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COMPLEXO SNARE NA SUSCEPTIBILIDADE AO TRANSTORNO POR USO DE ÁLCOOL

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Artigo Científico

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VAMP2 26 bp INSERTION/DELETION EFFECT ON ALCOHOL DEPENDENCE SUSCEPTIBILITY

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Abstract

Background: The etiology of Alcohol Dependence is multifactorial, resulting from the interplay of environmental and genetic factors, including variants in neurotransmission related genes. In this sense, the Soluble NSF-Attachment Protein Receptors (SNARE) complex, which acts on neurotransmitter release, could play an important role in this disorder. The aim of the present study was to evaluate polymorphisms in SNARE complex genes (*STX1A* rs2228607; *SYT1* rs1880867 and rs2251214; *VAMP2* 26 bp Ins/Del; *SNAP25* rs6108461 and rs8636) in relation to Alcohol Dependence susceptibility.

Methods: The sample comprised 115 men under inpatient treatment for with Alcohol Dependence and 308 non-dependent controls. Additionally, 278 men with Attention-Deficit/Hyperactivity Disorder (ADHD), of which 35 presented comorbidity with Alcohol Dependence were subsequently evaluated in order to replicate the association.

Results: A nominal association was found between the *VAMP2* 26 bp Ins/Del polymorphism and susceptibility to Alcohol Dependence, where the Ins/Ins genotype showed a risk to this disorder ($p = 0.012$; $OR = 2.21$). This result was supported by the ADHD replication sample, where the Ins/Ins genotype was also more frequent among Alcohol Dependent cases ($p = 0.008$; $OR = 5.26$). Other polymorphisms and haplotype analyses did not reveal significant differences between cases and controls.

Conclusion: Overall, the present study suggests that *VAMP2* 26 bp Ins/Del polymorphism, previously associated with ADHD, is also implicated in Alcohol Dependence susceptibility.

Key-words: SNARE, Alcohol Dependence, *VAMP2*.

1. Introduction

Alcohol Dependence (AD) is the most severe phenotype within Alcohol Use Disorders, being characterized by a set of symptoms that includes withdrawal, tolerance to alcohol and craving (DSM-IV, APA, 1994). The prevalence of AD in Brazil is estimated in 10.48% in men and 3.63% in women (Laranjeira, 2012). The high incidence of this phenotype impacts substantially in social and economic areas, and represents an important health problem, with estimates from the World Health Organization showing that, in 2012, about 5.9% of all global deaths were attributable to alcohol consumption (World Health Organization). In this context, efforts have been made to better elucidate the mechanisms for the development of dependence and its whole spectrum of manifestations. AD is a complex phenotype, in which both environmental and genetic factors are involved in its etiology (Bau, 2002). Since its heritability estimates are around 50% (Verhulst et al., 2015), association studies have sought to identify genetic factors involved in its susceptibility. The most consistent results involve genes responsible for alcohol metabolism, such as the genes encoding alcohol dehydrogenase (*ADH*) (Ehlers et al., 2012; Gelerneter et al., 2014; Marshall et al., 2014; Park et al., 2013; Peng et al., 2014; Zuo et al., 2014) and aldehyde dehydrogenase (*ALDH*) (Ehlers et al., 2012; Gelerneter et al., 2014; Peng et al., 2014; Zuo et al., 2014). Additionally, genes involved in neurotransmission are considered to contribute to the etiology of alcoholism, especially those related to the dopaminergic pathway (Bau, 2002; Contini et al., 2006; Mota et al., 2012; Wang et al., 2007).

Neurotransmitters' release (mostly epinephrine, serotonin and dopamine) on the reward-system pathways are related to the pathophysiology of addiction (Bear et al., 2007). Accordingly, alterations in the main components of neurotransmitter release such as Soluble NSF-Attachment Protein Receptors (SNARE) complex have already been implicated in several psychiatric disorders (reviewed in Cupertino et al., 2016) and could also be associated with AD susceptibility. The core components of the SNARE complex are Synaptosomal-Associated Protein of 25 kDa (SNAP-25), Syntaxin 1A (STX1A) and Vesicle-Associated Membrane Protein 2 (VAMP-2/Synaptobrevin-2). Neurotransmitter exocytosis also requires other accessory proteins, such as Synaptotagmin-1 (SYT1), located at the synaptic vesicle membrane.

