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Glucocorticoid receptor alpha translational isoforms as mediators of early adversities and negative emotional states

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Abstract

Childhood trauma (CT) increases the risk for psychopathology through disturbed acquisition and extinction of fear. The effects of CT are mediated by abnormalities of the hypothalamic-pituitary-adrenal axis and glucocorticoid receptor (GR). Since, the alterations in GR α translational isoforms have been documented in psychiatric disorders we sought to: 1) explore whether multiple GR α isoforms in the human peripheral blood mononuclear cells of two independent cohorts (whole cell $n = 40$; and nuclear extracts $n = 43$, adult subjects) mediate the effect of CT on negative affectivity (NA) measured by Depression, Anxiety and Stress Scales (DASS), and 2) examine their role/function during fear extinction in the animal model.

In multiple regression analysis, CT, nuclear 40-kDa GR α , their interactions and FKBP5 explained 22%–35% of variance in DASS scores. Structural equation modeling showed that CT had a significant direct effect on 40-kDa and DASS in both cohorts, and on the nuclear 25-kDa GR α . The association between 40-kDa and total DASS was significantly mediated by nuclear FKBP5, whereas on DASS anxiety, over FKBP5 in both cohorts and nuclear full length GR α . Nuclear 40-

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Contributors

All authors have materially participated in the research and/or article preparation, and have approved the final article. The contribution of each individual author is as follows: Miroslav Adzic designed the study, performed statistical analyses, interpreted results and wrote the paper. Milica J. Nesic, Marina Mihaljevic, Zorana Pavlovic, Nadja P. Maric recruited human subjects, collected blood samples, performed psychopathological evaluation of human participants. Minja Milosavljevic and Zeljka Brkic performed molecular analyses of human samples. Ivan Soldatovic did statistical analyses. Emilija Glavonic, Milos Mitic, Zorica Petrovic and Ester Francija carried out all animal experiments, including behavioral procedures and molecular analyses. Emilija Glavonic and Milos Mitic contributed to interpretation of animal data, and prepared MS for publication. Jelena Radulovic and Nadja P. Maric contributed to the design of the study as well as interpretation of the results.

Conflict of interest statement

All authors declare that they have no conflicts of interest.

Ethical statement

The human study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All experimental procedures were approved by the Ethical Committee of Vin a Institute of Nuclear Sciences and in compliance with the requirements of the European Council Guide for Care and Use of Laboratory Animals (2010/63/EU).

kDa GR α and its interaction with CT had a significant direct effect on DASS anxiety. In mice, the successful extinction learning was followed by nuclear translocation of 40-kDa GR α and induction of BDNF exon IV expression.

Our data revealed that the association between CT and adult NA in non-clinical subjects is mediated by the GR α translational isoforms, in particular 40-kDa GR α , and emphasized its role in fear extinction and neural plasticity.

Keywords

Childhood trauma; Glucocorticoid receptor; Fear conditioning; Plasticity

1. Introduction

Childhood trauma (CT) has been implicated in the onset and illness course of several psychiatric disorders such as posttraumatic stress disorder (PTSD) (Widom, 1999), depression (Widom et al., 2007), anxiety disorders (Copeland et al., 2007) and psychotic illness (Khashan et al., 2008; Day et al., 1987). A recent study emphasized that experiencing CT, particularly under seven years of age, significantly moderated the subsequent response to antidepressant therapy (Williams et al., 2016). Even in healthy subjects, with or without risk for psychiatric disorders, CT has great impact on cognition and learning ability (van Os et al., 2017). Indeed, individuals with a history of CT displayed more negative implicit self-associations than subjects without a history of CT (van Harmelen et al., 2010). Furthermore, disruptions in learning processes involved in the acquisition and extinction of conditioned fear are considered to be a central mechanism in the etiology of trauma-related psychopathology (Maren and Holmes, 2016). Several studies documented that glucocorticoids via glucocorticoid receptor (GR) modulate the strength to which memory for contextual fear conditioning is established and maintained (Cordero and Sandi, 1998; de Quervain et al., 2017). In addition, early life trauma induces persistent changes in the hypothalamic-pituitary-adrenal (HPA) axis and disturbs neurodevelopment processes which could make a person vulnerable to psychiatric diseases (Ridout et al., 2016). Indeed, individuals with CT experiences may exhibit persistent neuroendocrine and anatomical changes, including: glucocorticoid insensitivity, increased central corticotropin-releasing hormone activity, immune upregulation and reduced hippocampal volume (Hornung and Heim, 2014). Thus, a clear relationship exists between early life stress, HPA axis abnormalities and depression.

Potential molecular mechanisms that mediate this relationship have been a subject of many recent studies. Literature data revealed that the biological effects of environmental exposures during early life are mediated by epigenetic mechanisms such as methylation, which enables childhood exposures to have long-lasting effects (Tyrka et al., 2016). Substantial evidence from animal and human research emphasized that methylation of genes for GR and its co-chaperone FKBP5, are highly susceptible to environmental exposures in both the prenatal period and during early childhood (Kosten and Nielsen, 2014; Kertes et al., 2016; Tyrka et al., 2012; Tyrka et al., 2015; Binder, 2009).

Complex actions of glucocorticoids (GCs), the end products of HPA axis activity, may also be attributed to the significant diversity of GR transcripts and isoforms. Besides full-length, the GR α isoforms have been found to be expressed in the human prefrontal cortex also as smaller GR α isoforms (67-, 50-, 40- and 25-kDa) (Sinclair et al., 2011a) generated as a result of alternate translation start sites in exon 2 (Lu and Cidlowski, 2005). Several lines of evidence revealed the alteration of full-length GR α levels in cortical brain of schizophrenia, bipolar disorder and major depression patients (Knable et al., 2001), as well as a greater abundance of GR α -D1 (50-kDa) isoform in schizophrenia and bipolar disorder (Sinclair et al., 2011b). Furthermore, changes in abundance of multiple GR α protein isoforms across rodent and human (Sinclair et al., 2011a) development stages have been described (Galeeva et al., 2006). Dynamic patterns of GR isoforms expression across development favour the hypothesis that windows of vulnerability to stress exist across human cortical development, indicating neonatal and adolescent periods as critical windows of stress pathway development.

