UNIVERSITA' DEGLI STUDI DI MILANO
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Behavioural phenotype and electroencephalographic profile of adolescent and adult SNAP-25$^{+/-}$ mutant mice.

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ABSTRACT

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 N-ethylmaleimide-sensitive proteine (NSF) attachment protein receptor complex and modulates voltage-gated calcium channels activity. Snap25 gene has been associated with schizophrenia, and bipolar disorder, and lower levels of SNAP-25 have been described in patients with schizophrenia. In particular several SNAP-25 intronic single polymorphisms were linked to attention deficit hyperactivity disorder, one of the most common neuropsychiatric diseases among children and adolescents. The most animal models of this pathology, available until now, are characterized by reduced SNAP-25 level in the CNS: the coloboma mice, and the spontaneously hypertensive rats (SHR). However none of them can completely ricapitulate all the core features of the human patology.

Thus we used adult SNAP-25 heterozygous (SNAP-$25^{+/-}$) mice in comparison with age-matched wild type mice (SNAP-$25^{+/+}$) to investigate at which extent the reduction of the protein levels affects neuronal network function and mouse behavior, and the possible therapeutic effect of antiepileptic drugs. We also characterized adolescent SNAP-$25^{+/-}$ mice (6-7 weeks) in order to evaluate if they can be considered a new ADHD animal model.

Firstly we analysed general health, sensory and motor abilities, and emotional behaviour in our animals, without finding any abnormalities in heterozygous mice.

Since altered SNAP-25 level were associated with cognitive deficit, we performed T-maze test for the evaluation of spatial memory, latent inhibition test for attention, conditioned taste aversion and object recognition for associative memory. SNAP-$25^{+/-}$ resulted impaired in associative but not in spatial memory, probably because of the heterogeneous protein expression levels in different hippocampal areas, being more

expressed in CA3, known to play a key role in associative memory, than in CA1, critical for long-term spatial memory.

SNAP-25$^{+/-}$ has been associated to disease characterized by altered social behaviour, such as schizophrenia and bipolar disorder. We tested SNAP-25 mutant mice in sociability and social novelty test. Heterozygous showed impairment both in sociability and in social recognition.

Pathologies characterized by SNAP-25 alterations show significantly higher incidence of epilepsy. For this reason we recorded the cortical electric activity of mice and we found that SNAP-25 levels reduction was associated with network hyperexcitability, in terms of spike activity, which did not lead to spontaneous epileptiform behaviour. Acute treatment with antiepileptic drugs and Ca^{2+} antagonist normalized cerebral activity. Among these drugs, sodium valproate was more effective in blocking EEG and behavioural deficits. Since it is known the correlation between EEG alteration and cognitive deficits we can hypothesize that the mnemonic and social deficit are due to the abnormal EEG profile.

Adolescents SNAP-25$^{+/-}$ mice showed the same deficits found in the adults. They also were hyperactivite, and were not susceptible to d-amphetamine treatment. These results are in line with the characteristic phenotype of ADHD children, that display cognitive deficits, problems in socialization and hyperactivity, normalized by stimulants.

Recently EEG analysis was used as diagnostic tool to discriminate ADHD from other neuropsychiatric diseases. EEG in ADHD children is characterized by spikes and alteration in spectral power bands frequency. Spectral analysis heterozygous mice EEG recordings was caharacterized by spikes, a decrease in fast waves and a parallel increase in slow waves, as occur in ADHD children. Repeated exposition to a

VLP solution (0.1%) reduced all the behavioural deficit and was effective in blocking spike activity for two weeks, when discontinued.

SNAP-25$^{+/-}$ mice seem to be a promising ADHD animal model, able to recapitulate almost all the core symptoms of the human disease. Repeated treatment with VLP resulted effective in all the tests carried out in adolescents mice, in line with recent findings of its therapeutic effects on ADHD symptoms in x-fragile syndrome.

INTRODUCTION

SYNAPTOPATHIES

Synaptopathy is an increasingly popular term used to define key features of neurodegenerative and psychiatric diseases. It implies that disruptions in synaptic structure and function are potentially the major determinant of such brain diseases (Brose et al., 2010). An high number of neuropsychiatric diseases can be included in this category, such as schizophrenia, attention deficit/hyperactive disorder, bipolar disorder, epilepsy, autism, mental retardation, x-fragile syndrome, Alzheimer disease, Parkinson's disease.

The human synapse proteome is a highly complex collection of proteins that is disrupted by hundreds of gene mutations causing over 100 brain diseases. These synaptic diseases, or synaptopathies, cause major psychiatric, neurological and childhood developmental disorders through mendelian and complex genetic mechanisms (Grant, 2012). Evidence that synaptic mutations were important for behaviour was unequivocally demonstrated with mice carrying engineered mutations 20 years ago (Silva et al, 1992; Bayes et al., 2011), but only recently accumulating body of evidence showed for synaptic mutations a key role in human disease (Grant 2012). Surprisingly few mutations have been found in neurotransmitter receptor systems and far more have been found in other types of synaptic and neuronal proteins.

Mutations in synaptic proteins can occur both in pre and post-synaptic terminals. In the presynaptic terminal there are SNARE proteins complexes that form the vesicular release machinery, coupling the arrival of the action potential to the release of neurotransmitters. In the postsynaptic terminal there are signal transduction complexes that couple the neurotransmitter receptors to intracellular signaling to regulatory and metabolic processes.

For example, in humans, the MAGUK Associated Signaling Complexes are mutated in Autism, Schizophrenia, Intellectual Disability and many other diseases, and mice carrying orthologous mutations show relevant cognitive, social, motor and other phenotypes (Fernandez et al., 2009). Diseases with similar symptom spectrum arise from disruption of complexes and interacting proteins within the synapse proteome (Frank et al., 2011)

SCHIZOPHRENIA

Schizophrenia is a common mental disorder with a prevalence of approximately 0.5-1% (American Psychiatric Association, 1994) and significant heritability estimated about 85%(Cardno et al, 2000). The clinical symptoms can be classified into two main categories: psychotic or `positive' symptoms, and `negative' symptoms.

Positive symptoms are those that most individuals do not normally experience but are present in people with schizophrenia and generally respond well to medication (American Psychiatry Association, 2004) . They can include delusions, disordered thoughts and speech, and tactile, auditory, visual, olfactory and gustatory hallucinations, typically regarded as manifestations of psychosis (Kneisl and Trigoboff, 2009). Hallucinations are also typically related to the content of the delusional theme.

Negative symptoms are deficits of normal emotional responses or of other thought processes, they commonly include flat or blunted affect and emotion, poverty of speech (alogia), inability to experience pleasure (anhedonia), lack of desire to form relationships (asociality), and lack of motivation (avolition). Research suggests that negative symptoms contribute more to poor quality of life, functional disability, and the burden on others than do positive symptoms (Velligan, 2008). People with prominent negative symptoms often have a history of poor adjustment before the onset of illness, and response to medication is often limited (Smith, 2010). Difficulties in working and long-term memory, attention, executive functioning, and speed of processing also commonly occur (van Os and Kapur, 2009)

The disorder usually has its onset in early adulthood, although often cognitive and behavioural signs are present from childhood. In 40% of men and 23% of women diagnosed with schizophrenia, the condition manifested itself before the age of 19 (Cullen, 2008).

The first-line psychiatric treatment for schizophrenia is antipsychotic medication which can reduce the positive symptoms of psychosis in about 7–14 days. Antipsychotics, however, fail to significantly ameliorate the negative symptoms and cognitive dysfunction (Tandon et al, 2008). Long term use decreases the risk of relapse (Leucht et al, 2012).

Despite pharmacological treatments, outcomes are variable and approximately two-thirds

of affected individuals have persistent symptoms with only partial remission (American Psychiatric Association, 1994). Schizophrenia is thought to be a neurodevelopmental disease characterized by defective connectivity in various brain regions during early life (Rehn et al, 2005; Walsh et al, 2008). The neuropathophysiology of schizophrenia remains unclear, although alterations in dopaminergic and serotoninergic circuitry (as in the case of ADHD), as well as in glutamatergic transmission have been strongly implicated.

Particular attention has been paid to the function of dopamine in the mesolimbic pathway of the brain. This focus largely resulted from the accidental finding that phenothiazine drugs, which block dopamine function, could reduce psychotic symptoms. It is also supported by the fact that amphetamines, which trigger the release of dopamine, may exacerbate the psychotic symptoms in schizophrenia (Laruelle et al, 1996) The influential dopamine hypothesis of schizophrenia proposed that excessive activation of D2 receptors was the cause of (the positive symptoms of) schizophrenia (Jones et al, 2002).

Interest has also focused on the neurotransmitter glutamate and the reduced function of the NMDA glutamate receptor in schizophrenia, largely because of the abnormally low levels of glutamate receptors found in the postmortem brains of those diagnosed with schizophrenia (Konradi et al, 2003) and the discovery that glutamate-blocking drugs such as phencyclidine and ketamine can mimic the symptoms and cognitive problems associated with the condition (Lathi et al, 2001)

Numerous genetic linkage and association studies have identified regions of the genome that may harbour schizophrenia risk genes, but the complex genetics and symptomatology of the disease have limited progress in achieving a clear identification of the specific genetic determinants responsible for neurophysiological deficits that underpin the disorder (Harrison et al, 2005). Candidate genes that have been shown to be potentially associated with the disease include neuregulin, a protein belonging to the EGF family; dysbindin, a protein constituent of the dystrophinassociated protein complex; Catechol-o-methyltransferase (COMT), one of several enzymes that degrade catecholamines; the DISC1 (Disrupted-in-Schizophrenia-1) encoding a brain protein likely to play a role in cytoskeletal scaffolding;

RGS4 (Regulator of G-protein Signaling 4) that modulates the GTPase activity of heteromeric G-proteins; GRM3, a metabotropic glutamate receptor; and the gene G72, which encodes a protein in brain thought to modulate NMDA glutamate receptor function (Kirov et al, 2005).

EPILEPSY

Epilepsy is a group of heterogeneous neurologic disorders affecting almost 1% of the population. It is characterized by recurrent, unprovoked episodes of seizures, due to abnormal synchronous firing of groups of neurons, arising from periodic neuronal hyperexcitability. Clinical manifestations of epilepsy are varied and despite availability of a number of antiepileptic drugs, about one-third of epileptic patients are resistant to treatment. Antiepileptic drugs (AEDs) are able to act not only on recurring and sudden seizures, but also on cognitive and behavioural abnormalities associated to (Dudra-Jastrzębska et al, 2007). They are able to act by decreasing the frequency of seizures and modulating the activity of neurotransmitters and their psychotropic effect: they increase the post-synaptic inhibition and alter the synchronization of neuronal network, in order to decrease the excessive neuronal excitability associated with the development of seizures and, secondly, to limit the spread of epileptic activity in surrounding brain regions. However the excessive reduction of neuronal excitability may result in decrease of motor skills, attention and memory, common side effects of the blockade of Na + channels and an increase in the inhibitory GABAergic system (Shehata et al, 2009).

Several changes can occur in the brain undergoing pathological hyperactivity, at the level of both single cells and anatomically defined neuronal networks.

Based on electroencephalographic recordings, seizures can be classified in focal (originating in a single area of the brain) or generalized (involving both brain hemispheres). Generalized epileptic syndromes can be classified as symptomatic (caused by identifiable factors) or idiopathic (without a clear aetiology and partially caused by genetic defects) (Fisher et al, 2005).

Generalized syndromes consist of myoclonic seizures (sudden twitches of the limbs or

trunk muscle), absences (sudden suspension of consciousness for a period of 5-30 seconds) and generalized seizures (characterized by loss of consciousness, stiffening tonic clonic seizures and shock of all the muscles). The latter is the most important epileptic manifestation and challenging not only for the risk of trauma but also for respiratory and cardiovascular functions due the massive muscle contractions. Focal seizures are divided into two broad categories, the simple and the complex. The first is characterized by signs of paroxysmal activity in an area of the brain with specific functions, while the second by impaired consciousness. In the complex focal seizure the patient appears confused and sometimes shows automatic movements of the face and trunk (psychomotor crises). Complex focal seizures are typical when the firing originates fromn the temporal or frontal lobe of the cortex. All partial seizures, simple or complex, can spread to the entire brain and develop in a grand mal seizure.

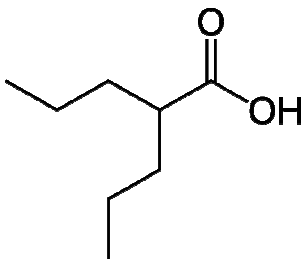
Recently it was discovered that many epileptic syndromes are related to mutations affecting genes for subunits of voltage-dependent or ligand-dependent channels, reinforcing the concept that epilepsy is a channelopathy. Some forms of mutations are: familial benign neonatal seizures, familiar benign infantile seizures, the family of the adult benign myoclonic epilepsy and the progressive myoclonic epilepsies (Steinlein, 2008).

Generalized idiopathic epilepsies include childhood absence and juvenile absence. These are characterized by a short, sudden impairment in consciousness and behavioural arrest, possibly accompanied by facial clonus. The electroencephalographic hallmark of absence epilepsy are spike-wave discharges (SWDs) that occur in the thalamo-cortical circuitry. Mutations in different genes, including genes codifying for GABA receptors, Na+, K+, and Ca2+ channels, have been associated to the familiar forms of generalized idiopathic epilepsy with a Mendelian inheritance (Mulley et al, 2003; Khosravani and Zamponi, 2007; Helbig et al, 2008). Analysis of absence epilepsy mouse models tottering/tg, lethargic/lh and stargazer/stg, bearing mutations in α1A, β4, γ2 Ca2+ channel subunits respectively, revealed altered high voltage activated (HVA) channels current density in tg and stg thalamic neurons, and increased low voltage activated (LVA) channels peak current and a depolarized shift of

the stady-state inactivation curves in tg, lh and stg thalamic neurons (Zhang et al, 2002). Furthermore, the association of mutations in human α1A (P/Q type) channels with absence epilepsy has been reported (Jouvenceau et al, 2001).

ANTIEPILEPTIC DRUGS

AEDs are drugs that prove to be able to act not only on recurring and sudden seizures, but also on cognitive and behavioural abnormalities, and associated with it (Dudra-Jastrzębska et al., 2007). They act by decreasing seizures frequency and modulating neurotransmitters activity and their psychotropic effects: they increase the post-synaptic inhibition and alter the synchronization of neuronal network, in order to decrease the excessive neuronal excitability associated with the development of seizure and, secondly, to limit the spread of epileptic activity in brain regions surrounding. However the excessive reduction of neuronal excitability may result in decrease of motor skills, attention and memory, common side effects of the blockade of Na + channels and an increase in the inhibitory GABAergic (Shehata et al., 2009).



Anticonvulsant property of Valproic Acid (VPA) was discovered in 1936 (Meunier et al, 1963) and currently is one of the most frequently used anti-epileptic drugs worldwide, and in addition is now being used for the treatment of many other conditions, including BD (bipolar disorder) (Emrich et al, 1981) and migraine (Calabresi et al, 2007). It has also been proposed in cancer (Blaheta et al, 2002), Alzheimer's disease (Tariot et al, 2002) and latent HIV treatment (Lehrman et al, 2005), in addition to having a variety of side effects, including hepatotoxicity and teratogenicity. Many of these effects of VPA are indirect and/or occur via an unknown mechanism (Lagace et al, 2005).

