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GENETIC SUSCEPTIBILITY TO POSTTRAUMATIC STRESS DISORDER: ANALYSES OF THE *OXYTOCIN RECEPTOR, RETINOIC ACID RECEPTOR-RELATED ORPHAN RECEPTOR A* AND *CANNABINOID RECEPTOR 1* GENES

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SUMMARY

Background: *Exposure to life-threatening events is common and everyone will most likely experience this type of trauma during their lifetime. Reactions to these events are highly heterogeneous and seems to be influenced by genes as well. Some individuals will develop posttraumatic stress disorder (PTSD), while others will not. In this study, our aim was to analyze the correlation between single nucleotide polymorphisms (SNPs) within the oxytocin receptor (OXTR) gene (rs53576 and rs2254298), the RAR-related orphan receptor A (RORA) gene (rs8042149) and the cannabinoid receptor 1 (CNR1) gene (rs1049353) and PTSD. All candidate genes have been previously associated with stress related disorders and the reaction to traumatic events.*

Subjects and methods: *Participants (N=719) have been exposed to war-related trauma during the war in South-Eastern Europe (Bosnia and Herzegovina, Croatia and Kosovo). We correlated the presence and absence of current and lifetime PTSD as well as PTSD severity (Clinician Administered PTSD scale (CAPS)) and current psychopathology (Brief Symptom Inventory (BSI) score) with the mentioned SNPs. DNA was isolated from whole blood and genotyped for OXTR rs2254298 and rs53576 following previously published protocols, for RORA rs8042149 via PCR-RFLP and CNR1 rs1049353 via KASP.*

Results: *Nominally significant results were found for OXTR rs53576 in connection with the CAPS and BSI scores within lifetime PTSD patients. The additive allelic model indicated that G allele carriers achieved lower CAPS (p=0.0090) and BSI (p=0.0408) scores than participants carrying one or two copies of the A allele. These results did not withstand correction for multiple tests. No significant results were observed for OXTR rs2254298, RORA rs8042149 and CNR1 rs1049353 although the results for RORA showed a slight tendency that rs8042149 may influence the level of BSI scores in current PTSD patients.*

Conclusions: *This study points to a role of the OXTR gene in PTSD and the related psychopathology following war related trauma.*

Key words: *posttraumatic stress disorder - oxytocin receptor gene - RAR-related orphan receptor A - cannabinoid receptor 1*

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INTRODUCTION

Posttraumatic stress disorder (PTSD) is a common and very often chronic disorder that can develop following exposure to a life-threatening or traumatic event. Trauma is an essential part of the diagnosis of PTSD, and approximately 70% of the population will experience at least one traumatic event during their lifetime (Benjet et al. 2015). An estimated 10% of exposed individuals will develop PTSD symptoms (Kessler et al. 2012). PTSD is highly heterogeneous and can manifest itself in different ways. The disorder is characterized by several symptom clusters as defined in the Diagnostic and Statistical Manual of Mental Disorders - V (APA, 2013), which include the following:

- Symptoms of re-experiencing in the form of intruding thoughts, nightmares, flashbacks, emotional distress and physical reactivity to traumatic reminders (criterion B).
- Symptoms of avoidance, which include avoiding traumatic reminders that trigger symptoms of re-experiencing (criterion C).
- Alterations in mood and cognition, which include a variety of symptoms such as dissociative amnesia, persistent negative beliefs about oneself and the environment, diminished interest in usual activities and affective changes (criterion D).
- Symptoms of hyperarousal that can be experienced as aggressive behavior including auto and hetero-aggression, sleep disturbance, impulsivity and cognitive deficits (criterion E).

Furthermore, symptoms have to be experienced for at least a month and cause a related change in functionality. Long term mental health consequences of war are observed in survivors even after decades and they still present a severe mental health burden. Priebe et al. (2010) found that PTSD prevalence in a sample of 640 participants from Bosnia and Herzegovina was 35.4%, which was the highest of all countries that were investigated (Croatia, Kosovo, Serbia and Republic of Macedonia). However, the key question of why some individuals develop symptoms of trauma related disorders following a traumatic event and why some do not still remains unsolved.

