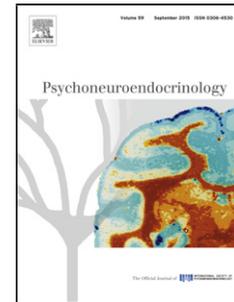


## Accepted Manuscript

Title: Trauma exposure relates to heightened stress, altered amygdala morphology and deficient extinction learning: Implications for psychopathology



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**Title:** Trauma exposure relates to heightened stress, altered amygdala morphology and deficient extinction learning: implications for psychopathology

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**Highlights**

(Manuscript title: Trauma exposure relates to heightened stress, altered amygdala morphology and deficient extinction learning: implications for psychopathology, by R. Cacciaglia et al.)

- We investigated amygdala morphology in healthy trauma-exposed individuals compared to unexposed controls
- People with previous trauma exposure displayed volume increase in the left lateral amygdala compared to unexposed controls
- Trauma-exposed individuals showed enhanced fear conditioning and deficient extinction, compared to healthy controls
- Compared to unexposed controls, traumatized people showed salivary cortisol hypo-suppression to dexamethasone
- We show multiform alterations in traumatized people which resemble symptoms of post-traumatic stress disorder and depression

**Abstract**

Stress exposure causes a structural reorganization in neurons of the amygdala. In particular, animal models have repeatedly shown that both acute and chronic stress induce neuronal hypertrophy and volumetric increase in the lateral and basolateral nuclei of amygdala. These effects are visible on the behavioral level, where stress enhances anxiety behaviors and provokes greater fear learning. We assessed stress and anxiety levels in a group of 18 healthy human trauma-exposed individuals (TR group) compared to 18 non-exposed matched controls (HC group), and related these measurements to amygdala volume. Traumas included unexpected adverse experiences such as vehicle accidents or sudden loss of a loved one. As a measure of aversive learning, we implemented a cued fear conditioning paradigm. Additionally, to provide a biological marker of chronic stress, we measured the sensitivity of the hypothalamus-pituitary-adrenal (HPA) axis using a dexamethasone suppression test. Compared to the HC, the TR group showed significantly higher levels of chronic stress, current stress and trait anxiety, as well as increased volume of the left amygdala. Specifically, we observed a focal enlargement in its lateral portion, in line with previous animal data. Compared

to HC, the TR group also showed enhanced late acquisition of conditioned fear and deficient extinction learning, as well as salivary cortisol hypo-suppression to dexamethasone. Left amygdala volumes positively correlated with suppressed morning salivary cortisol. Our results indicate differences in trauma-exposed individuals which resemble those previously reported in animals exposed to stress and in patients with post-traumatic stress disorder and depression. These data provide new insights into the mechanisms through which traumatic stress might prompt vulnerability for psychopathology.

**Keywords:** trauma, stress, amygdala, fear conditioning, extinction learning, post-traumatic stress disorder, depression.

## 1. Introduction

Stress can be broadly defined as any actual or perceived situation in which demands exceed one's personal resources, thus substantially altering homeostasis (Lazarus, 2006). Stress triggers important adaptive functions that promote health and improve performance (Smeets et al., 2009; Joëls et al., 2006). However, depending on its duration, type and lifetime onset, the load of stress can exceed the allostatic threshold and lead to long-lasting harmful outcomes (McEwen, 2007). This is especially the case for traumatic stressors, which are often precursors of mental disorders such as post-traumatic stress disorder (PTSD) or depression (McEwen and Gianaros, 2011). The amygdala, together with other brain regions such as the hippocampus and the prefrontal cortex, is a key region involved in the stress response system (Joëls and Baram, 2009) and displays both structural and functional alterations in PTSD and depression (McEwen et al., 2015). Animal research has provided compelling evidence that both acute and chronic experimental stressors cause patterns of dendritic remodeling in neurons of the amygdala. In rodents, chronic immobilization stress results in neuronal hypertrophy of the basolateral nucleus of the amygdala (BLA) (Henckens et al., 2015; Vyas

et al., 2002, 2006). Similarly, Mitra and Sapolsky (2008) reported larger BLA volumes in rats after chronic corticosterone administration. Remarkably, the authors found similar results already after a single acute dose of corticosterone, which might resemble the neuroendocrinological underpinnings of a traumatic event (Mitra and Sapolski, 2008). The increased dendritic hypertrophy in the BLA has been observed in various experimental settings using several different protocols of stress exposure (e.g., Padival et al., 2013; Cui et al., 2008) and has been shown to persist over time, that is, not regressing after a relatively long time of stress-free recovery (Vyas et al., 2004). These results are accompanied by coherent changes on the functional and behavioral levels, whereby stress enhances lateral amygdala activity (Rau et al., 2015; Rosenkranz et al., 2010), increases anxiety behaviors (Vyas et al., 2004; Chaby et al., 2015) and provokes enhanced fear learning (Marks et al., 2015; Monsey et al., 2014; Hui et al., 2004).

So far, most research conducted in humans has explored amygdala morphology in people who were diagnosed with PTSD or depression, and only few studies have focused on the structural amygdalar correlates of stress-exposure in pre-clinical samples (e.g., Gerritsen et al., 2015; Lupien et al., 2011). Moreover, whether traumatic stress in humans is associated with concurrent increased amygdala volume and enhanced amygdala-dependent learning remains unclear. In the present study, we assessed amygdala morphology in trauma-exposed individuals without a diagnosis of mental disorder compared to sex- and age-matched healthy controls. **We combined volumetric analysis with multivariate surface modelling in order to precisely localize potential group differences in amygdala volume, as previously performed (e.g., Kim et al., 2012). To characterize the psychological dimensions related to trauma,** in all subjects we evaluated chronic stress, current stress, and trait anxiety. Given its **association** to experienced stress earlier reported (Zavos et al., 2012; Schmidt et al., 2000; McLaughlin and Hatzenbuehler, 2009), we additionally assessed anxiety sensitivity, **an index of subjective distress related to one's anxiety symptoms (Reiss et al., 1986).** To determine potential group differences in aversive learning, we collected skin conductance responses and ratings of valence, arousal and stimulus contingency during a cued fear

conditioning paradigm, a task which greatly depends on the amygdala (Mahan and Ressler, 2012). Finally, to provide a biological marker of chronic stress, we assessed the sensitivity of the hypothalamus-pituitary-adrenal (HPA) axis, using a low-dose dexamethasone suppression test (Yehuda et al., 1993). Based on the literature reported above, we expected to find larger amygdala volume and enhanced fear conditioning in trauma-exposed individuals compared to controls as well as alterations in HPA axis reactivity.

