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## Oxytocin receptor and G-protein polymorphisms in patients with depression and separation anxiety

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**Abstract****Background**

The impact of combined variants of Oxytocin Receptor (OXTR) and G protein  $\beta 3$  subunit genes was investigated in relation to retrospective reports of childhood as well as contemporary adult separation anxiety (SA), based on evidence of a  $\beta/\gamma$  dimer-mediated signaling for OXTR.

**Methods**

A case-control association study (225 healthy adults and 188 outpatients with depression) was performed to establish Risk-Combined Genotype (RCG) of the studied variants (OXTR rs53576 and the functional G $\beta 3$  subunit rs5443). Current SA was evaluated by the ASA-27 and retrospective childhood symptoms by the SASI. GG genotype of OXTR rs53576 combined with T-carrier genotype of G $\beta 3$  rs5443 represented the RCG.

**Results**

Compared to non-RCG, those with RCG had significantly higher levels of childhood and adult SA. The RCG was significantly associated with childhood SA threshold score (OR=2.85, 90%CI: 1.08-7.50). Childhood SA was, in turn, strongly associated with a threshold SA score in adulthood (OR=15.58; 95% CI: 4.62-52.59).

**Limitations**

Although the overall sample size is sizable, comparisons among subgroups with specific combination of alleles are based on relatively small numbers.

**Conclusions:**

Our study indicates that variations in OXTR and G $\beta 3$  genes are specifically associated with presence and severity of SA in childhood and adulthood, but not with depression or anxiety in general. Because there is increasing interest in oxytocin in social behavior, the gene-SA associations identified have potential translational and clinical relevance.

Keywords: separation anxiety, oxytocin receptor, G $\beta 3$ , depression, polymorphisms

## 1. Introduction

Depressive disorders are thought to result from the interplay of multiple genes interacting with environmental factors (Swaab et al., 2005; Grippo et al., 2007). There is substantial literature suggesting the involvement of the oxytocin (OXT) in depression based on evaluation of its levels in plasmatic/cerebrospinal fluid and of OXT transcripts in post mortem tissues of depressed patients (Scantamburlo et al., 2007; Wang et al., 2008). From this perspective, the traditional view of OXT as an endocrine hormone acting on peripheral organs (i.e., to induce labor and milk ejection) has been revised (Gimpl, G., Fahrenholz, F., 2001). Oxytocin is now considered to be a neurotransmitter or neuromodulator with central actions in the limbic system, particularly the amygdala, a key structure involved in mood disorders (Kirsch, 2015). Previous studies have documented modifications of neural activity induced by OXT in limbic regions of depressed patients (Pincus et al., 2010) and in animal models of depression (Slattery & Neumann, 2010).

In numerous studies, depression has been found to be strongly associated with separation anxiety, a condition characterized by apprehension over rejection, abandonment and high sensitivity to real or perceived threats to relationships (Carnelley et al., 1994; Murphy and Bates, 1997; Mickelson et al., 1997; Bifulco et al., 2002; Shaver et al., 2005; Conradi and De Jong, 2008). Separation from close attachment figures induces anxiety, which is normal and adaptive in early childhood. If this emotional state persists in its more severe form into adulthood, the individual feels unable to function alone in the absence of a reference person with a negative impact on depressive symptomatology, quality of life and outcome of treatment for mood disorders (Pini et al. 2014; Milrod et al. 2014; Marnane and Silove, 2013). The estimated lifetime prevalence of adult separation anxiety disorder occurring alone is 4.8%, but rate of comorbidity with depressive disorders reaches about 30% (Shear et al., 2006; Pini et al. 2014; Silove et al., 2015). Most studies, which attempted to identify the biological foundations of separation anxiety, pointed out the important role of OXT. Milrod and colleagues (Milrod et al., 2014) reappraised all available data on OXT in this context, concluding that altered plasma OXT levels are associated with greater anxiety and relationship dissatisfaction in persons with separation anxiety disorder. As might be expected given the strong evolutionary conservation of the small nonapeptide

OXT, mutation analysis of the corresponding gene, as a whole, has shown no consistent disturbance in separation anxiety disorder in adulthood (Costa et al., 2009a). A positive genetic association with separation anxiety disorder in adulthood has been found however, for a single nucleotide polymorphism (SNP; rs53576) of the OXT Receptor (OXTR) gene (Costa et al., 2009b). Specifically, the GG genotype of this SNP has been linked to high levels of separation anxiety and insecure attachment in patients with major depression (Costa et al., 2009b). Other authors have found that an interaction between exposure to high levels of early adversity with another OXTR SNP (rs2254298) is associated with the level of separation anxiety (SA) symptoms in adolescents (Thompson et al., 2011). Although the precise genetic function of OXTR rs53576 remains to be established, a study suggests it may determine deficits in socio-behavioral domains such as separation sensitivity, attachment and positive affect (Tost et al., 2010). Interest in the potential psychiatric relevance of this genetic variant was furthered by studies indicating that rs53576A is over-transmitted in some families with offspring exhibiting autistic spectrum disorders and may represent a central component of haplotypes relevant to high-functioning autism (Werner et al., 2009).

The SNP rs53576 is located within the third intron of the OXTR gene which appears to be instrumental in the epigenetic regulation of OXTR expression (Muzimoto et al., 1997). The OXTR is a prototypical G protein-coupled receptor (GPCR) known to couple to heterotrimeric Gq/11 protein. Contrary to the classical perspective on GPCR signaling, the  $\beta/\gamma$  subunits are the major mediators of OXT-evoked activation of intracellular signaling, including both to peripheral cells and neurons (Zhong et al., 2011). In magnocellular neurons, the release of  $\beta/\gamma$  subunits induced by OXTR activation has been shown to play a prominent role in generating burst firing patterns, indicating that the dimer is crucial for pulsatile neuropeptide secretion (Wang and Hatton, 2007).

In our efforts to extend knowledge about the role of the OXT pathway in separation anxiety we explore the role of a functional SNP (rs5443) of  $\beta 3$  subunit of G protein gene in addition to that of OXTR rs53576 (Roskopf et al., 2003; Ruiz-Velasco et al., 2003), given that the former may be involved in a intracellular signaling pathway activated by the stimulation of OXTR. Our focus is also

influenced by the knowledge that the  $\beta 3$  subunit isoform of G protein is ubiquitously expressed in the brain, including in oxytoninergic regions (Liang et al., 1998; Zhong et al., 2003; Wang et al., 2007). The T allele of rs5443 causes an alternative splicing of exon 9 resulting in an in-frame deletion of 123-bp. G $\beta 3$  splice variant (termed G $\beta 3s$ ) is 41 amino acids shorter than the wild-type G $\beta 3$ . Although this issue has been not definitively clarified, there is evidence of an inability of G $\beta 3s$  to activate a variety of intracellular effectors (Siffert et al., 1998).

As described above, data from literature show a strong association between oxytocin pathways and depression. Parallel, converging evidences pointed out the essential role of OXT in the biological foundations of attachment behaviours of which separation anxiety represents one of most important clinical correlates among adult individuals (Milrod et al., 2016). Within this framework, in a previous study, we found a significant association between OXTR polymorphisms and several attachment dimensions among depressive patients (Costa et al., 2009b). All these data, prompted us to explore, in patients with depression, whether combined genotypes of OXTR rs53576 and G $\beta 3$  rs5443 were associated with either childhood or adult separation anxiety and their relationship with depressive symptoms.

## 2. Method

### 2.1. Subjects selection

The study sample comprised 413 participants, of which 225 were healthy individuals. The remaining sample included an overall group of 188 consecutive adult psychiatric outpatients with Axis I mood disorders as a principal diagnosis referred to the outpatients' clinic of the Department of Psychiatry at the University of Pisa. Patients with psychotic disorders, substance abuse and serious medical conditions were excluded from the analyses. The study sample represented an extension of a sample used in a previous our work (Costa et al., 2009b). All recruited patients and controls were Caucasians on the basis of a retrospective analysis of their genealogy departing from three generations behind, according to previous studies (Fuku et al., 2015; Tannorella et al. 2016). Healthy individuals were recruited among university personnel and were assessed by SCID-I-NP (First et al. 2002a) and additional medical screening. Volunteers with a current or past history of major psychiatric disorders were excluded. The University of Pisa Ethical Committee approved the study design. All subjects provided written informed consent prior to participation after being informed of the nature of study procedures.