The effects of many genes on the development and persistence of anxiety and stress-related disorders are now widely studied. An example is the oxytocin receptor (OXTR) gene, which seems to be an important genetic variable (Gottschalk & Domschke 2016). A large body of evidence links oxytocin (OXT) to stress regulation, and it has been found that the plasma levels of oxytocin seem to increase as a response to stress stimuli (Neumann et al. 2000, Onaka 2004). The first discoveries in humans revealed that plasma levels of oxytocin increased following exposure to uncontrollable noise and several types of psychosocial stressors (Sanders et al. 1990, Olff et al. 2013). The hypothesis is that during stressful stimuli or situations, oxytocin release serves to dampen physiological stress levels. The higher the basal levels of oxytocin, the lower the norepinephrine levels, blood pressure and heart rate in response to stress (Light et al. 2004). The probable stress regulation mechanism of oxytocin appears to be related to functional changes of the amygdala (Viviani et al. 2011). In support of these findings, neuroimaging studies have shown that intranasal administration of oxytocin lowered amygdala activity probably by enhancing amygdala-prefrontal cortex connectivity (Domes et al. 2007, Sripada et al. 2012).

The effects after oxytocin administration are important for the autonomic stress response (Koch et al. 2016), amygdala reactivity and anxiety levels, as well as beneficially impact socio-emotional processes and behavior (Preckel et al. 2014). Furthermore, the allelic status seems to play an important role in determining behavior patterns after stress, meaning that carrying different alleles such as the G allele of rs53576 increased prosociality (Kogan et al. 2011) and empathy (Rodrigues et al. 2009). This means that different allele carriers respond and behave differently after major traumatic events.

Furthermore, studies have shown amygdala size differences between PTSD and non-PTSD groups, with the PTSD cohort having smaller amygdala volumes (Morey et al. 2012). OXTR rs2254298 G homozygotes were found to have smaller volumes of corticolimbic system structures such as the amygdala, anterior cingulate cortex and the posterior brainstem than carriers of the A allele (Furman et al. 2011). Also, the OXTR rs2254298 A allele was positively correlated with bilateral amygdala volume. The same correlation was seen in the A/A genotype in rs53576, whereby significant association was observed (Wang et al. 2014). Allele-load-dependent changes in the hypothalamus are considered to be the oxytonergic "core" of the brain. This means that carrying a risk allele (rs53576 A allele) leads to anatomical and functional interaction change in structures that is a central fear and anxiety regulator (Tost et al. 2010). The association between OXTR polymorphisms and amygdala volume could be a potential explanation of the complex and very often severe psychopathology related to stress (Furman et al. 2011).

Studies have concluded that OXTR variants such as rs53576 play an important role in attachment styles and consequently influence anxiety levels as shown in studies of probands with social anxiety and PTSD (Notzon et al. 2016, Sippel et al. 2017). As such, the interaction between attachment styles and SNPs could present a promising factor that may contribute to vulnerability to PTSD (Sippel et al. 2017). Early traumatic experiences seem to influence the oxytocin system later in life, and the main consequences are seen in changes in attachment styles which become insecure and present a major risk factor for development of mental health disorders (Olff et al. 2013).

The Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORA) gene is also an intriguing target to investigate in in the context of trauma. RORA, whose function is complex and still poorly understood, belongs to the nuclear hormone receptors 1 subtype (NR1). The encoded protein seems to be related to brain development, neuroprotection and the regulation of circadian rhythms. RORA is widely expressed in cortical and subcortical neurons, where it protects them against oxidative stress and inflammation. These seem to be related to a possible mechanism linked to the effects of traumatic stress on the brain.

The correlation of RORA polymorphisms and traumatic stress was first evaluated a few years ago in genome-wide association study conducted by Logue et al. (2013). The study was conducted on a population of white non-Hispanic participants and their spouses and demonstrated a significant association between lifetime PTSD diagnosis and RORA rs8042149. Probands with low trauma exposure who are homozygous for the high-risk allele (G) have exhibited higher PTSD symptoms compared to carriers of the low risk allele (A). Another study demonstrated that the RORA gene was associated with higher PTSD symptoms, and this association was more pronounced in persons previously exposed to child abuse (Lowe et al. 2015). The main effects were observed in chronically elevated symptoms, which lead to the conclusion that RORA might influence the maintenance of subthreshold symptoms, causing patients not to remit on time. The underlying mechanism still seems to be more complex, but some promising results do exist. Miller et al. (2013) concluded that RORA might play a role in protection against neurodegeneration after oxidative stress. RORA has also been correlated with regulation of circadian rhythms and steroid hormone levels, both of which are important for the PTSD symptom spectrum (Germain 2013). The same mechanism was observed in another study, which included acute stressors only (Amstadter et al. 2013). The association after acute stressors was the same, meaning that carrying the G allele presented a risk factor for developing higher PTSD symptoms. Since the heritability of PTSD was mainly evaluated for chronic symptoms, this study underlines the assumption that the development of PTSD is genetically influenced. Yet other studies suggest that RORA might play an important role in general psychopathology, possibly through its relationship with other vulnerability factors such as neuroticism (Kim et al. 2017).

