Individual differences in the acquisition and generalization of fear: Testing the effects of the BDNF-val66met polymorphism and trait anxiety

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Contents

I. Introduction

1. Fear and anxiety

Fear is an emotion that serves an important adaptive function by allowing an animal to mobilize its resources in order to cope with an imminent threat. Fear is evoked by either stimuli that have posed a danger to the members of a particular species during the evolutionary past (e.g., snakes, angry conspecifics, or heights in humans) or by initially innocuous stimuli that have come to signal these innate threats or other painful outcomes. In humans, a fear response is characterized by negative thoughts about the imminent threat, high physiological arousal (e.g., heart racing, sweating, or trembling), and behavioral escape. Thanks to animal research, the neural substrates of fear are fairly well-known. The key structure involved in fear is the amygdala, which integrates sensory information about threatening stimuli and controls the fear response (Davis, 1998). In humans, evidence from both neuroimaging studies (Phelps & LeDoux, 2005) and studies using participants with brain lesions (Hamm & Weike, 2005) have also shown the central role of the amygdala in fear reactions.

Anxiety is a negative, future-oriented, mood state in response to distant or diffuse threats. In humans, it is characterized by worry about the potential threats, muscle tension, and avoidance of the situation that may lead to the threatening encounter. Anxiety appears to be sustained by neural substrates that are partially distinct to those involved in fear. Particularly, the bed nucleus of the stria terminalis plays a specific role mediating behaviors associated with anxiety (Davis, 1998).

Paralleling the predator imminence model of defensive responses in mammals (Fanselow, 1994), it has been suggested that in humans fear and anxiety play complementary adaptive roles as a function of threat proximity (e.g., Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001). Anxiety interferes with the ongoing activity so that an individual can be

vigilant about potential threats. As the danger comes closer, the anxious responding pattern gradually switches to fear, and thus the individual prepares to escape.

2. Acquisition of new fears: conditioning and generalization

As mentioned in the previous section, fear is elicited by specific stimuli that have threatened the survival of the members of a particular species in the evolutionary past. In addition, new fears may be acquired by means of Pavlovian fear conditioning, in which an innocuous stimulus comes to trigger a fear response after being temporally paired with a natural threat or another painful outcome (referred as unconditioned stimulus, US, because it is unconditionally aversive). The fear reaction acquired through this associative process is called conditioned fear response, and the initially innocuous stimulus is then referred as conditioned stimulus (CS).

Fear conditioning does not only operate through direct experience (Rachman, 1977). In vicarious conditioning an individual acquires a fear response by watching a conspecific reacting fearfully to a given stimulus. This form of conditioning has been observed in many species, including invertebrates (e.g., Guzmán, Tronson, Guedea, Huh, Gao, & Radulovic, 2009). In humans, the association between the CS and the US may also be verbally instructed. Both vicarious and instructed conditioning are common pathways for the transmission of fears from parents to children (Barrett, Rapee, Dadds, & Ryan, 1996; Gerull & Rapee, 2002). Regardless of the acquisition modality, fear conditioning plays an important adaptive role by signaling potentially harmful events and prompting appropriate defensive responses such as avoidance.

Generalization of conditioned fear is another associative mechanism by which fear extends to new stimuli. Specifically, stimuli that bear some resemblance (perceptual or conceptual; e.g., Dunsmoor, White, & LaBar, 2011) to a CS can also elicit the fear response, even though they have never been directly paired with the US. This allows the appropriate reaction to novel, potentially harmful, stimuli, based on previous experience with a related CS.

3. Fear-conditioning paradigms

Fear conditioning has been extensively investigated in the lab, both in animal and human subjects. Because the basic aspects of fear conditioning are common across most animal species, findings from animal research bear important implications for humans. Through the experimental study of fear conditioning, researchers have gained invaluable knowledge about the neural substrates of fear (e.g., Davis, Walker, Miles, & Grillon, 2010) and the etiology and treatment of pathological anxiety (e.g., Mineka & Zinbarg, 2006; see section 3). Several experimental paradigms are used to study fear-conditioning processes. Next is a brief description of the most common.

Simple and differential conditioning. The acquisition of fear is often studied using either a simple or a differential conditioning paradigm. In *simple conditioning*, a stimulus that does not elicit any significant response (e.g., a geometric shape) is presented repeatedly followed by an aversive US (e.g., a mild electric shock). The neutral stimulus becomes an excitatory conditioned stimulus (CS+), because its presentation comes to activate the neural representation of the US. By that means, the CS+ elicits a conditioned fear response directed to deal with the impending US. The acquisition of fear is measured by comparing fear responses upon the CS+ presentation relative to responses to the same stimulus before conditioning, or to responses during intertrial intervals. This paradigm does not allow to control for non-associative learning processes, such as the general increase in fear produced by the mere US presentation (i.e., sensitization). For this reason, an additional control stimulus is used in the *differential conditioning paradigm*. That stimulus is never paired with the US, and thus it comes to signal its absence, becoming an inhibitory conditioned stimulus (CS-). In differential conditioning, fear acquisition is measured by comparing responses to the CS+ with responses to the CS-.

Generalization. Once fear is acquired, stimuli that are similar to the CS+ (generalization stimuli, GSs) can be presented in order to test whether they are also capable of

evoking the fear response. In a typical *generalization paradigm*, a number of GSs gradually varying in perceptual similarity to the CS+ are used. Generalization of fear is indexed by a gradient that shows the magnitude of the fear response as a function of CS+ similarity. Typically, the magnitude of the fear response decreases as the GSs are less similar to the CS+. However, variables other than similarity also influence generalization. For example, responses evoked by GSs that are more intense than the CS+ (e.g., larger) are greater than responses evoked by GSs that are less intense (e.g., smaller), even though they may be equally similar to the CS+ (Ghirlanda & Enquist, 2003). In addition, the paradigm used during fear conditioning (simple vs. differential) may also affect the shape of the generalization gradient (Dunsmoor & LaBar, 2013).

Extinction. Given its logical applied implications, researchers are also interested in how an acquired fear can be reduced. This is done using an *extinction paradigm*, in which the conditioned fear response typically decreases when the CS+ is presented repeatedly in the absence of the US. Extinction of fear is measured by tracking the decline of responses across the non-reinforced presentations of the CS+. It is now established that fear extinction does not involve a destruction of the original CS-US association. Instead, the new CS-no US (inhibitory) association is stored together with the previous one, and the expression or inhibition of fear depends on the context in which the CS+ is presented again (Bouton, 2002). This accounts for the recovery of the conditioned fear response that usually happens when the CS+ is presented again after a period of time (i.e., spontaneous recovery), in a context different than the one in which extinction took place (i.e., renewal), or after the US is presented alone (i.e., reinstatement).

Each of the fear-conditioning paradigms introduced above may vary on a number of significant parameters, such as the conditioned response that is measured or the type of CS or US (see Lissek et al. 2005, tables 3 to 5). In addition, other fear-conditioning processes have been studied in humans using different paradigms. Among others, these include context

conditioning (Grillon & Davis, 1997), reconsolidation (Schiller et al., 2010), conditioned discrimination (Jovanovic et al., 2005), extinction recall (Milad et al., 2008), or generalization of extinction (Vervliet, Vansteenwegen, & Eelen, 2004).

4. Pathological anxiety

4.1. Aberrant fear in anxiety disorders

Despite playing an adaptive role, when fear conditioning develops in a dysfunctional way it may lead to pathological anxiety. In fact, individuals that are diagnosed with an anxiety disorder display fear or anxiety to objects or situations that do not pose any real danger, and this significantly interferes with their daily life. It has been suggested that excessive fear is the core feature of three of the current diagnostic categories of anxiety disorders: specific phobia, social phobia, and panic disorder (PD; Watson, 2005). In addition, the hallmark of posttraumatic stress disorder (PTSD) is the inability to suppress fear under safe conditions (Jovanovic et al., 2009).

In specific phobia, individuals experience an irrational fear to a given object or situation (e.g., dogs, storms, or darkness), which they try to avoid at any cost. In social phobia, the feared situations are those in which individuals might be evaluated or judged by others, and socially phobic individuals avoid such situations or endure them with a great distress. PD is characterized by unpredictable panic attacks (i.e., sudden feelings of terror, high physiological arousal, and tendencies to escape), which often lead to avoidance of situations in which patients perceive that escape might be difficult or embarrassing if they were to have an attack (i.e., agoraphobia). Finally, PTSD includes, among other symptoms, persistent increased arousal, and fear and avoidance of reminders of the traumatic event. The abovementioned symptoms show the of excessive fear in the phenomenology of anxiety disorders.

4.2. Abnormal fear conditioning in the etiology of pathological anxiety

The current fear-conditioning model of pathological anxiety proposes that abnormal fear-conditioning processes, together with other diathesis-stress factors, play a crucial role in the etiology of pathological anxiety (Mineka & Zinbarg, 2006). The simplest way in which fear conditioning and pathological anxiety may be related is the one in which a fear-conditioning episode, sustained by an intense aversive experience, originates an anxiety disorder. According to this idea, in specific phobia, a certain object (CS; e.g., a dog), that initially did not elicit a significant emotional response, comes to evoke a strong sense of fear and avoidance after being paired with a painful outcome (US; e.g., a bite). Similarly, in social phobia, the association is made between a social situation (CS) and humiliation or harassment (US; Mineka & Zinbarg, 2006). In PD, different exteroceptive and/or interoceptive cues (CSs; e.g., a shopping mall or heart palpitations) are associated with a panic attack (US; Bouton, Mineka, & Barlow, 2001). Finally, in PTSD, the CS is a certain context in which a life-threatening event (US; e.g., a physical assault) occurred. Traumatized individuals experience fear when they are exposed again to that context or even by merely thinking of it (Jovanovic & Ressler, 2010).

The causal relationship between a single fear-conditioning episode and the onset pathological anxiety has been deemed as too simplistic (for a review of criticisms, see Rachman, 1977). First, not everybody diagnosed with an anxiety disorder recalls an original aversive event. However, as mentioned above, fear conditioning does not only function through direct experience. Fears can also be acquired through vicarious or instructed conditioning. In fact, some studies have shown that individuals suffering from pathological anxiety report that their parents were particularly sensitive to the situations they fear (e.g., Bruch & Heimberg, 1994 on social phobia; Ehlers, 1993 on panic disorder).

A second criticism states that, as mentioned in section 2, fear conditioning essentially serves an adaptive function. Therefore, under normal circumstances fear conditioning should

not lead to pathological anxiety. This relates to the fact that not everybody that experiences a strong aversive event goes on to develop an anxiety disorder (Kessler et al., 2005). The key to solve this apparent contradiction is to consider that there are inter-individual differences in fear-conditioning processes. In other words, individuals consistently differ in the way they acquire, generalize, and/or extinguish, new fears. Those suffering from pathological anxiety would be characterized by abnormal fear-conditioning, which would act as a diathesis mechanism predisposing them to develop pathological anxiety upon the occurrence of an aversive event (Beckers, Krypotos, Boddez, Effting, & Kindt, 2013; Mineka & Oehlberg, 2008).

4.3. Potential mechanisms of abnormal fear conditioning

Based on findings from different fear-conditioning studies, four potential mechanisms of abnormal fear conditioning in individuals with pathological anxiety have been proposed.

Enhanced conditionability. In a differential fear-conditioning study with PTSD patients and trauma-exposed controls, Orr and colleagues (2000) found larger differential conditioning (i.e., greater fear responses to the CS+ than to the CS-) in PTSD patients than in healthy controls. In addition, the PTSD group continued to exhibit differential conditioning during the extinction phase. These findings have been interpreted as indicative of stronger excitatory conditioning, or enhanced ‘conditionability’, in individuals prone to pathological anxiety (e.g., Otto et al., 2007).

Deficits in contingency awareness. Grillon (2002) conducted a differential fear-conditioning study in healthy individuals that included two similar sessions separated by a period of time. After the first session, half of the participants were not aware of CS-US contingency (i.e., they could not verbalize that the CS+ preceded the US). Those unaware participants did not acquire differential conditioning as measured by physiological indices of fear. In addition, they showed signs of anxiety (i.e., high physiological reactivity during baseline conditions) throughout the first session and at the beginning of the second session.

Finally a larger number of unaware participants did not return for the second session. Based on these results and previous literature (e.g., the safety signal hypothesis of Seligman & Binik, 1977), Grillon suggested that deficits in contingency awareness would lead to increased contextual anxiety, because the inability to learn about threat signals (i.e., CS+) hampers the capacity to identify periods of safety. Thus, unaware individuals will remain in a chronic state of anxiety.

Impaired inhibitory fear mechanisms. Grillon and Morgan (1999) conducted a differential fear-conditioning study in war veterans with and without PTSD. The study consisted of two identical sessions separated by 4 or 5 days. In the first session, PTSD patients failed to show differential conditioning, and this was due to greater fear responses to both the CS+ and the CS- than responses during intertrial intervals. However, PTSD patients, as well as healthy controls, were aware of the CS-US contingency. These results would be indicative of impaired inhibitory fear mechanisms in PTSD patients despite showing intact contingency awareness. In fact, Davis, Falls and Gewirtz (2000) suggested that pathological anxiety results from a failure to inhibit fear responses to safety signals. Because a combination of excitatory and inhibitory associative mechanisms are thought to be involved in fear extinction (Bouton, 2002), difficulties in extinguishing the fear response observed in anxiety patients would also be consistent with this theory (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Michael, Blechert, Vriends, Margraf, & Wilhelm, 2007).

Overgeneralization of fear. An alternative explanation of the results reported by Grillon and Morgan (1999) is that PTSD patients generalized conditioned fear from the CS+ to the CS-, due to perceptual similarity between both stimuli (blue and green colored lights). This would be consistent with the observation that anxiety patients generalize symptoms of fear to stimuli that resemble those associated with the original aversive event, such as the broadening of the exteroceptive and interoceptive stimuli capable of eliciting a panic attack often observed in PD. In fact, Lissek et al. (2010) used a set of stimuli gradually varying in their similarity to a

CS+ to study fear generalization in PD patients. Compared to healthy controls, PD patients generalized the fear response to stimuli that were less similar to the CS+. These authors suggested that overgeneralization of fear might be a biomarker of PD. Moreover, findings of increased generalization of fear among individuals at risk for anxiety disorders (Haddad, Pritchett, Lissek, & Lau, 2012) suggest that overgeneralization of fear, reflecting a lower threshold of threat reactivity, may predispose to pathological anxiety.

In a meta-analysis of 20 fear-conditioning studies comparing clinical samples with healthy controls, Lissek and colleagues (2005) found evidence that individuals with anxiety disorders exhibited both enhanced conditionability to CS+ and impaired inhibition (or generalization) of fear to CS- and to extinguished CS+. Studies conducted more recently confirm these findings (reviewed in Craske et al., 2009). On the other hand, the hypothesis of reduced contingency awareness has not received support. Several studies have reported similar percentages of unaware participants among patients and controls (e.g., Jovanovic et al., 2009; Lissek et al., 2005, p. 1411). Furthermore, another study showed abnormal fear conditioning in anxiety patients that was not related to differences in awareness (Blechert et al., 2007).

5. Sources of individual differences in fear conditioning

5.1. Genetic polymorphisms

If abnormal fear conditioning constitutes a diathesis mechanism to clinical anxiety, it should be observed in individuals at risk for anxiety disorders. Moreover, genetic and environmental factors identified as diatheses for anxiety disorders could also influence and account for individual differences in fear conditioning (Mineka & Oehlberg, 2008). A fear-conditioning study conducted with monozygotic and dizygotic twins reported that genetic factors accounted for one third of the variability in the acquisition and extinction of fear. This indicates that fear conditioning is a moderately heritable trait in humans (Hettema, Annas, Neale, Kendler, & Fredrikson, 2003). Interestingly, a meta-analysis of family and twin studies

of anxiety disorders showed genetic contributions to PD (43%) and generalized anxiety disorder (32%) similar to those found for fear conditioning (Hettema, Neale, & Kendler, 2001).

Over the recent years, a considerable number of studies have examined the association between fear conditioning and different genetic polymorphisms. A functional genetic polymorphism is a variation in the DNA sequence that produces two or more different versions or alleles at a specific locus, leading to changes in gene expression. Some of these polymorphisms are of interest for researchers in fear conditioning, because they have been related to variation in the activity of neural structures and neurotransmitter systems implicated in fear (e.g., serotonin transporter gene polymorphism and amygdala reactivity; see Munafo, Brown, & Hariri, 2008). In a review of genetic association studies on human fear conditioning, clear evidence for the association between genetic polymorphisms and differences in fear conditioning was found only for the serotonin transporter and the catechol-O-methyltransferase genes. These polymorphisms were also linked to variation in risk for PTSD (Lonsdorf & Kalisch, 2011).

A polymorphism in the brain-derived neurotrophic factor (BDNF) gene has lately received substantial attention in relation to its potential influence on fear conditioning. The BNDF is involved in synaptic plasticity in the hippocampus and the amygdala, thereby participating in emotional memory formation (Rattiner, Davis, & Ressler, 2005). The BDNF polymorphism (BDNF-val66met) involves the substitution of the amino acid valine (val) by methionine (met) at codon 66. In humans, the met allele has been associated with reduced intracellular trafficking and activity-dependent secretion of the BDNF in the hippocampus, leading to poor memory performance (Egan et al., 2003; Hariri et al., 2003). Nevertheless, the existing studies of the association between the BDNF-val66met polymorphism and fear conditioning (Hajcak et al., 2009; Lonsdorf et al., 2010; Soliman et al., 2010) have yielded inconsistent findings (reviewed in Lonsdorf & Kalisch, 2011).

5.2. Trait anxiety

Trait anxiety, as measured with the trait scale of the State-Trait Anxiety Inventory (Spielberger, Gorsuch, & Lushene, 1982), is a general disposition to experience negative affect (Bados, Gómez-Benito, & Balaguer, 2010). High trait anxiety, or in general high negative affectivity, constitutes an established risk factor for mood and anxiety disorders (Clark, Watson, & Mineka, 1994; Hettema, Neale, Myers, Prescott, & Kendler, 2006; Jorm et al., 2000). However, the precise mechanisms by which high trait anxiety may evolve to pathological anxiety are not yet known (Ormel, Rosmalen, & Farmer, 2004).

An important body of literature indicates that both individuals with high trait anxiety and those with clinical anxiety show attention biases to threat-related stimuli and interpret ambiguous or uncertain stimuli as negative (see Bishop, 2007 for a review). Sandi and Richter-Levin (2009) put forward a model to explain the transition from high trait anxiety to anxiety disorders (as well as depression). According to this model, cognitive biases in individuals with high trait anxiety, which are linked to an overactive stress hormone axis (Portella, Harmer, Flint, Cowen, & Goodwin, 2005) and a hyper-responsive amydala (Etkin et al., 2004), constitute a diathesis for pathological anxiety. When individuals with high trait anxiety are faced with an important stressful event, their idiosyncratic patterns of neuroendocrine and cognitive functioning may escalate to more disruptive neural dysfunctions at amygdalar, prefrontal, and hippocampal levels. These dysfunctions would then manifest in both a proneness to recall negative or fear-related information and deficits in attention, working memory, and executive function.

Within this diathesis-stress model, abnormal fear conditioning may result from the interaction between the neuroendocrine and cognitive patterns that characterize high trait anxious individuals and the occurrence of a fear-provoking event, and it may be a mediating mechanism between high trait anxiety and the onset of pathological anxiety. In fact,

neuroimaging studies have shown a correlation between trait anxiety and amygdala activity – linked to fear expression– during acquisition and generalization of conditioned fear (Dunsmoor, Prince, Murty, Kragel, & LaBar; 2011) and during extinction (Barrett & Armony, 2009; Sehlmeyer et al., 2010). However, the current evidence of the association between high trait anxiety and abnormal fear conditioning is inconclusive, with some studies reporting null findings (Fredrikson & Georgiades, 1992; Otto et al., 2007; Pineles et al., 2009) whereas others have found positive results (Baas, 2013; Boddez et al., 2012; Chan & Lovibond, 1996; Gazendam, Kamphuis, & Kindt, 2013; Grillon & Ameli, 2001; Haddad, Pritchett, Lissek, & Lau, 2012).

5.3. Temporal stability of individual differences in fear conditioning

Before accepting that individual differences in fear conditioning may represent a pathogenic mechanism of anxiety disorders, it should be proven that these are stable over time. Despite the importance of this prerequisite, the temporal stability of fear-conditioning measures has rarely been tested.