### 2.2. Psychometric evaluation

All patients were assessed with the SCID-I (First et al., 2002) to establish a DSM-IV Axis-I diagnosis and patterns of psychiatric comorbidity and were specifically screened for lifetime separation anxiety disorder. Patients were evaluated by the Hamilton Depression Rating Scale (HAM-D) (Hamilton, 1960) to assess the severity of depression. Anxiety was assessed by the 'Anxiety' Factor score of the HAM-D (McClintock et al., 2011).

### 2.3. Assessment of Separation Anxiety

#### 2.3.1. Adult Separation Anxiety – Checklist (ASA-27)

The ASA-27 is a self-report questionnaire containing 27 items assessing relevant symptoms in adulthood (Manicavasagar et al., 2003). Participants respond to items on a four-point Likert scale,

ranging from “This has never happened” to “This happens very often”. Item scores (0–3) are added to yield a total score ranging from 0 to 81. The measure has shown a high level of internal consistency (Cronbach’s alpha = .89), and test–retest reliability ( $r = .86, p < .001$ ) (Manicavasagar et al., 1997; Silove and Marnane, 2013). A total score of 22 or higher provides a threshold for adult separation anxiety that has been shown to correspond closely to a diagnosis of separation anxiety disorder based on a structured clinical interview (Manicavasagar et al., 2003).

### 2.3.2. Separation Anxiety Symptom Inventory (SASI)

The SASI is a 15-item self-report measure assessing separation anxiety symptoms retrospectively, based on experiences prior to 18 years of age (Silove et al., 1993). Items are scored from 0 to 3 on a frequency scale. The SASI has been shown to have sound internal (Cronbach’s alpha = 0.88) and test-retest reliability over 24 months (intraclass correlation coefficient = 0.89). In the development of the measure, distributions were found to be skewed, a pattern adjusted for by applying a square root transformation. Hence, a raw score of 16 generates a transformed score of 4. Mean transformed SASI scores of 4 or more have been associated with reports of past childhood separation anxiety disorder and/or school refusal, offering some evidence of the concurrent validity of the measure.

### 2.4. Genotyping

The studied SNPs were rs53576 (G6930A) located in intron 3 of *OXTR* (ID:5021) and rs5443 (C825T) located in exon 10 of G protein  $\beta 3$  subunit gene, (Gene name: *GNB3*; ID:2784). In Caucasians, the *OXTR* rs53576 and *G $\beta 3$*  rs5443 minor allele is A (frequency=0.35) (Walum et al., 2011) and T (frequency= 0.30) (Roskopf et al., 2002), respectively. Genomic DNA from all subjects was isolated from peripheral whole blood cells with a QIAamp DNA Mini Kit (Qiagen). Genotyping analyses were performed by means of PCR-RFLP techniques, as previously described (Costa et al., 2009b; Willeit et al., 2003). Both the case and control status were blind to the operator during genotyping processes. Briefly, 25  $\mu$ l PCR contained 1.25 mmol/l MgCl<sub>2</sub>, 200  $\mu$ mol/l dNTPs, 50 ng of DNA, DNA polymerase (1 unit of Amplitaq Gold-Applied Biosystems for *OXTR* rs53576; 0.025 unit of Taq Gold Polymerase-Perkin Elmer for *G $\beta 3$*  rs5443), forward (Fwd) and reverse (Rev) primer (0.20  $\mu$ mol/l of



each primer for OXTR rs53576: Fwd: 5'-GCCACCATGCTCTCCACATC-3'; Rev: 5'-GCTGGACTCAGGAGGAATAGGGAC-3'; 5 pmol/l of each primer for Gβ3 rs5443: Fwd: 5'-TGACCCACTTGCCACCCGTGC-3'; Rev: 5'-GCAGCAGCCAGGGCTGGC-3'). Thermal cycling was performed with an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, then followed by a terminal extension of 7 min at 72 °C. For OXTR rs53576, the PCR products were digested with 2.5 Units BamHI (New England BioLabs) and separated by electrophoresis on 2.5% ultrapure agarose-1000 (Gibco BRL). Digestion at 37°C resulted in 120- and 220-bp products for minor allele A, and in a 340-bp undigested product for the major allele G. For Gβ3 rs5443, the PCR products were digested with 2.5 Units BseDI (New England BioLabs) and separated by electrophoresis in 2.5 % Nusieve GTG agarose gels (Cambrex). Digestion at 37°C resulted in 116- and 152-bp products for major allele C, and in a 268-bp undigested product for the minor allele T.

#### *2.4.1. SNP predictive analysis on transcription factor binding*

The OXTR rs53576 was analyzed to predict whether it affected the binding affinity of transcription factors to their nucleotide sequences. The analysis was performed by the use of the Transcription factor Affinity Prediction (TRAP) Web tools developed at the Max Planck Institute for Molecular Genetics and available at Web site (<http://trap.molgen.mpg.de/>). This program identifies sequence motifs to which the transcription factors bind using the database of TRANSFAC and JASPAR matrices. The program sTRAP (difference between two sequences) was chosen as an option. The analysis was conducted by the simultaneous insertion of the two nucleotide sequences (1001 bp each) containing the G allele or A allelic variant of the polymorphism (in fasta format). Three types of analyses were made selecting as a matrix and a background model: 1) “Transfac\_12.1 metazoans” and “chordate conserved elements”; 2) “Transfac\_10.1 vertebrates” and “human promoter”; 3) “Jaspar vertebrate” and “human promoter”, respectively.

### 2.5. Statistical analysis

Statistical analyses were performed using SPSS software (Version 20.0; SPSS Inc, Chicago, Ill). Hardy-Weinberg equilibrium (HWE) was tested by  $\chi^2$  test for goodness of fit based on the web program HWE test calculator for biallelic markers ([www.oege.org/software/hwe-mr-calc.shtml](http://www.oege.org/software/hwe-mr-calc.shtml)) (Rodriguez et al., 2009). The statistical package QUANTO 1.2.4.exe was used to evaluate the statistical power of the study. Given a case-control cohort of almost 200 subjects each and reported minor allele frequencies of 0.35 and 0.30 for G $\beta$ 3 rs5443 and OXTR rs53576 respectively (Rosskopf et al., 2002; Walun et al., 2012), the study had an a priori power higher than 80% to detect ORs of 1.5 or higher for each single SNP, and to detect ORs of 2.2 or higher for gene-gene interactions under the additive genetic model. For categorical comparisons and to assess differences in the genotype and allele distributions between groups, the  $\chi^2$  test was performed. Odds ratios have been calculated and expressed with 95% confidence intervals (CIs). The synergy index (S) based on the method of departure-from-additivity model introduced by Rothman (Rothman, 1974) was calculated to assess the joint effect between risk factors. This method showed that the independent risk factors adhered to an additive model and then assessed the joint effect based on the departure from this additivity model. The S value was estimated by the mathematical formula:  $S = [OR_{(+/+)} - 1] / [(OR_{(+/-)} - 1) + (OR_{(-/+)} - 1)]$ , where  $OR_{(+/+)}$ ,  $OR_{(+/-)}$ , and  $OR_{(-/+)}$  were odds ratio exposed to both two factors, only the first factor, and only the second factor, respectively. The S is 1.0 when there is no interaction between the two factors. When S is greater than 1, the joint effect of two risk factors on the disease is greater than the sum of independent effects of each of the two factors, which suggests the presence of an interaction. Comparisons for dimensional scales were expressed in terms of mean scores and standard errors (SE) by using two-tailed Student's t test. Linear regressions were performed in order to control for a possible confounding effect of sex on any association between the SASI or ASA-27 total scores and OXTR rs53576/G $\beta$ 3 rs5443 non risk (-/-) and risk (+/+) combined genotype. Dichotomized squared root-transformed SASI scores (threshold :  $\geq 4$  vs  $< 4$ ) (Silove et al., 1993) were used to calculate crude ORs representing associations between childhood separation anxiety and the combined genotype. For the association between adult separation anxiety and the combined genotype,

we used the dichotomized ASA-27 total score based on the pre-established threshold:  $\geq 22$  vs  $< 22$ )

(Silove et al., 2013).