Several studies have investigated the association between PTSD, major depressive disorder (MDD) and generalized anxiety disorder (GAD) and the cannabinoid system (Fani et al. 2012, Lindstrom et al. 2011, Sveen et al. 2009). Neumeister et al. (2013) have found that PTSD is associated with an increased cannabinoid receptor type 1 (CB1) availability in an amygdala-hippocampal-cortico-striatal neural circuit as well as in brain regions outside this circuit, suggesting a greater brain-wide CB1 receptor availability in individuals with PTSD relative to controls with and without of trauma histories exposure. Increased availability of the CB1 receptor in the amygdala was associated with increased attentional bias to threat, as well as symptoms associated with increased severity of trauma-related threat in humans with a broad dimensional spectrum of trauma-related psychopathology (Pietrzak et al. 2014). Another significant finding was that the relation between CB1 receptor availability in the amygdala and severity of threat symptomatology is controlled by attentional bias to it (Pietrzak et al. 2014).

Given the aforementioned and the numerous investigations of the mentioned gene variants within the realm of psychiatry, we set out to investigate the possible association between PTSD and OXTR rs2254298 and rs53576, RORA rs8042149 and CNR1 rs1049353 (1359 G/A).

SUBJECTS AND METHODS

Subjects

Participants were recruited between 2013 and 2015 at five research centers (Sarajevo, Prishtina, Tuzla, Zagreb and Mostar) in the context of a Stability Pact for the South Eastern Europe (SEE) collaborative research study "Molecular Mechanisms of Posttraumatic Stress Disorder", supported by DAAD (Deutscher Akademischer Austausch Dienst). Methods regarding recruitment, diagnostic assessment, inclusion and exclusion criteria, as well as sample size and gender distribution were previously described (Dzubur Kulenovic et al. 2016). Most of the volunteers have experienced traumatic events related to war and ethnic cleansing in the time frame from 1991-1999, while some of them had not experienced any trauma or never had symptoms of PTSD. Therefore the experimental sample of a total of 719 volunteers (mean age 49.4±7.9; 232 females and 487 males) was divided into three major groups (Table 1).

The experimental group comprised 218 participants with current PTSD (mean age 50.1±6.7; 61 females and 157 males), 151 participants with lifetime PTSD (mean age 49.5±8.2; 53 females and 98 males), and 350 participants with no diagnosable PTSD (mean age 48.8±8.5; 118 females and 232 males) (Dzubur Kulenovic et al. 2016).

Table 1. Participant Groups According to Trauma

Group 1	Group 2	Group 3
Trauma +	Trauma +	Trauma +/-
Current PTSD	Recovered or Lifetime PTSD	No PTSD in life course

Ethical Votes

Ethical votes at the participating clinical centers were obtained between 2011 and 2013 on the basis of local translations of an information and consent form designed by the Würzburg center. Participants thus were informed and gave written informed consent according to the principles of the Declaration of Helsinki (WMA 2013).

Psychometric Instruments

To clarify the presence or absence of PTSD symptoms, the Mini International Neuropsychiatric Interview (M.I.N.I.) was used. Categorization of PTSD symptoms on current and lifetime was conducted using the Clinician Administrated PTSD Scale (CAPS) (Blake et al. 1995), while general psychiatric symptoms were measured with the Brief Symptom Inventory (BSI) (Derogatis & Melisaratos 1983).

Molecular Analyses

Genomic DNA was isolated from frozen venous blood according to manufacturer's instructions using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). It was stored at -80°C until genotyping at the Laboratory of Functional Genomics in Würzburg.

OXTR rs2254298 genotyping was done following published protocols with minor modifications (Wu et al. 2005). DNA was first amplified by PCR in a 25 µl reaction volume containing 45-65 ng genomic DNA, 0.4 mM of each primer
(F: 5'-GCCCCACACCCGAAGTCAGC-3' and R: 5'-TGTTGCCCTGGTCTCTGGCCA-3'), 0.1 mM of each nucleotide, 1.5 mM MgCL2 and 0.3 U TaqTM DNA polymerase under the following cycler conditions: 5 min denaturation at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 66.4°C and 45 s at 72°C and a final extension step of 5 min at 72°C and afterwards digested for 1.5 h at 65°C with the restriction endonuclease BsrI (NEB, Frankfurt a. Main, Germany). Differentially sized fragments, representing each genotype, were separated on a 3% agarose gel by electrophoresis and visualized with ethidium bromide. Genotyping for OXTR rs53576was done according to published protocols (Ziegler et al. 2015).

