# Epileptiform Activity and Cognitive Deficits in SNAP-25$^{+/-}$ Mice are Normalized by Antiepileptic Drugs

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**Synaptosomal-associated protein of 25 kDa (SNAP-25) is a protein that participates in the regulation of synaptic vesicle exocytosis through the formation of the soluble NSF attachment protein receptor complex and modulates voltage-gated calcium channels activity. The *Snap25* gene has been associated with schizophrenia, attention deficit hyperactivity disorder, and bipolar disorder, and lower levels of SNAP-25 have been described in patients with schizophrenia. We used SNAP-25 heterozygous (SNAP-25$^{+/-}$) mice to investigate at which extent the reduction of the protein levels affects neuronal network function and mouse behavior. As interactions of genotype with the specific laboratory conditions may impact behavioral results, the study was performed through a multilaboratory study in which behavioral tests were replicated in at least 2 of 3 distinct European laboratories. Reductions of SNAP-25 levels were associated with a moderate hyperactivity, which disappeared in the adult animals, and with impaired associative learning and memory. Electroencephalographic recordings revealed the occurrence of frequent spikes, suggesting a diffuse network hyperexcitability. Consistently, SNAP-25$^{+/-}$ mice displayed higher susceptibility to kainate-induced seizures, paralleled by degeneration of hilar neurons. Notably, both EEG profile and cognitive defects were improved by antiepileptic drugs. These results indicate that reduction of SNAP-25 expression is associated to generation of epileptiform discharges and cognitive dysfunctions, which can be effectively treated by antiepileptic drugs.**

**Keywords:** epilepsy, memory, SNAP-25, valproate

## Introduction

Synapses are fundamental brain structures that mediate information transfer between neurons. Synaptic dysfunctions contribute to a large number of psychiatric diseases, including schizophrenia, autism, and intellectual disability, which are therefore called "synaptopathies" (Grant 2012). Synaptosomal-associated protein of 25 kDa (SNAP-25) is a soluble NSF attachment protein receptor protein, tethered to the plasma membrane via several cysteine-linked palmytoil chains, that participates in synaptic vesicle exocytosis through the formation of a complex with syntaxin and with the synaptic vesicle protein synaptobrevin/VAMP (Jahn and Scheller 2006; Südhof and Rothman 2009). SNAP-25 also interacts with and modulates the activity of various voltage-gated ion channels (VGCC) (Atlas 2001; Zamponi 2003; Catterall and Few 2008; Condliffe et al. 2010).

SNAP-25 is involved in different psychiatric disorders. Case–control- or family-based studies indicated that the *Snap25* gene is associated with attention deficit hyperactivity disorder (ADHD) (Barr et al. 2000; Kustanovich et al. 2003; Mill et al. 2004; Faraone et al. 2005; Feng et al. 2005). Accordingly, *Snap25* intronic single nucleotide polymorphisms (SNPs) have been linked to inattentive hyperactivity in a group of ADHD children (Zhang et al. 2010), and associated with hyperactivity in autism spectrum disorders (Guerini et al. 2011). Genome-wide linkage scan analysis for schizophrenia susceptibility genes suggested the chromosomal region 20p12.3-11, containing *Snap25*, as a candidate region for the disease (Lewis et al. 2003). Also, SNAP-25 levels are lower in the hippocampus (Young et al. 1998; Thompson et al. 2003) and in the frontal lobe (Thompson et al. 1998) of patients with schizophrenia. Finally, modifications of SNAP-25 levels occur in the brain of bipolar patients (Fatemi et al. 2001; Scarr et al. 2006), while one SNP variant in the promoter region, associated with higher SNAP-25 expression in prefrontal cortex, was linked with early onset of bipolar disorder (Etain et al. 2010).

The demonstration that SNAP-25 levels are altered in psychiatric diseases suggests that variations in the protein expression may have a pathogenic effect, possibly affecting synaptic function and network activity, and resulting in phenotype alterations. Indeed, homozygous mutant mice in which Ser187 of SNAP-25 is substituted with Ala display an altered emotional behavior (Kataoka et al. 2011), while replacement of the mature SNAP-25 b isoform with the SNAP-25 a isoform, which is present in early development, results in developmental defects, spontaneous seizures, and impaired short-term synaptic plasticity (Johansson et al. 2008).

So far, the only evidence that reduction of SNAP-25 expression may directly impact the behavioral phenotype derives from the analysis of the *coloboma* mouse, which is characterized by a hemizygous 2-centimorgan deletion of a segment on chromosome 2q, including the gene region encoding SNAP-25 (Hess et al. 1995). The *coloboma* mice, largely used as a model for ADHD (Wilson 2000; reviewed in Faraone et al. 2005; Russell 2007), display a hyperactive phenotype, which is reduced by the expression of the SNAP-25

transgene (Hess et al. 1996). However, *coloboma* mice may not be suited for investigating the neurophysiological and behavioral phenotype induced by reduction of SNAP-25 levels, because the 37 genes that are present in the deleted region on chromosome 2, including genes for phospholipase C beta-1 (Plcb1), *coloboma* (cm), Plcb4, and Jag1, may contribute to the mice phenotype (Gunn et al. 2011).

While SNAP-25 homozygous mutant mice die at birth from respiratory failure, heterozygous (SNAP-25$^{+/-}$) mice are viable (Washbourne et al. 2001). We used therefore SNAP-25$^{+/-}$ mice to investigate at which extent selective reduction of the protein levels, as occurring in psychiatric diseases, affects neuronal network function and mouse behavior. The large majority of behavioral tests were carried out in at least 2 of 3 European laboratories and generated reproducible results. We found that SNAP-25$^{+/-}$ mice display hyperactivity and show an abnormal EEG profile associated with cognitive defects, both normalized by treatment with valproate (VLP).

## Materials and Methods

### *Animals*

Male SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ C57BL/6 mice, originally from M.C. Wilson (University of New Mexico Health Sciences Center, Albuquerque, NM, USA), were provided by J. Sorensen (MPI, Goettingen). Mice were maintained and repeatedly backcrossed on C57BL/6 background for more than 10 generations.

Zero- to six-month-old age-matched littermate mice were used. 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. Mice 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 7:00 AM). Genotyping was performed by PCR as described (Washbourne et al. 2001).

### *Westen Blot Analysis*

Homogenates from cortices of E18, P7, P14, P30, and adult (3 months old) mice were analyzed by western blotting using anti-SNAP-25, 1:1000 (Chemicon, Temecula, CA., USA), anti-Calbindin (CB), 1:500 (Swant, Bellinzona, Switzerland), anti-beta-III-Tubulin 1:4000 (Promega Corporation, Madison, USA), anti-alpha-Tubulin 1:2000 (Sigma-Aldrich, St. Louis, MO), anti-vGlut1 1:2000 (Synaptic System, Gottingen, Germany). Antibodies against SNAP-47, SNAP-29, and SNAP-23 were a gift of R. Jahn (MPI, Gottingen). Immunoreactive bands were detected using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Rockford, IL), scanned with a Bio-rad GS-800™ calibrated densitometer, and analyzed with Image J software. Beta-III tubulin or alpha-tubulin was used as loading controls. For each developmental stage, SNAP-25/beta-III tubulin or SNAP-25/alpha tubulin optical densities were normalized to the average of controls.

### *Immunohistochemical Analysis*

Immunohistochemistry was performed on 5 SNAP-25$^{+/+}$ and 3 SNAP-25$^{+/-}$ mice at postnatal day (P) 2 and on 3 SNAP-25$^{+/+}$ and 3 SNAP-25$^{+/-}$ adult (P90) mice. The immunoperoxidase and immunofluorescence procedure was performed on free-floating sections (Moroni et al. 2008) using the following primary antibodies: anti-CB, 1:5000, anti-nonphosphorylated neurofilaments, 1:1000 (SMI311; Sternberger Monoclonals Incorporated, Lutherville, USA), anti-calretinin (CR), 1:3000, anti-neuropeptide Y (NPY; Peninsula Bachem, Bubendorf, Switzerland), anti-doublecortin, 1:800 (DCX; Cell Signaling Technology, Danvers, MA, USA), anti-cholecystokinin, 1:100 (CCK-8; Neomarkers, Fremont, CA, USA), anti-vGlut1, 1:1500 (Synaptic System, Gottingen), anti-vGlut2, 1:1000 (Synaptic System, Gottingen), and anti-vGat, 1:1000 (Synaptic System, Gottingen). For cytoarchitectonic analysis, selected sections were stained with thionin (0.1% in distilled water).

### *qRT-PCR Analysis*

Brain tissues from P7, P14, P30, and adult mice were used for real-time PCR analysis. Sample was homogenized prior to RNA extraction in 800 µL of Trizol. Total RNA was isolated using the NucleoSpin miRNA (Macherey-Nagel GmbH &Co., Düren, Germany) isolation kit according to the manufacturer's protocol. The RNA was eluted with 30-µL Rnase-free water. All RNA was quantified by spectrophotometer and optical density 260/280 nm ratios were determined. Reverse transcription was performed on 2-µg RNA using Superscript III First-Strand Synthesis System and random hexamer primers (Life Technologies, Carlsbad CA, USA). Real-time polymerase chain reaction (qRT-PCR) was performed using 7900 HT fast-real-time PCR system instrument (Life Technologies, Carlsbad CA, USA). The amplification was carried out in a total reaction volume of 11 µL, using the TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad CA, USA). Predeveloped TaqMan Assay Reagent (FAM-MGB) for SNAP25 and for GAPDH were purchased from PE Applied Biosystems. Each gene was analyzed in triplicate. Data analysis was performed with the ΔΔCt method. All RNA levels were normalized to Gapdh.