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### 3. Results

#### 3.1. Demographic and clinical characteristics of study samples

Of the 225 healthy controls, 153 were females and 72 were males. Among patients, 131 were females and 57 were males. Patients and healthy individuals did not differ in sex distribution (Chi-square: 0.13,  $p = 0.713$ ). The mean age of controls and patients were, respectively,  $42.2 \pm 11$  and  $42.5 \pm 12.4$  ( $t=0.260$ ,  $p=0.795$ ).

The HAM-D mean total score was  $11.2 \pm 7.22$  in the overall sample of patients and the mean HAM-D ‘Anxiety’ factor score was  $2.85 \pm 2.19$ . Demographic and clinical characteristics of study patients with comparisons between bipolar ( $N=95$ ) and unipolar subjects ( $N=93$ ) are displayed in Table 1.

#### 3.2. Allele and genotype distributions of OXTR rs53576 and Gβ3 rs5443 SNPs

Allele and genotype frequencies of OXTR rs53576 and Gβ3 rs5443 SNPs are summarized in Table 2.

In healthy individuals, genotype frequencies of both SNPs were in Hardy–Weinberg equilibrium ( $\chi^2=1.489$ ,  $df=1$ ,  $P=0.222$  for rs53576;  $\chi^2=0.982$ ,  $df=1$ ,  $P=0.322$  for rs5443). Allele frequencies (Tab.2) are consistent with those previously reported for Caucasians (Walum et al., 2012; Roskopf et al., 2002).

For OXTR rs53576, comparison of allelic variant frequencies between patients and healthy individuals revealed an excess of GG genotype in patient group ( $P=0.037$  vs GA genotype;  $P=0.049$  vs A-carriers; Tab. 2). These results are consistent with previously published data (Costa et al., 2009b). For Gβ3 rs5443, the comparison showed an excess of the TT genotype and T allele in the patient group

( $P=0.016$  vs CC genotype;  $P=0.025$  vs C allele; Tab. 2), as previously reported (Bondy et al., 2002; Lee et al., 2004).

For analyses of the combined OXTR rs53576/ Gβ3 rs5443 SNPs, the homozygous genotype carrying minor allele (rs53576 AA and rs5443 TT genotype) was relatively infrequent so that we collapsed homozygous and heterozygous genotypes to form one group (named A-carriers and T-carriers, respectively). For OXTR rs53576, the GG and A-carrier groups were considered as the risk (+) and non-risk (-) genotypes, respectively. For Gβ3 rs5443, the T-carrier and CC groups were considered as the risk (+) and non-risk (-) genotypes, respectively. Combinations of OXTR rs53576 and Gβ3 rs5443 genotypes were grouped into four categories: those containing both of the risk genotype (+/+), those containing one of the risk genotypes (+/-), or (-/+), and those containing none of the risk genotypes (-/-). As shown in Table 2, the genotype distribution comparison analyses revealed an increased number of individuals with both risk genotypes in the group of patients (OR=2.21, 95% CI=1.23-3.97,  $P=0.008$ ). Analysis of the joint effect of OXTR rs53576 and Gβ3 rs5443 using the method of departure-from-additivity model showed a S value significantly greater than 1, suggesting the presence of a synergic interaction between the two SNPs ( $S=2.37$ ; 95% CI =1.07-5.24).

### *3.3. Comparisons of SASI, ASA-27, HAM-D and HAM-D Anxiety Factor scores between OXTR rs53576/ Gβ3 rs5443 combined genotype*

In Figure 1 (Part A), the SASI mean squared-root scores were  $2.89\pm 0.21$  in (-/-) and  $3.79\pm 0.17$  in (+/+) combined ( $t=3.313$ ,  $df=$  p=0.001) genotypes respectively. Comparisons of SASI total scores between the genotype (-/+) or (+/-) and non risk genotype (-/-) were not statistically significant.

In Figure 1 (Part B), the ASA-27 total scores in the (-/-) ( $27.6\pm 2.22$ ) and (+/+) ( $35.4\pm 2.40$ ) groups are displayed, being comparison significant ( $t=2.313$ ,  $p=0.023$ ). Comparisons of ASA-27 total scores between the genotype (-/+) or (+/-) and non risk genotype (-/-) gave no statistically significant results.

In Figure 1 (Part C), HAM-D total scores were  $10.9 \pm 1.36$  in the (-/-) group and  $11.4 \pm 1.03$  in the (+/+) group ( $t=0.299$ ,  $p=0.767$ ). Comparisons of HAM-D total scores between the genotype (-/+ or +/-) and non risk genotype (-/-) were not statistically significant.

In Figure 1 (Part D), the HAM-D 'Anxiety' Factor scores were  $3.13 \pm 0.41$  in the (-/-) group and  $2.01 \pm 0.30$  in the (+/+) group ( $t=0.438$ ,  $p=0.662$ ). No significant differences emerged for comparisons of HAM-D 'Anxiety' Factor scores between the genotype (-/+ or +/-) and non risk genotype (-/-).

### *3.4. Associations (Odds Ratios) between OXTR rs53576/Gβ3 rs5443 combined genotype and separation anxiety*

#### *3.4.1. Childhood Separation Anxiety (SASI $\geq 4$ )*

In the patient group, as shown in Figure 2 (part A), amongst subjects with a SASI total score  $< 4$  (SASI-), 77.8% ( $n=28$ ) had a (-/-) and 55.1% ( $n=27$ ) had a (+/+) combined genotype. Subjects with a SASI total score  $\geq 4$  (SASI+) comprised 22.2% ( $n=8$ ) with a (-/-) combined genotype and 44.9% ( $n=22$ ) with (+/+) the combined genotype. In terms of odds ratios, using the (-/-) combined genotype as a reference category, the (+/+) combined genotype was significantly associated with assignment to the SASI+ group (OR=2.85, 90%CI: 1.08-7.50,  $p=0.034$ ).

#### *3.4.2. Adult Separation Anxiety (ASA-27 $\geq 22$ )*

As shown in Figure 2 (part B), of subjects with an ASA-27 total score  $< 22$  (ASA-), 38.9% ( $n=14$ ) had a (-/-) and 22.4% ( $n=11$ ) had a (+/+) combined genotype in patients group. Subjects with an ASA-27 total score  $\geq 22$  (ASA+) 61.1% ( $n=22$ ) were associated with the (-/-) combined genotype and 77.6% ( $n=38$ ) with the (+/+) combined genotype. Using the (-/-) combined genotype as the reference category, the (+/+) combined genotype showed an increased association with ASA+ (OR=2.20, 90%CI: 0.85-5.67,  $p=0.103$ ), although not reaching statistical significance.

Linear regression analyses were performed controlling control for sex in examining associations first between the SASI and then the ASA-27 total score and the (-/-) and (+/+) combined genotype of

OXTR rs53576/ Gβ3 rs5443 SNPs. Results showed no effect for sex with the SASI (+/+ genotype:  $\beta=0.351$ ,  $t=3.428$ ,  $p=0.001$ ; sex:  $\beta=0.53$ ,  $t=0.520$ ,  $p=0.605$ ), nor with the ASA-27 (+/+ genotype:  $\beta=0.251$ ,  $t=2.405$ ,  $p=0.018$ ; sex:  $\beta=0.180$ ,  $t=1.721$ ,  $p=0.089$ ).

