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ORIGINAL ARTICLE

Association between *SNAP-25* gene polymorphisms and cognition in autism: functional consequences and potential therapeutic strategies

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Synaptosomal-associated protein of 25 kDa (SNAP-25) is involved in different neuropsychiatric disorders, including schizophrenia and attention-deficit/hyperactivity disorder. Consistently, SNAP-25 polymorphisms in humans are associated with hyperactivity and/or with low cognitive scores. We analysed five *SNAP-25* gene polymorphisms (rs363050, rs363039, rs363043, rs3746544 and rs1051312) in 46 autistic children trying to correlate them with Childhood Autism Rating Scale and electroencephalogram (EEG) abnormalities. The functional effects of rs363050 single-nucleotide polymorphism (SNP) on the gene transcriptional activity, by means of the luciferase reporter gene, were evaluated. To investigate the functional consequences that SNAP-25 reduction may have in children, the behaviour and EEG of SNAP-25$^{+/-}$ adolescent mice (SNAP-25$^{+/+}$) were studied. Significant association of *SNAP-25* polymorphism with decreasing cognitive scores was observed. Analysis of transcriptional activity revealed that SNP rs363050 encompasses a regulatory element, leading to protein expression decrease. Reduction of SNAP-25 levels in adolescent mice was associated with hyperactivity, cognitive and social impairment and an abnormal EEG, characterized by the occurrence of frequent spikes. Both EEG abnormalities and behavioural deficits were rescued by repeated exposure for 21 days to sodium salt valproate (VLP). A partial recovery of SNAP-25 expression content in SNAP-25$^{+/-}$ hippocampi was also observed by means of western blotting. A reduced expression of SNAP-25 is responsible for the cognitive deficits in children affected by autism spectrum disorders, as presumably occurring in the presence of rs363050(G) allele, and for behavioural and EEG alterations in adolescent mice. VLP treatment could result in novel therapeutic strategies.

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INTRODUCTION

Recent evidences suggested that SNAP-25 (synaptosomal-associated protein of 25 kDa) is involved in different neuropsychiatric and neurological disorders.[1] SNAP-25 participates in the regulation of synaptic vesicle exocytosis through the formation of a soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE) complex[2] and interacts with different types of voltage-gated calcium channels,[3] inhibiting their function and thus reducing neuronal calcium responsiveness to depolarization.[4–6]

Interestingly, polymorphisms in the *SNAP-25* gene as well as altered expression of the protein have been associated with abnormal behavioural phenotype in both animal models[7–9] and humans. Polymorphisms in the *SNAP-25* gene have been found in patients affected by attention-deficit/hyperactivity disorder (ADHD),[10–13] schizophrenia[14–16] and autism spectrum disorders (ASDs).[17] In a group of Sardinian children who developed primary ASD, *SNAP-25* polymorphisms were associated with a more compromised clinical outcome,[17] and a significant correlation was observed between *SNAP-25* single-nucleotide polymorphisms (SNPs) rs363043 and the Childhood Autism Rating Scale (CARS). Notably, these correlations were predominantly with hyperactivity and one or more aspects of the executive functions. SNAP-25 was also shown to be involved in the differential cognitive ability of healthy subjects. In particular, four *SNAP-25* SNPs (rs363043, rs353016, rs363039 and rs363050) were associated with an increment of performance, but not of verbal intelligence quotient.[18]

Reduction of SNAP-25 expression has been described in brains of patients affected by either schizophrenia[14] or ADHD.[19] Reduction of protein expression was associated with the occurrence of frequent electroencephalographic spikes, suggesting a diffuse network hyperexcitability as shown in *coloboma* mouse[20] and *SNAP-25* heterozygous mice.[21] Interestingly, epilepsy is associated with several neurodevelopmental disorders including ADHD, ASD and intellectual disability.[22] Such co-occurrence may share a genetic basis.[23] Children and adolescents with epilepsy, in particular, tend to show an increased risk of ADHD,[24,25] suggesting a strong interrelationship between the ASD and ADHD phenotype and childhood epilepsy. Notably, the epileptiform activity, characterized by the occurrence of frequent electroencephalogram (EEG) spikes in 3-month-old *SNAP-25*$^{+/-}$ mice, was

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accompanied by cognitive deficits that were reverted by antiepileptic drugs.[21]

In an attempt to understand more in depth the role of SNAP-25 in human diseases characterized by an abnormal cognitive profile, we first analysed five *SNAP-25* gene polymorphisms (rs363043, rs363039, rs363050, rs3746544 and rs1051312) in a clinically characterized cohort of children affected by ASD; in particular, we evaluated possible associations between such SNPs and the clinical outcome of ASD. As we found a correlation between rs363050 SNP and cognitive deficits, the functional effects of this polymorphism on the gene expression was evaluated by means of the luciferase reporter gene confirming its involvement in gene transcriptional modulation. Moreover, given that SNAP-25 expression can be altered in childhood neuropsychiatric diseases and our previous work demonstrated behavioural and EEG deficits in adult *SNAP-25*$^{+/-}$ mice, we decided to verify whether similar deficits were present also during adolescence (6 weeks old), in order to highlight possible autistic or ADHD symptoms. Finally, to verify a possible therapeutic application of valproate (VLP), which was previously shown to rescue some behavioural and EEG deficits when acutely administered, we evaluated the effect of this antiepileptic drug after chronic exposure.

MATERIALS AND METHODS

Human studies

Subjects. Forty-four Italian ASD patients (40 males, 4 females, mean age 10.9 years; s.d.= 4.7 years) were enroled in the study. All subjects were born in peninsular Italy from families without Sardinian ancestry and were of Italian descent. All children underwent an in-depth examination that included clinical and neurological evaluations, mental status examination (covering the social interaction, imaginative play, language and communication domains), neuropsychological evaluation (using the Leiter-R, Wechsler Intelligence Scale for Children-R, Raven and Vineland Adaptive Behaviour Scales according to the specific clinical picture) and other diagnostic tools, such as the Modified Checklist for Autism in Toddlers, CARS, the Australian Scale for Asperger's syndrome, karyotype and DNA analysis for fragile X and MeC-P2, screens for inborn errors of metabolism (phenylketonuria), amino and organic acidopathies, EEG, brain-stem acoustic evoked potentials, visual evoked responses and computerized tomography or magnetic resonance imaging; some parents gave their consent only for computerized tomography rather than for magnetic resonance imaging. In-depth genetic analyses were performed as well in these children.[26,27]

ASD diagnosis was made according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) criteria[28] and the children were classified as follows: autistic disorder (33 cases, 75%), Asperger's syndrome (7 cases, 15.9%) and pervasive developmental disorder—not otherwise specified (4 cases, 0.09%). Children with identified causes of autistic symptoms (for example, fragile X syndrome) were excluded from the study. Informed consent was obtained from all participants/legal guardians prior to inclusion in the study. The study was approved by the institutional review board of the Don Carlo Gnocchi ONLUS Foundation, Milan.