RORA rs8042149 genotyping was conducted via PCR-RFLP as follows: DNA was amplified by PCR (25 µl reaction mix: 50 ng genomic DNA, 0.4 mM of each primer, 0.1 of each mM nucleotide, 1 mM $MgCL_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH9), 0.01% Tween 20 and 0.3 U TaqTM DNA polymerase) using the oligonucleotide primers
F: 5'- GGAGCACAACTGTTACTCCAG-3'and R: 5'-AACAGCAGGAGAGAAATGCCA-3' and the following cycler conditions: 3 min denaturation at 95°C, 35 cycles of 45 s at 95°C, 45 s at 59.4°C and 45 s at 72°C and a final extension step of 5 min at 72°C. PCR fragments were digested with the restriction endonuclease NLAIII (NEB, Frankfurt a. Main, Germany) for 3h at 37°C, which results in differentially sized fragments representing the respective genotypes. The fragments were separated on a 3% agarose gel by electrophoresis and visualized with ethidium bromide. Fragment lengths and resulting genotypes were determined by two independent investigators blinded for diagnosis.

CNR1 rs1049353 was genotyped using a custom designed KASPTM genotyping assay (LGC, Berlin, Germany). A PCR reaction including an end-point fluorescent read-out was done according to manufacturers' instructions in a CFX384 Touch Cycler (Biorad, Munich, Germany). Genotype analysis was performed using the CFX Manager Software.

Statistical Analyses

Statistics were performed using PLINK 1.9. All SNPs were polymorphous (minor allele frequency≥10%), reached a minimal genotyping call rate of 98% and did not deviate from Hardy-Weinberg equilibrium ($p \geq 0.1$). Logistic regression was used for case-control analyses by testing all patients of either subgroup, lifetime or current PTSD, in combination versus the control individuals. Within the two groups of patients, linear regression was carried out individually for analyses on CAPS and BSI scores. The additive allelic and the genotypic models were tested in all phenotypes. The significance level was Bonferroni adjusted for 23 variants that were analyzed in total within the entire project ($\alpha=0.002$).

RESULTS

Oxytocin Receptor

Significant associations for OXTR rs53576 were not found for the categorical phenotype of PTSD, but nominally significant results were obtained when correlated with CAPS scores within the lifetime PTSD group ($P_{allelic}=0.0090$; $P_{dominant}=0.0173$ and $P_{genotypic}=0.0309$; Table 2 and Figure 1), consistent with the minor (A) allele increasing the risk for developing more severe PTSD symptoms. With regard to the BSI score, the association with OXTR rs53576 was nominally significant within the lifetime PTSD group in the allelic model ($p=0.0408$; Table 2 and Figure 2). The genotypic model did not yield any significant results ($p \geq 0.05$; Table 2).

However, these nominal significant results for OXTR rs53576 and CAPS and BSI scores could not be replicated in patients with current PTSD symptoms and did not withstand correction for multiple tests. For OXTR rs2254298, no significant associations were found at all ($p_{all} \geq 0.05$).

Table 2. OXTR rs53576 associations, along with genotype and allele counts, for participants with lifetime and current PTSD symptoms and controls, lifetime CAPS and BSI means and standard deviations, as well as nominal p-values

OXTR_rs53576	Allelic Model		Genotypic Model			Dominant Model	
	A	G	AA	AG	GG	AA/AG	GG
Controls	214	484	37	140	172	177	172
$PTSD_{lifetime}$	82	218	13	56	81	69	81
$PTSD_{current}$	125	311	14	97	107	111	107
$P_{case-control}$-value	0.2961			0.3128		0.2627	
$CAPS_{lifetime}$ (mean±SD)	71.3±17.4	65.3±17.4	75.5±18.9	69.5±16.3	63.8±17.5	70.6±17.0	63.8±17.5
P_{CAPS}-value	*0.0090*			*0.0309*		*0.0173*	
$BSI_{current}$ (mean±SD)	82.4±51.4	70.0±48.1	94.3±54.6	77.2±49.1	67.5±47.5	80.3±50.6	67.5±47.5
P_{BSI}-value	*0.0408*			0.1151		0.0808	