Despite being important for several brain pathways, the SNARE complex has been poorly studied in relation to AD. In a study evaluating transcription levels of *STX1A* and *VAMP2* in rats with alcohol-intake preference, mRNA levels of both genes were increased

in frontal cortex in relation to the alcohol non-preferring rats (Worst et al., 2005). Linkage studies on alcohol use phenotypes have implicated regions in chromosome 7, containing *STX1A* (alcoholism - Strat et al., 2008) and chromosome 17, containing *VAMP2* (alcoholism - Hill et al., 2004; and alcohol consumption - Bergen et al., 2003). Furthermore, *SNAP25* polymorphisms and haplotypes were shown to influence impulsivity traits of healthy adults (Németh et al., 2013). Taken together, these studies suggest that genetic variants within SNARE components might exert a role on alcoholism. In this context, the present study aims to evaluate polymorphisms in genes of the SNARE complex - *STX1A* (rs2228607 SNP), *SYT1* (rs1880867 and rs2251214 SNPs), *VAMP2* (insertion/deletion polymorphism of 26 bp, referred to as 26 bp Ins/Del) and *SNAP25* (rs6108461 and rs8636 SNPs) - in relation to AD susceptibility in two independent samples.

2. Material and Methods

2.1. Subjects

The cases sample comprised a total of 115 men (mean age 41.35 ± 10.18 years) meeting the DSM-III-R criteria for AD (Diagnostic and Statistical Manual of Mental Disorders, 3th edition, revised version, APA, 1987), under an abstinence-controlled environment at Hospital Espírita de Porto Alegre, Brazil. Interviews were conducted based on the Semi-Structured Assessment for the Genetics in Alcoholism (SSAGA, Bucholz et al., 1994), developed by the Consortium on the Genetics of Alcoholism (COGA) of the US National Institute of Alcohol Abuse and Alcoholism.

The control sample comprised 308 men (mean age 29.15 ± 8.54 years) assessed in the blood donation center at Hospital de Clínicas de Porto Alegre, Brazil, screened negative for Alcohol Dependence and Abuse through an adapted version of the Structured Clinical Interview for DSM-IV Axis I Disorders, Patient Edition (SCID-I; First et al., 1998).

Additionally, a replication sample comprising 278 men who fulfilled DSM-IV criteria (APA, 1994) for Attention-Deficit/Hyperactivity Disorder (ADHD; mean age 32.42 ± 11.03 years) was ascertained in the ADHD Outpatient Clinic - Adult Division from Hospital de Clínicas de Porto Alegre, Brazil. These subjects were also evaluated for AD through SCID-I (First et al., 1998), totalizing 35 individuals with AD. The exclusion criteria were IQ score lower than 70, history of psychosis, presence of neurological disease,

head traumas, and/or neurodegenerative disorders. The ADHD sample was used as a replication sample for nominal associations reported in the main case-control analyses. For further description of this sample see Grevet et al. (2006) and Vitola et al. (2012).

The subjects included in the study were unrelated Brazilians of European descent, aged 18 years or older. Participants signed an informed consent form approved by the Ethics Committees of the corresponding institutions where volunteers were ascertained.

2.2. Polymorphisms selection and laboratory methods

The polymorphisms were selected based on previous evidence of association with psychiatric disorders, description of SNP functionality or minor allele frequency higher than 10%. DNA was extracted from peripheral blood by salting out method (Lahiri and Nurnberger, 1991). The *SXT1A*, *SYT1* and *SNAP25* SNPs were genotyped by Real Time PCR using Taqman allelic discrimination assays (Applied Biosystems, Foster City, CA, USA); while *VAMP2* 26 bp Ins/Del was genotyped by PCR followed by 3.5% agarose electrophoresis gel. Genotype distributions were in Hardy-Weinberg equilibrium ($p < 0.01$). The genotyping rate was higher than 90%, and the genotyping error rate was lower than 1%. Allelic and genotypic frequencies are shown in **Supplementary Table S1**.