SNP typing. Genomic DNA was isolated from peripheral human blood. Procedural details are reported in Supplementary Materials and Methods.

In vitro studies

Transient transfection and the luciferase assay. The SH-SY5Y human neuroblastoma cell line was grown in RPMI 1640 medium (Lonza Group, Basel, Switzerland), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine (Lonza Group). For further details see Supplementary Materials and Methods.

Animal studies

Subjects. All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 27/2010. All efforts were made to minimize the number of subjects used and their suffering. Male *SNAP-25*$^{+/+}$ and *SNAP-25*$^{+/-}$C57BL/6 mice were backcrossed and genotyped as previously reported.[21] All mice used were littermates from mated heterozygous. Animals were individually housed throughout the testing period with free access to food and water at controlled temperature (20–22 °C) with a 12-h light/dark cycle (lights on at 0700 hours). Each mouse was used only once. Animals were randomly assigned to each experimental group and tested at 6–7 weeks of age (adolescence).

Pharmacological treatment

Mice, at the age of 3 weeks, were exposed 24 h per day for 21 days to VLP solution (0.1% dissolved in plain water) (Sigma-Aldrich, St Louis, MO, USA) or water. Each day the bottles containing VLP (or water for the control animals) were weighted to obtain the daily amount of fluid intake, and refilled with fresh solution. Twenty-four hours after the last VLP exposure different groups of mice (10 each) were submitted to behavioural tests and EEG.

Behavioural assays

Spontaneous motor activity and amphetamine response. Motor function was evaluated using an activity cage (43 × 43 × 32 cm) (Ugo Basile, Varese, Italy), placed in a sound-attenuating room. The cage was fitted with two parallel horizontal infrared beams located 2 cm from the floor. Before the start of the test, each mouse (6–7 weeks of age) was habituated to the testing room for at least 1 h. Cumulative horizontal movement counts were recorded for 4 h before and 3 h after treatment. Animals were treated subcutaneously with saline or amphetamine sulphate (4 mg kg^{-1}) (Sigma-Aldrich) dissolved in 0.9% NaCl, according to Hess *et al.*[29]

Object recognition test. The test was conducted over a 2-day period in an open plastic arena (60 × 50 × 30 cm). Animals were habituated to the test arena for 10 min on the first day. After 1-day habituation, mice were subjected to familiarization (T_1) and novel object recognition (T_2). The novel object recognition task was performed as previously described[30] using a delay time of 120 min. The performance was evaluated by calculating a discrimination index ($N-F/N+F$), where N = time spent exploring the new object during T_2, F = time spent exploring the familiar object during T_2.

Conditioned taste aversion. *SNAP-25*$^{+/+}$ and *SNAP-25*$^{+/-}$ mice were singly housed during the conditioned taste aversion (CTA) test. After mice were adapted to a restricted drinking schedule (20 min h^{-1} per day for 4 days), they were exposed to a saccharin solution (conditioned stimulus, CS; 0.5%) followed 1 h later by a malaise-inducing injection of LiCl (unconditioned stimulus, US; 0.14 M, 2% body weight, intraperitoneally) according to Callaerts-Vegh *et al.*[31] After 24 h of wash-out (drinking water for 30 min), mice could freely choose to drink either saccharin solution or tap water during three daily choice tests. The mean amount of saccharin intake expressed as the percentage of total fluid consumed ((saccharin/saccharin +water) × 100) was taken as an aversion index.

Sociability and preference for the social novelty test. The sociability and preference for the social novelty test was used as previously described by Sala *et al.*[32] After 10 min habituation to the cage, an unfamiliar male mouse was enclosed in one side comportment, whereas the opposite side contained an empty wire cage. For the social novelty test, carried out in the same apparatus immediately after the sociability test, one side compartment contained the familiar mouse (from the previous sociability phase), and the other side an unfamiliar mouse. For both tests the time spent and the number of entries made in each chamber were recorded for 10 min. Data were expressed as the difference score between the time spent to explore the compartment containing the familiar mouse and that spent in the empty compartment (for sociability test), or containing the stranger animal and that for the familiar mouse (for social novelty test).

EEG

After surgery (See Supplementary Materials and Methods), EEG activity was recorded in a Faraday chamber, using a Power-Lab digital acquisition system (AD Instruments, Bella Vista, NSW, Australia; sampling rate 100 Hz) in freely moving mice.

Table 1. Distribution of five SNAP-25 SNPs (rs363050(A/G), rs363039(G/A) rs363043(C/T), rs3746544(T/G)) and rs1051312(T/C) genotypes in children with autism spectrum disorders

Genotype (number)	*CARS scores, mean (s.d.)*	*Autism scores, mean (s.d.)*	*Hyperactivity scores, mean (s.d.)*	*Cognitive scores, mean (s.d.)*	*Cognitive level frequencies*	
					1–3	*4–6*
rs363050						
AA (18)	41.4 (5.5)	5.3 (0.5)	2.9 (0.6)	3.5 (1.1)	0.44	0.56
AG (18)	37.2 (6.1)	2.7 (0.5)	2.4 (0.7)	4.5 (1.1)	0.11	0.89
GG (8)	37.8 (7.2)	3.2 (0.7)	2.6 (0.5)	3.1 (1.0)	0.75	0.25
	df = 2, c2 = 10.7, *P* = 0.005	df = 2, F = 2.5, *P* = 0.09	df = 2, F = 2.4, *P* = 0.11	df = 2, F = 6.8, *P* = 0.003	df = 2, c2 = 10.7, *P* = 0.005	
rs363039						
GG (18)	40.8 (5.4)	3.0 (0.5)	2.9 (0.6)	3.5 (1.0)	0.44	0.56
GA (22)	37.4 (6.1)	2.8 (0.6)	2.5 (0.6)	4.3 (1.2)	0.23	0.77
AA (4)	39.6 (9.9)	3.0 (0.8)	2.7 (0.5)	3.0 (0.8)	0.75	0.25
	df = 2, F = 1.5, *P* = 0.23	df = 2, F = 0.7, *P* = 0.49	df = 2, F = 2.1, *P* = 0.13	df = 2, F = 3.2, *P* = 0.05	df = 2, c2 = 4.8, *P* = 0.09	
rs363043						
CC (20)	37.5 (6.7)	2.9 (0.6)	2.6 (0.6)	3.9 (1.0)	0.40	0.60
CT (22)	39.9 (5.4)	2.8 (0.5)	2.8 (0.5)	3.9 (1.4)	0.32	0.68
TT (2)	45.0 (8.5)	3.5 (0.7)	3.5 (0.7)	3.5 (0.7)	0.50	0.50
	df = 2, F = 1.8, *P* = 0.18	df = 2, F = 1.2, *P* = 0.29	df = 2, F = 1.2, *P* = 0.29	df = 2, F = 0.1, *P* = 0.90	df = 2, c2 = 0.5, *P* = 0.79	
rs3746544						
TT (17)	40.6 (6.6)	2.9 (0.5)	2.8 (0.8)	3.6 (1.2)	0.35	0.65
TG (23)	38.1 (6.2)	2.9 (0.6)	2.5 (0.5)	4.1 (1.2)	0.30	0.70
GG (4)	37.7 (4.6)	3.1 (0.2)	2.9 (0.5)	3.5 (1.0)	0.75	0.25
	df = 2, F = 0.9, *P* = 0.43	df = 2, F = 0.3, *P* = 0.73	df = 2, F = 1.3, *P* = 0.28	df = 2, F = 0.9, *P* = 0.43	df = 2, c2 = 2.9, *P* = 0.23	
rs1051312						
TT (25)	39.5 (5.8)	2.9 (0.5)	2.6 (0.5)	3.8 (0.7)	0.40	0.60
TC (17)	37.1 (6.1)	2.8 (0.6)	2.5 (0.7)	3.9 (1.3)	0.35	0.65
CC (2)	48.7 (3.2)	3.5 (0.7)	3.0 (0.1)	4.5 (1.1)		01.0
	df = 2, F = 3.2, *P* = 0.05	df = 2, F = 1.4, *P* = 0.25	df = 2, F = 3.1, *P* = 0.06	df = 2, F = 0.4, *P* = 0.67	df = 2, c2 = 1.3, *P* = 0.52	