Considering that further identification of mechanisms that underlie the link between CT and psychopathology as well as factors that may buffer this risk is a crucial step in order to develop targets for preventive interventions, we sought to: 1) determine if expression levels of glucocorticoid receptor α isoforms in whole cell and nuclear extracts of peripheral blood mononuclear cells (PBMC) could be associated with CT and negative emotional states in healthy adults. The advantage of using nuclear extracts lies in providing insight into whether the potential association could be assigned to nuclear GR α isoforms and GR transcriptional activity. 2) explore if and how these isoforms mediate the effect of childhood trauma on negative emotional states in non-clinical adults and 3) determine the role/function of these isoforms in fear extinction learning during mouse development.

2. Materials and methods

2.1. Human study

2.1.1. Participants—The research was performed as a pilot within a larger study entitled: “The analysis of glutamatergic and glucocorticoid signalization in persons with depression and trauma-related psychopathologies” and the sample included in this study consisted of non-clinical adult subjects, recruited separately as two independent cohorts ($n = 40$ and $n = 43$, total 83). Subjects were recruited by a local advertisement. The study was approved by the Ethics Committee of the Clinical Centre of Serbia, Belgrade, and Faculty of Medicine, University of Belgrade. All participants signed a written consent before entering the study. Subjects with past or present medical disorders and those with prescribed or non-prescribed medication on either continual or acute basis (for at least one week) were excluded. For ensuring that participants are free of any clinically psychiatric disorder, trained psychiatrists used Mini-International Neuropsychiatric Interview (MINI) for diagnosing mental disorders (Sheehan et al., 1998). After completing the interview, subjects continued with self-assessment that included sociodemographic data, Childhood Trauma Questionnaire (CTQ) and Depression Anxiety Stress Scales (DASS). Blood samples were obtained at 8 AM. None of the participants met MINI criteria for the past or present psychiatric disorder, thus their symptoms (measured by DASS) were considered

subthreshold and/or transient. All subjects in the study were Caucasian. Women were mixed with regard to menstrual phase.

2.1.2. Questionnaires—Childhood Trauma Questionnaire (CTQ) (Bernstein et al., 2003) was used to assess adversities before the age of 17. CTQ presents a retrospective self-report questionnaire that measures five types of childhood adversities: emotional abuse, physical abuse, emotional neglect, physical neglect and sexual abuse. Each domain of trauma severity is covered by five items, rated on a 5-point Likert scale (1 = never true, 5 = very often true). We summed the mean scores and divided them with appropriate number of items: all 25 items to obtain the total trauma scores (CTQ-total); 15 items for abuse to assess the total abuse score (CTQ-abuse); 10 items for neglect to measure the total neglect score (CTQ-neglect).

DASS is a 42-item instrument with a 4-point Likert-type response scale and was used to measure the negative emotional states in the last 14 days (Lovibond and Lovibond, 1995). DASS assesses depression, anxiety and stress and each of these subscales also taps a more general dimension of psychological distress (Henry and Crawford, 2005). Scores for each subscale were calculated by summing the scores of the corresponding items, and results of DASS were interpreted according to the literature (Lovibond and Lovibond, 1995). All subjects had normal to moderate DASS scores. Symptoms measured by DASS were considered as sub-clinical and/or transient (in line with dimensional approach to psychopathology and the concept of subthreshold disorders (Regeer et al., 2006), because none of the participants met criteria for past or present psychiatric disorder).

2.1.3. Preparation of whole cell extracts—Peripheral blood (10 mL) from healthy subjects ($n = 40$) was obtained at 08:00 h in a Na₂EDTA BD Vacutainer tubes and peripheral blood mononuclear cell (PBMC) were purified using Lymphocyte Separation Medium (LSM, MP Biomedicals) on ice (4 °C) according to the manufacturer's protocol. All the samples went through the same procedure. After isolation, PBMC pellets were stored at -80 °C until further preparation.

Whole cell extracts from PBMC were prepared using RIPA buffer. PBMC pellets were suspended in 800 µL of ice-cold RIPA buffer containing 150 mM NaCl, 1% Triton x-1000, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris HCl (pH 7.4), 5 mM Na₂EDTA, 1 mM Na₂EGTA, protease inhibitors (1 mM PMSF, 5 µg/mL leupeptin, 2 µg/mL aprotinin) and phosphatase inhibitors (Na₃VO₄- S-6508 Sigma-Aldrich, and 5 mM NaF-S-1504 Sigma-Aldrich). Cells were incubated on ice for 30 min, the tubes were vortexed vigorously for 20 s and finally PBMC homogenates were centrifuged at 12000 xg for 15 min at 4 °C. Supernatant was used for preparing whole cell extracts.

2.1.4. Preparation of nuclear extracts—Nuclear extracts from PBMC ($n = 43$) were prepared according to mini-extraction protocol (31) commonly used in our laboratory. Briefly, PBMC pellets were suspended in 500 µL of ice-cold buffer A (10 mM HEPES, pH 7.9) containing 10 mM KCl, 0.1 mM Na₂EDTA, 0.1 mM Na₂EGTA, 1 mM DTT and protease and phosphatase inhibitors. Cells were incubated on ice for 15 min, after which 75 µL of 10% solution of Tween 20 was added, the tubes were vortexed vigorously for 20 s and

finally PBMC homogenates were centrifuged at $10000 \times g$ for 7 min at 4 °C. After pouring off the supernatant, nuclear pellets were suspended in 150 μ L of ice-cold buffer C (20 mM HEPES, 7.9 pH) containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and the same protein and phosphatase inhibitors as in buffer A. Samples were then incubated at 4 °C for 15 min on shaking platform followed by centrifugation at $10000 \times g$ for 10 min at 4 °C. Obtained supernatants were used as nuclear extracts.

After determining the protein concentration by BCA Assay kit (SERVA Electrophoresis), whole cell and nuclear extracts were boiled in sample loading buffer according to Laemmli (1970)) for 5 min.

2.1.5. Western blot analysis—Equal amounts of whole cell and nuclear extract proteins (60 μ g) were loaded on 10% SDS PAGE and separated by electrophoresis at constant voltage (100 V). Proteins were transferred onto a PVDF membrane (Immobilon-P transfer membranes, Millipore). After blocking for 1 h in 5% non-fat dry milk in phosphate-buffered saline (PBS), membranes were incubated with respective primary and secondary antibodies. For total GR (tGR) detection (95-kDa), M-20 antibody (Santa Cruz Biotechnology) was used and its α isoforms (25-kDa, 40-kDa, 50-kDa, 67-kDa) were detected by tGR P-20 antibody (Santa Cruz Biotechnology) (dilution 1:1000). β -actin was used as a loading control (Spiliotaki et al., 2006; Martinez-Gras et al., 2011), and detected by specific antibody (Abcam). All membranes were incubated with primary antibodies overnight at 4 °C followed by incubation with secondary anti-rabbit HRP-linked antibodies (Amersham) for 2 h at 4 °C. Signals were detected using chemiluminescence substrate Pico (Thermo Scientific).