## **2. Material and Methods**

### **2.1 Study participants**

Eighteen healthy persons with previous trauma experience without diagnosis of PTSD or depression (TR group, 8 female), and 18 non-exposed healthy matched controls (HC group, 8 female) took part in the study. The two groups did not significantly differ in age, sex or years of education. Sample characteristics are reported in Table 1. The participants were recruited in schools for rescue ambulance workers located in Baden-Württemberg and Rheinland-Pfalz, Germany, and were part of a larger sample monitored within the context of a longitudinal study on the consequences of stress exposure. Previous studies have documented that rescue workers are at higher risk for a later onset of stress-related mental disorders, due to the frequent traumatic situations they experience on a regular basis (McFarlane et al., 2009). Trauma exposure was evaluated using the Post-traumatic Stress Diagnostic Scale (Foa et al., 1997) and the Clinician-Administered PTSD scale (Blake et al., 1995), assessing: a) the presence of a traumatic event in the life of the person; b) the nature of the traumatic exposure; c) the severity of the trauma and d) the timing of the traumatic exposure. Exclusion criteria were gross brain structural abnormality and the presence of mental disorders such as major

depressive disorder, chronic or acute substance abuse, schizophrenia, or borderline personality disorder, as assessed with the German version of the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders-IV (Wittchen et al., 1997). Subjects were assigned to the TR group when they had experienced a traumatic event in their life, according to the diagnostic criteria for trauma experience provided by DSM-IV-TR (American Psychiatric Association, 2000). In the TR group, all participants experienced a single traumatic exposure (type 1 trauma), which occurred on average 7.41 (standard deviation = 5.55) years before the experiment. Of the traumas, 41.2% referred to a severe vehicle accident, 29.4% to traumatic loss of a loved one, 23.5% to domestic violence, and 5.9% to childhood abuse. All subjects provided written informed consent to participate in the study. The study protocol was approved by the Ethics Committee of the Medical Faculty Mannheim of the University of Heidelberg, and conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki, sixth revision, 2008).

## 2.2 Questionnaires

We used the Trier Inventory of Chronic Stress (Schulz and Schlotz, 1999) to assess chronic stress and the Daily Hassles Scale-R (Kanner et al., 1981) for the evaluation of current stress. The German version of the State-Trait Anxiety Inventory G-form X2 (Laux et al., 1981) and the Anxiety Sensitivity Index (Reiss et al., 1986) were used to assess trait anxiety and anxiety sensitivity.

## 2.3 Magnetic resonance imaging protocol and volumetric assessment

Structural magnetic resonance imaging (MRI) was performed with a 1.5 Tesla full body scanner (Siemens Magnetom VISION, Siemens GmbH Erlangen, Germany). A T1-weighted high resolution 3D Fast Low Angle Shot (FLASH) sequence was implemented to collect structural whole-head volumes (echo time [TE] = 5 ms, repetition time [TR] = 15 ms, field of view [FoV] = 220x220 mm, matrix 256x256x1, slice thickness = 1mm with no gap, flip angle = 30°, voxel size = 0,86 mm x 0,86 mm x 1 mm). Additionally, a T-2 weighted Turbo Spin Echo sequence

was used in two consecutive scanning sessions, which were subsequently reduced to a unique dataset by fusing the images and reducing the gap to zero (TE = 54ms, TR = 7280ms, FoV 220x220 = mm, matrix = 256x256x1, slice thickness = 2mm, slice gap = 2mm, flip angle = 180°, voxel size = 0,86 x 0,86 x 4mm). All images were three-dimensionally assembled and resampled to a 1mm<sup>3</sup> voxel size applying a tri-linear interpolation algorithm. Borders of the amygdalae were manually outlined using the software BRAINS2 (Magnotta et al., 2002). Tracing was performed in native space by a trained operator (R.C.), who was blind to the experimental conditions. The segmentation protocol was developed based on previously published standardized guidelines (Nacewicz et al., 2006; Pruessner et al., 2000) and it is described in detail in the online Supplementary Material. To control for individual brain size, prior to statistical analysis individual amygdala volumes were divided by total brain volume and multiplied by a factor 10<sup>3</sup>.

#### 2.4 Total brain volume

Total brain volume (sum of gray and white matter) was assessed with the Brain Extraction Tool (Smith, 2002) and the Automated Segmentation Tool (Zhang et al., 2001), from the Functional Magnetic Resonance Imaging of the Brain (FMRIB) software library (<http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/>).

#### 2.5 Amygdala shape analysis

Shape analysis was based on the use of spherical harmonic basis functions and conducted with the SPHARM-PDM toolbox (<https://www.nitrc.org/projects/spharm-pdm>). The shape analysis algorithm used in this study was proposed and described in detail by Styner et al. (2006). We used default parameters, as described by the authors. Binary images of the manually segmented amygdalae were pre-processed to fill small holes and minimally smoothed in order to ensure spherical topology. Afterwards, SPHARM-PDM generated a surface mesh. A triangulation mesh was then generated from each binary image and mapped to a unit sphere with 4002 surface vertices. The SPHARM coefficients were computed from

the mesh and its parameterization, using straightforward fitting to spherical harmonic functions up to the 15<sup>th</sup> degree. This cut-off was described in a previous study as optimal for studying the amygdala (Chung et al., 2010). The correspondences across all surfaces were established by aligning the first order ellipsoids of all the parameterizations. All parameterizations were sampled into triangulated meshes, which were spatially aligned by using a rigid transformation. In order to correct for total brain volume, the surfaces were scaled accordingly.

## 2.6 Cued fear conditioning paradigm

The study design was identical to the one described in Cacciaglia et al. (2015). Briefly, we used a delay cued aversive conditioning paradigm that consisted of four different phases (habituation, early and late acquisition, extinction). Two distinct geometric figures served as conditioned stimuli (CS+ and CS-), while a painful but tolerable electric stimulation produced by an electric stimulus generator (Digitimer, DS7A, Welwyn Garden City, UK), served as unconditioned stimulus (US). The US was applied to the participants' right thumb through a cupric (copper) electrode and the level of stimulation was individually determined. During habituation 10 CS+, 10 CS-, and 4 US trials were presented, where the CS+ and CS- occurred without any temporal contingency with the US. During early acquisition, 18 CS+ and 18 CS- trials followed in a pseudorandom order, with the constraint that no more than 3 CS of one type (e.g., 3 CS+) could appear in sequence. Of the 18 CS+, 9 were paired with the US (CS<sub>paired</sub>, 50 % reinforcement), while the remaining 9 CS+ were not reinforced (CS<sub>unpaired</sub>). The 9 CS<sub>paired</sub> co-terminated with the US (delay conditioning), while the CS- was never coupled to the US. The late acquisition phase had a similar structure as the early acquisition, with the same number of stimuli. Finally, during extinction, 18 CS+ and 18 CS- trials were again presented, while the US was omitted. The fear conditioning paradigm is described in detail in the online Supplemental Material. Skin conductance responses (SCRs) were recorded with VarioPort (BECKER MEDITEC, Karlsruhe, Germany) at a sampling rate of 16 Hz, using 13mm Ag/AgCl electrodes placed on the thenar and hypothenar eminence of the participants' left hand, on which 0.5 Volts current was applied. SCRs analysis was performed on averaged