Abbreviations: ANOVA, analysis of variance; ASD, autism spectrum disorder; CARS, Childhood Autism Rating Scale; df, degrees of freedom; SNP, single-nucleotide polymorphism; SNAP, synaptosomal-associated protein. Children with ASD (*N* = 44) were classified based on the CARS, autism, hyperactivity, degree of cognitive impairment and cognitive-level frequencies. ANOVA test was applied to mean score distribution of CARS, autism and hyperactivity, and cognitive scores are shown. Cognitive-level frequencies were classified as follows: 1: profound mental retardation; 2: severe mental retardation; 3: mild mental retardation; 4: moderate mental retardation; 5: borderline level; 6: normal functioning.

Basal 24-h recording. For basal cerebral activity, freely moving mice were recorded for 24 h. For each 24-h EEG recording, the mean number of spikes was evaluated in both genotypes. EEG traces were sampled at 100 Hz. [20,33]

Chronic VLP. Twenty-four hours after the last intake of VLP, given for 21 days, mice were recorded weekly for 2 h during 3 weeks.

Western blot analysis

Western blot was carried out as previously described.[21] Homogenates of hippocampi and prefrontal cortex from *SNAP-25*$^{+/-}$ mice (immediately after VLP withdrawal and after 3 weeks) were separated by electrophoresis, blotted on nitrocellulose membrane and analysed using monoclonal antibodies against SNAP-25 (Sternberger Monoclonals, Lutherville, MD, USA; 1:100 000) and anti-beta-III-tubulin (Promega, Madison, WI, USA, 1:4000). Membranes were washed and incubated for 1 h at room temperature with the secondary antibody IRDye 680-conjugated goat anti-mouse (LI-COR Biosciences, Lincoln, NE, USA; diluted 1:10 000) or IRDye 800-conjugated goat anti-rabbit (LI-COR Biosciences; diluted 1:10 000) and then scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). Alternatively, immunoreactive bands were detected using the Pierce ECL Western Blot Substrate (Thermo Fisher Scientific, Rockford, IL, USA), scanned with GS-800TM calibrated densitometer (Bio-Rad Laboratories, Segrate, Italy), and analysed with Image J Software (National Institutes of Health, Bethesda, MD, USA). For each sample, SNAP-25/beta-III-tubulin pixel values were normalized to the average of *SNAP-25*$^{+/+}$ controls.

Table 2. Distribution of the rs363050(A/G), rs363039(G/A) and rs363043(C/T), rs3746544(TG) and rs1051312(TC) genotypes evaluated in children with autism spectrum disorders

	EEG−/SEIZ−, N Freq	*EEG+/SEIZ−, N Freq*	*EEG+/SEIZ+, N Freq*	*df*	*c2*	P-*value*
rs363050						
AA (18)	15 (0.83)	2 (0.11)	1 (0.06)			
AG (18)	14 (0.77)	3 (0.17)	1 (0.06)			
GG (8)	6 (0.74)	1 (0.13)	1 (0.13)			
				3	0.73	0.95
rs363039						
GG (18)	16 (0.89)	1 (0.05)	1 (0.05)			
GA (22)	16 (0.73)	4 (0.18)	2 (0.09)			
AA (4)	3 (0.75)	0	1 (0.25)			
				3	3.58	0.46
rs363043						
CC (20)	15 (0.75)	3 (0.15)	2 (0.10)			
CT (22)	18 (0.82)	3 (0.14)	1 (0.04)			
TT (2)	2 (1.00)	0	0			
				3	1.07	0.89
rs3746544						
TT (17)	15 (0.88)	1 (0.06)	1 (0.06)			
TG (23)	17 (0.74)	4 (0.17)	2 (0.09)			
GG (4)	3 (0.75)	1 (0.25)	0			
				3	2.04	0.63
rs1051312						
TT (25)	20 (0.80)	4 (0.16)	1 (0.04)			
TC (17)	13 (0.76)	2 (0.12)	2	−12		
CC (2)	2 (1.00)	0	0			
				3	1.58	0.81

Abbreviations: df, degrees of freedom; EEG, electroencephalogram; SEIZ, seizures. Children (44) were classified based on the degree of cognitive impairment and on the degree of cortical electrical dysfunction. Cortical dysfunction: 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 seizure (EEG+/SEIZ+). *N* = number of patients. Values are expressed as frequencies. *P*-value with Bonferroni correction.

Statistical analyses

For human studies, χ^2-analysis was used to exclude any deviation of SNP genotype distribution from the Hardy–Weinberg equilibrium (*P*-value was >0.05 both in cases and in controls). The χ^2-statistics or Fisher's exact test, as appropriate, were applied to contingency table (2XN) 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 first evaluated in relationship with the SNP genotype by contingency table clustering as categorical variables with lower scores from 1 to 3 (1 = profound mental retardation, 2 = severe mental retardation, 3 = moderate mental retardation) and higher scores from 4 to 6 (corresponding to lower cognitive deficit, 4 = mild mental retardation, 5 = borderline cognitive level and 6 = normal functioning), and then a more in depth analysis was performed by numerical degrees scores association with SNPs by analysis of variance. All the numerical variables evaluated resulted normally distributed after the Kolmogorov–Smirnov test analysis. Analysis of variance calculation was applied to CARS, autism degree and hyperactivity score (two specific items derived from CARS). For luciferase and animal studies, data were given as mean ± s.e.m. and analysed by one-way or two-way analysis of variance for repeated measures followed by Tukey's or Bonferroni's *post hoc* tests, when appropriate. Pair-wise comparisons between genotypes or treatments were assessed using the Student's *t*-test. All statistical analyses were done with software Prism, version 6 (GraphPad, San Diego, CA, USA) using a critical probability of $P<0.05$. Statistical analyses performed for each experiment were summarized in each legend of the figures with the chosen statistical test, *n* and *P*-values, as well as degree of freedom and *F/t* values.