Densitometry of protein bands on X-ray film was performed by the ImageJ analysis PC software. In order to minimize the variations between different blots, and to compare the protein levels between different blots, an internal reference sample (IRS) was run on each gel (Day et al., 1987; Spiliotaki et al., 2006; Perisic et al., 2009). The IRS was made as a mixture of aliquots of the control samples from the study. Levels of each examined protein from all subjects were normalized to actine (i.e. tGR/actine) and then presented relative to the signals of IRS set as 100%, i.e. as a percentage of signals of IRS for respective protein. Regarding 95-kDa GR α isoform, we obtained and quantified only a single band that corresponded to a 95-kDa. We have previously verified this band by two different tGR antibodies in our published paper (Simic et al., 2013).

All samples were analyzed at least twice, and the average value was calculated. Four samples have been excluded and we used pairwise deletion of missing data in statistical analyses, because we did not obtain representative values for examined proteins on Western blot.

2.2. Animal study

Animal studies were performed on male and female C57BL/6 mice obtained from the Jackson laboratory. Mice were housed in groups of 4 in standardized cages and held under a 12 h light/dark cycle with ad libitum access to food and water. To eliminate litter-driven effects, animals from various litters were randomly placed in the experimental groups.

Separate cohorts of adolescent and adult mice of both sexes (postnatal days 29 and 70 respectively, P29 and P70) were used for all procedures. After they reached P29 and P70 of age, they were subjected to fear conditioning and extinction protocol that lasted 5 days in total. All experimental procedures were approved by the Ethical Committee of Vinca Institute of Nuclear Sciences and in compliance with the requirements of the European Council Guide for Care and Use of Laboratory Animals (2010/63/EU).

2.2.1. Rodent cued fear conditioning and extinction—Fear conditioning apparatus contained a square chamber with metal shock-delivering bars placed in a sound-attenuated box (TSE Systems, Inc). Animals were given 2 min to habituate to the conditioning chamber (sprayed with 0.1% (vol) peppermint extract dissolved in 70% EtOH) before being fear conditioned with three tone-shock pairings. Stimuli pairings consisted of a 30 s (5 kHz, 70 dB) tone presentation (conditioned stimulus, CS) that coterminated with a 0.7-mA foot shock (unconditioned stimulus, US) during the last second of tone duration. Intertrial interval (ITI) between stimuli was 30 s. After ending the final CS-US pairing, animals were left in the chamber to recover for 1 min before being returned to their home cages. The fear conditioning box was cleaned after each mouse with peppermint scented ethanol (0.1% (vol) in 70% EtOH). 24 h after conditioning, mice were subjected to fear extinction procedure in a new arena (green cylindrical chamber scented with 0.1% (vol) lemon extract dissolved in 70% EtOH) in order to eliminate the effect of context on extinction. After the initial acclimation period of 2 min, animals were exposed to five tone presentations each lasting 30 s in the absence of foot shock. ITIs between the tones were 30 s. After the final tone presentation, mice remained in the arena for an additional minute before being returned to their home cages. Fear extinction trials were repeated for 4 consecutive days. The extinction arena was cleaned after each animal with 70% ethanol scented with lemon (0.1% (vol) in 70% EtOH). 1 h after the last extinction session on 4th day of extinction, animals were killed by cervical dislocation and their tissues were used for further molecular analyses. The cued fear conditioning and extinction procedures were performed according to protocol established by Pattwell et al. (2012)).

All behavioral procedures were computer-controlled by Noldus software. Mice were videotaped for subsequent analysis by researchers blind to the experimental groups. Freezing behavior was quantified as a species-specific measure of fear that is defined as the absence of visible muscle movement except that necessary for respiration (Pattwell et al., 2012). Results are presented as percentage of time animals spend freezing.

2.2.2. Preparation of cytoplasmic and nuclear fractions—Different subcellular fractions were prepared from the frozen hippocampi. Extraction of cytoplasmic and nuclear fractions from frozen tissue was performed as previously described in Adzic et al., 2015 (Adzic et al., 2015). Briefly, previously frozen tissues were homogenized in ice-cold 20 mM Tris-HCl (pH 7.2) buffer that contained 10% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, protease and phosphatase inhibitors. Following homogenization, samples were centrifuged for 10 min at 2000 x g in order to separate cytoplasmic (contained in the supernatant) and nuclear fraction (contained in the pellet) and to obtain final cytosolic and nuclear fractions ready for subsequent molecular analyses.

2.2.3. Western blot analysis—For Western blot analysis, equal amounts of hippocampal cytosol and nuclear extracts (60 µg of proteins) were separated by electrophoresis on 10% SDS-PAGE at constant voltage (100 V). Protocol for protein detection, as well as the antibodies used were the same as previously described for human samples (tGR M-20; P-20; Santa Cruz Biotechnology). β-actin was used as a loading control. Densitometry of protein bands on X-ray films was performed by the ImageJ analysis PC software.

2.2.4. Gene expression analysis - RTqPCR—Total hippocampal RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, hippocampus was weighed and homogenized in 1 mL TRIzol® Reagent per 100 mg of tissue. Total RNA was isolated using chloroform-isopropanol extraction and quantification and quality of RNA samples was assessed using NanoDrop 1000 Spectrophotometer (Thermo Scientific). For cDNA synthesis, a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. The cDNAs were subsequently stored at – 20 °C until use.

Studied genes were amplified by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Real-time PCR System (Applied Biosystems) according to the manufacturer's protocols. Levels of mRNA were measured for BDNF exon IV, BDNF exon IX and FKBP5. Housekeeping genes cyclophilin (CF) and hypoxanthine phosphoribosyltransferase (HPRT) were used as endogenous controls. Sequences for each primer are listed as follows: BDNF E4 (F): 5'-CAG AGCAGCTGCCTTGATGTT-3', BDNF E4 (R): 5'-GCCTTGTCCTGGACG TTTA-3'; BDNF E9 (F): 5'-TGC AGG GGC ATA GAC AAA AG-3', BDNF E9 (R): 5'-TGAATCGCCAGCCAATTCTC-3'; FKBP5 (F): 5'-GCTGGCAA CAACACGAGAG-3'; FKBP5 (R): 5'-GAGGAGGGCCGAGTTCATT-3'; Cyclophilin (F): 5'-CCCACCGTGTCTTCGACA-5', Cyclophilin (R): 5'-CCAGTGCTCAGAGCTCG AAA-3'; HPRT (F): 5'-TCCTCCTCAGACCG CTTTT-3', HPRT (R): 5'-CCTGGTTCATCATCGCTA ATC-3'. cDNA was amplified under following conditions: hold 95 °C/10 min, 40 cycles of denaturation 95 °C/15 s, annealing 60 °C/1 min and extension 60 °C/1 min followed by final extension 72 °C/15 min. Analysis of melting curves was also performed, so that formation of a single PCR product could be confirmed.