electrodermal activity computed across 1"- 9" from stimulus onset, as recommended in previously published guidelines (Pineles et al., 2009). Data from 3 participants in the TR and 4 in the HC group could not be processed due to technical problems, and were replaced with the group mean values. Immediately after each experimental phase, the participants were asked to rate arousal and emotional valence of the two CSs using the self-assessment manikin (SAM) (Bradley and Lang, 1994). SAM scores were subsequently transformed to a numerical scale ranging from 1 to 9 points. CS-US contingency awareness was also evaluated immediately after each phase and was assessed by asking "how likely will this colored shape be followed by the painful stimulation". Participants had to move a cursor on a 10-point scale ranging from "extremely unlikely" to "extremely likely" by using an ergonomic portable keyboard connected to a PC workstation.

## 2.7 Salivary cortisol assessment

Salivary cortisol was assessed on two consecutive days at nine different time-points, using sterile cotton salivettes, which study participants held in the mouth for about one minute. The subjects were instructed to take the first cotton probe as soon as they would wake up, and then after 30', 45', 60', at 11:00h, 13:00h, 15:00h, 18:00h, 20:00h. This sampling strategy allows to quantify a full day cortisol profile, and therefore can return potential group differences in separate time-windows of the day (cfr., Törnhaage, 2009).

The sampling swabs were stored in a tube with an electric monitoring cap (MEMS, Aardex Ltd., Switzerland) that registered the exact time the tube was opened and swabs were removed. Subjects were instructed to take an oral dose of dexamethasone (0.5 mg) at 11:00 pm of day one, and to repeat the same measurement protocol on the day after. Concentrations of salivary free cortisol were measured using a commercially available chemiluminescence-immuno-assay (CLIA; IBL, Hamburg, Germany).

## 2.8 Statistical analyses

Statistical analyses were conducted using the Predictive Analytic Software for Windows, v.18.0.1 (PASW, SPSS Inc., Chicago, IL), except for the amygdala multivariate shape analysis, which is described below. Normality of distributions was inspected using the Kolmogorov-Smirnov test, which yielded nonsignificant results for all the variables assessed. Group differences in the psychological variables were assessed with independent-samples *t*-tests. In order to detect potential group differences in amygdala volume, a one-way analysis of variance (ANOVA) including group as fixed between-subjects factor and corrected volumes of total, right or left amygdala separately as dependent variables, was performed. To control for its potential influence, sex was included as covariate. To further disentangle the behavioral predictors of amygdala volume, we entered scores of chronic stress, current stress, trait anxiety and anxiety sensitivity as independent variables in a backward linear regression analysis.

In order to detect potential significant amygdala shape differences between groups, we used the non-parametric multivariate Hotelling's  $T^2$  statistics, which calculates mean differences among centroids of the generated meshes, and further corrected for multiple testing using a false discovery rate (FDR) approach. To determine the effectiveness of our conditioning paradigm and to test for potential group difference in fear conditioning, a repeated measures ANOVA including CS-type (2 levels: CS+<sub>unpaired</sub>, CS-) and phase (4 levels: habituation, early and late acquisition, extinction) as within-subject factors and group as between-subject factor, was performed. Upon significant 3-way interaction involving the factor group, we conducted Bonferroni corrected independent samples *t*-tests on the differential SCRs value (CS+<sub>unpaired</sub> - CS-) separately for each conditioning phase. Ratings of stimulus contingency, arousal, and emotional valence were analyzed similarly as the SCRs, with CS-type and phase as within-subject factors and group as between-subject factor.

To determine the effectiveness of the DEX-test and to detect potential group differences in salivary cortisol, we performed a repeated measures ANOVA, including day (2 levels) and time-points (9 levels) as within-subjects factors and group as between-subject factor. As a summary indicator of salivary cortisol, we computed the area under the curve both across the

whole day (total area under the curve: tAUC), and across the first four time-points (morning area under the curve: mAUC), using the following formula:

$$AUC_G = \sum_{i=1}^{n-1} \frac{(m_{(i+1)} + m_i) \cdot t_i}{2}$$

with  $t_i$  denoting the individual time distance between measurements,  $m_i$  the individual measurement and  $n$  the total amount of measures (Pruessner et al., 2003). A Bonferroni-corrected independent samples  $t$ -tests was then performed including group as independent factor and tAUC or mAUC as dependent variables. Relationships between amygdala volumes and conditioning as well as cortisol data were assessed with 2-tailed Pearson correlation analyses. For both repeated measures ANOVAs conducted on conditioning and cortisol data, confidence intervals were adjusted using Bonferroni correction and a Greenhouse-Geisser correction was applied in case of violation of sphericity.

### 3. Results

#### 3.1 Questionnaire data

Table 1 displays the results of the assessed psychological variables. The two groups did not significantly differ in anxiety sensitivity. However, compared to the HC, the TR group reported significantly higher levels of chronic and current stress as well as trait anxiety.

#### 3.2 Amygdala volume

Total brain volume did not significantly differ between the TR and the HC group ( $F_{1,35} = 0.06$ ,  $P = 0.81$ ). Compared to HC, TR showed significantly larger total amygdala volume ( $F_{1,35} = 4.23$ ,  $P = 0.04$ ). This result was significant for the left ( $F_{1,35} = 9.16$ ,  $P = 0.005$ ), but not for the right amygdala ( $F_{1,35} = 0.91$ ,  $P = 0.34$ ) (Fig. 1a). Sex was not significantly related to either left ( $F_{1,35} = 0.01$ ,  $P = 0.98$ ), or right amygdala volume ( $F_{1,35} = 0.73$ ,  $P = 0.41$ ). Backward linear

regression revealed one significant model after having excluded the non-significant variables ( $F_{3,35} = 3.85$ ,  $P = 0.01$ ), and identified anxiety sensitivity as a predictor of total amygdala volume (standardized  $\beta$ -value = 0.39,  $t_{35} = 2.51$ ,  $P = 0.02$ ) (Fig. 1b).

