that coincided with increased phosphorylation of the protein kinase B (AKT) in the basolateral amygdala 45 min after conditioning. No similar changes were observed after non-associative immediate shock presentations. Fear extinction of recent but not older fear memories was preserved. However, HAB mice were more prone to relapse of conditioned fear with the passage of time. HAB mice also displayed higher levels of contextual fear compared to NAB and LAB mice and exaggerated avoidance following step-down avoidance training. Interestingly, HAB mice showed lower and LAB mice higher levels of acoustic startle responses compared to NAB controls. The increase in arousal observed in LAB mice coincided with the general absence of conditioned freezing. Taken together, our results suggest that the genetic predisposition to high anxiety-related behavior may increase the risk of forming traumatic memories, phobic-like fear and avoidance behavior following aversive encounters, with a clear bias towards passive coping styles. In contrast, genetic predisposition to low anxiety-related and high risk-taking behavior seems to be associated with an increase in active coping styles. Our data imply changes in AKT phosphorylation as a therapeutic target for the prevention of exaggerated fear memories.



## 1. Introduction

Fear enables reflexive adaptation to threatening stimuli and situations. It is characterized by both active (e.g., startle, fight/flight) and passive (e.g., freezing, avoidance) responses. Exaggerated fear may become maladaptive and thereby contribute to the development of psychopathology (Maren, 2007; Rosen & Schulkin, 1998). For example, patients diagnosed with anxiety disorders show immoderate physiological reactions to aversive stimuli in comparison to healthy individuals (MacLeod, Rutherford, Campbell, Ebsworthy, & Holker, 2002; McTeague et al., 2010). In addition, they display stronger acquisition and slower extinction of learned fear behaviors (Lissek et al., 2005) and an increased return of fear after treatment (Rodriguez, Craske, Mineka, & Hladek, 1999). In particular, amygdala-based processes seem to contribute to the association between phobic fear and trait anxiety (Indovina, Robbins, Nunez-Elizalde, Dunn, & Bishop, 2011).

In the past three decades, the linkage between trait anxiety and learned fear has been broadly described in terms of neuroanatomical (Charney, 2003; Davis, 1992; Shin & Liberzon, 2010) and pharmacological (Santos, Gargaro, Oliveira, Masson, & Brandao, 2005) parallels. It is becoming increasingly apparent that the mechanisms underlying Pavlovian fear conditioning have much in common with human anxiety disorders (Bouton, Mineka, & Barlow, 2001; Marks & Tobena, 1990; Pitman, Orr, Shalev, Metzger, & Mellman, 1999; Rosen & Schulkin, 1998; Sullivan, Apergis, Gorman, &

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LeDoux, 2003). Due to close homologies in the anatomical and molecular signatures of the fear matrix between humans and rodents, classical fear conditioning in rats and/or mice may teach important lessons about acquisition, expression and extinction of conditioned fear that can then be extrapolated to humans (Davis, Walker, Miles, & Grillon, 2010; Delgado, Olsson, & Phelps, 2006; Monfils, Cowansage, Klann, & LeDoux, 2009; Ressler et al., 2004; Schiller et al., 2010; Walker & Davis, 2002).

In rodents, conditioned fear is assessed by pairing of an a priori neutral stimulus, such as a tone or a light signal (the conditioned stimulus, or CS), with a punishment, such as an electric foot shock (the unconditioned stimulus, or US). In consequence of the CS–US association, presentations of the CS alone is capable of eliciting a conditioned fear response (e.g., freezing or fear-potentiated startle). The formation of fear memories critically depends on the amygdala (Liang, Hon, & Davis, 1994). Repeated presentations of the CS in absence of the expected punishment leads to a gradual decline in fear responses, which is called fear extinction. In most cases, this fear extinction process cannot simply be explained by forgetting or erasure of the original memory trace, since conditioned fear may reappear with the passage of time (spontaneous recovery) and/or in a different test context (renewal) (Bouton & Moody, 2004; Bouton, Westbrook, Corcoran, & Maren, 2006; Myers & Davis, 2002; Quirk & Mueller, 2008).

Fear conditioning procedures often lead to parallel formation of elemental (i.e. auditory or visually cued) and configural (i.e. contextual) fear memories. The latter process may contribute to the development of avoidance behavior (Mowrer, 1960), another core feature of anxiety disorders (North, Suris, Davis, & Smith, 2009; Rosen & Schulkin, 1998). Under experimental conditions, avoidance behavior can be studied in inhibitory (e.g., step-down or step-through) avoidance tasks.

