UNIVERSITA' DEGLI STUDI DI MILANO

Dipartimento di Biotecnologie Mediche
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Corso di Dottorato in
Farmacologia, chemioterapia e tossicologia mediche
XXV Ciclo

CAUSES AND CONSEQUENCES OF REDUCED EXPRESSION OF SNAP-25 IN NEURONAL NETWORKS

G3R

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Tesi di Dottorato di

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Anno accademico 2011/2012

Summary

ABSTRACT

Synaptosomal-associated protein of 25 kDa (SNAP-25) is a protein that participates in the regulation of synaptic vesicle exocytosis and modulates voltage-gated calcium channels activity. Altered levels of SNAP-25 expression have been associated to different neurological and neuropsychiatric conditions, such as schizophrenia, ADHD, and bipolar disorder, and lower levels of SNAP-25 have been described in patients with schizophrenia. I used *Snap-25* heterozygous (*Snap-25*$^{+/-}$) mice, expressing reduced levels of SNAP-25, to investigate at which extent the reduction of the protein expression affects neuronal network function and mouse behaviour. *Snap-25*$^{+/-}$ mice displayed a moderate hyperactivity, which disappeared in the adult animals and showed an impairment in associative learning and recognition memory. Electroencephalographic recordings revealed the occurrence of frequent spikes, suggesting a diffuse network hyperexcitability. Moreover, SNAP-25$^{+/-}$ mice displayed higher susceptibility to kainate-induced seizures. Notably, treatment with antiepileptic drugs improve both EEG profile and cognitive defects. The abnormal EEG profile observed in SNAP-25$^{+/-}$ mice could contribute to the learning and memory defects. Based on the results i have obtained, showing the occurrence of cognitive disabilities in mice expressing reduced levels of SNAP-25, i conducted a functional analysis on SNP rs363050, which associates with low performance IQ and is located on intron 1 of the *Snap-25* gene, to investigate whether the presence of the parental or minor allele may affect the expression of SNAP-25. The presence of minor allele resulted in a reduction of the transcription capability, while the parental allele did not increase the basal activity of an heterologous promoter. These results indicate that reduction of SNAP-25 expression is associated to generation of epileptiform discharges and cognitive dysfunctions, which can be effectively treated by antiepileptic drugs and support the evidence that the rs363050 polymorphism might have a role in modulating *Snap-25* gene expression levels.

INTRODUCTION

THE SYNAPSE

In the central nervous system communication between neurons occurs in site termed *synapses*. The term synapse was originally introduced more than 100 years ago by Charles Sherrington and was then later adopted by Ramòn Cajal. However, since the introduction of electron microscopy and related techniques that allow subcellular and molecular resolution, our knowledge about these structures has steadily increased over the last 50 years. Synapses are the key elements for signal transduction and plasticity in the brain; chemical synapses are specialized sites of information exchange between neurons and their target cells. At the synapse, the arriving electrical signal is rapidly and efficiently transformed into a chemical signal through the regulated exocytosis of neurotrasmitter-filled synaptic vesicles. The most common arrangement is either the axo-dendritic or the axo-somatic synapse, in which the axon of the cell of origin makes its functional contact with the dendrites or the soma of the target neuron; contacts between adjacent cell bodies (soma-somatic) or overlapping dendrites (dendro-dendritic) are more rare.

Each synapse is a complex of several components: a presynaptic element, a cleft and a postsynaptic element. The presynaptic element, named *bouton*, is characterised by the presence of synaptic vesicles and a presynaptic thickening at the *active zone*; in this region, at any time a small number of vesicles are docked and presumably ready for fusion. The active zone is also enriched with voltage gated calcium channels (VGCCs), which are necessary to permit activity-dependent fusion and neurotransmitter release. The *synaptic cleft* is an essential space between the presynaptic and the postsynaptic elements; the width of the cleft (~20 μm) defines the volume in which the vesicles release its content, and therefore the peak concentration of the neurotransmitter upon release. On the flanks of the synapse, the cleft is spanned by adhesion molecules which are believed to stabilize the cleft (Fig. 1). The postsynaptic element is usually a portion of a soma or a dendrite, or rarely a part of an axon. It is marked by the presence of a thickening named *postsynaptic density* (PSD), which is associated with neurotransmitter receptors (e.g. NMDA glutamate receptors) and other molecules (e.g. the protein PSD95).

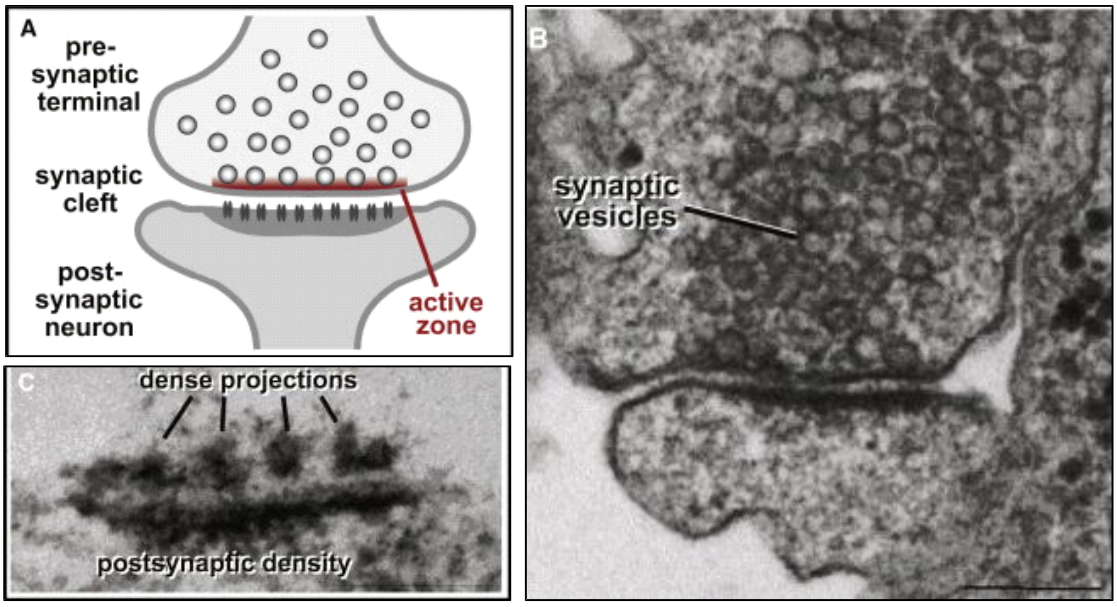


Figure 1. The synapses. (**A**) Schematic rappresentation of the synaspe. (**B**) Electron microscopy image of a synaptic bouton and the postsynaptic density (**C**) (from Sudhof 2012).

Synaptic transmission is initiated when an action potential reaches the presynaptic terminal, which depolarises the membrane in the synapse that leads to opening of voltage-gated Ca^{2+} channels. This causes a rapid influx of Ca^{2+} into the presynaptic terminal. This elevation of the intracellular Ca^{2+} concentration stimulates synaptic vesicles to fuse with the presynaptic membrane. In most synapses, release is stimulated by Ca^{2+} influx through P/Q- (CaV2.1) or N- (CaV2.2) type Ca^{2+} channels, whereas the related R- (CaV2.3) or the more distant L- (CaV1 series) type Ca^{2+} channels are involved only rarely (see Dietrich, 2003). Even at rest, synapses have a finite but low probability of release, causing spontaneous events of exocytosis that are reflected in electrophysiological recordings as *miniature postsynaptic currents* (Katz, 1969). Ca^{2+} influx triggers at least two components of release that are probably mechanistically distinct: a fast, synchronous phasic component is induced rapidly, in as little as 50 µsec after a Ca^{2+} transient develops (Sabatini and Regehr, 1996), and a lower asynchronous component continues for >1 sec, as an increase in the rate of spontaneous release after the action potential (Barrett and Stevens, 1972; Geppert et al. 1994; Goda and Stevens, 1994; Atluri and Regehr, 1998). Both components of release are strictly Ca^{2+} dependent.

Neurotransmitter release requires the fusion of synaptic vesicles with the surface membrane at the active zones, thereby releasing their contents (*exocytosis*); after this event, vesicles are re-endocytosed, recycled, and refilled with neurotransmitters. When a nerve terminal is stimulated, not all vesicles can be released immediately because synaptic

vesicles exist in functionally different pools. There are three distinct pools of vesicles: a readily-releasable pool (RRP) characterized by a small cluster of vesicles (10+) at the active zone, ready for release, a recycling pool (100+ vesicles) that supplies vesicles to the RRP, and a large reserve pool (several hundred vesicles) that supplies vesicles to the recycling pool (Rizzoli and Betz, 2004). At rest, molecules called synapsins tether reserve pool synaptic vesicles to the cytoskeleton. Upon strong stimulation, high calcium ion concentration in the terminal triggers the phosphorylation of synapsins; as a consequence, synaptic vesicles are released from the cytoskeleton and can move to the membrane and be primed for release.

The fusion of synaptic vesicles with the plasma membrane is under tight temporal and spatial control as synaptic vesicles only dock, fuse and release neurotrasmitters at a restricted and highly specialized area of the presynaptic plasma membrane called the active zone; this area is tightly associated with an electron dense cytoskeletal matrix, which is referred to as cytomatrix at the active zone (CAZ). Ultrastructural studies of synapses have revealed that the presynaptic active zone is closely and precisely aligned with the PSD and that the plasma membranes on both sites of the synaptic cleft appears as electron-dense structures, suggesting their proteinaceous nature. A variety of proteins have been identified that are associated with the CAZ, most of which are not restricted to active zone but can also be found in other compartments of the cell. Amomg these, proteins involved in synaptic vesicle fusion (syntaxin, SNAP-25, Munc18), cytoskeletal proteins (actin, tubulin, myosin, spectrin, β-catenin), scaffolding proteins (CASK, Mint, SAP97 and velis/MALS), voltage-gated calcium channels (VGCCs) and cell adhesion molecules (neurexin, cadherins, integrins and sidekicks). Emerging evidence suggests that five evolutionarily conserved proteins, RIM, Munc13, RIM-BP, α-liprin, and ELKS proteins form the core of active zones. In addition to these five core active zone proteins, piccolo and bassoon (two large homologous proteins) are associated in vertebrates with active zones (Dieck et al. 1998; Wang et al. 1999; Fenster et al. 2000; Limbach et al. 2011). These proteins form an interconnected network that is linked to synaptic vesicles, cytoskeletal proteins and calcium channels and are instrumental for the integration and organization of distinct steps of the synaptic vesicle cycle. For example, through Munc13, the CAZ is involved in the priming of synaptic vesicles and, through Piccolo, it might play a role in coordinating exocytic and endocytic steps (Fejtova and Gundelfinger, 2006). RIMs are central elements of active zone that interact with most other active zone proteins. Functionally, RIM proteins perform at least two essential functions. First, RIM proteins regulate the priming

activity of Munc13 (Deng et al. 2011). Second, RIM proteins tether Ca^{2+}-channels to the active zone, in fact only very recently the molecular mechanism recruiting Ca^{2+}-channels to active zones was shown to depend on RIM proteins (Kaeser et al. 2011) (Fig. 2).

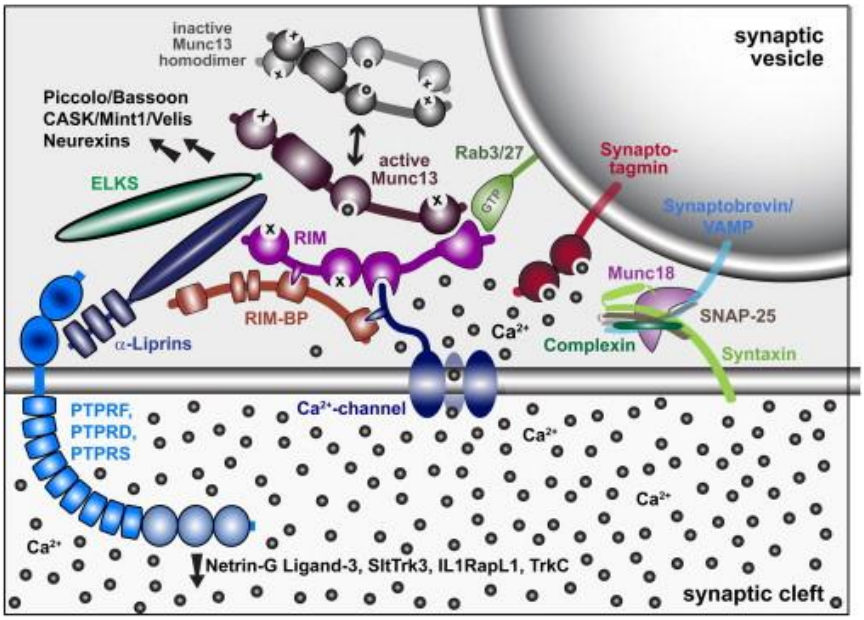


Figure 2. Molecular model of the active zone protein complex and its relation to the synaptic vesicle fusion machinery, Ca^{2+} channels, and synaptic cell-adhesion molecules (from Sudhof 2012).

Synaptic transmission is initiated when an action potential triggers neurotransmitter release from a presynaptic nerve terminal (Katz, 1969). An action potential induces the opening of Ca^{2+} channels, and the resulting Ca^{2+} transient stimulates synaptic vesicle exocytosis.

With a diameter of approximately 40 nm, SVs are small organelles, making them comparable in complexity to other supramolecular structures such as ribosomes, spliceosomes, or viruses.

Synaptic vesicles contain two classes of obligatory components: transport proteins involved in neurotransmitter uptake and trafficking and proteins that participate in synaptic vesicle exo- endocytosis and recycling. Transport proteins are composed of a vacuolar-type proton pump (V-ATPase) that generates the electrochemical gradient, which fuels neurotransmitter uptake and neurotransmitter transporters that mediate the actual uptake.

In 2006 Takamori proposed a model that integrates all quantitative data and includes structural models of abundant proteins (Fig. 3). Takamori et al. observed that a few proteins, essential for membrane traffic and neurotransmitter uptake, are very abundant in synaptic vesicles (synaptobrevin2/VAMP2 is present in 70 copies for vesicle,

synaptophysin1 in 30 copies per vesicle, synaptotagmin1 in 15 copies per vesicle); in contrast many proteins are present in only a few copies per vesicles (the proton pomp is the prominent example: only one to two copies for vesicle) (Takamori et al., 2006).

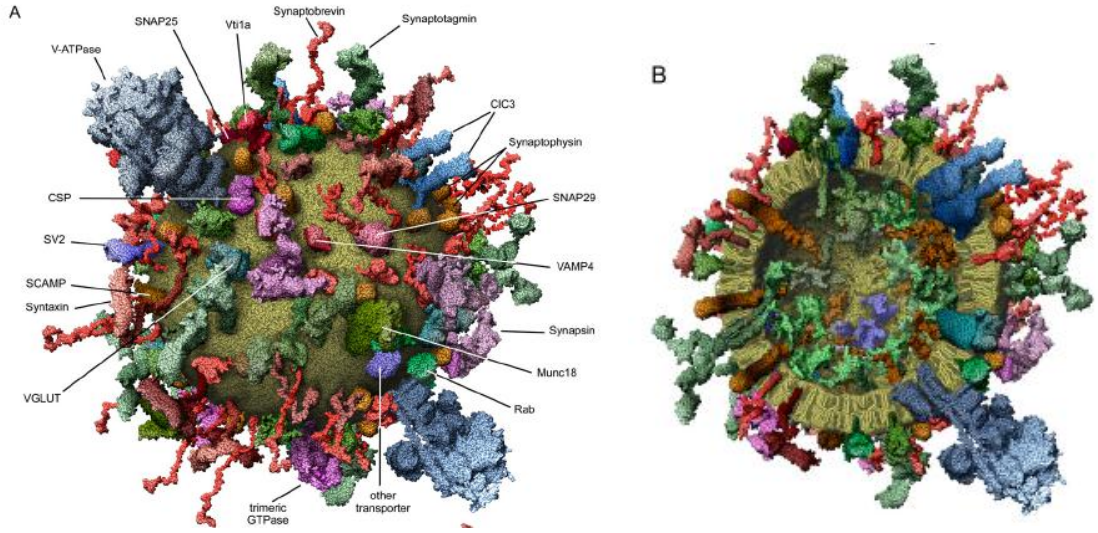


Figure 3. The model is based on space-filling models of all macromolecules at near atomic resolution. (**A**) Outside view of a vesicle. (**B**) View of a vesicle sectioned in the middle (the dark-colored membrane components represent cholesterol) (from Takamori et al., 2006).

Calculation of the relative occupancy of the vesicle membrane by lipids and protein transmembrane regions suggests that approximately 20% of the membrane is occupied by transmembrane regions. Given that each transmembrane region is presumably surrounded by a ring of fixed phospholipids, this finding implies that there is very little fluid phospholipid membrane present in synaptic vesicles. Thus, the membrane of the synaptic vesicles is more rigid than anticipated (Sudhof, 2006).

THE SYNAPTIC VESICLE CYCLE

Synaptic vesicles undergo a trafficking cycle in the nerve terminal that can be divided into sequential steps: first, neurotransmitters are actively transported into synaptic vesicles (step 1), second, synaptic vesicles cluster in front of the active zone (step 2). Then synaptic vesicles dock at the active zone (step 3), where the vesicles are primed (step 4) to convert them into a state of competence for Ca^{2+}-triggered fusion-pore opening (step 5) (Fig. 4).
After fusion-pore opening, synaptic vesicles endocytose and recycle probably by three alternative pathways, which are thought to coexist: (a) vesicles are reacidified and refilled with neurotransmitters without undocking, thus remaining in the readily releasable pool

(step 6, called “kiss-and-stay”); (b) vesicles undock and recycle locally (step 7, called “kiss-and-run”) to reacidify and refill with neurotransmitters (back to steps 1 and 2); or (c) vesicles endocytose via clathrin-coated pits (step 8) and reacidify and refill with neurotransmitters either directly or after passing through an endosomal intermediate (step 9). Most successive steps occur without much vesicle movement except for docking (step 3) and recycling (steps 7–9).