6. Aims

The aim of the present Ph D dissertation was to examine individual differences in the acquisition and generalization of conditioned fear as a function of allele variation in the BDNF-val66met polymorphism (study 1; Torrents-Rodas, et al., 2012) and trait anxiety (study 2; Torrents-Rodas, et al., 2013). To this end, we used a fear-conditioning and fear-generalization paradigm developed by Lissek et al. (2008). The fear response was measured by physiological indices (startle reflex and skin conductance response) as well as online ratings of US expectancies. Given the inconclusive findings of previous studies, we did not have any specific hypothesis about the direction of the association between the BDNF-val66met polymorphism and the acquisition and generalization of fear. On the other hand, we expected that individuals with high trait anxiety would show deficits in fear acquisition, mainly driven by impaired fear

inhibition to the safety signal or CS-, and overgeneralization of fear. Additionally, we tested the temporal stability of individual differences in the acquisition and generalization of conditioned fear (study submitted for publication, see the appendix).

II. Study 1: Acquisition and generalization of fear conditioning are not modulated by the BDNF-val66met polymorphism in humans

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Acquisition and generalization of fear conditioning are not modulated by the BDNF-val66met polymorphism in humans

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Abstract

Few studies have investigated the role of the BDNF-val66met polymorphism in fear conditioning in humans, and previous results have been inconsistent. In the present study, we examined whether the BDNF-val66met was associated with differences in the acquisition and generalization of fear during a differential conditioning paradigm in a large sample of participants ($N = 141$). Using three different indexes of fear learning (fear-potentiated startle, skin conductance response, and online risk ratings) no effects of the BDNF-val66met were found either on the acquisition or the generalization of conditioned fear. Taken together with previous data, our study suggests that the BDNF-val66met polymorphism has no effect on the acquisition or generalization of fear.

Descriptors: Genetics, Conditioning, Emotion, Individual differences

Fear conditioning is a form of associative learning by which a neutral stimulus becomes a conditioned stimulus (CS) that elicits a negative emotional response after being paired with an aversive unconditioned stimulus (US). Deficits in fear conditioning are involved in pathological anxiety (Lissek et al., 2005) and may constitute a useful biomarker in the study of anxiety disorders (Graham & Milad, 2011).

In recent years there has been an increased interest in the genetic aspects of fear conditioning in humans. Twin data support the role of genetic factors in fear conditioning and suggest that genes may have a different effect in the several components of this process (e.g., habituation or acquisition/extinction; Hettema, Annas, Neale, Kendler, & Fredrikson, 2003). But it has been only recently that the first studies reporting an association with specific genotypes have emerged (see Lonsdorf & Kalisch, 2011, for a review).

Among others, the brain-derived neurotrophic factor (BDNF) gene has lately received substantial attention in relation to its potential effects on conditioning. The BDNF is a neurotrophin widely expressed in the mammalian brain that has been involved in cognitive learning and memory in rodents (see Poo, 2001) and humans (Egan et al., 2003). Data in rodents indicate that the BDNF gene is expressed in the amygdala during fear conditioning (e.g., Chhatwal, Stanek-Rattiner, Davis, & Ressler, 2006) and is critical for the acquisition of fear (Rattiner, Davis, & Ressler, 2005). In the human BDNF gene, a common Single Nucleotide Polymorphism (SNP) has been identified at codon 66 (val66met), involving the substitution of valine (val) by methionine (met). This polymorphism affects the intracellular trafficking and secretion of BDNF, with met carriers showing decreased activity-dependent secretion of the neurotrophin (Chen et at., 2004). Consistent with animal studies, recent data suggest that this variation may be associated with anxiety or fear in humans. For example, Montag, Basten, Stelzel, Fiebach, and Reuter (2010) found an association between the BDNF met allele and anxiety-related traits (although data on this issue are not conclusive; see Frustaci, Pozzi, Gianfagna, Manzoli, & Boccia, 2008).

Three recent studies have looked at the role of the BDNF-val66met in fear conditioning in humans. Hajcak et al. (2009) used a differential conditioning paradigm where a CS was repeatedly associated with a shock (CS+) and several stimuli that ranged in perceptual similarity (20%, 40%, or 60%) to the CS+ were

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presented to assess fear generalization. In this study, BDNF met carriers ($n = 13$) displayed impaired acquisition, as shown by an attenuated fear-potentiated startle (FPS) to the CS+ compared to val/val homozygous ($n = 44$), the latter also showing increased FPS to perceptually similar stimuli (i.e., higher fear generalization). Lonsdorf et al. (2010) used also a differential conditioning paradigm followed by a delayed extinction phase and found reduced FPS (but not skin conductance response, SCR) to the CS+ in late acquisition and early extinction for the BDNF met carriers ($n = 9$) in comparison to val/val homozygous ($n = 39$). Finally, Soliman et al. (2010) compared BDNF val homozygous ($n = 36$) and met carriers ($n = 36$) in a differential conditioning paradigm and found no effect of the BDNF polymorphism on fear acquisition using SCR, but met-carrier individuals were slower in extinguishing conditioned fear responses.

The results on the modulation of fear conditioning in humans by the BDNF-val66met are therefore not conclusive, and previous studies have some important limitations. Because of the low frequency of the met allele, the group of individuals carrying this allele was generally small and, specifically, very few participants with the BDNF met/met genotype were included ($n = 3$ in the Hajcak et al., 2009, and Lonsdorf et al., 2010, studies, and $n = 5$ in the Soliman et al., 2010, study). Furthermore, Soliman et al. (2010) measured only SCR, and some data suggest that the FPS may be a better index of fear conditioning (see Hamm & Weike, 2005).

In the present study, we examined whether the functional genetic variation in the BDNF-val66met was associated with differences in the acquisition and generalization of fear during a differential conditioning paradigm. We addressed previous limitations using a large ($n = 141$) sample of individuals—with up to 50 met carriers—and indexing fear conditioning with SCR and FPS as well as subjective ratings.

Method

Participants

One hundred forty-one Caucasian healthy volunteers (38 men and 103 women; mean age = 22.29 years $\pm$ 2.54 *SD*) were recruited by advertisements. Exclusion criteria (assessed by an ad hoc structured interview) were lifetime or current drug abuse or dependence, smoking more than 10 cigarettes per day, current psychiatric or medical disorder, pregnancy, visual or auditory impairment, and current use of medication. Participants were asked to abstain from alcohol, tobacco, and any other drug 24 h before the experiment and from caffeinated drinks 12 h before the experiment.

The ethics committee of the Autonomous University of Barcelona approved the study, and participants received 15€ in exchange for their time.

Physiological Recordings

Physiological responses were recorded using a Biopac 150 polygraph (Biopac Systems, Inc.). The startle blink response was measured by recording the electromyographic activity (EMG) of the orbicularis oculi, using two 0.5-cm Ag/AgCl surface electrodes and following standard guidelines (Blumenthal et al., 2005). Impedance level was maintained below 5 kΩ. The raw EMG signal was sampled 2,000 times per second, filtered to reduce power line noise (analogue 50 Hz notch filter) and to attenuate the frequencies beyond the EMG spectrum (infinite impulse response band-pass filter, cutoff frequencies of 28 and 500 Hz), and then rectified and smoothed off-line (10-ms moving window average) using Acq-Knowledge v.3.9.0 (Biopac Systems).

Skin conductance was recorded from the distal phalanges of the index and the middle left-hand fingers by means of two Ag/AgCl electrodes filled with electrolyte. The GSR100C module (Biopac Systems) was used to provide a constant of 0.5 V and to amplify the recorded signal. The signal was sampled at a rate of 125 Hz.

Experimental Procedure and Stimuli

We used the generalization paradigm developed by Lissek and colleagues, which consists of a habituation phase followed by three experimental phases: pre-acquisition, acquisition, and generalization (cf. Lissek et al., 2008). The experimenter was blind to genetic data.

Ten rings of gradually increasing size were presented for 8 s on a computer monitor and served as CSs and generalization stimuli (GSs). The diameter for the smallest ring was 5.08 cm, and subsequent rings increased by 15%. The rings at the two extremes of this size continuum served as CSs. For half of participants, the smallest ring was the CS+, paired with the US before its offset, and the largest was the CS− (not paired with the US); for the other half this pairing was reversed. The intermediate rings were used to test generalization. A fixation cross appeared on the screen when no stimulus was presented (intertrial intervals, ITIs). The US was an electric shock of 100 ms duration delivered to the volar surface of the right forearm with an intensity adjusted for each participant after a workup procedure as being "highly uncomfortable but not painful." It was generated by a stimulator (Grass Instruments S48; West Warwick, RI), isolated (SIU5), and transmitted via a constant-current unit (CCU1) to a bipolar bar electrode (EP10-621, Technomed Europe; Beek, the Netherlands). Between 3 and 11 shocks ($M = 4.55 \pm 1.65$ *SD*) were applied to arrive at the final intensity. The acoustic startle probe was a 50-ms duration, 102-dB(A) burst of white noise, with a near instantaneous rise time, presented binaurally through headphones. Startle probes were presented 4 or 5 s after the beginning of odd trials; interprobe intervals ranged from 18 to 25 s. During even trials, online ratings of perceived risk of shock were obtained (1 = *no risk*, 2 = *moderate risk*, 3 = *high risk*), 1 or 2 s after trial onset. Stimulus timing and response recording were controlled by the commercial system Presentation (Neurobehavioral Systems, Inc.).

Upon arrival at the laboratory, participants were given written instructions about the experiment and signed the informed consent. They were not instructed about the CS-US contingency, but were told that they might learn to predict the shock if they attended to the presented stimuli. Next, the genetic sample was collected, the electrodes were placed, and the intensity of the US was adjusted. After placing the headphones, nine startle probes were presented to reduce initial startle reactivity (habituation; results not presented here). Pre-acquisition consisted of six CS+, six CS−, and six ITI trials presented in the absence of the US. Acquisition consisted of 12 CS+ (9 of them coterminating with US delivery), 12 CS−, and 12 ITI trials. Generalization consisted of 12 CS+ (6 of them coterminating with US delivery), 12 CS−, 12 ITI, and 6 trials from each of the eight GS sizes. Following Lissek et al. (2008), prior to analyses, responses to every two GSs were averaged, resulting in four classes of responses to GSs (Class 1, Class 2, Class 3, and Class 4). There was a 10-min break between the acquisition and the generalization. After the experiment, participants rated the discomfort produced by both the US and the startle probe on a 1 (*no discomfort*) to 10 (*maximum discomfort*) scale; answered a

multiple-choice question regarding contingency awareness ("The electric stimulus usually appeared: (a) in the presence of the smallest ring; (b) in the presence of the biggest ring; (c) randomly; (d) I don't know"); and completed the trait portion of the Spanish version of the State Trait Anxiety Inventory (STAI-T; Spielberger, Gorsuch, & Lushene, 1982).

Data Reduction and Response Definition

Scorers were blind to the stimuli presented or genotype data. The onset latency window for the startle response was 20–100 ms and the peak magnitude was determined within 150 ms of response onset. Startle amplitudes were computed in microvolts (mV) as the difference between the EMG value at response peak and the average EMG during the baseline period (50 ms preceding startle probe onset). In those trials in which no response was detected, amplitude was scored as 0 mV. After visual inspection, trials with excessive baseline activity were rejected (3.2% in the BDNF val/val group, 3.9% in the BDNF met-carrier group). The percentage of rejected trials did not differ between groups, $\chi^2(1) = 3.44$, $p = .064$. Previous to statistical analysis, all startle responses of one individual were T-transformed, resulting in a distribution with an overall mean of 50 and a standard deviation of 10.

SCR to the CSs were scored for half of the trials (those where risk ratings were not obtained). SCR magnitudes (in microsiemens, μS) were computed as the difference between the maximum SCR value and the value at response onset, occurring 1–5 s after CS onset. This time window was chosen to avoid interference of the SCRs elicited by the startle probes (Grillon & Ameli, 2001; Weike, Schupp, & Hamm, 2007). Trials in which no response could be detected or with a response magnitude <0.02 μS were considered nonresponse trials, and trials showing artifacts or excessive baseline activity were rejected (9.7% in the BDNF val/val group, 8.6% in the BDNF met-carrier group). The percentage of rejected trials did not differ between groups, $\chi^2(1) = 2.21$, $p = .137$. SCR data were square-root transformed to normalize the distribution.

Genotyping

Genomic DNA was extracted from buccal mucosa on a cotton swab using the Real Extraction DNA Kit (Durviz S.L.U., Valencia, Spain). The rs6265 SNP (val66met) of the BDNF gene was determined using the Taqman 5′ exonuclease assay (Applied Biosystems) and genotyped using Applied Biosystems (AB) TaqMan technology. The probe for genotyping the rs6265 was ordered through the TaqMan SNP Genotyping assays (code C_11592758_10) AB assay-on-demand service. The final volume of the polymerase chain reaction (PCR) reaction was 5 mL, which contained 10 ng of genomic DNA, 2.5 ml of TaqMan Master Mix, and 0.125 ml of 40× genotyping assay. The cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. PCR plates were read on an ABI PRISM 7900HT instrument with SDS v2.1 software (Applied Biosystems).

Data Analysis

One hundred twenty-nine individuals participated in the fear conditioning and generalization paradigm and 141 only in the fear conditioning paradigm. Three participants were excluded from the analyses of generalization because of technical problems. Additionally, participants were excluded from the analyses of the startle response or SCR in a particular experimental phase if all the trials for one type of stimulus in a given block were rejected ($n = 1$ [startle] and $n = 6$ [SCR] during acquisition; $n = 3$ [startle] and $n = 6$ [SCR] during generalization). Participants were excluded from the analyses of risk ratings in a particular experimental phase if they reported the same value throughout the phase or if there were technical problems ($n = 2$ during acquisition and $n = 2$ during generalization). Finally, participants were excluded from SCR analyses if they did not show any response ($n = 10$ during acquisition and $n = 6$ during generalization).

Data were analyzed with SPSS version 19.0. Differences between the two genotype groups in baseline characteristics were assessed with *t* tests and the Fisher's exact test. Data were analyzed separately for each experimental phase (pre-acquisition, acquisition, and generalization) and for each measure (startle blink response, SCR, and risk ratings), using repeated-measures analyses of variance (ANOVAs; GLM procedure). In pre-acquisition and acquisition, stimulus (CS+, CS-, and ITI for startle; CS+ and CS– for SCR and risk ratings) and genotype group (BDNF val/val and BDNF met carriers) were included as within- and between-subjects variables, respectively. In addition, the within-subjects variable block (first and second) was also taken into account in acquisition. Generalization included stimulus (CS–, Class 1, Class 2, Class 3, Class 4, and CS+) and genotype group (BDNF val/val vs. BDNF met carriers).

Simple contrasts were calculated to specify main or interaction effects. The level of significance was $p < .05$ (two-tailed). Greenhouse–Geisser corrections were applied when necessary. We report η^2 as an estimate of effect size.

Results

Genotype Frequencies and Sample Characteristics

Ninety-one out of 141 participants (64.5%) were homozygous for the BDNF val/val genotype, 40 (28.4%) were carriers of the val/met genotype, and 10 (7.1%) were homozygous for the met/met genotype (allele frequencies: val allele = 78.7% and met allele = 21.3%). Hardy–Weinberg equilibrium was verified for the present population, $\chi^2(2) = 1.18$, $p = .550$.

Because the BDNF met/met genotype ($n = 10$) had a much lower frequency than the val/met and val/val genotypes, and following previous studies (e.g., Hajcak et al., 2009; Lonsdorf et al., 2010), val/met and met-homozygous participants were combined as a BDNF met-carrier group ($n = 50$).

Characteristics of the two genotype groups are presented in Table 1. The two groups did not differ in gender distribution, STAI-T scores, selected intensity for the US, discomfort rating of the US, discomfort rating of the startle probe, or frequency of contingency-aware participants. Participants in the BDNF val/val group were slightly older than participants of the BDNF met-carrier group, $t(139) = 2.14$, $p = .034$, but the difference was very small (mean difference = 0.95 years, 95% confidence interval [0.07, 1.82]).

Pre-acquisition

Analysis of startle blink responses, SCR, and risk ratings revealed main effects of neither stimulus nor Stimulus × Genotype interaction effects (all Fs < 0.21, ps > .1).

Table 1. *Participant Characteristics*

	BDNF val/val ($n = 91$)	BDNF met carriers ($n = 50$)
Age in years	22.63 (2.68)	21.68 (2.18)
Men (n and %)	22 (24.2%)	16 (32.0%)
STAI-T score[a]	21.47 (11.48)	21.92 (12.32)
US intensity (in mA)	3.51 (0.98)	3.49 (0.83)
US discomfort[b]	6.66 (1.56)	6.50 (1.53)
Startle probe discomfort[b]	7.09 (1.70)	6.98 (2.13)
Contingency-aware individuals (n and %)	79 (86.8%)	46 (92.0%)

Note. Unless otherwise indicated, means and *SD* are provided.
[a]The range of scores in the Spanish version of the STAI-T scale goes from 0 to 60. [b]Ratings ranged from 1 (*no discomfort*) to 10 (*maximum discomfort*).

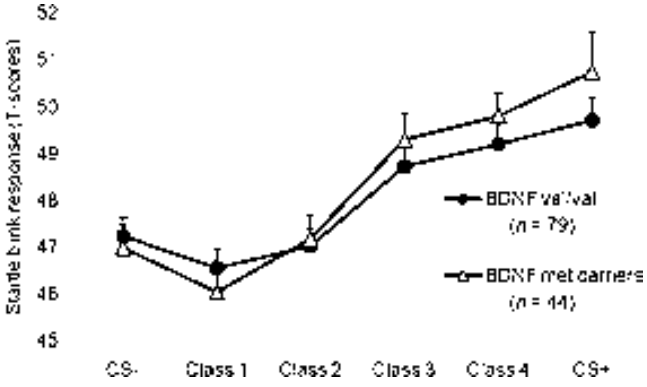

Figure 2. Startle blink magnitudes for each stimulus by genotype group, at generalization. Error bars reflect the standard errors of the mean.

Acquisition

Startle blink responses. During acquisition, the repeated-measures ANOVA revealed robust startle potentiation, as indicated by a significant main effect of stimulus, $F(2,276) = 24.22$, $p < .001$, $\eta^2 = .15$. Simple contrasts revealed that the significant main effect of stimulus was attributable to a significant CS+ potentiation, CS+ vs. ITI: $F(1,138) = 6.66$, $p = .011$, $\eta^2 = .05$, as well as CS discrimination, CS+ vs. CS−: $F(1,138) = 17.59$, $p < .001$, $\eta^2 = .11$ (see Figure 1, left panel). Genotype had no significant influence as a main effect, and there were no significant Genotype × Stimulus, Genotype × Block, or Genotype × Stimulus × Block interactions (all Fs < 1.74, ps > .1).

Skin conductance responses. The results for the SCR were similar to the startle data. There was evidence of differential conditioning, as shown by a significant main effect of stimulus, $F(1,123) = 25.73$, $p < .001$, $\eta^2 = .17$, in the expected direction, that is, larger SCR to the CS+ than to the CS− (see Figure 1, center panel). Again, none of the interactions including the genotype term were significant (all Fs < 1.28, ps > .1).

Risk ratings. For online risk ratings, there was also evidence of differential conditioning. A main effect of stimulus, $F(1,137) = 249.51$, $p < .001$, $\eta^2 = .65$, was found, with higher risk ratings to CS+ than to CS− (see Figure 1, right panel). Again, genotype did not interact with stimulus or block (all Fs < 2.21, ps > .1).

Generalization

Startle blink responses. Generalization of fear conditioning was evidenced by a main effect of stimulus, $F(4.41,533.76) = 19.90$, $p < .001$, $\eta^2 = .14$, during the generalization phase. As shown in Figure 2, startle responses decreased as the stimulus differed from the CS+. The pattern of this downward gradient did not differ across genotype groups, as shown by a nonsignificant Genotype × Stimulus interaction nor did it show a main effect of genotype (all Fs < 0.68, ps > .1).

Skin conductance responses. SCR during generalization followed a pattern similar to that of startle data. Again, a stimulus main effect was found, $F(3.27,366.68) = 12.63$, $p < .001$, $\eta^2 = .10$, but none of the interactions with genotype was significant (all Fs < 0.56, ps > .1).

Risk ratings. Also consistent with startle data, analyses of risk ratings revealed a main effect of stimulus, $F(2.30,288.02) = 212.77$, $p < .001$, $\eta^2 = .64$, and increasing levels of risk from CS− to CS+ according to the similarity to the CS+. Genotype was not significant as main effect or interacting with risk ratings (all Fs < 0.71, ps > .1).