In addition to the behavioral changes following fear conditioning, cellular and molecular mechanisms contributing to learned fear have also been identified, for instance, the protein kinases activity. In a recent review of signaling pathways underlying emotional states, distinct protein kinases and their downstream targets in the basolateral amygdala and hippocampus were proposed as mediators of fear conditioning and fear extinction (Tronson, Corcoran, Jovasevic, & Radulovic, 2012). Among them, CaMKII (Irvine, Vernon, & Giese, 2005), ERK (Lin, Yeh, Lu, & Gean, 2003), AKT (Lin et al., 2001) as well as its downstream GSK-3β and β-catenin (Maguschak & Ressler, 2008) deserve particular attention because of their potential involvement in the formation of fear memories.

Previous data have shown that rats selectively bred for high levels of trait anxiety took longer for extinguishing conditioned fear (Muigg et al., 2008). To gain further insights into the interrelation between trait anxiety and development and maintenance of fearful memories, we tested high (HAB), normal (NAB/CD1) and low anxiety-related behavior (LAB) mice (Landgraf et al., 2007) for their responses in a set of fear conditioning, inhibitory avoidance learning and acoustic startle response paradigms. Behavioral experiments were complemented by measurements of changes in protein kinase activity at the level of basolateral amygdala and dorsal hippocampus.

## 2. Material and methods

### 2.1. Animals

Male CD1 mice were purchased from Charles River (Sulzfeld, Germany) at an age of 5–8 weeks. Male HAB, NAB and LAB mice used in this study were selectively inbred in the animal facilities of the Max Planck Institute of Psychiatry as described previously (Krömer et al., 2005). Briefly, >250 animals from 25 litters of outbred Swiss CD1 mice purchased from Charles River were used as starting point for selective and bidirectional breeding for extremes in anxiety-related behavior on the elevated plus-maze (EPM). Males and females that spent either the least or most time on the open arms of the EPM were mated to establish the HAB and LAB mouse lines, respectively. The animals were routinely tested at the age of 7 weeks with HAB and LAB mice spending less than 15% and more than 65% of their time, respectively, on the open arms of the EPM. NAB mice are bred for intermediate anxiety-related behavior. As >80% of CD1 mice spent 30% to 45% of their time on the open arms of the EPM, this range was chosen for the selection of NAB mice without any overlap with HAB or LAB animals. Moreover, both CD1 and NAB mice could be used as controls in the present study. All mice were single-housed in macrolon type II cages ($L23 \times W16.5 \times H14\ cm^3$) 2 weeks prior the experiments under standard laboratory conditions with reversed 12 h/12 h light/dark cycle (light on at 9 pm), temperature 23 ± 1 °C, and food and water *ad libitum*. Laboratory animal care and experiments were conducted in accordance with the regulations of the current version of the German Law and Animal Protection. Animal protocols were approved by the Government of Bavaria.

### 2.2. Behavioral tasks

#### 2.2.1. Fear conditioning

The fear conditioning setup has been described and displayed in detail before (Kamprath & Wotjak, 2004). For fear conditioning, mice were placed into a cubic-shaped conditioning chamber with a metal grid for shock application, and the light was switched on. Three minutes later, a 20-s tone (CS: 80 dB, 9 kHz sine wave) was presented that co-terminated with a scrambled electric foot shock (US: 2 s, 0.7 mA). The conditioning procedure was repeated twice with inter-tone intervals of 30 s and 20 s, respectively. Animals were returned back to their home cages 1 min after the last foot shock. For immediate shock, mice were placed into the cubic-shaped chamber and the light was switched on. Two seconds later, three foot shocks were presented with inter-shock intervals of 1 s. Animals were returned back to their home cages 2 s after the last foot shock.

To test for auditory-cued fear memory, mice were placed into a neutral test context (cylinder), which differed from the original conditioning context in shape, texture, bedding and odor (Fanselow, 1980). The house light was switched on and the tone presentation was started 3 min later. Mice were returned to their home cages 1 min after termination of tone presentation. To test for the intensity of contextual fear memory, mice were placed back in the conditioning context for 3 min. The specificity of contextual fear was assessed by exposing the animals to a grid context, which differed from the shock context in shape, texture and odor (Fanselow, 1980), except for the presence of the grid floor. In addition, we compared freezing responses in the conditioning context with baseline freezing during the 3 min preceding the tone presentation in the test context as a measure of context generalization.

#### 2.2.2. Step-down avoidance

The apparatus consisted of a metal grid floor ($23 \times 21\ cm^2$, 42 metal bars with a diameter of 3 mm, spaced apart 0.5 cm) inserted in a clear Plexiglas box ($L25 \times W25 \times H50\ cm^3$). The cage was illuminated with a 30 W lamp during the experimental period resulting in ~300 lux measured at floor level. A plastic platform ($L10 \times W10 \times H2.5\ cm^3$) was placed into the center of the metal grid floor. Electric shocks (0.7 mA, 2 s) were delivered through the grid floor from a programmable animal shocker (San Diego Instruments, San Diego, CA, USA). The test consisted of a training session and a retention session done 1 day later. During the training session, each mouse was placed on the platform and