### *Spontaneous Motor Activity and Amphetamine Response*

Spontaneous motor activity was carried out as described in Supplementary Materials and Methods. Before the start of the test, SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (7–9 weeks of age) were habituated to the testing room for at least 1 h. Cumulative horizontal and vertical movement counts were recorded for 4 h before and 3 h after treatment., animals were treated subcutaneous (s.c.) with saline or amphetamine sulfate (4 mg/kg) dissolved in 0.9% NaCl. Activity measures began immediately after injection and lasted 3 h, according to Hess et al. (1996).

### *Electroencephalogram*

After surgery (for details see Supplementary Materials and Methods), Electroencephalogram (EEG) activity was recorded, in a Faraday chamber, using a Power-Lab digital acquisition system (AD Instruments, Bella Vista, Australia; sampling rate 100 Hz) in freely moving SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice ($n = 10$ mice per genotype). EEG traces were analyzed as described (Manfredi et al. 2009) for spike activity. Basal cerebral activity was recorded continuously for 24 h in freely moving mice. For each 24-h EEG recording, the mean number of spikes was evaluated in both genotypes. After the recordings, the EEG and video (through a video camera put inside the Faraday chamber) were analyzed for the incidence/duration of spontaneous cortical spike activity and the percentage of animals displaying spike activity, as previously described (Zhang et al. 2004; Manfredi et al. 2009).

### *Kainate-Induced Seizures*

SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice ($n = 10$ mice per genotype) received intraperitoneal (i.p.) kainic acid (KA, Sigma Aldrich, St. Louis, MO) dissolved in saline at 35-mg/kg body weight. Saline-injected animals of both genotypes were used as controls. Seizure severity was determined as described (Bozzi et al. 2000). The rating scale value was scored every 20 min for a maximum of 3 h, and data were used to calculate the time-course of seizure severity for each genotype (Schauwecker and Steward 1997; Tripathi et al. 2008). At the end of behavioral observation (3 h after KA), animals were returned to their home cages; animals were killed at 14 days after KA for histopathological analyses.

### *Quantification of NPY Staining*

The quantification method was adapted from that described in Antonucci et al., 2008. Four-eight NPY-stained sections through the dorsal hippocampus were analyzed in each KA-treated mouse (wild type [wt], $n = 5$; heterozygous [het], $n = 5$). Images of CA3 stratum radiatum

and of the overlying corpus callosum in each hemisphere were digitized (Zeiss Axiovision). Light intensity and microscope settings were optimized initially and then held constant. Care was taken to avoid saturation at either end of the pixel intensity range (0–255). Mean signal intensity in the CA3 stratum radiatum was divided by the background labeling in each section (calculated in the callosum of each hemisphere). For each animal, an NPY staining score was obtained by averaging the values obtained in individual sections. The NPY staining score was then correlated to the maximum behavioral seizure score recorded for each mouse following KA treatment. Pearson correlation analysis was performed using SigmaPlot 11.0.

#### *Two-Bottle Preference Tests and Latent Inhibition*

Two-bottle preference and latent inhibition test in a conditioned taste aversion (CTA) paradigm were performed as previously described (Bruno et al. 2007). For details, see Supplementary Materials and Methods.

#### *Conditioned Taste Aversion*

SNAP-25$^{+/+}$ ($n = 10$) and SNAP-25$^{+/-}$ ($n = 12$) mice were individually housed during the CTA test. After mice were adapted to a restricted drinking schedule (20 min/day for 4 days), they were exposed to a saccharin solution (0.5%) followed 1 h later by a malaise-inducing injection of LiCl (0.14 M, 2% body weight, i.p.). Beginning 48 h after conditioning, mice could freely choose to drink either saccharin solution or tap water during 3 daily choice tests (ct1–ct3). The amount of saccharin intake expressed as the percentage of total fluid consumed ([saccharin/saccharin + water] × 100) was taken as an aversion index.

#### *Object Recognition*

Ten SNAP-25$^{+/+}$ and 11 SNAP-25$^{+/-}$ mice were used. The novel object recognition task was performed as described in Supplementary Materials and Methods.

#### *Sociability and Preference for Social Novelty Test*

Ten SNAP-25$^{+/+}$ and 12 SNAP-25$^{+/-}$ mice were used. The sociability and preference for social novelty test was performed in a 3-chamber transparent polycarbonate box as described in Supplementary Materials and Methods.

#### *Pharmacological Treatment*

One week after basal EEG, animals were recorded 1 h before and for 2 h immediately after drug i.p. treatment: VLP sodium salt (250 mg/kg), ethosuximide (ETO; 200 mg/kg), carbamazepine (CBZ; 50 mg/kg), and nimodipine (NIMO; 10 mg/kg). VLP was given immediately before HCl exposure in the CTA test, 20 min before T1 in the object recognition test, and 20 min before sociability and social novelty test. All drugs were dissolved in saline, NIMO in 10% ethanol, and saline and CBZ in 1% Tween 80. The doses of ETO, VLP, and NIMO were chosen for their ability to suppress differently induced seizures in mice (Larkin et al. 1992; DeLorey et al. 1998; Liljelund et al. 2005; Shitak et al. 2006; Marrosu et al. 2007; Chung et al. 2009). All the drugs were given i.p. in a volume of 0.1 mL/10 g. Fresh drug solutions were prepared daily. Drugs were purchased from Sigma-Aldrich (St. Louis, MO).

#### *Data Analysis*

One-way ANOVA with repeated measures, 1-way factorial ANOVA design with genotype (SNAP-25$^{+/+}$, SNAP-25$^{+/-}$) or 2-way ANOVA as between subject factor were used. Post hoc analysis was done using Tukey's Bonferroni's or Holm Sidak's post hoc tests. Pairwise comparisons between genotypes or treatments were assessed with Student's *t*-test or Fisher exact probability tests. Correlation between the maximum behavioral seizure score and NPY was performed using SigmaPlot 11.0 and by Pearson correlation analysis. The significance threshold was set at $P < 0.05$. All statistical analyses were done with software Prism, version 5 (GraphPad, San Diego, CA).

## Results

### *Developmental Expression of SNAP-25 in Heterozygous Mice Brain*

Western blotting analysis of wild-type and heterozygous mice cortices showed that SNAP-25 and syntaxin progressively increase during brain development. To validate SNAP-25 levels and to minimize development-related artifacts possibly leading to erroneous data interpretation, both beta-III-Tubulin and alpha-Tubulin were used as a loading marker for SNAP-25 quantitation. Notably, SNAP-25 levels in heterozygous mice tended to progressively increase relatively to SNAP-25$^{+/+}$ mice, indicating a partial compensation of protein expression during postnatal development ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, *t*-test) (Fig. 1*A*,*C*). A comparable trend was observed when SNAP-25 levels were normalized to alpha-tubulin ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, *t*-test) (Fig. 1*D*).

The protein increase was not accompanied by a parallel increase in SNAP-25 mRNA levels, as assessed by qRT-PCR analysis (Fig. 1*B*). No significant difference in syntaxin (Fig. 1*E*) or GAP-43 (Fig. 1*F*) expression was found between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice during development. In the adult SNAP-25$^{+/-}$ brain, a reduced level of SNAP-25 was detected, in the absence of changes in SNAP-47, SNAP-29, SNAP-23, the calcium-binding protein CB, and the vesicular glutamate transporter vGlut1 (Fig. 1*G*).

### *Lack of Anatomical Alterations in SNAP-25$^{+/-}$ Brain*

We analyzed whether anatomical alterations occur in brains of SNAP-25$^{+/-}$ when compared with SNAP-25$^{+/+}$ mice. The main brain structures, cortex, hippocampus, and thalamus were comparable in SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ P2 mice, as indicated by thionin staining (Fig. 2*A*,*B*). Normal cortical plate and layers V and VI (Fig. 2*D*,*F*) and similar hippocampal CB expression (Fig. 2*C*,*E*) were observed. Consistently, no major differences in the main brain structures were detected between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ adult animals (Fig. 2*G*,*H*). The thickness of the cortices and the cortical lamination was comparable, as indicated by thionin staining (Fig. 2*I*,*J*) and immunostaining for SMI 311, which labels a subpopulation of pyramidal cells mainly located in layers II–III and V (Fig. 2*N*, *O*). The expression pattern of the calcium-binding proteins CB and CR, identifying the subfields of hippocampus (Fig. 2*L*, *M*,*R*,*S*) and of CCK and NPY (Fig. 2*P*,*Q*,*U*,*V*) was identical in SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice. Also, DCX staining in the subgranular zone, labeling migrating neuronal precursor cells that eventually integrate into hippocampal circuitry (Parent et al. 1997; van Praag et al. 2002), was comparable in amount and distribution (Fig. 2*W*,*X*). Also, the distribution of excitatory and inhibitory terminals in CA1 hippocampal region was not different between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (Fig. 2*K*,*T*). Finally, no major alterations were observed in the barrel cortex of SNAP-25$^{+/-}$ mice when compared with wild type, although further analysis may unveil slight segregation differences (Fig. 2*Y*,*Z*). A quantitation of vGAT and vGlut1-positive puncta, expressed as either a fraction of VAMP2-positive puncta or a reciprocal ratio, revealed no significant differences between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice, at

