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SNAP-25 single nucleotide polymorphisms are associated with hyperactivity in autism spectrum disorders

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ABSTRACT

Synaptosomal-associated protein of 25 kD (SNAP-25), a protein participating in the regulation of synaptic vesicle exocytosis and in calcium homeostasis, was recently involved in neuropsychiatric conditions. Because alterations affecting the homeostasis of calcium are described in patients affected by autism spectrum disorders (ASD) we investigated a possible involvement of SNAP-25 in ASD by evaluating five SNAP-25 gene polymorphisms in a cohort of 67 ASD children.

Data analyzed in relationship with clinical outcomes and compared to those of 205 healthy sex-matched children did not reveal significant differences. Further analyses nevertheless showed the presence of highly significant associations of the rs363043 (CT) genotype, localized in the intron 1 region that affects the transcription factor binding sites of the SNAP-25 gene, with both increasing CARS ($p = 0.001$) and hyperactivity scores ($p = 0.006$).

The finding that polymorphisms of the SNAP-25 gene, a gene involved in neurotransmission and regulation of calcium homeostasis, are associated with the degree of hyperactivity in children with ASD, reinforces the hypothesis that alterations of these mechanisms play a pivotal role in the events leading to ASD-associated behavioral impairment. Modulation of these processes could result in novel therapeutic strategies.

1. Introduction

Autism spectrum disorders (ASD) are severe pervasive developmental disorders characterized by specific impairements in social interaction and communication, as well as by stereotyped and repetitive behaviors and interests. ASD are heterogeneous disorders with a wide range of additional phenotypic characteristics and an intelligence quotient (IQ) ranging from mental retardation to above average. The incidence of ASD has dramatically risen from 2–5 to 15–60/10 000 children during the past two decades; broader diagnostic criteria and increased medical awareness have likely contributed to determine this trend [1,2]. Notably, ASD are often accompanied by co-morbidity including seizures (30%) and mental retardation (80%), but also attention deficit, hyperactivity and impulsivity which are clinical features shared with attention deficit hyperactivity disorder (ADHD) (40–78%) [3].

The etiopathogenesis of ASD is still unclear; nevertheless, several lines of evidence support a prenatal onset for developmental abnormalities that evolve to autism later on during infanthood [4]. Genetic factors strongly contribute to ASD. Several genotype–phenotype correlations between causal gene mutations or cytogenetic abnormalities, and behavioural or morphological phenotypes have been described, and mutations in different genes have been detected in monogenic forms of autism [2–6]. Post-mortem assessments have also unveiled a number of early neurodevelopmental alterations in brains of autistic patients including reduced programmed cell death and/or increased cell proliferation, altered cell migration, abnormal cell differentiation with reduced neuronal size and altered synaptogenesis [2,5]. A significant elevation of neocortical Ca^{2+} levels has recently been shown as well in post-mortem brains of such patients [7].

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We investigated the possibility that synaptosomal-associated protein of 25 kD (SNAP-25), a protein participating in the regulation of synaptic vesicle exocytosis through the formation of a SNARE complex [8], may be involved in the pathogenesis of ASD. SNAP-25 plays an important role in the release of neurotransmitter and interacts with different types of voltage-gated calcium channels [9], inhibiting their function and thus reducing neuronal calcium responsiveness to depolarisation [10–12]. Interestingly, changes in SNAP-25 levels have been detected in schizophrenic patients, with decreased levels of the protein in the hippocampus and in the frontal lobe Broadman's area 10 and elevated levels of SNAP-25 in prefrontal lobe Broadman's area 9 and cingulate cortex [reviewed in ref. 13]. Also, elevated SNAP-25 levels have been detected in the CSF of schizophrenic patient, thus suggesting that changes in the expression levels of the protein might correlate with schizophrenia [14]. Notably, a SNAP-25 promoter variant was also recently found to be associated with early-onset bipolar disorder and high protein expression in brain [15].

Whether changes in protein expression primarily impact on neurotransmitter release and/or intracellular calcium regulation is still to be defined. Importantly, however, whereas genetic reductions in SNAP-25 expression do not impact on SNARE-dependent neurotransmission [14,16,17], such a limited reduction results in a major enhancement of neuronal calcium responsiveness to stimuli [12,17], indicating that even modest alterations of the expression of this protein may result in significant changes of neuronal calcium homeostasis. Besides schizophrenia, SNAP-25 has also been involved in ADHD [18], a condition characterized by hyperactive behaviour and impaired attentive ability resulting in social dysfunction [19] and neuroticism, i.e. the tendency to experience negative emotions including anger, anxiety, and depression [20]. In particular, two specific SNPs localized in the 3′ untranslated region of SNAP-25 gene (rs3746544 and rs1051312) were associated to ADHD [21,22].