The 2-Propylpentanoic acid is a branched short-chain fatty acid with a 8 carbon atoms chain. Depending on the route of administration it is also used as sodium salt. It has several mechanisms of action that may underlie its ability to reduce neuronal activity. VPA is able to

block voltage-gated sodium channel by the indirect inhibition of sub-threshold slow Na^+ currents and membrane-depolarizing rectification (Tian et al, 1994) and T-type Ca^{2+} channels (Rosemberg , 2007). It decreases the levels of aspartate in the brain and also increases the levels of the inhibitory neurotransmitter GABA (Umka et al., 2010). This anticonvulsant drug elevates GABAergic tone by direct inhibition of GABA transaminase (Rosenberg et al, 2007) and succinic semialdehyde dehydrogenase (Mesdijan et al, 1982), which are enzymes responsible for GABA breakdown. It may also stimulate the synthesis of GABA, but the direct mechanism is not known (Umka et al., 2010). Sodium valproate has efficacy in all partial and generalised seizures, including absence seizures.

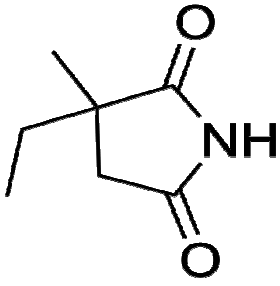
VPA can modulate gene expression through a direct inhibition of enzyme histone deacetylase (HDAC), since VPA inhibits class I and II HDAC enzymes directly (Lehrman et al, 2006). Inhibition of HDAC activity gives rise to hyperacetylation of gene promoters, and altered expression of approx. 2% of the genome (Van Lint et al, 1996). Inhibition of HDACs has been associated with tumour cell toxicity (Eyal et al, 2005) and was shown to be linked to teratogenicity (Phiel et al., 2001). Teratogenic effects have also been observed in patient studies, whereby VPA treatment during pregnancy is associated with an increased risk of neural tube defects such as spina bifida and other malformations (Nau et al, 1986). Children exposed to VPA during prenatal life have learning difficulties and behavioural problems, probably due to his activities as inhibitor of HDAC1 1 (Gurvich et al., 2005).

Exposure to VPA causes hyperacetylation of DNA leading to increased expression of growth arrest and pro-differentiation genes (Kostrouchova et al, 2007). A recent study has shown that chronic VPA treatment reduced cell proliferation and induced cell differentiation within the sub granular zone of the dentate gyrus in adult, resulting in deficits in learning and spatial memory (Hsieh et al., 2004). A number of reports have documented a range of mild to moderate cognitive impairments, including memory deficits, in adult patients taking VPA (Carpay et al., 2005; Cysique et al., 2006; Gualtieri and Johnson, 2006; Senturk et al., 2007). This effect of the drug is supported by reports of improvements in cognition when VPA is discontinued (Ristic et al, 2006; Masmoudi et al, 2006; Hommet et al, 2007; Lossius et al,

2008). The causes of these cognitive changes could be the generalized neuro-suppressant effect of this compound, but may also be due to more specific effects on those brain regions in which adult neurogenesis continues. Conversely in a model of rodent status epilepticus, VPA markedly decreased neuronal damage in the hippocampal formation and improved neurological and memory functions (Brandt et al, 2006).

Valproate is also used in the treatment of migraine and in the treatment of behavioural disorders and represents a possible alternative to traditional methods in the treatment of ADHD symptoms in fragile X syndrome patients, probably due his action of HDAC inhibition and reactivation of FMR1 gene, that is silenced in this pathology (Torrioli et al., 2010). A study published in August 2005 found that three of four patients treated with valproic acid in addition to highly active antiretroviral therapy (HAART) showed a mean 75% reduction in latent HIV infection (Lehrman et al, 2005). The enzyme histone deacetylase 1 (HDAC1) is needed for HIV to remain latent, or dormant, in infected cells. The idea was that VPA, by inhibiting HDAC1, forced HIV out of latency (reactivation) and antiretroviral drugs could then stop the virus. Subsequent trials, however, found no long-term benefits of valproic acid in HIV infection (Sagot-Lerolle et al, 2008).

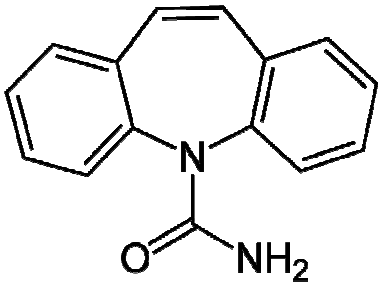
VPA is commonly used in bipolar disorders prophylaxis, but the mechanism of action of VPA remains unclear. The depletion of cellular inositol by the reduction of inositol recycling was proposed as a mechanism of action of lithium in BD (Berridge et al, 1989) and VPA have been shown to reduce InsP3 levels in several model systems (Shaltiel et al, 2007). It seems also effective in the depression phase in bipolar disorders by inhibiting the action of protein kinase C and therefore decreasing the levels of interleukin-6 that in that phase are increased (Coyle et al., 2003); the action of VPA in decreasing neuronal excitatory neurotransmission could represent another mechanism for its effectiveness in mania episodes (Johannessen et al., 2001).



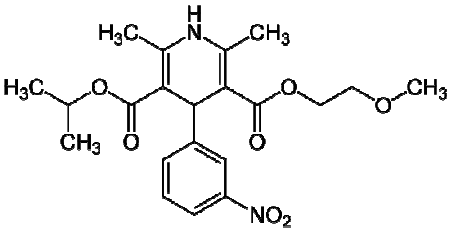
Ethosuximide (ETH) ((RS)-3-methyl-pyrrolidin-2,5-dione), is one of the oldest anticonvulsants. It is considered the first choice drug for treating absence seizures in part because it lacks the idiosyncratic

hepatotoxicity of the alternative anti-absence drug, valproic acid (Katzung, 2003). A study conducted on human neocortical cells removed during surgery for intractable epilepsy, the first using human tissue, found that ethosuximide had no effect on Ca2+ currents at the concentrations typically needed for a therapeutic effect (Sayer et al, 1993). Another study reported that ethosuximide had no effect on T-type currents, but decrease non-inactivating Na+ current by 60% and the Ca2+-activated K+ currents by 40% in rat and cat thalamocortical cells. It was concluded that the decrease in Na+ current is responsible for the anti-absence properties (Leresche et al, 1998). In 2001 was demonstrated that past studies were often done in isolated neurons that had lost most of their T-type channels (Gomora et al, 2001). Studies in rat thalamic neurons propose a model of action that is based on the inhibition of type T Ca2 + channels (Takechi et al., 2008).

Together with VPA, it represents one of the main drugs used successfully in the treatment of epileptic children, presenting very slight side effects, often only temporary. Effects such as decrease in attention and memory occur both in people presenting phenomena of seizure and in subjects who do not present (Glauser et al, 2010).



Carbamazepine (CBZ) is one of the most used drugs for the treatment of epilepsy. Its dibenzo-azepinic structure is derived from tricyclic antidepressants. The antiepileptic effect is due to its ability to block Na^+ channel and to inhibit the increase of intracellular Ca2+ induced by N-methyl-D-aspartate (NMDA) (Takechi et al., 2008). Its use may be associated with moderate cognitive dysfunction, including sedation and impaired attention, concentration and hand-eye coordination, and these effects are attributed to the carbamazepine action in blocking Na+ channel (Shehata et al, 2009). An effect on neuropathic pain, caused by injury or disease affecting the central or peripheral nervous system, especially trigeminal neuralgia, has been seen with CBZ and, along with valproate, it also had therapeutic effect on bipolar disorder (Landmark, 2008). Carbamazepine can cause severe skin reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis (Garcia et al, 2010).



Nimodipine (NIM) is a dihydropyridine calcium channel blocker, originally developed for the treatment of high blood pressure. It has shown good results in preventing a major complication of subarachnoid haemorrhage (a form of cerebral haemorrhage) termed vasospasm; this is now the main use of nimodipine (Choi et al, 2012). Nimodipine binds specifically to L-type voltage-gated calcium channels and has with high lipid solubility and permeability across the blood-brain barrier (Van den Kerckhoff and Drewes, 1985). Nimodipine is associated with low blood pressure, flushing and sweating, edema, nausea and other gastrointestinal problems, most of which are known characteristics of calcium channel blockers. It is contraindicated in unstable angina or an episode of myocardial infarction more recently than one month (Food and Drug Administration, 2009).

ADHD

Attention deficit hyperactivity disorder (ADHD) appears to be one of the most common neuropsychiatric disorders among children and adolescents. and it affects roughly 1 in 20 school-aged children in the U.S.A. (American Psychiatric Association, 1994). A recent analysis has revealed a worldwide prevalence of the disease of 8 to 12%, in which children are affected 3-4 times more than girls (Biederman et al., 2005). Symptoms of the disease may persist into adulthood for about half of the children who have been diagnosed with ADHD, although this estimate is difficult because of the lack of official diagnostic criteria for adults, with deleterious effects on educational, social and occupational outcomes and a higher risk of developing substance abuse. In adolescents, symptoms may differ from those of children due to adaptation processes learned during socialization.

The characteristic behaviors observed in patients are inattention, impulsivity and hyperactivity, but it is difficult to find objective criteria for discriminating behaviors "normal" from "abnormal" in these three parameters. Children with this disorder have difficulties in

social integration and learning at school, difficulties in adulthood can evolve into employment problems and phenomena of abuse (Faraone et al., 2003).

ADHD is considered to be a heterogeneous disorder with variable manifestation in symptoms. There is a classification, as described in the Diagnostic and Statistical Manual Of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994), which provides for three categories :

-Patients mainly inattentive (ADHD-PI): have difficulty focusing attention on organizing and completing a task or learning something new, they are also easily distracted and move from one activity to another without focusing on one in particular. They do not seem to listen when spoken to, and they have difficulty processing information as quickly and accurately than the others.

-Predominantly hyperactive-impulsive patients (ADHD-HI): they have difficulty staying seated and performing quiet activities, patients talk non-stop and play with everything they have at hand. Regarding impulsivity behaviors are more pronounced impatience, not thinking about the consequences of an action, and the difficulty awaiting the expected events.

-patients (ADHD-C) have characteristics of both (ADHD-HI) both (ADHD-PI) then this category is considered as a subtype of the two combined.

The syndrome can be considered as a complex disease in 70-80% of cases coexists with one or other disturbances, thus aggravating the symptoms and making complex both diagnosis and therapy. The most commonly associated are: oppositional defiant disorder, which consists of anti-social behavior such as stubbornness and aggression, conduct disorder, specific learning disorders (dyslexia, dysgraphia, etc.), memory disorders, anxiety disorders and, less frequently, depression, obsessive-compulsive disorder, which is believed to have a common genetic component, bipolar disorder and Tourette's syndrome (Cantwell, 1996). ADHD children are 2.7 times more likely to have epilepsy (Daviss et al. 2010), showing higher occurrence of subclinical epileptiform activity (Richer et al. 2002; Becker et al. 2004).

One of the more striking aspects of ADHD is its clear inheritance, reflected by a 2-8 fold

increased risk for either parents or siblings of ADHD-diagnosed individuals, and an estimate of 76% heritability based on twin studies (Faraone et al., 2005). The high heritability therefore shows that ADHD involves a complex interaction between genes (Faraone, 2004; Thapar et al., 2005). Several candidate genes have been implied and confirmed by linkage analysis, and several polymorphisms were found in genes coding for different subtypes of receptors and dopamine transporters (DRD4, DRD5 and DAT), serotonin (HTR1B and 5HTT), adrenaline (α2), norepinephrine (NET) and SNAP 25, (Russel, 2011). The multiplicity of these genes supports the idea that the biological heterogeneity may be a central factor to the clinical variability of the disorder (Smalley, 1997). The majority of genes evaluated for ADHD are involved in neurotransmission mediated by biogenic amines: dopamine, noradrenaline, and serotonin (Faraone et al, 2005). The rational for focusing on these genes is drawn largely from the well-documented therapeutic benefit of psychostimulant medication (e.g., methylphenidate and amphetamine) in ameliorating the behavioural impairments associated with ADHD (Castellanos, 1997). In particular, an impaired dopaminergic activity at the base of the symptoms of ADHD has been suggested. Dopamine is involved in the development, behavior and purpose in memory formation linked to a reward or reinforcement (Sagvolden et al., 2005, Johansen et al., 2009). The memory linked to a reward is an ability that, when consolidated, allows to carry out even complex tasks that require long lead times and different stages of reinforcement, and the failure to acquire, due to a deficit in dopamine release, explains the impulsivity and the inattention related to the disease.

Meta-analysis of linkage studies in case-controlled and family-based studies provide consistent evidence for the involvement of the dopamine D4 and D5 receptors, dopamine transporter, the synthetic enzyme dopamine β- hydroxylase, the serotonin transporter and HTR1B receptor (Faraone et al, 2005). Among these genes, only those encoding for D4 receptor (Falzone et al, 2002; Avale et al, 2004) and the dopamine transporter (Gainetdinov et al, 1999) have been tested in mouse genetic models. These studies found significant neurochemical and behavioural effects only in homozygous null mutants.

For what regards structural abnormalities, numerous studies have reported a decrease of

cerebral volume, in particular in the corpus callosum, in the cerebellum, basal ganglia and the prefrontal cortex of the right hemisphere of patients with ADHD (Castellanos et al., 1996, 2002; Hill et al., 2003; Durston et al., 2004; Valera et al., 2007). The functional abnormalities were also found in fronto-striatal circuits and fronto-parietal (Dickstein et al., 2006). In addition, studies of patients with lesions of the right frontal cortex showed a behaviour very similar to that with ADHD (Clark et al., 2006).

The role of environmental factors has been implicated in numerous studis, and have been estimated to account for the additional variability in penetrance reported in twin studies (Spencer et al, 2007; Faraone et al, 2005; Taphar et al, 2005). Several risk factors were identified, including: prenatal exposure to drugs, obstetric complications, head injury, and possibly lead exposure (Biederman and Faraone., 2005).

Experimental evidence indicates that prenatal exposure to ethanol has an effect on dopaminergic transmission, which in turn plays a key role in the onset of hyperactivity (Gibson et al., 2000) Moreover the children of smoking mothers possess an incidence greater in the onset of ADHD compared with controls (Neuman et al., 2007, Schmitz et al., 2006).

ADHD TREATMENT

Stimulant medications are the medical treatment of choice. There are a number of non-stimulant medications, such as atomoxetine, that may be used as alternatives (Wigal, 2009) There are no good studies of comparative effectiveness between various medications, and there is a lack of evidence on their effects on school performance and social behaviors (McDonagh et al, 2007).

The medications used to treat ADHD have pharmacological characteristics highly defined and can be classified according to the following criteria: the mechanism of action or target neurotransmitters.

Except for the α2 adrenergic receptor agonists (eg, guanfacine), all drugs used act indirectly by enhancing and/or prolonging the action of catecholamines. This aim can be achieved in several ways: stimulating the release of catecholamines directly from the pre-

synaptic terminal, inhibiting the reuptake of monoamines (dopamine and norepinephrine) or inhibiting catabolism blocking monoamine oxidase (MAO).