For each of the tested animal groups, qPCR was repeated at least three times for every gene and samples were always run in triplicate. Standard curves were included to estimate the efficiency of amplification and E values were obtained from the slope of cycle threshold versus log concentration curve (Rutledge and Cote, 2003). PCR efficiency values were similar among all primers (0.96–0.99) and calculations of the relative amount of transcripts were performed using the CT method (Livak and Schmittgen, 2001).

2.3. Statistical analyses

All human and animal data were analyzed using the SPSS software, version 20.00 (SPSS, Chicago, IL, USA). Main characteristics of human participants were presented as mean ± standard deviations (continuous/numeric variables) and as frequencies (percent).

Comparisons between results obtained from cohort I and cohort II were performed by a

nonparametric Mann–Whitney test for continuous variables or by a chi square (χ^2) test for categorical variables. Non-parametric Mann-Whitney *U* test was used due to small sample size and non-normal distribution. Variables with non-normal distribution were log transformed to obtain distribution as adequate as possible for SEM (assumption that all continuous variables are approximately normally distributed). Pearson 2-tailed correlation was used to analyze the relationship between CTQ (total), DASS (total and DASS components), GR and its isoforms. Pairwise deletion of missing data was used in correlation matrices.

Linear regression modeling (enter method) was used to determine whether CTQ, GR α isoforms and their interaction were significant predictors of negative affectivity measured by DASS. The statistical analyses that include GR α isoforms were controlled for age. The *p* values, < 0.05 were regarded as statistically significant.

Furthermore, we employed a structural equation modeling (SEM) approach to test the relationship among CTQ, DASS scores and GR α isoforms, using AMOS version 20.0. Several indices, such as chi square (X^2) statistic (X^2/df), values of comparative fit index (CFI), normed fit index (NFI), and the root mean square error of approximation (RMSEA) were used to determine whether the hypothesized model fit the observed data. The model fit is considered adequate when CFI and NFI values are > 0.90, and the RMSEA values are < 0.08 (Hu and Bender, 1999).

All values obtained from animals were presented relative to adolescent (P29) female group (choosing of this group was arbitrary). Regarding behavioral analyses, extinction efficiency parameter was analyzed using paired-samples *t*-test, whereas spontaneous recovery was assessed by General linear model (GLM). Molecular data from Western blot and real-time qPCR was analyzed using GLM with sex and age as independent factors. In order to simplify presentation of data, all statistically significant differences were given as *p* < 0.05 and presented as mean $\pm$ SEM.

3. Results

3.1. Human study

3.1.1. Sociodemographic and psychometric data—Demographic and clinical characteristics of healthy subjects are presented in Table 1a. No differences in childhood trauma and DASS scores between cohorts were found. The second cohort was significantly older than the first one, which has been taken into account in further analyses.

3.1.2. Western blots of GR α protein isoforms in healthy controls—GR α isoforms were detected in PBMC whole cell (cohort I) and nuclear (cohort II) protein extracts of healthy individuals at 95-kDa, 67- kDa, 50-kDa, 40-kDa and 25-kDa, normalized to the corresponding β actin as a loading control. Representative western blots are presented in Fig. 1.

3.1.3. CTQ total and DASS scores correlations—Correlation between childhood trauma measured by CTQ and negative emotional states (measured by DASS) is presented in

Table 1b. Significant correlation was observed between CT total and DASS – anxiety scores in both cohorts, while the second cohort also exhibited a significant correlation between CT total and DASS total scores ($p < 0.05$) (Table 1b.).

3.1.4. Correlations among childhood trauma, DASS scores and GR α

isoforms—As shown in Table 1b., whole cell 40-kDa GR α isoform was significantly correlated with CT scores. Furthermore, we observed a significant correlation between nuclear 40-kDa GR α and certain DASS scores in cohort II, as well as a significant correlation of GR α 25-kDa isoforms with specific DASS scores in both cohorts (Table 1b.). In particular, nuclear GR α -40 kDa was significantly negatively related to DASS – total ($r = -0.345$, $p = 0.049$) and DASS – depression scores ($r = -0.484$, $p = 0.004$). In addition, both whole cell and nuclear levels of GR α -25 kDa isoform were negatively related to DASS – total (I cohort $r = -0.473$, $p = 0.003$; II cohort $r = -0.439$, $p = 0.011$), and DASS – stress scores (I cohort $r = -0.509$, $p = 0.001$; II cohort $r = -0.387$, $p = 0.016$). Moreover, whole cell GR α 25-kDa isoform was significantly negatively related to DASS – anxiety scores (I cohort $r = -0.467$, $p = 0.003$), while nuclear levels of GR α 25-kDa isoform were negatively associated with DASS – depression scores (II cohort $r = -0.410$, $p = 0.011$).

3.1.5. Correlations among GR α isoforms and FKBP5—Correlation between GR α isoforms and FKBP5 is presented in Table 1c. We observed that whole cell GR α 95-kDa was significantly positively correlated with GR α 25-kDa (I cohort $r = 0.403$, $p = 0.011$) and FKBP5 (I cohort $r = 0.323$, $p = 0.045$). Regarding nuclear extracts, we found that FKBP5 was significantly positively correlated with GR α 40-kDa (II cohort $r = 0.362$, $p = 0.03$).

3.1.6. General linear model—The relative contributions of gender, age, GR α translational isoforms, CT and GR α isoforms x CT interaction and FKBP5 to variation in DASS scores were determined by linear multiple regression (enter method).

A significant model that incorporated gender, age, nuclear GR α 40-kDa isoform, CT and their interaction was able to explain 20% of the variance in DASS total scores. In this model, nuclear GR α 40-kDa isoform was significant predictor of DASS total scores (Table 2, cohort II). Further analyses revealed that CT and CT x (nuclear) GR α 40-kDa interaction were able to explain 22% of variance in DASS anxiety. Moreover, a model that included nuclear FKBP5 beside age, nuclear GR α 40-kDa isoform, CT and their interaction was able to explain 35% of the variance in DASS anxiety scores in II cohort. Neither of the models were significant in cohort I (Table 2, cohort I).