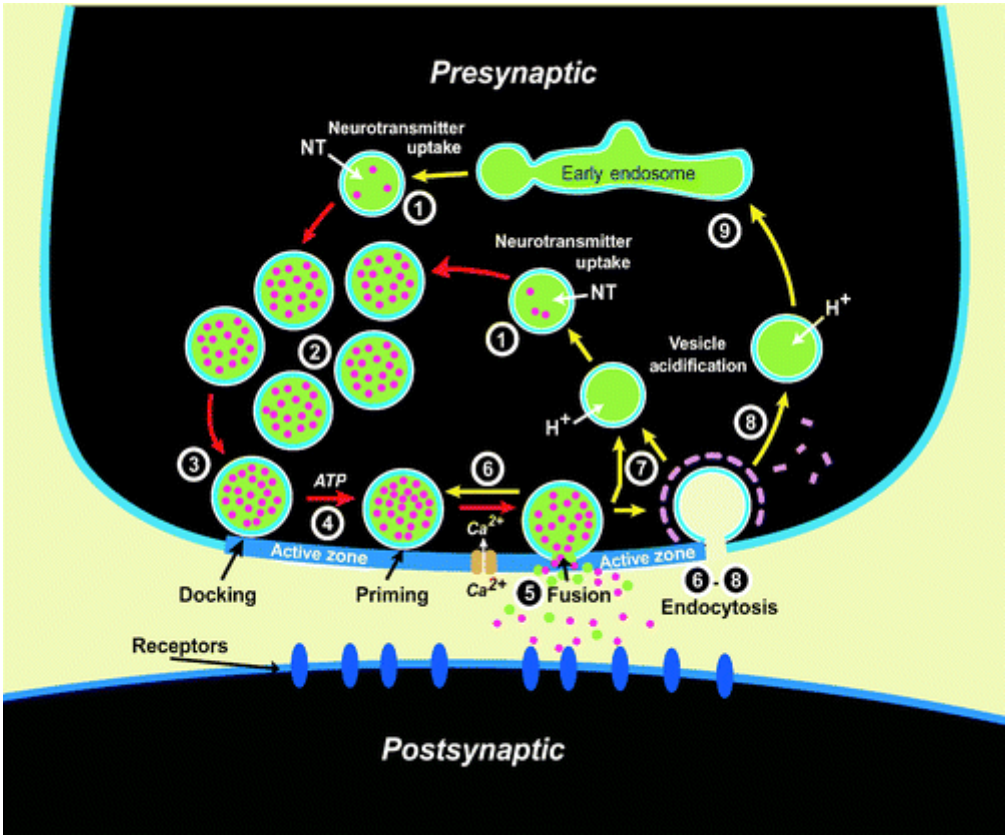


Figure 4. The synaptic vesicle cycle. Synaptic vesicles are filled with neurotransmitters by active transport (step 1) and form the vesicle cluster that may represent the reserve pool (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for $Ca2^{+}$ triggered fusion-pore opening (step5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via several routes: local reuse (step 6), fast recycling without an endosomal intermediate (step 7), or clathrin-mediated endocytosis (step 8) with recycling via endosomes (step 9). Steps in exocytosis are indicated by red arrows and steps in endocytosis and recycling by yellow arrows (from Sudhof, 2004).

Synaptic vesicle endocytosis is a calcium-dependent process; its speed is therefore increased in an activity-dependent manner, so the vesicle pool is replenished faster. The endocytotic pathway called kiss-and-run predicts that synaptic vesicles maintain their molecular identity, remain on the same spot at the active zone, are rapidly reloaded with neurotransmitter, and are ready for the arrival of a new action potential for a new round of exocytosis.

Synaptic vesicles, before the release of their contents, dock at the pre-synaptic membrane of active zones or ribbon synapses and undergo a priming reaction that prepares them for exocytosis/neurosecretion. Vesicle fusion and pore formation are facilitated by high-affinity interaction of a group of highly-conserved proteins, collectively called SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors). SNARE proteins associated with the vesicles are termed vesicle-SNAREs (v-SNAREs) and those on the presynaptic plasma membrane are called target-SNAREs (t-SNAREs). Vesicles are released synchronously as well as asynchronously, and their mode of release is determined by proteins such as synaptotagmins and complexins that interact and regulate conformational changes within the SNARE proteins (Krishnakumar et al., 2011). The first step in vesicle fusion is "tethering," where the vesicles are brought to the active zone to be attached to protein complexes at the presynaptic membrane (Whyte and Munro, 2002), facilitating contact between v-SNARE and t-SNARE proteins. At the presynaptic membrane of neurons, syntaxin 1A is attached to Munc-18, forming a closed structure (Smyth et al., 2010). The tethering process is thought to detach this complex and open syntaxin 1A for SNARE interaction. Munc-18 activates exocytosis/neurosecretion (Gracheva et al., 2010) with its dissociation from the syntaxin 1A closed form, freeing the SNARE motif for complex formation (Dulubova et al., 1999 and Shi et al., 2011). However, there is evidence for a continued association of Munc-18 with the amino terminus of syntaxin 1A in the assembled SNARE complex. Munc-18 may also be involved in chaperoning syntaxin 1A to the membrane (Shi et al., 2011). The tethering step is followed by docking of vesicles, where SNARE proteins come in contact with each other via the SNARE motifs in a calcium-independent manner. Docking is followed by vesicle priming, where the SNARE proteins form a stable complex via their SNARE motifs, rendering the vesicles competent for fusion (Fig. 5).

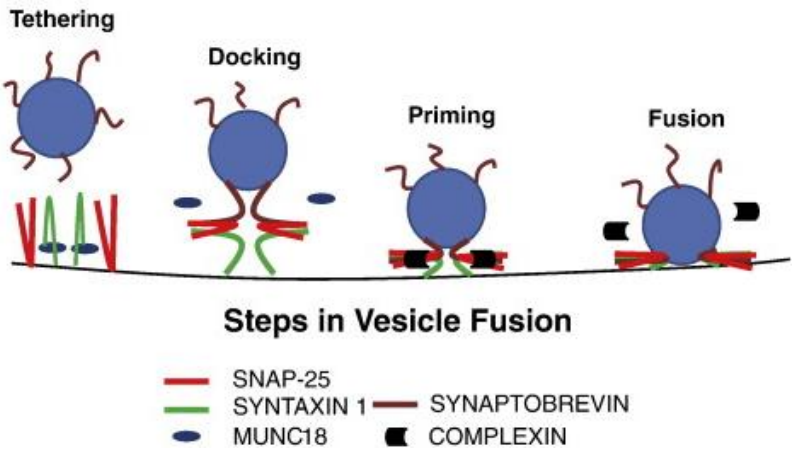


Figure 5. Steps in vesicle fusion include vesicle tethering, docking, priming and finally, fusion. These events are driven by high-affinity interaction between v-SNARE and t-SNARE proteins, regulated by calcium and calcium-binding proteins through their interaction with the SNARE complexes (Ramakrishnan et al., 2012).

The function of SNARE-proteins in fusion has been referred to as the "SNARE hypothesis", it describes a mechanistic model of membrane fusion based on the characteristics of plasma membranes and exocytosis/neurosecretion. It is assumed that the SNARE proteins present in the acceptor (plasma membrane) and donor (vesicle) membranes mediate the spatial specificity of the interaction between the vesicle and presynaptic membrane preceding fusion (Sollner et al., 1993). SNARE-proteins contain a characteristic sequence, referred to as the SNARE motif (Fig. 6), that comprises 60–70 residues composed of eight heptad repeats. Most SNARE proteins contain one SNARE motif, whereas SNAP-25 and its homologs (SNAP-23, -29, and -47) include two SNARE motifs.

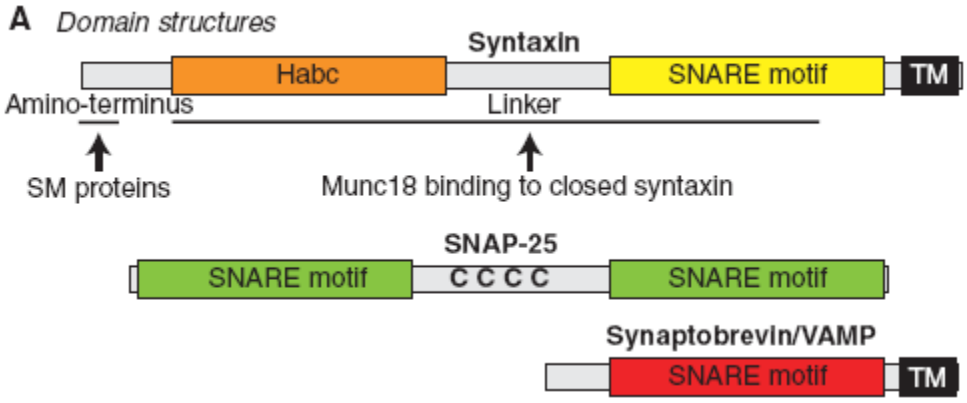


Figure 6. Schematic diagram of the domain structures of syntaxin, SNAP-25, and synaptobrevin/VAMP (Habc, Habc-domain; TM, transmembrane region) (from Sudhof and Rizo 2011).

The four SNARE motifs of synaptic SNARE proteins assemble into the four-helix coiled coil structure of the SNARE complex (Fig. 7) (Poirier et al. 1998; Sutton et al. 1998), with one SNARE motif each from synaptobrevin and syntaxin-1, and two SNARE motifs from SNAP-25.

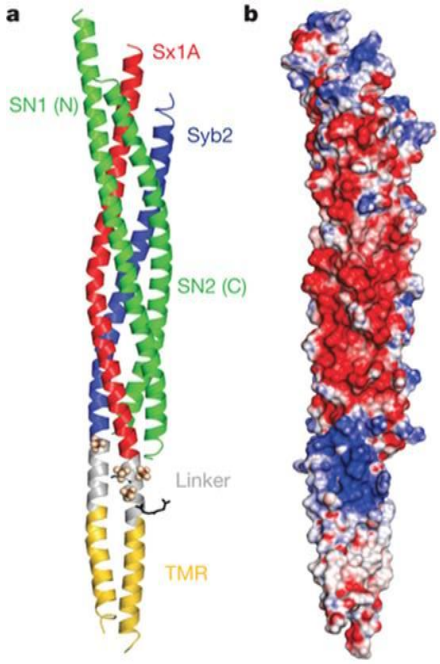


Figure 7. Synaptobrevin2 and syntaxin1A form continuous helices. (**a**) Ribbon plot of the synaptic SNARE complex including linkers and TMRs (colour coding as in Fig. 7; Sx1A, syntaxin1A; Syb2, synaptobrevin2; SN1, SN2, SNAP-25; TMR, trans-membrane region). (**b**) Surface plot showing the electrostatic potential of the synaptic SNARE complex (blue, positive charge; red, negative charge) (from Stein et al., 2009)

The SNARE cycle starts with the amino- to carboxy-terminal zippering of *trans*-SNARE complexes that bridge the gap between the membranes destined to fuse. Full zippering of *trans*-SNARE complexes likely produces fusion-pore opening, although it is possible that the full zippering only stresses the membranes and that fusion-pore opening occurs subsequently mediated by the SM protein. After fusion pore opening, the two membranes completely merge, and *trans*-SNARE complexes are converted into *cis*-SNARE complexes (i.e., SNARE complexes are on a single membrane), which are dissociated into monomers by NSF and SNAPs (Fig. 8).

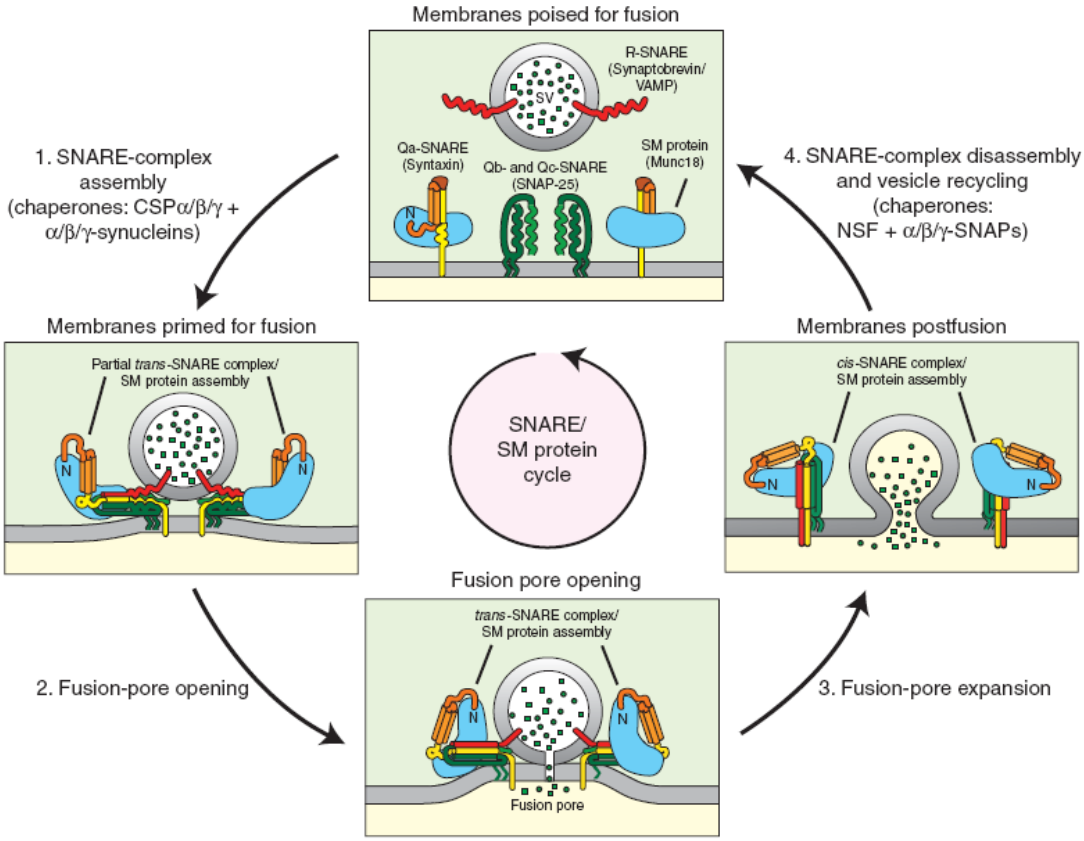


Figure 8. The SNARE/SM protein cycle. During priming, SNARE proteins partially zipper up into trans-complexes, and the SM protein associates with the trans-complexes by binding to the syntaxin amino terminus (left diagram). Full SNARE-complex assembly then pulls the membranes apart, opening the fusion pore (bottom diagram), which expands such that the vesicle membrane collapses into the target membrane, and the trans-SNARE complexes are converted into cis-SNARE complexes (right diagram). Afterward, cis-SNARE complexes are dissociated by the ATPase NSF acting in conjunction with its adaptors a/b/g-SNAPs, and vesicles recycle to start another round of the cycle (from Sudhof and Rizo 2011).

Several synaptic protein may regulate the assembly of SNARE complexes. Members of the mammalian Munc13 protein family have been found to be absolutely required for the priming process (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999; Brose et al., 2000). Its function is tightly regulated by Ca^{2+} (via the C2B-domain, diacylglycerol synthesis, and calmodulin) and RIM. In priming, Munc13 probably acts via a direct or indirect interaction of the MUN-domain with SNARE and/or SM proteins (Ma et al. 2011).

Complexins are small cytoplasmic proteins that bind via a central helix to a groove on the surface of the SNARE complex, which is formed by the helices of syntaxin and synaptobrevin. These act to modulate SNARE-mediated exocytosis by their calcium-dependent interaction with the assembled SNARE core complex (SNAREpin), thus

"clamping" the SNARE and temporarily arresting exocytosis (Bracher et al., 2002, Chen et al., 2002 and Giraudo et al., 2006). Clamping of SNAREpins by complexins 1 and 2 is necessary for synchronous exocytosis. Complexins also potentiate fusogenicity of vesicles by interacting with SNAREs through their N-termini (Xue et al., 2010). Further, it has been suggested that the complexin-SNARE interaction regulates the force that trans-SNARE complexes apply to the fusing membranes (Maximov et al., 2009).

Tomosyn, snapin and Munc18 are presynaptic proteins that can regulate the assembly of SNARE complexes. Tomosyn binds to both syntaxin and SNAP-25 to form a similar structure to the SNARE complex (Hatsuzawa et al., 2003; Pobbati et al., 2004). Both the SNARE complex and the trimeric tomosyn-syntaxin-SNAP-25 complex form at a similar rate, and synaptobrevin is unable to displace tomosyn from the existing complex, suggesting that the tomosyn-syntaxin-SNAP-25 complex represents an end product. As a consequence of preventing SNARE complex formation, tomosyn inhibits secretion in various neuroendocrine cells (Hatsuzawa et al., 2003; Yizhar et al., 2004; Zhang et al., 2006). Interestingly, the trimeric complex containing tomosyn cannot bind complexin (Pobbati et al., 2004) suggesting that, in a functional environment such as the presynapse, the formation of the SNARE complex containing synaptobrevin might still be favoured.

Snapin is another modulatory protein of SNARE complexes which binds to SNAP-25 *in vitro* (Ilardi et al., 1999) and it may stabilize the interaction between synaptotagmin and the SNARE complex (Tian et al., 2005).

The SM protein Munc18-1 is as essential for exocytosis of synaptic and secretory vesicles as the SNARE proteins. This protein forces syntaxin to adopt a closed conformation that prevents its participation in the SNARE complex, thus "negatively" regulating SNARE complex formation. Naturally, Munc18-1 has been assumed to dissociate from syntaxin before SNARE complex formation can occur. This hypothesis is supported by biochemical data showing that the priming factor Munc13 displaces Munc18-1 from syntaxin in vitro (Sassa et al., 1999). However, one can equally well assume that the Munc18-1/syntaxin interaction is only weakened, and still persists, during SNARE complex assembly. A second model for Munc18-1 action is based on the observation that SM proteins, including Munc18-1, protect their syntaxin partner from degradation (Bryant and James, 2001; Rowe et al., 2001), although 50-70% syntaxin persist in the absence of Munc18-1 (Voets et al., 2001; Weimer et al., 2003; Toonen et al., 2005). In a third model, synaptobrevin diplaces from membrane sheets by binding to the other SNARE proteins (Zilly et al., 2006), and Munc18-1 was found to accelerate vesicle fusion *in vitro* (Shen et al., 2007). The

controversy about Munc18-1 role in vesicle fusion machinery has been partially solved by Sorensen and colleagues (Gulyás-Kovács et al., 2007), who demonstrated in chromaffin cells that Munc18-1 participates in two different steps of exocytosis: during the docking phase, it binds to the closed conformation of syntaxin1 thus promoting vesicle docking and excluding a "negative" role in the fusion machinery; after vesicle docking, Munc18-1 appears to engage in a distinct interaction with the exocytotic machinery in a different conformation, thus promoting vesicle priming (Fig. 9) (Gulyás-Kovács et al., 2007). A kinetic role in which SM proteins cooperate with SNAREs by helping them assemble into productive topological arrangements at the interface of two membranes (such as ring-like arrangements that could facilitate the opening of fusion pores), possibly by restricting the diffusion of SNAREs into the space between fusing membranes, has also been proposed (Rizo et al., 2006; Sudhof and Rothman, 2009).