RESULTS

Human studies

The rs363050 gene polymorphism correlates with decreasing cognitive scores in autistic children.

The genotype distribution of the five analysed *SNAP-25* SNPs (rs363050(A/G), rs363039(G/A) rs363043(C/T), rs3746544(T/G) and rs1051312(T/C)) was in the Hardy–Weinberg equilibrium. *SNAP-25* genotypes were then examined in relationship with the CARS score[34] 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 define hyperactivity level and autistic core-behaviour on a seven-step 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.[28] Categorical variables were evaluated by contingency table and χ^2-evaluation.

Significant associations of the rs363050 polymorphism with altered cognitive scores was observed by the comparison of categorical cognitive score variables ($P=0.005$ after correction for 2 degrees of freedom (df)) (Table 1): in particular, rs363050(GG) genotype was more frequently observed in subjects with lower cognitive scores (1–3) than in subjects with higher cognitive scores (0.75 vs 0.25) (Table 1). Cognitive score association with *SNAP-25* polymorphism was further analysed by numerical degrees scores association with SNPs by analysis of variance. Also, in this case results showed a statistical association between rs363050 polymorphism distribution and cognitive scores, index of mental retardation, whereas no associations were observed between these SNPs and any of the other neuropsychiatric parameters analysed. Finally, the analysis of possible correlations between cortical electrical dysfunction (classified as: EEG abnormalities (+/−) and presence of seizures (SEIZ) (+/−) (EEG−/SEIZ−; EEG+/SEIZ−; EEG−/SEIZ+; EEG+/SEIZ+) and *SNAP-25* SNPs