3.1.7. Structural equation modeling—SEMs for cohort I and cohort II are displayed in Fig. 2a and b (left and right, respectively). Hypothesized models included the following variables: gender, total DASS (dependent variable), CT, whole cell or nuclear 25- and 40-kDa GR α isoforms and CT x GR α isoforms interactions. The 95-kDa GR α was also included in the analyses, but only significant models that incorporated this isoform were presented. Models showed very good fit to the data, as indicated by the following fit indices: cohort I: Chi-square 3.98, $p = 0.858$; CFI = 0.99; NFI = 0.979; RMSEA = 0.001 and cohort II: Chi-square 14.98, $p = 0.243$; CFI = 0.972; NFI = 0.901; RMSEA = 0.078.

In cohort I, total DASS scores were significantly positively related to CT and negatively to CT x whole cell 25-kDa GR α interaction, explaining 28% of variation in DASS scores. Neither 40- nor 25-kDa GR α isoform had a direct significant effect on total DASS scores. It should be noted that higher 40-kDa GR α levels were significantly related with higher FKBP5 levels. Similar results were found in cohort II. Namely, total DASS scores were significantly positively related to CT and negatively to nuclear 25-kDa GR α , explaining 40% of variation in DASS scores. Higher nuclear 40-kDa GR α levels were significantly related to higher FKBP5 levels. While whole cell FKBP5 levels showed no relation to total DASS scores, higher nuclear FKBP5 was negatively related to them. This model indicated that nuclear 40-kDa GR α exhibited significant indirect effect on total DASS scores.

Regarding DASS anxiety, SEMs for cohort I and cohort II are displayed in Fig. 2b (left and right, respectively). Besides the dependent variable (DASS anxiety), SEM for cohort I also included following variables: gender, CT, whole cell 25- and 40-kDa GR α isoforms, CT x GR α isoforms interactions and FKBP5. On top of these variables, model for cohort II additionally included 95-kDa GR α isoform. These two models showed very good fit to the data, as indicated by the following fit indices: cohort I: Chi-square 8.21, $p = 0.768$; CFI = 0.99; NFI = 0.973; RMSEA = 0.001 and cohort II: Chi-square 11.43, $p = 0.817$; CFI = 0.95; NFI = 0.953; RMSEA = 0.013.

In cohort I, DASS anxiety scores were significantly positively related to CT and negatively to CT x whole cell 25-kDa GR α interaction, explaining 41% of variation in DASS anxiety scores. In addition, higher whole cell 40-kDa GR α levels were significantly related to higher FKBP5, which exhibited a negative relation to DASS anxiety scores. In cohort II, DASS anxiety was significantly positively related to CT, 40-kDa GR α and 95-kDa GR α , whereas we found a negative link between DASS anxiety scores and CT x nuclear 40-kDa GR α interaction and FKBP5. These results accounted for 53% of variation in DASS anxiety scores. Moreover, higher levels of 95-kDa GR α were significantly related to lower FKBP5 levels and to CT x 25-kDa GR α interaction.

Finally, it is worth mentioning that in contrast to whole cell 40-kDa GR α , nuclear levels of this isoform were negatively related to CT.

By using the interaction plot tool (two-way interaction), we plotted the interaction obtained in the last model using the unstandardized regression coefficients for the dependent variable (DASS anxiety), independent variable (CT) and moderator (40-kDa GR α). Fig. 2c shows that upon low 40-kDa GR α levels, DASS anxiety increased in parallel with CT. On the other hand, upon higher 40-kDa GR α levels the association between CT and DASS is disrupted. This data indicated that nuclear 40-kDa GR α negatively moderated the relationship between CT and DASS-anxiety.

3.2. Animal study

3.2.1. Effect of age and sex on fear extinction—We monitored animals' freezing behavior in both sexes and calculated parameters related to distinct extinction learning processes. Extinction efficiency is a measure of overall success in extinction learning and it is defined as a difference in percentages of freezing between the start of the first and the start

of the final day of extinction. Additionally, spontaneous recovery of freezing allows us to evaluate animals' ability to consolidate memory of fear extinction. Higher spontaneous recovery indicates an increase in fear-associated behavior. In this experiment, spontaneous recovery was calculated by subtracting freezing percentage at the end of the previous day from the freezing percentage at the beginning of the next day of fear extinction.

Adult females were the only group that exhibited significant difference for extinction efficiency; $t(10) = 3.44$, $p < 0.05$ (Fig. 3a). Both adult female and male mice displayed lower spontaneous recovery of freezing compared to their adolescent counterparts. Statistically significant changes were detected in adult females that showed lower percentages of freezing recovery between the second and the third day of fear extinction (age: $F(1,40) = 12.25$, $p < 0.05$; Fig. 3a), while adult males had substantially decreased recovery between the first and the second day of tests (age: $F(1,40) = 9.34$, $p < 0.05$; Fig. 3a). Furthermore, adolescent males displayed a much higher spontaneous recovery than adolescent females between the first and the second day of extinction (sex: $F(1,40) = 5.32$, $p < 0.05$; Fig. 3a).

3.2.2. Effect of age and sex on protein expression—We examined the effect of age and sex on the expression of GR α isoforms in both cytoplasmic and nuclear hippocampal fractions. As can be seen in Fig. 3b, adult females had lower levels of all cytosolic GR α isoforms than adolescents: GR α 95 (age: $F(1,61) = 54.83$, $p < 0.05$), GR α 67 (age: $F(1,55) = 13.49$, $p < 0.05$), GR α 50 (age: $F(1,39) = 108.32$, $p < 0.05$) and GR α 40 (age: $F(1,41) = 36.17$, $p < 0.05$). For GR α 40-kDa, this decrease corresponded to its marked elevation in nuclear levels when compared to adolescent females (age x sex: $F(1,27) = 10.46$, $p < 0.05$; Fig. 3b), suggesting that translocation of GR α 40 to nucleus occurred.

Regarding adult males, analyses revealed that GR α 95 (age: $F(1,61) = 54.83$, $p < 0.05$), GR α 50 (age: $F(1,39) = 108.32$, $p < 0.05$) and GR α 40 (age: $F(1,41) = 36.17$, $p < 0.05$) levels in cytosol were reduced relative to male adolescents, as shown in Fig. 3b. Decrease in cytosolic GR α 95 levels was parallel to lower levels in nucleus when compared to adolescent males (age x sex: $F(1,34) = 19.95$, $p < 0.05$; Fig. 3b).