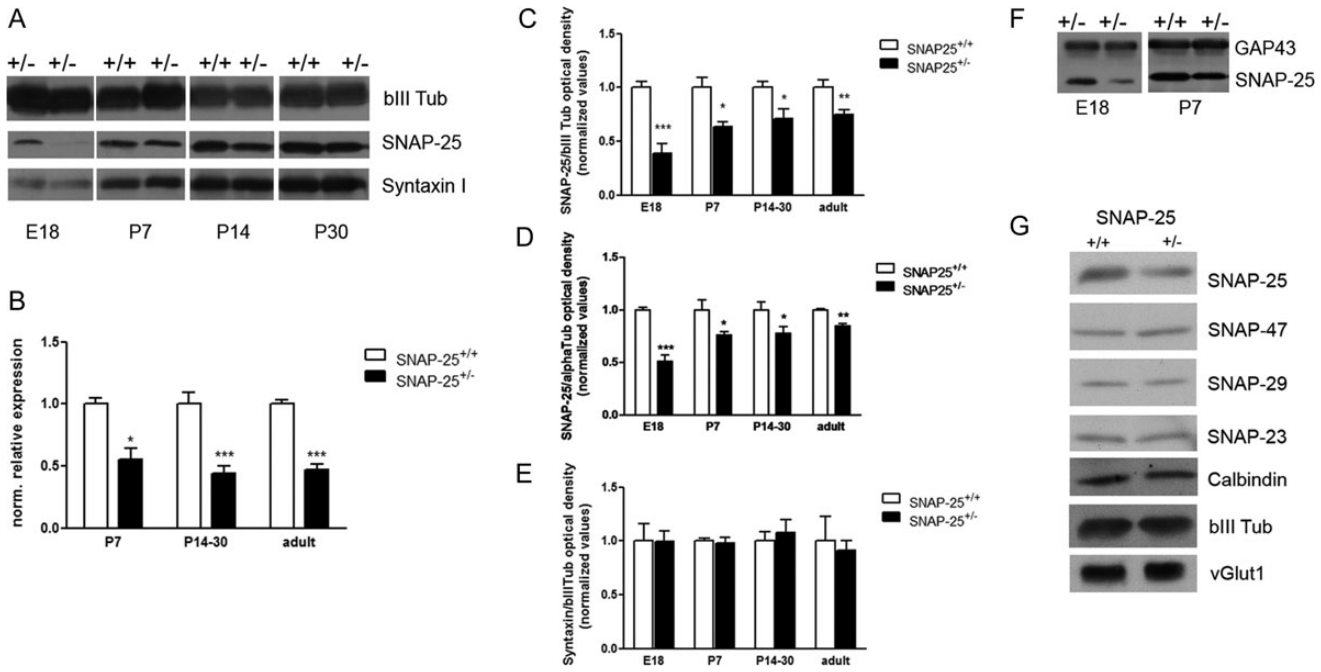


**Figure 1.** SNAP-25 levels in SNAP-25$^{+/-}$ cortices progressively increase during postnatal development. (*A*, *C*, and *D*) Western blotting analysis (*A*) and relative quantitation (*C* and *D*) of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ cortices from E18, P7, P14, P30 and adult (3 months old) mice reveals a progressively higher expression ratio in SNAP-25$^{+/-}$ mice during postnatal development (normalized SNAP-25 levels: (*C*) E18, SNAP-25$^{+/+}$ ($n = 6$) 1 ± 0.056, SNAP-25$^{+/-}$ ($n = 7$) 0.38 ± 0.089; P7, SNAP-25$^{+/+}$ ($n = 9$) 1 ± 0.091, SNAP-25$^{+/-}$ ($n = 5$) 0.63 ± 0.049; P14-30, SNAP-25$^{+/+}$ ($n = 8$) 1 ± 0.054, SNAP-25$^{+/-}$ ($n = 8$) 0.71 ± 0.092; adult, SNAP-25$^{+/+}$ ($n = 6$) 1 ± 0.068, SNAP-25$^{+/-}$ ($n = 7$) 0.75 ± 0.042. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, unpaired Student's *t*-test. (*D*) E18, SNAP-25$^{+/+}$ ($n = 5$) 1 ± 0.025, SNAP-25$^{+/-}$ ($n = 6$) 0.511 ± 0.061, $P < 0.0001$; P7, SNAP-25$^{+/+}$ ($n = 5$) 1 ± 0.095, SNAP-25$^{+/-}$ ($n = 5$) 0.763 ± 0.0312, $P < 0.05$; P14-30, SNAP-25$^{+/+}$ ($n = 9$) 1 ± 0.077, SNAP-25$^{+/-}$ ($n = 8$) 0.777 ± 0.065, $P < 0.05$; adult, SNAP-25$^{+/+}$ ($n = 3$) 1 ± 0.012, SNAP-25$^{+/-}$ ($n = 3$) 0.85 ± 0.022, $P < 0.01$. (*B*) RT-qPCR analysis reveals that SNAP-25 mRNA is about half in SNAP-25$^{+/-}$ mice at all developmental stages (normalized fold expression: P7, SNAP-25$^{+/+}$ ($n = 2$) 1 ± 0.044, SNAP-25$^{+/-}$ ($n = 2$) 0.551 ± 0.094; P14-30, SNAP-25$^{+/+}$ ($n = 4$) 1 ± 0.089, SNAP-25$^{+/-}$ ($n = 5$) 0.441 ± 0.056; adult, SNAP-25$^{+/+}$ ($n = 3$) 1 ± 0.034, SNAP-25$^{+/-}$ ($n = 3$) 0.470 ± 0.046. *$P < 0.05$; ***$P < 0.001$, unpaired Student's *t*-test). (*E*) Quantitation of syntaxin expression at different developmental stages shows no differences in the protein expression between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ animals (E18, SNAP-25$^{+/+}$ ($n = 2$) 1 ± 0.160, SNAP-25$^{+/-}$ ($n = 2$) 0.997 ± 0.095; P7, SNAP-25$^{+/+}$ ($n = 3$) 1 ± 0.023, SNAP-25$^{+/-}$ ($n = 3$) 0.978 ± 0.051; P14-30, SNAP-25$^{+/+}$ ($n = 6$) 1 ± 0.087, SNAP-25$^{+/-}$ ($n = 6$) 1.081 ± 0.115;adult, SNAP-25$^{+/+}$ ($n = 2$) 1 ± 0.227, SNAP-25$^{+/-}$ ($n = 2$) 0.909 ± 0.090) (*F*) Western blotting analysis of GAP43 expression in mice cortices at early developmental stages (E18, P7) reveals the absence of differences between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (E18, SNAP-25$^{+/+}$ ($n = 5$) 1 ± 0.099, SNAP-25$^{+/-}$ ($n = 5$) 1.183 ± 0.169; P7, SNAP-25$^{+/+}$ ($n = 4$) 1 ± 0.085, SNAP-25$^{+/-}$ ($n = 4$) 1.093 ± 0.102). (*G*) Western blotting analysis of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ adult cortices shows absence of major alterations in the expression of different brain markers.

least in the CA1 region of the hippocampus (vGAT/VAMP2 ratio: SNAP-25$^{+/+}$ 1 ± 0.080; SNAP-25$^{+/-}$ 0.972 ± 0.096; $P = 0.83$; vGlut1/VAMP2 ratio: SNAP-25$^{+/+}$ 1 ± 0.129; SNAP-25$^{+/-}$ 0.975 ± 0.137; $P = 0.89$; vGAT/vGlut1 ratio: SNAP-25$^{+/+}$ 1 ± 0.085; SNAP-25$^{+/-}$ 1.024 ± 0.122; $P = 0.88$). Also, no major difference was observed in vGlut2 distribution in the dentate gyrus of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (not shown).

### *SNAP-25$^{+/-}$ Mice, at the age of 7 Weeks, Show Motor Hyperactivity due to Lack of Habituation*

As a hyperactive phenotype has been described in *coloboma* mice, which is reduced by the expression of the SNAP-25 transgene (Hess et al. 1996), we monitored spontaneous motor activity in SNAP-25$^{+/-}$ mice at 7 weeks of age (Fig. 3) and in the adult (Supplementary Table S1). The time course of horizontal and vertical activity recorded every 10 min is given in Figure 3*A*,*B*. During the first 2-h recording, both genotypes showed a similar horizontal and vertical activity. However, during the following 2 h (120–240 min) SNAP-25$^{+/-}$ mice failed to habituate, thus resulting more active than wild-type littermates. S.c. injection of *d*-amphetamine (4 mg/kg) increased horizontal activity in SNAP-25$^{+/+}$ mice during the first hour after treatment (240–300 min), whereas in the following hour (300–360 min), the stimulant effect decreased (Fig. 3*A*). Conversely, *d*-amphetamine appears not to exert any effect on SNAP-25$^{+/-}$ mice in the first hour after treatment, whereas it significantly reduced motor activity in the following hour (Fig. 3*A*). However, it has to be noted that removal of animals from the cage to perform injection, induced an increase in motor activity. Indeed, a parallel group of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice, subjected to the same protocol, but treated with saline instead that amphetamine, showed an increase in motor activity of 250% and 680%, respectively. A recovery of motor function was obtained during the last period. Vertical activity (Fig. 3*B*) was reduced by treatment with *d*-amphetamine in both genotypes starting from the first hour and a partial recovery was reached at 360 min. The observed reduction of vertical movements was probably due to the intense horizontal activity. When the time course was statistically evaluated in terms of 1-h each blocks, significant differences were obtained for horizontal (Fig. 3*C*) and vertical activity (Fig. 3*D*) (see legend of Fig. 3*C*,*D*). A normal locomotor activity was found in SNAP-25$^{+/-}$ adult mice (Supplementary Table S1).