### 3.5 Relationship between Childhood Separation Anxiety (SASI $\geq 4$ ) and Adult Separation Anxiety (ASA-27 $\geq 22$ )

We found that 95.4% of subjects with a SASI score  $\geq 4$  had a total score  $\geq 22$  on the ASA-27. Conversely, among those with SASI score  $< 4$ , a similar percentage (57% and 43%, respectively) had ASA-27  $\geq 22$  and ASA-27  $< 22$ . In terms of Odds Ratios (OR), the presence of childhood SA was significantly predictive of having the same condition during adulthood (OR= 15.58; 95% CI: 4.62-52.59).

### 3.6 Predictive transcription factor binding analysis of OXTR rs53576.

The potential functionality of rs53576 in the third intron of *OXTR* is uncertain. However rs53576 is located inside a region containing sequences involved in the epigenetic transcriptional regulation and in the binding of nuclear proteins (not yet identified) capable of suppressing the transcription of *OXTR* gene (Mizumoto et al., 1997). In order to explore the potential functionality of OXTR rs53576, *in silico* analysis was used to predict differential transcription factor binding affinities of sequences containing the OXTR rs53576 SNP. Among the top 10 predictions were differences in binding affinities of the transcription factors p53 and p63. In particular, the risk G allele (differently from the non-risk A allele) altered p53 sequence motifs, causing the loss of p53 binding affinity. Details of this analysis are presented in Table 3.

The results were obtained using Transfac database. Analysis by the use of Jaspar database gave for the Matrix\_ID= MA0106.1, Matrix name=TP53: absolute difference  $\log(p) = -1.73$ ; P value for G allele= 0.0036; P value for A allele=6.67e-05.

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#### 4. Discussion

We found a positive association between OXTR rs53576/ Gβ3 rs5443 risk combined genotype (+/+) and separation anxiety specifically related to childhood manifestations, indicating a neural mechanism related to the oxytocinergic system that is not influenced by sex. Individuals with risk-combined genotype (+/+) were characterized by higher levels of childhood and adult separation anxiety (a trend for the latter) with respect to non-risk combined genotype (-/-). On the contrary, such associations were not found for levels of anxiety or depression in general, as measured by the Hamilton Depression Rating Scale, which, indeed, were not particularly severe at the time of the evaluation. These data suggest specificity of association between the two polymorphisms and separation anxiety. Rather than experiencing high levels of fluctuating anxiety, adults with SA report extreme anxiety about separations from major attachment figures (partner, children, or parents), fear that harm would befall those close to them and need to maintain proximity to them. Therefore, the nature of the social interaction and emotional response in relation to close attachment figures in patients with depression may be strongly related by these specific associations with separation anxiety (Milrod et al., 2016). These data align closely with previous evidences in this direction. Milrod et al. (2014), reviewing relevant literature on this issue, confirmed that separation anxiety is common among patients with depressive disorders and complicates course and negatively impact treatment response. Applying independent thresholds for childhood SA and adult SA (SASI score  $\geq 4$  and ASA-27 total score  $\geq 22$ , respectively), the association between the risk genotype and childhood SA was statistically significant and approached that level in adulthood. These data indicate a genetic substrate for separation anxiety, which may be particularly marked where onset is in childhood. We also found that the presence of childhood SA was strongly predictive of having the same condition during adulthood (OR= 15.58; 95% CI: 4.62-52.59) suggesting that a lifelong pattern of this form of anxiety, commencing in early life, may be most associated with the genetic pattern under study. In Figure 3, we integrate possible genetic and clinical relationships between childhood and adult separation anxiety on the basis of our data.

Taking into account our combined genetic and clinical findings, the data offer support for the hypothesis that the expression of SA in childhood is strongly related to the occurrence of the pattern in life, and that this lifelong pattern is related to alterations in oxytocinergic system (Eapen et al., 2014). It is possible, as suggested by our data, that the later the onset of SA, the less influenced the pattern is by genetic factors, environmental experiences, such as repeated traumatic losses or other adverse events perhaps playing a more decisive role.

From a molecular point of view, the present findings suggest that the risk of the combined genotypes studies might be reflected in the effects on oxytocinergic pathway functionality. The combination of the risk allelic variants of rs53576 and rs5443 may result in altered intracellular signaling pathways by acting at two specific levels: alteration of OXTR expression and the OXTR-mediated intracellular response, respectively. In Figure 3B, we offer a schema proposing how allelic variants could contribute to modulate the OXTR-mediated pathway, based on our findings and extant data in the literature. In particular, our *in silico* results provided evidence that the OXTR rs53576 risk G allele abolishes the binding affinity of the transcription factor p53, suggesting that there is a potential alteration in the regulation of OXTR expression p53 which is known to be the most potent transcription factor activated in response to cytotoxic insults and one that exerts pro-apoptotic activities (Tolstonog et al., 2010). However, there also is emerging evidence of an intriguing non-apoptotic role for p53 (axonal outgrowth and regeneration, synaptic remodeling) in the nervous system (Qin et al., 2009; Tedeschi et al., 2009; Buizza et al., 2013). Whether the *in silico* observed difference in p53 binding could account for patterns of altered OXTR expression in specific neuron populations represents a promising area for future research. Consistent with our hypothesis, additional SNPs located in intron 3 have been found to influence OXTR expression (Tansey et al., 2010; Dixon et al., 2007). Although the functionality of rs53576 remains unknown, there is evidence for its potential involvement in structural neural alterations in key oxytocinergic regions. Indeed, rs53576 has been associated with changes in the volume of the hypothalamus, and in the structural coupling of the hypothalamus and amygdala (Tost et al., 2010; Wang et al., 2013). Concerning the Gβ3 rs5443, emerging evidence supports its role in altered Gβ3 protein functionality (Roskopf et al., 2003; Ruiz-

Velasco et al., 2003). Although it has been not definitively clarified, there is evidence of an inability of the splice-variant protein Gβ3s derived from the risk T-allele in activating a variety of intracellular effectors (Siffert et al., 1998) (see Table 1 in Supplementary material).

### *Limitations*

Limitations of this study should be acknowledged. Comparisons among groups with specific combination of alleles are based on relatively small sizes and, therefore, confirmatory data from independent studies are needed. Use of data collected from university personnel as control group may be associated with differences between patients and controls in aspects like socio demographic background or intelligence, both of them potentially affecting levels of anxiety (Coplan et al., 2011). In addition, we did not perform a genetic control of Caucasian origin. However, Caucasian origin was based on a retrospective analysis of their genealogy departing from three generations behind. This strategy has been used in previous studies (Fuku et al., 2015; Tannorella et al. 2016).

The distinction made between childhood and adult separation anxiety based on the two measures used (the SASI and ASA-27) may be somewhat artificial in that it is possible that adults with heightened symptoms are prone to report similar emotional responses in early life and vice versa. Only longitudinal studies commencing in childhood are capable of addressing this issue critically. Finally, relationship of the oxytocin receptor gene with CSF or plasma oxytocin peptide has not been explored in this study. Therefore, it is difficult to integrate our findings into the general literature focusing on the measurement of the former indices across a range of mental disorders.

Our data add knowledge on the role of oxytocin in abnormal behaviours and psychopathology. Further studies are warranted to shed light on alterations of oxytocin pathway as a key mediator for a range of common mental disorders in childhood and adulthood. This is a promising avenue that may hasten the discovery of personalized treatment approaches based on oxytocin administration or other forms therapy that influence its expression.