The SNAP-25 gene lies in an area of previous suggestive linkage to intelligence (20p12–p11.2) [23]. Interestingly, a family-based genetic association test performed in two independent cohorts of Caucasian children and adults evidenced SNAP-25 single nucleotide polymorphisms (SNPs) association with variation in IQ phenotypes across both cohorts. The four SNPs (rs363043, rs353016, rs363039, rs363050) are localized within intron 1, in a region of about 13.8 kb, and are known to affect transcription factor binding sites [24].

We investigated the possibility that SNAP-25 plays a role in ASD by analyzing five SNAP-25 gene polymorphisms, rs363043, rs363039, rs363050, rs3746544 and rs1051312 in a well clinically characterized cohort of children affected by ASD, evaluating possible associations between such SNPs and the clinical outcome of ASD.

2. Methods and materials

2.1. Patients and controls

Sixty-seven ASD patients (51 male, 16 female, mean age 11.7 years; S.D. = 4.8 years) followed by the Pediatric Neuropsychiatry Institute at the University of Sassari were enrolled in the study. All children were thoroughly examined; in particular, all patients underwent in-depth clinical and neurological evaluations, mental status examination (covering the social interaction, imaginative play, language, and communication domains), neuropsychological evaluation (using the Leiter-R, WISC-R, Raven and Vineland Adaptive Behavior Scales according to the specific clinical picture), and other diagnostic tools such as the Modified Checklist for Autism in Toddlers (MCHAT), the Childhood Autism Rating Scale (CARS), the Australian Scale for Asperger's Syndrome (ASAS), karyotype and DNA analysis for fragile X and MEC-P2, screens for inborn errors of metabolism (phenylketonuria (PKU); amino and organic acidopathies), electroencephalogram (EEG), brain-stem acoustic evoked potentials (BAEP), visual evoked responses, and computerized tomography (CT) or magnetic resonance imaging (MRI); some parents gave their consent only for CT, rather than for MRI. In-depth genetic analyses were performed as well in these children [25,26].

The diagnosis was made according to DSM-IV-TR criteria [27] and the children were classified as follows: autistic disorder (45 cases, 66.2%), Asperger's syndrome (9 cases, 13.24%), CDD (2 cases, 2.94%), PDD–NOS (12 cases, 17.65%). Children with identified causes of autistic symptoms (e.g. fragile X syndrome) were excluded from the study. Informed consent was obtained from all participants/legal guardians prior to inclusion in the study.

A group of 205 healthy unrelated Italian subjects, sex-matched with the ASD patients (165 male, 40 female) were enrolled as control group. No age-matching was used, since no neuropsychological parameters but only genetic data were used to compare cases and controls.

The study was approved by the Institutional Review Board of the Don Carlo Gnocchi ONLUS Foundation, Milano.

2.2. SNP typing

Genomic DNA was isolated from peripheral blood by phenol–chloroform extraction using standard procedures. SNPs were typed using the Taqman SNP Genotyping Assays (Applied Biosystems) on an ABI PRISM 7000 Sequence Detection System. For rs363039, rs363043, rs363050, and rs3746544 respectively the C_327976_10, C_2488346_10 C_329097_10, and C_27494002_10. Human Pre-Designed Assays (Applied Biosystems) were used. The restriction enzyme polymorphism rs1051312 was genotyped by DdeI digestion as previously described [21]

2.3. Statistical analysis

Chi-square analysis was used to exclude any deviation of SNP genotype distribution from Hardy–Weinberg equilibrium (HWE); p value was >0.05 both in cases and in controls. The chi-squared statistics or Fisher's exact test, as appropriate, were applied to 2XN table to compare: case–control differences of SNP distributions, as well as EEG alteration with or without seizures in ASD patients in relationship with SNPs. Cognitive degree distribution was finally evaluated in relationship with SNP genotype by contingency table too.