### *SNAP-25$^{+/-}$ Mice Display an Altered EEG Profile and are More Susceptible to Kainate-Induced Seizures*

As SNAP-25 controls neurotransmitter release and VGCC activity, we recorded the EEG profile of SNAP-25$^{+/-}$ mice. 24-h cortical EEG recordings on freely moving animals

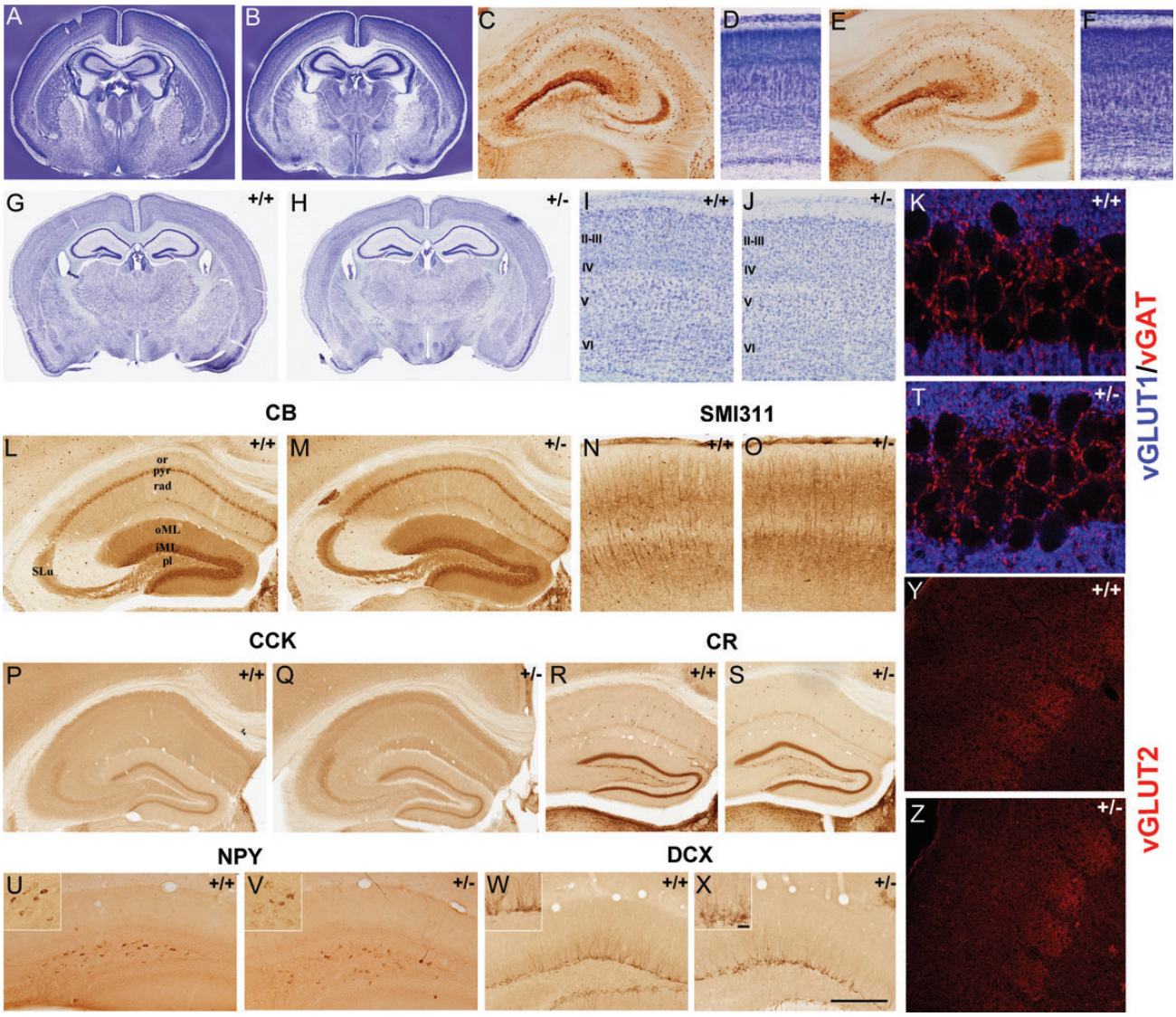


**Figure 2.** Cytoarchitectural analysis of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice brain. (*A–F*) Analysis of P2 brain. Photomicrographs of Thionin-stained coronal sections of brain from SNAP-25$^{+/+}$ (*A*) and SNAP-25$^{+/-}$ (*B*) mice show no difference in size and cytoarchitecture of the main brain structures. High magnification of developing cortices reveal comparable cortical lamination (*D* and *F*). CB expression in hippocampus shows the same pattern of distribution (*C* and *E*). (*G–Z*) Analysis of adult brain. Photomicrographs of Thionin-stained coronal sections of brain reveal comparable main brain structures, cortex, hippocampus and thalamus, in SNAP-25$^{+/+}$ (*G*) and SNAP-25$^{+/-}$ (*H*) mice. The thickness and layering of the cortices are not altered in SNAP-25$^{+/-}$ mice (*I* and *J*) as confirmed by immunostaining for the anti-nonphosphorylated neurofilaments SMI311 (*N* and *O*). Similar pattern of CB immunoreactivity is evident in SNAP-25$^{+/+}$ (*L*) and SNAP-25$^{+/-}$ (*M*): note CA1 and CA2 pyramidal layers, mossy fibers and dentate gyrus intensely stained. The labeling for CCK (*P* and *Q*) and CR (*R* and *S*), mainly localized in the molecular layer of dentate gyrus, is identical in SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice. High magnification images of dentate gyrus, show NPY (*U* and *V*) and DCX (*Y* and *X*) immunoreactivity in the polymorphic layer and in the subgranular zone respectively. No gross difference in synaptic excitatory (vGlut1, red) and inhibitory (vGAT, green) pattern is detectable in CA1 hippocampal regions (*K* and *T*). vGlut2 staining of the barrel cortex in SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (*Y* and *Z*). Scale bar = 3 mm for *A* and *B*; 300 $\mu$m for *C,E*; 400 $\mu$m for *D,F*; 2,4 mm for *G,H*; 330 $\mu$m for *I,J,N,O*; 20 $\mu$m for *K,T*; 450 $\mu$m for *L,M*; 510 $\mu$m for *P,Q*; 490 $\mu$m for *R,S*; 230 $\mu$m for *U-X*; 250 $\mu$m for *Y,Z*.

revealed that heterozygous mice displayed frequent spikes of high amplitude (Fig. 4*A–C*), which, however, did not lead to spontaneous seizures. In only one case (a het mouse displaying 365 spikes/24 h), we could observe occurrence of generalized seizures following handling. Abnormal EEG pattern was observed in all tested SNAP-25$^{+/-}$ mice. The percentage of SNAP-25$^{+/-}$ mice showing abnormal discharges was significantly larger than of SNAP-25$^{+/+}$ mice (Fig. 4*C*). Epileptiform discharges were also detected by EEG electrodes positioned at hippocampal level (not shown). Furthermore, SNAP-25$^{+/-}$ mice were more susceptible to seizures induced by kainate (KA). Figure 4*D* shows the time-course of the behavioral response of SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice to 35 mg/kg KA over a 3-h period after i.p. administration. In all mice, this dose of KA resulted within the first 10 min in immobility and staring, followed by head bobbing and isolated limbic motor (Stage 4) seizures, characterized by forelimb clonus and rearing. Overall, latency to the first Stage 4 seizure did not differ between SNAP-25$^{+/+}$ (18.5 ± 5.5 min) and SNAP-25$^{+/-}$ mice (18.2 ± 3.6 min; $P > 0.05$, unpaired *t*-test). However, while SNAP-25$^{+/+}$ animals only displayed isolated limbic motor seizures, SNAP-25$^{+/-}$ mice rapidly progressed to Stage