Another class of proteins that may regulate SNARE function at the synapse are synaptophysins, abundant synaptic vesicle proteins that bind directly to synaptobrevin (Johnston and Sudhof, 1990; Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). Synaptobrevin cannot simultaneously bind to synaptophysins and participate in the SNARE complex, which suggests that binding of synaptobrevin to synaptophysin restricts the availability of synaptobrevin for fusion (Edelmann et al., 1995, Becher et al., 1999). Consistent with this hypothesis, the synaptobrevin/synaptophysin complex is upregulated during development (Becher et al., 1999) and chronic blockade of glutamate receptors, which caused an increase in neurotransmitter release, decreases the formation of synaptobrevin/synaptophysin complex (Bacci et al., 2001).

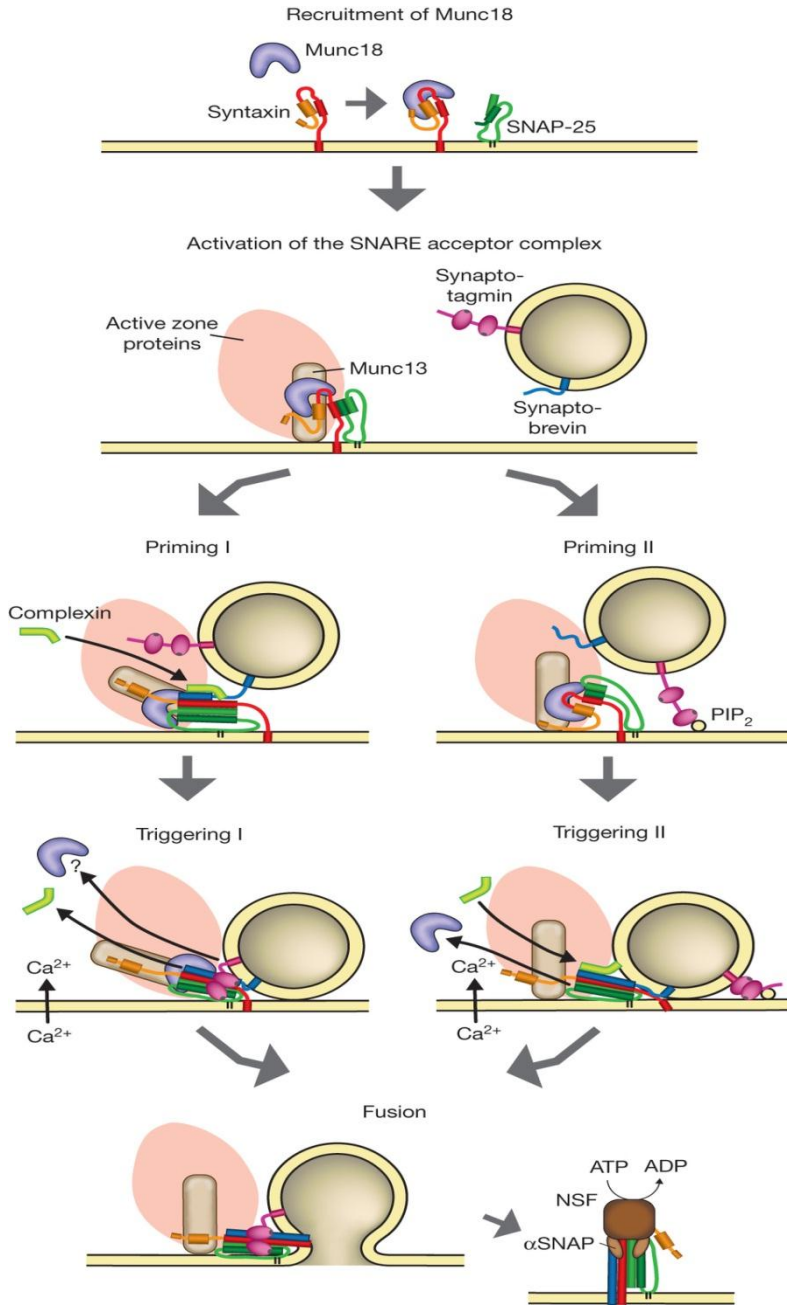


Figure 9. Priming I involves arrest of a partially zippered SNARE complex, here shown with bound Munc18, Munc13 and synaptotagmin. Calcium influx triggers binding of synaptotagmin to the SNARE complex and to the plasma membrane (involving $PI(4,5)P_2$, not shown here), associated with displacement of complexin and (possibly) Munc18 and/or Munc13. Priming II involves arrest after positioning of the vesicle with the aid of active zone components and (possibly) contact of synaptotagmin with $PI(4,5)P_2$ in the plasma membrane, but no contact between the SNAREs. Ca^{2+}-triggering pulls the vesicle closer via synaptotagmin-mediated cross-linking, resulting in SNARE assembly, associated with full opening of syntaxin and displacement of Munc18, and binding of complexin (from Jahn and Fausshauer 2012).

THE RAB3 CYCLE

Rabs constitute the largest family of small Ras-like GTPases with 11 identified in yeast and more than 60 members in humans and regulate intracellular transport. Synaptic vesicles contain members of at least three families of Rab proteins: Rab3 (Rab3A, 3B, 3C, and 3D) (Schluter et al., 2002), Rab5 (Fischer von Mollard et al., 1994), and Rab11 (Khvotchev et al., 2003). Of these, Rab3 proteins are the most abundant; Rab3A alone accounts for ~25% of the total Rab GTP binding in brain (Geppert et al., 1994). Rab3 molecules are attached to the membrane by a post-translational modification of the c-terminal residues consisting of two geranylgeranyl chain, which confer the properties of an intrinsic membrane protein of synaptic vesicles (Johnston et al., 1991). Rab3 undergoes a cycle of synaptic vesicle binding and dissociation in parallel with synaptic vesicle exo- and endocytosis (Fischer von Mollard et al., 1991). During or after synaptic vesicle fusion, GTP on Rab3 is hydrolyzed to GDP and Rab is then removed from the membrane by guanine nucleotide dissociation inhibitor (GDI) in preparation for the next cycle (Araki et al., 1990). The insertion of the Rab into the target membrane is mediated by a GDI dissociation factor (GDF) that releases the Rab from GDI. Rab3 dissociation from vesicles depends on Ca^{2+}-triggered exocytosis of synaptic vesicles (Fischer von Mollard et al., 1991), which suggests that the Rab3 cycle ensures directional interactions of Rab3 with effector proteins during exocytosis (Fig. 10).

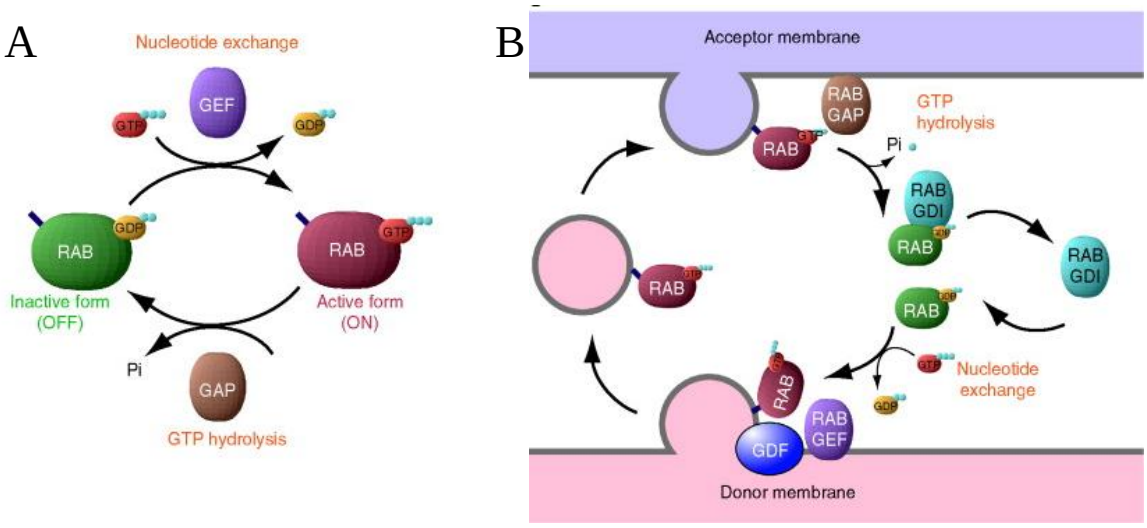


Figure 10. Model of RAB GTPase cycle. (**A**) RAB proteins cycle between the inactive (GDP-bound, green) and active (GTP-bound, magenta) forms. Two major classes of proteins are involved in this regulation. The RAB guanine nucleotide exchange factor (GEF, purple) exchanges bound GDP for GTP, and the RAB GTPase activating protein (GAP, brown) accelerates GTP hydrolysis on the active RAB protein. GTP, red; GDP, yellow. (**B**) RAB proteins cycle between the cytosol and various membranes (from Saito and Ueda 2009).

FUSION PORE

Porosomes have been demonstrated to be secretory portals at the plasma membrane in cells (Jena, 2005 and 2007). Porosomes are supramolecular lipoprotein structures at the cell plasma membrane, where membrane-bound secretory vesicles transiently dock and fuse to release inravesicular contents to the outside during cell secretion (Lee et al., 2012).

Lipids of the vesicles diffuse in the plasma membrane, whereas proteins remain in the vesicle (Taraska et al., 2003; Perrais et al., 2004; Fulop et al., 2005). Various lipids and proteins in the vesicle and plasma membranes have been revealed to be actively involved in the formation, stabilization, and pre-/post-fusional regulation of fusion pores. It has been suggested that a circular arrangement of five to eight syntaxin transmembrane segments could form proteinaceous fusion pores and that the lipid molecules might intercalate between them to complete the structure (Han et al. 2004). Fusion pore formation is thought to be associated with conformational change in the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complex that is formed between the vesicle and the plasma membranes (Michael et al. 2006; Schweizer and Ryan 2006).

The membrane spanning C -terminus of syntaxin has been shown to line the fusion pore (Han et al., 2004), since a change in the residues of syntaxin C-terminus facing the pore lumen disturbs catecholamine release. Synaptobrevin seems to regulate the timing and the dilation of the fusion pore. Indeed, in chromaffin cells deprived of synaptobrevin, exocytosis is supported by cellubrevin, a close homolog of synaptobrevin2, albeit with slower kinetics and with a prolonged foot signal (Borisovska et al., 2005). Finally, as expected from a protein without a transmembrane domain, the third SNARE, SNAP-25, is probably not involved in fusion pore formation or dilation, as no mutant studied so far has been shown to have any effect on these parameters (Becherer and Rettig, 2006). Nevertheless SNAP-25, interacting with synaptotagmin, can control fusion pore dynamics during the final steps of exocytosis (Bai et al., 2004). An important functional role of phosphatidylserine (PS) in Ca^{2+}-triggered exocytosis has been suggested. Increase in PS content in PC12 cells has two distinct and opposing effects on the kinetic of exocytosis, enhancing an early step leading to fusion pore opening, and slowing a later step when fusion pores dilate (Zhang et al., 2009).

Synaptotagmin 1 is thought to trigger exocytosis through its Ca^{2+}-dependent interactions with PS-containing liposomes and SNARE proteins (Chapman et al., 2002). Its isoforms and its mutants can influence the stability of initial fusion pores (Bai et al., 2004; Wang et al., 2001; Zhang et al., 2010), suggesting that it may interact with this fusion intermediate.

THE SYNAPTOTAGMIN

Synaptotagmins are synaptic and secretory vesicle proteins that contain a single N-terminal transmembrane region, and two C-terminal Ca^{2+}-binding C2-domains (Perin et al. 1990). There were identified 16 mammalian synaptotagmin isoforms, 8 of which bind Ca^{2+} with distinct apparent Ca^{2+}-affinities (Syt1-3, Syt5-7, Syt9, and Syt10) (Li et al.1995; Bhalla et al. 2005). Evidence accumulated over the past years demonstrates that the synaptotagmins are the major Ca^{2+} sensor in synchronous Ca^{2+}-dependent synaptic transmission (Brose et al., 1992; Davletov and Sudhof, 1993; Geppert et al., 1994; Sutton et al., 1995; Sudhof and Rizo, 1996; Desai et al., 2000; Fernandez-Chacon et al., 2001; Littleton et al., 2001). Synaptotagmins 1 and 2 are abundant synaptic vesicle proteins that are differentially expressed (Geppert et al., 1991; Ullrich et al., 1994). Synaptotagmins are anchored to the vesicle through an N-terminal transmembrane domain and possess tandem C2 domains (C2A and C2B) of ~135 amino acids in their cytoplasmic domains. These C2 domains bind to lipids containing phosphatidyl serine and phosphatidyl choline, and, with micromolar affinity, to Ca^{2+} (Bai and Chapman, 2004): the C2A domain of synaptotagmin 1 binds three Ca^{2+} ions (Ubach et al., 1998), and the C2B domain two Ca^{2+} ions (Fernandez-Chacon et al., 2001). The binding of synaptotagmin with phospholipids is essential for achieving a physiologically apparent Ca^{2+} affinity. Blocking Ca^{2+}-binding to the C2B-domain blocks synchronous exocytosis (Mackler et al. 2002), whereas blocking Ca^{2+}-binding to the C2A-domain decreases exocytosis ~40%, and additionally decreases the apparent Ca^{2+}-cooperativity of exocytosis ~40% (Shin et al.2009). Thus, the two C2-domains of synaptotagmins thus are not equivalent, but they cooperate with each other, with the C2B-domain playing the leading part.

There is clear evidence for a role of synaptotagmin in the docking, mediated by its interaction with SNAP-25 (Chieregatti et al., 2002), and in the fusion process (Wang et al., 2001; 2003; 2006). Synaptotagmins have been long considered Ca^{2+} sensors for evoked but not for spontaneous transmission, given that deletions of Syt1 and of Syt2 increase spontaneous release (Littelton et al., 1994; Maximov et al., 2005; Pang et al., 2006). However, it has been recently demonstrated that Syt1 deletion strongly decreased the fast component of AP-evoked release in synapses of neuron cultures from *Syt1* knockout (KO) mice. On the other hand, asynchronous release was increased at both types of synapses (Geppert et al., 1994; Nishiki et al., 2004; Maximov et al., 2005; Yoshihara et al., 2002). Similarly, spontaneous release was increased after *Syt1* genetic deletion in cultured neurons from *Syt1* KO mice, leading to the idea that Syt1 has a second function in

suppressing spontaneous release (‘release clamping’) in addition to its primary role as a Ca^{2+} sensor. By interacting with the SM protein Munc18-1, synaptotagmin also has a role in positioning vesicles at the synapse during the docking phasem; there were describe two alternative models.

Finally, a role of Syt in membrane bending has been recently demonstrated by utilizing membranes with different degrees of curvature (Hui et al., 2009); interaction of Syt with lipid bilayers serves to drive localized invagination of the target membrane to facilitate fusion (Monck and Fernandez, 1994) and constitutes a critical step in Ca^{2+} regulated fusion (Hui et al., 2009).

THE SNARE PROTEIN SNAP-25

SNAP-25 is a hydrophilic protein of 206 amino acids was originally identified as a cDNA corresponding to an mRNA abundantly expressed in mouse brain but not in non-neuronal tissue (Oyler et al., 1989). It has been demonstrated that SNAP-25 is widely but differently distributed both in the nervous system as well as in neuroendocrine and endocrine cells (Jacobsson et al., 1994, 1996, 1999; Bark et al., 1995; Gonelle-Gispert et al., 1999) and with an onset of gene expression that correlates to neuronal maturation (Catsicas et al., 1991). SNAP-25 associates with membranes through palmitoyl residues that are thioester-linked to four closely spaced cysteine residues at the centre of the protein. The amino- and carboxy-terminal domains are highly conserved and are predicted to form intermolecular coiled coils. The *Snap-25* gene is a single gene spanning more than 80 kb of genomic DNA and the polypeptide is encoded for by eight different exons spaced by quite large intron sequences (Bark, 1993). SNAP-25 exists in two isoforms, SNAP-25a and SNAP-25b, which arise from alternate splicing of exon 5, and are differentially expressed throughout the nervous system, both encoding for 39 amino acids (Bark and Wilson, 1994). SNAP-25a and SNAP-25b differ by only nine amino acids and include the region in the SNAP-25 protein that spans the last part of the N-terminal SNARE motif and the first part of the linker that separates the N- and C-terminal α-helices. Thus, the amino acid differences participating in forming the four α-helix coil–coil structure in the SNARE complex could possibly interfere with SNARE complex stability or ability to interact with accessory proteins. In addition, differences in the linker region distort the cystein quartet, and although all four cysteines are retained, they appear in a different organization and

sequence context (Bark, 1993; Bark and Wilson, 1994). Studies of gene expression of these two alternative SNAP-25 variants in mouse brain have demonstrated that the two mRNAs are differently expressed during development and their neuroanatomical distributions are individual and distinct. In rat brain, SNAP-25b protein expression increased dramatically during post-natal development and was similar in hippocampus, cerebellum and cortex, implying that changes in expression of this isoform are largely occurring at a global level. On the other hand SNAP-25a is mostly expressed at early stages of brain development (Oyler et al., 1991; Bark et al., 1995; Boschert et al., 1996), whereas during the post-natal period the increase in its expression was more modest and variable (Prescott et al., 2011). The increase in SNAP-25b expression coincides in particular with the specific elevation of the mRNA splice variant b (Bark et al., 1995; Boschert et al., 1996) and with the concomitant shift of the protein from cell bodies to cell processes and presynaptic terminals (Oyler et al., 1991; Bark et al., 1995; reviewed in Hepp and Langley, 2001). Examination of neuroexocytosis from chromaffin cells of *Snap-25* null mice clarified how alternative splicing of SNAP-25 may affect Ca^{2+} triggered exocytosis (Sorensen et al., 2003). Chromaffin cells contain two pools of releasable vesicles: the readily releasable pool (RRP) and the slowly releasable pool (SRP) (Voets et al., 1999). In this model, SNAP-25b-driven secretion is characterized by a more than 2-fold larger size of the SRP and the RRP as compared to SNAP-25a, suggesting that the progressive increase in SNAP-25b mRNA relative to SNAP-25a during the first week of postnatal brain development (Bark et al., 1995) could be required for increasing pools of primed vesicles to meet the needs of synaptic maturation (Sorensen et al., 2003).

Genetic experimental approaches have demonstrated that SNAP-25 is not required for stimulus-independent neurotransmitter release, but is essential for evoked synaptic transmission (Washbourne et al., 2002). Genetic ablation of the t-SNARE SNAP-25 abolished completely evoked release, without affecting spontaneous release. Moreover, *Snap-25* null-mutant neurons showed higher frequencies in spontaneous events as compared to wild-type neurons, suggesting a dual role in membrane fusion: SNAP-25 can act as positive regulator in action potential-dependent release, and simultaneously it can act as negative regulator to inhibit spontaneous events (Washbourne et al., 2002). Nevertheless very recently, Bronk et al. (2007) analyzed neurotransmission in hippocampal cultures from *Snap-25*-deficient mice. They confirmed the block of evoked glutamatergic transmission but they observed the persistence of spontaneous neurotransmission, although at a much lower frequency than in heterozygous cultures.