Additionally, statistical analyses showed that in adults, males had higher levels of cytosolic 95-kDa GR α (sex: $F(1,61) = 10.48$, $p < 0.05$), 67-kDa GR α (sex: $F(1,55) = 17.62$, $p < 0.05$) and 40-kDa GR α (sex: $F(1,41) = 19.89$, $p < 0.05$) when compared to females (Fig. 3b). On the other hand, adult females exhibited higher expression of 95-kDa GR α (sex: $F(1,32) = 4.46$, $p < 0.05$) and 40-kDa GR α (sex: $F(1,25) = 9.93$, $p < 0.05$; Fig. 3b) in nucleus.

3.2.3. Effect of age and sex on the expression of GR-regulated genes in the hippocampus—In adult females statistical analyses revealed increase in BDNF exon IV expression when compared to adolescents (age: $F(1,45) = 6.46$, $p < 0.05$; Fig. 3c), while for other genes we did not find any statistical differences. On the other hand, adult males expressed higher levels of BDNF exon IX (age: $F(1,57) = 7.80$, $p < 0.05$; Fig. 3), and FKBP5 (age: $F(1,57) = 33.19$, $p < 0.05$; Fig. 3c) in respect to adolescents, while BDNF exon IV showed no differences.

Regarding sex-specific differences in adult groups, females showed increased BDNF exon IV gene expression in comparison to males (sex x age interaction: $F(1,47) = 17.38$, $p < 0.05$; Fig. 3c), whereas males had higher levels of FKBP5 mRNA (sex x age interaction: $F(1,59) = 17.91$, $p < .05$; Fig. 3c). In adolescent animals only significant change was observed in BDNF exon IX where males had lower expression than females (sex x age interaction: $F(1,59) = 6.67$, $p < .05$; Fig. 3c).

4. Discussion

In this study, we provide preliminary evidence that GR α translational isoforms in PBMC mediate the effects of early life environmental exposure on the risk of developing stress-related psychiatric disorders. Our central finding is that the effects of CT on later negative affectivity are largely mediated through 40-kDa GR α isoform, whose effect on DASS is mediated through its direct or indirect action via modulating nuclear 95-kDa GR α and FKBP5. We further noticed that 40-kDa GR α isoform may have a significant role during extinction learning and that its nuclear levels are positively related to BDNF exon IV expression.

A growing body of literature highlighted childhood adversity as influential for the development and course of mental disorders (Bouton, 2004; McEwen, 2003; Chapman et al., 2004; Teicher et al., 2009; Crawford et al., 2009). Our results, obtained in two independent cohorts of non-clinical participants, confirm a positive association between CT and negative emotional states in later life.

Genetic and epigenetic studies yielded insights in a potential molecular mechanism through which CT influences the development of psychiatric disorders. They emphasized that a long-term consequence of early life trauma is biologically transmitted through specific epigenetic signature on the negative regulator of GR α function, FKBP5. However, since GR α translational isoforms are characterized by a dynamic pattern of expression during development, it is possible that alteration of their expression, particularly during the stress sensitive periods such as early childhood and adolescence, may alter the responsivity of the brain to circulating GCs and contribute to changes in the HPA axis, predisposing individuals to develop psychopathology. The current study tested this hypothesis by using linear regression and a multiple mediation model, linking CT, GR α isoforms and FKBP5 as CT mediators, and the outcome of negative emotional status.

Our SEM analyses indicated that CT could have an after-effect on individual affective status via GR α translational isoforms, in particular 25-kDa and 40-kDa GR α , either directly, or indirectly by modulating full length GR α and FKBP5 protein levels. As evidenced by our results, tGR is more tightly associated with negative affectivity. Furthermore, 40-kDa GR α isoform is the more direct mediator of CT than 25-kDa GR α and it seems that 40-kDa GR α negatively moderates the relationship between CT and negative emotional states. Functional characteristics of this isoform have not yet been described, although it was demonstrated that GR α translational isoforms could impact GR function by altering its transcriptional potential (Lu and Cidlowski, 2005). Thus, it is possible that alteration in the levels of GR α translational isoforms upon early life traumatic experience may disturb the action of GCs in

the brain. This may further contribute to changes in HPA axis activity directly or/and by affecting the function of key stress-regulating molecules, GR and FKBP5.

Recent data suggest that psychopathology following CT occurs through disturbed fear conditioning and alterations in neural circuitry underlying the acquisition and extinction of fear (Varese et al., 2012; Jovanovic and Ressler, 2010). The neural circuitry underlying fear conditioning is conserved across species (Lissek and van Meurs, 2015) and besides the amygdala and prefrontal cortex (PFC), it includes the hippocampus, brain structure that supports extinction learning and retrieval (Johansen et al., 2011; Hartley et al., 2011). In line with that, the current study examines relationship between hippocampal GR α isoforms and fear extinction in young adolescent and adult mice of both sexes. It is worth mentioning that the animal model was not intended to translate clinical findings, but rather to define the role of GR α isoforms during the process of extinction learning. However, future studies using an animal model of childhood abuse/neglect and examining behavioral phenotypes (animals with/without depressive-like behavior) in parallel with subjects experiencing early life adversities (non-clinical subjects and depressive patients) could more precisely define the role of GR α translational isoforms in adverse childhood experience and their relation to trauma-related psychopathologies. Our results identified different nuclear translocation of 40-kDa GR α isoform that may underlie sex and development-related differences in fear extinction. Namely, we found that nuclear translocation of 40-kDa GR α isoform in fear-extinguished female adult mice may provide an additional mechanism by which this GR α isoform mediates extinction. On the other hand, during young adolescence, we found sex unspecific decrease in fear extinction behavior compared to adults as well as in adult male mice compared to females. In addition, both female and male young adolescent animals exhibited significant spontaneous recoveries in freezing behavior, indicating that learning processes are perturbed in this developmental stage. This data are in agreement with earlier studies that have shown attenuated fear extinction in young male adolescent animals (Pattwell et al., 2012). Regarding GR α isoforms, our results suggested that a lack of 40-kDa GR α nuclear translocation in the hippocampus during adolescence may be associated with blunted regulation of fear extinction during this stage of development.

To demonstrate that nuclear 40-kDa GR α isoform is functional in the hippocampus, we analyzed the expression of GR responsive genes BDNF (exons IV and IX) and FKBP5. Our results indicated that increased nuclear 40-kDa GR α isoform in adult females was paralleled with increased expression of BDNF exon IV. This is in agreement with several studies indicating that hippocampal BDNF is necessary for fear extinction (Hanson et al., 2015; Heldt et al., 2007; Sakata et al., 2013). Taken together, these findings suggest that 40-kDa GR α isoform may have direct implications in the regulation of BDNF and neuronal plasticity.