2.3. Statistical analyses

Case-control analyses were performed using binary logistic regression in SPSS software version 18.0 (IBM Corp, 2011). Age was included as covariate, since it was associated ($p < 0.2$) with both the outcome (AD) and some of the evaluated polymorphisms. In order to prevent type I error due to multiple tests, Bonferroni correction was applied considering 6 independent tests (number of evaluated polymorphism), changing the significance threshold to $p < 0.05/6 = 0.008$.

Association findings were subsequently replicated in the ADHD sample, using also binary logistic regression with age as covariate. In order to better estimate the size effect of the results, a meta-analytic approach was performed comprising both findings (i.e. main and replication associations). Heterogeneity was evaluated through Cochrane's Q statistic and I^2 test. The appropriate meta-analytic model was chosen based on these results (i.e. fixed-model if $P_{Cochrane's} > 0.05$; random-model if $P_{Cochrane's} < 0.05$). These procedures were conducted using PLINK version 1.07 (Purcell et al., 2007).

All tests were performed grouping genotypes according to their frequencies (i.e. heterozygotes and homozygotes for the minor allele were assembled and compared to homozygotes for the major allele). For the *STX1A* SNP (rs2228607), however, the homozygotes have similar frequencies and thus were grouped based on previous significant reports (i.e. AA+AG and GG).

Haplotype analyses were performed for *SYT1* (rs1880867-rs2251214) and *SNAP25* (rs6108461-rs8636) SNPs. Significant results were corrected under a 10,000-permutation [max(T)] procedure. The haplotype tests were conducted on PLINK software.

3. Results

The case-control analyses revealed that the Ins/Ins genotype of *VAMP2* 26 bp Ins/Del was associated with increased risk to AD ($p = 0.012$; OR = 2.21; **Table 1**). This result, however, did not remain significant after Bonferroni correction.

The observed nominal association between *VAMP2* 26 bp Ins/Del and AD was subsequently replicated in the sample of patients with ADHD with and without comorbid AD. In this analysis, the Ins/Ins genotype of the *VAMP2* 26 bp Ins/Del was also associated with a higher frequency of AD ($p = 0.008$; OR = 5.26; **Table 2**).

These results were compiled through a meta-analytic approach. After a literature search for other studies regarding this polymorphism and AD, no results were found. Thus, the meta-analysis was restricted to the present data. Since no significant heterogeneity was detected ($P_{Cochrane's} = 0.219$; $I^2 = 33.69$), a fixed-effect model was applied. Such meta-analytic algorithm was used to obtain a better comprehension of the effect size of *VAMP2* 26 bp Ins/Del, supporting the association of Ins/Ins genotype ($p < 0.001$; OR = 2.64) with an increased susceptibility to Alcohol Dependence **(Supplementary Table S2)**.

The haplotype analyses for *SYT1* (rs1880867-rs2251214) and *SNAP25* (rs6108461-rs8636) SNPs did not reach significant thresholds ($P_{global} = 0.155$ and $P_{global} = 0.102$, respectively).

4. Discussion

This study expands the association of the *VAMP2* 26 bp Ins/Del polymorphism, previously reported in ADHD (Kenar et al., 2014), to Alcohol Dependence. Additionally,

this finding was replicated in a sample of patients with ADHD with or without AD. Our results reinforce the influence of SNARE complex genes in psychiatric disorders (reviewed in Cupertino et al., 2016).