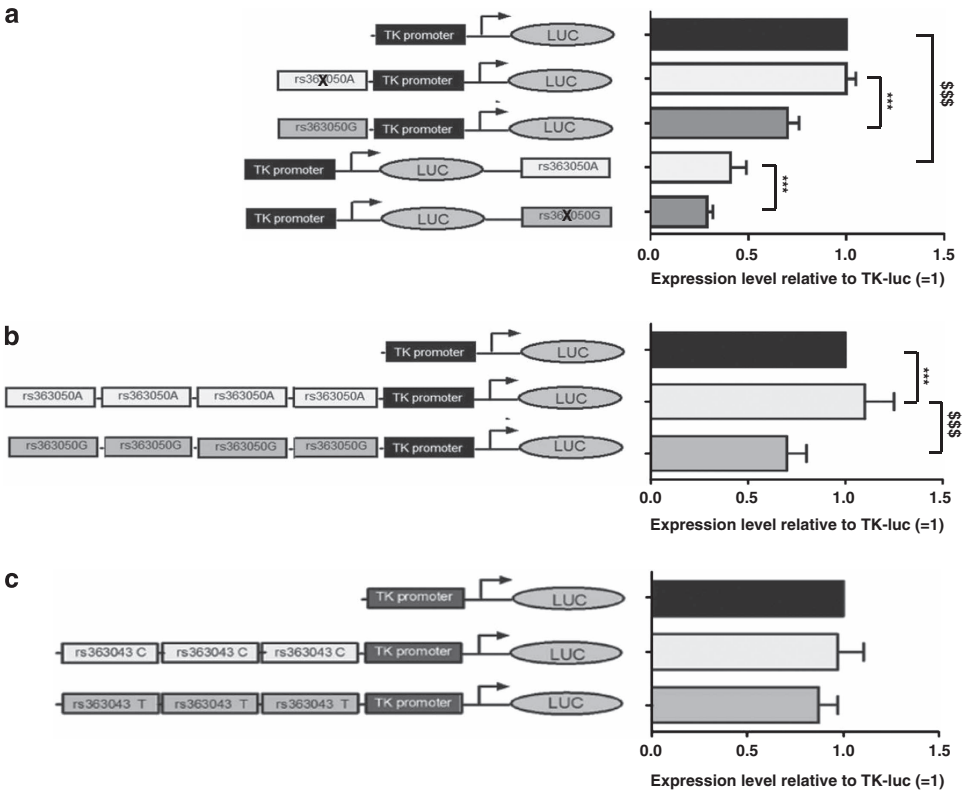


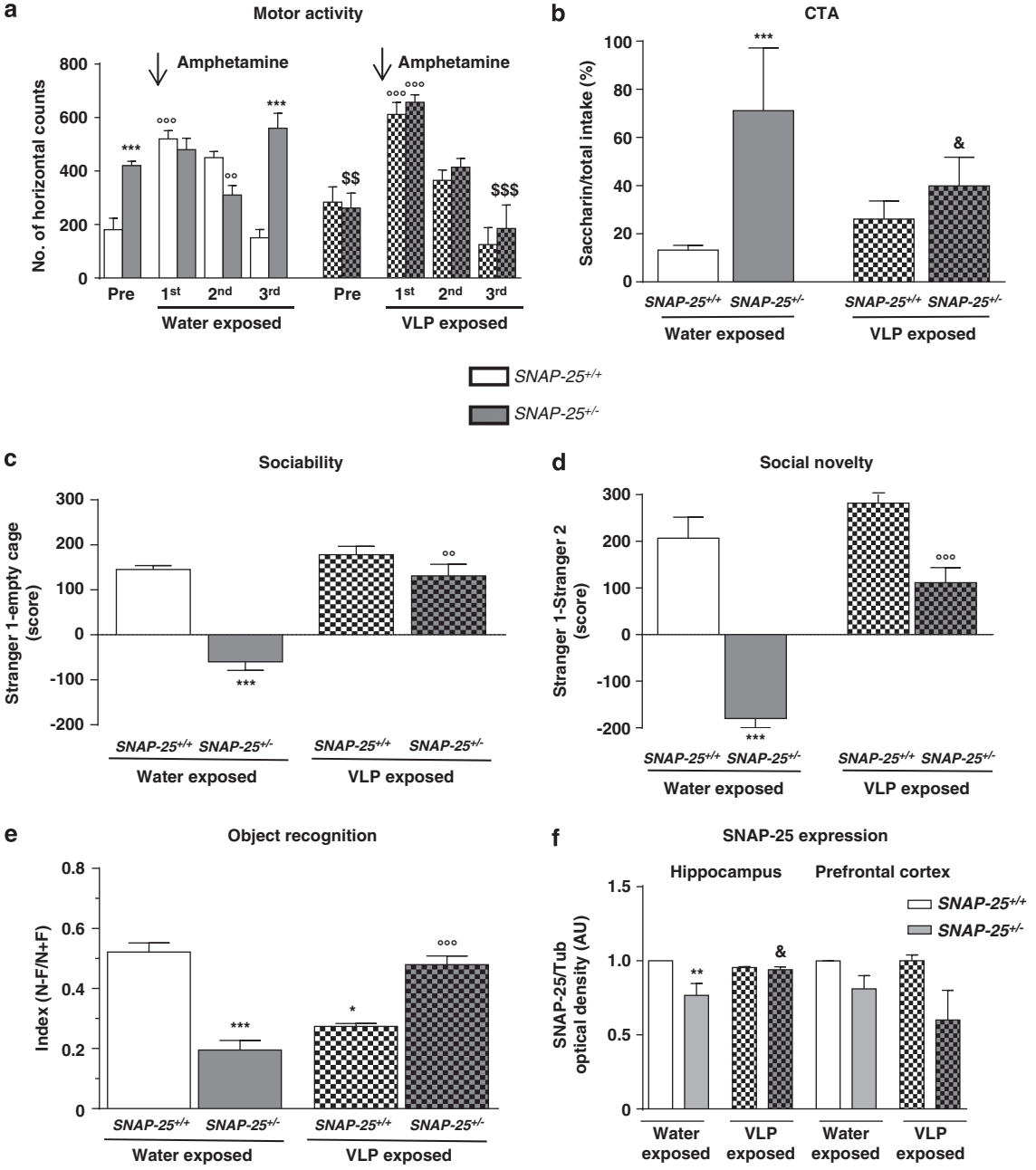
Figure 1. Functional effect of rs363050 single-nucleotide polymorphism (SNP). SNP rs363050 contains a regulatory element leading to synaptosomal-associated protein of 25 kDa (SNAP-25) expression decrease. (**a**) The presence of the parental allele (A), when cloned in single copy upstream the thymidine kinase (TK) promoter (construct rs363050A-TK-luc) did not increase the activity of the heterologous TK promoter (white bar vs black bar), whereas the presence of the minor allele (G) (construct rs363050G-TK-luc) significantly affected by 33% the activity of the promoter ($F(4,10) = 234.8$, $P = 0.001$, one-way analysis of variance (ANOVA)). (**b** and **c**) The region surrounding the rs363050 and rs363043 SNP was cloned in four and three concatenated copies, respectively. Left: schematic illustration of the constructs. The black boxes represent the HSV-TK promoter, the white boxes in panel A the 747 bp region of *SNAP-25* gene intron 1 spanning rs363050 SNP, the grey oval represent the *Firefly* luciferase reporter gene (luc). The black cross indicates the (A) to (G) change; the arrows indicate the transcription start site. Right: luciferase assays. SH-SY5Y cells were transiently transfected with the constructs shown on the left. The bars show the transcriptional activity of the constructs expressed as a relative expression level of luciferase normalized to that of renilla with respect to the TK-luc construct (=1), and expressed as mean values ± s.d. of at least three independent experiments performed in triplicate. The three asterisks indicate a statistically significant difference between rs363050A and rs363050G constructs (one-way ANOVA). In **a**, $$$ indicates a statistically significant difference between TK-luc/pGL4 basic and TK-luc-rs363050A construct (one-way ANOVA, $P < 0.001$); in **b** it indicates a statistically significant difference between four rs363050A and rs363050G copy constructs (one-way ANOVA, $P < 0.001$).

as categorical variables, by contingency table and χ^2-evaluation (Table 2), did not reveal the presence of any further association.

In vitro studies

SNP rs363050 encompasses a regulatory element leading to SNAP-25 expression decrease.

The results obtained in humans studies reported a strong association between *SNAP-25* SNP rs363050 polymorphism and cognitive functions. rs363050 SNP is localized in intron 1 of the gene coding for SNAP-25; as regulatory elements are often present in introns of genes,[35–38] we conducted, by means of the luciferase reporter gene assay, an analysis of rs363050 SNP functional effect on transcriptional activity with the aim to define whether it could have a key role in the *SNAP-25* gene expression. A series of plasmids, in which the region encompassing the rs363050 SNP and carrying either the parental (A) or the minor allele (G) was inserted in single or four concatenated copies upstream and/or downstream the thymidine kinase (TK) promoter in pGL4 basic vector, were originated (Figures 1a and b). Their activity was tested by means of transient transfection of the human neuroblastoma SH-SY5Y cell line and compared with that of the plasmid without the cassette (Figures 1a and b, TK-luc/pGL4 basic). The A allele did not affect transcription regulation of the reference plasmid (Figure 1a, rs363050A-TK-luc vs TK-luc) when present in single copy, but had a slight, although statistical significant increase when in four copies (Figure 1b). The presence of the susceptible G allele resulted in a statistical significant decrease in luciferase (luc) activity of 30% (Figure 1a, $P < 0.001$) and 42% (Figure 1b, $P < 0.001$), respectively. When the same cassettes were inserted downstream the luc reporter gene, both constructs (TK-luc-rs363050A and TK-luc-rs363050G) showed a marked decrease in the capability of driving the expression of the reporter gene of 60% ($P < 0.001$) with respect to the TK promoter (Figure 1a, grey and hatched bar vs black bar). However, the reduced transcription capability of the minor allele (G) with respect to the parental allele (A) was again observed (Figure 1a, construct TK-luc-rs363050A vs TK-luc-rs363050G, 31% reduction, $P < 0.001$). As a control, three concatenated copies of the sequence, surrounding the rs363043 polymorphic site, were tested (Figure 1c). No effect on transcription was measured in the presence of both the parental allele (C) and minor allele (T).



Animal studies

Experiment 1. Adolescent *SNAP-25*$^{+/-}$ mice are impaired in motor activity, memory, social interaction and show reduced SNAP-25 expression: therapeutic effect of VLP.

Given the SNP rs363050(G) was strongly associated with cognitive scores in ASD children and since the rs363050 minor allele displayed a reduced transcription capability, we evaluated the behavioural profile of 6-week-old *SNAP-25*$^{+/-}$ mice in order to define whether reductions of the protein levels could affect the cognitive profile in young individuals. Furthermore, as acute treatment with VLP has been previously demonstrated to abolish associative memory defects in adult *SNAP-25*$^{+/-}$ mice,[21] animals were exposed to plain water or VLP, for 21 days, 24 h per day. The mean daily intake was not different between VLP- and water-exposed mice. The VLP amount corresponded to 250–270 mg·kg^{-1} per day (Supplementary Figure 2).

Analysis of motor activity was carried out in mice of both genotypes in basal conditions and after chronic VLP treatment. The time course of horizontal activity, recorded every 10 min for 4 h (baseline) and after D-amphetamine injection for 3 h after water or VLP pre-exposure, is reported in Supplementary Figure 2. Analysis of the time course (Figure 2a), evaluated as blocks of 1-h each, showed that *SNAP-25*$^{+/-}$ mice were hyperactive. Amphetamine treatment significantly increased the number of horizontal movements in *SNAP-25*$^{+/+}$ water-exposed mice, but it was ineffective in *SNAP-25*$^{+/-}$ mice. Any difference among the groups on the mean horizontal activity counts after VLP was shown, confirming that the drug normalized motor activity in the *SNAP-25*$^{+/-}$ mice.