Furthermore, even though FKBP5 expression was elevated in adult males, its expression was not related to any of the GR α translational isoforms. Interestingly, this increase was opposite to full length GR α levels, which is known to be a positive transcriptional regulator of FKBP5 gene (Chen et al., 2017). However, besides GR α , expression of FKBP5 gene is under tight regulation by other steroids, such as androgen and progesterone via androgen response elements (ARE) and progesterone response elements (PRE) sequences (Zannas et

al., 2016; Hubler and Scammell, 2004). It seems that increase of FKBP5 expression in adult males in our study could result from elevated AR activity. In addition to its known function in the regulation of GR action and HPA axis activity, FKBP5 is also associated with fear extinction. Indeed, increased expression of FKBP5 in infralimbic medial PFC was shown to be associated with attenuated extinction of fear (Magee et al., 2006). Therefore, the absence of fear extinction learning in adult males could be attributed to the heightened FKBP5 expression in the hippocampus.

Our results should be interpreted considering several limitations. Data were cross-sectional and the study does not warrant causal inferences. Prospective studies with larger samples are needed to support causality, particularly during the development stages. Furthermore, the contraceptive use was not considered and should be included in future analyses. In our study further stratification of women (cohort I $n = 22$, cohort II $n = 17$) in respect to contraceptive use, as a new variable, would not give a sufficient number of cases necessary for a valid conclusion. In addition, the GR translational isoforms were analyzed using PBMC, and it is questionable how these results reflect GR isoforms in the central nervous system. Our preliminary data obtained in PBMC of psychotic patients indicated that specific GR α isoform predicted SCH diagnosis as it was reported for that GR α isoform in the brain (Sinclair et al., 2012). Moreover, several animal studies indicated that GR of various brain regions and GR from immune system tissues are similarly regulated by corticosterone (Lowy, 1991; Spencer et al., 1991). Additionally, it has been demonstrated that peripheral blood cells and brain share > 80% of the transcriptome with comparable molecular processes between these two tissues (Gladkevich et al., 2004; Liew et al., 2006). Besides this, it is known that inflammatory signals involved in regulation of chronic stress response and negative affectivity (NA) could interact with GR signaling and alteration of GR function (Miller et al., 2008). This interaction between GR and immune system, could point out to association between GR signaling in the periphery and central nervous system function and behavior (Dantzer et al., 2008).

The strengths of this study lie in: a) the testing of the hypotheses on distinct samples of two cohorts of non-clinical subjects, which greatly increases study significance and adds stronger support to the finding that CT effects on mood are significantly mediated by 40-kDa GR α ; b) using standardized methods (blood sampling at the same time as the psychometric assessment; standardized multiple variable modeling analysis; validated psychometric scales; GR α isoform analysis in one laboratory) warranting validity of obtained data and c) employing human and animal approach to examine the role of GR α isoforms in trauma-related psychopathology.

5. Conclusion

This study elucidates a comprehensive model of interaction between CT and negative emotional states, emphasizing the role of GR α isoforms as mediating factors. Our data obtained in two independent cohorts of healthy subjects indicated that the effects of CT on mood may be mediated by GR α translational isoforms, particularly nuclear 40-kDa GR α , which then could affect negative emotional states through direct and indirect paths involving full length GR α and FKBP5. The animal results demonstrated that 40-kDa GR α

additionally could be involved in fear extinction learning by affecting BDNF mRNA expression and neuronal plasticity in a sex-dependent manner. Our results provide insight into pathophysiology related to childhood trauma by focusing on GR α translational isoforms, which could be considered in the development of new treatments.

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Fig. 1.

Glucocorticoid receptor (GR) α protein expression in whole cell (a) and nuclear lysate (b) of the peripheral blood mononuclear cells in healthy subjects. The full length GRα (95-kDa) was detected using M-20 antibody, whereas other GRα translational isoforms were detected by P-20 antibody. Levels of all proteins were adjusted to β-actin, which served as a loading control, and presented as the percentage of IRS of each protein. c) The purity of subcellular fractions was assayed by anti-β tubulin and anti-lamin B for the cytosolic and nuclear compartments, respectively.

Fig. 2.

Structural equation model of (a) total DASS scores and (b) DASS Anxiety, CT, gender, age, whole cell (left panel) and nuclear (right panel) FKBP5, 25- and 40-kDa GR α isoforms and CT x GR α isoforms interactions for healthy subjects (cohort I $N=40$, cohort II $N=43$). Boxes present the measurement variables and the arrowed lines between boxes mark significant paths (black line) where the numbers denote path coefficients (standardized regression coefficients β) and the arrows show the directions of influences. The bold dotted lines (right panel) present the direct effect of CT, 40-kDa GR α isoform and their interaction on DASS anxiety levels. (c) Two-way interaction plot illustrating the differential relationship between CT and DASS anxiety, as a function of high versus low 40-kDa GR α levels. High and low are defined as 1 SD above or below the mean value for both CT and the 40-kDa GR α , based on the approach described by Hayes and Matthes (2009) (Hayes and Matthes, 2009). In subjects with high 40-kDa GR α levels the relation between CT and DASS anxiety is disrupted.

Fig. 3.

a left - Percentages of freezing behavior at the beginning of the first day (D1T1 [Day 1, Tone 1]) and the fourth day of fear extinction (D4T1 [Day 4, Tone 1]). Values are presented as mean ± SEM and analyzed by paired samples *t*-test. Statistically significant difference within each experimental group was taken at $p < .05$. Fig. 3a right - Percentages of spontaneous recovery of freezing behavior: D2T1-D1T5 ([Day 2, Tone1] - [Day 1, Tone 5]), D3T1-D2T5 ([Day 3, Tone1] - [Day 2, Tone 5]) and D4T1-D3T5 ([Day 4, Tone1] - [Day 3, Tone 5]). Fig. 3b - Levels of GRα translational isoforms in the cytosol and the nucleus of the hippocampus of female and male adolescent and adult mice with representative blots. Fig. 3c - Relative expression of GR-regulated genes BDNF (exons IV and IX) and FKBP5 in the hippocampus of adolescent and adult mice of both sexes. Fig. 3a right, b, and c - Values are calculated and presented as mean ± SEM of values for control group (P29 females) and analyzed by General linear model (GLM). Statistically significant differences between the groups were regarded as $p < .05$. * P29 vs. P70, # males vs. females.

Table 1a

Demographic and clinical characteristics of the subjects.