While stimulants and atomoxetine are generally safe, there are side-effects and contraindications to their use (Wigal, 2009) Medications are not recommended for preschool children, as their long-term effects in such young people are unknown (Greenhill et al, 2008) There is very little data on the long-term benefits or adverse effects of stimulants for ADHD (King et al, 2006), however they have the potential for abuse and dependence (Drug Class Review, 2009). Every drug used for ADHD may have adverse drug reactions such as psychosis and mania (Mosholder et al, 2009).

Amphetamine is a psychostimulant and was the first drug used for the treatment of ADHD. In the sixties, the discovery of the therapeutic potential of amphetamine on the disease was completely accidental. There are 2 isomers amphetamine (D and L), the one most marketed today is the D-isomer (Dexedrine ®) which is three times more potent. The primary therapeutic effect of amphetamine is the increased release of monoamines. This is achieved because amphetamine is chemically and structurally similar to dopamine and norepinephrine, thus acting as a competitive substrate for the transporters responsible for the reuptake of dopamine (DAT) and noradrenaline (NET) within the pre-synaptic terminal. Once released in cytoplasm through transporters, amphetamine induces release of catecholamines into the inter-synaptic space, in which they can't be neither reuptaken nor catabolized because amphetamine has occupied respectively the active dopamine transporter (DAT), norepinephrine transporter (NET) and monoamine oxidase (MAO). It may also happen that the amphetamine binds to SERT, a non-selective serotonin transporter, and that affect the levels of this neurotransmitter (Heal et al., 1998). The neurotransmitters "imprisoned" in the space inter-synaptic cause greater stimulation and the efflux of dopamine is increased by 4000-5000% of normal after treatment with D-amphetamine at doses above the therapeutic (Cheetham et al., 2007; Heal et al., 2009). Side effects of D-amphetamine are: weight loss, insomnia, nausea, vomiting, abdominal cramps, and increased blood pressure. Both these effects and the therapeutic action in the treatment of ADHD are caused by the action of the

drug at the central level, so the only way to achieve a balance between risks and benefits is to optimize the doses.

Methylphenidate has a chemical piperidine structure, with two chiral centers which give rise to 4 stereoisomers. The commercial version is the most widely used Ritalin® (methylphenidate-DL-stereo). The mechanism of its pharmacological action is the inhibition of reuptake of dopamine and norepinephrine transporters, acting on DAT and NET. This would suggest a moderate power and a modest and gradual improvement, whereas microdialysis experiments in rats show that the pharmacodynamics corresponds to that of amphetamine, in terms of speed in reaching the peak release, non-existence of a dose that establishes a plateu and release of dopamine and noradrenaline (Cheetham et al. 2007, Heal et al., 2009). Heal (2008) has hypothesized that methylphenidate acts as an allosteric modulator of DAT behaving as inverse agonist. The side effects induced by methylphenidate coincides with the profile outlined for amphetamine.

Atomoxetine is a potent and selective inhibitor of the reuptake of norepinephrine and is one of those non-stimulant medications used to treat attention deficit disorder and hyperactivity. The pharmacological action of atomoxetine in the prefrontal cortex (CPF) is different from that carried out in other districts. This is due to the organization of neuronal CPF and is on the basis of the effectiveness of the treatment and makes undesirable effects less powerful than those caused by amphetamines. The etiology of ADHD may in fact relate to a dysregulation of striatal-cortical catecholaminergic system. In the prefrontal cortex the dopaminergic innervation is poor and moreover the density of DAT transporters is low (Hitri et al., 1991), for this reason the diffusion of dopamine through the appropriate transporter is slow (Cass et al. 1995). Thus the excess of dopamine induce the NET transporter, which is poorly selective for the substrate, to store dopamine in noradrenergic terminals. This process explains how atomoxetine may indirectly act on dopamine levels in the CPF. The drug do not have the same effect in the striatum and nucleus accumbens where dopaminergic neurons are more abundant and organized. Comparing atomoxetine with methylphenidate and amphetamine the release of dopamine and norepinephrine product is lower in terms of

quantity and speed and can reach a plateau phase because the neuronal firing is reduced and operative feedback inhibition of autoreceptors can act. Side effects are common to psychostimulants but with lower intensity.

Bupropion is a weak and moderately selective inhibitor of dopamine reuptake, developed as an antidepressant used against smoking. Preclinical studies have demonstrated its effectiveness in the treatment of ADHD (Casat et al., 1987) in children but not adolescents (Daviss et al., 2001). Given the structural characteristics of the prefrontal cortex bupropion should intervene only at the level of dopamine blocking DAT. However It was found, by means of microdialysis experiments (Li et al., 2002), that with increased levels of noradrenaline, bupropion also inhibits norepinephrine transporter (NET) (Hasegawa et al., 2005). In the striatum and nucleus accumbens dopamine concentrations increase but with less extent than with amphetamine. The less effective, however, leads to decrease the potential for abuse and side effects.

The guanfacine is classified as α2 adrenergic agonist, in particular, it shows a greater affinity for the α2A subtype compared with subtypes B and C. The therapeutic efficacy in the treatment of disorders related to ADHD is given by the activation of postsynaptic receptors in the prefrontal cortex (PFC) (Arnsten, 2006) since PFC has an unusual neural organization that provides multiple receptor α2 A adrenergic on the post-synaptic membrane. Several research groups have found that the release of dopamine and noradrenaline is influenced by the change in the function of the receptors in the CPF, in particular with microdialysis experiments it was found increased levels of norepinephrine release and a decrease of dopamine. However the effect of adrenergic agonists is induced by the action on autoreceptors that have the function of inhibiting neurotransmitter release. The pharmacological profile obtained with the guanfacine differs from that of other drugs. This could be the reason for which is not very effective for certain types of illness.

Doctors usually recommend a psychostimulant drug, considered "first-line". These, despite the well-documented side effects, including possible phenomena of abuse, are used for their

efficacy and tolerability accumulated over many decades. There are patients for whom stimulants can be problematic and inappropriate, such as those with a history of substance abuse disorder or Tourette's. In this case are taken into consideration treatments not-stimulants: atomoxetine or guanfacine. Although there are no comparative trials between the two drugs, doctors generally recommend the guanfacine for young people with tics or with particular difficulties regarding impulsivity and hyperactivity and inattention predominate if atomoxetine or anxiety.

The Bupropion is considered a drug of second choice, not approved by the FDA (Food and Drug Administration) because of its limited efficacy.

In conclusion all available treatments are still imperfect: psychostimulants, which result the more effective, effects can "fade" and not all patients can tolerate them.

BIPOLAR DISORDER

Bipolar disorder is a serious, chronic and characterized by recurrent episodes of mania, hypomania, depression, sometimes in conjunction with each other and cyclic (four or more episodes per year) affects 1-5% of the world population (Etain et al., 2008). Mania is the defining feature of bipolar disorder. Mania is a distinct period of elevated or irritable mood, which can take the form of euphoria, and lasts for at least a week. People with mania commonly experience an increase in energy and a decreased need for sleep (DSMV IV, 1994). Hypomania is a mild to moderate level of elevated mood, characterized by optimism, pressure of speech and activity, and decreased need for sleep. Generally, hypomania does not inhibit functioning as mania does. Signs and symptoms of the depressive phase of bipolar disorder include persistent feelings of sadness, anxiety, guilt, anger, isolation, or hopelessness. In severe cases, the individual may become psychotic, a condition also known as severe bipolar depression with psychotic features. Such neurological disorder is divided into two types: the bipolar type I, characterized by the classic alternation of mania and depression phases, and type II, characterized by the presence of episodes of hypomania and depression (Landmark, 2008).

Different pathophysiological mechanisms have been proposed to explain the development of the syndrome, such as alterations of the functionality of dopaminergic neurons (Greene, 2006), interference with the mechanisms of intracellular signaling and decrease of glutamatergic neurotransmission. The hypothesis of the involvement of inositol in the pathology assumes primary meaning, also because of the use of lithium, as a drug treating the disorder, which acts by inhibiting the enzymatic degradation of inositol phosphate to inositol free and therefore inhibiting the signal mediated by inositol in the cell and the neurotransmission (Rogawsky et al., 2004). Also the intracellular Ca2+ level, important in synaptic plasticity, apparently seems to be involved in the pathogenic mechanisms of bipolarity (Anmann et al., 2005). Another proposed mechanism involves the expression of several genes, including early genes, such as c-fos and c-jun, which encode for several intracellular protein regulators (Brunello et al., 2003). Bipolar disorder, as well as other neurological disorders such as schizophrenia, phobias, post-traumatic stress, neuropathic pain, and anxiety myotonias are determined by changes in the excitability of the central and peripheral nervous system and striated muscles. Recent studies have investigated the effects of anticonvulsant drugs such as valproate, carbamazepine and lamotrigine in the treatment of the disease, with good results. The main pharmacological mechanisms, though still not fully understood, to the success of antiepileptic drugs in the treatment of other neurological diseases, is represented by their action on GABAergic and glutamatergic neurotransmission, on voltage-gated ion channels and intracellular signaling pathways (Landmark, 2008).

SNARE PROTEINS *(solubile NSF (N-ethylmaleimide-sensitive) fusion protein)*

In the central nervous system (CNS), neural activity is mediated by the synaptic release of neurotransmitters, a mechanism that needs the fusion of vesicles, containing that chemical signals, to the membrane of the pre-synaptic neuron, and the release of the neurotransmitters in the synaptic cleft, were they can reach and bind the selective receptors. This mechanism, that allows the communication between the neurons, is defined chemical synapsis. More in details, this process begins when an action potential reaches the pre-synaptic neuron termination inducing a variation in the membrane potential and the opening of Ca^{2+} voltage gate dependent ion channels (VGCC) in the presynaptic membrane. By means of the different concentration of Ca^{2+} ions between the two side of the presynaptic membrane (10^{-3} inside, 10^{-7} outside), the opening of ion channels induces a rapid increase of calcium inside the neuron. The high calcium concentration activates a set of calcium-sensitive proteins attached to vesicles, allowing their fusion with the pre-synaptic membrane and the neurotransmitters release in the synaptic space (Katz, 1969). The released transmitters diffuse across the synaptic cleft and bind to specific receptors on the membrane of the postsynaptic neuron. The binding of the neurotransmitter to receptor, induces a change in the conformation of the channels in the postsynaptic membrane, by changing the ability of the ions to pass it (incoming or outgoing). The resulting current flow changes the conductance and the membrane potential of the postsynaptic cell, by increasing or decreasing the probability that the neuron triggers an action potential. In this way the information is transferred from one neuron to another (Purves et al., 2005). Although the mechanism through which the Ca2+ triggers the exocytosis has not been fully elucidated, experimental evidences demonstrate how specific proteins, on the surface of the synaptic vesicle and elsewhere on termination synaptic, mediate this process (Augustine et al. ,1999). It is generally accepted that the SNARE proteins (soluble NSF (N-ethylmaleimide-sensitive) fusion protein) isolated from Rothman in 1994, are directly involved in the fusion of vesicles to the cell membrane of the presynaptic neuron forming a complex of crucial importance, the SNARE complex (Graham et al., 2002). It consists of two protein components: a vesicular (v-

SNARE) and a membrane (t-SNARE) component. To the first group belong the synaptobrevin or VAMP (vesicle-associated membrane protein), while syntaxin and SNAP-25 (soluble NSF-attachment proteins) to the second group (Loranger et al., 2002). In the Q/R nomenclature for organizing SNARE proteins, VAMP/synaptobrevin belongs to R-SNAREs, so named for the presence of an arginine at a specific location within the primary sequence of the protein, while Syntaxin and SNAP-25 are Q-SNARES, since they contain a glutamine in the formation of the zero ionic layer in the assembled core SNARE complex instead of the arginine. Syntaxin and VAMP have a carboxy-terminal transmembrane domain (Lin et al., 2000), while SNAP-25 is connected to the membrane by the palmitoylation of four cysteine residues located in the middle of the polypeptide chain (Hess et al., 1992). Like all proteins involved in eso-/endocytosis, SNARE proteins are initially inserted into the membrane of the endoplasmic reticulum (ER), from which they are transported to their destination compartment. Because of their particular structural features, however, the SNARE are inserted into the membrane of the reticulum through a mechanism different from that used by other membrane proteins. The SNARE are part of a class of proteins called "tail-anchored". These proteins are characterized by the presence of a transmembrane domain very close to the carboxy-terminal; most of the mass of the protein is cytosolic and is anchored to the lipid bilayer by the C-terminal tail. Almost all of the membrane proteins use the co-translational pathway to integrate into the phospholipid bilayer of the ER. This path is based on the co-translational bind of a signal sequence to the nascent polypeptide, the signal recognition particle (SRP); SRP brings the ribosome with the nascent polypeptide to the ER membrane and is then integrated in the membrane, through the channel Sec61. This co-translational mechanism is not usable from "tail-anchored" protein, including the SNARE, because the hydrophobic sequence, being near the C-terminus, remains masked by the ribosome and not accessible to SRP. The inclusion of protein "tail-anchored" in the ER membrane is then forcibly post-translationally. SNARE proteins are capable of forming a highly stable complex in vitro, which may represent the event immediately preceding the fusion of the membranes in vivo. The complex is generated by a four helix bundle of about 12 nm with an almost

parallel orientation: two arising from SNAP-25, one from VAMP and last one from syntaxin (Graham et al., 2002). The assembly of complex v-/t-SNARE in cells appears to be under the control of regulatory proteins; between these there are several members of the family of Rab GTPases and Sec1 (Weber et al., 1998). These proteins have also been observed in various cell types with non-neuronal regulated exocytosis, and also in organisms such as yeast (Elferink et al., 1989; Bennet et al., 1993)

This complex is in the trans configuration respect the two transmembrane domains, one in the vesicle and the other in the cell membrane: in this conformation the surface of the complex has the largest number of furrows and charges exposed, allowing the interaction with other proteins and then the fusion of the membranes. After the fusion, the complex switch mode cis with both transmembrane domains in the same membrane (Montecucco et al., 2005). Due to the extreme stability of the heterotrimeric complex that is formed (the t / 2 for disassembly spontaneous was estimated in 1010 years), the intervention of other proteins (NSF, NEM-sensitive fusion protein, and SNAPs other) to dissolve the bond that forms between the four helices and recover from the synaptic vesicle membrane is necessary (Fasshauer et al., 2002; Sorensen, 2005).