The Kolmogorov–Smirnov (K–S) test was applied to verify normal distribution of numerical variable scores. CARS scores, which resulted normally distributed, were shown as mean and standard deviation (S.D.) and analysis of variance ANOVA was calculated in relationship with SNPs distribution. Two other variables, namely autism degree and hyperactivity score (two specific items derived from CARS), were not normally distributed according to Kolmogorov–Smirnov test (<0.01); for these variables, mean ranks were reported and Kruskall–Wallis test was applied.

3. Results

Sixty-seven children with a diagnosis of ASD were molecularly genotyped for five SNPs within the SNAP-25 gene. In particular, the following SNPs were examined: rs363050(A/G), rs363039(G/A) rs363043(C/T), rs3746544 (T/G) and rs1051312 (T/C). The genotype distribution of the five SNPs was in Hardy–Weinberg equilibrium. Genotype and allelic distribution were compared to those obtained in 205 age- and sex-matched healthy subjects of Caucasian origin (165 male, 40 female) (Fig. 1). No significant difference was revealed between the two groups.

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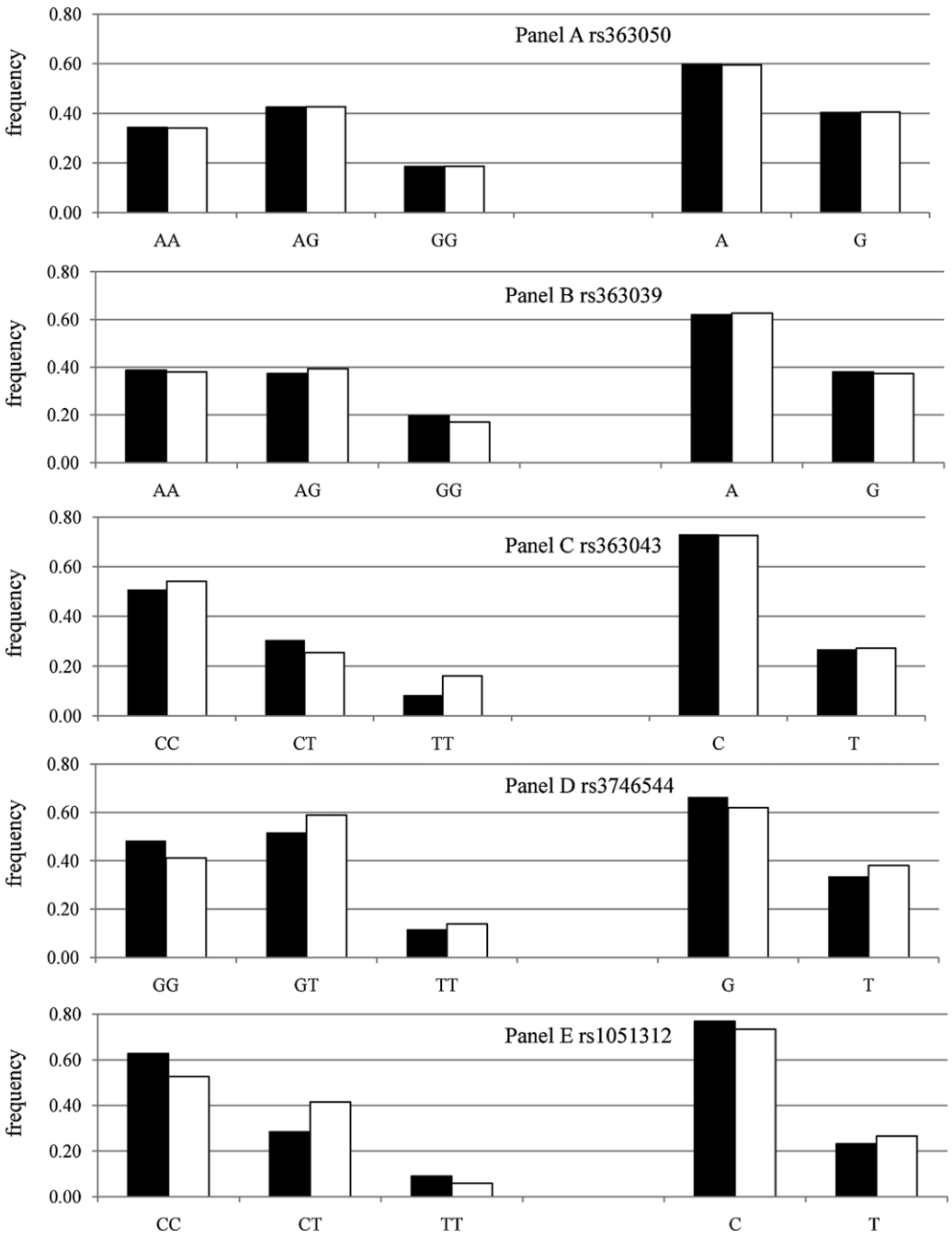

Fig. 1. Genotype and allelic frequency distribution in 68 ASD (black bars) subjects and 205 healthy control (white bars) of SNPs rs363050 (A), rs363039 (B), rs363043(C) rs3746544 (D) and rs1051312 (E).