	Cohort I (n = 40)	Cohort II (n = 43)	Z values	P values
Age (years)	30.2 ± 6.4	36.3 ± 10.3	-2.79	0.006
Gender (male/female)	18 M. 22F	16 M. 27F		0.398 ^a
CTQ^b total	5.99 ± 1.35	5.95 ± 0.92	-0.88	0.379
DASS^c total	18 ± 16.11	16.12 ± 13.67	-0.67	0.506
DASS – depression	3.64 ± 5.09	4.03 ± 4.33	-0.58	0.56
DASS – anxiety	4.38 ± 5.21	4.15 ± 5.12	-0.49	0.961
DASS – stress	9.97 ± 7.71	8.05 ± 6.16	-1.287	0.98

Note: Data are presented as mean ± sd.

Comparisons between two cohorts were performed by a Mann–Whitney test for continuous variables (Z values and the corresponding *p* values are given) or chi square (χ^2) test for categorical variables.

^a chi square (χ^2) test.

^b Childhood Trauma Questionnaire.

^c Depression Anxiety Stress Scale.

Table 1b

Pearson's correlations of DASS scores with CT (childhood trauma) and GR α translational isoforms.

		DASS total	DASS-depression	DASS-anxiety	DASS-stress	CT
Cohort I						
CT	Correlation	0.272	0.191	0.383	0.272	1.000
95-kDa	Correlation	-0.258	-0.055	-0.231	-0.277	0.100
67-kDa	Correlation	0.750	-0.142	0.068	0.121	0.252
50-kDa	Correlation	-0.055	-0.120	-0.204	-0.022	-0.166
40-kDa	Correlation	-0.105	-0.216	-0.106	-0.072	0.405
25-kDa	Correlation	-0.473	-0.175	-0.467	-0.509	-0.190
Cohort II						
CT	Correlation	0.299	0.260	0.435	0.175	1.000
95-kDa	Correlation	0.301	0.160	0.294	0.359	0.057
67-kDa	Correlation	-0.208	-0.214	-0.139	-0.170	0.231
50-kDa	Correlation	0.187	0.031	-0.082	0.137	-0.091
40-kDa	Correlation	-0.345	-0.484	-0.265	-0.215	0.012
25-kDa	Correlation	-0.439	-0.410	-0.275	-0.387	0.103

Note: All p values were accepted as significant at < 0.05 and were not included in the table. Statistically significant effects are bolded. The correlation coefficient was listed in the table. Cohort I refers to whole cell, cohort II to nuclear lysates.

Table 1c

Pearson's correlations between GR α translational isoforms and FKBP5.

		67-kDa	50-kDa	40-kDa	25-kDa	FKBP5
Cohort I						
95-kDa	Correlation	0.194	-0.134	0.189	0.403	0.323
FKBP5	Correlation	0.166	0.172	0.360	0.110	1.000
Cohort II						
		67-kDa	50-kDa	40-kDa	25-kDa	FKBP5
95-kDa	Correlation	0.046	0.188	0.186	0.08	-0.270
FKBP5	Correlation	0.174	-0.057	0.362	0.242	1.000

Note: All p values were accepted as significant at < 0.05 and were not included in the table. Statistically significant effects are bolded. The correlation coefficient was listed in the table. Cohort I refers to whole cell, cohort II to nuclear lysates.

Table 2

Linear regression modeling (enter method) of the effects of gender, CT, 95-,40- and 25-kDa GR α isoforms and their interactions on total DASS and DASS anxiety.

	Dependent variable	Predictor(s)	B(SE)	beta	t	F(p)	Adjusted R ²
Cohort I	Total DASS	Constant				NS	
		Gender					
		Age					
		95-kDa GR α					
		CTQ					
Cohort I	Total DASS	CTQ $\times$ 95				NS	
		Constant					
		Gender					
		Age					
		40-kDa GR α					
	Total DASS	CTQ				NS	
		CTQ $\times$ 40					
		Constant					
		Gender					
		Age					
	DASS anxiety	25-kDa GR α				NS	
		CTQ					
		CTQ $\times$ 25					
		Constant					
		Gender					
	DASS anxiety	Age				NS	
		95-kDa GR α					
		CTQ					
		CTQ $\times$ 95					
		Constant					
	DASS anxiety	Gender				NS	
		Age					

Dependent variable	Predictor(s)	B(SE)	beta	t	F(p)	Adjusted R ²
DASS anxiety	40-kDa GRα					
	CTQ					
	CTQx40					
	Constant				NS	
	Gender					
	Age					
	25-kDa GRα					
	CTQ					
	CTQx25					
	Constant				NS	
Total DASS	Gender					
	Age					
	95-kDa GRα					
	CTQ					
	CTQx95					
	Constant	1.571 (0.619)	-	2.539	2723	0.202
	Gender	0.102(0.280)	0.058	0.365	(0.039)	
	Age	0.02(0.013)	0.236	1.494		
	40-kDa GRα	-0.320(0.141)	-0.365	-2.265		
	CTQ	0.254(0.140)	0.279	1.808		
Total DASS	CTQx40	-0.191(0.139)	-0.213	-1.367		
	Constant				NS	
	Gender					
	Age					
	25-kDa GRα					
	CTQ					
	CTQx25					
	Constant				NS	
	Gender					
	Age					
DASS anxiety	95-kDa GRα					

Dependent variable	Predictor(s)	B(SE)	beta	t	F(p)	Adjusted R ²
DASS anxiety	CTQ					
	CTQx95					
	Constant	1.275 (0.583)	-	2.188	3.001	0.223
	Gender	-0.074(0.341)	-0.037	-0.218	(0.038)	
	Age	0.009(0.017)	0.094	0.541		
	40-kDa GRα	-0.210(0.171)	-0.211	-1.231		
DASS anxiety	CTQ	0.403(0.173)	0.391	2.329		
	CTQx40	-0.426(0.172)	-0.420	-2.481		
	Constant	0.959 (0.701)	-	1.367	3.463	0.345*
	Gender	-0.029(0.319)	-0.015	-0.092	(0.015)	
	Age	0.007(0.015)	0.071	0.658		
	40-kDa GRα	-0.052(0.169)	-0.052	-0.307		
DASS anxiety	CTQ	0.362(0.160)	0.351	2.263		
	CTQx40	-0.452(0.158)	-0.445	-2.853		
	FKBP5	-0.411(0.166)	0.414	-2.478		
	Constant				NS	
	Gender					
	Age					
	25-kDa GRα					
	CTQ					
	CTQx25					