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Insert Fig. 1 about here  
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### 3.3 Amygdala shape analysis

Fig. 2 illustrates the grand averages of the three-dimensional mesh reconstruction for both the right and the left amygdalae, while the Hotelling's T-square test is projected over the shapes. As shown in the color-code map, the group difference in volume was accounted for by a focal variation in the lateral and anterior portions of the left amygdala ( $P_{\text{uncorrected}} < .01$ ) (Fig. 2a). After applying the FDR correction, a smaller region of the left lateral amygdala remained significant ( $P_{\text{FDR-corr}} < 0.01$ ) (Fig. 2b). Shape analysis for the right amygdala did not reveal any significant results.

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Insert Fig. 2 about here  
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### 3.4 Cued fear conditioning

#### 3.4.1 *Skin conductance responses*

Fig. 3a and 3b show the group mean skin conductance responses (SCRs) across the four phases of the conditioning paradigm. Repeated measures ANOVA revealed significant main effects of CS-type ( $F_{1,34} = 42.19$ ,  $P < 0.001$ ) and phase ( $F_{3,102} = 4.27$ ,  $P = 0.02$ ) as well as a

significant CS-type x phase interaction ( $F_{3,102} = 3.24$ ,  $P = 0.04$ ), indicating that across the four phases, both groups showed significantly different electrodermal responses to the CS+<sub>unpaired</sub> compared to the CS-. We also found a significant CS-type x phase x group interaction ( $F_{3,102} = 7.88$ ,  $P = 0.001$ ), indicating that such differences in the SCRs were related to group membership. In either group, the SCRs did not significantly differ between CS+<sub>unpaired</sub> and CS- during habituation (HC group:  $t_{34} = -0.08$ ,  $P = 0.93$ ; TR group:  $t_{34} = 0.61$ ,  $P = 0.54$ ), but did significantly differ during early acquisition (Acq1) (HC group:  $t_{34} = 3.12$ ,  $P = 0.004$ ; TR group:  $t_{34} = 3.29$ ,  $P = 0.003$ ). During late acquisition (Acq2) and extinction (Ext), the HC did not show significant differences in SCRs (Acq2:  $t_{34} = -0.96$ ,  $P = 0.34$ ; Ext:  $t_{34} = 0.48$ ,  $P = 0.62$ ), while the TR continued to significantly differentiate CS+<sub>unpaired</sub> and CS- (Acq2:  $t_{34} = 5.09$ ,  $P < 0.001$ ; Ext:  $t_{34} = 5.11$ ,  $P < 0.001$ ). Compared to the HC, the TR group showed significantly higher differential SCRs during late acquisition ( $t_{34} = 5.23$ ,  $P < 0.001$ ) and extinction ( $t_{34} = 4.58$ ,  $P < 0.001$ ) (Fig 3c). This group difference was explained by a higher SCR exhibited by the TR group only to the CS+<sub>unpaired</sub> in both phases (Acq2:  $t_{34} = 5.66$ ,  $P < 0.01$ ; Ext:  $t_{34} = 4.96$ ,  $P < 0.01$ ), while not showing a lower SCR for the CS- (Acq2:  $t_{34} = 0.43$ ,  $P = 0.67$ ; Ext:  $t_{34} = 0.56$ ,  $P = 0.58$ ). The greater differential SCR values during the extinction phase in the TR compared to the HC group appeared to depend mainly on the last extinction trials (Supplementary Figure 3).

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Insert Fig. 3 about here  
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### 3.4.2 Verbal reports

For ratings of CS-US contingency, we found significant main effects of CS-type ( $F_{1,34} = 131.93$ ,  $P < 0.001$ ) and phase ( $F_{3,102} = 50.82$ ,  $P < 0.001$ ) as well as a significant CS-type x phase interaction ( $F_{3,102} = 57.19$ ,  $P < 0.001$ ). For arousal ratings, we observed a significant main effect of CS-type ( $F_{1,34} = 5.14$ ,  $P = 0.03$ ) but not phase ( $F_{3,102} = 1.16$ ,  $P = 0.33$ ), although the CS-type

x phase interaction was significant ( $F_{3,102} = 5.29, P = 0.002$ ). Finally, for emotional valence, we found significant main effects of CS-type ( $F_{1,34} = 23.49, P < 0.001$ ) and phase ( $F_{3,102} = 9.21, P < 0.001$ ) as well as a significant CS-type x phase interaction ( $F_{3,102} = 15.74, P < 0.001$ ). The 3-way interaction involving the factor group was not significant in any of the three rating categories, therefore we did not further investigate possible groups differences in the ratings.

### 3.4.3 Relationship between amygdala volume and conditioning data

We did not find any significant relationship between the volumes of either right or left amygdala and differential SCRs elicited during early acquisition (right amygdala:  $r_{34} = -0.18, P = 0.29$ ; left amygdala:  $r_{34} = -0.16, P = 0.36$ ), late acquisition (right amygdala:  $r_{34} = 0.17, P = 0.33$ ; left amygdala:  $r_{34} = 0.30, P = 0.07$ ), or extinction (right amygdala:  $r_{34} = 0.04, P = 0.84$ ; left amygdala:  $r_{34} = 0.30, P = 0.08$ ) phases. Similarly, there were no significant correlations between amygdala volumes and ratings of arousal, valence or CS-US contingency.

### 3.5 Baseline salivary cortisol

Fig. 4a shows the baseline salivary cortisol profile on day one across the nine measurement points, before DEX administration. A significant main effect of time-point was found ( $F_{8,272} = 80.96, P < .001$ ), which did not significantly interact with group ( $F_{8,272} = 1.81, P = 0.15$ ), indicating that the amount of endogenous salivary cortisol significantly varied across the nine measurements, independently of group membership. We did not find significant group differences in the total area under the curve (tAUC) ( $t_{34} = -1.95, P = 0.06$ ) or in the morning area under the curve (mAUC) ( $t_{34} = -0.15, P = 0.87$ ), although for the tAUC the result just missed significance, possibly suggesting a higher salivary cortisol concentration in the TR compared to the HC group. For baseline salivary cortisol, no significant correlations were found between amygdala volumes and tAUC or mAUC in either group.