When tested for CTA with saccharin (Figure 2b), *SNAP-25*$^{+/-}$ mice exhibited a significantly attenuated CTA, as they avoided the saccharin solution to a lesser degree compared with *SNAP-25*$^{+/+}$ mice during choice tests. Repeated treatment with VLP induced a

Figure 2. Behavioural profile and synaptosomal-associated protein of 25 kDa (SNAP-25) expression of *SNAP-25*$^{+/+}$ and *SNAP-25*$^{+/-}$ mice evaluated 24 h after sodium valproate salt (VLP) (1 mg ml^{-1}) or water exposure for 21 days. (**a**) The mean (± s.e.m.) of horizontal movements recorded every 10 min in the last 2 h of basal (pre) and every hour after amphetamine (4 mg kg^{-1}) treatment for 3 h (two-way analysis of variance (ANOVA) (genotype as a between-subject factor: $F(3,126) = 4.06$; $P = 0.01$; time as a within-subject factor: $F(3,126) = 30.95$; $P = 0.0001$; treatment × genotype interaction: $F(9,126) = 20.10$, $P < 0.0001$). $^{***}P < 0.001$ vs SNAP-25$^{+/+}$ water-exposed mice, same interval; $^{°°}P < 0.01$, $^{°°°}P < 0.001$ vs corresponding pre; $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs *SNAP-25*$^{+/-}$ water-exposed mice, same interval (Bonferroni *post hoc* test). (**b**) Conditioned taste aversion (CTA). Mice were exposed to saccharin solution (0.5%) followed, 1 h later, by a malaise-inducing injection of LiCl (0.14 M, 2% body weight, intraperitoneally). A lack of CTA was shown in *SNAP-25*$^{+/-}$ mice (genotype as a between-subject factor: $F(1,36) = 73.38$; $P < 0.0001$; treatment as a within-subject factor: $F(1,36) = 4.82$, $P = 0.03$; genotype × treatment: $F(1,36) = 27.84$; $P < 0.0001$, two-way repeated measure ANOVA). $^{***}P < 0.001$ compared with SNAP-25$^{+/+}$ water-exposed mice; $^{\&}P < 0.05$ compared with *SNAP*$^{+/-}$ water-exposed mice (Bonferroni *post hoc* test). (**c**–**d**) Difference score (mean ± s.e.m.) in terms of time difference spent in the chamber associated with the never-seen-before mouse and empty cage or familiar mouse (preference for social novelty test). There was a difference among groups for sociability: (genotype as a between-subject factor: $F(1,36) = 42.94$; $P < 0.0001$; treatment as a within-subject factor: $F(1,36) = 33.93$; $P < 0.0001$; genotype × treatment: $F(1,36) = 16.88$; $P = 0.0002$, two-way ANOVA for repeated measures of variance). There was a difference among groups for social novelty (genotype as a between-subject factor: $F(1,36) = 188.8$; $P < 0.0001$; treatment as a within-subject factor: $F(1,36) = 32.09$; $P < 0.0001$; genotype × treatment: $F(1,36) = 6.62$; $P = 0.001$, two-way repeated measure of variance). $^{***}P < 0.001$ compared with water-exposed *SNAP-25*$^{+/+}$ mice; $^{°°}P < 0.01$, $^{°°°}P < 0.001$ compared with *SNAP*$^{+/-}$ water-exposed mice (Bonferroni *post hoc* test). (**e**) Object recognition task. Discrimination index (mean ± s.e.m.), evaluated 120 min after mice were presented with a familiar and a new object, was different among groups (two-way ANOVA: genotype as a between-subject factor: $F(1,36) = 0.50$, $P = 0.48$; treatment as a within-subject factor: $F(1,36) = 5.16$; $P = 0.02$; genotype × treatment: $F(1,36) = 100.9$, $P = 0.0001$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with water-exposed *SNAP-25*$^{+/+}$ mice; $^{°°°}P < 0.001$ vs *SNAP-25*$^{+/-}$ water exposed (Bonferroni *post hoc* test). (**f**) SNAP-25 expression in the hippocampus and prefrontal cortex of 6-week-old mice pre-exposed to water or VLP for 21 days. Western blotting analysis and relative quantitation, carried out in *SNAP-25*$^{+/-}$ water-exposed mice, showed reduced SNAP-25 expression, significantly only in hippocampus, and a recovery of SNAP-25 expression after VLP treatment (genotype as a between-subject factor: $F(1,17) = 9.66$, $P = 0.006$; treatment as a within-subject factor $F(1,17) = 2.56$; $P = 0.12$; genotype × treatment $F(1,17) = 7.53$; $P = 0.01$). $n = 5/6$ for each group (genotype as a between-subject factor: $F(1,16) = 7.01$, $P = 0.001$; treatment as a within-subject factor $F(1,16) = 0.88$; $P = 0.36$; genotype × treatment $F(1,16) = 0.88$; $P = 0.35$). $n = 5/6$ for each group. No significant changes in prefrontal cortex were shown $^{**}P < 0.001$ compared with *SNAP-25*$^{+/+}$ water-exposed mice; $^{\&}P < 0.05$ vs *SNAP-25*$^{+/-}$ water exposed (Bonferroni test).

significant decrease in saccharin intake in the *SNAP-25*$^{+/-}$ mice compared with the water-exposed controls without affecting the intake of sweet solution in wild-type animals, suggesting a recovery in CTA. Mutant and control mice drank comparable amounts of fluid during the conditioning sessions (not shown), excluding a general alteration of fluid consumption or taste.

When tested for sociability, *SNAP-25*$^{+/+}$ water-exposed mice appeared normal, spending longer time to explore the compartment with the stranger mouse than the empty cage. Conversely, SNAP-25$^{+/-}$ mice, pre-exposed to water, spent more time in the empty compartment (Figure 2c). When tested for social recognition (Figure 2d), *SNAP-25*$^{+/-}$ mice remained close to the familiar stranger for the same time, suggesting an impaired social recognition. Both genotypes spent equal time in the central compartment. Pre-treatment with chronic VLP significantly rescued both sociability and social novelty deficits.

SNAP-25$^{+/-}$ adolescent mice were impaired in episodic memory, as indicated by the altered discrimination index in the novel object recognition test (Figure 2e). *SNAP-25*$^{+/-}$ mice exposed to water exhibited a reduced discrimination index compared with their littermates. VLP exposure slightly, but not significantly, decreased *SNAP-25*$^{+/+}$ performance, whereas it was able to significantly recover *SNAP-25*$^{+/-}$ mice deficit.