DIFFERENT ISOFORMS OF SNAP-25

Multiple isoforms and homologous proteins, originated by differential splicing of single genes or codified by different genes of the same family, respectively, have been described for VAMP, syntaxin and SNAP-25. The homologous forms of SNAP-25 comprise SNAP-23, SNAP-29 and SNAP-47 proteins (Fig. 11).

SNAP-23 shows 58 % identity at the aminoacidic level with SNAP-25 and it is ubiquitously expressed. Similarly to SNAP-25, SNAP-23 is localized to the plasma membrane (Wang et al. 1997), and assumes the essential functions in a variety of exocytic reactions in non-neuronal cells, nevertheless it has been detected in cortical neurons and in purified synaptic vesicles (Bragina et al. 2007; Takamori et al. 2006). SNAP-23 is proposed to bind another synaptotagmin isoform, syt7, which binds calcium with ~10-fold higher affinity (Shin and Sugita et al. 2002) and allows SNAP-23 to mediate granule docking and fusion at resting calcium levels (Chieregatti et al. 2004). Thus, SNAP-23 can support synaptic vesicle fusion in the absence of SNAP-25 and may function in a SNARE complex driving asynchronous and/or spontaneous neurotransmitter release.

SNAP-29 displays 39% similarity relative to SNAP-25; it has been proposed to be a ubiquitous cytoplasmic SNARE protein involved in general membrane trafficking (Hohenstein, 2001). Recent studies suggested that SNAP-29 is present at synapses and can act as a negative modulator for neurotransmitter release, probably by slowing recycling of the SNARE-based fusion machinery and synaptic vesicle turnover (Pan, 2005).

SNAP-47 revealed ubiquitous tissue distribution, with particularly high levels in the brain. In neurons, SNAP-47 shows a widespread distribution on intracellular membranes and is also enriched in synaptic vesicle fractions. *In vitro*, SNAP-47 can functionally substitute for SNAP-25 in SNARE complex formation with the neuronal SNAREs syntaxin 1a and synaptobrevin 2, and it also substituted for SNAP-25 in proteoliposome fusion. However, neither complex assembly nor fusion was as efficient as with SNAP-25 (Holt, 2006).

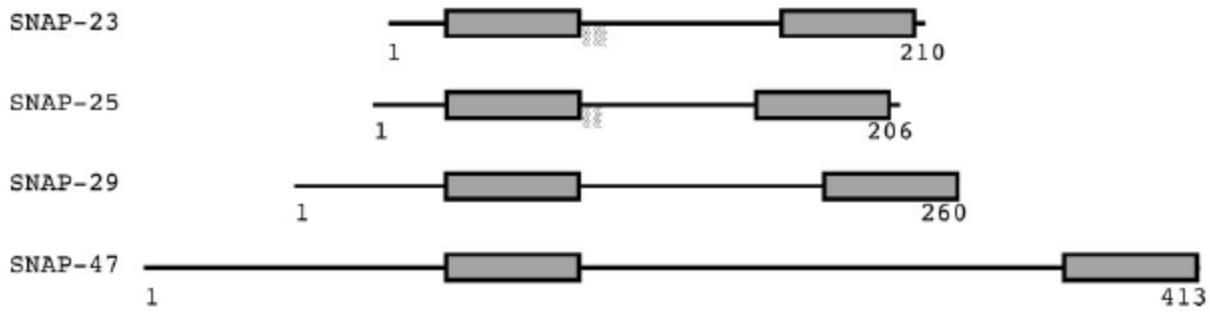


Figure 11. Schematic view of the domain structure of SNAP-25 isoforms. All SNAP-25 homologues contain tandem SNARE motifs (*boxed*). Note that SNAP-47 has an extended N-terminal domain and linker region compared with other Q-SNAREs. Sequences are illustrated at scale. Note the palmitoylation sites in the linker region indicated on SNAP-23 and SNAP-25.

SNAP-25 EXPRESSION IN EXCITATORY AND INHIBITORY NEURONS

Normal brain function relies on the fine balance of activity between the two most diffuse fast synapse populations in the CNS, the inhibitory GABAergic and the excitatory glutamatergic terminals. In the past years, evidences have accumulated demonstrating that GABAergic and glutamatergic neurons express different molecular components involved in neuronal development, intracellular signalling and pre-postsynaptic functions. In 2004, in a paper of our group, initial evidence emerged demonstrating that mature GABAergic inhibitory synapses express much lower levels of SNAP-25 than excitatory glutamatergic synapses (Verderio et al., 2004).

In 1989 Oyler and colleagues described the isolation and characterization of cDNA clones encoding a novel 25-kDa synaptosomal protein, SNAP- 25, showing that the proteinwas heterogeneously expressed in distinct populations of neurons (Oyler et al., 1989). This report was subsequently followed by other studies indicating that SNAP-25 is expressed in specific subtypes of conventional synapses, but not ribbon synapses (Catsicas et al., 1992), that variable SNAP-25 expression occurs in autonomic vasoconstrictor and vasodilator neurons in central and peripheral inputs to sympathetic neurons (Morris et al., 2000; Gibbins et al., 2003), and that heterogeneous levels of the protein are present in VAChT-containing varicosities of the ventral horn of the spinal cord (Hellstrom et al., 1999). Duc and Catsicas (1995) in particular reported that only a subset of central nervous system synapses display SNAP-25 immunostaining at the electron microscopy level.

In 2004, our group reported the lack of SNAP-25 immunoreactivity at inhibitory synapses of mature rat hippocampal cultures and adult rat hippocampus (Verderio et al., 2004). To determine the expression of SNARE proteins in excitatory and inhibitory hippocampal neurons in primary culture we used immunocytochemistry. Staining with various antibodies directed against SNAP-25 and recognizing both the a and b isoforms (Bark and Wilson, 1994) revealed the specific presence of the protein in processes of glutamatergic neurons. In contrast, processes of GABAergic neurons, identified by their labelling for the neurotrasmitter, or for the GABA vesicular transporter vGAT, or for the GABA-

synthesizing enzyme glutamic acid decarbosilase (GAD), lacked the immunoreactivity for SNAP-25 (Fig. 12).

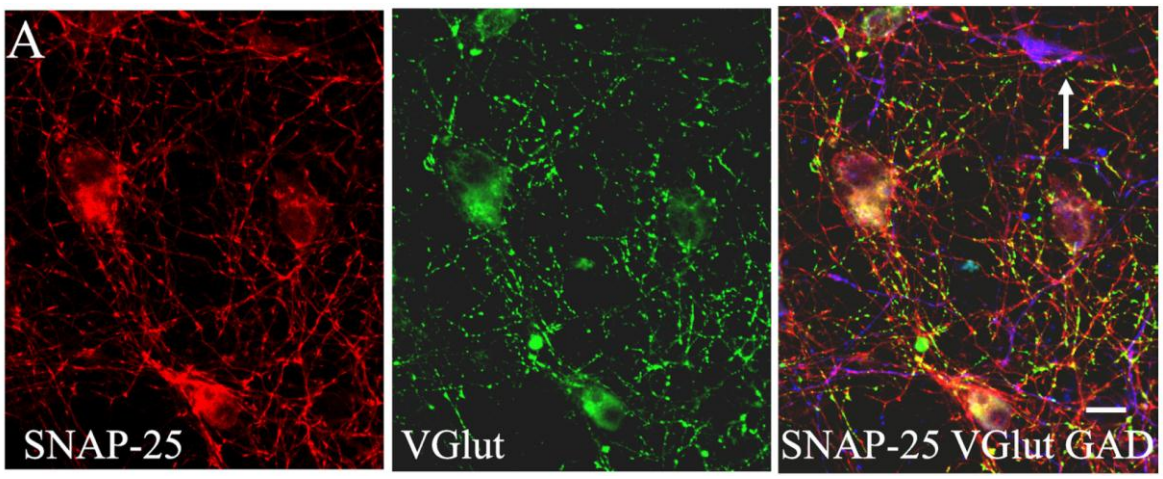


Figure 12. A triple immofluorescence labeling of hippocampal neurons for SNAP-25 (red), VGlut (green), and glutamic acid decarboxylase (GAD; blue). Arrow indicates a GAD-positive GABAergic neuron expressing lower levels of SNAP-25 (from Condliffe et al. 2010).

This finding was validated in slices from adult rat hippocampus, where SNAP-25, was found to be largely excluded from inhibitory terminals in CA1 region. One year later, Frassoni et al. (2005) investigated whether distinct interneuron subpopulations may differ for their content in SNAP-25, and this was evaluated in sections from adult rat hippocampus. One of the most useful characterizations of interneuron subtypes has been based on the presence of calcium-binding proteins such as parvalbumin (PV) and calretinin (CR). It was found that SNAP-25 immunoreactivity was undetectable in the large majority of PV-positive synapses in the *stratum pyramidale* of rat hippocampus while the same terminals were instead immunopositive for the plasma membrane SNARE syntaxin-1; SNAP-25 was also undetectable in CR-immunoreactive terminals. The lack of SNAP-25 immunoreactivity was also demonstrated in axon terminals of CCKpositive cells and in SOM-positive axon terminals (Frassoni et al., 2005).

This observation was subsequently extended to adult mouse hippocampus (Frassoni et al., 2005). Notably, a partial colocalization between SNAP-25 and GAD immunoreactivities at early postnatal stages (P0–P5) of development, when GAD staining is concentrated primarily in fine processes with periodic varicosities within the developing stratum radiatum (Dupuy and Houser, 1996). this colocalization became undetectable in the adult, suggesting a developmentally regulated down-regulation of the protein. The expression of

SNAP-25 was also examined in cultures of rat hippocampal neurons at different stages of development: colocalization between SNAP-25 and GABAergic markers was present in developing neurons at early stages in culture indicating a developmentally-regulated disappearance of SNAP-25 immunoreactivity during interneuron differentiation (Frassoni et al., 2005) (Fig. 13).

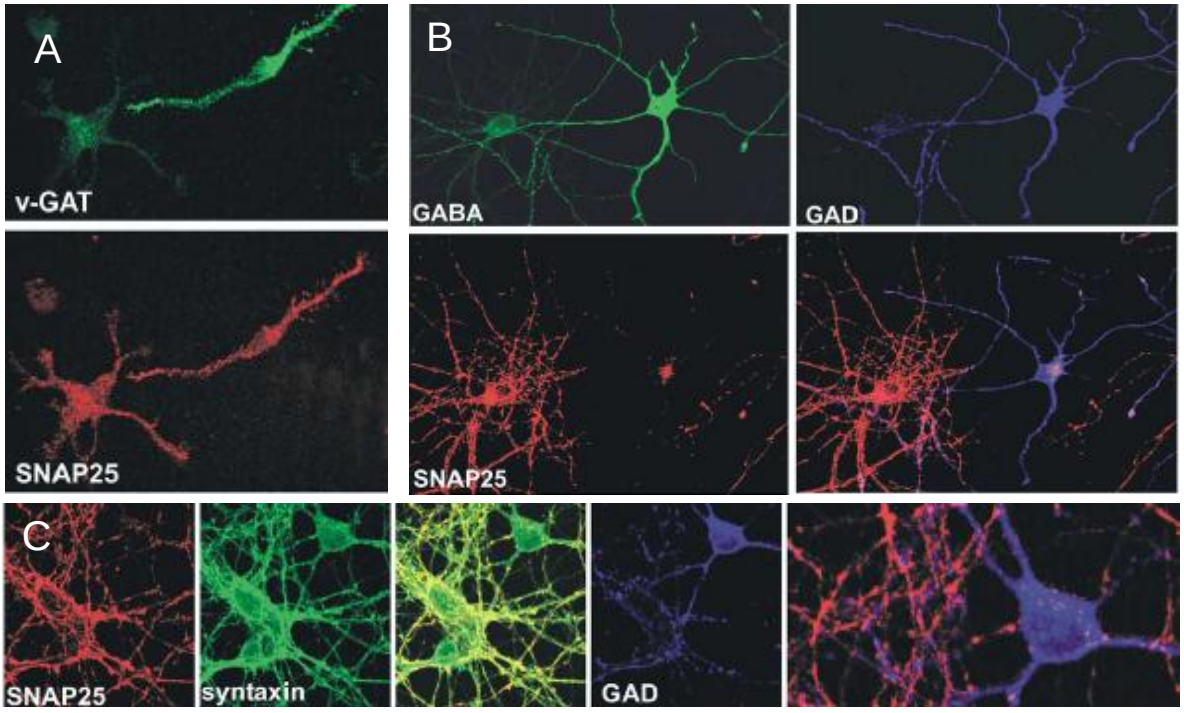


Figure 13. SNAP-25 immunoreactivity in rat cultured neurons at different developmental stages. (**A**) 2 DIV neurons double stained for vGAT and SNAP-25. Note that both the glutamatergic and the GABAergic neurons express detectable levels of SNAP-25. (**B**) Triple labeling of a 6 DIV culture for GABA (green), GAD (human serum, blue) and SNAP-25 (red). Note that, differently from the glutamatergic neuron, processes belonging to a multipolar GABAergic neuron, are virtually devoid of SNAP-25 immunoreactivity. SNAP-25 staining is instead detectable in the Golgi area and at the growth cone. (**C**) Triple labeling of a 15 DIV culture, established from E18 rat hippocampi and maintained in the presence of an astrocyte feeding layer, for SNAP-25 (red), syntaxin (green) and GAD (human serum, blue). Note that the GAD-positive neuron does not express detectable levels of SNAP-25, while it is immunopositive for syntaxin. SNAP-25 immunoreactivity appears to be excluded from GAD-positive presynaptic boutons (see red and blue merged image). Scale bars=18 µm, A; 12 µm, B; 10 µm, C. (from Frassoni et al., 2005).

Bragina and colleagues (2007) confirmed the absence of SNAP-25 immunoreactivity in adult GABAergic synapses in the cerebral cortex, also Garbelli and colleagues (Garbelli et al., 2008), who combined pre-embedding immunolabeling for SNAP-25 with post-embedding immunogold localization of the neurotransmitter GABA, and Mandolesi and colleagues (Mandolesi et al., 2009), who did not detect SNAP-25 labeling in GABAergic terminals of both the molecular and granular layers of the cerebellum. Moreover, poor expression of SNAP-25 was observed in vGlut2 positive glutamatergic cerebellar neurons,

while high levels of the protein were detected in vGlut1 positive cells, and a correlation between SNAP-25 expression and the release probability has been hypothesised (Mandolesi et al., 2009). On the contrary, Tafoya and colleagues (Tafoya et al., 2006) reported the presence of SNAP-25 immunoreactivity in developing cultured neurons from mouse and also showed the presence of high SNAP-25 levels at perisomatic basket cell terminals on CA1 pyramidal cells of the adult mouse hippocampus. The discrepancy between these results have not been clarified and have been attributed to either methodological differences (Bragina et al., 2007) or to alterations in tissue preservation (Garbelli et al., 2008).

Several studies were performed to understand whether GABAergic synaptic transmission is functional in the absence of SNAP-25 protein. These studies (Tafoya et al., 2006; Bronk et al., 2007; Delgado-Martinez et al., 2007) were carried out in brain slices or primary cultures from *Snap-25* deficient fetal mice and provided essentially identical conclusions.
Tafoya et al. (2006), performing whole-cell patch clamp recordings, showed the absence of detectable field stimulation-evoked GABAergic transmission in fetal brain slices from *Snap-25* null mutants and a complete lack of stimulus-evoked synaptic vesicle exocytosis in *Snap-25* null cultured interneurons. Bronk et al. (2007) demonstrated the block of evoked glutamatergic transmission in hippocampal cultures from *Snap-25*-deficient mice and the persistence of spontaneous neurotransmission, albeit at a much lower frequency than in heterozygous cultures. They also examined evoked GABAergic transmission in the same cultures and they concluded that SNAP-25 plays an equally critical role in excitatory and inhibitory neurotransmission, at least throughout the synaptic maturation process in vitro (Bronk et al., 2007). Finally, Delgado-Martinez et al. (2007) found that inhibitory neurotransmission in *Snap-25* null hippocampal or striatal cultures was rescued by lentiviral expression of SNAP-25a or SNAP-25b, with no significant differences in the IPSC amplitude, thus indicating that SNAP-25 supports inhibitory neurotransmission in developing cultures.
The above results indicate that SNAP-25 is functionally required at most, if not all, GABAergic terminals at early developmental stages. This conclusion is in line with the pattern of SNAP-25 expression during neuronal differentiation (Frassoni et al., 2005). However, they do not provide conclusive evidence on the function of SNAP-25 as a SNARE protein in mature synapses. As the *Snap-25* null mutants are not viable, the possibility indeed remains that SNAP-25 can play a role in GABAergic neurons

specifically at early developmental stages (Frassoni et al., 2005; Delgado-Martinez et al., 2007).

SNAP-25 AS A CELLULAR CALCIUM MODULATOR

Evidence indicates an additional function of SNAP-25 in the modulation of various ion channels. In particular, the interaction of SNAP-25 with different types of voltage-gated calcium channels (VGCCs), including N-type (Sheng et al., 1996), P/Q-type (Rettig et al., 1996; Martin-Moutot et al., 1996) and L-type (Wiser et al., 1999) has been demonstrated in non-neuronal cells. The synaptic protein interaction (synprint) site of the voltage-gated Ca^{2+} channel (VGCC) α1 subunit can interact with proteins involved in exocytosis, and it is therefore thought to be essential for exocytosis of synaptic vesicles (Fig. 14). This interaction has been shown to alter channel function by inhibiting L-type channel currents (Ji et al., 2002), reducing N-type channel current (Wiser et al., 1996), or the activity of P/Q-type Ca^{2+} channels by negatively shifting the steady state voltage dependence of inactivation (Zhong et al., 2001).

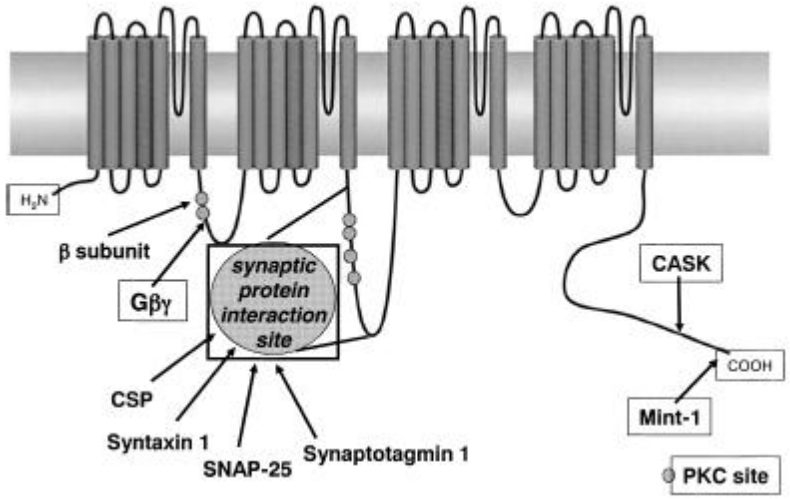


Figure 14. Transmembrane topology of the N-type calcium channel α1- subunit illustrating major protein interactions sites. The domain II – III linker region of vertebrate N-type channels contains a synaptic protein interaction site that interacts with syntaxin 1, synaptotagmin 1, and SNAP-25 (from Zamponi et al., 2003).