### 3.6 Dexamethasone suppression test

Fig. 4b depicts the time course of suppressed salivary cortisol on day two. Significant main effects of day ( $F_{1,34} = 252.03$ ,  $P < 0.001$ ) and day x time-point interaction ( $F_{8,272} = 67.61$ ,  $P < 0.001$ ) were detected, indicating that dexamethasone significantly reduced endogenous salivary cortisol, and this effect was time-point dependent. Tests of the within-subjects contrasts showed a significant time-point x group interaction for the first (+30' vs. awakening) and the fifth (1:00 pm vs. 11:00 am) contrast ( $F_{1,34} = 5.64$ ,  $P = 0.02$ ;  $F_{1,34} = 5.31$ ,  $P = 0.03$ ). Compared to the HC, the TR group showed significantly higher tAUC ( $t_{34} = -2.29$ ,  $P = 0.03$ ) and mAUC ( $t_{34} = -2.21$ ,  $P = 0.04$ ) values. Across groups, no significant correlations were found between amygdala volume and tAUC. However, across groups the volume of the left amygdala significantly positively correlated with mAUC ( $r_{36} = 0.39$ ,  $P = 0.02$ ).

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Insert Fig. 4 about here  
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#### 4. Discussion

In the present study we sought to determine whether the presence of a past traumatic event is associated with altered amygdala morphology and abnormal fear conditioning in a group of healthy young individuals (TR group) compared to matched non-exposed controls (HC group). **Our questions were motivated by previous data reported in animal models of traumatic stress which consistently indicated a persisted basolateral amygdala hypertrophy (Vyas et al., 2002, 2006; Cui et al., 2008; Padival et al., 2013) as well as greater fear conditioning (Marks et al., 2015; Monsey et al., 2014; Hui et al., 2004) following the administration of different experimental stressors. Therefore, we hypothesized to find larger amygdala volumes and enhanced acquisition of conditioned fear in the TR compared to HC group. In order to provide a biological marker of chronic stress and given the importance of the amygdala in modulating the stress response system (Joëls**

**and Baram, 2009), we also examined the sensitivity of the HPA axis and its relationship with amygdala volume.**

First, we have reported significantly higher scores of chronic stress, current stress and trait anxiety in the TR compared to the HC group. In line with our hypothesis, the TR compared to the HC group showed a significantly larger total amygdala volume. This result was dependent on the left but not the right amygdala. Additionally, we observed that across subjects, anxiety sensitivity significantly predicted total amygdala volume. Anxiety sensitivity indexes the level of subjective distress related to one's anxiety symptoms, thus providing a measure of cognitive bias towards feelings of anxiety. Previous research has shown that anxiety sensitivity positively relates to the occurrence of lifetime stressors, and has been regarded as a mechanism linking stressful life events to the development of anxiety disorders and potentially depression (Zavos et al., 2012; Schmidt et al., 2000; McLaughlin and Hatzenbuehler, 2009). Although we did not find a direct significant relationship between amygdala volume and chronic or current stress, the relationship with anxiety sensitivity potentially indicates that the observed volumetric group difference was related to experienced stress.

Using multivariate surface modeling, we could determine that the volumetric group difference was accounted for by a focal variation in the lateral portion of the left amygdala. We also detected a non-corrected significant volumetric difference in the anterior portion of the left amygdala, which most likely overlaps with the basolateral nuclei, as reported by histological and imaging studies of the human brain (Sah et al., 2003; Tamburo et al., 2009).

Our morphometric data confirm previously reported results on the increased dendritic arborization of the lateral and basolateral amygdala in rodents exposed to prolonged stress (Vyas et al., 2002, 2006; Padival et al., 2013), single stress (Cui et al., 2008) or corticosterone administration (Mittra and Sapolsky, 2008), and extend such findings to human participants. Our data are also in agreement with studies conducted in humans which documented larger amygdala volume in healthy adolescents who experienced chronic stress due to prolonged hospitalization (Metha et al., 2009; Tottenham et al., 2010) and in children exposed to adverse rearing conditions (Lupien et al., 2011). Another study recently reported larger amygdala

volume associated with total number of negative life events in healthy humans (Gerritsen et al., 2015), which further supports our data. Other studies investigated amygdala volume in trauma-exposed individuals without PTSD or depression, reporting larger left amygdala volume in traumatized persons compared to controls without, however, reaching significance (Hara et al., 2008; Schmahl et al., 2009), although in the study of Schmahl and colleagues (2009) trauma-exposed persons had comorbid borderline personality disorder.

The molecular mechanisms through which stress affects neuronal morphology in the amygdala are not yet fully understood, although a prominent role is played by a glucocorticoid-driven expression of brain-derived neurotrophic factor (Bennett and Lagopoulos, 2014) together with other neurochemical modulators including endocannabinoids (Hill et al., 2009) that act under epigenetic control (McEwen et al., 2015).

Although our subjects were not diagnosed with any mental disorder, the increased amygdala volume resembles changes in brain morphology that are similar to that observed in patients with depression. Indeed several studies documented larger amygdala volumes in depressed patients (van Eijndhoven et al., 2009; Weniger et al., 2006; Frodl et al., 2002; Lange and Irle, 2004), especially in early stages of the disorder (Lorenzetti et al., 2009). While we cannot exclude that the TR group had larger left amygdala volume already prior to the trauma, it is likely that the increased amygdala volume represents one mechanism through which traumatic stress confers vulnerability for depression. This interpretation is supported by our salivary cortisol data. The enhanced baseline cortisol amount we found in both groups within the first hour after awakening is consistent with the typical response pattern known as cortisol awakening rise (CAR), which has previously been reported in humans (Kudielka et al., 2007; Lasikiewicz et al., 2008; Pruessner et al., 1997). We did not find significant differences between the groups in baseline salivary cortisol secretion. However, after the DEX-test, the TR compared to the HC group showed a significantly reduced cortisol suppression, indexed by greater morning and total areas under the curve. This finding matches previous data on salivary cortisol hypo-suppression to exogenous corticosteroids in traumatized people without psychiatric disorders (e.g., Carpenter et al., 2009). Our data also resemble those reported in

patients with major depressive disorder, who show cortisol hypo-suppression in response to DEX administration (Yehuda et al., 2004; Holsboer, 2000; Holsboer et al., 1995). On the other hand, people suffering from PTSD show opposite patterns of response characterized by a cortisol hyper-suppression in response to DEX (Griffin et al., 2005; Yehuda et al., 1993). Thus, taken together, our data on increased amygdala volume and cortisol hypo-suppression in the TR compared to the HC group, point to a neuroendocrinological profile similar to that reported in patients with depression. This is further substantiated by the significant positive correlation we found between left amygdala volume and the suppressed morning area under the curve (mAUC). This latter finding, besides confirming the existence of an excitatory drive exerted by the amygdala on HPA axis negative feedback (Joëls and Baram, 2009), suggests that the two variables account for a unique dimension potentially acting as biological risk for depression.