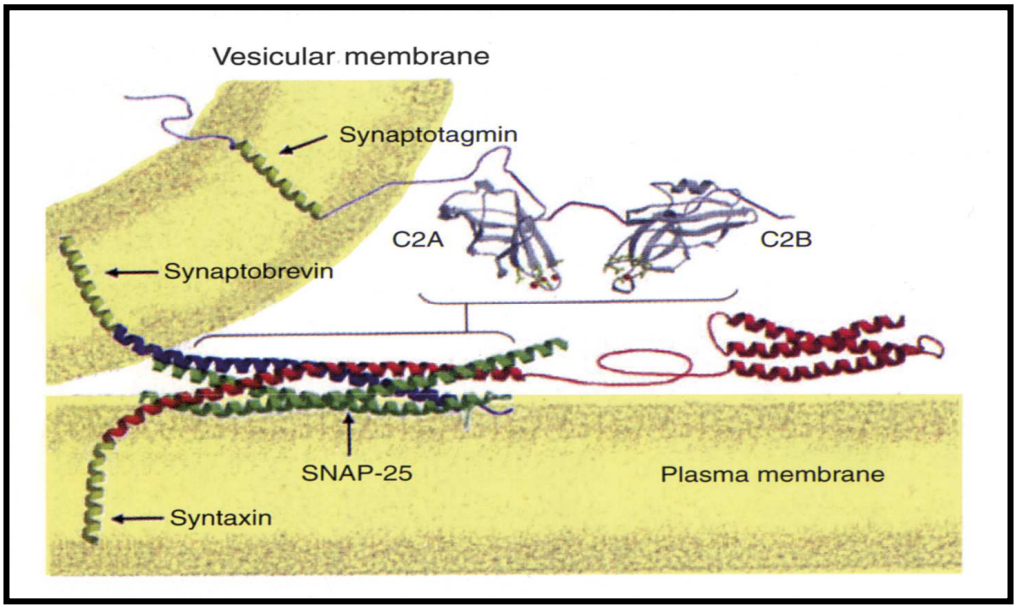


Fig A: SNARE and synaptotagmin (from *Cellular and Molecular Neurophysiology*, 2008)

In recent years it has been assumed that various SNARE complexes are connected through bonding with SNAP-25, to form a rosette around the fusion pore, a ring of SNARE

complexes necessary for the regulated rapid exocytosis (Weber et al., 1998 , Montecucco et al., 2005).

The SNARE proteins do not bind Ca^{2+}, so other molecules must intervene in the regulation of Ca^{2+}-dependent neurotransmitter release. Several presynaptic proteins (calmodulin, CAPS and Munc-13 for example) are able to bind this ion, however, the main candidate for the regulation of neurotransmitter release Ca^{2+}-dependent is synaptotagmin (which has been demonstrated to interact with the protein SNAP-25) (Schiavo et al., 1997). The synaptotagmin is a membrane protein of synaptic vesicles, equipped with two repeated structures, called domains C2 (C2A and C2B), through which binds Ca2 + (Augustine, 2001). In support of this hypothesis has been demonstrated that changes in the activity of synaptotagmin in mice, fruit flies, squid and other animals, alter the Ca2 +-dependent release of neurotransmitters. In fact, the deletion of even one of the 12 genes of synaptotagmin constitutes a lethal mutation (Littleton et al., 2001). It is not clear how the binding of Ca2 + to the molecule can lead exocytosis, but it is known that Ca2+ changes the chemical properties of synaptotagmin, allowing it to inserts into the membrane and interacts with other proteins. A possible model predicts that the SNARE proteins cause the intimate juxtaposition of the two membranes, and Ca2+-induced structural changes in synaptotagmin mediate the final stages of the fusion of synaptic vesicles (Purves et al., 2005).

VAMP or SYNAPTOBREVIN

VAMP is a protein consisting of 120 amino acids. There are two isoforms, VAMP-1 and VAMP-2, both integral membrane proteins of synaptic vesicles (Elferink et al., 1989). They are formed by a N-terminal proline-rich sequence, isoform-specific; a segment conserved among different isoforms and that contains a short α-helix that is inserted in the region at the interface of the lipid membrane, and is also a phosphorylation site for the Ca2 + / calmodulin kinase type II; by a transmembrane domain and a tail intraluminal poorly conserved and with variable length, due to alternative splicing. On the membrane vesicular VAMP may be associated with synaptophysin, membrane protein, and with the v-ATPase (Calakos et al.,

1994). In contrast to syntaxin and SNAP-25, mainly distributed in the central nervous system and endocrine VAMP has a ubiquitous distribution: VAMP-like proteins are expressed in adipocytes, where they regulates the sequestration of intracellular glucose transporter GLUT-4 (Bennet et al., 1993). An homologue of synaptobrevin was also identified, the cellubrevine (McMahon et al., 1993), for example expressed in the basolateral membrane of epithelial cells, where it seems, however, involved in the processes of exo-/endocytosis (Fields et al. 2007).

SYNTAXIN

Syntaxin has more than 20 isoforms in mammals and homologues in plants and yeast. In the nervous tissue two isoforms were characterized: syntaxin1 and syntaxin2. They are linked to the presynaptic membrane by a transmembrane segment connected to a short C-terminal extracellular domain and to a large cytosolic portion. The N-terminal domain is formed by three long α-helices that are involved in the protein-protein interaction (Chen et al., 2008). In the active zones of the synaptic terminal, syntaxin is associated with several types of Ca2+ channels (Davies et al., 2011) and interacts with proteins, Sec-1/Munc-18, involved in the intracellular events of membrane fusion. It is hypothesized that this interaction is responsible for the mechanisms of attack and vesicle fusion mediated by the SNARE complex (Li et al., 2011). During the long-term potentiation, isoforms may undergo a complex pattern of alternative splicing. This observation suggests a possible involvement of syntaxin in the mechanisms of synaptic plasticity (Kennedy et al., 2010). As for the synaptobrevin, also for the syntaxin were identified several non-neuronal counterparts (Franck et al., 2011), thus suggesting that the SNARE proteins include many protein families.

SNAP-25

SNAP-25 is a membrane-assocated protein located in neuronal presynaptic plasma membrane, conserved from yeast to man. Given the absence of a transmembrane domain, it is thought that its localization is mediated by palmitoylation of certain cysteine residues in the middle of the polypeptide chain (Hess et al., 1992); SNAP-25 is able to self-assemble into a

dimer linked by a disulfide bridge both in vitro and in vivo (Sadoul et al., 1997). The encoding gene was mapped on chromosome 2 and is active in different neuronal populations, such as neurons of the neocortex, the anterior thalamic nuclei, hippocampus and granular layer of the cerebellum (Oyler et al., 1989).

SNAP-25 is essential for the release of neurotransmitters (Augustine et al., 1999), in axonal growth during development and in the synaptic plasticity. SNAP-25 has the characteristic of being observed only in the CNS at the synaptic level unlike the other SNARE proteins; numerous isoforms of syntaxin and VAMP have been found in recent studies on CD8+ cytotoxic lymphocytes even during the attack phase of target cells (Pattu et al., 2011). There are two different isoforms of SNAP-25, SNAP-25a and SNAP-25b, arising from alternative splicing in exon 5 of the gene encoding the protein and the two isoform are different for only nine amino acids. The first of these two isoforms is more expressed during embryonic development, while the second in the adult brain (Bark et al., 1995). SNAP-25 has a key role in the formation of the SNARE complex, contributing with two of its four alpha-helical chains. Generation of SNAP-25 null mutant mice revealed that SNAP-25 is not required for stimulus-independent neurotransmitter release, but is essential for evoked synaptic transmission (Washbourne et al., 2002). Furthermore, clostridial toxins, which specifically cleave selected components of the SNARE complex, have unequivocally demonstrated the requirement of SNAP-25, syntaxin and synaptobrevin in vesicle exocytosis (Jahn et al., 2003; Schiavo et al., 2000). Besides its cognate SNARE proteins, SNAP-25 also interacts with the synaptic vesicle protein synaptotagmin I (Schiavo et al., 1997), providing an essential mechanism for triggering the Ca^{2+}-dependent membrane fusion (Zhang et al., 2002) and for controlling fusion pore dynamics during the final steps of exocytosis (Bai et al., 2004).

Increasing evidence supports that SNAP-25 also seems to regulate the activity of several voltage-gated ion channels: specifically interacts with different types of ion channels of voltage-gated Ca2+ (VGCCs), including N, L and P / Q N type, through a channel region known as the synaptic protein interaction (synprint) site, modulating the release of neurotransmitters (Corradini et al., 2009). It has been demonstrated that SNAP-25 controls

negatively neuronal Ca2 +-dependent responsiveness to depolarization (Verderio et al., 2004), inhibiting specifically, as a result of phosphorylation of Serine 187 by the Protein Kinase C, the neuronal VGCC channels (Pozzi et al., 2008). A further confirmation of this role of SNAP-25 derives from the studies on mutant mice with reduced total expression of the protein and with a dominance of the embryonic isoform. Is hypothesized that the altered kinetics of Ca2 + associated with the presence of SNAP-25a isoform, can be the cause of the increased level of hippocampal synaptic plasticity (Scullin et al., 2012). SNAP-25 plays the inhibitory action on the vesicular release in glutamatergic synapses, while seems to have no influence in the GABAergic synapses (Matteoli et al., 2009). Genetic reductions in SNAP-25 expression do not impact on SNARE-dependent neurotransmission (Bronk et al 2007, Delgado-Martínez et al., 2007), but in glutamatergic neurons lead to an augmentation of VGCC activity, slower inactivation kinetics, and a significant depolarizing shift in the voltage dependence of inactivation of the dominant P/Q-type current (Condliffe, 2010).

Therefore, reduced levels of SNAP-25 are sufficient to support exocytosis, but variations in SNAP-25 expression level alter VGCC function, which would impact on network excitability.

SNAP-25 therefore represents a multifunctional protein involved in the control of neurotransmitter secretion via several interactions. The concept that SNAP-25 plays additional functions besides formation of the pore complex is in line with the evidence that the protein, which is probably the most abundant protein in the brain, making up 1% of brain protein (Walch-Solimena et al., 1995), is not exclusively localized at synaptic sites but is also present all along axons and dendrites (Galli et al., 1995)

Clinical studies in children have shown that SNAP-25 is associated with working memory, which is the ability to store information on a task that is under completion. Children who express a specific polymorphism of SNAP-25 have abnormalities in gray matter density in the posterior cingulate cortex, which is normally involved in the regulation of attention, and also for this reason its involvement in ADHD has been suggested (Söderqvist et al., 2010). SNAP-25 in hippocampus CA1 area is involved in the consolidation processes of spatial and fear

memory. In addition, blocking SNAP-25 at the level of the area CA3, a role of this protein even in long-term memory and learning has been documented (Hou et al., 2006).

SNAP and ADHD

Two of the most studied animal models of ADHD are characterized by an altered expression of SNAP-25 gene. Coloboma mutant mouse has a reduced expression of the genes, and also the SHR rats have a decreased gene expression of SNAP-25 in the prefrontal cortex (Li, 2009). Polymorphisms at the SNAP25 gene locus in humans have been examined and association of SNAP-25 with ADHD has been determined in a number of linkage studies, (Barr et al, 2000; Brophy et al, 2002; Mill et al, 2002; Kustanovich at al, 2003; Mill et al, 2004; Feng et al, 2005; Choi et al, 2007)33-39 a finding that has been further confirmed by meta-analysis.18 Case-control studies of families indicates that the gene coding for SNAP-25 is associated with ADHD (Mill et al., 2004; Faraone et al., 2005; Feng et al., 2005). Consequently single nucleotides polymorphisms (SNPs) at the level of the introns of the gene coding for SNAP-25 have been linked to hyperactivity and inattention in a group of children suffering from ADHD (Zhang et al., 2011), while the single nucleotide polymorphisms on intron 1, which probably regulates the levels of expression of the protein, are associated with hyperactivity in autism-related disorders (Guerini et al., 2011). In a recent study, 61 SNPs across the SNAP-25 gens have been assayed and six of them were found to associate with ADHD and co-morbid major depressive disorder (Kim at al, 2007). The environmental lead exposure is a risk factor for ADHD (Braun et al., 2006). Maternal mouse exposure to lead reduced the expression of SNAP-25 in the hippocampus of pups (Li et al, 2009), suggesting an association of SNAP-25 with lead exposure and ADHD (Ganizadeh, 2011).

SNAP and SCHIZOPHRENIA

A recent meta-analysis of 20 separate genome-wide linkage scans for schizophrenia susceptibility genes reported significant linkage to the chromosomal region 20p12.3-11, which contains SNAP25, and suggested 20p12.3-11 as a strong candidate region for the

disease (Lewis et al, 2003). Evidence from several immunohistochemical and Western blot studies in postmortem brains implicated reductions in SNAP-25 expression in the etiology of schizophrenia. Decreased levels of SNAP-25 were found in the hippocampus (Young et al, 1998) and in the frontal lobe Broadman's area 10 of patients with schizophrenia in comparison with controls. In contrast, elevated levels of SNAP-25 were found in prefrontal lobe Broadman's area 9 (Thompson et al, 1998) and cingulate cortex (Gabriel et al, 1997). It has been suggested that these discrepancies in altered expression between different brain regions may not only reflect the depressed functionality of certain neural circuits, but also the hyperactivity of other pathways that may possibly result from compensatory mechanisms elicited through the neuropathophysiology of this disorder (Thompson et al, 2003). The analysis of two genetic models has recently supported the involvement of SNAP-25 in the neuropsychopathology of schizophrenia. Firstly, the mutant hDISC1 transgenic mouse, which has been proposed as an animal model of schizophrenia characterized by spontaneous locomotor activity, impaired spatial reference memory evaluated in the Morris water maze and abnormal social interaction, as shown by the decreased social non-aggressive activity, has been recently shown to lead to a significant reduction of SNAP-25 expression (Pletnikov et al, 2008). Secondly, a chemical mutagen induced dominant mutation (I67T missense) in a highly conserved domain of Snap25 of the blind-drunk mutant mouse, appears to give rise to a schizophrenic phenotype that includes impaired sensorimotor gating, an important component of the schizophrenia phenotype related to altered sensory processing, anxiety and apathetic behaviour - phenotypic elements which appear to replicate aspects of the negative symptomatology of the disease (Jeans et al, 2007).

SNAP and EPILEPSY

Interestingly, the mutant mouse coloboma (Cm/+), which has been implicated as a model of ADHD (see above), has also provided evidence for the involvement of SNAP-25 in epilepsy. In addition to their hyperkinetic activity, Cm/+ mice display frequent spontaneous bursts of bilateral cortical SWDs that are accompanied by behavioral arrest, which is typical

of absence epilepsy. These seizures could be completely blocked by intraperitoneal injection of the antiepileptic drug ethosuximide (Zhang et al, 2004). Analysis of calcium current amplitude in thalamocortical neurons of this mutant, moreover, revealed an increase in the peak current density of LVA currents in Cm/+ cells compared to wild type mice. It is important that this calcium increase is not itself seizure-induced, as it precedes the developmental onset of SWDs (Zhang et al, 2004), suggesting that the seizures may arise from abnormalities in calcium transients caused by a SNAP-25 deficiency in modulating presynaptic voltage-gated calcium channels (Pozzi et al, 2008). The role of SNAP- 25 in the etiopathology of epilepsy may involve alterations of synaptic formation/refinement during development. The epileptic phenotype of SNAP-25 mutant mice may be produced by a defect in the overall brain connectivity consequent to the reduced expression of the protein, which is known to play a crucial role in neurite extension (Osen-Sand et al., 1996). Furthermore SNAP-25 is expressed at much higher levels at excitatory vs. inhibitory synapses (Bragina et al, 2007) and is able to negatively modulate calcium dynamics (Pozzi et al, 2008). Thus, reduced levels of SNAP-25 may lead to increased calcium currents (Condliffe et al., 2010) and consequently to the onset of epileptic discharges (Bozzi et al, 2012).