### **Statement of interest**

None to declare

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## References

- Bifulco, A., Moran, P.M., Ball, C., Bernazzani, O., 2002. Adult attachment style. I. Its relationship to clinical depression. *Soc.Psychiatry Psychiatr. Epidemiol.* 37, 50—59.
- Bondy, B., Baghai, T.C., Zill, P., Bottlender, R., Jaeger, M., Minov, C., Schule, C., Zwanzger, P., Rupprecht R., Engel, R.R., 2002. Combined action of the ACE D- and the G-protein beta3 T-allele in major depression: a possible link to cardiovascular disease? *Mol Psychiatry*, 7, 1120-1126
- Buizza, L., Prandelli, C., Bonini, S.A., Delbarba, A., Cenini, G, Lanni, C., Buoso, E., Racchi, M., Govoni, S., Memo, M., Uberti, D., 2013. Conformational altered p53 affects neuronal function: relevance for the response to toxic insult and growth-associated protein 43 expression. *Cell Death Dis.* Feb 7;4:e484. doi: 10.1038/cddis.2013.13.
- Carnelley, K.B., Pietromonaco, P.R., Jaffe, K., 1994. Depression, working models of others, and relationship functioning. *J. Pers. Soc. Psychol.* 66, 127—140.
- Conradi, H.J., de Jonge, P., 2008. Recurrent depression and the role of adult attachment: a prospective and a retrospective study. *J. Affect. Disord.*, doi:10.1016/j.jad.2008.10.027.
- Coplan, J. D., Hodulik, S., Mathew, S. J., Mao, X., Hof, P. R., Gorman, J. M., & Shungu, D. C. (2011). The Relationship between Intelligence and Anxiety: An Association with Subcortical White Matter Metabolism. *Frontiers in Evolutionary Neuroscience*, 3, 1-7.  
<http://doi.org/10.3389/fnevo.2011.00008>

- Costa, B., Pini, S., Martini, C., Abelli, M., Gabelloni, P., Ciampi, O., Muti, M., Gesi, C., Lari, L., Cardini, A., Mucci, A., Bucci, P., Lucacchini, A., Cassano, G.B., 2009. Mutation analysis of oxytocin gene in individuals with adult separation anxiety. *Psychiatry Res.*, 168, 87-93 (a).
- Costa, B., Pini, S., Gabelloni, P., Abelli, M., Lari, L., Cardini, A., Muti, M., Gesi, C., Landi, S., Galderisi, S., Mucci, A., Lucacchini, A., Cassano, G.B., Martini, C., 2009. Oxytocin receptor polymorphisms and adult attachment style in patients with depression. *Psychoneuroendocrinology* 34, 506-514 (b).
- Dixon, A.L., Liang, L., Moffatt, M.F., Chen, W., Heath, S., Wong, K.C., Taylor, J., Burnett, E., Gut, I., Farrall, M., Lathrop, G.M., Abecasis, G.R., Cookson, W.O., 2007. A genome-wide association study of global gene expression. *Nat Genet.*, 39, 1202–1207
- Eapen, V., Dadds, M., Barnett, B., Kohlhoff J., Khan, F., Radom, N. et al. Separation anxiety, attachment and inter-personal representations: disentangling the role of oxytocin in the perinatal period. *PLoS One*. E-pub ahead of print 12 September 2014. doi: 10.1371/journal.pone.0107745.
- First, M.B., Spitzer, R.L., Gibbon, M., Williams, J.B.W. Structured clinical interview for DSM-IV-TR Axis I disorders, research version, patient edition. Edited by New York, Biometrics Research, New York State Psychiatric Institute, 2002
- First, M. B., Spitzer, R.L, Gibbon M., and Williams, J.B.W.: Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Non-patient Edition. (SCID-I/NP) New York: Biometrics Research, New York State Psychiatric Institute, November 2002.
- Fuku, N., He, Z.H., Sanchis-Gomar, F., Pareja-Galeano, H., Tian, Y., Arai, Y., Abe, Y., Murakami, H., Miyachi, M., Zempo, H., Naito, H., Yvert, T., Verde, Z., Venturini, L., Fiuza-Luces, C., Santos-Lozano, A., Rodriguez-Romo, G., Ricevuti, G., Hirose, N., Emanuele, E., Garatachea, N., Lucia, A. 2015. Exceptional longevity and muscle and fitness related genotypes: a functional in vitro analysis and case-control association replication study with SNPs THRrs7832552, IL6 rs1800795, and ACSL1 rs6552828. *Front Aging Neurosci.* 7:59.

- Gimpl, G., Fahrenholz, F., 2001. The oxytocin receptor system: structure, function, and regulation. *Physiol. Rev.* 81, 629-83.
- Grippe, A.J., Gerena, D., Huang, J., Kumar, N., Shah, M., Ughreja, R., Carter, C.S., 2007. Social isolation induces behavioral and neuroendocrine disturbances relevant to depression in female and male prairie voles. *Psychoneuroendocrinology* 32, 966—980.
- Hamilton, M., 1960. A rating scale for depression. *J Neurol Neurosurg Psychiatry* 23, 56–62
- Lee, H.J., Cha, J.H., Ham, B.J., Han, C.S., Kim., Y.K., Lee, S.H., Ryu, S.H., Kang, R.H., Choi, M.J., Lee, M.S., 2004. Association between a G-protein beta 3 subunit gene polymorphism and the symptomatology and treatment responses of major depressive disorders. *Pharmacogenomics J.*, 4, 29-33
- Liang, J-J, Cockett, M., and Khawaja, X.Z. , 1997. Immunohistochemical Localization of G Protein b1, b2, b3, b4, b5, and g3 Subunits in the Adult Rat Brain. *Journal of Neurochem.*, 71, 345-355
- Manicavasagar, V., Silove, D., Curtis , J., 1997. Separation anxiety in adulthood: a phenomenological investigation. *Compr Psychiatry*, 38, 274-282
- Manicavasagar, V., Silove, D., Wagner, R., Drobny, J. A., 2003 self-report questionnaire for measuring separation anxiety in adulthood. *Compr Psychiatry*, 44, 146-153
- Marnane, C., Silove, D., 2013. DSM-5 allows separation anxiety disorder to grow up. *Aust N Z J Psychiatry*, 47,12-15.
- McClintock, S.M., Husain, M.M., Bernstein, I.H., Wisniewski, S.R., Trivedi, M.H., Morris, D., Alpert, J., Warden, D., Luther, J.F., Kornstein, S.G., Biggs, M.M., Fava, M., Rush, A.J., 2011. Assessing anxious features in depressed outpatients. *Int J Methods Psychiatr Res.* 20, 69-82.
- Mickelson, K.D., Kessler, R.C., Shaver, P.R., 1997. Adult attachment in a nationally representative sample. *J. Pers. Soc. Psychol.* 73, 1092—1106.

- Milrod, B., Markowitz, J.C., Gerber, A.J., Cyranowski, J., Altemus, M., Shapiro, T., Hofer, M., Glatt, C., 2014. Childhood separation anxiety and the pathogenesis and treatment of adult anxiety. *Am J Psychiatry*, 171, 34-43.
- Milrod, B., Altemus, M., Gross, C., Busch, F., Silver, G., Christos, P., Stieber, J., Schneier, F., 2016. Adult separation anxiety in treatment nonresponders with anxiety disorders: delineation of the syndrome and exploration of attachment-based psychotherapy and biomarkers. *Compr Psychiatry*, 66, 139-45.
- Mizumoto, Y., Kimura, T., Ivell, R., 1997. A genomic element within the third intron of the human oxytocin receptor gene may be involved in transcriptional suppression. *Mol Cell Endocrinol*. 135, 129-138.
- Murphy, B., Bates, G.W., 1997. Adult attachment style and vulnerability to depression. *Person. Individ. Diff.* 22, 835—844.
- Pini, S., Abelli, M., Troisi, A., Siracusano, A., Cassano, G.B., Shear K.M., Baldwin, D., 2014. The relationships among separation anxiety disorder, adult attachment style and agoraphobia in patients with panic disorder. *J Anxiety Disord.* 28, 741-746.
- Qin, Q., Baudry, M., Liao, G., Noniyev, A., Galeano, J., Bi, X., 2009. A novel function for p53: regulation of growth cone motility through interaction with Rho kinase. *J Neurosci.* 29, 5183–5192
- Rodriguez, S., Gaunt, T.R., Day, INM., 2009. Hardy-Weinberg Equilibrium Testing of Biological Ascertainment for Mendelian Randomization Studies. *Am J Epidemiol.* 169, 505-514
- Roskopf, D., Koch, K., Habich, C., Geerdes, J., Ludwig, A., Wilhelms, S. Jakobs, K.H., Siffert, W., 2003. Interaction of Gbeta3s, a splice variant of the G-protein Gbeta3, with Ggamma- and Galpha-proteins. *Cell Signal.* 15, 479-488.
- Roskopf, D., Manthey, I., Siffert, W., 2002. Identification and ethnic distribution of major haplotypes in the gene GNB3 encoding the G-protein beta3 subunit. *Pharmacogenetics.* 12, 209-220.