CAPS = Clinician Administered PTSD Scale; BSI = Brief Symptom Inventory; OXTR = Oxytocin Receptor; SD = standard deviation; *Italics indicates p≤0.05*

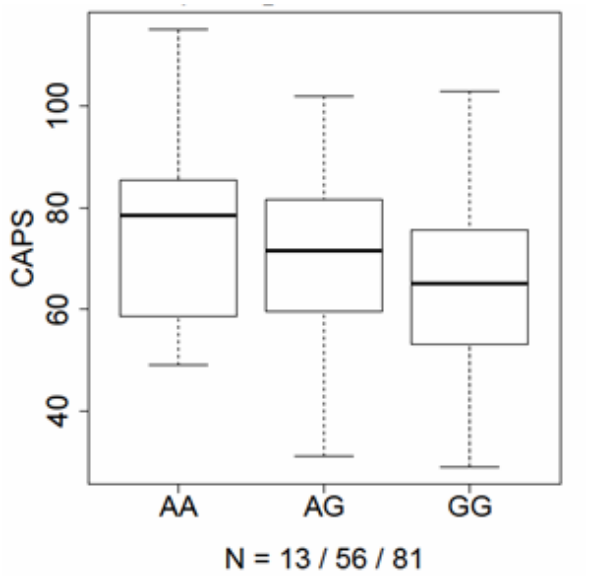

Figure 1. Distribution of lifetime CAPS values according to genotypes in the additive allelic model for OXTR rs53576 (p=0.0090)

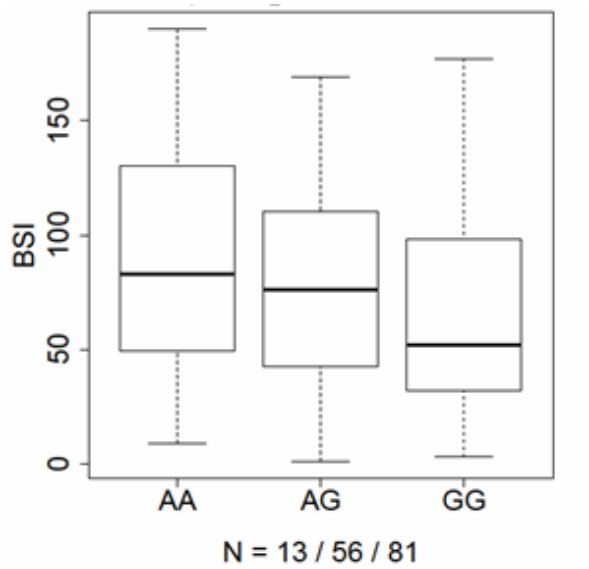

Figure 2. Distribution of lifetime BSI values according to OXTR rs53576 genotypes in the additive allelic model (p=0.0408).

Retinoic Acid Receptor-Related Orphan Receptor Alpha and Cannabinoid Receptor Type 1

Nominally significant observations were not found for RORA rs8042149 and for CNR1 rs1049353 in any of the models (p_{all}≥0.05). However, RORA rs8042149 showed a slight but not nominally significant (p=0.057) influence on BSI within the current PTSD patient subgroup in the recessive model. Carriers of at least one copy of the G allele demonstrated higher scores than T allele homozygotes.

DISCUSSION

The objective of this study was to examine the correlation between OXTR rs53376 and rs2254298, RORA rs8042149 and CNR1 rs1049353 and the development of trauma- and stress-related symptoms and general psychopathology. These genes have been previously associated with stress reactivity and possible psychopathological changes and PTSD symptoms. Nominally significant results were only found for OXTR rs53576, which modified the severity of PTSD symptoms and psychopathology in correlation with the allelic status. Specifically, the major allele (G) seems to be a protective factor for the development of PTSD symptoms and the related psychopathology as carriers of the mentioned G allele had lower CAPS and BSI scores. On the other hand, the A allele seems to aggravate symptom severity, resulting in higher CAPS and BSI scores. Changes have only been observed in the PTSD lifetime (remitted) group, and this may imply that OXTR modulates the reaction and the recovery from stress.