Accordingly with these data, it has been previously reported that SNAP-25 can modulate intracellular calcium dynamics, and indeed differential SNAP-25 expression in hippocampal neurons is reflected by different calcium responsiveness; in particular, it was demonstrated that the exogenous expression of SNAP-25 significantly reduces calcium

responses to depolarizing stimuli in hippocampal neurons in cultures, while the silencing of the expression of the protein significantly increases neuronal calcium responsiveness (Verderio et al., 2004). Interestingly, hippocampal GABAergic neurons are characterized by a higher calcium responsiveness to depolarization compared to excitatory neurons and exogenous expression of SNAP-25 in interneurons significantly reduces calcium responses to depolarizing stimuli, which is consistent with the differential expression of the protein in excitatory and inhibitory neurons (Verderio et al., 2004). These data suggested that different levels of SNAP-25 expression in excitatory versus inhibitory neurons may profoundly modulate, in a concentration-dependent manner, neuronal responses to synaptic stimuli and that SNAP-25 is involved in the regulation of neuronal excitability.

Pozzi et al. (2008) studied the SNAP-25 role as a modulator of calcium responsiveness in primary hippocampal cultures from *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ transgenic mice. The results revealed that cultures from *Snap-25*$^{+/-}$ mutants display levels of SNAP-25 intermediate between *Snap-25*$^{+/-}$ and wild-type neurons (Fig. 15), confirming previously reported data (Washbourne et al., 2002). Moreover, neuronal responsiveness to 30 mM KCl was inversely proportional to the amount of the expressed SNARE, with the highest and lowest calcium responsiveness being detectable in *Snap-25*$^{-/-}$ and wild-type neurons, respectively (Fig. 15B) (Pozzi et al., 2008), which is in line with a concentration-dependent modulation of calcium dynamics (Verderio et al., 2004).

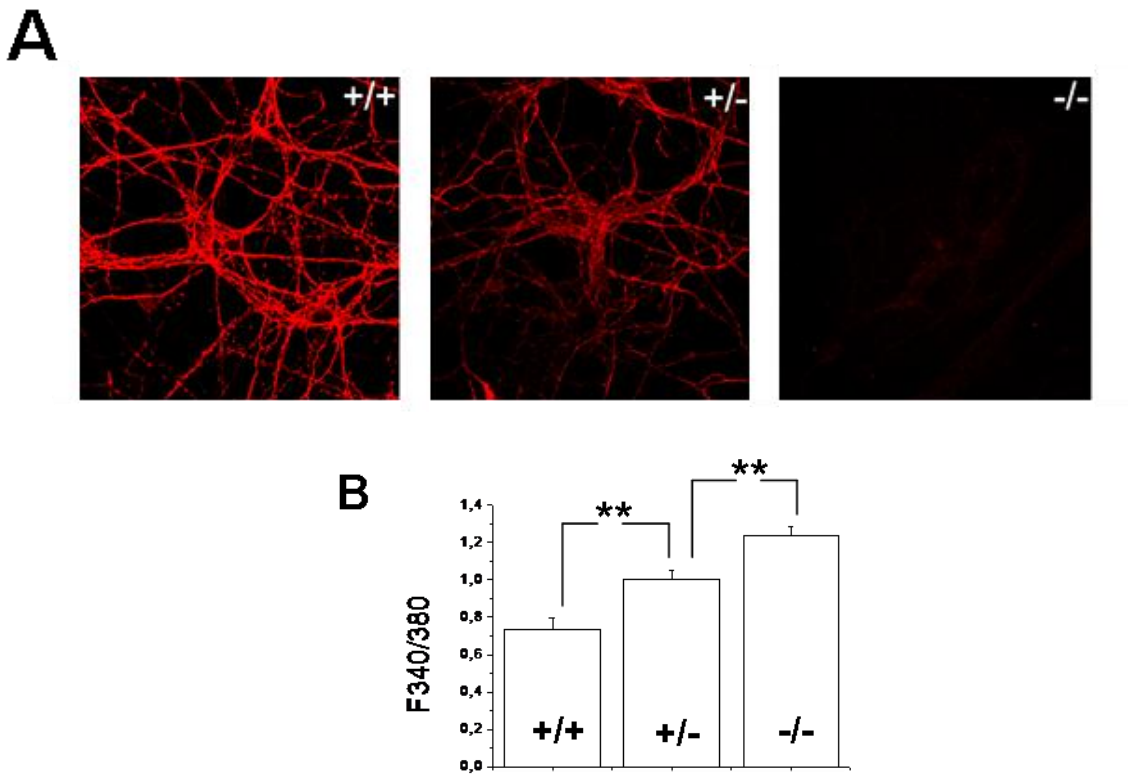


Figure 15. Calcium responsiveness to depolarization in wild-type, *Snap-25*$^{+/-}$ and *Snap-25*$^{-/-}$ neurons. (**A**) Immunofluorescence for SNAP-25 confirms the reduction and the abolishment of protein levels in heterozygous and knock-out neurons, respectively. (**B**) Calcium recordings from *Snap-25*$^{+/-}$ and $^{-/-}$ cultures display a significant increase in the responses upon depolarization with 30 mM KCl both in $^{+/-}$ and in $^{-/-}$ neurons. Noticeably, the increase in responses in inversely proportional to SNAP-25 expression levels (modified from Pozzi et al., 2008).

The crucial region of SNAP-25 that controls neuronal calcium dynamics (residues 180–197) contains a residue, ser187, which can undergo phosphorylation by PKC in an activity-dependent manner. Pozzi et al. demonstrated that negative control of VGCCs by SNAP-25 requires phosphorylation of the protein on Ser187 residue, which is activity-dependent both *in vitro* and *in vivo*; therefore, this mechanism may mediate a negative feedback modulation of neuronal activity during sustained activation (Fig. 16).

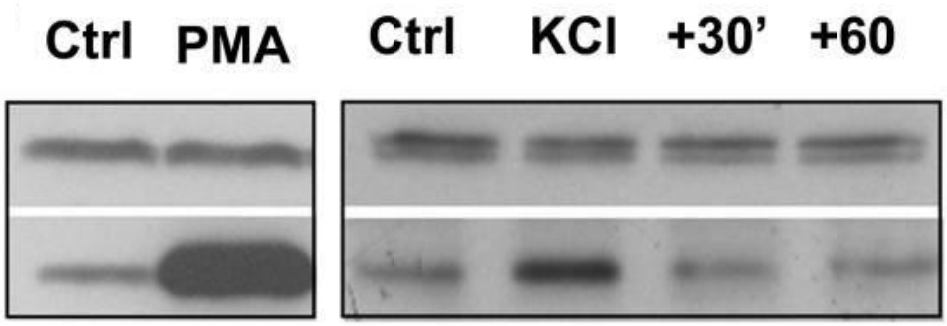


Figure 16. SNAP-25 phosphorylation is modulated by network activity. (*left*) Western blot analysis of SNAP-25 phosphorylation in hippocampal cultures (12 DIV) in control conditions or after stimulation with 1 uM PMA (PKC activator) for 30 min. (*right*) Time course of SNAP-25 phosphorylation in cultures exposed to 30 mM KCl for 5 min, immediately solubilized (KCl), or washed and solubilized after 30 or 60 min (from Pozzi et al., 2008).

Given the differential expression of SNAP-25 in glutamatergic and GABAergic neurons, in a recent paper of our group (Condliffe et al., 2010) it was investigated the regulatory role of endogenous SNAP-25 on native voltage-gated calcium channels in cultured hippocampal neurons.

Cultured glutamatergic and GABAergic neurons showed markedly different VGCC properties. Glutamatergic neurons had significantly lower VGCC current densities, enhanced inactivation rates and shifts in the voltage-dependence of activation and steady-state voltage-dependent inactivation curves compared to GABAergic neurons (Fig. 17). Also, VGCC current inactivated at a faster rate in glutamatergic neurons and the voltage dependence of activation and inactivation of VGCC current varied between glutamatergic and GABAergic neurons.

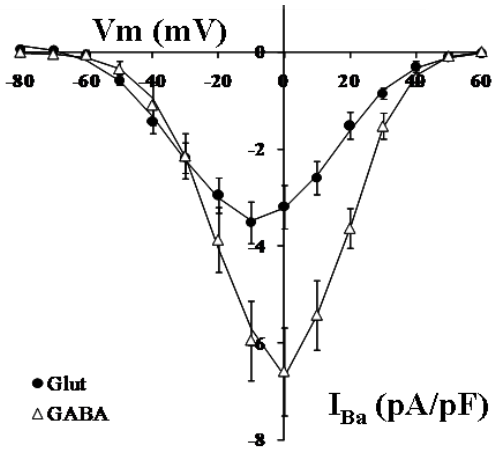


Figure 17. Reduced VGCC current density in glutamatergic compared to GABAergic neurons.

These results suggested that diverse VGCC properties are the consequence of differential levels of endogenous SNAP-25 physically interacting with somatodentritic VGCCs to alter channel function. This conclusion is supported by the fact that silencing of endogenous SNAP-25 in glutamatergic neurons increased VGCC properties towards GABAergic levels while over-expressing SNAP-25 in GABAergic neurons reduced inward VGCC current and inactivation kinetics (Fig. 18).

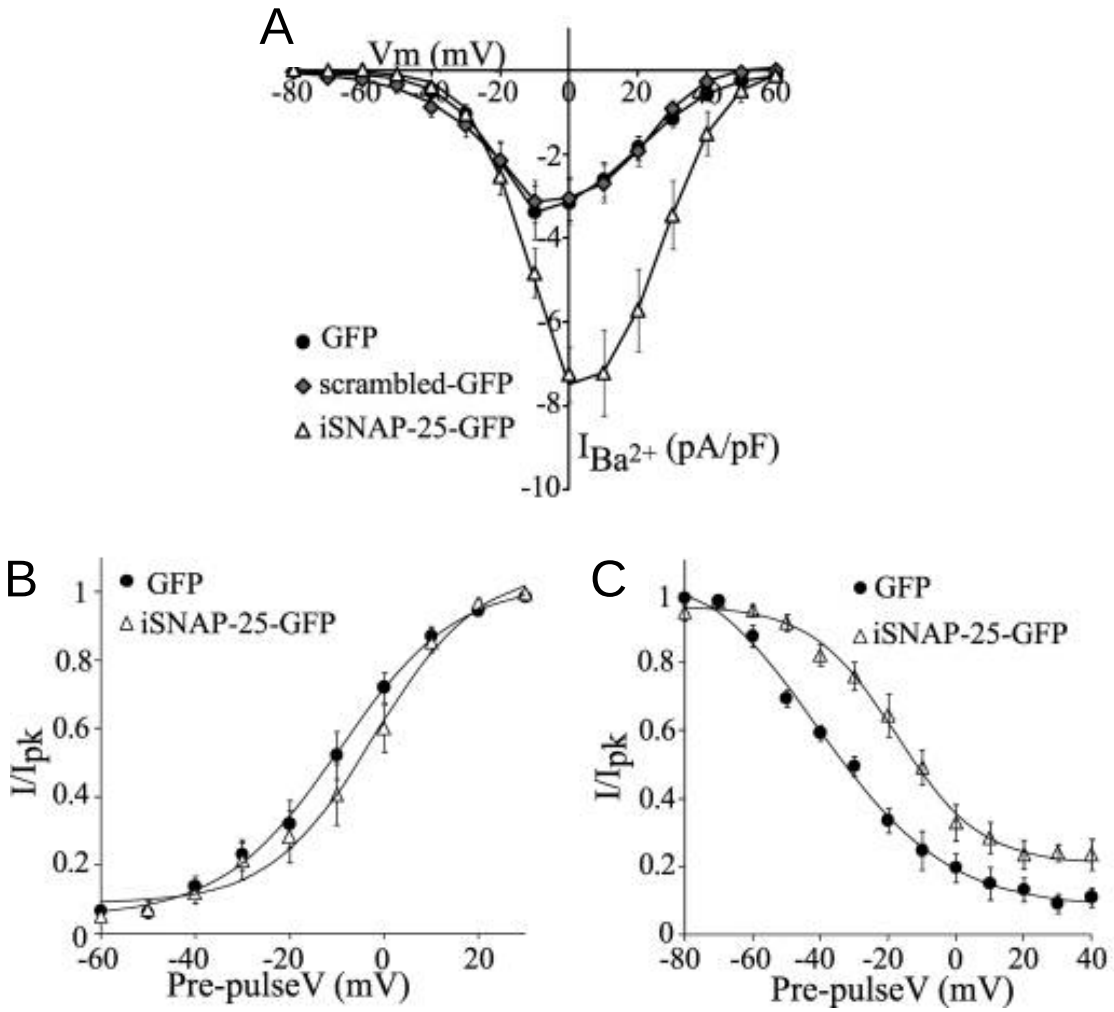


Figure 18. Silencing of endogenous SNAP-25 in glutamatergic neurons augments VGCC properties. (**A**) Relationships of I_{Ba} current density in glutamatergic neurons co-transfected with pSuper SNAP-25 siRNA and GFP, pSuper scrambled siRNA and GFP, or GFP alone. (**B**) Voltage dependence of steady-state activation of isolated P/Q-type current in glutamatergic neurons co-transfected with pSuper SNAP-25 and GFP or GFP alone. (**C**) Voltage dependence of steady-state inactivation of isolated P/Q-type current in glutamatergic neurons co-transfected with pSuper SNAP-25 and GFP or GFP alone.

Moreover it was found that VGCC current densities in glutamatergic neurons from *Snap-25*$^{+/-}$ mice were significantly elevated compared to wild type glutamatergic neurons, while VGCC current densities in GABAergic were similar between wild type and *Snap-25*$^{+/-}$ neurons (Fig. 19). Notably, no difference in the expression of the $Ca_V2.1$ P/Q channels or the $\alpha_2\delta_2$ auxiliary subunit of VGCCs were detected in membrane fractions obtained from wild-type or heterozygous mouse brains, thus excluding the possibility that reduced SNAP-25 levels may induce altered VGCC expression, leading to the increased calcium currents observed in *Snap-25*$^{+/-}$ neurons (Fig. 20).

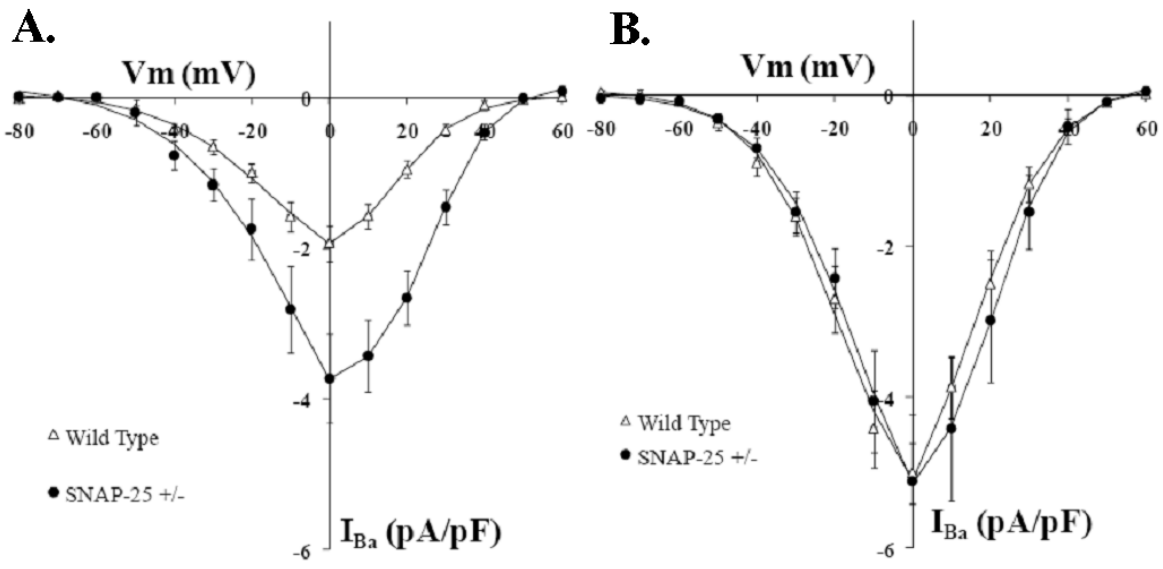


Figure 19. VGCC properties in wild type and *Snap-25* heterozygous mouse neurons. (**A**) Mean I-V relationships of I_{Ba} current density in wild type (n = 12) and *Snap-25*$^{+/-}$ (n = 12) glutamatergic neurons. (**B**) Mean I-V relationships of I_{Ba} current density in wild type (n = 9) and *Snap-25*$^{+/-}$ (n = 9) GABAergic neurons.

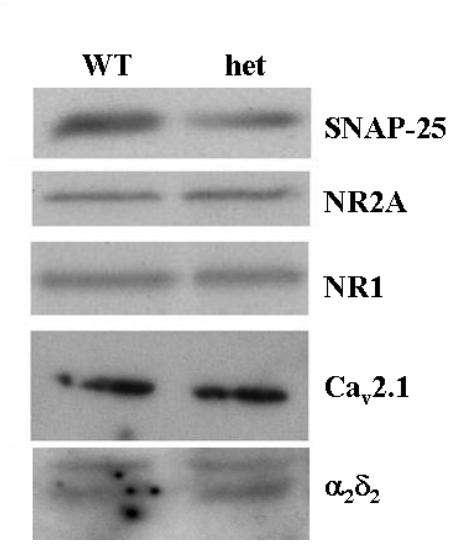


Figure 20. Membrane fractions prepared from heterozygous samples (het) contain reduced levels of SNAP-25 but comparable levels of the NMDA receptor subunits NR1 and NR2A, relative to wild type (WT). Note the equivalent expression of the $Ca_V2.1$ P/Q channels and the $\alpha_2\delta_2$ auxiliary subunit of VGCCs in wild-type and heterozygous fractions.