Western blotting analysis of SNAP-25 expression in the hippocampi and prefrontal cortex of 6–7-week-old mice, water exposed, revealed a reduction of about 30 and 20% expression in SNAP-25$^{+/-}$ tissue relative to wild type, respectively (Figure 3f, right), in line with previously reported findings evaluated in the cortex.[21] Chronic treatment with VLP completely rescued, only in the hippocampi, expression in SNAP-25$^{+/-}$. These findings open the possibility that the behavioural rescue observed in *SNAP-25*$^{+/-}$ mice upon VLP treatment might be at least partially associated with increased protein levels.

Experiment 2. Basal EEG activity is characterized by spike activity in adolescent *SNAP-25*$^{+/-}$ mice: VLP normalizes at least for 1 week the EEG recording.

In basal conditions, adolescent *SNAP-25*$^{+/-}$ mice displayed EEG abnormalities in terms of spike activity during 24-h recording (Figure 3a), as previously shown also in the adult heterozygous mice.[21] Two representative traces (Figure 3b) of one *SNAP-25*$^{+/+}$ (left) and one *SNAP-25*$^{+/-}$ mouse (right) are reported. In basal conditions SNAP-25$^{+/+}$ tracing appeared normal, whereas that of *SNAP-25*$^{+/-}$ mouse was characterized by an abnormal and recurrent increase of amplitude. Twenty-four hours after chronic 21-day VLP exposure, heterozygous tracing was normalized. VLP pre-exposure had no effects on the wild-type EEG profile. The quantitative analysis of the mean number of spikes, evaluated for 2 h and in the following 3 weeks after VLP withdrawal in both genotypes (Figure 3c, left), revealed a reduction of spikes in *SNAP-25*$^{+/-}$ mice during the first week, which progressively returned to basal value within the third week. Accordingly, a 15% decrease of SNAP-25 expression in heterozygous hippocampi was shown after VLP withdrawal.

DISCUSSION

The goal of the present study was to obtain supportive evidence for association between *SNAP-25* gene and ASD-specific scores, using multiple strategies. First, we found that among five SNPs in the *SNAP-25* gene, the SNPs rs363050 showed significant relation with altered cognitive scores in ASD children. Our findings agree with others,[39] where an association between rs363050 putative risk allele with intellectual disability traits was suggested. Different *SNAP-25* gene polymorphisms have been suggested to associate with related traits of autism[17] and ADHD,[40] working memory ability[41] including short-/long-term memory and visual attention.[16] We cannot exclude a linkage disequilibrium effect or an ethnic effect, which may explain the involvement of different SNPs in the same *SNAP-25* genetic locus. For this reason replication studies using larger case studies are warranted.

In this study, we performed a more in depth analysis of the possible functional role of rs363050 SNP to better understand its involvement in SNAP-25 expression. The analysis of transcriptional activity revealed that SNP rs363050 spans a region containing a regulatory element whose function is dependent on its position; furthermore, the presence of the minor allele rs363050(G) influences the transcription of the *SNAP-25* gene. This could be