Analyses Using Three Genotype Groups

Because this study includes the largest sample of met-homozygous so far published in a fear conditioning study ($n = 10$) and given

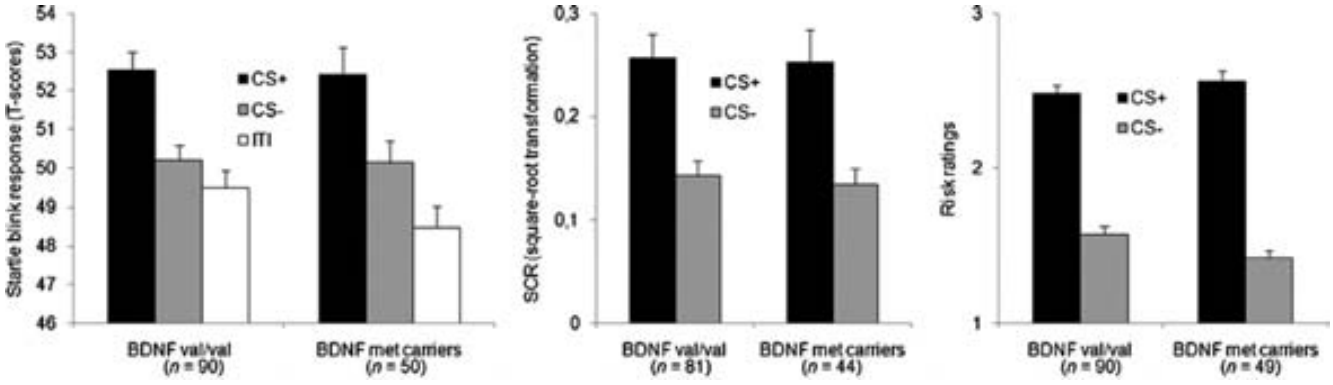

Figure 1. Responses to each CS by genotype group, at acquisition. Error bars reflect the standard errors of the mean.

previous results showing a dose relationship between number of alleles and anxiety (see Frielingsdorf et al., 2010), we repeated our analyses using three genotype groups (val/val, val/met, and met/met).

Results were similar to the analyses with two groups, and again none of the Stimulus × Genotype interactions nor the main effect of genotype were significant for any of the measures on fear acquisition or generalization (all Fs < 1.64, ps > .1).

Post hoc analyses

Given prior studies showing that BDNF effects on some emotional responses (e.g., Shalev et al., 2008) and mental disorders (e.g., Verhagen et al., 2010) are modulated by gender, we repeated the reported ANOVAs with two genotype groups, adding gender as a between-subjects variable. The results showed that, during acquisition, gender interacted with block for startle blink responses, $F(1,136) = 4.32$, $p = .040$, $\eta^2 = .03$, and had a main effect for SCR, $F(1,121) = 7.18$, $p = .008$, $\eta^2 = .06$, and for risk ratings, $F(1,135) = 13.75$, $p < .001$, $\eta^2 = .09$, but did not affect differential conditioning per se, as the Gender × Stimulus or the Gender × Stimulus × Block interactions were nonsignificant. Furthermore, in these additional analyses there were no significant interactions with—or a main effect of—genotype (all Fs < 0.84, ps > .1).

During generalization and for startle blink responses, there was a significant Gender × Genotype interaction effect, $F(1,119) = 4.49$, $p = .036$, $\eta^2 = .04$. Simple contrasts revealed that men had higher startle responses than women only for the BDNF val/val group, $t(119) = 5.54$, $p = .013$. There was also a significant main effect of gender on SCR, $F(1,110) = 18.73$, $p < .001$, $\eta^2 = .15$, and risk ratings, $F(1,120) = 5.75$, $p = .018$, $\eta^2 = .05$. However, fear generalization was not affected by gender, as shown by the nonsignificant interactions of Gender × Stimulus (all Fs < 1.49, ps > .1, η^2 range from .00 to .01). Again, there were no significant interactions with—or main effect of—genotype (all Fs < 0.23, ps > .1).

Given the significant (albeit small) age differences between genotype groups, all analyses were conducted again using age as a covariate, but the results remained unchanged.

Discussion

In this study, using a rather large sample and three different indexes of fear conditioning, we found no evidence that genetic variation in the BDNF-val66met is associated with differences in the acquisition or generalization of fear using a differential conditioning paradigm.

Our results on fear acquisition are in agreement with Soliman et al. (2010), who also found no effect of the BDNF polymorphism on fear acquisition measured by SCR in humans. They are also consistent with results from the animal literature, where no differences between knock-in BDNF met and wild-type mice have been found in cue-dependent fear (Chen et al., 2006; Liu, Lyons, Mamounas, & Thompson, 2004; Soliman et al., 2010). In contrast, Lonsdorf et al. (2010) did find a deficit in fear acquisition in human BDNF met carriers. However, these results were based on a small sample of met carriers ($n = 9$), were only evident for one of two fear-conditioning indexes (i.e., for FPS but not SCR), and were only significant later during the acquisition process. Some methodological differences between the present study and the Lonsdorf et al. study are also worth noting: (a) The CS+ used are qualitatively rather different: Lonsdorf et al. used facial pictures as CSs, whereas geometric figures were used here. Previous research has suggested that facial expressions might show enhanced "conditionability" (Canli & Lesch, 2007; Öhman, 2009) and therefore could explain the differences between these two studies. (b) Only individuals aware of the CS-US contingency were included in the Londsorf et al. study, whereas all participants taking part in the conditioning paradigm were included here (see below).

Our results also did not replicate the only previous study on the BDNF polymorphism and fear generalization in humans (Hajcak et al., 2009), where evidence for impaired fear generalization was found among BDNF met carriers compared to val homozygous, although these differences were only shown for FPS but not for risk ratings. The paradigm and methods used by Hajcak et al. were similar to ours, with two relevant exceptions: (a) In the later study, risk ratings were obtained retrospectively at the end of the experimental session, whereas in the present report these were obtained *during* the experimental procedure, and therefore they are a better estimate of the learning process. (b) Participants in the Hajcak et al. study were informed of the contingency between the US and the CS prior to the experimental session, whereas no specific information about the association was given to participants here (although they were told that they might learn to predict the shock). However, this is unlikely to account for the differences between our results and those obtained by Lonsdorf et al. (2010) and Hajcak et al. given that in our study the percentage of aware individuals was similar for both genotype groups.

An obvious limitation of reporting negative results is that they could be due to a lack of statistical power rather than a real lack of effect in the population. However, considering that the present study reports on the larger sample ($n = 141$) so far included in similar studies, we should have expected to find at least the same effects as previously reported (Hajcak et al., 2009; Lonsdorf et al., 2010); that not being the case suggests that differences between previous and present results might have more to do with other methodological issues that we tried to address here, rather than being a power problem. Our negative results on acquisition and generalization were further supported by a comparison of participants carrying none, one, or two BDNF met alleles, which confirmed the absence of effects of the genetic variation in the two fear processes under study. Anyway, these data should be seen with caution, given the small sample size ($n = 10$) of participants homozygous for the met allele. Another limitation of the present study is that startle probes were always presented after trials where risk ratings were performed, and therefore their occurrence was predictable. Although we believe that the probability of this affecting our genotype results is low, this should be taken into account in future studies using this paradigm.

Taken together with the results of previous studies, our data suggest that variation in the BDNF gene is not associated with fear acquisition and has little effect (if any) on fear generalization. However, our results do not fully invalidate the involvement of the BDNF gene in fear conditioning. The present report focuses on the acquisition and generalization of fear through cue differential conditioning. Previous research has linked the BDNF gene with hippocampus-dependent learning (Frielingsdorf et al., 2010), and it could be the case that the val66met polymorphism of the BDNF gene has a stronger effect on context conditioning—meant to depend on hippocampal function—rather than on cue conditioning like the one used here, where activity in the amygdala has been suggested to have a prominent role, as indicated by Lonsdorf and Kalisch (2011). In fact, animal research has strengthened the link

between the BDNF gene and contextual fear conditioning, showing that the loss of one copy of the BDNF gene in mice leads to impaired contextual fear conditioning and that an infusion of BDNF protein into the hippocampus may partially restore this deficit (Liu et al., 2004).

Another process where BDNF may play a role is fear extinction. In at least two studies, BDNF met/met mice have shown impaired extinction learning after fear conditioning (Soliman et al., 2010; Yu et al., 2009). In the Lonsdorf et al. (2010) study in humans, the impaired acquisition of met carriers carried over to an early (but not late) extinction phase 24 h later, but this was not attributed to extinction per se but rather to differences in memory acquisition that were transmitted to the following day.

Finally, in the study by Soliman et al. (2010), human met carriers were slower than val carriers in extinguishing conditioned fear responses (in the absence of differences in the acquisition). The psychophysiological results were complemented by neuroimaging (functional magnetic resonance imaging) data showing changes in activation of the brain areas allegedly involved in extinction (ventromedial prefrontal cortex and amygdala). This experiment also used a differential conditioning paradigm, but there was a reversal phase (i.e., the CS was repeatedly presented unpaired with the US) between the acquisition and the extinction, which complicates the interpretation (see Lonsdorf & Kalisch, 2011, for a critical review of this study). The results of these studies suggest, therefore, that the BNDF polymorphism may have a significant role in extinction, and recent research has pointed out the possible clinical implications of this. For example, met carriers with anxiety disorders could benefit more from non-exposure-based treatments (which rely on extinction) or need additional strategies to "enhance" extinction, leading to more personalized interventions (see Fullana et al., 2011). It must be noted, however, that there are other molecular mechanisms not related to the BDNF that could account for differences in extinction, including the NMDA receptors and voltage-gated calcium channels (see Chhatwal et al., 2006).

To sum up, our results suggest that the BDNF-val66met polymorphism has no effect on fear acquisition and generalization in humans through differential conditioning, but that does not rule out the role of this gene on fear conditioning. Future research should focus on alternative processes in which this polymorphism could have a greater effect, such as context conditioning or extinction.

References

Blumenthal, T. D., Cuthbert, B. N., Filion, D. L., Hackley, S., Lipp, O. V., & Van Boxtel, A. (2005). Committee report: Guidelines for human startle eyeblink electromyographic studies. *Psychophysiology*, *42*, 1–15. doi: 10.1111/j.1469-8986.2005.00271.x

Canli, T., & Lesch, K. P. (2007). Long story short: The serotonin transporter in emotion regulation and social cognition. *Nature Neuroscience*, *10*, 1103–1109. doi: 10.1038/nn1964

Chen, Z. Y., Jing, D., Bath, K. G., Ieraci, A., Khan, T., Siao, C. J., . . . Lee, F. S. (2006). Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science*, *314*, 140–143. doi: 10.1126/science.1129663

Chen, Z. Y., Patel, P. D., Sant, G., Meng, C. X., Teng, K. K., Hempstead, B. L., & Lee, F. S. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *Journal of Neuroscience*, *24*, 4401–4411. doi: 10.1523/JNEUROSCI.0348-04.2004

Chhatwal, J. P., Stanek-Rattiner, L., Davis, M., & Ressler, K. J. (2006). Amygdala BDNF signaling is required for consolidation but not encoding of extinction. *Nature Neuroscience*, *9*, 870–872. doi: 10.1038/nn1718

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A., . . . Weinberger, D. R. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*, *112*, 257–269. doi: 10.1016/S0092-8674(03)00035-7

Frielingsdorf, H., Bath, K. G., Soliman, F., Difede, J., Casey, B. J., & Lee, F. S. (2010). Variant brain-derived neurotrophic factor Val66Met endophenotypes: Implications for posttraumatic stress disorder. *Annals of the New York Academy of Sciences*, *1208*, 150–157. doi: 10.1111/j.1749-6632.2010.05722.x

Frustaci, A., Pozzi, G., Gianfagna, F., Manzoli, L., & Boccia, S. (2008). Meta-analysis of the brain-derived neurotrophic factor gene (BDNF) Val66Met polymorphism in anxiety disorders and anxiety-related personality traits. *Neuropsychobiology*, *50*, 163–170. doi: 10.1159/000182892

Fullana, M. A., Alonso, M. P., Gratacós, M., Jaurrieta, N., Jiménez-Murcia, S., Segalàs, C., . . . Menchón, J. M. (2011). Variation in the BDNF Val66Met polymorphism and response to cognitive-behavior therapy in obsessive-compulsive disorder. *European Psychiatry*. doi: 10.1016/j.eurpsy.2011.09.005

Graham, B. M., & Milad, M. R. (2011). The study of fear extinction: Implications for anxiety disorders. *American Journal of Psychiatry*. Advance online publication. doi: 10.1176/appi.ajp.2011.11040557

Grillon, C., & Ameli, R. (2001). Conditioned inhibition of fear-potentiated startle and skin conductance in humans. *Psychophysiology*, *38*, 807–815.

Hajcak, G., Castille, C., Olvet, D. M., Dunning, J. P., Roohi, J., & Hatchwell, E. (2009). Genetic variation in brain-derived neurotrophic factor and human fear conditioning. *Genes, Brain and Behavior*, *8*, 80–85. doi: 10.1111/j.1601-183X.2008.00447.x

Hamm, A. O., & Weike, A. I. (2005). The neuropsychology of fear learning and fear regulation. *International Journal of Psychophysiology*, *57*, 5–14. doi: 10.1016/j.ijppsycho.2005.01.006

Hettema, J. M., Annas, P., Neale, M. C., Kendler, K. S., & Fredrikson, M. (2003). A twin study of the genetics of fear conditioning. *Archives of General Psychiatry*, *60*, 702–708.

Lissek, S., Biggs, A. L., Rabin, S. J., Cornwell, B. R., Alvarez, R. P., Pine, D. S., & Grillon, C. (2008). Generalization of conditioned fear-potentiated startle in humans: Experimental validation and clinical relevance. *Behaviour Research and Therapy*, *46*, 678–687. doi: 10.1016/j.brat.2008.02.005

Lissek, S., Powers, A. S., McClure, E. B., Phelps, E. A., Woldehawariat, G., Grillon, C., & Pine, D. S. (2005). Classical fear conditioning in the anxiety disorders: A meta-analysis. *Behaviour Research and Therapy*, *43*, 1391–1424. doi: 10.1016/j.brat.2004.10.007

Liu, I. Y., Lyons, W. E., Mamounas, L. A., & Thompson, R. F. (2004). Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. *Journal of Neuroscience*, *24*, 7958–7963. doi: 10.1523/JNEUROSCI.1948-04.2004

Lonsdorf, T. B., & Kalisch, R. (2011). A review on experimental and clinical genetic associations studies on fear conditioning, extinction and cognitive-behavioral treatment. *Translational Psychiatry*, *1*, e41. doi: 10.1038/tp.2011.36.

Lonsdorf, T. B., Weike, A. I., Golkar, A., Schalling, M., Hamm, A. O., & Öhman, A. (2010). Amygdala-dependent fear conditioning in humans is modulated by the BDNFval66met polymorphism. *Behavioral Neuroscience*, *124*, 9–15. doi: 10.1037/a0018261

Montag, C., Basten, U., Stelzel, C., Fiebach, C. J., & Reuter, M. (2010). The BDNF Val66Met polymorphism and anxiety: Support for animal knock-in studies from a genetic association study in humans. *Psychiatry Research*, *179*, 86–90. doi: 10.1016/j.psychres.2008.08.005

Öhman, A. (2009). Of snakes and faces: An evolutionary perspective on the psychology of fear. *Scandinavian Journal of Psychology*, *50*, 543–552. doi: 10.1111/j.1467-9450.2009.00784.x

Poo, M. (2001). Neurotrophins as synaptic modulators. *Nature Reviews Neuroscience*, *2*, 24–32. doi: 10.1038/35049004

Rattiner, L. M., Davis, M., & Ressler, K. J. (2005). Brain-derived neurotrophic factor in amygdala-dependent learning. *The Neuroscientist*, *11*, 323–333. doi: 10.1177/1073858404272255

Shalev, I., Lerer, E., Israel, S., Uzefovsky, F., Gritsenko, I., Mankuta, D., . . . Kaitz, M. (2008). BDNF Val66Met polymorphism is associated with HPA axis reactivity to psychological stress characterized by genotype and gender interactions. *Psychoneuroendocrinology*, *34*, 328–388. doi: 10.1016/j.psyneuen.2008.09.017

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S. S., . . . Casey, B. J. (2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science*, *327*, 863–866. doi: 10.1126/science.1181886

Spielberger, C. D., Gorsuch, R. R., & Lushene, R. E. (1982). *STAI. Cuestionario de ansiedad Estado/Rasgo* (1st ed.). Madrid: Técnicos Especialistas Asociados (TEA).

Verhagen, M., van der Meij, A., van Deurzen, P. A., Janzing, J. G., Arias-Vásquez, A., Buitelaar, J. K., & Franke, B. (2010). Meta-analysis of the BDNF Val66Met polymorphism in major depressive disorder: Effects of gender and ethnicity. *Molecular Psychiatry*, *15*, 260–271. doi: 10.1038/mp.2008.109

Weike, A. I., Schupp, H. T., & Hamm, A. O. (2007). Fear conditioning requires awareness in trace but not delay conditioning. *Psychophysiology*, *44*, 170–180. doi: 10.1111/j.1469-8986.2006.00469.x

Yu, H., Wang, Y., Pattwell, S., Jing, D., Liu, T., Zhang, Y. B., . . . Chen, Z. Y. (2009). Variant BDNF Val66Met polymorphism affects extinction of conditioned aversive memory. *Journal of Neuroscience*, *29*, 4056–4064. doi: 10.1523/JNEUROSCI.5539-08.2009

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III. Study 2: No effect of trait anxiety on differential fear conditioning or fear generalization

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No effect of trait anxiety on differential fear conditioning or fear generalization

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ABSTRACT

Previous studies have shown that individuals with anxiety disorders exhibit deficits in fear inhibition and excessive generalization of fear, but little data exist on individuals at risk from these disorders. The present study examined the role of trait anxiety in the acquisition and generalization of fear in 126 healthy participants selected on the basis of their trait-anxiety scores. Measures of conditioning included fear-potentiated startle, skin conductance response and online risk ratings for the unconditioned stimulus. Contrary to our hypotheses, trait anxiety did not have any effect either on the acquisition or the generalization of fear. Our results suggest that these fear conditioning processes are not impaired in individuals at risk from anxiety.

1. Introduction

Fear conditioning is a form of associative learning by which a neutral stimulus becomes a conditioned stimulus (CS) that elicits a fear response after being paired with an innately aversive stimulus (unconditioned stimulus, US). This process allows the organism to respond appropriately to stimuli that signal a potential threat. However, when fear is expressed in non-threatening situations, such as in anxiety disorders, it becomes pathological.

Fear conditioning has been widely studied as a source of individual differences in the pathogenesis of anxiety. In a recent quantitative meta-analysis, Lissek et al. (2005) reviewed 20 empirical studies that compared patients with anxiety disorders and healthy individuals with regard to fear conditioning. The studies reviewed used either a simple or a differential conditioning paradigm. In the former, a single CS is paired with the US (CS+), whereas in the latter, a CS is paired with the US (CS+) and another CS is presented in the absence of the US, thus becoming a safety signal (CS−). Relative to healthy individuals, anxious patients displayed stronger fear responses to the CS+ in simple conditioning studies. They also showed increased fear both to the CS+ and the CS− in studies using differential conditioning. In addition, anxious patients displayed stronger fear responses during extinction (the phase following acquisition in a typical fear conditioning experiment, when the CS is no longer followed by the US).

These difficulties in suppressing fear responses to the CS− during acquisition and to the CS+ during extinction may be taken as evidence of deficits in fear inhibition processes among anxious patients (Davis et al., 2000). Recent studies (not included in the aforementioned meta-analysis) have also provided evidence consistent with deficits in fear inhibition to the CS− in patients with posttraumatic stress disorder (PTSD; Jovanovic et al., 2009, 2010) and panic disorder (Lissek et al., 2009).

The difficulties in suppressing fear responses to the CS− observed among anxious patients have also been related to the generalization of fear (Craske et al., 2009; Jovanovic et al., 2010; Lissek et al., 2005, 2009). Generalization of conditioned fear is also an adaptive process by which learned fear transfers to novel stimuli that are similar to the original CS. As in a differential conditioning paradigm, the CS+ and the CS− share many perceptual characteristics, anxious individuals would tend to transfer fear from one stimulus to the other. In fact, an excessive fear generalization (e.g., expression of fear to stimuli that resemble those present during a traumatic event or a first panic attack) may be characteristic of certain anxiety disorders (American Psychiatric Association, 2000). In the only study conducted on this topic in a clinical sample thus far, Lissek et al. (2010) found that individuals with

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panic disorder displayed greater fear generalization than healthy controls.

Most studies on individual differences in fear conditioning have been conducted in clinical samples. However, this research strategy has some limitations. First, results may be contaminated by the frequent comorbidity of different anxiety disorders, and of anxiety disorders and other psychiatric conditions (Merikangas & Swanson, 2010). Second, patients with anxiety disorders often follow pharmacological treatments that may interfere with measures of fear conditioning (e.g., Grillon & Baas, 2003) or with the conditioning process itself (Kindt et al., 2009). An alternative to circumvent these limitations is to study fear conditioning in individuals who are at risk from anxiety but do not suffer from a clinically defined anxiety disorder.