The 26 bp Ins/Del polymorphism is located at an intergenic region, at 2 kb from 3' end of *VAMP2* (Falbo et al., 2002), and has been investigated in a few studies. Concerning other neurological diseases and psychiatric disorders, this polymorphism was investigated in relation to Alzheimer Disease, where no association was found (Edgünlü et al., 2012), and to ADHD, where the Ins/Ins genotype was significantly more frequent in Turkish adults with ADHD compared to controls (Kenar et al., 2014). In the present study, we report a nominal association with AD as previously reported for ADHD. Despite being essential to scientific research (Anonymous, 2012; McNutt, 2014), replication has been a challenge in psychiatry studies (Kapur et al., 2012) mainly due to the expected small effect size and high heterogeneity observed in mental disorders. Thus, the replication within ADHD subjects reinforces the observed effect of *VAMP2* 26 bp Ins/Del on alcoholism. Since there is a small sample size and a wide range interval of Odds Ratio in the replication findings, the meta-analytic approach provided a more reliable effect size estimate, narrowing the confidence interval. Considering the overall scenario, it could be hypothesized that the association found in the Turkish sample might be due to subjacent endophenotype possibly related to both ADHD and AD susceptibilities.

These findings support the relationship between ADHD and Alcohol Dependence, indicated by the high rates of this comorbidity (Ohlmeier et al., 2008; Young et al., 2015). The present results of an effect of 26 bp Ins/Del on alcoholism, taken together with its previous association reported with ADHD (Herken et al., 2014), corroborates the idea of shared genetic factors underlying psychiatric disorders (Arcos-Burgos et al., 2012), as well as neurobiological similarities in terms of neurotransmission, mainly through the reward system (Vendruscolo and Takahashi, 2011).

Some limitations of this study need to be considered. The sample size of case and controls groups, and especially of the ADHD replication sample, is relatively small and limited the statistical power. In this sense, the relative weak statistical power could possibly be related to the lack of association findings regarding other SNARE complex polymorphisms evaluated. The lack of functional studies on *VAMP2* 26 bp Ins/Del polymorphism limits the interpretation of our findings in terms of biological mechanisms. Finally, neuroimaging and other putative endophenotype analyses could disentangle the possible links between this genetic variation, ADHD and AD.

As a conclusion, the current findings suggest that the *VAMP2* 26 bp Ins/Del should be considered in future genetic studies of AD. Additionally, the common factors underlying ADHD and AD must be included in the search for endophenotypes and susceptibility genetic factors.

Conflict of interest

No conflict declared.

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Contributors

Cibele Edom Bandeira, Renata Basso Cupertino and Claiton Henrique Dotto Bau designed the study; Eduardo Schneider Vitola and Eugenio Horácio Grevet collected the sample; Cibele Edom Bandeira, Renata Basso Cupertino, Alana Eduarda de Castro, Diana Müller extracted DNA from blood and genotyped the sample; Cibele Edom Bandeira and Renata Basso Cupertino conducted statistical analyses; Cibele Edom Bandeira and Renata Basso Cupertino drafted the first version of the manuscript. All contributors have made a substantial intellectual contribution to the work. All authors have participated in manuscript writing by reviewing drafts and approving this final version.

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Table 1. Case-control analyses of effects of SNARE complex gene variants on Alcohol Dependence susceptibility

Gene – Polymorphism	Genotype groups (n (%))			
	Reference	Test	OR (CI 95%)	P-value
STX1A - rs2228607	GG	AA + AG		
Cases (n=114)	23 (20.2)	91 (79.8)	0.96 (0.52-1.80)	0.903
Controls (n=305)	66 (21.6)	239 (78.4)		
SYT1 - rs1880867	TT	GT + GG		
Cases (n=115)	62 (53.9)	53 (46.1)	0.81 (0.49-1.34)	0.410
Controls (n=307)	140 (45.6)	167 (54.4)		
SYT1 - rs2251214	GG	AA + AG		
Cases (n=115)	69 (60.0)	46 (40.0)	0.69 (0.41-1.17)	0.166
Controls (n=306)	182 (59.5)	124 (40.5)		
VAMP2 – 26 bp Ins/Del	Del/Del + Ins/Del	Ins/Ins		
Cases (n=115)	21 (18.3)	94 (81.2)	2.21 (1.19-4.13)	**0.012***
Controls (n-308)	89 (28.9)	219 (71.1)		
SNAP25 - rs6108461	GG	AA + AG		
Cases (n=115)	37 (32.2)	78 (67.8)	0.77 (0.45-1.33)	0.345
Controls (n=308)	83 (26.9)	225 (73.1)		
SNAP25 - rs8636	CC	CT + TT		
Cases (n=114)	44 (38.6)	70 (61.4)	1.09 (0.65-1.84)	0.735
Controls (n=306)	120 (39.2)	186 (60.8)		