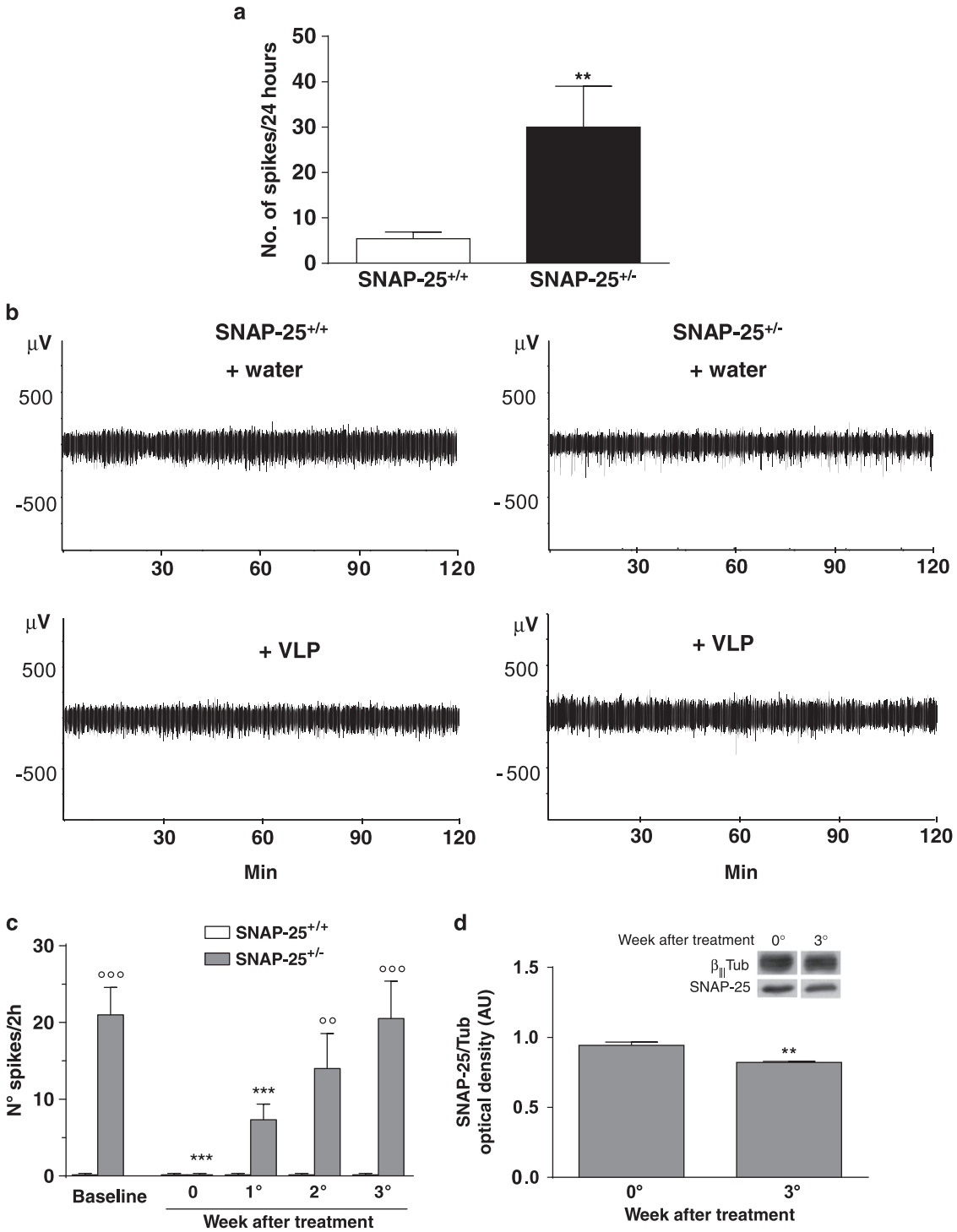


Figure 3. Synaptosomal-associated protein of 25 kDa (SNAP-25$^{+/-}$) mice showed abnormal electroencephalogram (EEG), which was rescued by VLP at least for 1 week. (**a**) Spike activity (mean ± s.e.m.) in basal conditions evaluated every hour during the 24-h recording. $^{**}P < 0.01$ compared with SNAP-25$^{+/+}$ mice. (**b**) Effect of exposure to sodium valproate salt (VLP) or water for 21 days evaluated in two representative mice for 2 h, evaluated 24 h after the end of the exposure. Two-way analysis of variance (ANOVA) showed differences among the groups (genotype as a between-subject factor: $F(1,40) = 62,51$, $P < 0.0001$; time as a within-subject factor: $F(4,40) = 6,415$; $P < 0.001$; genotype × time $F(4,40) = 6.39$; $P < 0.001$). (**c**) Quantitative analysis of mean (± s.e.m.) number of spikes evaluated every week for 3 weeks after exposure cessation. $^{***}P < 0.001$ vs baseline *SNAP-25*$^{+/-}$ mice. $^{\circ\circ}P < 0.05$, $^{\circ\circ\circ}P < 0.001$ vs corresponding SNAP-25$^{+/+}$ mice (Bonferroni *post hoc* test). (**d**) Western blotting analysis and relative quantification of SNAP-25 levels in SNAP-25$^{+/-}$ hippocampi evaluated immediately and 3 weeks after VLP withdrawal. $N = 5$ for each genotype. $^{**}P < 0{,}0038$ (Student's *t*-test).

due to the impairment of binding of factors involved in the modulation of the *SNAP-25* gene expression level or in the binding of other factors, different from the ones that recognize the sequence of the parental allele, acting as a repressor. Due to the importance that the presence of the minor allele can have on SNAP-25 expression levels, experiments aimed to identify and characterize this factor will be further exploited. SNAP-25 has a central role in synaptic transmission and plasticity.[4,40,42,43] The protein forms a complex with syntaxin and the synaptic vesicle proteins (synaptobrevin and synaptotagmin), which is required for the Ca++-mediated exocytosis of the neurotransmitter into the synaptic cleft. The protein is also involved in the processes of axon growth[44] and dendritic spine morphogenesis.[45] Changes in the levels of expression of the SNAP-25 protein may influence the functioning of synaptic circuits associated with cognitive functions.

Our findings on adolescent *SNAP-25*$^{+/-}$ mice strongly corroborate the association between *SNAP-25* and cognitive function. A significant impairment in different forms of memory was found when SNAP-25 protein expression was reduced. Memory deficits were observed in three different forms of memory: episodic, aversive and social. About the latter, social recognition disability was accompanied by a decrease of social interaction, suggesting an autistic-like trait.

ASD are often accompanied by co-morbidity including hyperactivity (40%) and seizures (30%), which are clinical features shared with ADHD.[46] A significant correlation between *SNAP-25* gene polymorphism and hyperactivity in a cohort of children with a diagnosis of ASD has been previously found.[17] Interestingly, the behavioural profile of adolescent *SNAP-25*$^{+/-}$ mice confirmed hyperactivity, as previously shown in mice of the same age,[21] and we believe revealed for the first time abnormal EEG characterized by frequent spikes of high amplitude, already in very young mice. Notably, comparing the behavioural deficit previously observed in adults,[21] adolescent *SNAP-25*$^{+/-}$ mice showed a similar cognitive deficit but a greater impairment of social behaviour and social recognition. In contrast, EEG abnormality was less pronounced in adolescent *SNAP-25*$^{+/-}$ mice. These findings are in line with what happens in autistic patients. Findings on intellectual functioning outcome in autistic adulthood revealed that intelligence quotient scores tend to remain stable overtime in the majority of studies.[47] More difficult is the comparison between mice and patients about social behaviour: large individual differences were found, although autism-related symptoms and behaviour generally improve with age.[47] Epilepsy increases with age[48,49] and the cumulative risk of epilepsy in adults with autism is estimated at 20–35%.[50] The abnormal EEG activity could be a factor contributing to learning (see Corradini *et al.*[21] for discussion) and is indeed well established that epileptiform EEG discharges unaccompanied by overt clinical change may be associated with transitory cognitive impairment detectable by appropriate psychological testing;[51] also, a decline in intelligence quotient score in patients affected by epilepsy has been reported.[52]

Repeated exposure to VLP, largely normalized, as expected, the altered EEG profile but, surprisingly, it exerted positive effects on all behavioural deficits, including hyperactivity. VLP is known to have a broad spectrum of actions: it reduces neuronal excitability regulating the glutamate/GABA neurotransmitters system and modulating the synaptic/inhibitory balance. This modulation has been recently linked to its indirect action through astrocytes.[53] In addition, VLP is an histone deacetylase inhibitor and also induces brain-derived neurotrophic factor and glial cell-line-derived neurotrophic factor NF transcription/expression in astrocytes to provide a consequent neuroprotective effect *in vitro*.[54]

We observed detrimental effects induced by prolonged exposure to VLP in SNAP-25$^{+/+}$ mice on object recognition and slightly on CTA. VLP can interfere with learning and memory processes both in experimental models[55,56] and patients.[57,58] Indeed, it has been reported to alter spatial learning in immature rats via the activation of protein kinase Cγ in hippocampal neurons.[54] Altered dendritic morphology and impaired social transmission of food preference in epileptic mutant mice,[56] after 3 months of exposure to oral VLP (100 mg per 100 ml), have also been found. On the other hand, VLP reduced seizures, mortality and rescued a normal hippocampal long term potentiation in the same mice, in which severe seizures occurred in the early period of their life.[56]

Interestingly, in our experiments, VLP was able to rescue the decreased SNAP-25 expression into the hippocampus, suggesting the possibility of a direct effect on the protein expression levels. The mechanism by which VLP acts on SNAP-25 expression remains unclear. Hippocampus is an area strongly involved in different forms of memory. Future studies will be needed to evaluate the SNAP-25 level expression in other areas involved in motor function and sociability, such as striatum and amygdala.

In conclusion, our current data, together with a previous study carried out in adult *SNAP-25*$^{+/-}$ mice,[21] suggest that reduced levels of SNAP-25 protein expression are responsible for behavioural and EEG alterations in adolescent mice. Given rs363050(G), which associates with altered cognitive scores in ASD children, leads to reduced protein expression, one could support our previous hypothesis that the reduction of SNAP-25 expression could be involved in the cognitive and neuropsychological deficits in children affected by ASD.[59]

Notably, beside the autistic symptoms, *SNAP-25*$^{+/-}$ mice show a broad spectrum of deficits that mimic the well-known neuropsychiatric or neurologic disorders, such as ADHD, schizophrenia and epilepsy. In this context, VLP appears to gain significant attention for its potential therapeutic use. The potential mechanism through which alterations in SNAP-25 may have a direct role in the aetiology as well as contribute to the pathology of these disorders requires future studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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