To investigate whether the changes in VGCC current between glutamatergic and GABAergic neurons (Condliffe et al., 2010 and 2011) could impact neurotransmission, synaptic properties were investigated in *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ hippocampal cultures

(Antonucci et al., submitted). This recent paper of our group demonstrated that there is a selective enhancement of evoked glutamatergic currents in 14 DIV old *Snap-25* heterozygous neurons, but no alterations in spontaneous glutamatergic release (Fig. 21), synaptic vesicle readily releasable pool, and the overall number of synapses was observed.

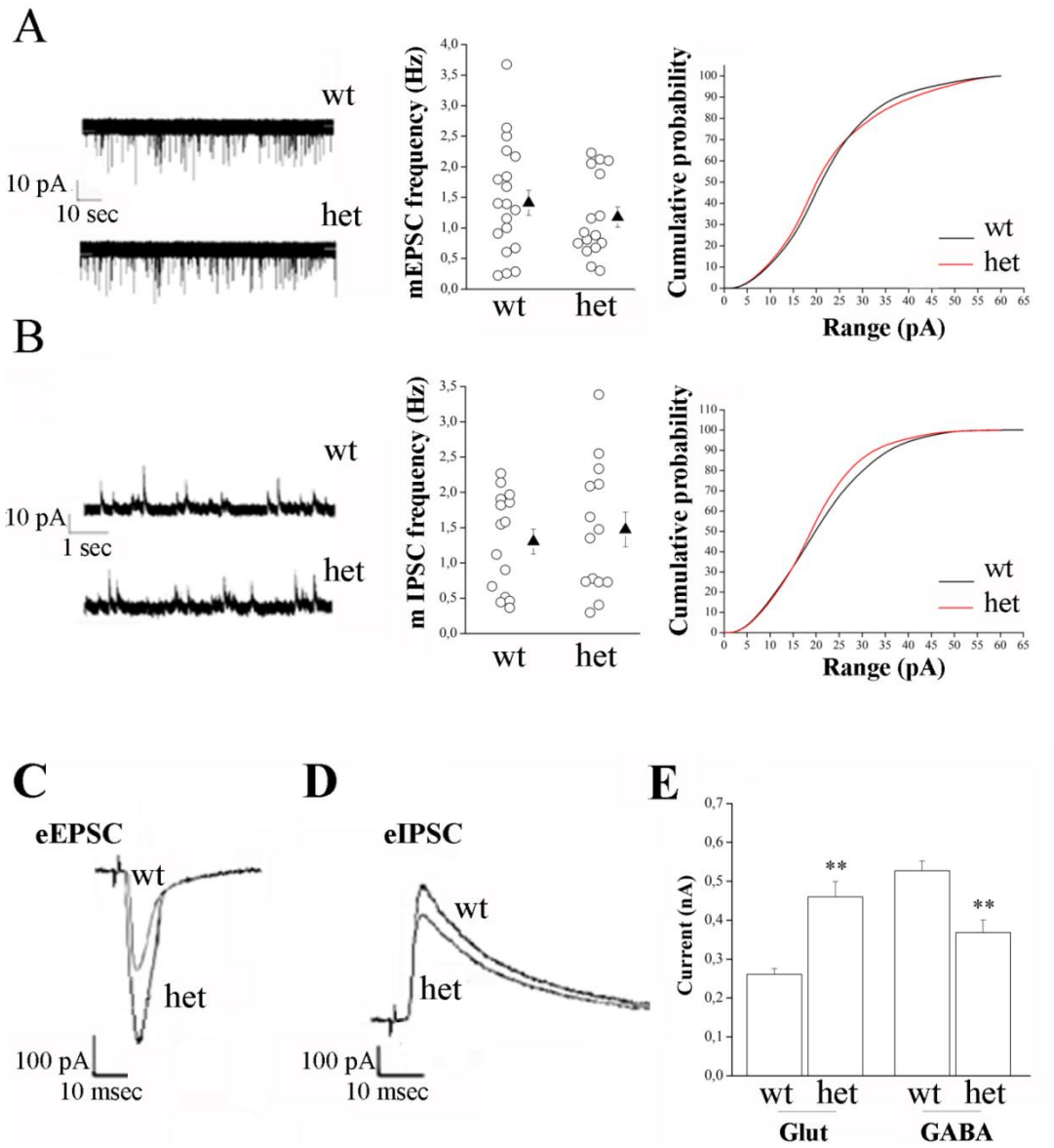


Figure 21. (**A,B**) Representative traces of mEPSCs and mIPSCs from 14 DIV wt or het neurons followed by the quantitative analysis of miniature frequency and amplitude. (**C,D**) Representative traces of eEPSCs (**C**) and eIPSC (**D**) from div 14 - wt or het neurons. (**E**) Quantitative analysis of evoked events after a single depolarizing stimulus (from Antonucci et al., submitted).

Furthermore, it was noticed a switch from paired pulse facilitation to paired pulse depression in *Snap-25* heterozygous neurons, indicating an increase in presynaptic release probability (Fig. 22) . The change in paired pulse ratio was a direct consequence of lower SNAP-25 levels in the presynaptic neuron, as acute reduction of the protein by siRNA specifically in the presynaptic, but not in the postsynaptic element, shifts paired pulse facilitation to paired-pulse depression. These data indicated that substantial reductions of SNAP-25 expression mediate increased probability of release specifically at glutamatergic but not GABAergic synapses, possibly leading to a prominent imbalance between inhibitory and excitatory neurotransmission.

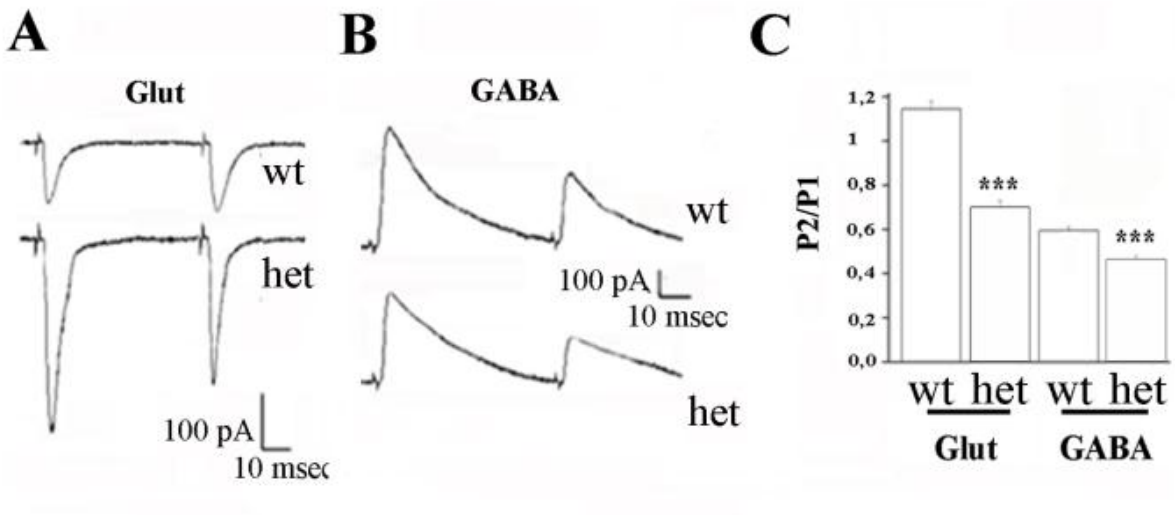


Figure 22. Shift from paired pulse facilitation to paired pulse depression at 14 DIV hippocampal *Snap-25* het synapses. **(A, B)** Representative traces of short term plasticity experiments performed in DIV 14 synaptic pairs where the presynaptic glutamatergic or GABAergic neuron was stimulated by two consecutive stimuli. (**C**) Measurement of Paired Pulse Ratio (PPR= P2/P1) at glutamatergic synapses (N=4, t-test p<0,001) and at GABAergic synapses (N=4, t-test p<0,001).

THE *SNAP-25*$^{+/-}$ MOUSE MODEL

In order to investigate the physiological role of SNAP-25 and the different functions of the alternatively spliced isoforms SNAP-25a and SNAP-25b, a number of gene-targeted mouse mutants for *Snap-25* have been generated (reviewed in Bark, 2009).

In particular, Washbourne and colleagues have examined the involvement of SNAP-25 in neuronal exocytosis by generating SNAP-25 null mutant mice. The mutation was generated by targeted gene replacement: the region containing both exon 5a and 5b, defined by *NsiI* and *AvrII* sites, was replaced by PGKneo, thus disrupting both alternatively spliced isoforms of the protein (Fig. 23). The targeting construct was transfected into ES cells and four ES cell lines which appeared to have undergone homologous recombination were injected into C57BL/6 blastocysts and the chimeric mice were backcrossed onto a C57BL/6J background for 4-5 generations (Washbourne et al., 2002). Mice heterozygous for the *Snap-25* mutation are robust, fertile and phenotypically indistinguishable from wild-type littermates. In contrast, no homozygous mutants can be obtained from heterozygote crosses, consistent with an embryonic lethal phenotype; examination of mutant diaphragms shows that while muscle fibers are innervated by the respiratory phrenic nerve, they failed to exhibit stimulus-evoked contraction, suggesting that the lethality at birth is a consequence of respiratory failure (Washbourne et al., 2002).

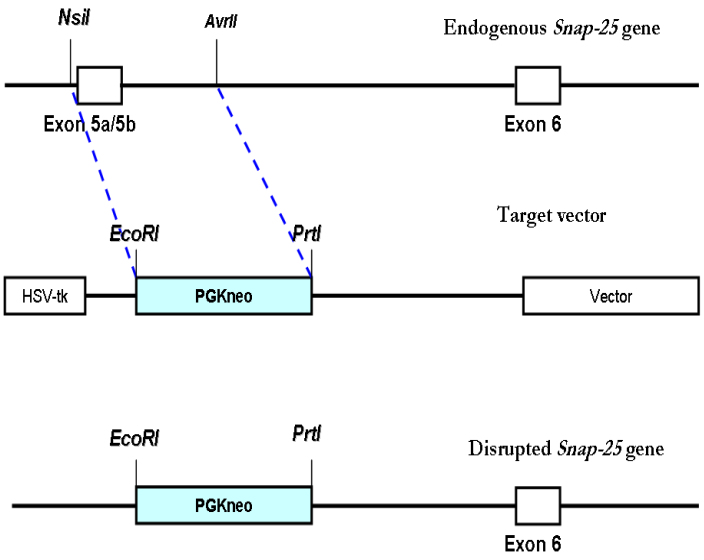


Figure 23. Generation of *Snap-25* mutated construct. Both alternatively spliced forms of exon 5 were disrupted in the target construct, thus ensuring a reduction in the production of both SNAP-25a and SNAP-25b proteins (modified from Washbourne et al., 2002).

Western blot analysis of E17.5 brains shows a dose-dependent loss of SNAP-25 protein in heterozygote and homozygote mutants, while neither the abnormally spliced mRNA (abnormal splicing of exon 4 to 6 in the absence of exon 5 should lead to a shift in open reading frame with termination after one codon into exon 6) nor the truncated polypeptide accumulate to a significant level in mutant fetal brain. Moreover, a survey of presynaptic proteins reveales no detectable difference in the expression among wild-type, heterozygote and homozygous mutants (Washbourne et al., 2002) (Fig. 24).

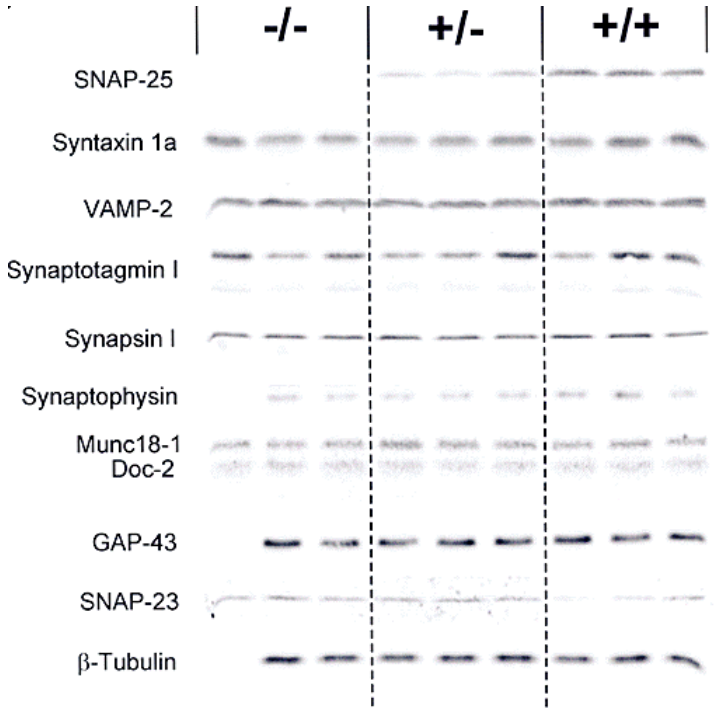


Figure 24. Western blot analysis of E17.5 forebrain shows no dramatic differences in levels of expression of synaptic proteins. Importantly, SNAP-23, the non-neuronal homolog of SNAP-25, is not significantly induced in the *Snap-25* null mutant brain (from Washbourne et al., 2002).

SNAP-25 IN NEUROPSYCHIATRIC DISORDERS

In the last years data accumulated suggesting that SNAP-25 is involved in different neuropsychiatric and neurological disorders; alterations in SNAP-25 isoform expression have been linked to diseases with developmental onset such as schizophrenia, attention deficit hyperactivity disorder, epilepsy and autism. Recent genetic studies of human populations and of some mouse models implicate that alterations in *Snap-25* gene structure, expression and/or function may contribute directly to these distinct neuropsychiatric and neurological disorders. Further studies are required to clarify if the changes in SNAP-25 expression may rapresent a cause or be a consequence of the pathologies.

SNAP-25 IN ADHD

Attention-deficit/hyperactivity disorder (ADHD) is a frequently diagnosed neuropsychiatric disorder in childhood, with worldwide prevalence estimated as 5.3% (Polanczyk et al., 2007). Although for many years it was believed to be a disorder of childhood and adolescence, it is now recognized to also occur in adulthood. It is characterized by a developmentally inappropriate and pervasive expression of inattention, impulsivity, and hyperactivity. Evidence from several neurobiological studies supports the idea that dysregulation of the catecholaminergic systems underlies ADHD neurobiology (Genro et al., 2010; Volkow et al., 2009). Neuroimaging studies in children with ADHD also show neurodevelopmental brain anomalies, such as reduced cortical volume and folding and gray matter heterotopia, which indicate that, neuroanatomically, ADHD is a disorder of early brain development (Shaw et al., 2009). Although the involvement of dopamine in ADHD has been consistently suggested in the literature, genetic linkage and association studies have identified neurodevelopmental genes other than dopamine-related genes as possible candidates for this disorder.

The primary cause for ADHD is hypothesized to be altered neurotransmission, therefore genes encoding synaptic proteins involved in the release of neurotransmitters into the synaptic cleft are particularly interesting candidates (Brookes et al. 2005). One of these candidates is the gene for the synaptosomal associated protein SNAP-25. Human *Snap-25* gene is located on chromosome 20p11.2. The gene for SNAP-25 is suggested as a candidate gene for ADHD based on the mouse mutant strain *Coloboma*. The mutation in the *Coloboma* mouse involves a 2 cM deletion of chromosome 2p, a region that encompasses several genes, including *Snap-25*. This deletion of the *Snap-25* 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 littermates. The specific role of SNAP-25 in the hyperactive phenotype has been confirmed by the amelioration of the hyperactivity when the deleted *Snap-25* gene is replaced with a transgene of *Snap-25* (Hess et al., 1996). The mechanisms underlying the behavior of these mice appear to be the result of dysregulation of the controlled release of neurotransmitters in selected brain regions. Another interesting finding of the *Coloboma* strain is that administration of dextroamphetamine, reduces locomotor activity (Raber et al., 1997; Jones et al., 2001). Based on this first observation and supported by further behavioural and neurochemical analyses in the *Coloboma* mutant mouse (Heyser et al., 1995; Bruno et al., 2007), polymorphisms at the *Snap-25* gene locus in humans have been examined and association of SNAP-25 with ADHD has been determined in a number of linkage studies (Corradini et al., 2009; Zhang et al., 2011; Sarkar et al., 2012).

SNAP-25 IN SCHIZOPHRENIA

Schizophrenia is a chronic debilitating mental disorder affecting approximately 1% of the population worldwide, with the onset of symptoms in late adolescence or early adulthood. The clinical symptoms can be classified into two main categories: psychotic or 'positive' symptoms, including hallucinations, altered emotional activity, and disorganized behaviour, and 'negative' symptoms, such as delusions, reduced interest and motivation and cognitive impairment. 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 and Rees, 2005; Lang et al., 2007; Walsh et al., 2008), but the neuropathophysiology of the disorder remains unclear, although as in the case of ADHD alterations in dopaminergic and serotoninergic circuitry, as well as in glutamatergic transmission have been strongly implicated. Recent studies provide evidence that there is a marked reduction, damage and loss of oligodendrocytes in the prefrontal cortex in schizophrenia (Vostrikov et al., 2008) and that the use of antipsychotics can influence subsequent myelination (Bartzokis et al., 2009). On the other side Harrison and

Weinberger (2005) have supposed that genetic factors influence the schizophrenic phenotype by a functional effect on synaptic plasticity and the development of cortical microcircuitary. The chromosome region which includes the *Snap-25* gene (20p12.3-11), emerged as a strong candidate region for schizophrenia as a result of genome-wide association studies for susceptibility genes in schizophrenia (Lewis et al., 2003). Studies of different parts of the brain in patients with schizophrenia (Hurt et al., 2002) demonstrated low levels of SNAP-25 in the cerebellum and hippocampus. Increased levels of this protein were seen in the cerebrospinal fluid of schizophrenia patients, and correlations were found between the level of this protein and the severity of psychotic symptoms and the occurrence of thought disorders (Thompson et al., 2003). Decreases in the mRNA level were also found in various parts of the brains in post mortem studies of patients with bipolar affective disorder (Scarr et al., 2006). 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).

Recently, it has been characterized the blind-drunk mutant mouse which show a dominant mutation in a highly conserved domain of SNAP-25. This mutation results in increased SNARE binding affinities, impairment of the exocytotic vesicle recycling process, reduced glutamate release and a reduction in evoked cortical excitatory post-synaptic potentials (EPSPs). This mouse also exhibits many aspects associated with schizophrenia, like impairment in sensorimotor gating, high anxiety and apathetic behaviors (Jeans et al., 2007). Another study on the schizophrenia susceptibility gene *DTNBP1* demonstrated that the overexpression of dysbindin increase the expression of SNAP-25 in primary cortical neuronal culture (Numakawa et al., 2004).