Possible associations between SNAP-25 genotypes and different clinical patterns were evaluated next. To this end, SNAP-25 genotypes were examined in relationship with CARS score [28] in toto (range: 15–60), as well as in relationship with the specific scores assigned to hyperactivity (CARS' item 13) and autistic core-behaviour (CARS' item 15). The latter are specific items of the CARS, meant to separately measure observed hyperactivity level and autistic core-behaviour on a 7 steps scale (ranging from 1, meaning "normal for child's age", to 4, "severely abnormal for child's age", with increases of 0.5). Moreover, based on the hypothesis that brain dysfunctions could be inferred from a lower cognitive functioning and/or from cortical electrical abnormalities, patients were subsequently classified according to their cognitive levels using the DSM IV TR criteria [24], which assign a numerical score (1–6) to identify normal functioning (1), borderline cognitive level (2), mild mental retardation (3), moderate mental retardation (4), severe mental retardation (5) or profound mental retardation (6), or the degree of "cortical electrical dysfunction" (classified as lack of EEG abnormalities and absence of seizures (EEG−/SEIZ−), presence of EEG abnormalities without seizures (EEG+/SEIZ−), seizures without interictal EEG abnormalities (EEG−/SEIZ+) and presence of both EEG abnormalities and seizures (EEG+/SEIZ+).

The Kolmogorov–Smirnov test was applied to the results to evaluate quantitative data distributions of CARS, autism and hyperactivity scores. CARS data resulted normally distributed, therefore ANOVA test of mean score distribution was calculated to evaluate possible relationships between SNPs distribution and CARS (range: 21–60) (Table 1).

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Table 1
Distribution of five SNPs of the SNAP 25 gene (rs363050 (A/G), rs363039(G/A) rs363043(C/T), rs3746544 (T/G) and rs1051312 (T/C) genotypes) in 67 children with autism spectrum disorders. ANOVA test applied to mean score distribution of CARS and cognitive evaluations and Kruskall–Wallis test for autism and hyperactivity scores (not normally distributed) are presented.

Genotype	Rs363050			Rs363039			Rs363043			Rs3746544			Rs1051312		
	AA (*N* = 23)	AG (*N* = 34)	GG (*N* = 10)	GG (*N* = 26)	GA (*N* = 31)	AA (*N* = 10)	CC (*N* = 34)	CT (*N* = 30)	TT (*N* = 3)	TT (*N* = 29)	TG (*N* = 31)	GG (*N* = 7)	TT (*N* = 42)	TC (*N* = 19)	CC (*N* = 6)
CARS mean (S.D.)	40.2 (9.9)	40.1 (9.2)	39.3 (9.7)	38.3 (8.5)	41.7 (9.9)	39.3 (9.7)	**38.5**[a] (7.5)	**43.3**[a] (9.9)	**24.8**[a] (2.3)	40.3 (9.7)	40.2 (9.7)	38.1 (7.3)	41.4 (9.7)	37.7 (8.9)	37.7 (7.5)
Autism mean rank	34.1	33.6	35.2	31.4	35.8	35.2	33.1	37.2	12.2	35.6	33.5	30.6	36.1	30.9	29.0
Hyperactivity mean rank	34.5	35.8	26.9	29.4	40.2	26.9	**30.6**[b]	**40.3**[b]	**9.3**[b]	34.3	35.4	26.9	36.5	32.1	22.3

N: number of patients; S.D.: standard deviation; bold numbers correspond to significant p values ($p < 0.01$).
[a] $p = 0.001$.
[b] $p = 0.006$

A significant association of the rs363043(CT) genotype with increasing CARS scores ($p = 0.001$) was detected. The degree of autism was higher as well in ASD patients carrying the presence of the rs363043(CT) genotype, albeit these differences did not reach statistical significance. Notably, hyperactivity scores were significantly higher in ASD patients with rs363043(CT) genotype ($p = 0.006$). No association was revealed for these SNPs in relationship with other neuropsychiatric parameters.