Elevated trait anxiety is an important risk factor for anxiety disorders (Chambers et al., 2004; Hettema et al., 2006). However, few studies have aimed to examine individual differences in fear conditioning as a function of trait anxiety. Consistent with the above-mentioned studies in clinical samples, Grillon and Ameli (2001) found deficits in fear inhibition in the presence of safety signals among individuals with high trait anxiety, although the goal of this study was to develop a paradigm to study conditioned inhibition, and the sample size was rather small (only 18 participants were highly anxious individuals). In addition, two recent neuroimaging studies found a significant positive association between trait anxiety and the activation of brain areas that mediated the expression of conditioned fear (Dunsmoor et al., 2011; Indovina et al., 2011).

In the present study, we examined the role of trait anxiety in the acquisition and generalization of fear. Based on previous research, we predicted that individuals with high trait anxiety would show deficits in fear inhibition (i.e., inability to suppress fear responses to CS− in a differential conditioning paradigm). We also predicted that individuals with high trait anxiety would generalize conditioned fear to a greater extent than non-anxious individuals (i.e., would show a more gradual decline in conditioned fear when stimuli ranging in perceptual similarity to the CS+ were presented). We used fear-potentiated startle (FPS), skin conductance response (SCR), and online ratings of risk for US, as measures of fear conditioning.

2. Method

2.1. Participants

Nine-hundred and ninety-two undergraduates were screened with the trait scale of the State-Trait Anxiety Inventory (STAI-T, Spanish version; Spielberger et al., 1982). The final sample consisted of 126 individuals selected on the basis of their mean trait anxiety score on two separate administrations of the STAI-T (separated by a period of 10 months on average). Three groups were thus formed (low anxiety: percentiles 1–20; medium anxiety: percentiles 36–65; and high anxiety: percentiles 81–100). Participants were screened for exclusion criteria (lifetime/current drug abuse or dependence, smoking more than 10 cigarettes per day, current psychiatric or medical disorder, pregnancy, visual/auditory impairment, and current use of medication, as per self-report) with an ad hoc structured interview conducted by a research psychologist. They were asked to abstain from alcohol, tobacco, and any other drug 24 h before the experiment, and of caffeinated drinks 12 h before the experiment. Table 1 shows the basic characteristics of each group.

The study was approved by the university ethics committee, and participants received 15€ in exchange for their time.

Table 1
Participants' characteristics and variables related to the experimental procedure (unless otherwise indicated, means and standard deviations are provided).

	Low ($n = 39$)	Medium ($n = 47$)	High ($n = 40$)
Male sex, n (%)	10 (26%)	12 (26%)	12 (30%)
Age (years)	22.77 (2.71)	21.96 (2.22)	22.26 (2.74)
STAI-T[a]	8.41 (2.30)	20.11 (1.99)	37.92 (4.08)
Contingency-unaware individuals[b], n (%)	4 (10%)	7 (15%)	4 (10%)
Shock intensity (mA)[c]	3.68 (0.18)	3.57 (0.14)	3.31 (0.13)
Shock discomfort (1–10)[c]	6.41 (0.23)	6.57 (0.25)	6.93 (0.22)
Startle probe discomfort (1–10)[c]	7.13 (0.28)	6.70 (0.28)	7.25 (0.30)

[a] State-trait anxiety inventory, Trait version. Scores range from 0 to 60 in the Spanish version of the STAI-T.
[b] $p > .05$. Pearson's χ^2.
[c] $p > .05$. F ratio.

2.2. Stimuli and procedure

We used the paradigm developed by Lissek et al., which consists of three experimental phases (pre-acquisition, acquisition, and generalization) preceded by nine startle habituation trials (cf. Lissek et al., 2008) and which allows the study of both fear conditioning and generalization.

Ten rings of gradually increasing size were presented for 8 s on a computer monitor and served as conditioned stimuli (CSs) and generalization stimuli (GSs). The diameter of the smallest ring was 5.08 cm and subsequent rings increased by 15%. The rings at the two extremes of this size continuum served as CSs. For half of the participants in each anxiety group, the smallest ring was the CS+ (paired with the US before its offset) and the largest was the CS−; for the remaining participants the pairing was reversed. The intermediate rings were used to test conditioned generalization. A fixation-cross appeared on the screen when no stimulus was presented (inter-trial interval, ITI). The US was an electric shock of 100 ms duration, with an intensity adjusted for each participant after a workup procedure as being "highly uncomfortable but not painful", delivered to the volar surface of the right forearm. It was generated by a stimulator (Grass Instruments S48; West Warwick, RI), isolated (SIU5), and transmitted via a constant-current unit (CCU1) to a bipolar bar electrode (EP10-621, Technomed Europe; Beek, NL). Between 3 and 11 shocks ($M = 4.56 \pm 1.73$ SD) were applied in order to arrive at the final intensity. The acoustic startle probe was a 50 ms duration, 102 dB(A) burst of white noise with a near instantaneous rise time, presented binaurally through headphones. Startle probes were presented 4 or 5 s after the beginning of odd trials, inter-probe intervals (IPIs) ranged from 18 to 25 s. ITI durations (9 to 17 s) were adjusted to keep IPIs within the specified range. During even trials, online ratings of perceived risk of shock for each stimulus were obtained (1 = no risk, 2 = moderate risk, 3 = high risk). One or 2 s after trial onset, a question at the top of the screen (Level of risk?) cued participants to respond as quickly as possible using a computer keyboard. Stimulus timing and response recording were controlled by the commercial system Presentation (Neurobehavioral Systems Inc).

Upon arrival at the laboratory, participants read the instructions for the experiment and signed the informed consent. They were not instructed about the CS-US contingency, but were told that they might learn to predict the shock if they attended to the presented stimuli. Next, the electrodes were placed, and the intensity of the shock was adjusted. After placing the headphones, nine startle probes were presented to reduce initial startle reactivity (habituation; results not presented here). Pre-acquisition consisted of six CS+ and six CS− trials presented in the absence of the US; Acquisition consisted of 12 CS+ (nine of them co-terminating with US delivery) and 12 CS− trials. Generalization consisted of 12 CS+ (six of them co-terminating with US delivery), 12 CS−, and six trials from each of the eight GS sizes. Trials for all the phases of the study were presented in quasi-random order with the restriction that no more than two stimuli of the same class appeared consecutively. Furthermore, to ensure an even distribution of trial types, the trials were arranged into two and six blocks for acquisition and generalization phases, respectively. In addition, an equal number of each trial type was used for the recording of psychophysiological measures (recorded in odd trials) and risk ratings (recorded in even trials). ITI trials were intermixed with CS and GS trials across the experimental session (six in pre-acquisition, 12 in acquisition and generalization). In half of the ITI trials, startle probes were also presented. Following Lissek et al. (2008), prior to analyses, responses to every two sizes of GSs were averaged in four classes of responses to GSs (class 1, class 2, class 3, and class 4). There was a 10 min break between the acquisition and generalization. After the experiment, participants rated the discomfort produced both by the US and the startle probe on a 1 (no discomfort) to 10 (maximum discomfort) scale; and answered a multiple-choice question (based on Dawson & Reardon, 1973) regarding contingency awareness ("The electric stimulus usually appeared: (a) in the presence of the smallest ring; (b) in the presence of the biggest ring; (c) randomly; (d) I don't know"). Individuals who correctly identified the stimulus that co-occurred with the US were considered contingency-aware.

2.3. Physiological recordings

Physiological responses were recorded using a Biopac 150 polygraph (Biopac Systems, Inc). The startle blink response was measured by recording the electromyographic activity (EMG) of the orbicularis oculi, using two 0.5 cm Ag/AgCl surface electrodes and following standard guidelines (Blumenthal et al., 2005). Impedance level was maintained below 5 kΩ. The raw EMG signal was sampled at a rate of 2000 Hz, filtered to reduce power line noise (analog 50 Hz notch filter) and to attenuate the frequencies beyond the EMG spectrum (infinite impulse response band-pass filter, cut-off frequencies of 28 and 500 Hz), and then rectified and smoothed off-line

(10 ms moving window average), using the AcqKnowledge v.3.9.0 software (Biopac Systems, Inc).

Skin conductance was recorded from the distal phalanges of the index and the middle left-hand fingers by means of two Ag/AgCl electrodes filled with electrolyte. The GSR100C module (Biopac Systems) was used to provide a constant voltage of 0.5 V and to amplify the recorded signal. The signal was sampled at a rate of 125 Hz.

2.4. Data reduction and response definition

Scorers were blind to the stimuli presented.

The onset latency window for the startle response was 20–100 ms and the peak magnitude was determined within 150 ms of response onset. Startle amplitudes were computed in microvolts (μV) as the difference between the EMG value at response peak and the average EMG during the baseline period (50 ms preceding startle probe onset). In those trials in which no response was detected, amplitude was scored as 0 μV. After visual inspection, trials with excessive baseline activity were rejected [3.34% in the low, 3.10% in the medium, and 4.23% in the high anxiety group; $\chi^2(2) = 5.41$, $p > .05$]. Prior to statistical analysis, startle responses of each participant were T-transformed.

SCRs to the CSs were analyzed for half of the trials (those where risk ratings were not obtained). SCR magnitudes in microsiemens (μS) were computed as the difference between the maximum SCR value occurring 1–5 s after CS onset and the value at response onset.[1] Trials in which no response could be detected or with a response magnitude of <0.02 μS were considered non-response trials. SCRs were visually inspected to ensure that responses were related to stimulus presentation and were not due to artifacts (defined as fluctuations in the skin conductance level occurring prior to stimulus onset which could interfere with the detection of a response). Trials showing artifacts were rejected [10.66% in the low, 9.10% in the medium, and 7.52% in the high anxiety group; $\chi^2(2) = 10.00$, $p < .01$. The percentage of rejected trials across CSs and GS classes ranged between 8.39% and 10.60%; $\chi^2(5) = 3.53$, $p > .05$]. SCR data were square-root transformed to normalize the distribution.

2.5. Data analysis

One-hundred and twenty-six individuals participated in the fear conditioning paradigm, of which 114 also took part in the generalization paradigm.[2] Four participants were excluded from the analyses of generalization because of technical problems. Additionally, participants were excluded from the analyses of the FPS or SCR in a particular experimental phase if all the trials for one type of stimulus in a given block were rejected [$n = 1$ (FPS) and $n = 2$ (SCR) during acquisition; $n = 2$ (FPS) and $n = 5$ (SCR) during generalization]. Participants were excluded from the analyses of online risk ratings in a particular experimental phase if they reported the same value throughout the phase or due to technical problems ($n = 2$ during acquisition and $n = 2$ during generalization). Finally, participants were excluded from SCR analyses if they did not show any response ($n = 9$ during acquisition and $n = 5$ during generalization).

Data were analyzed separately for each experimental phase (pre-acquisition, acquisition, and generalization) and for each measure (FPS, SCR, and risk ratings), using repeated-measures ANOVAs (GLM procedure). In pre-acquisition and acquisition, stimulus (ITI, CS− and CS+ for FPS; CS− and CS+ for SCR and risk ratings) and anxiety group (low, medium, and high) were included as within- and between-subjects factors, respectively. Generalization included stimulus (CS−, class 1, class 2, class 3, class 4, and CS+) as within-subjects, and anxiety group (low, medium, and high) as between-subjects factors. Ring size (larger ring as the CS+ and smaller ring as the CS+) was initially included as a between-subjects factor in all the analyses, but was maintained only in those analyses in which it had a significant effect. Gender was excluded from the final analyses because it did not interact with the other factors, indicating that participants' gender did not affect fear acquisition or generalization. FPS was analyzed using T-scores as well as raw data. Because similar results were obtained, only T-scores are presented. For main effects and interactions involving repeated measures, Greenhouse–Geisser's ε is reported together with the uncorrected degrees of freedom. We report η_p^2 as a measure of effect size for main and interaction effects. Data were analyzed with SPSS 13.0.

3. Results

Preliminary analyses showed that there were no differences between groups regarding awareness of the CS-US contingency, selected intensity of the US, and ratings of the discomfort produced by the shock or the startle probe (Table 1). In addition, groups did not differ in overall startle reactivity, $F(2, 105) = 2.75$, $p > .05$, $\eta_p^2 = .05$, or in startle habituation across the experimental session, $F(6, 315) = 1.05$, $p > .05$, $\eta_p^2 = .02$; Greenhouse–Geisser $\varepsilon = .80$. This was assessed by a repeated-measures ANOVA of startle responses during ITI trials (raw data). Factors were phase/block (pre-acquisition, first block of acquisition, second block of acquisition, and generalization) and anxiety group (low, medium, and high).

3.1. Pre-acquisition

Although during pre-acquisition, participants displayed overall greater responses to the CS− than to the CS+, both for startle [$F(1, 122) = 10.24$, $p < .01$, $\eta_p^2 = .08$] and skin conductance data [$F(1, 112) = 7.07$, $p < .01$, $\eta_p^2 = .06$], this difference disappeared for the last pre-acquisition trial of each CS [FPS: $F(1, 115) = 1.85$, $p > .05$, $\eta_p^2 = .02$; SCR: $F(1, 98) = 1.35$, $p > .05$, $\eta_p^2 = .01$]. This effect is possibly related to the order in which the stimuli were presented, as in pre-acquisition, CS− generally came before CS+. Risk ratings showed the opposite pattern, being greater for the CS+ than for the CS− also during the last trial. A significant Stimulus × Ring size interaction revealed that participants from the group in which the large ring served as the CS+ reported greater risk to the CS+ than to the CS− ($M = 1.78$, SD = 0.60; vs. $M = 1.36$, SD = 0.45), $t(63) = 5.53$, $p < .001$. In contrast, participants from the group in which the small ring served as the CS+ did not show those differences (CS+: $M = 1.68$, SD = 0.56; vs. CS−: $M = 1.65$, SD = 0.66), $t(59) = 0.39$, $p > .05$ (note that during pre-acquisition, the US was not presented). However, and more importantly, the Stimulus × Group interaction was not significant for any of the three measures, indicating that the three anxiety groups did not differ from each other before the acquisition phase started.

3.2. Acquisition

3.2.1. FPS

A main effect of stimulus was found, $F(2, 244) = 21.92$, $p < .001$, $\eta_p^2 = .15$. Follow-up contrasts revealed that all participants showed differential conditioning across acquisition, with greater startle responses during the presentation of the CS+ ($M = 52.31$, SD = 4.42) than during the presentation of the CS− ($M = 50.24$, SD = 3.67), $t(1, 123) = 4.17$, $p < .001$. Relative to ITIs ($M = 49.13$, SD = 4.13), startle responses were potentiated both by the CS+, $t(123) = 6.49$, $p < .001$, and the CS−, $t(123) = 2.32$, $p < .05$. However, the Stimulus × Group interaction was not significant, indicating that differential conditioning was similar across anxiety groups (see Fig. 1, top panel).

3.2.2. SCR

As with FPS, the whole sample acquired differential conditioning, with greater SCRs to the CS+ ($M = 0.24$, SD = 0.20) than to the CS− ($M = 0.14$, SD = 0.12), $F(1, 112) = 37.51$, $p < .001$, $\eta_p^2 = .25$. The absence of any significant effect involving an interaction of Stimulus × Group indicated that differential conditioning was similar for all anxiety groups (see Fig. 1, middle panel).

3.2.3. Risk ratings

The whole sample reported greater risk to the CS+ ($M = 2.52$, SD = 0.49) than to the CS− ($M = 1.52$, SD = 0.44), $F(1, 118) = 257.07$, $p < .001$, $\eta_p^2 = .69$. Despite a significant Stimulus × Group interaction [$F(2, 118) = 5.20$, $p < .01$, $\eta_p^2 = .08$], post hoc analyses revealed that again all three groups clearly discriminated between CSs (all $ts > 7.47$, $ps < .001$). The Stimulus × Group interaction was significant because participants in the low anxiety group reported lower risk to the CS− than participants in the medium anxiety group [$M = 1.35$, SD = 0.36; vs. $M = 1.64$, SD = 0.48; $t(83) = 3.15$, $p < .01$], not

[1] Given concerns that SCRs to the startle probe – which was presented 4 s after CS onset – could have affected the SCRs of interest, all the analyses were repeated reducing the time window to 1–4 s after CS onset. The results remained unchanged.

[2] The rest of the participants ($n = 12$) followed a slightly different procedure during generalization and were therefore not included in this current study.

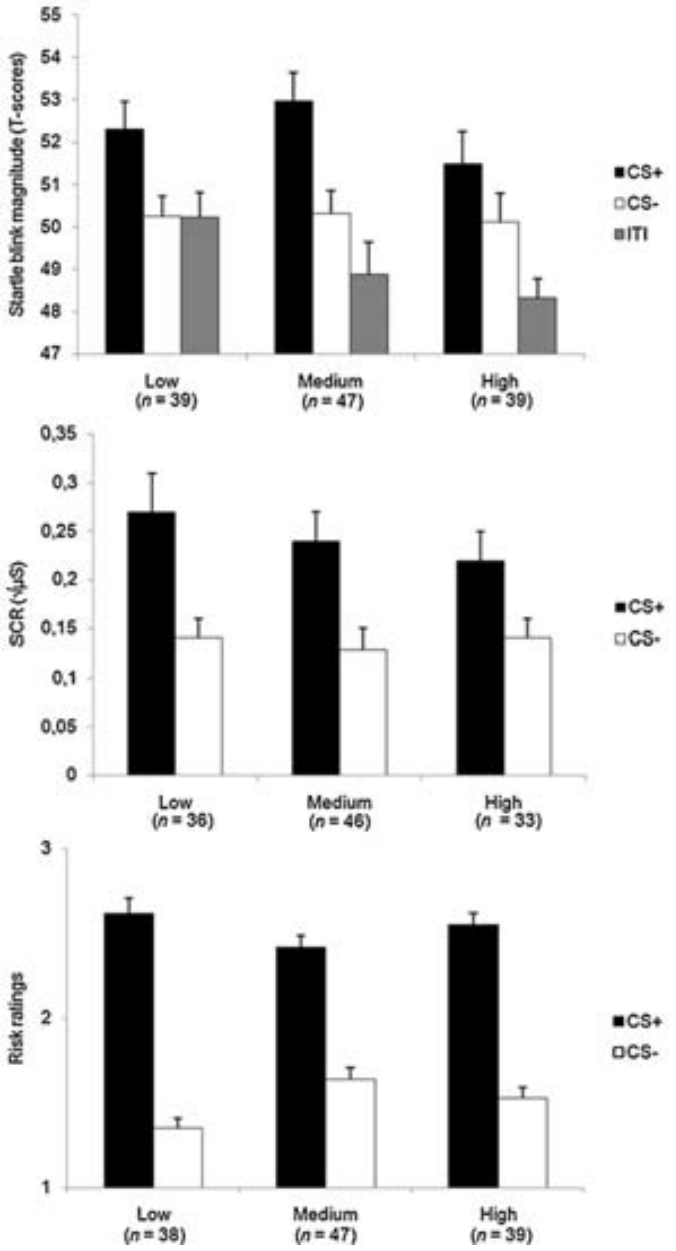

Fig. 1. Responses to each CS by anxiety group, at acquisition. Bars represent standard errors of the mean.

because of differences between groups in CS+ or differential (CS+ vs. CS−) responding.

3.3. Generalization

3.3.1. FPS

For the whole sample, startle responses varied as a function of the stimuli presented during the generalization test, $F(5, 525) = 16.12$, $p < .001$, $\eta_p^2 = 13$; Greenhouse–Geisser $\varepsilon = .90$. Generalization of FPS across stimuli was analyzed by comparing responses elicited during the presentation of the CS- with responses elicited during each GS (Lissek et al., 2008), with α adjusted by means of a Bonferroni correction ($p < .05/5 = .01$). These analyses showed that startle responses elicited during the presentation of the CS+ were potentiated in comparison to responses elicited during the CS−, $t(107) = 4.76$, $p < .001$. Responses elicited during the class 4 and class 3 GSs were also potentiated in relation to those elicited during the CS−, both $ts(107) > 3.30$, $ps \leq .001$. In contrast, responses elicited during the presentation of the class 2 and class 1 GSs did not differ from responses elicited during the CS−, both $ts(107) < 2.07$, $ps > .01$. Thus, conditioned fear, as measured by startle potentiation, extended to the class 4 and class 3 GSs. In addition, the pattern of the generalization gradient did not differ across anxiety groups, as indicated by a non-significant Stimulus × Group interaction (see Fig. 2, left panel).

3.3.2. SCR

As occurred with FPS, SCR varied depending on the type of stimulus presented, $F(5, 480) = 9.87$, $p < .001$, $\eta_p^2 = 09$; Greenhouse–Geisser $\varepsilon = .68$. Comparisons of SCRs to each stimulus with SCRs to the CS− (α set at $p < .01$) revealed that SCRs to the CS+ were greater than SCRs to the CS−, $t(98) = 2.82$, $p < .01$, and that SCRs to the class 4 GS were also greater than SCRs to the CS−, $t(98) = 3.96$, $p < .001$. However, SCRs to the class 3, class 2, and class 1 GSs did not differ from SCRs to the CS-, all $ts < 2.13$, $ps > .01$. Thus, differential responding, as measured by SCR, extended only to the class 4 GS. Again, the pattern of responses as a function of the stimuli was not different across anxiety groups, as indicated by a non-significant Stimulus × Group interaction (see Fig. 2, middle panel).