SNAP-25 IN EPILEPSY

Epilepsy is one of the most common neurological disorders that affects more than 65 million people worldwide of all ages, with onset most often occurring in childhood and older adulthood. It is characterized by the repeated occurrence of spontaneous bursts of neuronal overactivity, known as seizures. These seizures differ from person to person with respect to their cause and severity, the areas of the brain involved, the location and functions of the body affected, the effectiveness of medications and other treatments, and

many other factors. Based on electroencephalographic recordings, seizures can be classified into two major categories: (1) focal seizures that originate in a network of neurons limited to one hemisphere of the brain and (2) generalized seizures that originate in a network of neurons that is distributed to both brain hemispheres (Berg et al., 2010). Generalized epileptic syndromes can be further classified into symptomatic (caused by identifiable factors) or idiopathic (without a clear ethiology and partially caused by genetic defects) (Fisher et al., 2005). Generalized idiopathic epilepsies include childhood absence epilepsy and juvenile absence epilepsy. These are typically short in duration and manifest themselves as sudden behavioral arrest and impaired consciousness The electrographic hallmark are spike and wave discharges (SWDs), that arise from synchronous firing of thalamocortical networks (Blumenfeld et al., 2005). Several analysis on absence epilepsy revealed a strong causal link to mutations in various types of voltage-gated and ligand-gated ion channels, including voltage-gated potassium, sodium, and calcium channels, as well as $GABA_A$ receptors (Mulley et al., 2005; Helbig et al., 2008). Analysis in absence epileptic mice models, like Tottering, Leaner and Rolling Nagoya mice, revealed mutation that results in decreased P/Q-type current density (Fletcher et al., 1996; Wakamori et al., 1998).

Interestingly, the mutant mouse *Coloboma* (*Cm/+*), which has been implicated as a model of ADHD, has also provided evidence for the involvement of SNAP-25 in epilepsy. *Cm/+* mice display robust cortical cortical spike-wave discharges and increased thalamic T-type currents (Zhang et al., 2004), two typical features of absence epilepsy (Tsakiridou et al., 1995; Coenen and Van Luijtelaar, 2003). SNAP-25 is expressed at much higher levels at excitatory respect to inhibitory synapses (Verderio et al., 2004; Bragina et al., 2007). Thus, hyperexcitability could result from perturbations of the processes that balance the developmental assembly of inhibitory and excitatory circuits. Recent evidence indicates an additional function of SNAP-25 in the modulation of various ion channels, in particular this protein have a role in the regulation of calcium homeostasis in neurons. It has been shown that phosphorylated SNAP-25 negatively modulates calcium dynamics by inhibiting voltage-gated calcium channels (Verderio et al., 2004; Pozzi et al., 2008). In addition Condliffe et al. demonstrated that endogenous SNAP-25 negatively regulates native VGCC in glutamatergic neurons, therefore the regulation of SNAP-25 may have a crucial role in the control of normal neuronal network activity (Condliffe et al., 2010).

Recently, based on the conclusion that the phosphorylation of SNAP-25 plays an important role in synaptic function, it was generated a mutant mouse, substituting Ser^{187} of SNAP-25

with Ala (Kataoka et al., 2011). The most striking effect of the mutation was observed in behavior. The homozygous mutant mice froze readily in response to environmental change, and showed strong anxiety-related behavior in general activity and light and dark preference tests. In addition, the mutant mice sometimes exhibited spontaneously occurring convulsive seizures. All these results confirm the relevance of PKC-dependent SNAP-25 phosphorylation.

SNAP-25 IN AUTISM

Autism spectrum disorders (ASD) represent complex neurodevelopmental disorders characterized by limited social interactions, abnormal use of language and stereotyped patterns of behavior, interests, and activities. ASD are heterogeneous disorders with a wide range of additional phenotypic characteristics and an intelligence quotient (IQ) ranging from mental retardation to above average. There are three main subtypes of ASD: classical autism (autistic disorder), asperger disorder (high functioning) and pervasive developmental disorder not otherwise specified (PDD-NOS) (American Psychiatric Association 2000). The prevalence of affecting is 1 in 110 children with a 4.5:1 male to female ratio. The first behavioral signs of social impairment may appear usually between 1 and 2 years of age and the final diagnosis is commonly made by 2-4 years of age (Chourchesne et al., 2007). Symptoms of ASD are frequently accompanied by intellectual disability (ID) (75%), dysmorphic features, epilepsy (25%), but also attention deficit, hyperactivity and impulsivity which are clinical features shared with attention deficit hyperactivity disorder (ADHD) (40–78%) (Murray et al. 2010). The etiology of ASD is still not clear, but several evidence show a diversity of morphological, functional, genetic, and neurotransmitter systems alterations. Family studies suggest that genetic factors contribute significantly to autism (up to 90%). In less than 20 percent of subjects are identifiable monogenic causes, the remaining individuals have other genetic or multigenic causes and/or epigenetic influences which are environmental factors altering gene expression without changing the DNA sequence (Piven et al., 1997). Post-mortem studies have demonstrated altered early neurodevelopmental alterations in brains of autistic patients including reduced programmed cell death and/or increased cell proliferation, altered cell migration, abnormal cell differentiation with reduced neuronal size and altered synaptogenesis (Persico et al., 2006; Bauman et al., 2005). Furthermore it has been proposed that dysregulated Ca^{2+} levels may be a broadly influential factor in causing autism (Napolioni et al., 2011). A very recent paper (Guerini et al., 2011) investigated the

possibility that SNAP-25 plays a role in ASD by analyzing five *Snap-25* gene polymorphisms, rs363043, rs363039, rs363050, rs3746544 and rs1051312, in a well clinically characterized cohort of children affected by ASD. They found a significant association of the rs363043 (C/T) genotype with increasing Childhood Autism Rating Scale (CARS) scores in a cohort of children with a diagnosis of ASD, supporting a role for *Snap-25* as a new gene involved in the genetic background of ASD (Fig. 25).

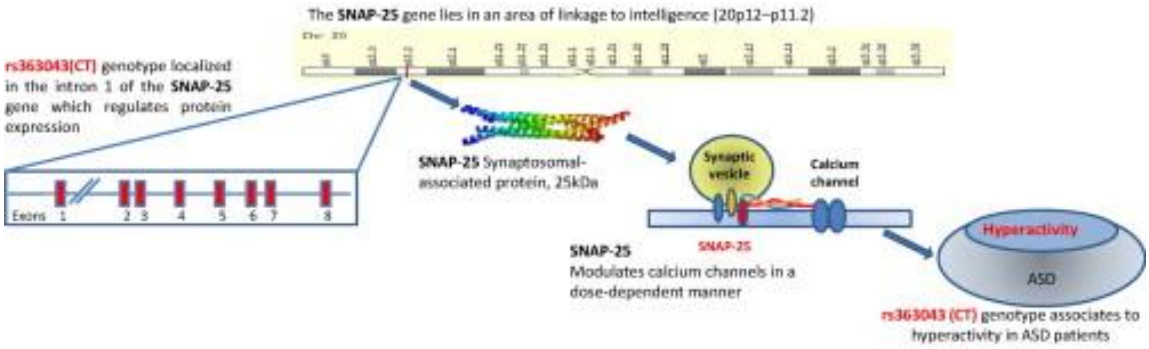


Figure 25. Polymorphism rs363043 is localized in the intron 1 of the *Snap-25* gene which regulates protein expression (from Guerini et al., 2011).

POLYMORPHISMS IN *SNAP-25* GENE

Point of variation or polymorphisms are the source of genetic variation that contribute to differences between individuals, and, thus, are the focus of attention for molecular genetic studies. There are several types of polymorphisms, though two commonly studied types are repeat sequences and single-nucleotide polymorphisms (SNPs). Repeat sequences consist of a set of bp that can be short in length (i.e., 2-4 bp) or quite long (i.e., 10-60 bp), and the different variants of the polymorphism, or alleles, are defined as how many times the sequence is repeated (e.g., two-repeats vs. four-repeats vs. seven-repeats). A SNP consists in a DNA sequence variation that occurs when a single nucleotide (A,T,C,or G) in the genome sequence is altered; to be classified as polymorphism, the variation must occur in at least 1% of the population.

In the human genome SNPs occur in just about every 3000 base pairs (Cargill et al., 1999) and the frequency of occurrence of the different alleles changes in different populations. The human genome is estimated to contain around 25’000 genes, each of which is

responsible for the production of a specific protein. The structure of a gene consists of a promoter region that is involved in the initiation of transcription of the gene, a process that ultimately leads to the production of the gene product, and the gene sequence itself. The gene sequence consists of exons, which are elements responsible for the coding of proteins, and introns, which are not involved in the coding of proteins. As a result, polymorphisms that lie within the exons are the most likely to result in functional changes in the gene product, though recent research suggests that polymorphisms in the promoter region and introns may also result in functional changes in the gene product and may play a role in regulating gene expression. A number of polymorphisms have been implicated in different neuropsychiatric and neurological disorder. Polymorphisms in neurological disorder with a central immune component are well described, mainly due to their role in increasing neurodegeneration. In contrast, polymorphisms resulting in or affecting psychiatric disorders are less well studied and frequently are not replicated by meta-analysis. Furthermore, even if a significant association has been confirmed, the role of the identified polymorphism in causing and/or augmenting the disorder is often difficult to rationalize.

Recently mounting evidence has revealed an association between SNAP-25 and ADHD in humans, although the alleles and polymorphisms associated with this disorder were not consistent across studies. Initially Barr and collegues identified two polimorphisms mapped to *Snap-25*, 1065 (T/G) and 1069 (T/C), as possible sites for association between ADHD and *Snap-25* gene (Barr et al., 2000). In 2002, Mill suggested that tetranucleotide repeat polymorphism (TAAA - 13 repeats) may have a role in the expression or function of *Snap-25* gene and that there could be an association between the rs363006 SNP and ADHD (Mill et al., 2002 and 2004). In contrast Feng et al., (2005) did not identify any linkage with this polymorphism, but showed a strong relation with rs362549. Later the first report of an association between SNAP-25 and ADHD in Chinese subjects of Han descen was published. Zhang's group detected the transmission disequilibrium phenomenon of ADHD's different subtypes on rs362549 fragment of SNP; they hypothesized that the allele G in rs362549 fragments may be a *Snap-25* risk allele which may cause increased hyperactivity. Other similar association tests were performed between SNPs of *Snap-25* gene and schizophrenia. One previous single-marker association study was negative (Tachikawa et al., 2001), however few years later Lezheiko studied two markers (MnlI and DdeI, or T1065G and T1069C, respectively) and an association between the T1065G polymorphism of the *Snap-25* gene with the effectiveness of performing neurocognitive

tests was seen not only in schizophrenia patients, but also in mentally healthy subjects (Lezheiko, 2007). Following a paper revealed an linkage between one of markers (DdeI) and the overall level of cognitive functioning in patients and measures of executive functions and verbal memory (Spellmann et al., 2008).

Learning and memory are key components of human intelligence and the *Snap-25* gene lies in an area of previous suggestive linkage to intelligence (Posthuma et al. 2005). Recently, a family-based genetic association test, performed in two independent cohorts of Caucasian children and adults, has evidenced association between four *Snap-25* single nucleotide polymorphisms (SNPs) and variation in IQ phenotypes across both cohorts. The four SNPs (rs363043, rs353016, rs363039, rs363050) are localized within intron 1, in a region of about 13.8 kb, and are known to affect transcription factor binding sites (Gosso et al. 2008). The SNP rs363050, in particular, is associated with better results in the performance subtests of the Weschler intelligence scale and the increased allelic frequency of this SNP was associated with an increase of nonverbal cognitive skills (Gosso et al. 2006). Furthermore, in recent years, it has been proposed that a different distribution of SNAP-25 protein in male or female brains may be involved in gender differences that characterize many neuropsychological skills in neuropsychiatric diseases such as ADHD and ASD (Ghezzo et al. 2009).

AIM OF THE PROJECT

SNAP-25 is a member of the SNARE protein complex that participates in synaptic vesicle exocytosis. Previous studies have demonstrated that SNAP-25, which is expressed at high levels in glutamatergic but not GABAergic terminals of hippocampal neurons (Verderio et al., 2004), regulates intracellular calcium dynamics by negatively modulating neuronal voltage-gated calcium channels upon SNAP-25 activity-dependent phosphorylation on Ser187 (Pozzi et al., 2008, Condliffe et al., 2010). Reductions of SNAP-25 expression, inducing increases of calcium responsiveness specifically in glutamatergic neurons, may thus lead to unbalances in neuronal network activity, possibly resulting in a pathological state. In line with this possibility, alterations of SNAP-25 expression have been associated to several neurological diseases, such as schizophrenia, ADHD, and bipolar disorder, and lower levels of SNAP-25 have been described in patients with schizophrenia (Thompson et al., 2003). During my PhD project, I used *Snap-25* heterozygous (*Snap-25*$^{+/-}$) mice, expressing reduced levels of SNAP-25, to investigate at which extent the reduction of the protein expression affects neuronal network function and mouse behaviour. Since reduced levels of SNAP-25 in humans might be a consequence of the presence of polymorphisms regulating transcriptional activity of the gene, and given a strong association between three polymorphisms in the *Snap-25* gene (rs363039, rs363050 and rs363043) and intelligence has been recently demonstrated (Posthuma et al. 2005), in the second part of my PhD I carried out a functional analysis on SNP rs363050, located on intron 1 of the *Snap-2*5 gene, to investigate whether the presence of the parental or minor allele may affect the expression of SNAP-25.

EXPERIMENTAL PROCEDURES

ANIMALS

Male *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ C57BL/6 mice, originally from M.C. Wilson (University of New Mexico Health Sciences Center, Albuquerque, NM, USA), were provided by J. Sorensen (MPI, Goettingen). Mice were maintained and repeatedly backcrossed on C57BL/6 background for more than 10 generations. Zero- to six-month-old age-matched littermate mice were used. All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 27/2010. All efforts were made to minimize the number of subjects used and their suffering. Mice were individually housed throughout the testing period with free access to food and water at controlled temperature (20–22 °C) with a 12-h light/dark cycle (lights on at 7:00 AM). Genotyping was performed by PCR, using DNA extracted by tail biopsy.

Gene	**Primer sense**	**Primer Antisense**
Snap-25	CGAAGAAGGCATGAACCATATCAAC	GCCCGCAGAATTTTCCTAGTTCCG
Neomycin	GCACGCAGGTTCTCCGGCCGC	TCAAGCGTATGCAGCCGCCGC

WESTERN BLOTTING

Detection of neuronal proteins in cortex homogenates from E18, P7, P14, P30, and adult (3 months old) mice. Homogenates of mouse brain cortex obtained from *Snap-25*$^{+/-}$ and control mice were separated by electrophoresis, blotted, and incubated with primary antibody, followed by HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), and developed by ECL (Amersham Biosciences, Amersham, UK). The following primary antibodies were used: monoclonal antibdsies directed against SNAP-25 (Sternberger Monoclonals Inc., Baltimore, MD), PSD95 (NeuroMab, Davis, CA), β_{III}Tubulin (Promega, Milano), alpha-Tubulin (Sigma-Aldrich, St. Louis, MO), NMDAR1 (SySy, Goettingen, Germany), vGlut1 1:2000 (Synaptic System, Gottingen, Germany); polyclonal antibodies against Calbindin (Swant, Bellinzona, Switzerland), NMDAR2A (Zymed Laboratories, San Francisco, CA). Antibodies against SNAP-47, SNAP-29, and

SNAP-23 were a gift of R. Jahn (MPI, Gottingen). Beta-III tubulin or alpha-tubulin was used as loading controls.

qRT-PCR ANALYSIS

Brain tissues from P7, P14, P30, and adult mice were used for realtime PCR analysis. Sample was homogenized prior to RNA extraction in 800 µL of Trizol. Total RNA was isolated using the NucleoSpin miRNA (Macherey-Nagel GmbH &Co., Düren, Germany) isolation kit according to the manufacturer's protocol. The RNA was eluted with 30-µL Rnase-free water. All RNA was quantified by spectrophotometer and optical density 260/280 nm ratios were determined. Reverse transcription was performed on 2 µg RNA using Superscript III First-Strand Synthesis System and random hexamer primers (Life Technologies, Carlsbad CA, USA). Real-time polymerase chain reaction (qRT-PCR) was performed using 7900 HT fast-real-time PCR system instrument (Life Technologies, Carlsbad CA, USA). The amplification was carried out in a total reaction volume of 11 µL, using the TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad CA, USA). Predeveloped TaqMan Assay Reagent (FAM-MGB) for *Snap-25* (Mm00456921_m1) and for GAPDH (4352339E) were purchased from PE Applied Biosystems. Each gene was analyzed in triplicate. Data analysis was performed with the ΔΔCt method. All RNA levels were normalized to Gapdh.

BRAIN SLICES AND IMMUNOHISTOCHEMISTRY

Experiments were performed on 5 *Snap-25*$^{+/+}$ and 3 *Snap-25*$^{+/-}$ mice at postnatal day (P) 2 and on 3 *Snap-25*$^{+/+}$ and 3 *Snap-25*$^{+/-}$ adult (P90) mice. All the experiments were undertaken in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC). All efforts were made to reduce the number of animals used and to minimize their suffering. Animals were anesthetized with chloral hydrate (4%; 1 ml/100 g body weight, i.p.) and perfused transcardially with 4% paraformaldehyde in 0,1 M phosphate buffer, pH 7.2 (see Frassoni, 2000 for details). The forebrains were dissected out and coronally cut with a Vibratome or with a freezing microtome in serial sections. The following antibodies were used: monoclonal anti-non-phosphorilated neurofilaments (SMI311, Sternberger Monoclonals Inc., Baltimore, MD), vGlut1 (Synaptic System, Gottingen), vGlut2 (Synaptic System, Gottingen); polyclonal

antibodies against the calcium-binding proteins calbindin and calretinin (CB and CR; Swant, Bellinzona, Switzerland), doublecortin (DCX, Cell Signalling, Beverly, MA), the neuropeptide Y (NPY) (Bachem, Weil am Rhein, Germany) and vGat (Synaptic System, Gottingen). Free-floating sections were preincubated for 45 min in 0.01 M phosphate buffered saline pH 7.4, containing 10% normal goat serum and 0.2% Triton X-100 and then incubated with primary antibodies. Subsequently the sections were incubated in the corresponding secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and signals were revealed with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) followed by avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB). For cytoarchitectonic analysis, selected sections were stained with thionin (0.1% in distilled water).

BEHAVIORAL TESTS

SPONTANEOUS MOTOR ACTIVITY

Spontaneous motor activity was carried out as previously described (Braida and Sala, 2000) in 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. Cumulative horizontal movement counts were recorded for 15 min. Before the start of the test, *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice (7–9 weeks of age) were habituated to the testing room for at least 1 h. 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). Cumulative horizontal and vertical movement counts were recorded for 4 h.