Categorical variables were evaluated by contingency table and chi square evaluation.

Cortical electrical dysfunction (classified as: EEG abnormalities (+/−) and presence of seizures (+/−) (EEG−/SEIZ−; EEG+/SEIZ−; EEG−/SEIZ+; EEG+/SEIZ+)) as well as different cognitive level distribution were also evaluated in relationship with different SNPs, as categorical variables, by contingency table and chi square evaluation (Table 2). No correlations emerged from this analysis.

4. Discussion

Data herein describe the presence of a significant correlation between rs363403(CT) polymorphism in the SNAP-25 gene and CARS score in a cohort of children with a diagnosis of ASD; the rs363403(CT) genotype was more frequent as well in the same children, even if the differences did not reach statistical significance. Importantly, hyperactivity was not only confirmed by parents' interviews, but also considered by parents and teachers as a relevant additional problem in their everyday interaction with these children.

SNAP-25 was shown to be involved in the differential cognitive ability of healthy subjects. In particular, four SNAP-25 SNPs (rs363043, rs353016, rs363039, rs363050) were associated with an increment of performance but not of verbal IQ [24]. A recent report also suggested a strong correlation of SNAP-25 expression with the early onset of bipolar disorders [15]. Individuals homozygous for one variant located in the promoter region showed a significant higher SNAP25b expression level in the prefrontal cortex, possibly implying a contribution of the SNARE protein in altered presynaptic vesicle fusion and neurotransmitter release [15]. Recently, an intronic SNP of SNAP-25 was also shown to be associated with inattentive hyperactivity in a Chinese group of ADHD children [29].

Our results support these observations and indicate that SNAP-25 is likely involved in the pathogenesis of at least some phenotypes among ASD as well. Notably, the correlations observed were predominantly with hyperactivity or at least with one or more aspects of the Executive Functions, i.e. the ability to evaluate, plan and adequate reactions to external stimuli: we can therefore speculate that SNAP-25 might be one of the genetic factors involved in these neuropsychological functions, all essentially related with frontal lobes activity [30] and studied as relevant factors in the definition of intelligence and in the clinical phenotype of many neuropsychiatric disorders (e.g. ADHD, ASD, schizophrenia and mood disorders).

SNAP-25 participates to the formation of the SNARE complex, which is required for synaptic vesicle fusion [8]. Therefore, the possibility emerges that polymorphisms in the *SNAP25* gene may influence one or more neurotransmission systems in specific brain areas. Notably, the coloboma mice, which is hemizygous for the deletion of the *SNAP-25* gene, shows a dysregulation of the evoked release of neurotransmitters in selected brain regions, with reduced glutamate release in the neocortex and reduced dopamine release from dorsal but not ventral striatum [31]. SNAP-25 also regulates neuronal calcium responsiveness to stimuli [10–12] and is notable that the different neuropsychiatric alterations where SNAP-25 has been involved are characterized by a dysregulation of calcium homeostasis [32,33]. Indeed, an enhanced calcium response through L type channels has been described in the SHR model

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Table 2
Contingency table. Distribution of the rs363050 (A/G), rs363039(G/A), rs363043(C/T), rs3746544 (TG) and rs1051312 (TC) genotypes evaluated in 67 children in relationship with autism spectrum disorders with cognitive degree (1 normal functioning; 2: borderline level; 3: moderate mental retardation; 4: mild mental retardation; 5: severe mental retardation; 6: profound mental retardation) and with the degree of "cortical electrical dysfunction" (classified as lack of EEG abnormalities and absence of seizures (EEG−/SEIZ−), presence of EEG abnormalities without seizures (EEG+/SEIZ−), seizures without interictal EEG abnormalities (EEG−/SEIZ+) and presence of both EEG abnormalities and seizures (EEG+/SEIZ+).