3.3.3. Risk ratings

As occurred with the other measures, participants' responses varied as a function of the stimuli presented, $F(5, 525) = 210.80$, $p < .001$, $\eta_p^2 = .67$; Greenhouse–Geisser $\varepsilon = .45$. Comparisons of risk ratings to each stimulus with ratings to the CS− (α set at $p < .01$) revealed that participants reported greater risk ratings to the CS+ than to the CS−, $t(107) = 18.17$, $p < .001$, and that risk ratings to the class 4, class 3, and class 2 GSs were also greater than ratings to the CS−, all $ts(107) > 4.39$, $ps < .001$. Risk ratings to the class 1 GS were the only ones that did not differ from ratings to the CS−, $t(107) = 0.59$, $p > .05$. In addition, the Stimulus × Group interaction was also significant, $F(10, 525) = 2.90$, $p < .05$, $\eta_p^2 = .05$; Greenhouse–Geisser $\varepsilon = .45$. However, trend analyses revealed no differences across anxiety groups in the patterns of the generalization gradients, with all three groups showing significant linear and quadratic trends, all $Fs > 13.44$, all $ps < .001$. Rather, this interaction was attributable to the fact that participants with low anxiety reported lower risk to the CS−, and to the class 1 and class 2 GSs, than the other participants. Comparisons of the ratings reported by each anxiety group to each type of stimulus (Bonferroni-corrected α set at $p < .05/3 = .017$) showed that participants with low anxiety reported lower risk to the CS− and to the class 2 GS than participants with high anxiety, both $ts > 2.45$, $ps < .017$. They also reported lower risk to the class 1 and class 2 GSs than participants with medium anxiety, both $ts > 2.49$, $ps < .017$ (see Fig. 2, right panel). Finally, there was a main effect of group, $F(2, 105) = 3.30$, $p < .05$, $\eta_p^2 = .06$, with participants with low anxiety reporting overall lower levels of risk than participants with high anxiety, $t(73) = 2.52$, $p < .05$.[3]

4. Discussion

The present study aimed to examine the role of trait anxiety in the acquisition and generalization of fear. We expected individuals with elevated trait anxiety to exhibit abnormal fear conditioning (specifically, inability to suppress fear responses to CS−). Our

[3] All the analyses were repeated excluding contingency-unaware participants. The results remained unchanged except for the Stimulus × Group interaction observed in risk ratings of generalization, which now became only marginally significant ($p = .08$).

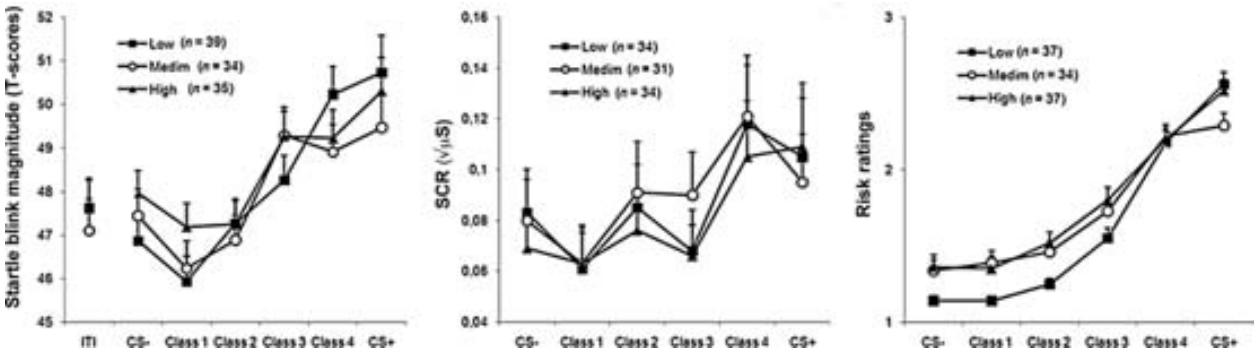

Fig. 2. Generalization gradients by anxiety group. Bars represent standard errors of the mean.

results, based on a rather large sample ($n \geq 39$ per group) and using three different indices of fear, do not support this idea.

Previous work has shown that anxiety may play a role in fear acquisition both in clinical (see Lissek et al., 2005) and non-clinical samples. In non-clinical samples, trait anxiety (or similar constructs such as neuroticism or harm avoidance) has been associated with fear acquisition in studies using different experimental paradigms, including conditioned inhibition (Chan & Lovibond, 1996; Grillon & Ameli, 2001), context conditioning (Baas, 2012), and a blocking procedure (Boddez et al., 2012). However, there are at least three previous studies that found no effect of trait anxiety in fear acquisition (Fredrikson & Georgiades, 1992; Otto et al., 2007; Pineles et al., 2009). These studies used a differential conditioning paradigm, clear unambiguous CSs (geometric shapes), and a high reinforcement rate (100%) and are therefore methodologically closer to ours.

A careful review of the previous literature suggests that studies that found trait anxiety effects used relatively ambiguous conditioning tasks with rather complex CSs, low reinforcement rates, and/or without clear experimental instructions about stimulus contingencies (see also Beckers et al., 2012). One interesting possibility is that this ambiguity may foster the emergence of threat-related cognitive biases in participants with high trait anxiety, which in turn could modulate trait anxiety effects on fear conditioning.

Another variable that may play a role in explaining previous research inconsistencies is contingency awareness. Previous studies have found a higher (or marginally higher) number of unaware participants among individuals with high as compared to low trait anxiety (Chan & Lovibond, 1996; Baas et al., 2008; Grillon, 2002). Furthermore, lack of contingency awareness has been deemed important in some accounts of pathological anxiety, because it is associated with increased subjective and physiological signs of anxiety (Baas et al., 2008) as well as increased behavioral avoidance (Grillon, 2002). However, although these studies found a relationship between contingency awareness and trait anxiety, they generally failed to demonstrate a direct effect of trait anxiety on other fear conditioning outcomes. It must be noted, however, that there is evidence of fear conditioning abnormalities (specifically, deficits in fear inhibition) among individuals with PTSD, despite the fact that they are most often aware of the CS-US contingency (Grillon & Morgan, 1999; Jovanovic et al., 2009).

We also examined the role of trait anxiety on fear generalization. The characteristics of the generalization task, with intermediate generalization stimuli that were perceptually similar to both the CS+ and the CS−, might have created a particular ambiguous situation regarding the prediction of threat (Britton et al., 2010). According to the above-mentioned studies, individual differences in fear-responding as a function of trait anxiety should have emerged in such an ambiguous situation, at least at a cognitive level. However, this was not the case, and the hypothesized greater fear generalization for the high trait anxiety group was not found.

To explain this apparent contradiction, we suggest that the successful acquisition of differential conditioning by all three anxiety groups produced subsequent similar gradients of fear generalization, regardless of trait anxiety. In other words, as all our participants acquired a differential response to the CS+ and CS−, the generalization pattern was similar. This would be in line with accumulated evidence indicating that generalization gradients are basically determined by the perceptual properties of the generalization stimuli and the previous differential learning (Ghirlanda and Enquist, 2003; Vervliet et al., 2011).

However, in the aforementioned study by Lissek et al. (2010), PD patients showed greater fear generalization than healthy controls despite similar fear acquisition.

This may indicate that maladaptive generalization of fear is specific to individuals suffering from panic disorder, as suggested by Lissek et al. (2010).

Finally, two other results deserve brief consideration. First, participants with low anxiety reported lower levels of perceived risk to the CS−, and the GSs more similar to the CS−, than the other participants. Nonetheless, these effects did not translate into different patterns of differential conditioning or generalization. Second, participants reported greater risk when the CS+ was represented by the larger ring. This may be interpreted as evidence of a proneness to associate danger with larger stimuli. In fact, animal studies of generalization have reported biases toward greater responding to stimuli that are larger than the CS+ (Ghirlanda and Enquist, 2003).

In conclusion, our results indicate that individuals with high trait anxiety do not show the abnormalities in the acquisition and generalization of fear typical of some clinical populations. This may suggest that these abnormalities might be acquired as a result of pathological processes rather than being a predisposing vulnerability mechanism. Future studies should examine the potential effects of other factors that characterize individuals with high trait anxiety, such as treat-related cognitive biases, in the development of both pathological anxiety and abnormalities in fear conditioning.

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References

American Psychiatric Association, 2000. Diagnostic and Statistical Manual of Mental Disorders, 4th ed., text rev. ed. American Psychiatric Press, Washington, DC.

Baas, J.M.P., 2012. Individual differences in predicting aversive events and modulating contextual anxiety in a context and cue conditioning paradigm. Biological

Psychology, http://dx.doi.org/10.1016/j.biopsycho.2012.02.001, Advance online publication.
Baas, J.M.P., van Ooijen, L., Goudriaan, A., Kenemans, J.L., 2008. Failure to condition to a cue is associated with sustained contextual fear. Acta Psychologica 127, 581–592.
Beckers, T., Krypotos, A.M., Boddez, Y., Effting, M., Kindt, M., 2012. What's wrong with fear conditioning? Biological Psychology, http://dx.doi.org/10.1016/j.biopsycho.2011.12.015, Advance online publication.
Blumenthal, T.D., Cuthbert, B.N., Filion, D.L., Hackley, S., Lipp, O.V., Van Boxtel, A., 2005. Committee report: guidelines for human startle eyeblink electromyographic studies. Psychophysiology 42, 1–15.
Boddez, Y., Vervliet, B., Baeyens, F., Lauwers, S., Hermans, D., Beckers, T., 2012. Expectancy bias in a selective conditioning procedure: trait anxiety increases the threat value of a blocked stimulus. Journal of Behavior Therapy and Experimental Psychiatry 43, 832–837.
Britton, J.C., Lissek, S., Grillon, C., Norcross, M.A., Pine, D.S., 2010. Development of anxiety: the role of threat appraisal and fear learning. Depression and Anxiety 20, 5–17.
Chambers, J.A., Power, K.G., Durhama, R.C., 2004. The relationship between trait vulnerability and anxiety and depressive diagnoses at long-term follow-up of generalized anxiety disorder. Journal of Anxiety Disorders 18, 587–607.
Chan, C.K.Y., Lovibond, P.F., 1996. Expectancy bias in trait anxiety. Journal of Abnormal Psychology 105, 637–647.
Craske, M.G., Rauch, S.L., Ursano, R., Prenoveau, J., Pine, D.S., Zinbarg, R.E., 2009. What is an anxiety disorder? Depression and Anxiety 26, 1066–1085.
Davis, M., Falls, W.A., Gewirtz, J., 2000. Neural systems involved in fear inhibition: extinction and conditioned inhibition. In: Myslobodsky, M.S., Weiner, I. (Eds.), Contemporary Issues in Modeling of Psychopathology. , 1st ed. Kluwer Academic Publishers, Boston, pp. 113–142.
Dawson, M.E., Reardon, P., 1973. Construct validity of recall and recognition post-conditioning measures of awareness. Journal of Experimental Psychology 98, 308–315.
Dunsmoor, J.E., Prince, S.E., Murty, V.P., Kargel, P.A., LaBar, K.S., 2011. Neurobehavioral mechanisms of human fear generalization. Neuroimage 55, 1878–1888.
Fredrikson, M., Georgiades, A., 1992. Personality dimensions and classical conditioning of autonomic nervous system reactions. Personality and Individual Differences 13, 1013–1020.
Ghirlanda, S., Enquist, M., 2003. A century of generalization. Animal Behaviour 66, 15–36.
Grillon, C., 2002. Associative learning deficits increase symptoms of anxiety in humans. Biological Psychiatry 51, 851–858.
Grillon, C., Ameli, R., 2001. Conditioned inhibition of fear-potentiated startle and skin conductance in humans. Psychophysiology 38, 807–815.
Grillon, C., Baas, J., 2003. A review of the modulation of the startle reflex by affective states and its application in psychiatry. Clinical Neurophysiology 114, 1557–1579.
Grillon, C., Morgan III, C.A., 1999. Fear-potentiated startle conditioning to explicit and contextual cues in Gulf War veterans with posttraumatic stress disorder. Journal of Abnormal Psychology 108, 134–142.
Hettema, J.M., Neale, M.C., Myers, J.M., Prescott, C.A., Kendler, K.S., 2006. A population-based twin study of the relationship between neuroticism and internalizing disorders. American Journal of Psychiatry 163, 857–864.
Indovina, I., Robbins, T.W., Núñez-Elizalde, A.O., Dunn, B.D., Bishop, S.J., 2011. Fear-conditioning mechanisms associated with trait vulnerability to anxiety in humans. Neuron 69, 563–571.
Jovanovic, T., Norrholm, S.D., Blanding, N.Q., Davis, M., Duncan, E., Bradley, B., et al., 2010. Impaired fear inhibition is a biomarker of PTSD but not depression. Depression and Anxiety 27, 244–251.
Jovanovic, T., Norrholm, S.D., Fennell, J.E., Keyes, M., Fiallos, A.M., Myers, K.M., et al., 2009. Posttraumatic stress disorder may be associated with impaired fear inhibition: relation to symptom severity. Psychiatry Research 167, 151–160.
Kindt, M., Soeter, M., Vervliet, B., 2009. Beyond extinction: erasing human fear responses and preventing the return of fear. Nature Neuroscience 12, 256–258.
Lissek, S., Biggs, A.L., Rabin, S.J., Cornwell, B.R., Alvarez, R.P., Pine, D.S., et al., 2008. Generalization of conditioned fear-potentiated startle in humans: experimental validation and clinical relevance. Behaviour Research and Therapy 46, 678–687.
Lissek, S., Powers, A.S., McClure, E.B., Phelps, E.A., Woldehawariat, G., Grillon, C., et al., 2005. Classical fear conditioning in the anxiety disorders: a meta-analysis. Behaviour Research and Therapy 43, 1391–1424.
Lissek, S., Rabin, S.J., McDowell, D.J., Dvir, S., Bradford, D.E., Geraci, M., et al., 2009. Impaired discriminative fear-conditioning resulting from elevated fear responding to learned safety cues among individuals with panic disorder. Behaviour Research and Therapy 47, 111–118.
Lissek, S., Rabin, S., Heller, R.E., Lukenbaugh, D., Geraci, M., Pine, D.S., et al., 2010. Over-generalization of conditioned fear as a pathogenic marker of panic disorder. American Journal of Psychiatry 167, 47–55.
Merikangas, K.R., Swanson, S.A., 2010. Comorbidity in anxiety disorders. In: Stein, M.B., Steckler, T. (Eds.), Behavioral Neurobiology of Anxiety and its Treatment. , 1st ed. Springer-Verlag, Berlin, pp. 37–59.
Otto, M.W., Leyro, T.M., Christian, K., Deveney, C.M., Reese, H., Pollack, M.H., et al., 2007. Prediction of fear acquisition in healthy control participants in a de novo fear-conditioning paradigm. Behavior Modification 31, 32–51.
Pineles, S.L., Vogt, D.S., Orr, S.P., 2009. Personality and fear responses during conditioning: beyond extraversion. Personality and Individual Differences 46, 43–53.
Spielberger, C.D., Gorsuch, R.R., Lushene, R.E. (Eds.), 1982. STAI. Cuestionario de ansiedad Estado/Rasgo [STAI. State/trait anxiety questionnaire] (. , 1st ed. Técnicos Especialistas Asociados (TEA), Madrid.
Vervliet, B., Iberico, C., Vervoort, E., Baeyens, F., 2011. Generalization gradients in human predictive learning: effects of discrimination training and within-subjects testing. Learning and Motivation 42, 210–220.

IV. General discussion

The present Ph D dissertation is framed within the fear-conditioning model of pathological anxiety. According to this model, clinical anxiety emerges from abnormal fear-conditioning processes and other related diathesis-stress factors (Mineka & Zinbarg, 2006). This has been supported by studies showing abnormal fear conditioning in individuals diagnosed with anxiety disorders (Lissek et al., 2005), but evidence of these abnormalities in individuals at risk for clinical anxiety is rather scarce (Beckers, Krypotos, Boddez, Effting, & Kindt, 2013). Thus, we examined individual differences in the acquisition and generalization of conditioned fear in healthy participants characterized by different levels of susceptibility to pathological anxiety. Two sources of variability in vulnerability to anxiety were investigated: allele variation in the BDNF-val66met polymorphism (study 1) and trait anxiety (study 2).

Despite the important body of research conducted recently on the relationship between the BDNF-val66met polymorphism and pathological anxiety, it is far from clear which of the polymorphism alleles is associated with increased risk for anxiety disorders (Frustaci, Pozzi, Gianfagna, Manzoli, & Boccia, 2008; Lonsdorf & Kalish, 2011). Thus, we expected that variation in BDNF-val66met polymorphism would lead to differences in the acquisition and generalization of fear, but we did not have any hypothesis about the direction of this association. On the other hand, and given that high trait anxiety represents an established risk factor for pathological anxiety (Clark, Watson, & Mineka, 1994; Hettema, Neale, Myers, Prescott, & Kendler, 2006; Jorm et al., 2000), we expected that individuals with high trait anxiety would show abnormal fear acquisition, mainly driven by impaired fear inhibition to the safety signal or CS-, and overgeneralization of fear. Contrary to our expectations, we did not find any effect of the BNDF-val66met polymorphism or trait anxiety on the acquisition and generalization of fear.

For the three measures (startle reflex, skin conductance response, and online ratings of US expectancies), participants from the two studies exhibited differential conditioning during acquisition and the normal generalization gradient, with a gradual decrease in fear as the GSs were less similar to the CS+. Note that the samples from studies 1 and 2 partially overlapped (i.e., data from 120 participants were included in the sample of the two studies; *n* study 1 = 141, and *n* study 2 = 126). Therefore, it is not surprising that the experimental paradigm yielded similar results in the overall sample of both studies.

As already discussed in study 1, and considering the three previous reports on the association between the BDNF-val66met polymorphism and fear conditioning, our null results are not particularly surprising. Hajcak et al. (2009) and Lonsdorf et al. (2010) found impaired differential conditioning in met-carriers, but not in val-homozygous participants. However, the number of met-carriers in both studies was rather small (n = 13 and n = 9, in the former and the later study, respectively). Moreover, Soliman et al. (2010), with a larger subsample of met-carriers (n = 36), reported differential conditioning in both the val-homozygous and the met-carrier participants. In addition, Hajcak et al. (2009), who also examined generalization, found similar fear responses to the GSs in both allele groups.

In the discussion of the second paper, we reviewed studies that also reported no effect of trait anxiety on fear conditioning (Fredrikson & Georgiades, 1992; Otto et al., 2007; Pineles et al., 2009) as well as studies that did find positive results (Baas, 2013; Boddez et al., 2012; Chan & Lovibond, 1996; Grillon & Ameli, 2001). The experimental tasks in this latter group of studies possibly represented more threat-ambiguous situations, thereby fostering the emergence of the threat-related cognitive biases that characterize participants with high trait anxiety (Bishop, 2007). Two studies published after study 2 was submitted for publication also found effects of trait anxiety on fear conditioning. Gazendam, Kamphuis, and Kindt (2013) reported that participants with high trait anxiety showed deficits in fear inhibition to a CS- during acquisition, as well as during 24-h-delayed extinction and reextinction. However, the results

concerning extinction and reextinction were based on planned comparisons and *near significant* interaction effects of the trait-anxiety factor. On the other hand, Haddad, Pritchett, Lissek, and Lau (2012) found that participants with high trait anxiety showed similar fear responses to a CS+ and a CS- that was perceptually similar to the CS+. These authors concluded that this constituted evidence of exaggerated fear generalization in individuals with high trait anxiety. However, these effects were limited to a particular block of acquisition, and were not observed during extinction. In addition, separate analyses by trait-anxiety group were conducted despite the fact that trait-anxiety did not significantly interact with the other factors included in the analysis.

All in all, part of the previous research on the influence of trait anxiety on fear conditioning is in accordance with our results. Moreover, the effects reported in the trait-anxiety studies that found positive results do not appear to emerge as ‘easily’ as the effects observed in individuals with clinical anxiety (Lissek et al., 2005). This may suggest that the occurrence of an important stressful event, that usually precedes the onset of clinical symptoms and possibly triggers a cascade of dysfunctional neuroendocrine patterns in vulnerable individuals (Sandi & Richter-Levin, 2009), may be necessary for the emergence of severe abnormalities in fear conditioning.

Finally, as additional material, we include an unpublished study on the temporal stability of individual differences in the acquisition and generalization of fear (see the appendix). Our estimates suggest that a moderate part of the variability in these processes is influenced by stable individual characteristics. Our sample consisted of healthy participants, and therefore we observed consistent individual variation in the acquisition and generalization of fear at premorbid level.