On the basis of previous literature reporting increased fear conditioning in stressed rodents (Marks et al., 2015; Monsey et al., 2014; Hui et al., 2004), we assessed conditioned fear and extinction learning using a cue delay conditioning paradigm. We found that the TR showed enhanced late acquisition and reduced extinction compared to the HC group, as determined with SCRs. Since previous studies reported SCRs to rapidly decrease during within-session extinction in healthy humans (Birbaumer et al., 2005; Milad et al., 2010), we interpret our findings in terms of deficient extinction learning manifested by the TR group. The increased differential SCRs we found in the TR group during late acquisition, together with the nonsignificant group differences in CS-US contingency learning, is consistent with our recent findings reporting the impact of amygdala volume on the SCRs but not on declarative learning in healthy people who underwent fear conditioning (Cacciaglia et al., 2015). **This is further indicative of a dissociation between autonomic responses and verbal reports of conditioning with respect to amygdala volume, and suggests that SCRs and contingency awareness represent two independent learning processes which may differentially contribute as liability factors to psychopathology.**

Our conditioning data match recent observations of enhanced fear acquisition and impaired fear extinction in subjects with high levels of anxiety (Acheson et al., 2015). On the other hand,

deficient extinction learning has previously been reported in PTSD patients (Peri et al., 2000; Wessa and Flor, 2007). The literature on extinction learning in depression is mixed, with one study reporting deficient (Dibbets et al., 2015) and another enhanced extinction (Kuhn et al., 2014). Thus, our SCR data are more consistent with previously reported data on patients with PTSD.

It should be noted that our conditioning results diverge from two studies where healthy trauma-exposed persons reported similar SCRs compared to non-exposed controls during aversive conditioning (Blechert et al., 2007; Milad et al., 2009). However, in those studies, a substantially different design was employed. In particular, the conditioning procedure used by Milad et al. (2009) included pictures with varied spatial context, rather than classical cued delay conditioning, as used here. In the study of Blechert et al. (2007) the participants' age range was considerably different with respect to ours, having twice the mean age of our sample, which might have affected associative learning mechanisms underlying conditioning (Rogers and Gilbert, 1997).

We did not find significant relationships between volumes of either the right or left amygdala and differential SCRs in any conditioning phase, although the left amygdala volume was positively associated to SCRs in both late acquisition and extinction on a trend level. This negative result was somehow unexpected, given that we previously reported an association between amygdala volume and SCRs during cued fear conditioning (Cacciaglia et al., 2015). However, unlike our previous work, the present study includes people who suffered a traumatic experience, and this might have affected the relationship between the two variables. Moreover, the sample size of the current study is considerably smaller than the previous one.

This study has several limitations. First, with the employed methodology we could not clearly establish a temporal course of the observed effects. For example, we could not determine whether the enlarged volume of the left amygdala was affected by the deficient inhibition of the HPA axis, or vice versa. To this aim, longitudinal studies are required as well as more extensive animal research. Second, while we focused solely on the amygdala morphological aspects, other brain regions are critically implicated in the stress-response system, such as the

hippocampus and the medial prefrontal cortex (Joëls and Baram, 2009; McEwen et al., 2016). Therefore, a systematic investigation of the temporal course of hippocampal and cortical prefrontal morphology after trauma exposure is of great relevance and should be addressed thoroughly. Another limitation of our study is that we did not assess childhood trauma, a factor that is potentially associated with altered brain morphology and variability in fear learning (Lanius et al., 2010), therefore we cannot draw conclusions on a potential effect of this variable in our study. Future studies should take this variable into consideration.

Taken together, our results display multiform alterations in trauma-exposed individuals, which resemble those previously observed in patients suffering from depression and PTSD.

As already proposed previously (Goldsmith et al., 2013; Badour and Feldner, 2013; Ehring and Quack, 2010), we suggest that that traumatic stress may result in maladaptive emotional responses, which may in turn, depending on additional genetic and environmental factors, ultimately precipitate anxiety disorders, depression, or both (Bardeen et al., 2013; Elhai et al., 2011). The two diagnoses are in fact often comorbid (Gorman, 1996; Ressler and Mayberg, 2007) and are thought to share partially common pathophysiological mechanisms (Boyer, 2000; Goodwin, 2015). In support of this, previous structural MRI findings have reported similar changes in anxious and depressed patients (van Tol et al., 2010).

Thus, our data suggest that traumatic stress can serve as biological priming for consequent psychopathology, representing a risk factor over time, possibly depending on genetic predisposition. Elucidating the mechanisms that link stress exposure to consequent psychopathology will help to devise specific treatment needs for the affected patients.

### **Conflict of interest**

(Manuscript title: **Trauma exposure relates to heightened stress, altered amygdala morphology and deficient extinction learning: implications for psychopathology**, by R. Cacciaglia et al.)

All co-authors agree with the contents of the manuscript and there is no financial interest to report.

### **Contributors**

(Manuscript title: **Trauma exposure relates to heightened stress, altered amygdala morphology and deficient extinction learning: implications for psychopathology**, by R. Cacciaglia et al.)

Raffaele Cacciaglia wrote the manuscript, collected and analyzed the data

Frauke Nees, analyzed the data

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Slawomira J. Diener, collected the data