SNAP and BIPOLAR DISORDER

Recently six gene regions that potentially confer susceptibility to the development of bipolar disorder have been identified (Coyle et al., 2003). Among these the 20p12 region, identified by three independent studies, containing the gene encoding the protein SNAP-25 (Cichon et al., 2001). Several evidences led to hypothesize SNAP-25 as a candidate gene for the development of the pathology: it is a protein essential for presynaptic anchoring of vesicles containing neurotransmitters (Augustine et al., 1999) and post-mortem studies show a decrease in certain regions of the brain of bipolar patients (Fatemi et al., 2001 Scarr et al., 2006). The abnormalities found in patients in the neurotransmission of dopamine, serotonin and norepinephrine are due to an altered exocytosis of these neurotransmitters, caused in

particular by alterations of SNAP-25 isoform b. In human the polymorphism in the promoter region of the gene for SNAP-25, variant-523c/A, is associated with higher SNAP-25 expression in the prefrontal cortex and with early-onset bipolar disorder (Etain et al., 2010).

ANIMAL MODELS OF ADHD

The development of animal models of a given human pathology is very important in order to investigate its pathogenesis, in particular in the case of neuropsychiatric disorders. Direct studies on patient are not possible due to obvious ethical reasons and limitations of available techniques. It is however difficult to reflect the human psychiatric disorders in animals: only the basic cognitive processes such as memory or socializing can be evaluated, but the complex ones can't be represented, as language.

You need three minimum requirements to be considered a valid animal model to represent a human disease (Willner, 1986): the first (face validity) provides that the model mimics the core symptoms of human disease, the second (construct validity), requires an aetiology which is similar to the human disease, the third characteristic (predictive validity) provides that the treatment with the drugs used for human results equally effective and so is possible to investigate the molecular causes of the disorders and test the possible innovative therapies (Sagvolden et al., 2000, 2005).

Several animal models of ADHD were developed, obtained by crosses (spontaneously hypertensive rat SHR), genetic mutations (knockout mouse for the dopamine transporter DAT, coloboma mutant mice, mice mutated receptor for thyroid TRβ1, mice mutated for the α4β2 nicotinic receptor) or by exposure to chemicals, such as ethanol, nicotine and polychlorinated biphenyls, or to environmental factors, such as anoxia and social isolation.

The rat model (SHR) is the most widely studied. It was developed crossing consanguineous Wistar-Kyoto (WKY) and is thus a natural model of ADHD. It has all the major symptoms of the disease: impulsivity, hyperactivity and inability to maintain attention. For what regards the hyperactivity the SHR rat shows this behaviour only in familiar surroundings but not in new environments (Sagvolden et al., 2000, 2005), these results are in

line with the studies of humans with the disease. In addition to behavioural changes, in SHR rats there is an altered release of dopamine in the prefrontal cortex, nucleus accumbens and striatum (Russell et al., 1995) and an increased release of noradrenaline glutamate-dependent (Russell et al., 2000). To validate the model SHR, the hyperactive rats were treated with methylphenidate and D-amphetamine (two psychostimulant used in the treatment of human disease) (Sagvolden et al., 1992) and with a α2A-adrenoceptor agonist (guanfacine) that is found to be effective in improving clinical impulsivity, hyperactivity and inattention (Hunt et al., 1995). In both cases SHR rats showed an improvement in symptoms (Sagvolden et al., 2006). The SHR model, however, is characterized by high blood pressure, which is not however observed in human subjects with ADHD, and it is likely that this alteration in blood pressure the underlying cause of hyperactivity observed in these animals. An analysis of WKHA rats (SHR and WKY obtained by crossing) showed that loosing hypertension the hyperactivity does not improve with the use of methylphenidate (Drolet et al., 2002).

Within the transgenic models, there is the transgenic mouse models mutant for TRβ1 (thyroid receptor expressed primarily in the brain). It was found in fact that children with high TSH (thyroid stimulating hormone) show the symptoms of ADHD (Burd et al., 2003). This is explained by considering that the thyroid hormones are associated with the control of the development of certain brain systems, in particular those related to attention, motor activity, motivation and impulsive behavior. The result is that the mutant mice for the receptor TRβ1 show the same symptoms of ADHD and it is possible to reduce hyperactivity with methylphenidate. A second example is found in the mouse model with deletion of the β2 subunit of the nicotinic receptor. It has the classic symptoms of ADHD (inattention, hyperactivity and loss of inhibitory control). A4β2 nicotinic receptor agonists in fact reduce the symptoms ADHD-like in the animal model (Granon et al., 2006). These two models essentially mimic the symptoms of the human disease, but the genetic deletions that characterize these models does not coincide with the polymorphisms found in clinical trials, then the etiology will be different. Two other mouse models, instead, were developed by

means of genetic manipulation of genes that genetic studies on human ADHD patients have reported as polymorphisms: mouse knockout (KO) for the DAT gene and the coloboma mouse.

In the mouse model knockout (KO) for the DAT gene (responsible for the reuptake of dopamine in the pre-synaptic terminal) extracellular dopamine levels are 10 times higher than the wild type (WT) in the nucleus accumbens and striatum and there is a down regulation of the autoreceptors (DRD1, DRD2) (Gainetdinov et al., 1999; Shen et al., 2004). DAT is also the target of amphetamine and methylphenidate and there has been a strong association between this gene and ADHD (Thapar et al., 2005). The DAT KO mice unlike clinical cases also possess hypermotility in new environments, while the deficits in learning and memory are similar to the human symptoms (Gainetdinov et al., 1999). As regards the responses to drugs, there has been a reduction in hyperactivity with methylphenidate and amphetamine, and also using selective inhibitors of the reuptake of norepinephrine (atomoxetine) or the α2A adrenergic receptor agonists; although the mechanism of action of certain drugs in this model remains unclear. The limits of KO mice DAT are due to the fact that the increased levels of dopamine in the striatum and nucleus accumbens is not found in patients and that, given the deletion of the gene coding for DAT, can't be explained how methylphenidate and amphetamine can act.

Coloboma mutant mouse (Cm/+) is heterozygous for a neutron irradiation induced semidominant deletion mutation, spanning 4.6 Mb on mouse chromosome 2, that encompasses 37 genes, including Snap25, Il-1a (interleukin 1a), Prn-p (prion protein), Pax-1 (paired box gene-1) phospholipase C beta-1 (Plcb1), coloboma (cm), Plcb4, and Jag1, (Hess et al., 1996). In coloboma mice, deletion of the Snap25 gene results in 50% lower amounts of the SNAP-25 mRNA and protein expression compared to wild-type mice. Interestingly, coloboma mutant mice possess several phenotypic characteristics that parallel ADHD symptoms. Coloboma mice exhibit normal circadian rhythm and, as children with ADHD, they are hyperactive during their active (nocturnal) phase, with locomotor activity averaging three fold the activity of control littermate (Hess et al, 1992) and also shows impulsivity and

inattention (Wilson, 2000; Bruno et al., 2007). Two key observations have led to the proposal that this mutant with reduced level of SNAP-25 expression represents a model for at least the hyperkinetic component of ADHD: 1) the hyperactivity was ameliorated by a low dose (4mg/kg, i.p.) of the psychostimulant amphetamine (AMPH), and 2) genetic rescue of the hyperactivity (but not the eye defect, or head bobbing) was obtained by crossing the mutant with SNAP-25 overexpressing transgenic mice (Hess et al, 1996). Interestingly, transgenic rescue of SNAP-25 function also restores normal dopaminergic transmission (Steffensen et al, 1999).

Coloboma mice have some characteristics that are not present in the human pathology. The hyperactivity is reduced with amphetamine but not with methylphenidate (Hess et al., 1996; Wilson, 2000) The difference in effect is likely to be due to the different actions of these two drugs. Both increase the extracellular concentration of catecholamines through blockade of the dopamine and norepinephrine transporters, but d-amphetamine also increases the release of these neurotransmitters (Russel, 2007). They have retinal degeneration and are thus functionally blind, they show an increased frequency of head bobbing and circling compared to wildtype littermates (Heyser et al, 1995). Furthermore Cm+/- mice have severe sensory, motor coordination and balance deficits, which may preclude them as a model of ADHD (Gunn et al, 2011). The re-insertion of the snap-25 gene in Cm mice did not stop circling or head-bobbing behaviors (Hess, 1996; Steffensen et al, 1999), suggesting that other genes in the deletion region have significant effects on the Cm mouse behavioral phenotype.

AIM

SNAP-25 is a cytosolic protein that is necessary, along with other proteins, for the SNARE complex formation. It which plays a key role in the phenomenon of exocytosis of synaptic vesicles (Augustine et al., 1999) and negatively modulates the calcium responsiveness in glutamatergic neurons (Matteoli et al., 2009). Experimental evidence underlies how alterations in SNAP-25 expression may be involved in diseases and mental disorders such as ADHD, schizophrenia, epilepsy and bipolar disorder (Corradini et al, 2009). Thus the first aim of the present study was to investigate at which extent selective reduction of the protein levels, as occurring in psychiatric diseases, affects neuronal network function and mouse behaviour. While SNAP-25 homozygous mutant mice die at birth from respiratory failure, heterozygous (SNAP-25+/−) mice are viable (Washbourne et al. 2001).

Behavioural and electroencephalographic (EEG) characterization were carried out on adult male mice heterozygous for SNAP-25 (protein expression reduced by 50%), kindly provided by Prof. Michela Matteoli.

Firstly, general health (fur, pilo-erection, body tone, skin colour), reflexes (righting, corneal, pinna, tail pinch) and sensory abilities (vision, hearing, smell and pain) will be investigated. Spontaneous motor function will be analysed through a motor activity cage; rotarod for motor coordination and hanging wire for muscle strength. Emotional reactivity will be evaluated in the elevated plus maze and in the hole board for anxiety-like behaviour and in the forced swim and tail suspension test for depressive symptoms.

Then we will focus on mnemonic, social and EEG profile, since in human altered levels of SNAP-25 are linked to diseases characterized by alteration in these parameters (Nagy et al, 2009). Latent inhibition test will be used to highlight any attention deficit, while memory will be investigated by means of T-maze (spatial memory), object recognition (episodic memory), and conditioned taste aversion (associative memory). We will perform sociability and preference for social novelty test in order to evaluate the social behaviour of our mice and finally we will analyse their EEG profile in awake animals, recording their cortical activity for 24 hours after surgical implantation of electrodes in the parieto-occipital cortex. We are also

planning to study the effect of anticonvulsant drugs such as valproate, carbamazepine and ethosuximide and of a calcium antagonist, Nimodipine, on EEG. The most active compounds in normalizing EEG profile will be used to evaluate the possible recovery in memory and social deficits, since is known that alteration in cortical activity could induce cognitive deficits (Aldenkamp et al, 2010).

SNAP-25 is strongly associated to the onset of one of the most common neuropsychiatric disorders among children and adolescents, ADHD (Faraone et al, 2005), whose symptoms are hyperactivity, inattention, impulsivity, cognitive deficits and EEG alterations. Thus the second aim will be to characterize, from a behavioural and EEG point of view, a possible new animal model of disease, generated by heterozygous SNAP-25 mice evaluated at 6-7 weeks of age, corresponding to timing of disease onset in humans. Animals will be divided in two groups, one exposed to plain water, the other to an antiepileptic solution, for 21 days, in order to mimic the human posology. The antiepileptic drugt will be chosen on the basis of the previous experiments performed on adult animals.

We will investigate mnemonic, social and EEG profile by means of the same tests used in adult mice. Furthermore, to test the possible hyperactivity, the spontaneous motor activity will be evaluated by means of an activity cage for the recording of horizontal movements. Horizontal movements will be recorded for 7 hours, the first 4h as a baseline and the last 3h following treatment with amphetamine (first-line drug in the treatment of ADHD) to determine whether the latter is able to decrease the possible hyperactivity in SNAP-$25^{+/-}$, as observed in humans ADHD patients (Elia et al, 1991). Moreover, since is known that ADHD patients display EEG abnormalities, not only in terms of spike activity (Hughes et al, 2000), but also for what regards the % of each frequency bands in the spectral power (Loo and Makeig, 2012) we will analyse also this parameters in the adolescent mice.

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. All mice used were littermates from mated heterozygous and were genotyped by PCR. Animals were randomly assigned to each experimental group and tested at 3 month of age (adults) or at 6-7 weeks of age (adolescents). Animals were singly housed throughout the testing period with free access to food and water at controlled temperature (20-22℃) with a 12-h light/dark cycle (lights on at 7:00 A.M.). 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

General health and reflexes.

Each mouse was observed and any abnormalities in the general appearance of the fur (3-point scale), piloerection, body tone (3-point scale), skin color were recorded according to Lee et al (2008). In addition, mice were observed for the empty cage behaviour (nest-building behaviour) (Deacon, 2006). Neurological reflexes, including righting reflex (4-point-scale) corneal (8-point scale), pinna (8-point scale), and tail pinch (8-point scale) (Irwin, 1968) were assessed.

Sensory abilities

Hearing was evaluated using Preyer's reflex testing through a handclap sound. The reflex was considered positive when a rapid movement of the whole body of the animal was clearly noticed as previously described (Jero et al., 2001).

Visual acuity was performed using the visible cliff test (Brandewiede, 2005). The apparatus consisted of a wooded laminated box. A platform was installed adjacent to a wall 50 cm above the box floor. The platform and the inner surface of the box were covered with

black and white checkerboard contact paper that emphasized the ledge drop-off. A piece of clear Plexiglas covered the platform and spanned the ledge so that there was no actual drop-off but only the visual appearance of the cliff. Mice were placed on the platform close to the wall. Mice that stopped at the “edge” and explored the plexiglas floor before walking forward were considered to have good sight.

Olfactory test was carried out as described (Moy et al., 2004). Two days before the test, an unfamiliar food (Kellogg’s cereal) was placed overnight in the home cage of the subject mice. On the test day, each mouse was placed in a large cage containing 3 cm deep sawdust and allowed to explore for five min. The animal was removed from the cage, and one cereal was buried in the cage bedding. The animal was then returned to the cage and given 15 min to locate the buried food. Measures were taken of percent of uncovered buried food and latency to find the cereal.

Pain sensitivity was evaluated by means of the hot-plate assay, performed at 52℃. The time elapsing to the first pain response (licking or jumping) was scored. A maximal latency of 40 s was used (Crawley, 2000).

Motor functions

Spontaneous motor activity 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 the proband mice were habituated to the testing room for at least 1 h. Cumulative horizontal movement counts were recorded for 1h.

Coordination and balance were evaluated during four consecutive trials by quantifying the ability to maintain balance on a rotating cylinder using the Rotarod test (Dauge et al., 2001). The latency to fall was recorded and the percent of animals remaining on the rod during the last trial for 120 s was monitored.

Muscle strength was measured using the hanging wire cage test. Mice were placed on the underside of a standard wire rat cage top approximately 20 cm above a large cage

containing soft wood-chip bedding. The latency to release was recorded, with a maximum latency of 60 s (Crawley, 2000).

Emotional profile.