V. Conclusions

Based on our results, we conclude that:

- The BDNF-val66met polymorphism is not associated with abnormal acquisition or generalization of conditioned fear.

- High trait anxiety is not associated with deficits in the acquisition of differential fear conditioning or increased fear generalization.

- Individual differences in the acquisition and generalization of fear show modest temporal stability.

- Rather than a preexisting diathesis for pathological anxiety, abnormal acquisition and generalization of fear may be the result of other pathogenic factors that lead to the onset of an anxiety disorder.

VI. References of the introduction and general discussion

Baas, J. M. P. (2013). Individual differences in predicting aversive events and modulating contextual anxiety in a context and cue conditioning paradigm. *Biological Psychology,92*, 17-25.

Bados, A., Gómez-Benito, J., & Balaguer, G. (2010). The state-trait anxiety inventory, trait version: Does it really measure anxiety? *Journal of Personality Assessment, 92*, 560-567.

Barrett, P. M., Rapee, R. M., Dadds, M. M., & Ryan, S. M. (1996). Family enhancement of cognitive style in anxious and aggressive children. *Journal of Abnormal Child Psychology, 24*, 187-203.

Beckers, T., Krypotos, A. M., Boddez, Y., Effting, M., & Kindt, M. (2013). What's wrong with fear conditioning? *Biological Psychology,92*, 90-96.

Bishop, S. J. (2007). Neurocognitive mechanisms of anxiety: An integrative account. *Trends in Cognitive Sciences, 11*, 307-316.

Blanchard, D. C., Hynd, A. L., Minke, K. A., Minemoto, T., & Blanchard, R. J. (2001). Human defensive behaviors to threat scenarios show parallels to fear- and anxiety-related defense patterns of non-human mammals. *Neuroscience and Behavioral Reviews, 25*, 761-770.

Blechert, J., Michael, T., Vriends, N., Margraf, J., & Wilhelm, F. H. (2007). Fear conditioning in posttraumatic stress disorder: Evidence for delayed extinction of autonomic, experiential, and behavioural responses. *Behaviour Research and Therapy, 45*, 2019-2033.

Boddez, Y., Vervliet, B., Baeyens, F., Lauwers, S., Hermans, D., & Beckers, T. (2012). Expectancy bias in a selective conditioning procedure: Trait anxiety increases the threat value of a blocked stimulus. *Journal of Behavior Therapy and Experimental Psychiatry, 43*, 832-837.

Bouton, M. E. (2002). Context, ambiguity, and unlearning: Sources of relapse after behavioral extinction. *Biological Psychiatry, 52*, 976-986.

Bouton, M. E., Mineka, S., & Barlow, D. H. (2001). A modern learning theory perspective on the etiology of panic disorder. *Psychological Review, 108*, 4-32.

Bruch, M. A., & Heimberg, R. G. (1994). Differences in perceptions of parental and personal characteristics between generalized and nongeneralized social phobics. *Journal of Anxiety Disorders, 8*, 155-168.

Chan, C. K. Y., & Lovibond, P. F. (1996). Expectancy bias in trait anxiety. *Journal of Abnormal Psychology, 105*, 637-647.

Clark, L. A., Watson, D., & Mineka, S. (1994). Temperament, personality, and the mood and anxiety disorders. *Journal of Abnormal Psychology, 103*, 103-116.

Craske, M. G., Rauch, S. L., Ursano, R., Prenoveau, J., Pine, D. S., & Zinbarg, R. E. (2009). What is an anxiety disorder? *Depression and Anxiety, 26*, 1066-1085.

Davis, M. (1998). Are different parts of the extended amygdala involved in fear versus anxiety? *Biological Psychiatry, 44*, 1239-1247.

Davis, M., Falls, W. A., & Gewirtz, J. (2000). Neural systems involved in fear inhibition: Extinction and conditioned inhibition. In M. S. Myslobodsky, & I. Weiner (Eds.),

Contemporary issues in modeling of psychopathology (First ed., pp. 113-142). Boston: Kluwer Academic Publishers.

Davis, M., Walker, D. L., Miles, L., & Grillon, C. (2010). Phasic vs sustained fear in rats and humans: Role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology, 35*, 105-135.

Dunsmoor, J. E., & LaBar, K. S. (2013). Effects of discrimination training on fear generalization gradients and perceptual classification in humans. *Behavioral Neuroscience.* Advanced online publication. doi: 10.1037/a0031933

Dunsmoor, J. E., Prince, S. E., Murty, V. P., Kargel, P. A., & LaBar, K. S. (2011). Neurobehavioral mechanisms of human fear generalization. *Neuroimage, 55*, 1878-1888.

Dunsmoor, J. E., White, A. J., & LaBar, K. S. (2011). Conceptual similarity promotes generalization of higher order fear learning. *Learning and Memory, 18*, 156-160.

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M., Lu, B., & Weinberger, D. R. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell, 112*, 257-269.

Ehlers, A. (1993). Somatic symptoms and panic attacks: A retrospective study of learning experiences. *Behavior Research and Therapy, 31*, 269-278.

Etkin, A., Klemenhagen, K. C., Dudman, J. T., Rogan, M. T., Hen, R., Kandel, E. R., & Hirsch, J. (2004). Individual differences in trait anxiety predict the response of the basolateral amygdala to unconsciously processed fearful faces. *Neuron, 44*, 1043-1055.

Fanselow, M. S. (1994). Neural organization of the defensive behavior system responsible for fear. *Psychonomic Bulletin and Review, 1*, 429-438.

Fredrikson, M., & Georgiades, A. (1992). Personality dimensions and classical conditioning of autonomic nervous system reactions. *Personality and Individual Differences, 13*, 1013-1020.

Frustaci, A., Pozzi, G., Gianfagna, F., Manzoli, L., & Boccia, S. (2008). Meta-analysis of the brain-derived neurotrophic factor gene (BDNF) Val66Met polymorphism in anxiety disorders and anxiety-related personality traits. *Neurophsychobiology, 50*, 163-170.

Gazendam, F. J., Kamphuis, J. H., & Kindt, M. (2013). Deficient safety learning characterizes high trait anxious individuals. *Biological Psychology, 92*, 342-352.

Gerull, F. C., & Rapee, R. M. (2002). Mother knows best: Effects of maternal modelling on the acquisition of fear and avoidance behaviour in toddlers. *Behavior Research and Therapy, 40*, 279-287.

Ghirlanda, S., & Enquist, M. (2003). A century of generalization. *Animal Behaviour, 66*, 15-36.

Grillon, C. (2002). Associative learning deficits increase symptoms of anxiety in humans. *Biological Psychiatry, 51*, 851-858.

Grillon, C., & Ameli, R. (2001). Conditioned inhibition of fear-potentiated startle and skin conductance in humans. *Psychophysiology, 38*, 807-815.

Grillon, C., & Davis, M. (1997). Fear-potentiated startle conditioning in humans: Explicit and contextual cue conditioning following paired versus unpaired training. *Psychophysiology, 34*, 451-458.

Grillon, C., & Morgan III, C. A. (1999). Fear-potentiated startle conditioning to explicit and contextual cues in gulf war veterans with posttraumatic stress disorder. *Journal of Abnormal Psychology, 108*, 134-142.

Guzmán, Y. F., Tronson, N. C., Guedea, A. L., Huh, K. H., Gao, C., & Radulovic, J. (2009). Social modeling of conditioned fear in mice by non-fearful conspecifics. *Behavioural Brain Research, 201*, 173-178.

Haddad, A. D. M., Pritchett, D., Lissek, S., & Lau, J. Y. F. (2012). Trait anxiety and fear responses to safety cues: Stimulus generalization or sensitization? *Journal of Psychopathology and Behavioral Assessment, 34*, 323-331.

Hajack, G., Castille, C., Olvet, D. M., Dunning, J. P., Roohi, J., & Hatchwell, E. (2009). Genetic variation in brain-derived neurotrophic factor and human fear conditioning. *Genes, Brain and Behavior, 8*, 80-85.

Hamm, A. O., & Weike, A. I. (2005). The neuropsychology of fear learning and fear regulation. *International Journal of Psychophysiology, 57*, 5-14.

Hettema, J. M., Annas, P., Neale, M. C., Kendler, K. S., & Fredrikson, M. (2003). A twin study of the genetics of fear conditioning. *Archives of General Psychiatry, 60*, 702-708.

Hettema, J. M., Neale, M. C., & Kendler, K. S. (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *American Journal of Psychiatry, 158*, 1568-1578.

Hettema, J. M., Neale, M. C., Myers, J. M., Prescott, C. A., & Kendler, K. S. (2006). A population-based twin study of the relationship between neuroticism and internalizing disorders. *American Journal of Psychiatry, 163*, 857-864.

Jorm, A. F., Christensen, H., Henderson, A. S., Jacomb, P. A., Korten, A. E., & Rodgers, B. (2000). Predicting anxiety and depression from personality: Is there a synergistic effect of neuroticism and extraversion. *Journal of Abnormal Psychology, 109,* 145-149.

Jovanovic, T., Keyes, M., Fiallos, A., Myers, K. M., Davis, M., & Duncan, E. J. (2005). Fear potentiation and fear inhibition in a human fear-potentiated startle paradigm. *Biological Psychiatry, 57*, 1559-1564.

Jovanovic, T., Norrholm, S. D., Fennell, J. E., Keyes, M., Fiallos, A. M., Myers, K. M., Davis, M., & Duncan, E. J. (2009). Posttraumatic stress disorder may be associated with impaired fear inhibition: Relation to symptom severity. *Psychiatry Research, 167*, 151-160.

Jovanovic, T., & Ressler, K. J. (2010). How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. *American Journal of Psychiatry, 167,* 648-662.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication. *Archives of General Psychiatry, 62,* 593-602.

Lissek, S., Biggs, A. L., Rabin, S. J., Cornwell, B. R., Alvarez, R. P., Pine, D. S., & Grillon, C. (2008). Generalization of conditioned fear-potentiated startle in humans: Experimental validation and clinical relevance. *Behaviour Research and Therapy, 46,* 678-687.

Lissek, S., Powers, A. S., McClure, E. B., Phelps, E. A., Woldehawariat, G., Grillon, C., & Pine, D. S. (2005). Classical fear conditioning in the anxiety disorders: A meta-analysis. *Behaviour Research and Therapy, 43*, 1391-1424.

Lissek, S., Rabin, S., Heller, R. E., Lukenbaugh, D., Geraci, M., Pine, D. S., & Grillon, C. (2010). Overgeneralization of conditioned fear as a pathogenic marker of panic disorder. *American Journal of Psychiatry, 167*, 47-55.

Lonsdorf, T. B., & Kalisch, R. (2011). A review on experimental and clinical genetic associations studies on fear conditioning, extinction and cognitive-behavioral treatment. *Translational Psychiatry, 1*, 1-13.

Lonsdorf, T. B., Weike, A. I., Golkar, A., Schalling, M., Hamm, A. O., & Öhman, A. (2010). Amygdala-dependent fear conditioning in humans is modulated by the BDNFval66met ploymorphism. *Behavioral Neuroscience, 124*, 9-15.

Michael, T., Blechert, J., Vriends, N., Margraf, J., & Wilhelm, F. H. (2007). Fear conditioning in panic disorder: Enhanced resistance to extinction. *Journal of Abnormal Psychology, 116*, 612-617.

Milad, M. R., Orr, S. P., Lasko, N. B., Chang, Y., Rauch, S. L., & Pitman, R. K. (2008). Presence and acquired origin of reduced recall for fear extinction in PTSD: Results of a twin study. *Journal of Psychiatric Research, 42*, 515-520.

Mineka, S., & Oehlberg, K. (2008). The relevance of recent developments in classical conditioning to understanding the etiology and maintenance of anxiety disorders. *Acta Psychologica, 127*, 567-580.

Mineka, S., & Zinbarg, R. (2006). A contemporary learning theory perspective on the etiology of anxiety disorders. it's not what you thought it was. *American Psychologist, 61*, 10-26.

Munafò, M. R., Freimer, N. B., Ng, W., Ophoff, R., Veijola, J., Miettunen, J., Järvelin, M. R., Taanila, A., & Flint, J. (2009). 5-HTTLPR genotype and anxiety-related personality

traits: A meta-analysis and new data. *American Journal of Medical Genetics. Part B Neuropsychiatric Genetics, 150*, 271-281.

Ormel, J., Rosmalen, J., & Farmer, A. (2004). Neuroticism: A non-informative marker of vulnerability to psychopathology. *Social Psychiatry and Psychiatric Epidemiology, 39*, 906-912.

Orr, S. P., Metzger, L. J., Lasko, N. B., Macklin, M. L., Peri, T., & Pitman, R. K. (2000). De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *Journal of Abnormal Psychology, 109*, 290-298.

Otto, M. W., Leyro, T. M., Christian, K., Deveney, C. M., Reese, H., Pollack, M. H., & Orr, S. P. (2007). Prediction of "fear" acquisition in healthy control participants in a de novo fear-conditioning paradigm. *Behavior Modification, 31*, 32-51.

Phelps, E. A., & LeDoux, J. E. (2005). Contributions of the amygdala to emotion processing: From animal models to human behavior. *Neuron, 48*, 175-187.

Pineles, S. L., Vogt, D. S., & Orr, S. P. (2009). Personality and fear responses during conditioning: Beyond extraversion. *Personality and Individual Differences, 46*, 43-53.

Portella, M. J., Harmer, C. J., Flint, J., Cowen, P., & Goodwin, G. M. (2005). Enhanced early morning salivary cortisol in neuroticism. *American Journal of Psychiatry, 162*, 807-809.

Rachman, S. (1977). The conditioning theory of fear-acquisition: A critical examination. *Behavior Research and Therapy, 15*, 375-387.

Rattiner, L. M., Davis, M., & Ressler, K. J. (2005). Brain-derived neurotrophic factor in amygdala-dependent learning. *The Neuroscientist, 11*, 323-333.

Sandi, C., & Richter-Levin, G. (2009). From high anxiety trait to depression: A neurocognitive hypothesis. *Trends in Neurosicences, 32*, 312-320.

Schiller, D., Monfils, M. H., Raio, C. M., Johnson, D. C., LeDoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans usings reconsolidation update mechanisms. *Nature, 463*, 49-53.

Sehlmeyer, C., Schöning, S., Zwitserlood, P., Pfleiderer, B., Kircher, T., Arolt, V., & Konrad, C. (2009). Human fear conditioning and extinction in neuroimaging: A systematic review. *Plos One, 4*, e5865.

Seligman, M. E., & Binik, Y. M. (1977). The safety signal hypothesis. In H. Davis, & H. M. B. Hurwitz (Eds.), *Operant-pavlovian interactions* (pp. 165-187). New York: Hillsdale.

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S. S., Jing, D., Tottenham, N., Amso, D., Somerville, L. H., Voss, H. U., Glover, G., Ballon, D. J., Liston, C., Teslovich, T., Van Kempen, T., Lee, FS., & Casey, B. J. (2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science, 327*, 863-866.

Spielberger, C. D., Gorsuch, R. R., & Lushene, R. E. (Eds.). (1982). *STAI. cuestionario de ansiedad Estado/Rasgo* (1st ed.). Madrid: Técnicos Especialistas Asociados (TEA).

Torrents-Rodas, D., Fullana, M. A., Arias, B., Bonillo, A., Caseras, X., Andión, O., Mitjans, M., Fañanás, L., & Torrubia, R. (2012). Acquisition and generalization of fear conditioning are not modulated by the BDNF-val66met polymorphism in humans. *Psychophysiology, 49*, 713-719.

Torrents-Rodas, D., Fullana, M. A., Bonillo, A., Caseras, X., Andión, O., & Torrubia, R. (2013). No effect of trait anxiety on differential fear conditioning or fear generalization. *Biological Psychology, 92*, 185-190.

Vervliet, B., Vansteenwegen, D., & Eelen, P. (2004). Generalization of extinguished skin conductance responding in human fear conditioning. *Learning and Memory, 11*, 555-558.

Watson, D. (2005). Rethinking the mood and anxiety disorders: A quantitative hierarchical model for DSM-V. *Journal of Abnormal Psychology, 114*, 522-536.

VII. Appendix.

Study submitted for publication: Temporal stability of individual differences in the acquisition and generalization of fear

Temporal stability of individual differences in the acquisition and generalization of fear

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Abstract

Abnormalities in the acquisition and generalization of fear have been suggested as being either a predisposing factor or a consequence of clinical anxiety. Although a prerequisite of these ideas is that individual differences in these processes should show temporal stability, no previous studies specifically address this question. Thus, 71 participants were tested twice on a fear-conditioning and fear-generalization paradigm (8 months between sessions). Half of the participants were presented with new stimuli in session 2. ANOVAs showed the expected patterns of acquisition and generalization, and no differences were seen between sessions or as a function of the stimuli. Generalizability coefficients showed modest stability of individual differences in these processes. However, some acquisition measures showed poor stability when the same stimuli were used again. The results are discussed in terms of methodological and applied implications.

Fear conditioning is an adaptive process by which an organism learns to predict aversive outcomes, thereby allowing the development of appropriate responses to situations that involve potential danger. In a typical fear-conditioning paradigm, a neutral stimulus (the CS+) is repeatedly paired with an innately fear-provoking stimulus (the unconditioned stimulus, US). After several pairings, the CS+ becomes capable of eliciting a fear response. In addition, novel stimuli that are similar to the CS+ may also produce some degree of fear. This phenomenon is known as fear generalization and allows the organism to react appropriately to other potentially dangerous situations.

Abnormalities in fear conditioning (e.g., greater fear responses to CS+ and/or impaired inhibition of fear to safety signals or CS-) have been associated with anxiety disorders (Lissek et al., 2005), and research based on fear conditioning has provided a valuable insight into the aetiology (Mineka & Oehlberg, 2008) and treatment (e.g., Kircher et al., 2012) of these disorders. In addition, over recent years, fear generalization has attracted the interest of researchers, as it has been suggested that overgeneralization of fear may be the hallmark of some anxiety disorders (e.g., Lissek et al., 2012).

The above-mentioned abnormalities in acquisition and generalization of fear may either reflect neurocognitive dysfunctions that predispose to pathological anxiety (e.g., Bishop, 2007) or be a consequence of the pathology (e.g., Milad et al., 2008). Both of these accounts imply that individual differences in these fear-related processes are trait-like patterns of responding, and thus are stable over time –even if they are a result of the pathology, they should last as long as the pathology persists. Despite its importance, this prerequisite has generally been assumed to be true, but empirical efforts to test it remain scarce. Before widely accepting that individual patterns of acquisition and generalization of fear are trait-like characteristics, it is important to

establish that the measures of these processes show stability over time or, in other words, test-retest reliability.

To the best of our knowledge, only two previous studies have investigated the test-retest reliability of fear-conditioning measures, concluding that they show individual stability over time (Fredrikson, Annas, Georgiades, Hursti & Tersman, 1993; Zeidan et al., 2011). These studies used the skin conductance response (SCR) to index fear. However, in recent years, the startle reflex has been incorporated as a measure in many studies of human fear-conditioning. While the SCR is an index of general arousal, the startle reflex is sensitive to the valence of the stimuli presented (i.e., it is potentiated during negative stimuli relative to neutral or positive stimuli; Lang, Bradley, & Cuthbert, 1990). In addition, Fredrikson et al. (1993) did not take into account differential fear responding in their test-retest analyses,[1] and none of these studies examined the temporal stability of fear generalization.

The aim of the present study was to examine the temporal stability (i.e., test-retest reliability) of individual differences in the acquisition and generalization of fear. Participants underwent two identical experimental sessions separated approximately by 8 months. The conditioned fear response was measured by the startle reflex, the SCR, and online ratings of US expectancies.

In order to avoid potential retention of conditioning in session 2, which is often reported in retest sessions (e.g., Grillon, 2002), and following a report showing that the emotion-modulated startle –a psychophysiological measure of emotional processing–

[1] Both studies included a differential fear-conditioning procedure in which another stimulus is presented in addition to the CS+. This stimulus, called CS-, is never paired with the US and serves as a control stimulus for non-associative learning effects, such as a general increase of fear produced by the US presentation. Differential fear conditioning is measured by comparing responses to the CS+ with responses to the CS-.

was stable only when the experimental stimuli changed between sessions (Larson, Ruffalo, Nietert, & Davidson, 2000), half of the participants in our study were presented with new conditioned and generalization stimuli (CSs and GSs, respectively) in session 2. Our hypotheses were that participants in the new stimulus set group would show less retention of conditioning in session 2 and greater temporal stability of acquisition and generalization of fear.