Claudia Liebscher, collected the data

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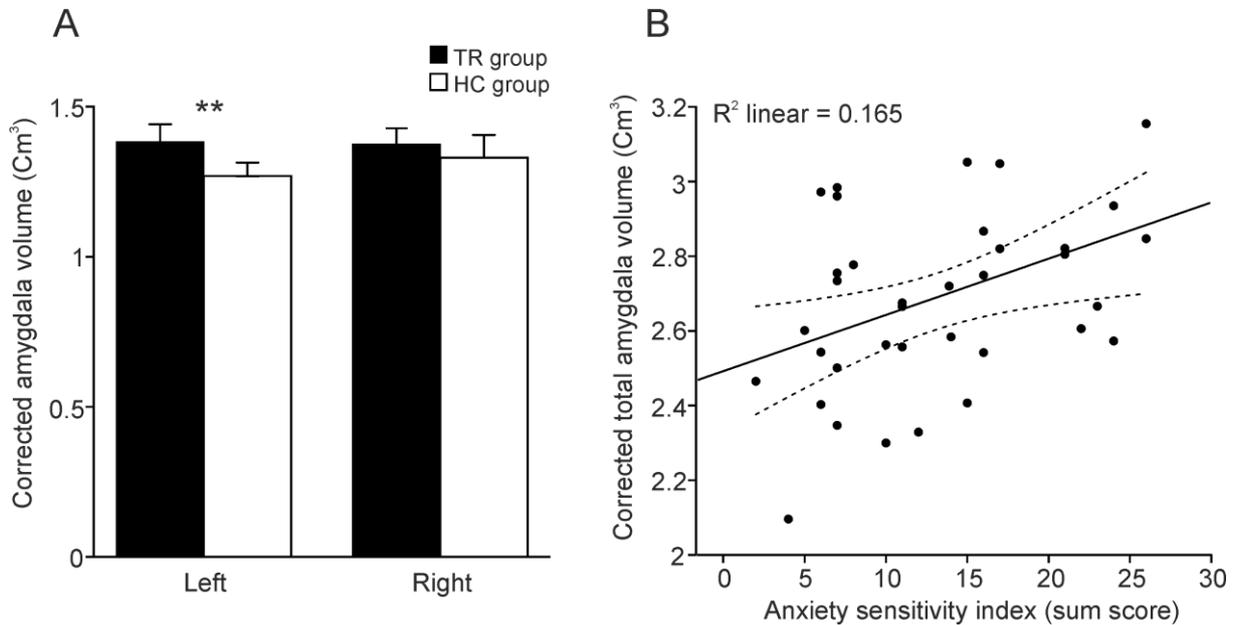
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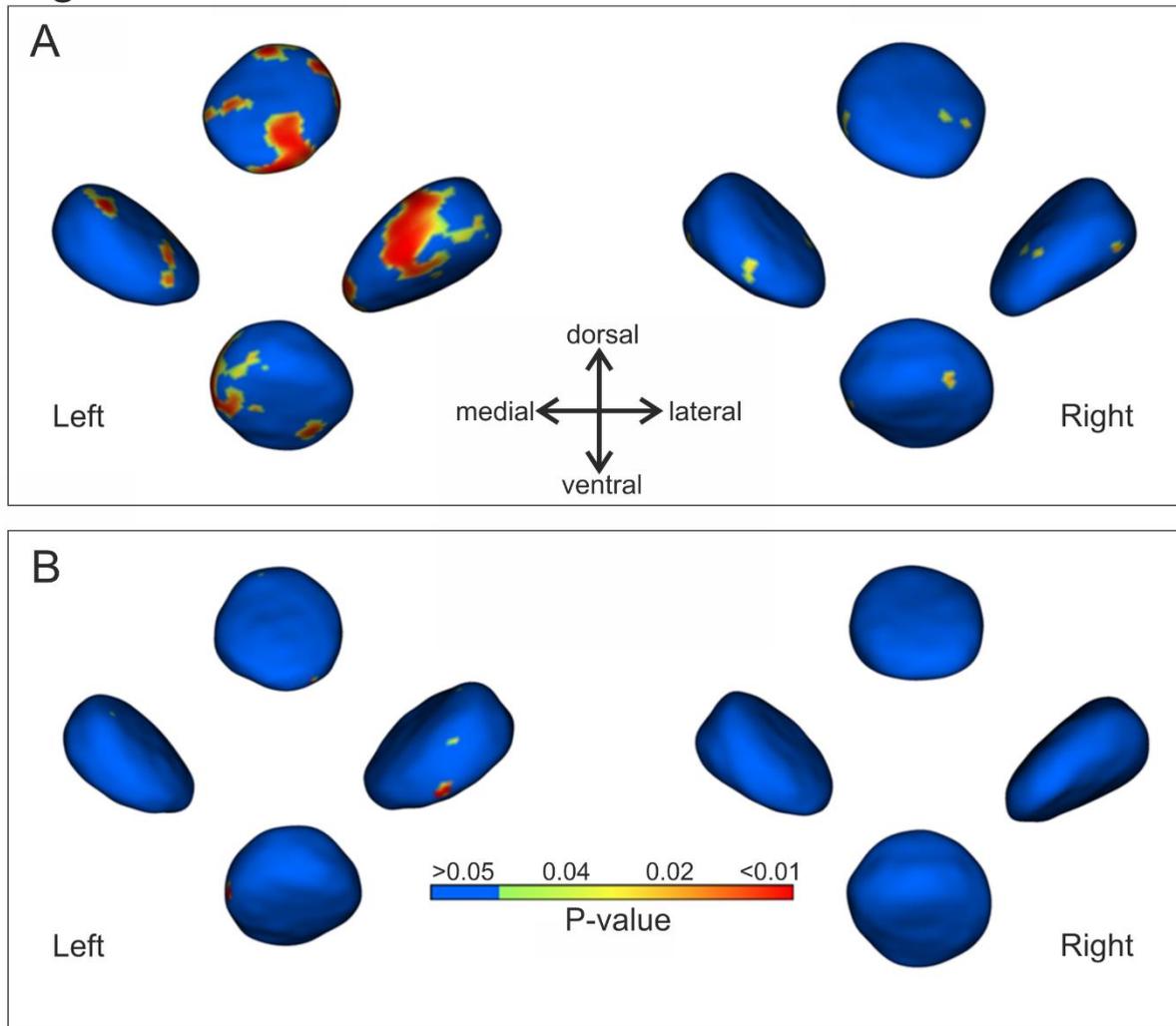
## Figure captions

Fig.1

**Figure 1. Amygdala volume in trauma-exposed individuals and non-exposed controls**

**A)** Left, but not right amygdala volume was significantly larger in trauma-exposed persons (TR group), compared to non-exposed healthy controls (HC group). \*\*indicates significance level  $P < 0.01$ . Error bars indicate standard error of the mean (s.e.m.) **B)** Across groups, scores of Anxiety Sensitivity significantly predicted total amygdala volume.