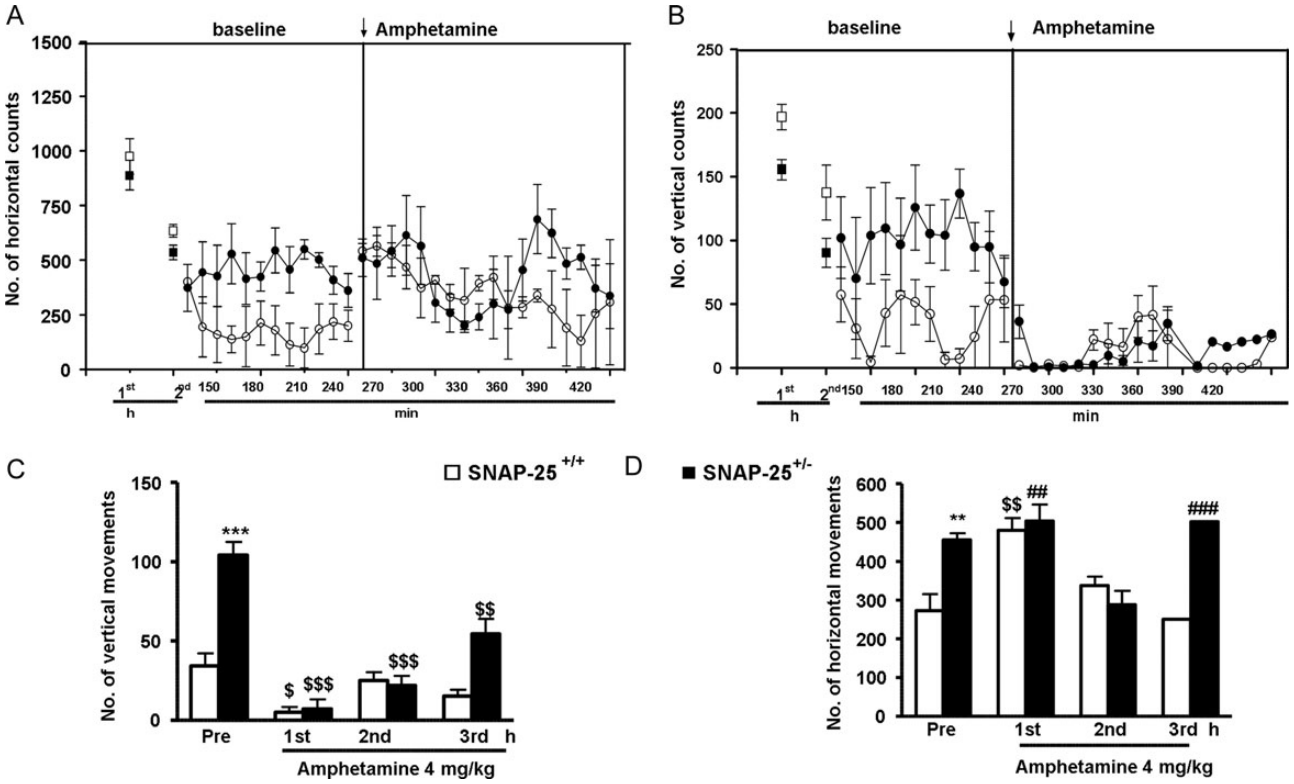


**Figure 3.** SNAP-25$^{+/-}$ mice display hyperactivity at 7 weeks of age. (*A* and *B*) Time-course of locomotor activity evaluated before (4 h of baseline) and after (3 h) amphetamine injection. After 2 h habituation, SNAP-25$^{+/-}$ mice exhibited an increased horizontal (*A*) and vertical (*B*) activity in comparison to SNAP-25$^{+/+}$ mice. Photocell beam interruptions were recorded every 10 min. During both the first and second hour, the number of counts, recorded every 10 min, were pooled and shown as mean ± standard error of means (SEM). (*C* and *D*) The time-course obtained in *A* and *B* was also evaluated as mean (±SEM) of horizontal and vertical activity counts in blocks of 1-h each period. Acute *d*-amphetamine treatment (4 mg/kg), given s.c. at 240 min (arrow), reduces the number of movements only in SNAP-25$^{+/-}$ mice at the 2 h and a complete recovery was observed at the 3 h. (*C*) Mean horizontal counts are higher in SNAP-25$^{+/-}$ mice before amphetamine administration (pre) (average of the number of counts during the 120–240 min period), whereas no significant change is observed after amphetamine injection. Conversely, in SNAP-25$^{+/+}$ mice, amphetamine increases horizontal activity during the first hour and returns to baseline within the 3 h. Genotype as between subject factor and treatment as within factor, genotype: $F_{1,56} = 113$, $P < 0.0001$; time : $F_{3,56} = 61.57$, $P < 0.0001$; genotype X time $F_{3,56} = 52.47$, $P < 0.0001$, 2-way repeated measure of variance, Bonferroni's post hoc analysis. SNAP-25$^{+/+}$ mice vs SNAP-25$^{+/-}$ mice, Pre: $^{**}P < 0.01$; SNAP-25$^{+/+}$ mice first hour vs pre: $^{\$\$}P < 0.01$; SNAP-25$^{+/-}$ mice 1 h and 3 h vs 2 h vs: $\#\#P < 0.001$, $\#\#\#P < 0.0001$. (*D*) The mean vertical activity counts evaluated over 1 h-period (mean ± SEM) are higher in SNAP-25$^{+/-}$ mice before amphetamine (pre), whereas a significant reduction occurs after amphetamine injection during the first and second hour. In SNAP-25$^{+/+}$ mice amphetamine decreases horizontal activity during the first hour only. Partial recovery was obtained at the 3 h in SNAP-25$^{+/-}$ while a complete recovery was found at the 3 h in Snap-25$^{+/+}$ mice. Genotype as between subject factor and treatment as within factor, genotype: $F_{1,56} = 32.06$, $P < 0.0001$; time : $F_{3,56} = 30.83$, $P < 0.0001$; genotype X time $F_{3,56} = 12.79$, $P < 0.0001$, 2-way repeated measure of variance, Bonferroni's post hoc analysis. SNAP-25$^{+/+}$ mice vs SNAP-25$^{+/-}$ mice, Pre: $^{***}P < 0.001$; SNAP-25$^{+/+}$ or SNAP-25$^{+/-}$ mice vs corresponding Pre: $^{\$}P < 0.01$,$^{\$\$\$}P < 0.01$, $^{\$\$\$}P < 0.01$. $n = 8$ per group.

5 (status epilepticus) and showed continuous generalized activity lasting for about 2 h. The average time of Stage 5 seizure onset in SNAP-25$^{+/-}$ mice was 45.7 ± 6.5 min. Mortality rate following KA administration was comparable between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (SNAP-25$^{+/+}$, 4/10; SNAP-25$^{+/-}$, 3/10). SNAP-25$^{+/-}$ mice had significantly higher behavioral scores than SNAP-25$^{+/+}$ mice starting from 40 min after KA (Fig. 4*D*). Thus, the progression of clinical signs following KA treatment was dramatically different in SNAP-25$^{+/+}$ and mutant animals. Saline-injected animals of both genotypes showed no behavioral seizures during the whole period of observation (not shown). NPY immunostaining revealed extensive loss of interneurons in SNAP-25$^{+/-}$ when compared with controls (Fig. 4*E*), indicating that the higher seizure scores of SNAP-25$^{+/-}$ mice are paralleled by loss of hilar neurons. Moreover, there was a robust upregulation of NPY immunoreactivity in the mossy fiber pathway of all SNAP-25$^{+/-}$ mice treated with KA (Fig. 4*E*). Indeed, the quantitative analysis indicated that NPY labeling in the CA3 stratum radiatum of KA-treated het mice was significantly upregulated with respect to KA-treated wt animals (*t*-test, $P = 0.009$; Fig. 4*F*). Examination of individual animals revealed a significant correlation between NPY intensity values and the maximum behavioral seizure score recorded after KA (Pearson correlation coefficient = 0.69, $P = 0.025$; Fig. 4*G*). In keeping with the well-known resistance of C57Bl/6 mouse strains to KA-induced pyramidal damage (Schauwecker and Steward 1997), CA1/CA3 cell loss was not found in Nissl-stained sections from KA-treated SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice (not shown).

### *SNAP-25$^{+/-}$ are Impaired in Nonspatial Associative Learning Tasks*

Before performing memory tasks SNAP-25 mice were initially checked for their general health, reflex, sensory abilities and emotional-like response (for methodological details see Supplementary Materials and Methods). As reported in Supplementary Table S1 and Supplementary Results,