Method

Participants

Seventy-five healthy participants were recruited by advertisement. Exclusion criteria were assessed by a research psychologist using an *ad hoc* structured interview and included those aged over 30, lifetime or current drug abuse (including smoking more than 10 cigarettes per day), current psychiatric or neurological disorder, and current use of medication acting on the central nervous system. Participants were asked to abstain from alcohol, tobacco, or other drugs 24 h before each session, and from caffeinated drinks in the previous 12 h. Four participants were excluded due to technical problems. The final sample consisted of 71 participants (52 women; age: $M = 22.35$ years, ± 2.61 *SD*). The study was approved by the university ethics committee, and participants were paid 35€.[2]

[2] In addition to the participants included in the present study, a number of individuals ($n = 49$) declined to participate in session 2. Comparisons between those individuals and the final sample did not show any significant difference in age, gender distribution, contingency awareness, and scores on the state and trait scales of the State-Trait Anxiety Inventory (STAI-S and STAI-T, respectively; Spanish version; Spielberger, Gorsuch, & Lushene, 1982). Moreover, these two groups did not differ in acquisition and generalization of fear during session 1.

Stimuli and procedure

We used a fear-conditioning and fear-generalization paradigm developed by Lissek et al. (2008), which consisted of three phases: pre-acquisition, acquisition, and generalization. Two sessions were conducted, separated approximately by 8 months (time between sessions: M = 7.7 months, ranging from 5.8 to 9.0). Session 2 was scheduled at approximately the same time of the day as session 1 (difference between times of the day: M = 0.25 h, ± 2.10 SD). Session 2 differed from session 1 only in that a new set of CSs and GSs was used in half of the participants (assigned randomly to this condition, n = 36). Data from session 1 were used in two previous studies (Torrents-Rodas et al., 2012; Torrents-Rodas et al., 2013).

Ten rings with gradually varying diameters (from 5.08 to 11.94 cm, each type increasing by 15%) were used as CSs and GSs. They were presented 8 s each time on a computer screen. The smallest and the largest ring served as CSs. The CS+ co-terminated with the US presentation in 75% of the acquisition and 50% of the generalization trials. The CS- was never paired with the US. The eight intermediate rings were presented during generalization together with the CSs. Six, 12, and 12 CSs of each type were presented during pre-acquisition, acquisition, and generalization, respectively. In addition, six GSs of each type were presented during generalization. The stimulus order was pseudo-randomized with the restriction that no more than two stimuli of the same type were presented consecutively. The new stimulus set used in session 2 for half of the participants consisted of 10 lines with gradually varying slopes (from 12º to 84º, the slope of each line increasing 8º). As with the rings, the extreme stimuli served as CSs and the intermediate stimuli as GSs. The size of the ring or the slope of the line that served as the CS+ was counterbalanced across participants. For those participants in the same stimulus set group, the size of the ring that was used as

the CS+ was the same in the two sessions. The US was an electric shock of 100-ms duration, with an intensity adjusted for each participant as being "highly uncomfortable but not painful". It was generated by a stimulator (Grass Instruments S48; West Warwick, RI), transmitted via a constant-current unit (CCU1), and delivered to the volar surface of the right forearm by a bipolar bar electrode (EP10-621, Technomed Europe; Beek, NL).

Four or 5 s after the beginning of the odd trials, the startle reflex was elicited by presenting a 50-ms, 102-dB(A), probe of white noise. Startle probes were also presented during inter-trial intervals (ITIs), when no CSs or GSs were displayed on the screen (three during pre-acquisition, six during acquisition, and six during generalization; 9 to 17-s duration). Inter-probe intervals ranged from 18 to 25 s. During even trials, online ratings of US expectancies (risk ratings) for each CS or GS were obtained. A question presented on the top of the screen 1 or 2 s after the trial onset (*Level of risk?*) cued participants to respond as quickly as possible using a computer keyboard (1 = *no risk*, 2 = *moderate risk*, 3 = *high risk*). Stimulus timing and response recording were controlled by the commercial system Presentation (Neurobehavioral Systems Inc).

The two sessions followed the same procedure. Upon arrival at the laboratory, participants read the instructions about the experiment and signed the informed consent. They were not instructed about the CS-US contingency, but were told that they might learn to predict the shock if they pay attention to the presented stimuli. Next, the recording and shock electrodes were attached to the participant, the intensity of the US was adjusted, and the headphones for the startle probes were placed. After participants answered the STAI-S, nine startle probes were presented to reduce initial startle reactivity, and the pre-acquisition and acquisition phases started. The generalization test was conducted after a 10-min break. After the experiment, participants reported the

discomfort produced by both the US and the startle probe on a 1 (*no discomfort*) to 10 (*maximum discomfort*) scale, answered the STAI-T, and answered a multiple-choice question (based on Dawson & Reardon, 1973) regarding contingency awareness ("The electric stimulus usually appeared: a) in the presence of the smallest ring/most vertical line; b) in the presence of the biggest ring/flattest line; c) randomly; d) I don't know"). Individuals who correctly identified the stimulus that co-occurred with the US were considered contingency-aware.

Physiological recordings and response definition

Physiological responses were recorded using a Biopac 150 polygraph (BIOPAC Systems, Inc). The startle blink response was measured by recording the electromyographic activity (EMG) of the orbicularis oculi, using two 0.5 cm Ag-AgCl surface electrodes. The raw EMG signal was sampled at a rate of 2.000 Hz, filtered (analogue 50-Hz notch filter; and digital infinite impulse response band-pass filter, cut-off frequencies of 28 and 500 Hz), and rectified and smoothed (10-ms moving window average) offline. The SCR was recorded from the distal phalanges of the index and the middle left-hand fingers by means of two Ag-AgCl electrodes filled with electrolyte. The signal was sampled at a rate of 125 Hz.

The onset latency and the peak magnitude windows for the startle reflex were defined within 20 to 100 and 20 to 150 ms after the startle probe, respectively. Startle amplitudes were computed in microvolts (μV) as the difference between the EMG value at response peak and the average EMG during the 50 ms preceding the probe (baseline). If no response was detected in a given trial, the amplitude was scored as 0 μV. After visual inspection, trials with excessive noise were rejected: 3.7% and 4.3% in sessions 1 and 2, respectively; $\chi^2(1) = 2.52$; $p > .05$.

The SCR to the CSs and GSs was analyzed for half of the trials (those where the risk ratings were not obtained). SCR magnitudes in microsiemens (μS) were computed as the difference between the maximum SCR value and the value at response onset, occurring 1 to 5 s after stimulus onset. This time window was chosen to avoid interference of the SCR by the startle probe (Weike, Schupp, & Hamm, 2007). Trials in which no response could be detected or with a response magnitude of < 0.02 μS were considered non-response trials (Dunsmoor, Mitroff, & LaBar, 2009), and trials showing interferences or excessive baseline activity were rejected after visual inspection: 11.7% and 6.2% in sessions 1 and 2, respectively; $\chi^2(1) = 64.50$; $p < .001$. SCR data were square-root transformed to normalize the distribution.

Scorers were blind to the stimuli presented.

Data analysis

Participants were excluded from the startle reflex or the SCR analyses of a particular phase if they had more than one rejected trial for one stimulus in either the first or the second half of that phase, and in either session 1 or session 2 (startle reflex: *n* acquisition = 2, *n* generalization = 9; SCR: *n* pre-acquisition = 9, *n* acquisition = 10, *n* generalization = 31). In addition, participants were excluded from the risk rating analyses if they reported the same value throughout an experimental phase (*n* pre-acquisition and acquisition = 1, *n* generalization = 2). Participants that did not show any SCR in a particular phase, in both session 1 and session 2, were also excluded from the SCR analysis of that phase (*n* pre-acquisition = 2, *n* acquisition = 1, *n* generalization = 1).

Acquisition and generalization of fear, and potential differences in these results between sessions, were analysed by means of repeated-measures ANOVAs for each experimental phase (pre-acquisition, acquisition, and generalization) and each measure

(startle reflex, SCR, and risk ratings). Two within-subjects factors were included in the analyses: stimulus (CS- vs. CS+ in pre-acquisition and acquisition[3]; and CS-, class 1 GS, class 2 GS, class 3 GS, class 4 GS, and CS+ in generalization[4]) and session (1 vs. 2). In addition, to test the potential effects of using a new stimulus set in session 2, stimulus set (same vs. new) was included as a between-subjects factor. Where appropriate, degrees of freedom were corrected using Greenhouse-Geisser's ε, and η_p^2 are reported as a measure of effect size for main and interaction effects.

We used the generalizability theory (G theory; Cronbach, Gleser, Nanda, & Rajaratnam, 1972) to study the temporal stability of individual differences in the acquisition and generalization of fear. By means of analysis of variance components, the G theory allows specifying the relative contribution of different sources of variance to a given measure. In addition, generalizability coefficients (G coefficients) can be calculated to determine different types of reliability (e.g., consistency across observers, internal consistency, or temporal stability). Aside from the usual psychometric applications, G coefficients have been used to estimate the reliability of different psychophysiological measures (Burgess & Gruzelier, 1996; Di Nocera, Ferlazzo, & Borghi, 2001; Hinz, Hueber, Schreinicke, & Seibt, 2002). Our analysis of variance components included seven sources of variation: participant, stimulus, session, Participant × Stimulus, Participant × Session, Stimulus × Session, and Participant × Stimulus × Session. Individual differences in the acquisition and generalization of fear were accounted for by the component Participant × Stimulus; with the component Participant × Stimulus × Session reflecting changes between sessions in the

[3] Startle reflex analyses included an additional level (ITI) accounting for startle responses during ITIs.

[4] Following Lissek et al. (2008), responses to every two consecutive sizes of GSs were averaged in four classes of responses to GSs.

idiosyncratic patterns of acquisition and generalization (plus unspecified sources of random error, see Strube & Newman, 2007). Following previous research on the temporal stability of psychophysiological response patterns (Hinz et al., 2002), a G coefficient was computed to determine the generalizability across time (i.e., temporal stability) of individual differences in the acquisition and generalization of fear. This coefficient varied from 0 (absent generalizability) to 1 (full generalizability across time):

$$E\rho^2 = \sigma^2_{\text{Participant} \times \text{Stimulus}} / (\sigma^2_{\text{Participant} \times \text{Stimulus}} + \sigma^2_{\text{Participant} \times \text{Stimulus} \times \text{Session}})$$

For the whole sample and for each type of measure, separate variance components and G coefficients were computed for acquisition and generalization. In addition, in order to test the effect of using a new stimulus set in session 2, these analyses were also performed separately for the two groups of stimulus set. The variance components were computed using the program developed by Mushquash and O'connor (2006). Ninety-five % confidence intervals (95% CIs) are reported for all the G coefficients (Brennan, 2001, pp. 198-199). All the analyses were conducted with SPSS 13.0.

Results

Preliminary results

There were no differences between sessions 1 and 2 regarding most experimental variables (Table 1).

Acquisition and generalization of fear across sessions

Pre-acquisition

Responses to the CS+ were not different from responses to the CS-, for either the startle reflex or the SCR, in either session 1 or session 2, or as a function of the stimulus

set (same vs. new). However, risk ratings to the CS+ were greater than ratings to the CS-, $F(1, 68) = 19.97$, $p < .001$, $\eta_p^2 = .23$. Furthermore, overall ratings decreased from session 1 to session 2, $F(1, 68) = 4.71$, $p < .05$, $\eta_p^2 = .07$ (Figure 1a). Despite the absence of significant effects involving stimulus set in the overall ANOVA, and given that we hypothesized less retention of conditioning in session 2 in the new stimulus set than in the same stimulus set group, we conducted separate Stimulus × Session ANOVAs for each group. These analyses did not show retention of conditioning in any of the groups. Thus, for the startle reflex and the SCR, no significantly greater responses to the CS+ than to the CS- were found in any of the sessions for any of the groups. Regarding risk ratings, the difference between ratings to the CS+ and the CS- found in session 1 did not increase in session 2 in either the same stimulus set or the new stimulus set group.

Acquisition

Differential conditioning (greater responses to the CS+ than to the CS-) was evident for the three measures [startle reflex: $F(1.60, 106.92) = 23.18$, $p < .001$, $\eta_p^2 = .26$; SCR: $F(1, 58) = 39.31$, $p < .001$, $\eta_p^2 = .40$; and risk ratings: $F(1, 68) = 305.95$, $p < .001$, $\eta_p^2 = .82$].[5]

The absence of significant Stimulus × Session interactions for the startle reflex and the SCR data indicated that differential conditioning was similar in both sessions. Conversely, the Stimulus × Session interaction was significant for the risk ratings, $F(1, 68) = 5.62$, $p < .05$, $\eta_p^2 = .08$. Nevertheless, the magnitude of differential conditioning

[5] Although significantly greater risk ratings to the CS+ than to the CS- were already present in pre-acquisition, this difference increased during acquisition [indicated by a Stimulus × Phase interaction, $F(1, 69) = 182.52$, $p < .001$, $\eta_p^2 = .73$. Follow-up comparisons showed an increase in risk ratings to the CS+, $t(69) = 15.98$, $p < .001$, but no differences in ratings to the CS-, $t(69) = 0.21$, $p > .05$].

did not differ between sessions, both *t*s (69) > 12.56, $p < .001$, but there was a decrease in the risk ratings to the CS- from session 1 to session 2, $t(69) = 2.48$, $p < .05$. In addition, overall SCRs decreased across the two sessions, $F(1, 58) = 14.08$, $p < .001$, $\eta_p^2 = .20$ (see Figure 1b).

The Stimulus × Session × Stimulus Set interactions were not significant for any of the measures. Nevertheless, given that we hypothesized greater temporal stability of fear acquisition in the new stimulus set than in the same stimulus set group, we conducted separate Stimulus × Session ANOVAs for each group. For the startle reflex and the SCR, results revealed significant differential conditioning in both groups, which did not differ between sessions (significant stimulus effects, all *F*s > 10.86, *p*s < .01, but non-significant Stimulus × Session interactions). Nonetheless, an overall decrease across sessions in the SCR reactivity was observed specifically in the same stimulus set group, $F(1, 31) = 11.46$, $p < .01$, $\eta_p^2 = .27$. For the risk ratings, a Stimulus × Session interaction appeared in the same stimulus set, $F(1, 33) = 4.88$, $p < .05$, $\eta_p^2 = .13$, but not in the new stimulus set group. Follow-up tests indicated that in the former group ratings to the CS- decreased from session 1 to session 2, $t(33) = 2.26$, $p < .05$. Thus, the decrease across sessions in overall SCR, and in the ratings to the CS-, reported above for the whole sample appeared to be more specific to the same stimulus set group.

Generalization

For the three measures, responses during generalization varied as a function of the type of stimulus presented [startle reflex: $F(3.73, 233.53) = 14.49$, $p < .001$, $\eta_p^2 = .20$; SCR: $F(1.97, 72.85) = 13.07$, $p < .001$, $\eta_p^2 = .26$; and risk ratings: $F(2.86, 191.28) = 309.11$, $p < .001$, $\eta_p^2 = .82$]. Pair-wise comparisons between the CS- and the other stimuli (Bonferroni-corrected $\alpha \leq .01$) indicated that differential conditioning was still

present in this phase and that it generalized to those GS classes more similar to the CS+ (see Figure 2).

The absence of significant Stimulus × Session interactions for any of the measures indicated that the pattern of generalization did not differ between sessions. In addition, none of the Stimulus × Session × Stimulus Set interactions were significant, and separate Stimulus × Session ANOVAs for each of the stimulus set groups did not reveal any difference across sessions in the pattern of generalization.

Given that the pre-acquisition analyses did not show retention of differential conditioning at the beginning of session 2, we tested whether differential conditioning extinguished by the end of generalization of session 1. The analyses showed that differential conditioning persisted for the three measures in the second half of generalization, all Fs > 6.20, ps < .05, $\eta_p^2 = .14$. Taking into account only the last generalization trial of each CS, differential conditioning was still present for the startle reflex and the risk ratings, both Fs > 13.45, ps < .01, $\eta_p^2 = .19$, but not for the SCR. However, the absence of a significant difference for the SCR might be due to a lack of statistical power or to general habituation of the SCR (M_{CS+} = 0.10 ± 0.19 *SD* vs. M_{CS-} = 0.06 ± 0.10 *SD*).

Temporal stability of individual differences in the acquisition and generalization of fear

Table 2 shows the variance estimates for the seven sources of variation in our study. The relative contribution of each of the sources to the overall variance depended on whether the measures were physiological (the startle reflex or the SCR) or self-reported (risk ratings). For the startle reflex and the SCR, participant and the Participant × Session interaction were the largest sources of variation. This indicated important differences across participants in physiological reactivity, and a certain variation across

sessions in each participant's reactivity. The component of variance associated with stimulus –indicative of differences in responding to the CSs during acquisition, and the CSs and GSs during generalization– was somewhat more modest, but the small amount of variation due to the Stimulus × Session interaction suggested that this effect remained constant across sessions, as seen in the ANOVAs. For the risk ratings, the largest source of variation was stimulus, with the Stimulus × Session interaction again playing again a negligible role. The component of variance associated with participant was nil during acquisition and modest during generalization. This indicated that the overall reported risk was very similar for all the participants.

More important to our analyses was the component of variance associated with Participant × Stimulus. For the startle reflex and the SCR, this source of variation was generally larger than stimulus, indicating that individual differences had an important effect in responding to the CSs during acquisition, and to the CSs and GSs during generalization. For the risk ratings, the component of variance associated with Participant × Stimulus was smaller than that associated with stimulus, but it was still non-negligible.

Table 3 shows G coefficients indicating the degree of generalizability across time (i.e., temporal stability) of individual differences in the acquisition and generalization of fear. The G coefficients of fear acquisition ranged between 0.32 and 0.38 for the three measures. The G coefficients of fear generalization were somewhat smaller, ranging from 0.20 to 0.32. However 95% CIs computed for each of the G coefficients overlapped with each other, and none of them included 0.

Table 4 shows G coefficients computed separately for the two groups of stimulus set.[6] Ninety-five % CIs of the G coefficients for each group were rather wide and overlapped with each other. However, in the same stimulus set group, 95% CIs of the G coefficients of fear acquisition for the SCR and the risk ratings included 0, indicating compromised temporal stability. On the other hand, the G coefficients of fear generalization for the startle reflex and the SCR appeared to be smaller in the new stimulus set group, but none of these coefficients included 0 in their corresponding 95% CIs.

Discussion

In the present study, we sought to examine the temporal stability of individual differences in the acquisition and generalization of fear. To that end, 71 healthy participants took part in two identical sessions of a fear-conditioning and fear-generalization paradigm separated by 8 months. Modest temporal stability of individual differences in fear acquisition and fear generalization was found. However, some acquisition measures showed poor stability when the same CSs were used again. In addition, no differences in fear acquisition and fear generalization were found between sessions.

Regarding the absence of differences between sessions, it is noteworthy that participants presented with the same CSs and GSs in both sessions did not show

[6] As a new stimulus set can only be presented if the measurement is conducted again, at least on a second session, we believed that it was more appropriate to explicitly tie the generalizability conclusions to a two-session measurement. Therefore, the Participant × Stimulus × Session term in the denominator of the G coefficients computed separately for the two groups was divided by 2 (see Strube & Newman, 2007). Because a two-session measurement is more reliable than a single-session measurement, these G coefficients are greater than those computed for the whole sample.

retention of conditioning in pre-acquisition of session 2. This happened despite the fact that conditioning was still present at the end of generalization of session 1, and is in contrast with previous results of fear-conditioning studies using different time intervals between sessions (4-5 days, Grillon & Davis, 1997; 1 week or 1 month, Grillon 2002; 1 or 28 days, Hammond, Baer, & Fuhrer, 1980; 1 or 6 months, Schell, Dawson, & Marinkovic, 1991). Given the large time span between sessions in our study, a possible explanation is that participants forgot the CS-US contingency and this prevented retention of conditioning (Hammond et al., 1980; Schell et al., 1991). Alternatively, our participants might have learned from session 1 that the CS-US contingency were dependent on the experimental phase (e.g., pre-acquisition vs. acquisition). Thus, they expected the US upon the CS+ presentation only after a number of trials from the beginning of the experiment. Our rather long pre-acquisition phase, and the experimental demand asking participants to try to predict the US occurrence during the experiment, might have boosted the realization that the CS-US contingency changed across the experimental phases. By using the startle reflex as a measure of fear, and given that the startle reflex was not greater during either of the CSs than during ITIs, we were able to show that the absence of conditioning during pre-acquisition in session 2 was not due to the generalization of fear to the CS-. Thus, we did not observe generalization of fear over time, which is often reported in animal research (e.g., Jasnow, Cullen, & Riccio, 2012).

In addition to the ANOVAs, G coefficients were computed to determine the temporal stability in the individual patterns of acquisition and generalization of fear. For the three measures (startle reflex, SCR, and risk ratings), the G coefficients of fear acquisition ranged between 0.32 and 0.38, while G coefficients of fear generalization ranged between 0.20 and 0.32. None of the 95% CIs of these coefficients included 0,

thus indicating a certain degree of temporal stability in all the measures. It is important to bear in mind that, even though the analyses are based on two test sessions, the G coefficients for the whole sample refer to the temporal stability of a single measurement. We chose to analyse the temporal stability of a single measurement because most fear-conditioning studies test participants only once. From a reliability perspective, testing an individual more than one time and aggregating the results across sessions would presumably increase the temporal stability of the measures reported here (Strube & Newman, 2007). In addition, a rather large interval between sessions was used in the present study. Temporal stability could be higher with shorter intervals.