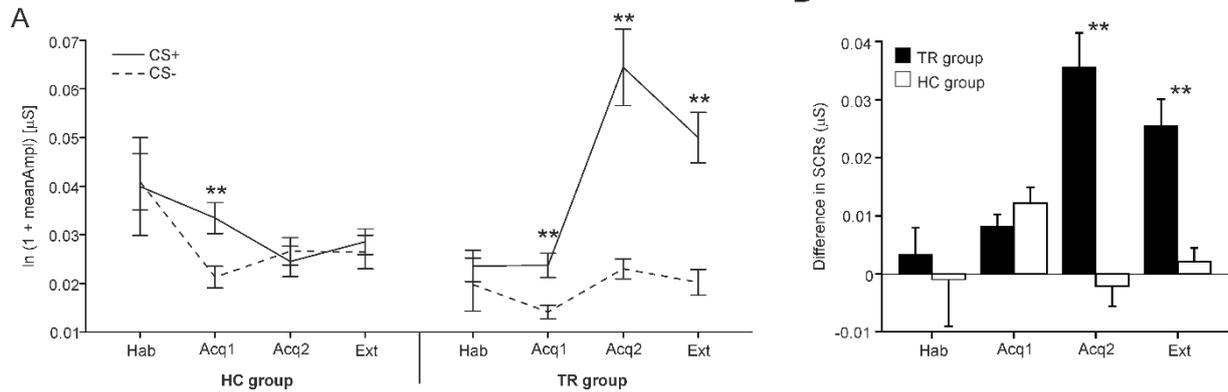
Fig. 2



**Figure 2. Grand average of the three-dimensional mesh reconstruction for the right and the left amygdala.** Prior to the shape analysis, the average amygdala mesh was computed for each group, and an overall average structure was computed over the two groups, which was then used in the shape analysis as the template object. The shape analysis was then computed by testing the local distances at every boundary point. A color-code significance map is projected over the shape, using the non-parametric Hotelling's  $T^2$  statistics. Colored areas indicate P-values < 0.05, with warmer colors showing smaller P-values. Blue indicates  $P > 0.05$ . **A)** P-value surface maps showing patterns of significant group difference between HC and TR using uncorrected statistical threshold. **B)** P-value surface maps showing patterns of significant group difference between HC and TR, after correcting for multiple testing using a

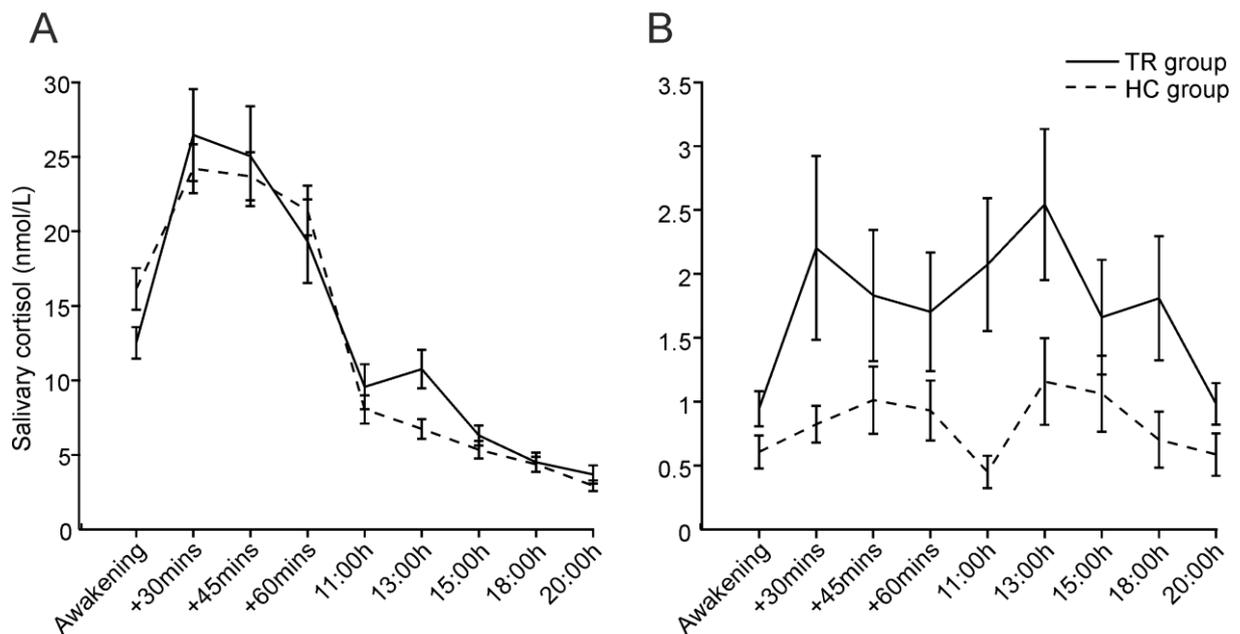
false discovery rate (FDR) approach. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

Fig.3



**Figure 3. Skin conductance responses during the fear conditioning paradigm and relationship with amygdala volume. A)** Group mean skin conductance responses (SCRs) are shown separately for the HC and the TR group during the four phases of the conditioning paradigm (Hab: habituation; Acq1: early acquisition; Acq2: late acquisition phase; Ext: extinction). The HC group showed a significant difference between the mean SCRs for CS+<sub>unpaired</sub> and those for CS- in the early acquisition phase, while not showing significant differences for the remaining phases. The TR group displayed significantly different SCRs between the CS+<sub>unpaired</sub> and CS- for Acq1, Acq2, as well as for Ext. **B)** Arithmetic differences between SCRs values computed for CS+<sub>unpaired</sub> and CS-. During habituation and early acquisition, no significant differences emerged between the two groups. However, compared to the HC, the TR group displayed significantly higher differential SCRs during Acq2 and Ext, suggesting enhanced aversive learning and deficient extinction. \*\*indicates significance level  $P < 0.01$ . Error bars indicate standard error of the mean (s.e.m.)

Fig. 4



**Figure 4. Salivary cortisol before and after a dexamethasone suppression test**

- A)** Baseline salivary cortisol daily profile on day one. No significant differences emerged between the groups in the area under the curve, computed across the whole day (tAUC) or across the morning (first four time-points, mAUC). **B)** Salivary cortisol after dexamethasone suppression test on day two. Compared to HC, the TR group showed significantly higher tAUC and mAUC. Error bars represent standard error of the mean (s.e.m.)

**Table 1 - Sample characteristics**

	Total Sample		TR group		HC group		<i>t</i> -value	<i>P</i> -value
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>		
Age <sup>1</sup>	22.25	3.79	22.83	4.41	21.67	3.07	0.92	0.36
Education <sup>1</sup>	12.78	0.92	12.65	1.13	12.89	0.68	0.86	0.48
TICS	2.42	0.48	2.64	0.33	2.19	0.53	3.01	0.005**
DHS	12.58	7.93	15.8	9.92	9.38	5.51	2.85	0.007**
STAI	34.95	7.97	38.41	8.84	31.05	5.66	2.62	0.02*
ASI	13.08	6.85	13.88	7.06	12.28	6.95	0.06	0.49

TICS: Tier Inventory for Chronic Stress; DHS: Daily Hassles Scale; STAI: State-Trait Anxiety Inventory; ASI: Anxiety Sensitivity Index

TR group: trauma-exposed individuals; HC group: non-exposed healthy controls

<sup>1</sup>indicated in years

\*\*indicates significance level  $p < 0.01$

\*indicates significance level  $p < 0.05$