	rs363050			rs363039			rs363043			rs3746544			rs1051312		
	AA ($N=23$)	AG ($N=34$)	GG ($N=10$)	GG ($N=26$)	GA ($N=31$)	AA ($N=10$)	CC ($N=34$)	CT ($N=30$)	TT ($N=3$)	TT ($N=29$)	TG ($N=31$)	GG ($N=7$)	TT ($N=42$)	TC ($N=19$)	CC ($N=6$)
Genotype frequencies															
EEG−/Seiz−	0.65	0.56	0.40	0.69	0.52	0.40	0.56	0.57	0.67	0.73	0.45	0.43	0.50	0.63	0.83
EEG+/Seiz−	0.22	0.32	0.40	0.23	0.32	0.40	0.38	0.20	0.33	0.17	0.39	0.43	0.33	0.26	0.17
EEG−/Seiz+	0	0.06	0.20	0	0.06	0.20	0.06	0.07	0	0.03	0.06	0.14	0.1	0	0
EEG+/Seiz+	0.13	0.06	0	0.08	0.10	0	0	0.16	0	0.07	0.1	0	0.07	0.11	0
			$pc=0.224$ 6 df			$pc=0.240$ 6 df			$pc=0.223$ 6df			$pc=0.335$ 6df			$pc=0.557$ 6df
Cognitive level frequencies															
1	0.39	0.23	0.40	0.34	0.26	0.40	0.35	0.23	0.67	0.28	0.35	0.29	0.30	0.32	0.33
2	0.04	0.18	0.10	0.08	0.16	0.10	0.15	0.10	0	0.14	0.10	0.14	0.12	0.16	0
3	0.31	0.18	0.10	0.31	0.16	0.10	0.20	0.20	0.33	0.31	0.16	0	0.12	0.32	0.50
4	0.04	0.12	0.30	0.11	0.06	0.20	0.15	0.10	0	0.03	0.10	0.57	0.17	0	0.17
5	0.09	0.20	0.10	0.08	0.23	0.10	0.15	0.17	0	0.17	0.16	0	0.17	0.16	0
6	0.13	0.09	0	0.08	0.13	0	0	0.20	0	0.07	0.13	0	0.12	0.04	0
			$pc=0.298$ 10df			$pc=0.364$ 10df			$pc=0.323$ 10df			$pc=0.028$ 10df			$pc=0.324$ 10df

N: number of patients; values are expressed as frequencies. *pc*: *p* value with Bonferroni correction; df: degree of freedom.

of ADHD, where a reduction of SNAP-25 also occurs [34]. Abnormalities in calcium transients, caused by a SNAP-25 deficiency in modulating presynaptic voltage-gated calcium channels, also seem to be causative of the slow wave discharges (SWD) in the coloboma model of absence epilepsy [16]. Finally, and even more interestingly, an altered calcium homeostasis has been described in brain of patients affected by autism-spectrum disorders [6,35]. The evidence that calcium dysregulation occurs in the different neuropsychiatric disorders where SNAP-25 has been involved, suggests that the role of the protein as a calcium channel modulator, more than its role as controller of synaptic vesicle fusion, may be relevant for these pathologies.

Since intron may play a role in regulation of gene expression, as also stated by Zhang et al. [29], we can speculate that rs363043 SNP may affect levels of SNAP-25 expression, as also indicated by the localization of the rs363403 SNP in the intron 1 region, a region which is known to affect the transcription factor binding sites of the SNAP-25 protein [24]. Changes in SNAP-25 expression levels may in turn lead to alterations of neurotransmitter release [31] or impairments in calcium homeostasis [11,12,17].

We cannot affirm that the SNAP 25 polymorphisms are susceptibility factors for ASD development: larger studies will be needed to exclude or confirm this possibility. Nevertheless, our results, although stemming from analyses preformed in a relatively small groups of children, seem to indicate that in those individuals who develop "primary ASD", the presence of SNAP-25 polymorphisms correlates with a more compromised clinical outcome.

This seems to be strongly related to hyperactivity, the neuropsychological trait showing a significant association to specific SNAP-25 polymorphisms and reported to be enhanced in the SNAP-25 deficient coloboma mice [reviewed in ref. 31]. It is possible that, through altered calcium homeostasis the SNP results in a differential modulation of synaptic activity. The finding that cortical electrical activity alteration did not correlate with any specific SNAP-25 polymorphism does not rule out this hypothesis, because some subgroups do contain too few patients to be statistically significant; this aspect needs to be investigated in larger studies.

5. Conclusions

In conclusion, these data support a role for SNAP-25 as a new gene involved in the genetic background of ASD. The role of SNAP-25 in controlling calcium homeostasis suggests that pharmacologic interventions targeting calcium flow and/or the activity of calcium channels could represent interesting therapeutic strategies in ASD.

Conflict of interest

There is no conflict of interest.

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