Our results address an important question that has been scarcely investigated. Regarding the temporal stability of individual differences in fear acquisition, Fredrikson et al. (1993) computed separate correlations for SCRs to CS+ and SCR to CS- (two conditioning sessions separated by 3 weeks), and concluded that first interval SCRs (occurring 1 to 4 s after CS onset) to both CSs showed adequate temporal stability (Pearson's correlation coefficients ranged from 0.37 to 0.85). However, these authors did not analyze conditioning by taking into account differential responding to the CS+ and the CS-, which allows the control of non-associative learning effects. In contrast, our G coefficients included the component of variance associated with Participant × Stimulus, which accounted for individual differences in responding across stimuli (the CS+ and the CS- during acquisition, and the CS+, the CS- and the GSs during generalization). These differences preclude any meaningful comparison between these studies. On the other hand, Zeidan et al. (2011) analyzed the temporal stability of individual differences in fear acquisition by correlating the maximum SCR to CS+ presented during an acquisition phase (three occasions separated by 3 months). An intraclass correlation (ICC) of 0.68 was interpreted as indicative of reliable conditioning

scores, but again, that index of conditioning did not control for non-associative learning effects. Nonetheless, they also computed an ICC for differential SCRs (CS+ minus CS-). This ICC was slightly greater than our G coefficient of fear acquisition for the SCR (0.43 vs. 0.32, respectively; ICC and G coefficients have a similar interpretation). The time span between test sessions, 3 months in Zeidan et al. vs. 7 months in the present study, could explain the greater value found in the former study. Although it has been suggested that fear generalization is a robust marker of pathological anxiety (Lissek, 2012), no previous studies have examined the temporal stability of individual differences in this process. Thus, our results regarding fear generalization cannot be compared.

A second aim of the present study was to test whether using a new set of CSs and GSs in session 2 increased the temporal stability of our measures of individual differences in the acquisition and generalization of fear. Separate G coefficients for each group indicated that this was the case for fear acquisition, as measured by the SCR and the risk ratings. For those measures, 95% CIs of the G coefficients included 0 in the same stimulus set but not in the new stimulus set group, thus indicating poor stability in the former group. Separate ANOVAs for each group showed that these differences might be related to the fact that, when the same CSs were presented in session 2, there was a decrease in the overall SCR and in the risk ratings to the CS-. On the other hand, the G coefficients of fear generalization for the startle reflex and the SCR appeared to be somewhat smaller in the new stimulus than in the same stimulus set group. However, none of the 95% CIs of these coefficients included 0, and separate ANOVAs did not show differences between groups in the generalization patterns of both sessions.

The present study has some limitations. First, the small number of male participants in our sample, and its distribution across groups (*n* same stimulus set group

= 11 vs. *n* new stimulus set group = 8), prevented us from conducting analyses of gender effects. In addition, despite some evidence suggesting that menstrual cycle and oral contraceptive consumption may influence some fear-conditioning processes in female participants (Mertz et al., 2012; Milad et al. 2006), we did not take these variables into account. Finally, the interval between sessions was rather variable.

The present study indicates that individual differences in the acquisition and generalization of fear show some stability over time. However, the obtained G coefficients, referring to a single measurement, were rather modest. When using acquisition and generalization measures to assess trait-like characteristics, it is important to take this caveat into account, at least with the paradigm used in the present study. Testing individuals more than once and aggregating the outcomes might increase the temporal stability of the acquisition and generalization measures reported here. In that case, using new CSs across tests would prevent the effects of previous experience on measures of fear acquisition.

Before asserting that differences in fear-conditioning processes are stable individual characteristics, researchers should show evidence that the different experimental paradigms provide consistent individual measures across time. Finally, as some anxiety disorders are associated with abnormal patterns of acquisition and generalization of fear (e.g., impaired differential conditioning or overgeneralization), it would be interesting to test the temporal stability of these processes in individuals diagnosed with pathological anxiety.

References

Bishop, S. J. (2007). Neurocognitive mechanisms of anxiety: An integrative account. *Trends in Cognitive Sciences, 11*, 307-316. doi:10.1016/j.tics.2007.05.008

Brennan, R. L. (2001). *Generalizability theory*. New York: Springer.

Burgess, A. P., & Gruzelier, J. H. (1996). The reliability of event-related desynchronization: A generalisability study analysis. *International Journal of Psychophysiology, 23*, 163-169. doi: 10.1016/S0167-8760(96)00046-3

Cronbach, L. J., Gleser, G. C., Nanda, H., & Rajaratnam, N. (1972). *The dependability of behavioral measurements: Theory of generalizability of scores and profiles*. New York: Wiley.

Dawson, M. E., & Reardon, P. (1973). Construct validity of recall and recognition postconditioning measures of awareness. *Journal of Experimental Psychology, 98*, 308-315.

Di Nocera, F., Ferlazzo, F., & Borghi, V. (2001). G theory and the reliability of psychophysiological measures: A tutorial. *Psychophysiology, 38*, 796-806. doi:10.1111/1469-8986.3850796

Dunsmoor, J. E., Mitroff, S. R., & LaBar, K. S. (2009). Generalization of conditioned fear along a dimension of increasing fear intensity. *Learning and Memory, 16*, 460-469. doi:10.1101/lm.1431609

Fredrikson, M., Annas, P., Georgiades, A., & Hursti, T. (1993). Internal consistency and temporal stability of classically conditioned skin conductance responses. *Biological Psychology, 35*, 153-163. doi:10.1016/0301-0511(93)90011-V

Grillon, C. (2002). Associative learning deficits increase symptoms of anxiety in humans. *Biological Psychiatry, 51*, 851-858. doi:10.1016/S0006-3223(01)01370-1

Grillon, C., & Davis, M. (1997). Fear-potentiated startle conditioning in humans: Explicit and contextual cue conditioning following paired versus unpaired training. *Psychophysiology, 34*, 451-458. doi:10.1111/j.1469-8986.1997.tb02389.x

Hammond, G. S., Baer, P. E., & Fuhrer, M. J. (1980). Retention of differential autonomic conditioning and memory for conditional stimulus relationships. *Psychophysiology, 17*, 356-362. doi:10.1111/j.1469-8986.1980.tb00163.x

Hinz, A., Hueber, B., Schreinicke, G., & Seibt, R. (2002). Temporal stability of psychophysiological response patterns: Concepts and statistical tools. *International Journal of Psychophysiology, 44*, 57-65. doi:10.1016/S0167-8760(01)00191-X

Jasnow, A. M., Cullen, P. K., & Riccio, D. C. (2012). Remembering another aspect of forgetting. *Frontiers in Psychology, 3*, 1-7. doi:10.3389/fpsyg.2012.00175

Kircher, T., Arolt, V., Jansen, A., Pyka, M., Reinhardt, I., Kellermann, T., . . . Straube, B. (2013). Effect of cognitive-behavioral therapy on neural correlates of fear conditioning in panic disorder. *Biological Psychiatry, 73*, 93-101. doi: 10.1016/j.biopsych.2012.07.026

Lang, P. J., Bradley, M. M., & Cuthbert, B. N. (1990). Emotion, attention, and the startle reflex. *Psychological Review, 97*, 377-395. doi:10.1037/0033-295X.97.3.377

Larson, C. L., Ruffalo, D., Nietert, J. Y., & Davidson, R. J. (2000). Temporal stability of the emotion-modulated startle response. *Psychophysiology, 37*, 92-101. doi:10.1111/1469-8986.3710092

Lissek, S. (2012). Toward an account of clinical anxiety predicated on basic, neurally mapped mechanisms of pavlovian fear-learning: The case for conditioned overgeneralization. *Depression and Anxiety, 29*, 257-263. doi:10.1002/da.21922

Lissek, S., Biggs, A. L., Rabin, S. J., Cornwell, B. R., Alvarez, R. P., Pine, D. S., & Grillon, C. (2008). Generalization of conditioned fear-potentiated startle in humans: Experimental validation and clinical relevance. *Behaviour Research and Therapy, 46*, 678-687. doi:10.1016/j.brat.2008.02.005

Lissek, S., Powers, A. S., McClure, E. B., Phelps, E. A., Woldehawariat, G., Grillon, C., & Pine, D. S. (2005). Classical fear conditioning in the anxiety disorders: A meta-analysis. *Behaviour Research and Therapy, 43*, 1391-1424. doi:10.1016/j.brat.2004.10.007

Merz, C. J., Tabbert, K., Schweckendiek, J., Klucken, T., Vaitl, D., Stark, R., & Wolf, O. T. (2012). Oral contraceptive usage alters the effects of cortisol on implicit fear learning. *Hormones and Behavior, 62*, 531-538. doi:10.1016/j.yhbeh.2012.09.001

Milad, M. R., Goldstein, J. M., Orr, S. P., Wedig, M. M., Klibanski, A., Pitman, R. K., & Rauch, S. L. (2006). Fear conditioning and extinction: Influence of sex and menstrual cycle in healthy humans. *Behavioral Neuroscience, 120*, 1196-1203. doi:0.1037/0735-7044.120.5.1196

Milad, M. R., Orr, S. P., Lasko, N. B., Chang, Y., Rauch, S. L., & Pitman, R. K. (2008). Presence and acquired origin of reduced recall for fear extinction in PTSD: Results of a twin study. *Journal of Psychiatric Research, 42*, 515-520. doi:10.1016/jpsychires.2008.01.017

Mineka, S., & Oehlberg, K. (2008). The relevance of recent developments in classical conditioning to understanding the etiology and maintenance of anxiety disorders. *Acta Psychologica, 127*, 567-580. doi:10.1016/j.actpsy.2007.11.007

Mushquash, C., & O'Connor, B. P. (2006). SPSS and SAS programs for generalizability theory analyses. *Behavior Research Methods, 38*, 542-547. doi:10.3758/BF03192810

Schell, A. M., Dawson, M. E., & Marinkovic, K. (1991). Effects of potentially phobic conditioned stimuli on retention, reconditioning, and extinction of the conditioned skin conductance response. *Psychophysiology, 28*, 140-153. doi:10.1111/j.1469-8986.1991.tb00403.x

Spielberger, C. D., Gorsuch, R. R., & Lushene, R. E. (Eds.). (1982). *STAI. cuestionario de ansiedad Estado/Rasgo* (1st ed.). Madrid: Técnicos Especialistas Asociados (TEA).

Strube, M. J., & Newman, L. C. (2007). Psychometrics. In J. T. Cacioppo, L. G. Tassinary & G. G. Bernston (Eds.), *Handbook of psychophysiology* (3rd ed., pp. 789-811). New York: Cambridge University Press.

Torrents-Rodas, D., Fullana, M. A., Arias, B., Bonillo, A., Caseras, X., Andión, O., . . . Torrubia, R. (2012). Acquisition and generalization of fear conditioning are not

modulated by the BDNF-val66met polymorphism in humans. *Psychophysiology, 49*, 713-719. doi:10.1111/j.1469-8986.2011.01352.x

Torrents-Rodas, D., Fullana, M. A., Bonillo, A., Caseras, X., Andión, O., & Torrubia, R. (2013). No effect of trait anxiety on differential fear conditioning or fear generalization. *Biological Psychology, 92*, 185-190. doi:10.1016/j.biopsycho.2012.10.006

Weike, A. I., Schupp, H. T., & Hamm, A. O. (2007). Fear conditioning requires awareness in trace but not delay conditioning. *Psychophysiology, 44*, 170-180. doi:10.1111/j.1469-8986.2006.00469.x

Zeidan, M. A., Lebron-Milad, K., Thompson-Hollands, J., Im, J. J. Y., Dougherty, D. D., Holt, D. J., . . . Milad, M. R. (2012). Test-retest reliability during fear acquisition and fear extinction in humans. *CNS Neurosciences and Therapeutics, 18*, 313-317. doi:10.1111/j.1755-5949.2011.00238.x

Table 1

Descriptive statistics for each session (unless otherwise indicated, means and standard deviations are provided).

	Session 1	Session 2
STAI-S [a]	14.51 (7.32)	13.47 (6.66)
Shocks presented during the work-up procedure [b]	4.65 (1.60)	4.08 (1.42)
Shock intensity (mA) [b]	3.56 (0.84)	3.29 (0.79)
Shock discomfort (1-10)	6.49 (1.44)	6.63 (1.32)
Startle probe discomfort (1-10)	6.72 (1.84)	6.90 (1.85)
Contingency-unaware individuals, *n* (%)	6 (8.5%)	2 (2.8%)

[a] State-Trait Anxiety Inventory, State version. Scores range from 0 to 60 in the Spanish version.

[b] $p < .05$. *t* test.

Table 2

Variance components (%) for each phase, each type of measure, and for the whole sample and separated by group of stimulus set.

	Startle reflex			SCR			Risk ratings		
	Whole sample	Same stimulus set	New stimulus set	Whole sample	Same stimulus set	New stimulus set	Whole sample	Same stimulus set	New stimulus set
Acquisition	(*n* = 69)	(*n* = 35)	(*n* = 34)	(*n* = 60)	(*n* = 31)	(*n* = 29)	(*n* = 70)	(*n* = 34)	(*n* = 36)
Participant	70.8	61.7	76.8	18.7	23.9	13.1	0.0	2.3	0.0
Stimulus	1.8	2.1	1.5	16.3	13.5	18.9	73.6	69.4	75.9
Session	0.0	0.0	0.0	6.4	10.7	1.4	0.0	0.0	0.0
Participant × Stimulus	2.8	2.4	3.1	12.8	9.0	18.2	8.3	6.1	11.1
Participant × Session	19.9	29.1	13.9	18.0	19.1	15.6	0.0	0.0	0.0
Stimulus × Session	0.1	< 0.1	< 0.1	1.2	0.0	2.6	1.1	2.3	0.0
Participant × Stimulus × Session	4.6	4.7	4.6	26.6	23.8	30.2	17.1	20.0	13.0
Generalization	(*n* = 62)	(*n* = 31)	(*n* = 31)	(*n* = 39)	(*n* = 23)	(*n* = 16)	(*n* = 70)	(*n* = 34)	(*n* = 36)
Participant	76.6	65.7	82.1	45.1	49.6	39.9	10.3	9.7	10.6
Stimulus	0.8	0.9	0.7	5.0	2.8	8.2	60.3	58.9	61.4
Session	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0
Participant × Stimulus	1.2	1.4	1.0	8.8	10.2	6.4	5.3	5.5	5.3
Participant × Session	16.8	28.1	11.1	21.5	21.3	20.7	6.4	6.9	6.1
Stimulus × Session	0.0	0.0	0.0	0.5	0.5	0.1	0.2	0.0	0.7
Participant × Stimulus × Session	4.7	3.9	5.1	19.1	15.5	23.9	17.5	19.0	15.9

Table 3

Generalizability coefficients ($E\rho^2$*) indicating the temporal stability of individual differences in the acquisition and generalization of fear. Values between brackets indicate 95% confidence intervals.*

	Startle reflex	SCR	Risk ratings
Acquisition	0.38 [0.19, 0.58] (*n* = 69)	0.32 [0.07, 0.52] (*n* = 60)	0.33 [0.10, 0.52] (*n* = 70)
Generalization	0.20 [0.09, 0.30] (*n* = 62)	0.32 [0.20, 0.45] (*n* = 39)	0.23 [0.13, 0.33] (*n* = 70)

Table 4

Generalizability coefficients ($E\rho^2$*) indicating the temporal stability of individual differences in the acquisition and generalization of fear by group of stimulus set. Values between brackets indicate 95% confidence intervals.*

	Startle reflex		SCR		Risk ratings	
	Same stimulus set	New stimulus set	Same stimulus set	New stimulus set	Same stimulus set	New stimulus set
Acquisition	0.51 [0.02, 0.75] (*n* = 35)	0.58 [0.15, 0.79] (*n* = 34)	0.43 [-0.20, 0.72] (*n* = 31)	0.55 [0.06, 0.79] (*n* = 29)	0.38 [-0.25, 0.69] (*n* = 34)	0.63 [0.28, 0.81] (*n* = 36)
Generalization	0.42 [0.20, 0.58] (*n* = 31)	0.28 [0.01, 0.47] (*n* = 31)	0.57 [0.27, 0.66] (*n* = 23)	0.35 [0.05, 0.62] (*n* = 16)	0.37 [0.14, 0.53] (*n* = 34)	0.40 [0.19, 0.55] (*n* = 36)

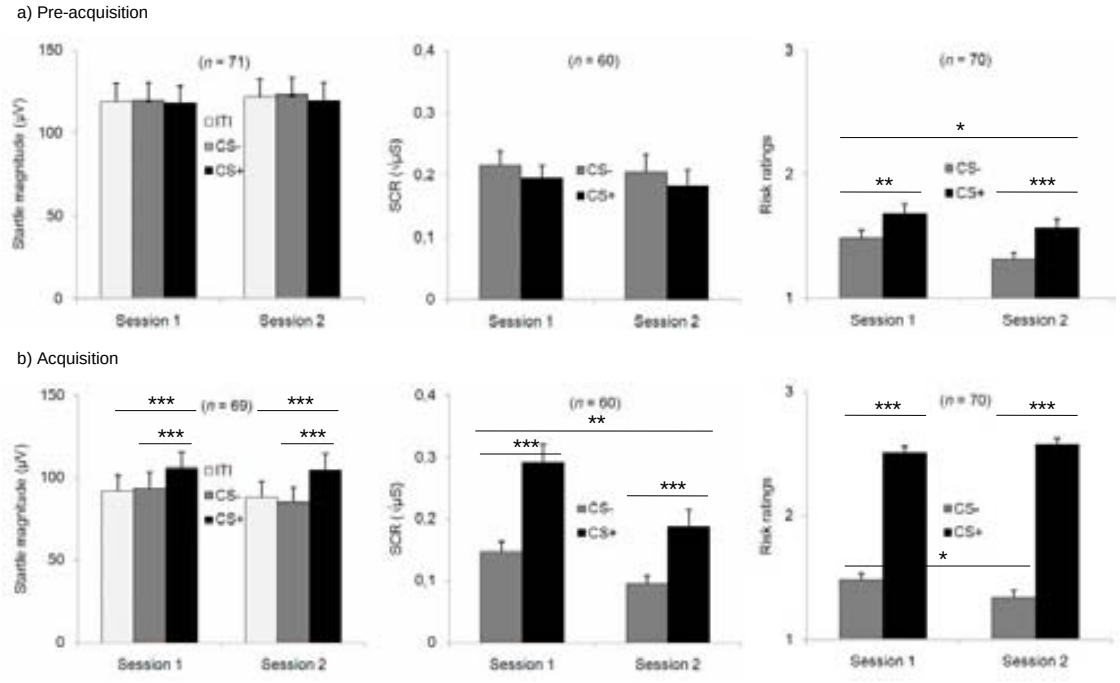

Figure 1. Responses to each CS by sessions during a) pre-acquisition and b) acquisition phase. Bars represent standard errors. *$p < .05$, ** $p < .01$, *** $p < .001$.

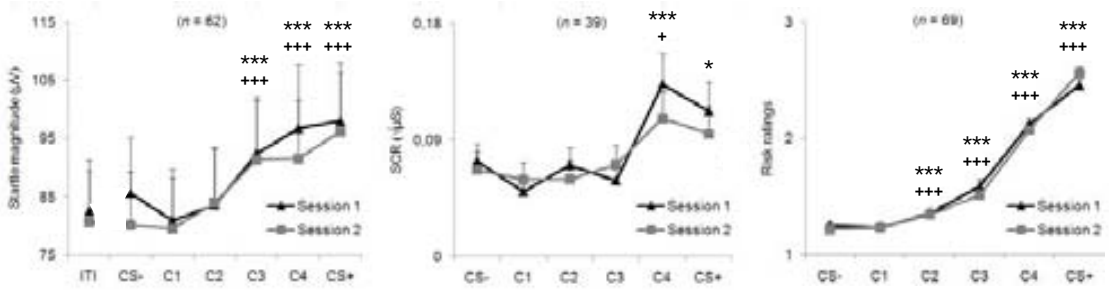

Figure 2. Generalization gradients for sessions 1 (black triangles) and 2 (grey squares). Bars represent standard errors. For each session, pair-wise comparisons were conducted between all the stimuli and the CS-. C1, C2, C3, and C4 refer to class 1 GS, class 2 GS, class 3 GS and class 4 GS; respectively. $*/^{+}$ $p < .05$, $**/^{++}$ $p < .01$, $***/^{+++}$ $p < .001$. Asterisks refer to session 1, whereas plus signs refer to session 2.