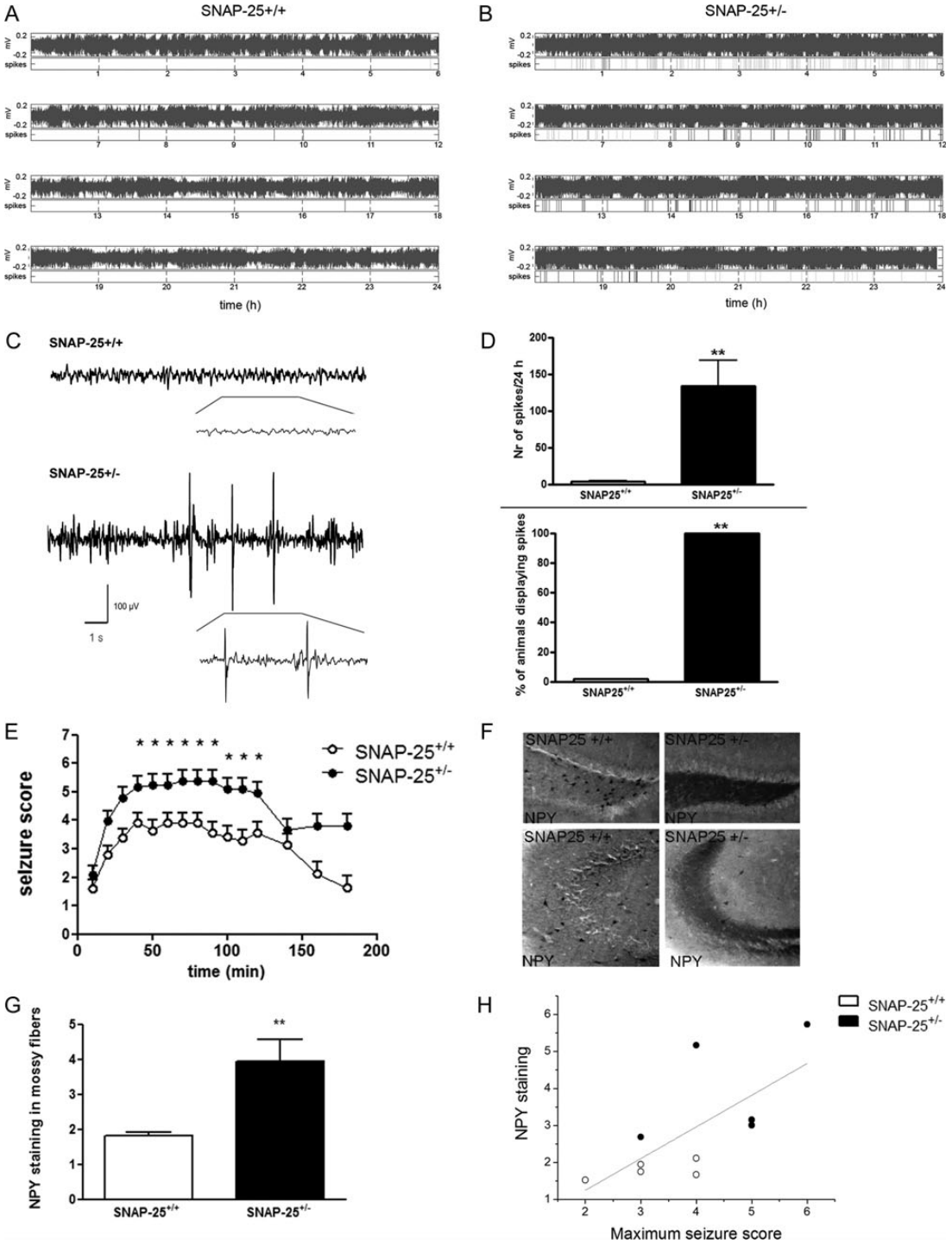


**Figure 4.** SNAP-25$^{+/-}$ mice display abnormal EEG profile and are more susceptible to kainate-induced convulsions. (*A*) 24-h representative EEG trace from a SNAP-25$^{+/+}$ (left) and a SNAP-25$^{+/-}$ (right) mouse. The SNAP-25$^{+/-}$ mouse shows increased spike frequency, as indicated by the quantitative evaluation in the bottom portion of the trace. For each EEG recording, the histogram of the maximum positive increments over overlapping 20 ms windows was derived. Increments above a threshold determined according to the increments distribution through an unsupervised approach (Manfredi et al. 2009) and whose amplitude was greater than twice the background were considered as spikes. (*B*) Magnification of representative EEG traces (30-s) from one SNAP-25$^{+/+}$ and one SNAP-25$^{+/-}$ mouse. (*C*) The average spike number (±SEM) recorded for 24 h is significantly higher in SNAP-25$^{+/-}$ versus SNAP-25$^{+/+}$ (**$P < 0.01$, *t*-test) (top) and the percentage of SNAP-25$^{+/-}$ mice showing abnormal discharges is greater (**$P < 0.01$, Fisher exact probability test) (bottom). (*D*) Increased susceptibility to KA-induced seizures in SNAP-25$^{+/-}$ mice. Progression of behavioral changes after systemic KA administration (35 mg/kg, i.p.) in SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice over a 3- h observation period. Genotype as between-subject factor and time within subject factor, genotype: $F_{1,18} = 6.39$, $P = 0.02$; effect of time: $F_{1,13} = 10.85$ $P < 0.001$; genotype x time interaction: $F_{1,30} = 0.71$, $P = 0.70$, 2-way repeated measure analysis of variance, $P < 0.05$, Holm Sidak post hoc test. Data represent mean seizure scores ± SEM. SNAP-25$^{+/+}$ mice versus SNAP-25$^{+/-}$ mice: *$P < 0.05$. (*E*) Upregulation of NPY following seizures in SNAP-25$^{+/-}$ mice. Coronal sections from the dorsal hippocampus of KA-treated animals (left, SNAP-25$^{+/+}$ mouse; right, SNAP-25$^{+/-}$ mouse). NPY staining in the dentate gyrus (top panels) and CA3 region (bottom panels): note loss of interneurons and strong upregulation of NPY in the mossy fiber pathway in SNAP-25$^{+/-}$ mice. Scale bar = 150 µm. (*F*) Quantification of NPY staining intensity in KA-treated mice. The histograms report average ± SEM of the NPY signal-to-background ratio (intensity of NPY label in CA3 stratum radiatum divided by the background staining) in SNAP-25$^{+/+}$ (open bars) and SNAP-25$^{+/-}$ animals (filled bars). There is a significant upregulation of NPY labeling in KA-treated het mice. **, $P < 0.01$, Student's *t*-test. (*G*) Scatter plot showing NPY staining values and maximum KA-induced seizure score for each individual mouse (wt, open circles; het, filled circles). There is a significant correlation between the 2 variables (Pearson correlation coefficient = 0.69, $P = 0.025$).

SNAP-25$^{+/-}$ mice displayed normal gross behavior and emotional-like reactivity (Supplementary Results and Fig. S1). SNAP-25$^{+/-}$ mice were not impaired in spatial learning and memory (Supplementary Materials and Methods, Results, and Fig. S2).

SNAP-25$^{+/-}$ and SNAP-25$^{+/+}$ mice were then tested for implicit associative learning (Fig. 5). There was a significant difference among different taste solutions intake in the 2-bottle preference test (Fig. 5*A*, top panel) in SNAP-25$^{+/-}$ mice. A strong preference for water over quinine and for saccharin over plain water, suggesting a normal taste sensitivity, was found. There was no significant difference in HCl (0.008 M) intake between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice. When mice were tested for the latent inhibition of CTA (Fig. 5*A*, middle panel), LiCl injection did not induce any CTA in SNAP-25$^{+/-}$ mice (41% less HCl consumed by non-pre-exposed SNAP-25$^{+/+}$ mice during the test trial than during the conditioning trial vs. 91% consumed by SNAP-25$^{+/-}$ mice). Pre-exposed mice of both genotypes consumed nearly as much HCl (98%, SNAP-25$^{+/+}$ mice, and 112%, SNAP-25$^{+/-}$ mice) during the test trial, as they did during the conditioning trial, excluding an impairment in viscerally related associative learning and memory (Fig. 5*A*, middle panel). CTA impairment was not due to the poor discrimination between HCl and plain water. Indeed, when tested for CTA with saccharin, SNAP-25$^{+/-}$ mice exhibited a significantly attenuated CTA, as they avoided the saccharin solution to a lesser degree compared with SNAP-25$^{+/+}$ controls during choice tests (Fig. 5*A*, bottom panel). 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 the novel object recognition (Fig. 5*B* and Supplementary Fig. S3), no significant difference was detected in the amount of time the mice spent exploring the 2 objects during the familiarization (T1) phase (data not shown), indicating that both genotypes had the same motivation to explore the object. However, SNAP-25$^{+/-}$ mice spent significantly less time exploring the novel object compared with the familiar one, as shown by a significant decrease of the discrimination index. This was not due to altered sensorial parameters as all mice appeared in health, displaying normal motor coordination, sensory abilities and were not aggressive (Supplementary Table S1).

When tested for sociability, SNAP-25$^{+/+}$ mice behaved normally, spending longer time to explore the compartment with the stranger mouse than the empty cage. Conversely, SNAP-25$^{+/-}$ mice spent the same amount of time in the 2 compartments (Fig. 5*C*, top panel). Moreover, when subjected to a social recognition test, SNAP-25$^{+/-}$ mice remained close to the new or old stranger for the same time, suggesting altered social recognition (Fig. 5*C*, bottom panel). Both genotypes spent equal time in the central compartment.

### *Antiepileptic Drugs Normalize the EEG Profile and Improve the Cognitive Defects in SNAP-25$^{+/-}$ Mice*

We next investigated whether treatment with antiepileptic drugs was able to normalize the altered EEG profile of SNAP-25$^{+/-}$ mice. Treatment with different antiepileptic drugs significantly reduced epileptiform activity (Fig. 6*A*). The most potent effect was observed after treatment with VLP (95% reduction) and ETO (80% reduction), whereas a partial but significant reduction was obtained with CBZ (60% reduction) or with the calcium antagonist NIMO (35% reduction) (Fig. 6*A*). The mean number of spikes was significantly reduced in SNAP-25$^{+/-}$ mice treated with VLP (Fig. 6*B*,*C*). Notably, VLP, given 2 h before test performance, significantly reversed the cognitive deficit of SNAP-25$^{+/-}$ mice in the object recognition task (Fig. 6*D*). Treatment with VLP per se slightly, but not significantly, worsened cognitive abilities in SNAP-25$^{+/+}$ mice (not shown), in agreement with previous reports indicating impaired nonspatial hippocampal memory in mice (Sgobio et al. 2010) and reduced mental speed in humans (Mula and Trimble 2009) treated with VLP. Treatment with VLP also reversed the CTA impairment (Fig. 6*E*) and normalized the defects in social memory (Fig. 6*G*), being however ineffective in restoring sociability (Fig. 6*F*). Treatment with VLP slightly reduced the cognitive performance in SNAP-25$^{+/+}$ mice (data not shown).

## Discussion

Human genetic data link SNAP-25 to a range of psychiatric and neurologic conditions. In particular, reductions of protein expression at levels comparable to those occurring in the SNAP-25$^{+/-}$ mice have been described in psychiatric patients, with 49% less SNAP-25 immunoreactivity detected, for example, in the hippocampus of patients with schizophrenia compared with the control group (Thompson et al. 2003). SNAP-25$^{+/-}$ mice provide therefore a useful model to better understand the role of the gene and test potential new therapies. As a relevant added value, the study was carried out in 3 distinct European laboratories, thus reducing the possibility that interactions of genotype with the specific laboratory conditions may impact behavioral results.

Like *coloboma* mice (Hess et al. 1996) and homozygous mutant mice in which Ser187 of SNAP-25 is substituted with Ala (Kataoka et al. 2011), SNAP-25$^{+/-}$ mice display hyperactivity, although this alteration occurs at 7 postnatal weeks and is normalized in the adult age. However, SNAP-25$^{+/-}$ mice are not anxious and, differently from *coloboma*, they do not display motor coordination impairment and PPI deficit (Gunn et al. 2011). This could result from the severe sensory deficits of *coloboma* mice, which are blind and deaf, while SNAP-25$^{+/-}$ mice display no sensory abnormalities. Also, *Coloboma* mice show a disrupted latent inhibition, a measure of selective attention, without CTA deficits. SNAP-25$^{+/-}$ mice, conversely, exhibit defects in implicit associative learning. All together, these data suggest that the behavioral profile of *coloboma* mice may be influenced by the other genes deleted besides SNAP-25 (Gunn et al. 2011).