Hole Board test. Exploratory behaviour was assessed using the board-hole test (File et al., 1998) placed in a dimly illuminated room. The apparatus consisted of a square with 16 holes (2 cm diameter), regularly spaced on the surface, at 3.5 cm from edges. Mice were placed in the centre of the plate, and the number of head dips was immediately counted for 5 min.

Elevated plus maze. Anxiety was measured in an elevated plus-maze (Lister, 1987). Each animal was placed onto the centre of the apparatus and the time spent on and entries onto each arm was noted for 5 min. The percentage of time spent in the open arms and the percent of open-arm entries were used as a measure of anxiety.

Forced swim test. This procedure was performed as previously reported (Porsolt et al., 1977). A 2-day procedure in which mice swim under conditions where escape was not possible, was used. On the first day, mice were forced to swim for 15 min. Twenty-four h later, they were re-tested for 5 min in the same conditions and time spent immobile was measured.

Tail suspension. The test is based on the observation that a mouse suspended by the tail alternates periods of immobility and agitation. It was conducted in according to Steru et al (1985): mice were suspended by the tail with an adhesive tape and left dangling in the air for 6 min. The duration of immobility (s) was measured.

T –maze.

Animals were food-deprived until reaching 85% to 90% of their free-feeding body weight. Mice were then habituated to a black wooden T-maze (stem length 41 cm; arm length 91 cm, each section was 11 cm wide with 19 cm high side walls). Mice were shaped to obtain food in the T-maze for 5 days (Moy et al., 2008). In the acquisition phase, for each mouse one

arm was designated as reinforced, where one palatable food reward (Kellogg's cereal) was available for each of 10 daily trials. The reinforced arm was on the left side for half of the mice, and on the right side for the other half. At the beginning of each trial each mouse was placed at the maze start and given a free choice to enter either arm. If the mouse made the correct choice, it was given time to consume the pellet and then guided back to the start for the next 9 daily trials. Incorrect choices were not rewarded or punished. The number of days to reach the criterion - that is, showing 80% of correct choices for 3 days - was recorded.

Each mouse that met the criterion for acquisition was then tested using a reversal procedure, in which the reinforced location was switched to the opposite arm, following the same method as described above. The number of days taken to reach the criterion was recorded also in the reversal phase.

Latent inhibition.

Latent inhibition was assessed in a conditioned taste aversion paradigm (CTA) (Bruno et al., 2007). Mice of both genotypes were water-deprived overnight starting at 5:00 P.M. the day before the experiment began. For 6 days, mice had access to unflavoured distilled water for 30 min each morning (9:30–10:00). HCl consumption during the test trial (after the aversive LiCl pairing) was expressed as a percentage of HCl intake during the conditioning trial (before the LiCl pairing). The mice were divided into 4 groups (two groups for each genotype). One SNAP-25$^{+/+}$ and one SNAP-25$^{+/-}$ group (pre-exposed groups), had access to 0.008 M HCl (Sigma-Aldrich, St.Louis, MO), while the remaining SNAP-25$^{+/+}$ and SNAP25$^{+/-}$ groups had access to unflavored water (non-pre-exposed groups) for 30 min in the morning. On Day 5, the conditioning day, all mice had access to 0.008 M HCl for 30 min in the morning, and, immediately afterwards, all mice were injected s.c. with 0.15 M LiCl (0.1 ml/10g) (Sigma-Aldrich, St. Louis, MO). On Day 6, all groups had access to unflavored water in the morning. On Day 7, the test day, all mice had access to 0.008 M HCl for 30 min in the morning. In addition to the morning drinking sessions, mice had access to unflavored distilled water for 1 h each afternoon (4:00–5:00 P.M.) throughout the experiment. Liquid intake was

measured by weighing water bottles before and after each drinking session.

Two-bottle preference tests.

2-bottle preference tests were performed as previously described (Bruno et al., 2007). Mice had access to plain water and 0.008 M HCl for 30 min each morning for 4 consecutive days, under the same deprivation schedule used in the latent inhibition experiment. The cohort was then tested for water vs. 0.8 mM quinine and for plain water versus 0.1% saccharin. Consumption (ml/10g) was measured for 3 days (days 2–4).

Conditioned taste aversion (CTA).

SNAP-25$^{+/+}$ (n = 10) and SNAP-25$^{+/-}$ (n = 12) mice were singly housed during the CTA test. After mice were adapted to a restricted drinking schedule (20 min per day for four days), they were exposed to a saccharin solution (0.1%) followed 1 h later by a malaise-inducing injection of LiCl (US; 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 three daily choice tests (ct1–ct3). The amount of saccharin intake expressed as the percentage of total fluid consumed [(saccharin/saccharin + water) x 100] was taken as an aversion index.

Object recognition.

The novel object recognition task was performed as previously described (Pan et al., 2008), with slight modifications. The test was conducted over a two-day period in an open plastic arena (60 x 50 x 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 (T1) and novel object recognition (T2). During the initial familiarization stage, two identical objects were placed in two corner of the arena equidistant from the walls and from each other. Each mouse was placed in the centre of the arena between the two objects for 10 min. Object recognition was scored when the animal was within 1 cm of an object with its nose toward the object. Exploration was not scored if a mouse reared above the object with its nose in the

air or climbed on an object. Mice were returned to the home cage after familiarization and retested 120 min later. and in the arena a novel object (never seen before) took the place of one of the two familiar. Scoring of object recognition was performed in the same manner as during the familiarization phase. From mouse to mouse the role (familiar or new object) as well as the relative position of the two objects were counterbalanced and randomly permuted. The objects for mice to discriminate consisted of white plastic cylinders, colored plastic Lego stacks of different shape and a metallic miniature car. The arena was cleaned with 70% ethanol after each trial. The basic measure was the time (in sec) taken by the mice to explore the objects in the two trials. 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 (Pitsikas et al., 2001).

Sociability and Preference for Social Novelty Test.

The Sociability and Preference for Social Novelty Test was used as described by Moy et al. (2004), with slight modifications. The apparatus was a rectangular, three-chamber transparent polycarbonate box (width = 42.5 cm; height = 22.2 cm; central chamber, length = 17.8; side chambers, length = 19.1 cm). The test procedure consisted in three separated phases.

Habituation: the proband mouse was placed in the middle compartment and allowed to explore all three chambers for 10 min. Each of the two side compartements contained an empty wire cage. The time spent in each compartment was recorded.

Sociability: after the habituation period, the test mouse was enclosed in the central compartment of the box and an unfamiliar male mouse (stranger 1, a never-seen-before adult male mouse), was enclosed in one of the wire cages placed in one of the side chambers. Stranger 1 was placed into the wire cage located in the less-preferred compartment by the test mouse during the habituation. Following placement of stranger 1, the doors were opened and the subject was allowed to explore the apparatus for 10 minutes. The time spent and the number of entries made in each chamber were recorded. The

difference score for sociability was calculated as difference between the time spent in the compartments containing stranger 1 and the time spent in the empty compartment according to DeVito et al (2009).

Preference for social novelty: at the end of the sociability test, each mas tested in a further 10-min session to quantify preference to spend time with a new stranger. A new unfamiliar mouse (stranger 2) was placed in the wire cage that had been empty in the previous phases. The test mouse had to choose between the first, already investigated, familiar mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The time spent and the number of entries made in each chamber were recorded. The difference score for social novelty was calculated as difference between time spent in the compartment containing the unfamiliar mouse (stranger 2) and time spent in the compartment containing the familiar mouse (stranger 1) according to DeVito et al (2009). Strangers belonged to the DBA/2J strain (Charles River, Calco, Italy) chosen for their very low scores of native aggression (Crawley, 1997).

Electroencephalogram (EEG).

Surgery. Mice were anesthetized with intraperitoneal injection of 5% chloral hydrate dissolved in saline and given a volume of 10 ml/kg. Four screw electrodes (Bilaney Consultants GMBH, Dusseldorf, Germany) were inserted bilaterally through the skull over cortex (anteroposterior,+2.0–3.0 mm; left–right 2.0 mm from bregma) as previously described (Manfredi et al., 2009) according to brain atlas coordinates (Paxinos and Franklin, 2004) A further electrode was placed into the nasal bone as ground. The five electrodes were connected to a pedestal (Bilaney, Dusseldorf, Germany) and fixed with acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy). Animals were allowed a week for recovery from surgery before the experiment.

Procedure. After surgery, 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 mice. The signal was transmitted from the test mouse, through the

electrodes, to an amplifier (Bio Amp) and then to a PC which allowed, by means of a software (Chart5, ADInstruments, Castle Hill, Australia) to show the cortical variations of the electric potentials in a graphical elaboration through the monitor. By means of the same PC software it was also possible to perform a quantitative analysis of spectral power, processing the signals for fast Fourier transform spectral analysis. Each 1-h spectral power was calculated as the mean of six 10-min recordings intervals. Spectral powers were calculated between 0 and 25 Hz, with a 0,2 Hz resolution, using the standard spectral power distribution: delta (0-4 Hz), teta (4.2-8 Hz), alpha (8,2-13 Hz) and beta (13.2-25 Hz).

Alterations in the behavior of the mice were continuously observed during the experiments, through a video camera placed inside the Faraday chamber. EEG traces were analyzed as elsewere described (Manfredi et al., 2009) for spike activity. For each EEG recording, the histogram of the maximum positive increments 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.

Twenty-four h recording. For the assessment of basal cerebral activity, freely moving mice were recorded continuously 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. After the recordings, the EEG and video (through a videocamera 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).

Acute treatment recording. For the evaluation of anticonvulsants effectiveness, freely moving mice were recorded continuously for 3 h, (one before and two after the treatment). Animals were injected at the end of the 1h basal and immediately after they were recorded for 2h.

Pharmacological treatments.

One week after basal EEG, animals were recorded 1 h before and for 2 h immediately after drug i.p. treatment: valproate sodium salt, VPA (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, 20 min before the sociability and social novelty test. All drugs were dissolved in saline, nimodipine in 10% ethanol and saline and carbamazepine 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,m 2007; Chung et al., 2009). All the drugs were given i.p. in a volume of 0.1ml/10 g. Fresh drug solutions were prepared daily. All the drugs were purchased from Sigma- Aldrich (St.Louis, MO).

For the repeated treatment, adolescents mice were exposed 24h/day to a VLP solution (0.1% dissolved in plain water). Each day the bottles containing the VLP solution (or water for the control animals) were weighted in order to obtain the daily amount of fluid intake, and refilled with fresh solution.

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 the proband mice (7–9 weeks of age) were 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. As previously shown for coloboma mouse (Hess et al., 1996), animals were treated s.c. with saline or amphetamine sulphate (4mg/kg) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% NaCl. Activity measures began immediately after injection and lasted 3 h. Amphetamine, a psychostimulant that acts at the presynaptic terminal to promote catecholamine release, was used since it is effective in ameliorating the hyperactivity expressed in ADHD-affected

children (Barkley, 1977; Shaywitz and Shaywitz, 1984) and in coloboma mouse (Hess et al., 1996).

Data analysis.

One-way ANOVA with repeated measures, or two-way ANOVA were used. *Post-hoc* analysis was done using Tukey's or Bonferroni's *post-hoc* tests. Pair-wise comparisons between genotypes or treatments were assessed with Student's *t*-test or Fisher exact probability tests. The significance threshold was set at $p < 0.05$. All statistical analyses were done with software Prism, version 5 (GraphPad, San Diego, CA).

RESULTS

ADULT SNAP-25$^{+/-}$ AND SNAP-25$^{+/+}$ MICE

Phenotypical characterization

General phenotypic and sensory abilities

In table1 the general phenotypical characteristics of SNAP-25$^{+/+}$ and SNAP$^{+/-}$ mutant mice is reported. No significant differences were found in weight, fur condition, piloerection, body tone and skin color. Nest building abilities did not did not differ between the two different genotypes or did general reflexes (righting, corneal, pinna and tail pinch). No difference was found in visual, olfactory, hearing and pain sensitivity (fig 1) as tested in the visual cliff test (fig 1A), buried pellet test (fig 1B), auditory test (fig 1C) and hot plate test (fig 1D), respectively. For the visual cliff test a significant difference between the time spent on the plain and check boarded floor side was found (2way repeated measure ANOVA with side as between-subject factor: $F_{(1,\ 18)}$=425, P<0.0001, and genotype as within-subject factor: not significant, side x genotype interaction: not significant followed by Bonferroni post hoc analysis).

Motor functions

Motor abilities of SNAP-25$^{+/+}$ and SNAP-25$^{-/-}$ mutant mice are shown in fig 2. The mean number of horizontal counts (fig 2A) was not different among genotypes. No differences among groups were found in the hanging wire test performance as measured by the latency to fall from the overturned grid (fig 2B). Fig 2C reports results obtained in the rotarod test. In all of the four trials there was no difference in the performance of heterozygous (HE) compared to the wild-type (WT) mice. The 2way repeated measure ANOVA (with trial number as between-subject factor: $F_{(3,\ 64)}$=5.3, P<0.001. and genotype as within-subject factor: not significant, trial x genotype interaction: not significant) followed by Bonferroni post hoc analysis, found a significant increase in the time spent on the rotarod in the fourth trial compared to the first trial, both in SNAP-25$^{+/+}$ (P<0,05) and in SNAP-25$^{+/-}$ (P<0,01), demonstrating that animals learned across the experiment to move on the rotating bar.

Emotional behaviour

No changes in anxiety and exploratory behaviour emerged in our studies. In the elevated plus maze the number (fig 3A) and the time spent (fig 3B) in the open arms did not differ between genotypes. In the Hole Board test SNAP-25$^{+/-}$ mice made a similar number of explorations compared to WT. To evaluate the depressive-like behavior, forced swim test (fig 4A) and the tail suspension test (fig 4B), were evaluated in terms of time spent immobile. No difference in immobility time was shown between the two genotypes.

Memory

Spatial Memory

The reduced level of SNAP-25 did not induce any significant change in the spatial memory assessed using the T-maze test. SNAP-25$^{+/+}$ and SNAP-25$^{+/-}$ mutant mice performed similarily during acquisition and reversal phase of the task (fig 5), in terms of number of animals reaching the criterion during acquisition (A) and reversal (B). The number of days needed to reach the criterion is shown in panel C. During both the acquisition and reversal phase, the two genotypes performed statistically similarly even if SNAP-25$^{+/-}$ mice showed a slightly delayed learning. However both genotypes took the same number of days to reach the criterion during the two phases.