- Rothman, K. J., 1974. Synergy and antagonism in cause-effect relationships. *Am J Epidemiol* 99, 385–388.
- Ruiz-Velasco, V., Ikeda, S.R., 2003. A splice variant of the G protein beta 3-subunit implicated in disease states does not modulate ion channels. *Physiol Genomics*.13, 85-95.
- Scantamburlo, G., Hansenne, M., Fuchs, S., Pitchot, W., Marechal, P., Pequeux, C., Anseau, M., Legros, J.J., 2007. Plasma oxytocin levels and anxiety in patients with major depression. *Psychoneuroendocrinology* 32, 407—410.
- Shear, K., Jin, R., Ruscio, A.M., Walters, E.E., Kessler, R.C., 2006. Prevalence and correlates of estimated DSM-IV child and adult separation anxiety disorder in the National Comorbidity Survey Replication. *Am J Psychiatry*. 163, 1074-1083.
- Shaver, P.R., Schachner, D.A., Mikulincer, M., 2005. Attachment style, excessive reassurance seeking, relationship processes, and depression. *Pers. Soc. Psychol. Bull.* 31, 343—359.
- Siffert, W., Roskopf, D., Siffert, G., Busch, S., Moritz, A., Erbel, R. et al., 1998. Association of a human G-protein beta3 subunit variant with hypertension. *Nat Genet.* 18, 45-48.
- Silove, D., Alonso, J., Bromet, E., Gruber, M., Sampson, N., Scott, K., Andrade, L., Benjet, C., Caldas de Almeida, J.M., De Girolamo, G., De Jonge, P., Demyttenaere, K., Fiestas, F., Florescu, S., Gureje, O., He, Y., Karam, E., Lepine, J.P., Murphy, S., Villa-Posada, J., Zarkov, Z., Kessler, R.C., 2015. Pediatric-Onset and Adult-Onset Separation Anxiety Disorder Across Countries in the World Mental Health Survey. *Am J Psychiatry*. 172, 647-56.
- Silove, D., Mamane, C., 2013. Overlap of symptom domains of separation anxiety disorder in adulthood with panic disorder-agoraphobia. *J Anxiety Disord.* 27, 92-97.
- Silove, D., Manicavasagar, V., O'Connell, D., Blaszczyński, A., Wagner, R., Henry, J., 1993. The development of the Separation Anxiety Symptom Inventory (SASI). *Aust N Z J Psychiatry.* 27, 477-88.
- Slattery, D. A., & Neumann, I. D., 2010. Oxytocin and Major Depressive Disorder: Experimental and Clinical Evidence for Links to Aetiology and Possible Treatment. *Pharmaceuticals*, 3, 702–724.



- Swaab, D.F., Bao, A.M., Lucassen, P.J., 2005. The stress system in the human brain in depression and neurodegeneration. *Ageing Res.Rev.* 4, 141—194.
- Tansey, K.E., Brookes, K.J., Hill, M.J., Cochrane, L.E., Gill, M., Skuse, D., Correia, C., Vicente, A., Kent, L., Gallagher, L., Anney, R.J., 2010. Oxytocin receptor (OXTR) does not play a major role in the aetiology of autism: genetic and molecular studies. *Neurosci Lett.* 474, 163-167.
- Tedeschi, A., Nguyen, T., Puttagunta, R., Gaub, P., Di Giovanni, S.A., 2009. p53-CBP/p300 transcription module is required for GAP-43 expression, axon outgrowth, and regeneration. *Cell Death Differ.* 16, 543–554.
- Thompson, R.J., Parker, K.J., Hallmayer, J.F., Waugh, C.E., Gotlib, I.H., 2011. Oxytocin receptor gene polymorphism (rs2254298) interacts with familial risk for psychopathology to predict symptoms of depression and anxiety in adolescent girls. *Psychoneuroendocrinology.* 36, 144-147.
- Tolstonog, G.V., Deppert, W., 2010. Metabolic sensing by p53: keeping the balance between life and death. *Proc Natl Acad Sci U S A.* 107, 13193–13194.
- Tannorella P, Stoccoro A, Tognoni G, Bonuccelli U, Migliore L, Coppedè F. Association study between the DNMT3A -448A>G polymorphism and risk of Alzheimer's disease in Caucasians of Italian origin. *Am J Neurodegener Dis.* 2016 Mar 1;5(1):85-93.
- Tost, H., Kolachana, B., Hakimi, S., Lemaitre, H., Verchinski, B., Mattay, V.S. et al., 2010. A common allele in the oxytocin receptor gene (OXTR) impacts prosocial temperament and human hypothalamic-limbic structure and function. *Proc Natl Acad Sci U S A* 2010. 107, 13936–13941.
- Walum, H., Lichtenstein, P., Neiderhiser, J.M., Reiss, D., Ganiban, J.M., Spotts, E.L., Pedersen, N.L., Anckarsäter, H., Larsson, H., Westberg, L., 2012. Variation in the oxytocin receptor gene is associated with pair-bonding and social behavior. *Biol Psychiatry.* 71, 419-426.
- Wang, J., Qin, W., Liu, B., Wang, D., Zhang, Y., Jiang, T. et al., 2013. Variant in OXTR gene and functional connectivity of the hypothalamus in normal subjects. *Neuroimage.* 81, 199–204.

- Wang, S.S., Kamphuis, W., Huitinga, I., Zhou, J.N., Swaab, D.F., 2008. Gene expression analysis in the human hypothalamus in depression by laser microdissection and real-time PCR: the presence of multiple receptor imbalances. *Mol. Psychiatry* 13, 786—799.
- Wang, Y.F., Hatton, G.I., 2007. Dominant role of betagamma subunits of G-proteins in oxytocin-evoked burst firing. *J Neurosci.* 27, 1902-1912.
- Wermter, A.K., Kamp-Becker, I., Hesse, P., Schulte-Körne, G., Strauch, K., Remschmidt, H., 2009. Evidence for the involvement of genetic variation in the oxytocin receptor gene (OXTR) in the etiology of autistic disorders on high-functioning level. *Am J Med Genet B Neuropsychiatr Genet.* 153, 629–639.
- Willeit, M., Praschak-Rieder, N., Zill, P., Neumeister, A., Ackenheil, M., Kasper, S., Bondy, B., 2003. C825T polymorphism in the G protein beta3-subunit gene is associated with seasonal affective disorder. *Biol Psychiatry.* 54, 682-686.
- Zhong, M., Yang, M., Sanborn, B.M., 2003. Extracellular signal-regulated kinase  $\frac{1}{2}$  activation by myometrial oxytocin receptor involves Galpha(q)Gbetagamma and epidermal growth factor receptor tyrosine kinase activation. *Endocrinology.* 144, 2947-2956.

### Figure legends

**Figure 1.** Comparisons for dimensional scales between oxytocin receptor (OXTR) rs53576/G $\beta$ 3 rs5443 risk (+/+) (N=49) and non-risk (-/-) (N=36) combined genotype groups. Figure also shows comparisons between the genotype (-/+ (N=60) or (+/-) (N=39) and non-risk genotype (-/-). **A)** **B)** and **C)** Bar graphs show results obtained for the Separation Anxiety Symptoms Inventory (SASI) mean squared-root, the Adult Separation Anxiety – 27 Items (ASA-27) and the Hamilton Depression Rating Scale (HAM-D) total scores, respectively. **D)** Bar graph shows results for HAM-D ‘Anxiety’ Factor. Data are represented as mean scores and standard errors (SE). Boldface indicates statistically significant results.