Our results are similar to what was found previously, namely that carrying the A allele leads to numerous deficits in social functioning, especially in terms of empathy (Gong et al. 2017), attachment (Sippel et al. 2017), lower self-esteem and negative affect in males (Lucht et al. 2009). Considering the definition and clinical presentation of PTSD, these parameters can be

correlated with the symptoms of the subjects used within this research and possibly suggest that they indirectly influence the course of illness. In a sample of 2163 US war veterans, Sippel et al. (2017) found that the A allele of rs53576 was related to PTSD lifetime symptoms especially in conjunction with insecure attachment styles. The actual connection of insecure attachment on responsiveness to social support has been observed before (Ditzen et al. 2008). Furthermore, Chen & Johnson (2012) found that individuals carrying the G allele had lower cortisol levels in stress situations only if they received social support. This effect was not found in an environment without social support, suggesting that this genetic variation impacts the degree in which social support buffers the consequences of stress.

Sociality is important for PTSD recovery in general. A meta-analysis reported that this SNP seemed to be related to interpersonal sociality (Li et al. 2015). Again, G homozygotes seem to be more sociable than A homozygotes in terms of general sociality but not sociality in close relationships. If social support is perceived in the right way and if a person behaves prosocial following trauma, this seems to be at least the "easier" way to recovery (Maercker & Hecker 2016). It seems that the OXTR gene may modulate the social behavior reactions after trauma. Since the research group in the present study mostly involved probands who had or have chronic PTSD, it is plausible that the level of sociality and the perception of social support as individual factors could lead to poorer symptom recovery. This has been proposed in previous studies (Laffaye et al. 2008, Beck et al. 2009).

Earlier studies connected OXTR rs53576 with psychological resources not only in correlation with stress but as impacts on stress response as well (Saphire-Bernstein et al. 2011). In this study, G allele carriers benefited from their allelic state in the way that they reported higher scores for psychological resources such as mastery, self-esteem, optimism and depression. In experimental environments, carriers of the A allele were more sensitive to stress situations and they were less adept to the reaction to it (Rodrigues et al. 2009). It is evident that most studies confirm OXTR's influence on the reaction to stress and point to the fact that this most probably leads to differences in responses on a social, behavioral and emotional level. Therefore, it may be proposed that OXTR does not necessarily reduce stress, but that it rather influences the reaction to it by shaping sociality, empathy, self-esteem, optimism and other social behaviors.

Although our results may suggest that the G allele of RORA rs8042149 might possibly be associated with higher BSI scores, no significant results were observed with RORA rs8042149, and this could be due to the fact that no detailed analyses regarding symptom clusters have been done. Previous studies have shown that specific symptom clusters seem to be related to genetic variations in this SNP, however these could be symptoms such as anhedonia, negative affect and dysphoria – rather those related to fear – that have previously been affected by this polymorphism. In previous studies, RORA, specifically rs8042149, has been associated with PTSD following acute (Amstadter et al. 2013) and long term stress exposure (Logue et al. 2010). Studies have shown that RORA mainly influences the persistence of subthreshold and chronically elevated symptoms (Lowe et al. 2015). However, this model seems to be more complex as other factors like comorbid psychiatric disorders and childhood trauma influence the gene expression. Most of the studies consider RORA to be a gene that presents a general risk factor for psychopathology development after distress, rather than an isolated candidate PTSD gene. As previous research has demonstrated, it seems that a dynamic interplay between genetic and environmental factors exists.

Although CNR1 has been proposed to be involved in contextual fear learning and been found associated with affective and anxiety phenotypes, we could not observe any association with any of our phenotypes studied. This argues against a major role for CNR1 rs1049353 in the pathogenesis of or recovery from PTSD.

There are several limitations to this study. The major limitation is the relatively small sample size, which, when divided into three groups becomes even lower. This lack of statistical power may explain why the observed OXTR finding did not withstand Bonferroni correction and why the result for RORA did not pass the threshold defining a nominal significance. Therefore, these findings have to be considered preliminary and replication in larger cohorts is required. This study also did not allow to control for other related or comorbid mental disorders such as depression (Saphire-Bernstein et al. 2011), autism (Wu et al. 2005) or social anxiety (Notzon et al. 2016). Similarly, we did not control for the influence of earlier traumatic events, especially those dating back to childhood, although they have been reported to influence the adult oxytocin (Smearman et al. 2016) as well as cannabinoid systems.

CONCLUSIONS

Our data provide tentative evidence for a role of OXTR genetic variation in PTSD. However, given the limitations of single-gene approaches and that all socio-emotional behaviors are influenced by multiple genes, future research e.g. using genome-and epigenome-wide approaches in larger cohorts should address how these genes may interact with others involved in social and emotional processing.

Contribution of individual authors:

Each author has actively participated in the international research project (see Acknowledgments) and, therefore, has substantially contributed to the development and publication of this manuscript.

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