A major accomplishment of our study is the finding that SNAP-25 levels reduction is associated with diffuse network hyperexcitability, which does not lead to spontaneous convulsive behavior. Our data are in line with the significantly higher incidence of epilepsy in pathologies characterized by SNAP-25 alterations. In particular, the incidence of epilepsy is about 6 times higher in patients with schizophrenia than in controls (Chang et al. 2011) and ADHD children are 2.7 times more likely to have epilepsy (Davis et al. 2010), also showing higher occurrence of subclinical epileptiform activity (Richer et al. 2002; Becker et al. 2004). Although the mechanisms by which reduced levels of SNAP-25 lead to network

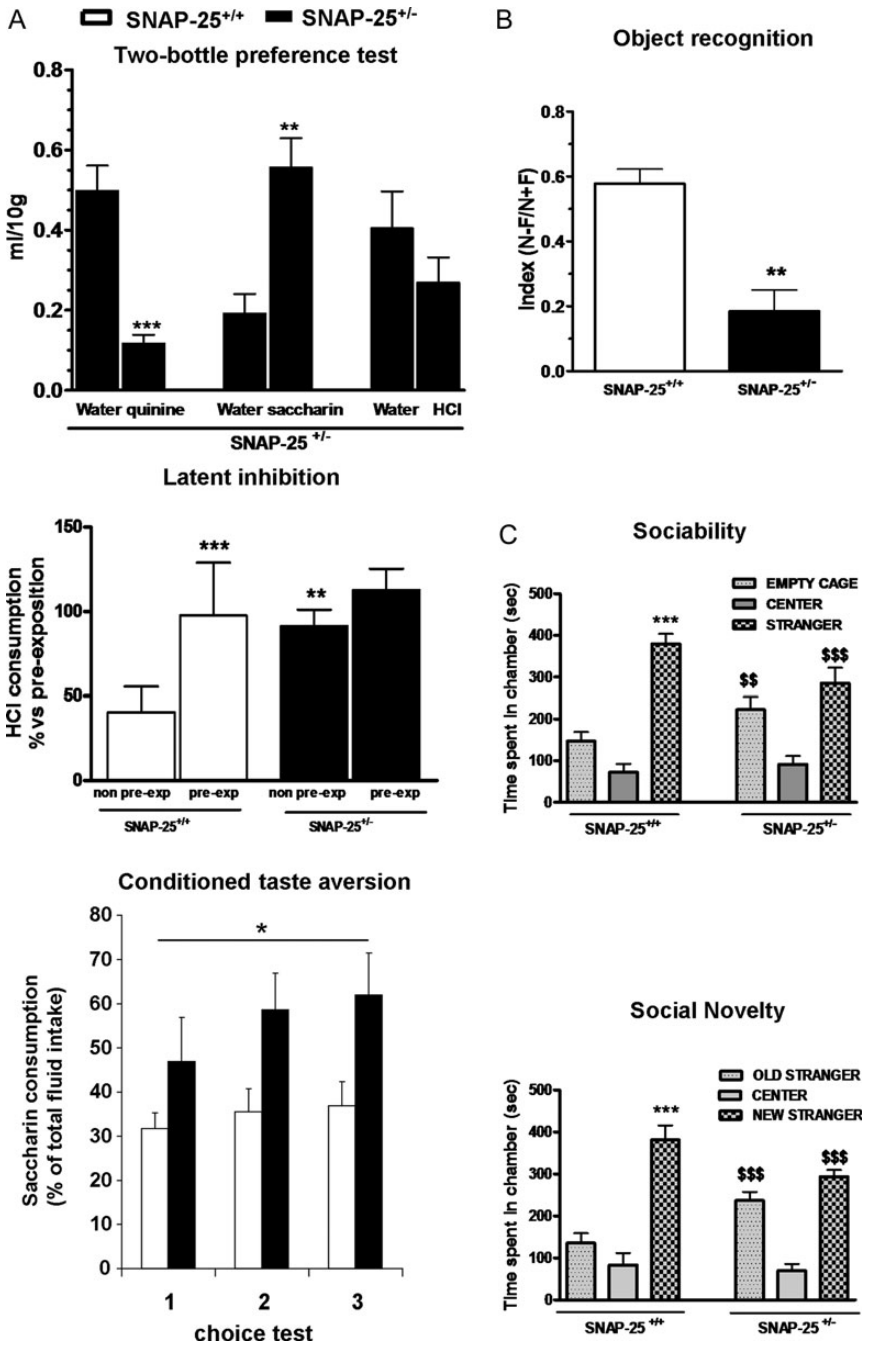


**Figure 5.** SNAP-25$^{+/-}$ mice are impaired in implicit associative learning tasks. (*A*) Two-bottle test (top). SNAP-25$^{+/-}$ mice consume less quinine than water (***$P < 0.0001$) and more saccharin than plain water (**$P = 0.0003$) (*t*-test). There is no significant difference in HCl (0.008 M) intake between SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mice ($P = 0.23$). Latent inhibition of CTA response (middle panel). SNAP-25$^{+/+}$ non-pre-exposed mice show a significant decrease of HCl intake in comparison with pre-exposed animals, indicating a latent inhibition. No significant difference in HCl consumption between non-pre-exposed and pre-exposed SNAP-25$^{+/-}$ mice occurs, indicating the lack of CTA. Genotype as between subject factor and treatment as within factor, genotype: $F_{1,36} = 3.52$, $P = 0.07$; treatment : $F_{1,36} = 13.11$, $P = 0.001$; genotype X treatment $F_{1,36} = 3.87$, $P = 0.05$, 2-way repeated measure of variance, Bonferroni's post hoc analysis. SNAP-25$^{+/+}$ or SNAP-25$^{+/-}$ mice versus SNAP-25$^{+/+}$ non-pre-exposed: **$P < 0.01$, ***$P < 0.001$, Bonferroni's post hoc analysis. CTA test (bottom panel). SNAP-25$^{+/-}$ mice drink significantly more saccharin - as expressed in percentage of total fluid intake - than SNAP-25$^{+/+}$ mice during the 3-daily choice tests following aversive conditioning ($F_{1,40} = 6.32$, $P = 0.02$, one-way repeated measure of variance, Tukey's post hoc test). *$P < 0.05$. (*B*) Object recognition test. Mice were allowed to explore an identical pair of objects and after 120 min they are presented with a familiar and a new object. SNAP-25$^{+/-}$ mice show no net preference between novel and familiar objects, as shown by the reduced discrimination index (**$P < 0.0001$, unpaired *t*-test). (*C*) Sociability and social novelty test. SNAP-25$^{+/-}$ mice show impaired social interaction (top), as they spend the same amount of time exploring a stranger mouse or an empty cage in a social choice paradigm. Genotype as between subject factor and compartment as within factor, genotype: $F_{1,36} = 0.77$, $P = 0.38$; compartment: $F_{1,36} = 27.88$, $P < 0.0001$; genotype X compartment $F_{1,36} = 4.68$, $P = 0.03$, 2-way repeated measure of variance, Bonferroni's post hoc analysis. SNAP-25$^{+/+}$, stranger versus SNAP-25$^{+/+}$ mice, empty cage and center: ***$P < 0.01$, SNAP-25$^{+/-}$ mice versus center $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$. Social novelty (bottom). SNAP-25$^{+/-}$ mice fail to show any preference for a novel mouse in a social recognition task. In contrast, SNAP-25$^{+/+}$ mice spend more time exploring the new stranger versus old stranger and center. Genotype as between subject factor and compartment as within factor, genotype: $F_{1,36} = 0.3$, $P = 0.8$; compartment: $F_{1,36} = 22.44$, $P < 0.0001$; genotype X compartment $F_{1,36} = 19.09$, $P = 0.0001$, 2-way repeated measure of variance, Bonferroni's post hoc analysis. SNAP-25$^{+/+}$ mice: new stranger versus old stranger and center, ***$P < 0.001$. SNAP-25$^{+/-}$ old or new stranger versus center: $^{\$\$\$}P < 0.001$. Error bars in all figures refer to mean ± SEM of 8-16 for each group.