**Figure 2.** Associations (Odds Ratios) between OXTR rs53576/G $\beta$ 3 rs5443 combined genotype and separation anxiety. **A)** Dichotomized squared root-transformed SASI scores (threshold for caseness:  $\geq 4$  vs  $< 4$ ) were used to calculate by crude ORs the association between childhood separation anxiety disorder and combined genotype. **B)** For the

association between adult separation anxiety disorder and combined genotype, the dichotomized ASA-27 total score (threshold for caseness:  $\geq 22$  vs  $< 22$ ) was used. Boldface indicates statistically significant results. (N are reported in Figure)

**Figure 3. A)** Relationship between OXTR rs53576/G $\beta$ 3 rs5443 combined risk genotype (+/+) and Threshold (SASI $\geq 4$ ) Childhood Separation Anxiety (CSAD) and Threshold (ASA-27 $\geq 22$ ) Adult Separation Anxiety (ASAD). **B)** Interaction between the two OXTR rs53576/G $\beta$ 3 rs5443 risk

**Table 1.** Demographic and clinical characteristics of study sample (N=188)

	Bipolar Patients N=95	Unipolar Patients N=93	t Test	P value
Age	40.92 $\pm$ 11.7	44.3 $\pm$ 12.5	1.905	0.058
HAM-D total score	11.0 $\pm$ 7.5	9.9 $\pm$ 6.2	-.932	0.35
HAM-D anxiety factor	2.91 $\pm$ 2.2	2.79 $\pm$ 2.2	.351	0.73
ASA-27 total score	31.4 $\pm$ 14.2	29.9 $\pm$ 15.3	-.711	0.48
SASI total score	3.3 $\pm$ 1.3	3.3 $\pm$ 1.4	-.142	0.89
Age at onset of mood disorder	27.6 $\pm$ 8.7	34.2 $\pm$ 12.4	3.048	0.003
Age at onset of separation anxiety	11.1 $\pm$ 11.8	13.0 $\pm$ 11.6	.657	0.51
			Chi square	P value
Sex (M/F)	26/69	31/62	0.79	0.37
Anxiety disorders comorbidity <sup>1</sup>	45 (48.9%)	37 (39.8%)	1.56	0.21
SASI $\geq 4$ <sup>2</sup>	25 (27.2%)	24 (25.8%)	0.04	0.83

<sup>1</sup>Panic disorder or social phobia or obsessive/compulsive disorder

<sup>2</sup>calculated on 92 bipolar patients

**Table 2.** Comparison of OXTR and G $\beta$ 3 SNP distributions between patients and healthy individuals.

<b>OXTR rs53576</b>	Patients with depression (n=188)	Healthy individuals (n=225)	OR [95% CI]	P
<i>Genotype</i>	<i>n (%)</i>	<i>n (%)</i>		
GG	90 (47.9)	86 (38.6)	reference	
GA	76 (40.4)	113 (50.2)	0.64 [0.47-0.97]	<b>0.037</b>
AA	22 (11.7)	26 (11.6)	0.81 [0.43-1.53]	0.515
A-carriers	98 (53.0)	139 (61.8)	0.67 [0.45-0.99]	<b>0.049</b>
Allele <sup>a</sup>				
G	256 (68.1)	285 (63.3)		
A	120 (31.9)	165 (36.7)	0.81 [0.61-1.08]	0.153
<b>G<math>\beta</math>3 rs5443</b>				
<i>Genotype</i>	<i>n (%)</i>	<i>n (%)</i>		
CC	77 (41.0)	110 (48.9)	reference	
CT	85 (45.2)	99 (44.0)	1.23 [0.81-1.85]	0.330
TT	26 (13.8)	16 (7.1)	2.32 [1.17-4.62]	<b>0.016</b>
T-carriers	111 (59.0)	115 (51.1)	1.38 [0.93-2.04]	0.107

Allele <sup>b</sup>					
C	239 (63.6)	319 (70.9)	reference		
T	137 (36.4)	131 (29.1)	1.40 [1.04-1.87]		<b>0.025</b>
<b>OXTR rs53576/Gβ<sub>3</sub>rs5443</b>					
<i>Combined Genotypes<sup>c</sup></i>	<i>n (%)</i>	<i>n (%)</i>			
(-/-)	38 (20.2)	61 (27.1)	reference		
(+/-)	39 (20.7)	49 (21.8)	1.28 [0.71-2.29]		0.411
(-/+)	60 (31.9)	78 (34.7)	1.23 [0.73-2.09]		0.432
(+/+)	51 (27.1)	37 (16.4)	2.21 [1.23-3.97]		<b>0.008<sup>d</sup></b>

<sup>a</sup>OXTR rs53576 allele frequencies in Caucasians (n=2309): G=0.65 A=0.35 (Walun et al., 2012).

<sup>b</sup>Gβ<sub>3</sub> rs5443 allele frequencies in Caucasians (n=1855): C=0.70, T allele= 0.30 (Roskopf et al., 2002).

<sup>c</sup>For OXTR rs53576: GG= risk genotype (+), GA or AA= non-risk genotype (-); For Gβ<sub>3</sub> rs5443: CT or TT=risk genotype (+), CC= non-risk genotype.

<sup>d</sup>significant difference after Bonferroni's correction (0.05/3=0.017). Rothman synergy index: S=2.37; 95% CI [1.07-5.24]. Boldface indicates statistically significant results.

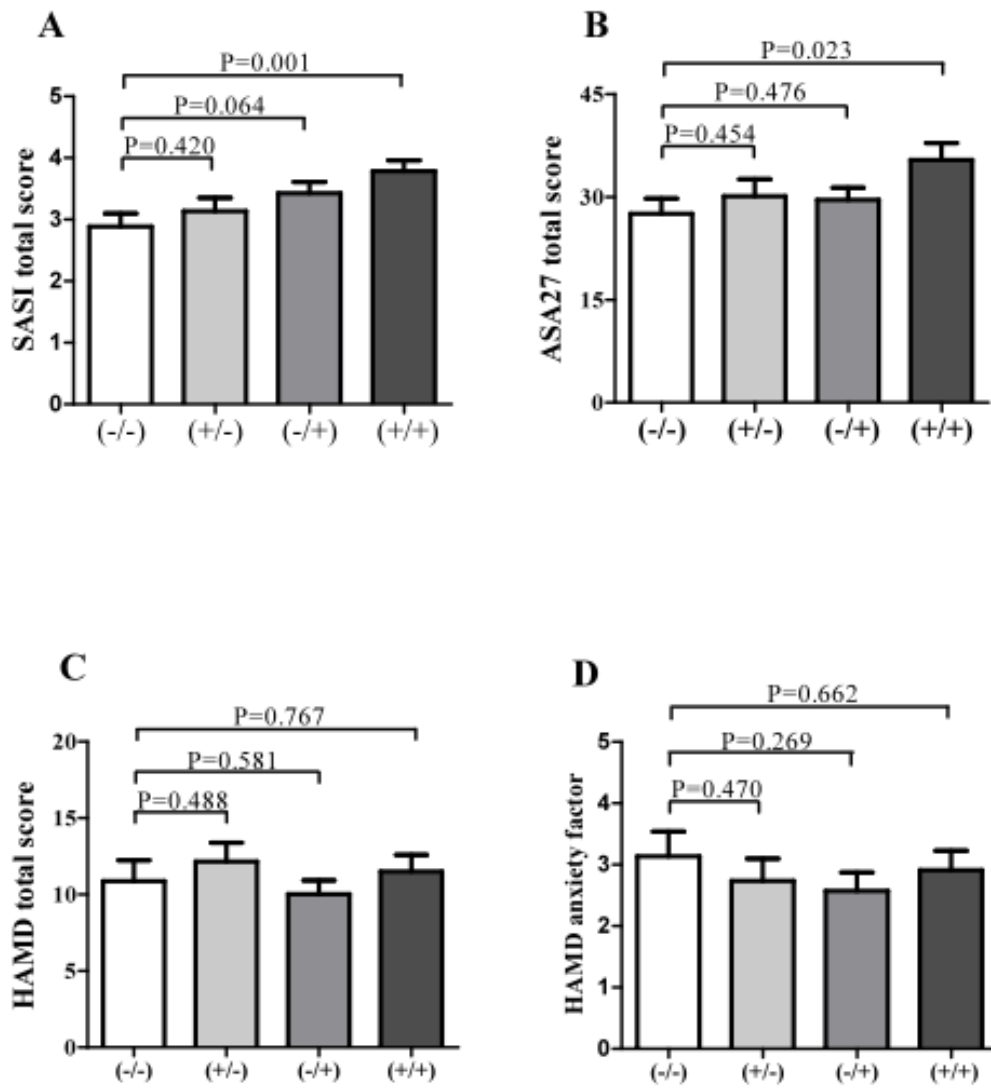
**Table 3:** Top 10 predicted transcription factors with differential affinities to the two alternative sequences of OXTR rs53576 using sTRAP. The table is sorted from the highest to the lowest 'Absolute difference log(p) for two sequences'.