Associative and learning memory

1) Latent inhibition and conditioned Taste Avesrion

Latent inhibition was assessed in a conditioned taste aversion paradigm. Latent inhibition occurs when a stimulus is repeatedly presented without reinforcement or consequence and is then uses as a conditioned stimulus (CS) in a conditioning paradigm (Lubow and Moore, 1959). Results (fig 6A), expressed as % of HCl solution intake in the test day compared to conditioning day, showed an impairment in conditioned taste aversion (CTA) in heterozygous mice that did not allow to evaluate any attention deficit. A significant difference in % of

solution intake was found by 2way repeated measure ANOVA (Genotype as between subject factor: $F_{(1,36)}$=3.52, P=0.07, and treatment as within factor: $F_{(1,36)}$ =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). Non-pre-exposed control mice consumed ~55% less HCl during the Test trial than during the Conditioning trial (Fig. 6A); mice learned the association between HCl and LiCl during the Conditioning trial. Pre-exposed control mice consumed a significantly higher % of HCl than non-pre-exposed control mice during the Test trial (P<0.001). Pre-exposed control mice consumed nearly as much HCl during the Test trial as they did during the Conditioning trial. The failure to reduce consumption during the Test trial after pre-exposure to the solution indicates latent inhibition. The SNAP25$^{+/-}$ mice, both pre-exposed and non-pre-exposed, showed an intake in the test phase similar to that observed in the conditioning phase. In particular non-pre-exposed heterozygous mice took a significant higher amount than non-pre-exposed wild-type (P<0.01), indicating that these animals are not able to associate the never before tasted solution with the malaise, showing an impairment in the conditioned taste aversion. This means that the high acid solution intake of the pre-exposed heterozygous mice, can't be seen as result of a good latent inhibition. To be sure that this deficit in CTA was not due to a deficit in taste discrimination, we performed a two-bottle preference test, in which the animals had to choose between water and differently tasted solutions (fig 6B). SNAP-25$^{+/-}$ mice consumed less quinine than water (P<0,0001) and more saccharin than plain water (P=0,0003, t-test). They consume more water than acid solution, but there was no significant difference in HCl and water intake. Thus, we repeated the CTA test changing the never tasted solution, using saccharine instead of HCl. The results (fig 6C) confirmed the previous results. SNAP-25$^{+/-}$ mice are not able to associate the taste solution with the malaise induced during the conditioning day. In fact their saccharine intake in the test day is significantly higher compared to the WT mice (P<0,01, t-test).

2) Episodic memory

When tested for the novel object recognition test (Fig. 7), no significant difference was detected in the amount of time the mice spent exploring the 2 objects during the familiarization (T1) phase (panel A), indicating that both genotypes had the same motivation to explore the object. However, after a delay of 120 min, 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 ($P<0.01$, unpaired t-test). This was not due to altered sensorial parameters as all mice appeared in health, displaying normal motor coordination, sensory abilities and were not aggressive (Table 1).

3) Social behaviour

In fig 8 the results obtained with the sociability and preference for social novelty test, carried out in order to evaluate social behaviour of SNAP-25 mutant mice, are shown. The mean time spent by the mice in the three compartments during sociability (8A) and social novelty (8B) respectively, is shown. The 2way repeated measure ANOVA revealed a significant difference among groups both in sociability (Genotype as between subject factor: not significant; compartment as within factor: $F_{(1,36)} = 27.88$, $P < 0.0001$; genotype X compartment: $F_{(1,36)} = 4.68$, $P = 0.03$, 2-way repeated measure of variance) and social novelty (Genotype as between subject factor: not significant; compartment as within factor: $F_{(1,36)}=22.44$, $P<0.0001$; genotype X compartment $F_{(1,36)}=19.09$, $P = 0.0001$). Bonferroni post hoc analysis showed that SNAP-25$^{+/+}$ mice behaved normally, spending longer time to explore the compartment with the stranger mouse than the empty cage ($P<0,0001$) or the familiar mouse ($P<0,0001$) during the sociability and social novelty test, respectively. Conversely, SNAP-25$^{+/-}$ mice spent the same amount of time in the 2 compartments in the sociability test. 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. Both genotypes spent equal time in the central compartment, in both phases of the test. The corresponding mean difference scores obtained by SNAP-25 mutant mice in

sociability and social novelty are reported in panels C and D. The results confirm that the heterozygous mice display an alteration in social behaviour, with a significantly lower difference score both in sociability (P<0,01) and social novelty test (P<0,01) when compared to the SNAP-25$^{+/+}$ mice (unpaired t-test).

EEG profile

As SNAP-25 controls neurotransmitter release and VGCC activity, we recorded the EEG profile of SNAP-25+/− mice. In figure 9 two 24-h representative EEG traces from a SNAP-25$^{+/+}$ (9A) and a SNAP-25$^{+/-}$ (9B) mouse, obtained in freely moving animals, are shown. The SNAP-25$^{+/-}$ mouse shows an increased frequency of high amplitude spike, as indicated by the quantitative evaluation in the bottom portion of the trace which, however, did not lead to spontaneous seizures. In only one case (a heterozygous mouse displaying 365 spikes/24 h), we could observe occurrence of generalized seizures following handling. The average spike number (±SEM) recorded for 24 h (Fig 9D) is significantly higher in SNAP-25$^{+/-}$ versus SNAP-25$^{+/+}$ (**P< 0,01, t-test). Abnormal EEG pattern was observed in all tested SNAP-25$^{+/-}$ mice: the percentage of SNAP-25$^{+/-}$ mice showing abnormal discharges (Fig. 9C) was significantly larger than of WT mice (**P< 0,01, Fisher exact probability test).

Anticonvulsant and Ca^{2+} antagonist effect on EEG profile

We next investigated whether treatment with antiepileptic drugs was able to normalize the altered EEG profile of SNAP-25+/− mice. Exemplificative trace of SNAP-25+/+ and SNAP-25+/- 1 hour before and 2 hours after treatment with the antiepileptic drugs are shown in fig 10. All the drugs used: sodium valproate (250 mg/kg) (VLP), etosuximide (200 mg/kg), carbamazepine (50 mg/kg) and nimodipine (10 mg/kg) were able to inhibit the spike activity, as indicated by the quantitative evaluation in the bottom portion of the trace, without affecting the SNAP +/+ trace. The percentage of spike inhibition, recorded during the first hour after treatment, on basal spike activity (Fig 11) was significant for all the drugs used (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). The Tukey's post hoc test revealed that sodium valproate was the most effective drug (95% reduction) (P<0,001) followed by etosuximide (80%) (P<0,001), whereas a partial but significant reduction was obtained with carbamazepine (60%) (P<0,01) or with the calcium antagonist nimodipine (35%) (P<0,01).

Sodium valproate effects on behavioural deficits

Since sodium valproate showed to be the most effective drug, among the substances we used in normalizing the cerebral activity, we tested the drug in the behavioural tasks in which SNAP-25$^{+/-}$ were impaired to investigate a possible therapeutic effect(Fig 12).

The discrimination index in the novel object recognition test is shown in panel A. The reduced discrimination index (P<0,01 Bonferroni's post hoc analysis), found in SNAP-25$^{+/-}$ mice, was reversed by pretreatment with VLP, given 20 min before T1 trial (P<0,05, Bonferroni's post hoc analysis) while treatment with VLP per se slightly, but not significantly, worsened cognitive abilities in SNAP-25$^{+/+}$ mice (genotype as between subject factor: not significative; treatment as within factor: not significative; genotype X treatment: $F_{(1,36)}$ = 11,44, P<0.01, 2-way repeated measure of variance).

Sodium valproate also reversed the CTA impairment (Fig. 12B) already seen in the SNAP-25$^{+/-}$ mice compared to SNAP-25$^{+/-}$, after saline injection (P<0,01). In fact pretreatment with the anticonvulsant drug, given immediately before HCl exposure, fully reversed the lack of CTA (P<0,01) (genotype as between subject factor: $F_{(1,36)}$ = 4.34, P = 0.04; treatment as within factor: $F_{(1,36)}$ = 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)

The difference score in the sociability (panel C) and preference for social novelty test (panel D) performed after the saline or sodium valproate i.p. injection is shown in fig.12. A difference among groups were detected both in sociability (genotype as between subject factor: $F_{(1,36)}$ = 55.48, P < 0.0001; treatment as within factor: not significant; genotype X treatment: not significant; 2-way repeated measure of variance) and social novelty (genotype as between subject factor: $F_{(1,36)}$ = 23.37, P < 0.0001; and treatment as within factor: $F_{(1,36)}$ =

14.76, P = 0.0005; genotype X treatment: $F_{(1,36)}$ = 25.42, P<0.0001, 2-way repeated measure of variance). Post hoc test confirmed the previous results: saline treated heterozygous mice displayed social interaction impairment and social memory deficit compared to wild-type, (P<0,01 Bonferroni's post hoc analysis).

Sodium valproate normalized the defects in social memory (P<0,001, Bonferroni's post hoc analysis), being however ineffective in restoring sociability. Treatment with VLP slightly reduced the social performance in SNAP-$25^{+/+}$ mice.

ADOLESCENT SNAP-$25^{+/-}$ AND SNAP-$25^{+/+}$ MICE

Before to start with the behavioural and electroencephalographic characterization of the 6-7 week aged SNAP-$25^{+/-}$ mice, we divided the animals in two sub-groups, one exposed to plain water, the other to a sodium valproate solution 0,1%, for 21 days, 24 hours/day. The mean solution intake throughout the exposition, related to animal weight, is shown in fig 13 (panel A), whereas in the panel B the area under curve (AUC) is shown. No difference were found in mean intake in all observed groups. Average animal intake (slightly less than 0,3 ml/g) of sodium valproate corresponds to the dose acutely given in the adult mice in our previous experiment (250 mg/kg, right axes in the panel A).

Behaviour

CTA:

Conditioned taste aversion test, performed using saccharin solution, showed in adolescent mice the same impairment found in the adult mutant mice. The results are reported in fig 14A, in terms of % of sweet solution intake during the conditioning day (genotype as between subject factor: $F_{(1,36)}$= 7.104, P<0.05; treatment as within factor: $F_{(1,36)}$= 7.107, P<0.05; genotype X treatment: $F_{(1,36)}$ = 10.93, P<0.01, 2-way repeated measure of variance). A significant increase of saccharin intake in the water-exposed heterozygous mice compared to the wild type (P<0,001, Bonferroni post hoc analysis) was shown. Repeated treatment with sodium valproate induced a significant decrease in saccharin intake in the

SNAP-25$^{+/-}$ compared to the water-exposed mice (P<0,05) without affecting the wild-type intake of sweet solution.

Object recognition:

When tested for episodic memory SNAP-25$^{+/-}$ adolescent mice had a significant decreased discrimination index in the novel object recognition test compared to the age matched WT mice (P<0.05, Bonferroni post hoc analysis), while sodium valproate exposition was able to significantly recover that deficit (P<0.05, Bonferroni post hoc analysis). Anticonvulsant slighty decreased SNAP-25$^{+/+}$ performance, but not significantly (genotype as between subject factor and treatment as within factor: not significant; genotype X treatment: $F_{(1,36)}$ = 10,71, P<0.01, 2-way repeated measure of variance).

Social behaviour:

The social behaviour, evaluated by means of sociability and preference for social novelty test, is shown in fig 15 (panel A and B). In both test differences among the groups were found (genotype as between subject factor: $F_{(1,36)}$= 7.104, P<0.05; treatment as within factor: $F_{(1,36)}$= 7.107, P<0.05; genotype X treatment: $F_{(1,36)}$ = 10.93, P<0.01, 2-way repeated measure of variance). The post-test highlighted deficit in the SNAP-25$^{+/-}$ water-exposed both in sociality (P<0,001, Bonferroni post hoc analysis) and social memory (P<0,05, Bonferroni post hoc analysis), as occurred in adult heterozygous mice. Sodium valproate, which per se didn't affect social behaviour in WT mice, was able to increase the time spent by heterozygous adolescent mice in the never seen before mice compartment both in sociability (P<0,01) and social novelty (P<0,05).

Spontaneous motor activity

As a hyperactivity is one of the core symptoms in ADHD, we monitored spontaneous motor activity in SNAP-25$^{+/-}$ and SNAP-25$^{+/+}$ mice at 7 weeks of age (Fig. 16). The time course of horizontal activity recorded every 10 min before (4h of baseline) and after (3h) amphetamine injection after water or sodium valproate solution exposition is given in Figure 17 (panel A and panel B). During the first 2-h recordings, both genotypes showed a similar horizontal activity, independently to their previous treatment. However, during the following 2

h (120–240 min) SNAP-25$^{+/-}$ water-exposed mice failed to habituate, thus resulting more active than wild-type littermates. As expected s.c. injection of d-amphetamine (4 mg/kg) (arrow) increased horizontal activity in SNAP-25$^{+/+}$ mice during the first hour after treatment (240–300 min), whereas in the following two hours (300–420 min), the stimulant effect decreased. Conversely, d-amphetamine appeared 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. A recovery of motor function was obtained during the last period. Spontaneous motor activity of the animals exposed for 21 days to sodium valproate is shown in the panel B. The time course of horizontal activity of heterozygous mice had the same trend of the wild-type. During the first two hours animals displayed a high number of horizontal movements but in the last two hours they habituated and the movements decreased in the same manner as occurred in the water-exposed SNAP-25$^{+/+}$. Also after treatment with amphetamine the trend appeared similare in both genotypes. Animals increased their motor activity in the first hour after treatment (240-300min) but the hyperactivity induced by amphetamine decreased in a time dependent manner during second and third hour.

The time-course (panel A and B) was also evaluated as mean (±SEM) of horizontal activity counts in blocks of 1-h each period, for water-exposed (panel C) and sodium valproate-exposed animals (panel D). 2-way ANOVA carried out in water exposed mice showed difference among the groups (genotype as between subject factor: $F_{(1,56)} = 113$, $P < 0.0001$; time as within factor: $F_{(3,56)} = 61.57$, $P < 0.0001$; genotype X time $F_{(3,56)} = 52.47$, $P < 0.0001$). SNAP-25$^{+/-}$ mice displayed hypermotility. Their mean horizontal counts was higher before amphetamine administration (pre) than that of SNAP-25$^{+/+}$ mice ($P<0,01$, Bonferroni post hoc test).

Acute d-amphetamine treatment (4 mg/kg), given s.c. at 240 min (arrow), significantly increase the horizontal movement in SNAP-25$^{+/+}$ mice in the first hour ($P<0,01$ Bonferroni post hoc analysis), and in the following tho hours the motor activity is not different if compared to the pre. Conversely amphetamine in SNAP-25$^{+/-}$ mice seems to be ineffective in

the first hour and reduces the number of movements at the second hour compared to their pre (P<0,01 Bonferroni post hoc analysis) and a complete recovery was observed at the last period, in which heterozygous mice returned hyperactive compared to wild-type (P<0,001 Bonferroni post hoc analysis).

Statistical analysis performed on the mean (±SEM) of horizontal activity counts for sodium valproate-exposed mice (panel D) didn't revealed any difference among the groups, confirming that the drug solution normalized motor activity in the SNAP-25$^{+/-}$ mice. Moreover, when compared to water-exposed mice, sodium valproate-exposed SNAP-25$^{+/-}$ displayed significative lower number of horizontal movements in the pre (P<0,01 Bonferroni post hoc analysis) and in the third hour after d-amphetamine treatment (P<0,001 Bonferroni post hoc analysis). (Exposition as between subject factor: not significant; time as within factor: $F_{(3,56)}$ = 9,8, P<0.0001; exposition X time: $F_{(3,56)}$ = 10,15, P<0.0001)

EEG

Adolescent SNAP-25$^{+/-}$ mice displayed EEG alterations in terms of spikes as occurred in the adult heterozygous mice. Two representative traces are reported in fig 17. In basal conditions SNAP-25$^{+/-}$ trace (B) was characterized by abnormal and rapid increase of amplitude (spike) that was not present in the wild-type trace (A). Immediately after the end of sodium valproate-exposition, heterozygous trace was normalized and any spike was detected (D). Two weeks after the end of the treatment, the EEG abnormalities returned and the trace was similar to that observed during the pre-treatment (F). Exposition to sodium valproate had no effects on wild-type EEG profile (C and E).