SNARE complex genes evaluated included Syntaxin 1A (*STX1A*), Synaptotagmin (*SYT1*), Vesicle-Associated Membrane Protein (*VAMP*) and Synaptosomal-associated protein of 25kD (*SNAP25*). OR: Odds Ratio; CI: Confidence Interval. Age was used as covariate in case-control analysis.

*P-value did not remain significant after Bonferroni correction (P<0.008).

Table 2. Influence of *VAMP2* 26 bp Ins/Del on Alcohol Dependence susceptibility in individuals with ADHD

Gene – Polymorphism	**Genotype groups (n (%))**			
	Reference	Test	OR (CI 95%)	P-value
***VAMP2* -26 bp Ins/Del**	Del/Del + Ins/Del	Ins/Ins		
Cases (n=35)	3 (8.57)	32 (91.43)	5.26 (1.54-18.03)	**0.008**
Controls (n=243)	73 (30.04)	170 (69.96)		

OR: Odds Ratio; CI: Confidence Interval. Age was used as covariate in case-control analysis.

Supplementary Table S1. Allele and genotype frequencies of SNARE complex gene variants

	Alcohol (n = 115)	Control (n= 308)
STX1A – rs2228607		
G	49.1 %	49.7 %
A	50.9 %	50.3 %
GG	23 (20.2)	66 (21.6)
AG	65 (57.0)	171 (56.1)
AA	26 (22.8)	68 (22.3)
SYT1 – rs1880867		
G	27.0 %	33.1 %
T	73.0 %	66.9 %
GG	8 (7.0)	36 (11.7)
GT	45 (39.1)	131 (42.7)
TT	62 (53.9)	140 (45.6)
SYT1 – rs2251214		
A	22.6 %	23.5 %
G	77.4 %	76.5 %
AA	6 (5.2)	19 (6.2)
AG	40 (34.8)	105 (34.3)
GG	69 (60.0)	182 (59.5)
VAMP2 – 26 bp Ins/Del		
Del	9.6 %	14.6 %
Ins	90.4 %	85.4 %
Del/Del	0 (0.0)	1 (0.3)
Ins/Del	21(18.3)	88 (28.6)
Ins/Ins	94 (81.7)	219 (71.1)
SNAP25 – rs6108461		
A	44.3 %	46.1 %
G	55.7 %	53.9 %
AA	24 (20.9)	59 (19.2)
AG	54 (47.0)	166 (53.9)
GG	37 (32.1)	83 (26.9)
SNAP25 – rs8636		
T	38.6 %	37.1 %
C	61.4 %	62.9 %
TT	18 (15.8)	41 (13.4)
CT	52 (45.6)	145 (47.4)
CC	44 (38.6)	120 (39.2)

STX1A, SYT1, VAMP2 and *SNAP25*: genes coding Syntaxin 1A, Synaptotagmin 1, VAMP-2 and SNAP-25, respectively. Genotypic data are represented as *n (%).*

Supplementary Table S2. Meta-analysis of the association studies of *VAMP2* 26 bp Ins/Del in Alcohol Dependence

Sample	P-value	OR[a] (CI 95%)
Genotype Group		
Discovery sample	**0.012**	2.21 (1.19-4.13)
Replication sample	**0.008**	5.26 (1.54-18.03)
Meta-analysis	**<0.001**[b]	2.64 (1.51-4.60)

CI: Confidence Interval.

[a] Odds Ratio (OR) referred to Ins/Ins genotype.

[b] $P_{Cochrane's} = 0.219$; $I^2 = 33.69$.