Rank order	Difference log(p) for the two sequences	P value rs53576 G allele	P value rs53576 A allele	Matrix_ID	Matrix name
1	-1.428	0.0121	0.0004	M00034	V\$P53_01
2	-1.160	0.1436	0.0099	M01655	V\$P53_05
3	0.609	0.1538	0.6258	M01045	V\$AP2ALPHA_02
4	-0.522	0.3747	0.1126	M01651	V\$P53_03
5	0.486	0.0249	0.0763	M01002	V\$DEAF1_02
6	-0.461	0.2828	0.0979	M01652	V\$P53_04
7	-0.440	0.3858	0.1401	M01656	V\$P63_01
8	-0.288	0.8862	0.4562	M00619	V\$ALX4_01
9	0.230	0.2687	0.4562	M00470	V\$AP2GAMMA_01
10	0.164	0.3703	0.5407	M0046	V\$AP2ALPHA_01

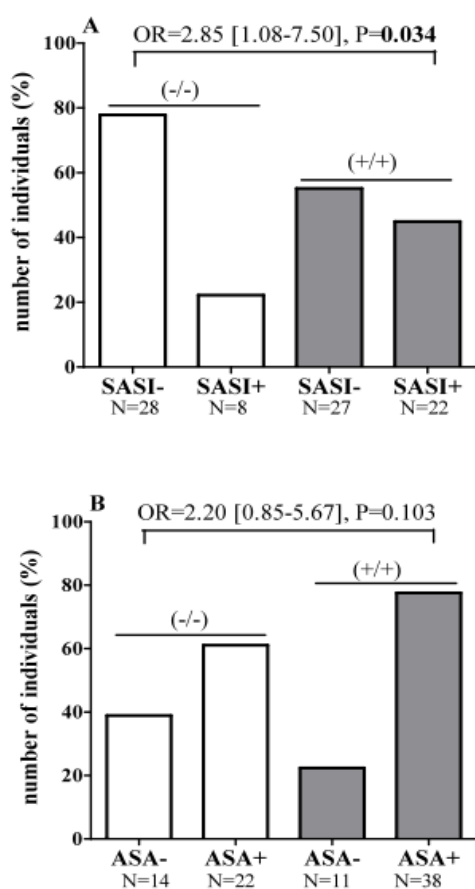
## Highlights

- Variations in OXTR and Gβ3 genes are associated with presence and severity of separation anxiety
- Variations in OXTR and Gβ3 genes are not associated with depression or anxiety in general
- Gene-separation anxiety associations have potential translational and clinical relevance

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**Figure 1.** risk (+/+), (+/-), (-/+) and non risk (-/-) combined genotype groups

**Figure 2.** Associations (Odds Ratios) between OXTR rs53576/Gβ3 rs5443 combined genotype and separation anxiety.



**Footnote:** **A)** Dichotomized squared root-transformed SASI scores (threshold for caseness:  $\geq 4$  vs  $< 4$ ) were used to calculate by crude ORs the association between childhood separation anxiety disorder and combined genotype. **B)** For the association between adult separation anxiety disorder and combined genotype, the dichotomized ASA-27 total score (threshold for caseness:  $\geq 22$  vs  $< 22$ ) was used. Boldface indicates statistically significant results. (N are reported in Figure)

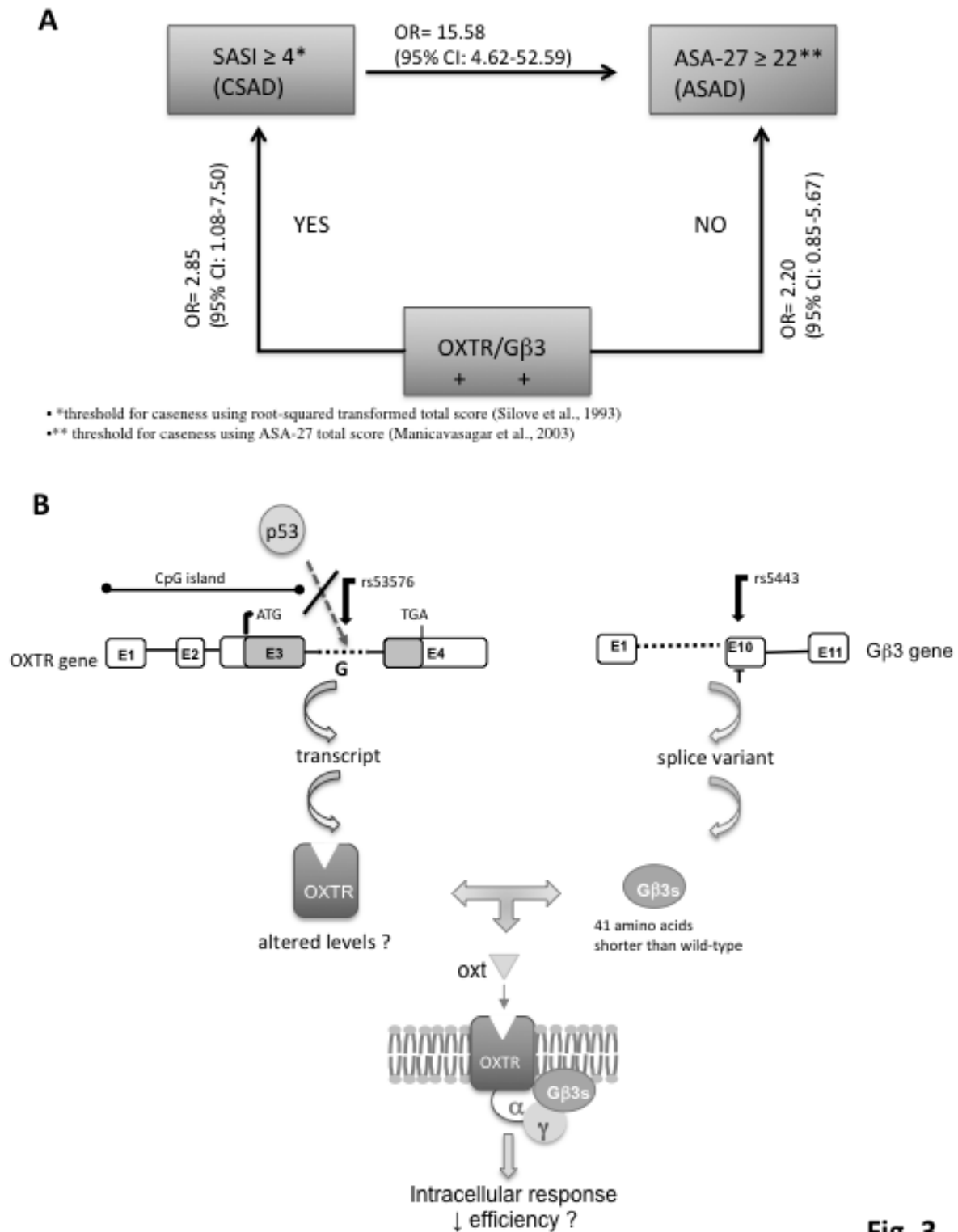


Fig. 3