In fig 17G the quantitative analysis of the mean number of spikes found before, immediately after and in the following three weeks after the treatment in both genotypes, is reported. 2-way repeated misure of variance found significative difference among the groups (Genotype as between subject factor: $F_{(1:40)}$= 62,51, P<0.0001; time as within factor: $F_{(4:40)}$= 6,415, P<0.001; genotype X time $F_{(4:40)}$=6,415, P<0.001). In basal condition SNAP-25$^{+/-}$ mutant mice displayed a higher number of spikes compared to the wild-type mice (P<0,001),

and treatment with the antiepileptic was able to significantly reduce the mean spike number, immediately (P<0,001) and one week after the end of the treatment (P<0,001). Its protective action lasted for one week, and starting from the second week the mean number of spikes was not different compared to the basal recording, returning significantly higher compared to the WT in the second (P<0.01) and in the third week (P<0.001).

In fig. 18 spectral power of the relative frequency bands is reported. *Delta* band (panel A), with a frequency between 0 and 4 Hz, is increased in SNAP-25$^{+/-}$ mice compared to WT (P<0.05), while *theta* band was not affected (panel B). The two fast bands, *alpha* (panel C), with a frequency in the range between 8 and 13 Hz, and *beta* (D) 13-25 Hz were significantly decreased in SNAP-25$^{+/-}$ mutant mice (P<0.01 and P<0.05 respectively), indicating in these animal an increase of slow waves and a parallel decrease of high frequency waves.

DISCUSSION

Genetic studies on human populations and on animal models demonstrate that alterations in SNAP-25 gene structure, expression and/or function may contribute directly to a range of psychiatric and neurological conditions, such as schizophrenia, ADHD, bipolar disorder and epilepsy (Corradini et al, 2009). In particular, SNAP-25 has been found to be strongly linked to ADHD, one of the most common neuropsychiatric diseases among children and adolescent (Guerini et al, 2011). Thus, in our study we characterized, from a behavioural and electroencephalographic point of view, heterozygous mutant mice, with a reduced expression (50%) of SNAP-25, compared to age-matched control mice. We planned to evaluate mutant mice not only during their adult age, in order to check the role of altered SNAP-25 gene expression in behaviour and cortical electric activity, but also during adolescence (6-7 weeks) in order to investigate a possible new ADHD animal model.

Firstly we investigated their general health, reflexes and sensory abilities, and any anxious or depressive-like behaviour, without finding any abnormalities in heterozygous mice. Also the motor abilities were not affected, and SNAP-25$^{+/-}$ adult mice had a normal spontaneous motor activity, motor coordination and muscular strength. Another animal model characterized by a reduced expression of SNAP-25, coloboma mice, shows severe sensory deficits and motor coordination impairment (Gunn et al, 2011), making difficult to assess the typical characteristics of ADHD (inattention, impulsivity, hyperactivity).

Secondly, memory function were investigated. Heterozygous mice, tested in a T-maze, showed a normal explicit spatial memory and flexibility, but they have deficit in implicit associative learning, having a poor performance in two test based on this kind of learning and memory ability, the conditioned taste aversion (CTA) and the object recognition 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).

In CTA test, an associative gustative memory is needed to associate the never taste solution with the visceral malaise. This kind of memory is mediated by insular gustative cortex, and its acquisition by protein synthesis (Merhav et al., 2010). Recently a role for NMDA (*N-methyl-D-aspartate)* receptor in the formation of taste memory has been suggested, since the CTA acquisition induce a prolonged phosphorylation on the regulatory NMDA 2B subunit and tyrosin-kinase inhibitors can block the formation of aversive memory, included CTA (Li et al., 2010). Since is known that SNAP-25 negatively modulates voltage-gated Ca^{2+} channels in glutammatergic neurons (Matteoli et al., 2010), probably the reduced protein level in SNAP-$25^{+/-}$ mice can affect taste memory acquisition. The results we obtained in the latent inhibition test, and conditioned taste aversion in our mutant mice are quite different from those obtained in coloboma mutant mice. Coloboma mice show a disrupted latent inhibition, a measure of selective attention, without CTA deficits (Bruno et al, 2006). These discrepancies are probably due to the deletion of other genes, besides SNAP-25, that may influence the behavioural profile of coloboma mice (Gunn et al, 2011).

Cognitive deficits seen in our animals are corroborated by studies that correlated SNAP-25 with impairment in learning and memory, both in humans (Gosso et al., 2008) and in mice (Li et al., 2010).

In our study we found that reduction in SNAP-25 expression is associated with diffuse network hyperexcitability, which does not lead to spontaneous convulsive behaviour. Heterozygous mice display EEG alteration, in terms of higher number of spikes, compared to the control mice. Our data are in line with the significantly higher incidence of epilepsy in pathologies characterized by SNAP-25 alterations. Notably, the incidence of epilepsy is about 6 times higher in patients with schizophrenia (Chang et al. 2011). Although the mechanisms by which reduced levels of SNAP-25 lead to network hyperexcitability are not defined, a defective modulation of VGCCs by reduced SNAP-25 levels may be implicated (Condliffe et al. 2010).

Interestingly, also coloboma mice showed a similar hyperexcitability, by means of robust cortico-cortical spike-wave discharges and increased thalamic T-type currents, reminiscent of

absence epilepsy (Zhang et al, 2004). In particular, analysis of calcium current amplitude in thalamocortical neurons of coloboma mice revealed an increase in the peak current density of low voltage-activated (LVA), but not of high voltage-activated currents (HVA), that precede the onset of spike-wave discharges (Zhang et al. 2010). Ethosuximide, acting on LVA currents (Coulter *et al*., 1989), blocked that EEG alteration in coloboma mice, suggesting the key role of LVA current in this kind of hyperexcitability. The same authors concluded that the increased peak density of LVA current in thalamic neurons of coloboma mice are due to the reduced expression of SNAP-25. They hypothesized that the alteration in synaptic transmission induced by lack of SNAP-25, can induce an increase in the low voltage-activate channel (T-channel) synthesis (Pozzi *et al*., 2008) or can influence the transcription of different T-channel isoform (Bertolesi *et al*., 2003).

In vitro studies demonstrated that acute down-regulation 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).

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, contributing to the learning deficits. It is known that in humans, the presence of spikes and seizures can cause damage in the organization of the cortical network and brain function (Shewmon et al., 1988) damaging processes that play a key role in the phenomena of learning and memory (Shatskikh et al., 2006). Although the relationship between EEG abnormalities and cognitive defects continues to be debated (Rapin, 1995; Aldenkamp et al., 2004, Fonseca et al., 2005), many clinical and experimental data support this association. 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, 2010).

Children with epilepsy show a slower mental development than children of the same age. They can have problems in reading, writing and arithmetic, with a progressive decline in their IQ (Neyens et al., 1999). Other studies, conducted in epileptic patients during the appearance of spikes, detected defects in the short-term memory (Aarts et al., 1984; Binnie et al., 1987; Kasteleijn et al., 1990), in visual perception and reaction time (Shewmon et al., 1989). Cognitive defects were also found in normal rats in which spikes were artificially induced in the CA1 region of the hippocampus. In particular, these animals showed deficits in spatial reference working memory, evaluated by means of the radial arm maze task and object recognition test (Shatskikh et al., 2006). Recently it has been also demonstrated, in rat pups, that the induction of interictal spikes, without seizures, can result in long-standing spatial cognitive impairment (Khan et al, 2010). The fact that the memory deficits may be due to multiple factors (Niemann et al., 1985; Ellenberg et al., 1989; Holmes, 1991; Mandelbaum et al., 1997; Tromp et al ., 2003), makes very difficult to study the mechanisms by which the electroencephalographic abnormalities lead to cognitive defects (Stafstrom et al., 2002). Nevertheless, the most likely hypothesis concerns the inhibition of neural network that connects the hippocampus to the entorhinal cortex (Barbarosie et al., 1997), since it is known that the hippocampus is a region with a high level of convergence sensory which plays a central role in the consolidation and stabilization of memory (Scoville et al., 1957; Squire, 1992; Eichenbaum, 1999).

In our study treatment with antiepileptic drugs, or with the calcium antagonist NIMO, largely normalized the altered EEG profile of SNAP-25$^{+/-}$ mice. The larger beneficial effects were produced by VLP and ETO, that are 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 blocked 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 children 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).

Acute administration of sodium valproate was able to block not only the spike activity but also the behavioural deficit displayed by heterozygous mutant mice. Treated SNAP-25$^{+/-}$ mice significantly increased their performances in associative memory tasks as object recognition, conditioned taste aversion and social memory.

These results are in line with experimental data in which sodium valproate has been shown to reduce neuronal damage associated with epileptic activity. In experimental models of rats mimicking status epilepticus, was demonstrated that valproate significantly reduces the neuronal damage in the hippocampal formation, increasing nerve functionality and memory (Brandt et al., 2006). It is hypothesized that the anticonvulsant effect of valproate is the result of combining multiple effects that the drug seems to have on the central nervous system, which include the channel block of voltage-dependent Ca^{2+} T-type, probably involved in the development of spikes and seizures (White, 1999), the inhibition of transamination of GABA, the reduction in NMDA-mediated neuronal excitability, the inhibition of histone deacetylases (HDACs) and glycogen synthase kinase (GSK) -3 (Faden et al., 1989). Previous studies also show how valproate is able to stimulate neuritis and dendrites growth (Yuan et al., 2001) and to promote hippocampal neurogenesis (Hao et al., 2004). Valproate can improve cognition, normalizing the increased neurogenesis in an animal model of epilepsy (Jessberger et al., 2007) and suppresses seizure-like behavior and improves learning ability in adult zebrafish treated with PTZ (Lee et al, 2010). Furthermore, valproic acid is able to restore the normal cognitive functions in patients suffering from epileptic encephalopathies, underling the fact that spikes and seizures may be related to cognitive alterations (Asarnow et al. 1997; Matsuzaka et al., 2001; Zupanc, 2003; Besag, 2004). The dose we used (250 mg/kg) gives rise to a plasma concentration, in the first hour following the

injection, similar to the therapeutic concentration in humans (40-100 µg/ml).

In SNAP-25$^{+/+}$ mice, VLP had a slightly worsening effect on associative memory. Recent studies in rats sub-chronically treated with high dose of sodium valproate, have shown negative effects on spatial working memory due to its inhibitory action on neuronal proliferation in the subgranular zone of the dentate gyrus (Umka et al., 2010). A number of reports have documented a range of mild to moderate cognitive impairments, including memory deficits, in adult patients taking VPA (Senturk et al., 2007; Cysique et al., 2006; Gualtieri and Johnson, 2006; Carpay et al., 2005). The memory decline in WT animals may depend by the inhibitory action on brain electrical activity exerted by valproate (Lee et al, 2010).

Our data directly correlate the reduction of SNAP-25, the 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.

We tested also adolescents SNAP-25$^{+/-}$ mice, in order to develop a new model of ADHD. We found the same alterations seen in the adult. Adolescents mice showed deficit in implicit associative memory, tested by means of object recognition test and CTA, and in social behaviour. This is in line with the human pathology. In fact 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). The performance of children with ADHD on a temporo-visuospatial working memory task was worse during a memory delay for inverse digits compared to non-affected children (Miranda-Casas et al, 2006). Children with ADHD have deficits in inhibitory control, working memory, short-term memory (Stevens et al, 2002) and executive function (Doyle, 2006). In a recent study, ADHD children showed worst performance in sustained attention, rapid serial naming

of figures and colours, comprehension of written instructions, word dictation, number comparison, arithmetical problems, visual working memory and long term memory (Yáñez-Téllez et al, 2012).

One of the ADHD core symptom is the hyperactivity. Differently from the adults, we found a spontaneous hyperactivity in adolescent SNAP-25$^{+/-}$ mice, due to lack of habituation. As expected amphetamine treatment significantly increased the motor activity in WT mice, while in heterozygous mice reduced the mean number of movements. This result suggests a common aetiology in hyperactivity in mice and humans, since is well known as psychostimulants act by reducing the hyperactivity seen in children suffering from the disease (Elia et al., 1991).

As reported in the literature also the mouse model coloboma expresses hypermotility, clearly correlated to the reduced expression of SNAP-25 (Hess et al., 1996). In addition, the hyperkinetic behaviour can be corrected by crossing coloboma mice with SNAP-25 over-expressing mice. In coloboma mice, however, the hypermotility was associated with a state of anxiety and motor coordination deficits, induced by sensory deficits (vision and hearing) caused by other genes deletion (Gunn et al., 2011).

Hyperactivity was also seen in homozygous mutant mice in which Ser187 of SNAP-25 is substituted with Ala (Kataoka et al. 2011). The possibility that the reductions in the levels of SNAP-25 may be involved in the hyperactivity is further supported by the observation that SHR rats, hyperactive, show reduced levels of SNAP-25 in the prefrontal cortex, a defect that disappears after repeated injections of amphetamine (Li et al., 2009). Our data on adult SNAP-25+/- mice did not show a significant difference in the number of horizontal movements when compared with WT mice of the same age. This feature of our model reflects the human pathology, in which there is, in adulthood, a hyperactivity remission, to a greater extent compared with attention deficits associated with this disease (Faraone et al., 2000).

The EEG profile of adolescent mice showed an abnormal cerebral activity in heterozygous mice, close to that of the adults and this is another feature that is shared with

the human disease. There is an high comorbidity between ADHD and epilepsy. 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). For more than 40 years, EEG research has attempted to characterize and quantify the neurophysiology of ADHD (Satterfield et al, 1974). Recently EEG have been used in the assessment, diagnosis and treatment of ADHD, and also used to describe and quantify the underlying neurophysiology of the disease (Loo and Barkley, 2005). Thus we analyzed the spectral power of the relative frequency bands, finding some alterations in the band distribution. SNAP-25$^{+/-}$ showed an increase in the slow frequency bands (*delta*) and a reduction in the fast bands (*alpha* and *beta*). The same results were obtained in one of the most studied ADHD animal model, the SHR rat, that displayed slower cortical and hippocampal EEG compared to control rats, characterized by increased delta and decreased alpha/beta activity (Vorobyov et al, 2012). Our data are in line with EEG studies on ADHD children, that found increase of slow-wave activity and reduced activity in *alpha* and *beta* bands compared to normal controls (Loo and Makeig, 2012).

Repeated treatment with VLP was able to restore all the behavioural deficit seen in adolescent heterozygous mice, probably blocking the EEG alteration. VLP has been recently used as treatment for ADHD symptoms in fragile X syndrome, with an improvement in the adaptive behavior, defined as the performance of daily activities required for personal and social competence, due to a significant reduction in hyperactivity after VPA treatment (Torrioli et al, 2010).

Concluding, our results suggest that SNAP-25$^{+/-}$ adolescent mice could represent a promising ADHD animal model, with a phenotype characterized by hypermotility, associative and social deficit and EEG alteration with an high homology to that observed in affected children. However to completely determine the ADHD profile of SNAP-25$^{+/-}$ mice it will be necessary to test the impulsivity, a feature of ADHD patients which includes both cognitive and motor components.

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FIGURES