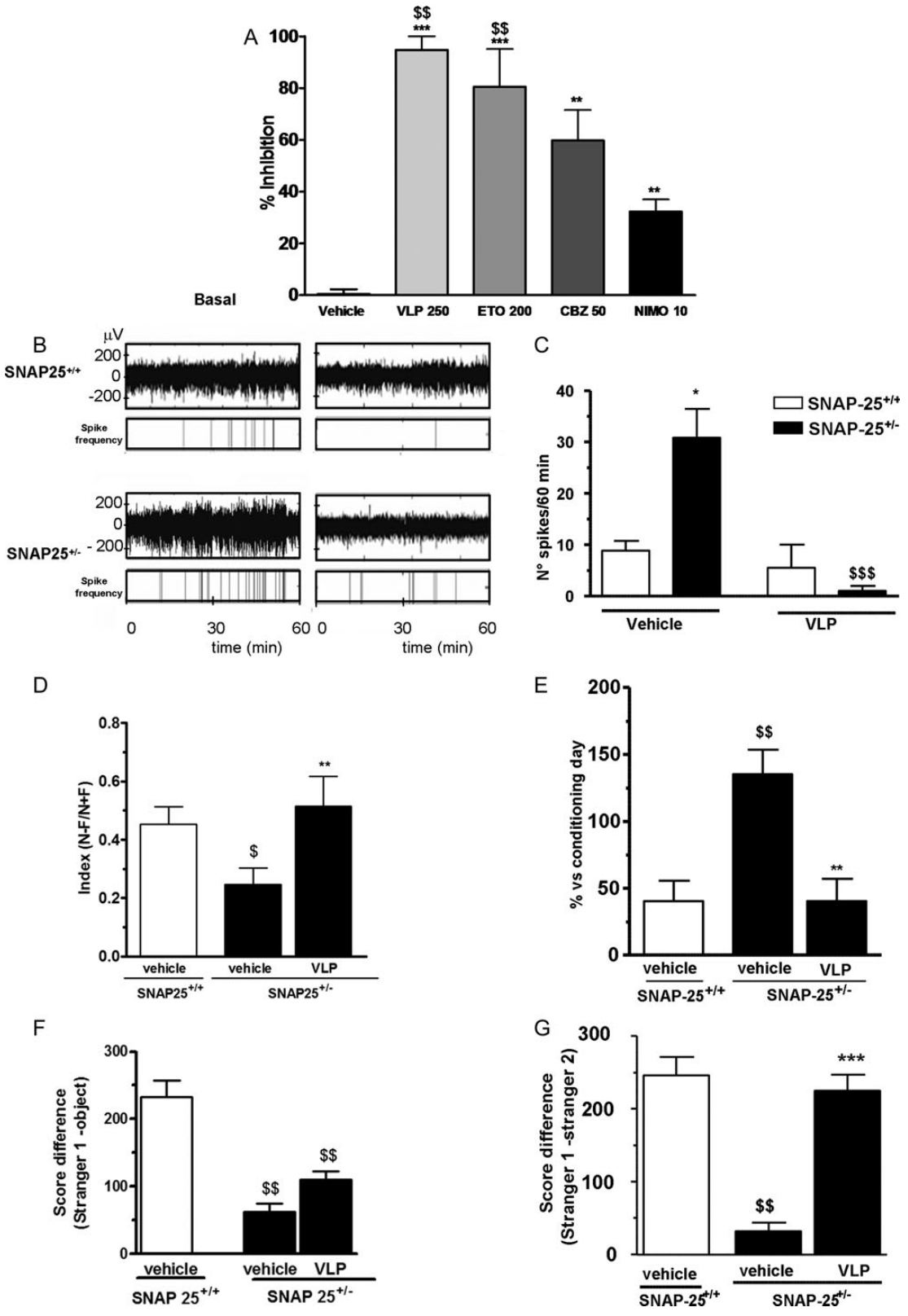


**Figure 6.** Antiepileptic drugs reverse EEG alterations and learning defects in SNAP-25$^{+/-}$ mice. (*A*) Treatment with VLP (250 mg/kg), ethosuximide (ETO) (200 mg/kg), carbamazepine (CBZ) (50 mg/kg), and nimodipine (NIMO) (10 mg/kg) to SNAP-25$^{+/-}$ mice significantly reduces spike activity (genotype as between subject factor and treatment as within factor, genotype: $F_{4,45} = 20.85$, $P = 0.0001$, one-way repeated measure of variance, Tukey's post hoc test) ($^{**}P < 0.001$, $^{***}P < 0.001$ compared with vehicle; $^{\$\$}P < 0.01$ compared with NIMO). (*B*) EEG recording of one representative SNAP-25$^{+/-}$ mouse treated with VLP showing a decreased spike activity in comparison to one SNAP-25$^{+/+}$ mouse. (*C*) The mean number of spikes recorded for 60 min in SNAP-25$^{+/-}$ mice is significantly reduced by VLP compared with vehicle. ($F_{1,36} = 5.44$, $P = 0.2$; treatment: $F_{1,36} = 19.65$, $P < 0.0001$; genotype X treatment: $F_{1,36} = 12.50$, $P = 0.001$, Bonferroni's post hoc analysis). SNAP-25$^{+/+}$ versus SNAP-25$^{+/-}$ mice: $^{*}P < 0.05$; VLP-treated SNAP-25$^{+/-}$ mice versus vehicle-treated SNAP-25$^{+/-}$ mice: $^{\$\$\$}P < 0.0001$. (*D*) VLP reverses the defect in object recognition test. The reduced discrimination index, found in SNAP-25$^{+/-}$ mice, is reversed by pretreatment with VLP given i.p. at the dose of 250 mg/kg, 20 min before T1 trial (genotype as between subject

hyperexcitability are not defined, a defective modulation of VGCCs by reduced SNAP-25 levels may be implicated (Atlas 2001; Zamponi 2003; Catterall and Few 2008; Pozzi et al. 2008; Condliffe et al. 2010). Indeed, analysis of calcium current amplitude in thalamocortical neurons of *coloboma* mice revealed an increase in the peak current density of low voltage-
activated currents that precedes the onset of spike-wave discharges (Zhang et al. 2010). Notably, acute downregulation of SNAP-25 in glutamatergic neurons by siRNA reduces calcium currents, and consistently, SNAP-25$^{+/-}$ glutamatergic but not GABAergic neurons display larger calcium current density (Condliffe et al. 2010). In line with these results, electrophysiological analysis of cultured neurons has demonstrated that SNAP-25$^{+/-}$ glutamatergic neurons shift from paired pulse facilitation to paired pulse depression, thus indicating an increase in presynaptic release probability (F. Antonucci, R. Morini and M. Matteoli, unpublished observations). Although a direct correlation between electrophysiological properties of cultured neurons and mice behavior cannot be easily drawn, these data suggest that an excitatory–inhibitory imbalance could be at the basis of the SNAP-25$^{+/-}$ phenotype. Addressing this possibility will require an in depth analysis of excitatory and inhibitory neurotransmission in different areas of the SNAP-25$^{+/-}$ brain. Indeed, quite surprisingly, cognitive deficits in SNAP-25$^{+/-}$ mice occur predominantly in implicit associative learning tasks, whereas no defects were found in explicit spatial orientation tasks. This may be related to the heterogeneous SNAP-25 levels in distinct neuronal hippocampal subpopulations. Indeed, 3-4-fold higher protein levels occur in CA3 region compared with CA1 (Oyler et al. 1989; Geddes et al. 1990), with CA3 possibly serving as predominant associative memory network, and CA1 being critical for long-term spatial memory (Nakazawa et al. 2003). Notably, ADHD children display impaired associative implicit learning, mediated by frontal-striatal-cerebellar circuits, but normal spatial contextual learning depending upon the medial temporal lobes (Barnes et al. 2010). Under this respect, an analysis, in SNAP-25$^{+/-}$ mice, of the synapses where SNAP-25 expression is in fact lower at early developmental stages could be useful for identifying the substrate of impaired forward signaling even if compensated by later network maturation.

The abnormal EEG activity of SNAP-25$^{+/-}$ mice could be a factor contributing to the learning deficits. Indeed, it has been proposed that the cognitive effects of epileptiform discharges may be very similar to the cognitive impact of short epileptic seizures (Aarts et al. 1984). Furthermore, a decline in IQ scores, similar to that seen in patients with nonconvulsive epileptic seizures, was reported in patients with frequent episodes of epileptiform discharges (Brinciotti et al. 1989; Aldenkamp et al. 2005, 2010). Interestingly, treatment with antiepileptic drugs, or with the calcium antagonist NIMO, largely normalize the altered EEG profile of SNAP-25$^{+/-}$ mice, the larger beneficial effects produced by VLP and ETO, that is, drugs effective at controlling absence seizures. It is possible that a common mechanism of action is shared by these antiepileptic drugs. At clinically relevant concentrations, ETO inhibits calcium T currents in thalamic neurons (Coulter 1997); CBZ has been proposed to modulate L- and P-type VGCC (Yoshida et al. 2007), whereas VLP blocks the voltage-gated sodium channels and T-type calcium channels (Rosenberg 2007). Finally, the calcium channel blocker NIMO (Mikati et al. 2004), acting as aspecific antagonist on L-, N-, P/Q-, R-, and T-type VGCC, has been indicated by preclinical and clinical studies potentially useful in the treatment of various disorders of the central nervous system (Choudhary et al. 2006). Along this line, it is notable that CBZ has been reported to exert a positive effect on a child with ADHD and subclinical EEG discharges without seizures (Laporte et al. 2002), whereas treatment with VLP appeared to ameliorate ADHD symptoms in fragile X syndrome boys (Torrioli et al. 2008). Our data offer a logical frame for explaining these findings, by directly linking reduction of SNAP-25, generation of subclinical epileptiform discharges and learning impairments. They also suggest that human genetic variations, resulting in the reduction of protein expression, may create a hyperexcitable physiopathological background susceptible to functional failures and demonstrate the beneficial effect of antiepileptic drugs in ameliorating the abnormal excitability and cognitive impairments linked to reduction of SNAP-25 levels.


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factor and treatment as within factor, genotype: $F_{1,36} = 23.37$, $P < 0.0001$; treatment: $F_{1,36} = 14.76$, $P = 0.0005$; genotype X treatment: $F_{1,36} = 25.42$, $P < 0.0001$, 2-way repeated measure of variance Bonferroni's post hoc analysis). SNAP-25$^{+/+}$ mice, vehicle versus SNAP-25$^{+/-}$ mice, vehicle: $^{\$}P < 0.05$; Valproate-treated SNAP-25$^{+/-}$ mice versus saline-treated SNAP-25$^{+/-}$ mice: $^{**}P < 0.001$. (*E*) VLP reverses CTA impairment. SNAP-25$^{+/-}$ mice are unable to associate sucrose intake with a malaise induced by LiCl given subcutaneously (63 mg/kg) in the conditioning day. Pretreatment with VLP, given immediately before HCl exposure, fully reverses the lack of CTA (genotype as between subject factor and treatment as within factor, genotype: $F_{1,36} = 4.34$, $P = 0.04$; treatment: $F_{1,36} = 4.45$, $P = 0.04$; genotype X treatment: $F_{1,36} = 3$, $P = 0.05$, 2-way repeated measure of variance. Bonferroni's post hoc analysis). SNAP-25$^{+/+}$ versus SNAP-25$^{+/-}$ mice: $^{\$\$}P < 0.01$; Valproate-treated SNAP-25$^{+/-}$ mice versus vehicle-treated SNAP-25$^{+/-}$ mice: $^{**}P < 0.001$. (*F*) Effect of VLP in sociability in SNAP-25$^{+/-}$ mice. VLP do not reverse the impaired social interaction (genotype as between subject factor and treatment as within factor, genotype: $F_{1,36} = 55.48$, $P < 0.0001$; treatment: $F_{1,36} = 1.45$, $P = 0.22$; genotype X treatment: $F_{1,36} = 1.45$, $P = 0.2$, 2-way repeated measure of variance, Bonferroni's post hoc analysis). SNAP-25$^{+/+}$ versus SNAP-25$^{+/-}$: $^{\$\$}P < 0.01$. (*G*) VLP is effective in improving putative social memory (genotype as between subject factor and treatment as within factor, genotype: $F_{1,36} = 23.37$, $P < 0.0001$; treatment: $F_{1,36} = 14.76$, $P = 0.0005$; genotype X treatment: $F_{1,36} = 25.42$, $P < 0.0001$, 2-way repeated measure of variance, Bonferroni's post hoc analysis). SNAP-25$^{+/+}$ vehicle mice versus SNAP-25$^{+/-}$ vehicle mice: $^{\$\$}P < 0.01$). SNAP-25$^{+/-}$ VLP-treated versus SNAP-25$^{+/-}$ vehicle-treated mice: $^{***}P < 0.001$. Bars represent the mean ± SEM of 8–12 animals for each group.

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