EEG RECORDINGS

Mice were anesthetized with intraperitoneal (i.p.) injection of 5% chloral hydrate dissolved in saline and given in a volume of 10 ml/kg body mass. 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) 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). The animals were allowed a week of recovery from surgery before starting the experiment and acclimatized to a sound-attenuated Faraday chamber for a period of 3 days. For the assessment of basal cerebral activity, freely moving mice were recorded continuously for 24h using PowerLab System (ADInstruments, Castle Hill, Australia). For each 24-h EEG recording, the mean number of spikes was evaluated in both genotypes. After the recordings, the EEG and video (through a video camera put inside the Faraday chamber) were analyzed for the incidence/duration of spontaneous cortical spike activity and the percentage of animals displaying spike activity, as previously described (Zhang et al. 2004; Manfredi et al. 2009).

BEHAVIORAL OBSERVATION OF KA-INDUCED SEIZURES

Kainic acid (KA, Sigma) was dissolved in saline and administered i.p. at 35 mg/kg body weight. Saline-injected animals of both genotypes were used as controls. Ten wild-type and 10 *Snap-25*$^{+/-}$ mice were used. In all experiments, the experimenter was blind to the genotype of the animals. Seizure severity was determined according to Racine's scale (Racine, 1972): stage 0: normal behavior; stage 1: immobility; stage 2: forelimb and/or tail extension, rigid posture; stage 3: repetitive movements, head bobbing; stage 4: forelimb clonus with rearing and falling (limbic motor seizure); stage 5: continuous rearing and falling; stage 6: severe whole body convulsions (tonic-clonic seizures); stage 7: death. For each animal, the rating scale value was scored every twenty minutes for a maximum of 3 hr after KA administration. The maximum rating scale values reached by each animal over each 20 min interval were used to calculate the rating scale value (± SE) for each treatment group. Statistical analysis was performed by two-way repeated measures ANOVA followed by post-hoc Holm-Sidak test. At the end of behavioral observation (3 hr after KA), animal were returned to their home cages; animals were killed at 14 days after KA for histopathological analyses.

QUANTIFICATION OF NPY STAINING

The quantification method was adapted from that described in Antonucci et al., 2008. Four-eight NPY-stained sections through the dorsal hippocampus were analyzed in each KA-treated mouse (wild type [wt], n = 5; heterozygous [het], n = 5). Images of CA3 stratum radiatum and of the overlying corpus callosum in each hemisphere were digitized (Zeiss Axiovision). Light intensity and microscope settings were optimized initially and

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

TWO-BOTTLE PREFERENCE TEST

2-bottle preference test were performed as previously described (Bruno et al. 2007). A cohort of 10 *Snap-25*$^{+/-}$ mice had access to plain water and 0.008 M HCl for 30 min each morning for 4 consecutive days. 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) for each test and the mean consumption was analyzed using one-way ANOVA.

NOVEL OBJECT RECOGNITION

The test was conducted over a two-day period in an open plastic arena (60 x 50 x 30 cm), as previously described (Pan et al. 2008). Ten *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice were used. 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 the centre 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 a maximum of 10 min or until it had completed 30s of cumulative object exploration. Object recognition was scored when the animal was within 0.5 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, coloured 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 s) 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 T2, F= time spent exploring the familiar object during T2 (Pitsikas et al. 2001).

CONDITIONED TASTE AVERSION

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

SOCIABILITY AND SOCIAL NOVELTY TEST

The apparatus was a rectangular, three-chamber transparent polycarbonate box (width=42.5 cm; height=22.2 cm; centre chamber, length=17.8; side chambers, length=19.1 cm). The proband mouse was firstly placed in the middle compartment and allowed to explore all three chambers for 10 min (habituation) (Moy et al. 2004, Sala et al. 2011). An unfamiliar adult DBA/2J male mouse was placed in one side compartment whereas the opposite contained an empty wire cage. For the social novelty test, carried out in the same apparatus without any cleaning, immediately after sociability test, one side compartment contained the familiar mouse (from the previous sociability phase), the other side an unfamiliar mouse. The new unfamiliar mouse was placed in the wire cage that had been empty during the prior 10-min session. Familiar and unfamiliar animals from different home cages had never been in physical contact with the subject mice or with each other. For both tests, the time spent in each chamber and the number of entries into each chamber were recorded for 10 min. Data are expressed as time spent in each chamber or the difference score between the time spent to explore the compartment containing the conspecific and that spent in the empty compartment (for sociability test), or containing the stranger animal (unfamiliar) and that for the familiar mouse (for social novelty test) (DeVito et al. 2009).

PHARMACOLOGICAL TREATMENT

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

CELL LINES AND CULTURES

The SH-SY5Y human neuroblastoma cell line was grown in RPMI 1640 medium, 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Lonza).

PLASMID CONSTRUCTIONS

A 747 bp fragment, spanning region of *Snap-25* intron 1 containing the polymorphism A/G (rs363050), was amplified by PCR from human genomic DNA (forward primer: 5'- GGA TCC TTA GAC TAG GAT TCA CCA GGC C - 3'; reverse primer: 5'- GGA TCC CCC AAA GCA GGT GGT AA – 3', in which the *Bgl II* site was added and cloned upstream the TK promoter and downstream the luciferase gene in the TK-luc/pGL4 basic reporter vector. All restriction enzymes were purchased from NEB (New England Biolabs). At first we obtained the plasmid pTK-luc/pGL4basic by digesting the pGL4 basic plasmid DNA (Promega) and the TK-luc/pGL3 with *Bgl II* and *Hind III* enzymes. The DNA fragment containing the TK promoter was inserted into the *BglII / HindIII*-digested pGL4b. The rs363050A-TK-luc and the rs363050G-TK-luc constructs were obtained by cloning the 747 bp fragment into the *Bgl II site* (upstream the TK promoter) and into the *Bam HI* site (downstream the luciferase gene; TK-luc-rs363050A and TK-luc-rs363050G) of pTK-luc/pGL4b. The correct orientation and the identity of the inserts were verified by restriction analysis and DNA sequencing.

The constructs containing multimerized copies of a cassette spanning the rs363050 SNAP-25 polymorphism were obtained by cloning in the *Bgl II* and *Hind III site*, respectively, the following oligonucleotides:

```
gatcttgtgaatgagtgg/atcgggcagcca                    Bgl II
    aacacttactcaccagcccgtcggtctag

agctttgtgaatgagtgg/atcgggcagcca
    aacacttactcaccagcccgtcggttcga                 Hind III
```

in a ligation reaction mix containing the plasmid pTKluc/pGL4 basic digested with Bgl II or Hind III and an excess of oligonucleotides (oligonucleotide:vector, 10:1 ratio) to force the insertion of multiple copies. The orientation and the number of inserted copies were verified by DNA sequencing.

TRANSIENT TRANSFECTIONS

The cells were transiently transfected by means of lipofection using 2 x 10^5 SH-SY5Y cells. Briefly, the cells were plated onto a well of a six-multiwell tissue culture plate (Euroclone) the day before transfection. 100 ng of pRL-TK were mixed with an equimolar amount of the construct of interest and 2 µl of FUGENE HD (Promega). After 20 minutes of incubation at room temperature , the DNA-lipid mixture was added to the cells and incubated for 48 hr, when luciferase activity was measured. The pRL-TK plasmid expresses the Renilla luciferase reporter gene under the control of the thymidine kinase minimal promoter and was cotransfected in each sample to normalize for transfection efficiency.

FIREFLY LUCIFERASE AND RENILLA LUCIFERASE ASSAYS

Firefly and Renilla luciferase activities were detected by using the Dual-Luciferase Reporter Assay System (Promega). The cells were harvested, washed twice in phosphate-buffered saline (PBS), and detached by means of PBS/EDTA (1mM). After a brief centrifugation, the pellet was washed once and lysed in 60 µl of Passive Lysis Buffer (Promega) for 20 min at room temperature. The lysate was clarified by centrifugation and

20 µl were added to 95 ul of luciferase assay substrate to be tested for Firefly luciferase activity in a Berthold L1920 luminometer for 12 s. The quenching of Firefly luciferase luminescence and the concomitant activation of Renilla luciferase were accomplished by adding 95 µl of Stop & Go Reagent to the sample tube immediately after quantitation of the Firefly luciferase reaction.

DATA ANALYSIS

The results are given as the mean values ± standard deviation (SD) of at least three independent experiments. The data were analysed by means of a paired two-tailed Student's *t* test using GraphPad Prism 4 Software (GraphPad Software, Inc.); p values of <0.05 were considered significant.

EMSAs (electrophoretic mobility-shift assays)

The double-stranded oligonucleotides used in the EMSA were labelled with α^{32}P-dCTP by fill-in and purified on a G-25 Sephadex column (Hoffmann-La Roche). Reaction mixtures containing 2 μg of BSA, 10 μl of 2× binding buffer [1× binding buffer is 20 mM Hepes, pH 7.9, 2 mM MgCl2, 4%Ficoll, 0.5 mM dithiothreitol], 2 μg of double-stranded poly(dI-dC) (dI-dC) (Sigma-Aldrich), 100 mM final salt concentration (NaCl+KCl)] and 5 µg of nuclear extract were assembled in a final volume of 20 μl and pre-incubated for 15 min on ice; 10000–20000 c.p.m. of the labelled probes (1 fmol) were added to each reaction. After 15 min of incubation on ice, the reaction mixtures were loaded onto 0.5× Tris/borate/EDTA, 5% non-denaturing polyacrylamide gels (acrylamide/bisacrylamide, 29:1) and run at a constant voltage of 150 V. Competition experiments and supershifts were carried out by pre-incubating the reaction mixtures with the appropriate amounts of unlabelled oligonucleotides or antibodies. All the oligonucleotides used in the EMSAexperiments were purchased from Sigma-Aldrich.. All of the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A).

SNAP-25 rs363050_A 5' TGACCTGTGAATGAA**TGAGTGA**TCGGGCAG 3'
3' GGACACTTACTT**ACTCACT**AGCCCGTCGG 5'

SNAP-25 rs363050_G 5' TGACCTGTGAATGAA**TGAGTGG**TCGGGCAG 3'
3' GACACTTACTT**ACTCACC**AGCCCGTCGG 5'

RESULTS

EFFECTS OF SNAP-25 REDUCTION *IN VIVO*

As previously described, SNAP-25 is not only involved in the formation of the SNARE complex, but it also acts as modulator on native VGCCs (Pozzi et al., 2008; Condliffe et al, 2010). Reduction of the protein levels causes a significant increase of calcium currents and calcium dynamics in excitatory, but not inhibitory neurons, generating a prominent imbalance between excitatory and inhibitory neurotransmission. Moreover, recent data obtained in our lab (Antonucci et al., submitted) show that substantial reductions of SNAP-25 at the presinaptic level enhance evoked glutamatergic neurotransmission. For this reason we focused on the possible effects that altering SNAP-25 expression may have *in vivo*.

Developmental Expression of *Snap-25* in Heterozygous Mice Brain

The heterozygous mice for *Snap-25* (*Snap-25*$^{+/-}$) generated by Washbourne et al. (2002) result in 50% lower amounts of the SNAP-25 mRNA and protein expression compared to wild-type mice at embryonic day 17.5 (E17.5; Washbourne et al., 2002). In order to confirm SNAP-25 reduction during brain development, we analyzed the levels of the protein at different developmental stages. Western blotting analysis of wild-type and heterozygous mice cortices revealed that SNAP-25 and syntaxin progressively increase during pre- and postnatal development of the brain. BetaIII-Tubulin and alpha-Tubulin were used as loading markers for SNAP-25 quantitation to minimize development-related artifacts. The results showed a physiological increase of SNAP-25 in wild-type mice and, notably, an enhanced increase of the protein levels in heterozygous mice relatively to *Snap-25*$^{+/+}$ mice, indicating a partial compensation of protein expression during postnatal development. The protein increase was not accompanied by a parallel increase in SNAP-25 mRNA levels, as assessed by qRT-PCR analysis, suggesting that post-transcriptional mechanisms may be involved. No significant difference in syntaxin or in growth-associated protein GAP-43 expression was found between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice during development (Fig. 26).

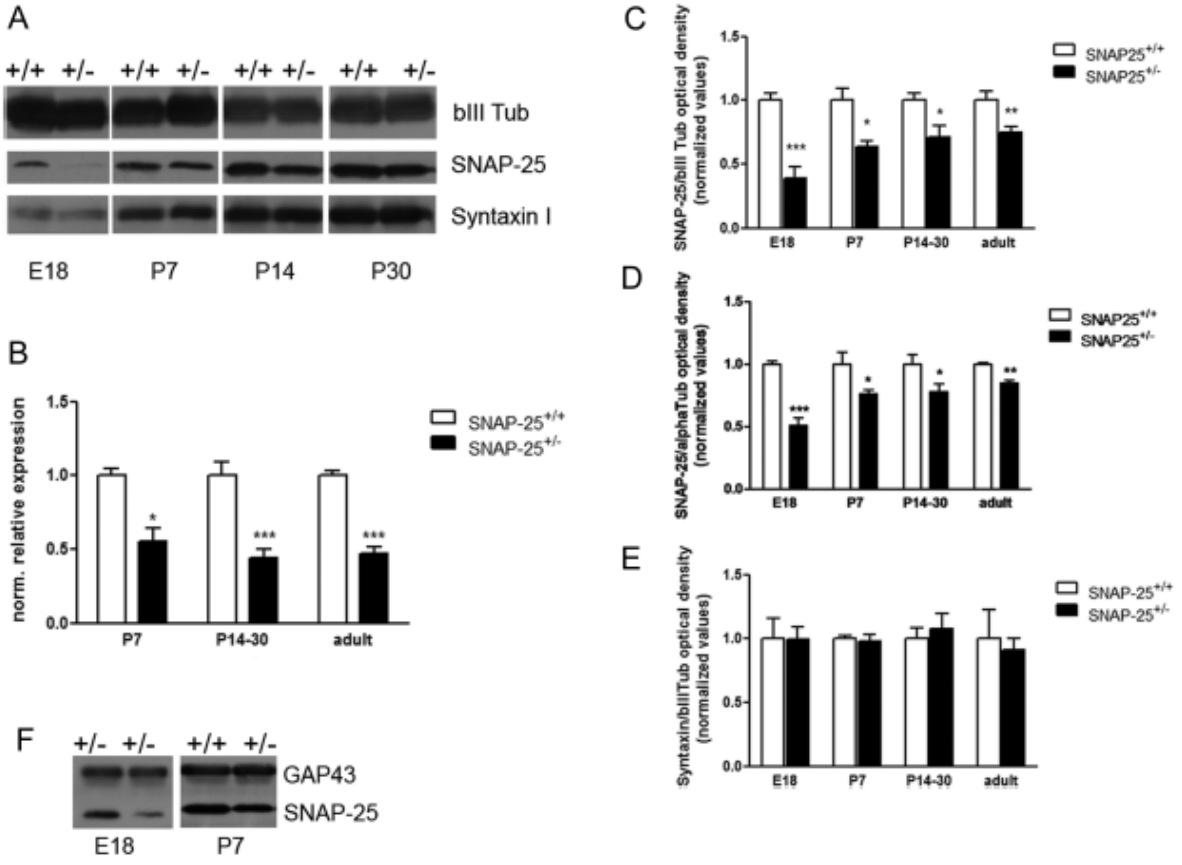


Figure 26. SNAP-25 levels in *Snap-25*$^{+/-}$ cortices progressively increase during postnatal development. (A, C, and D) Western blotting analysis (A) and relative quantitation (C and D) of *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ cortices from E18, P7, P14, P30 and adult (3 months old) mice reveals a progressively higher expression ratio in *Snap-25*$^{+/-}$ mice during postnatal development. (B) RT-qPCR analysis reveals that SNAP-25 mRNA is about half in *Snap-25*$^{+/-}$ mice at all developmental stages. (E) Quantitation of syntaxin expression at different developmental stages shows no differences in the protein expression between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ animals. (F) Western blotting analysis of GAP43 expression in mice cortices at early developmental stages reveals the absence of differences between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice.

Morphological characterization of *Snap-25*$^{+/-}$ mouse brains

Mice heterozygous for the *Snap-25* mutation were robust, fertile and phenotypically indistinguishable from wild-type littermates. To evaluate the possible effects of SNAP-25 reduction on brain anatomy, morphological characterization of *Snap-25*$^{+/-}$ pup (post-natal day 2, P2) and adult (P60) brains was performed. Thionine staining revealed the absence of major alterations in the main brain structures, cortex, hippocampus, and thalamus of both P2 (Fig. 27 A,B) and P60 (Fig. 27 G,H) *Snap-25*$^{+/-}$ mice. We observe normal cortical plate and layers V and VI (Fig. 27 D,F), moreover the thickness of the cortices was comparable, as indicated by thionin staining (Fig. 27 I,J).

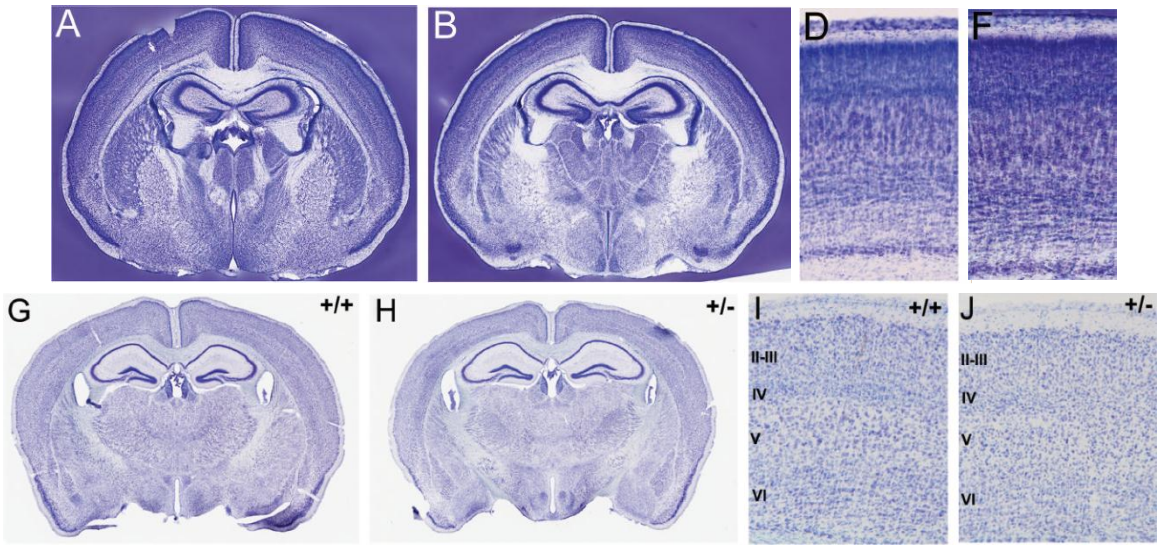


Figure 27. Thionine staining of pup (P2) and adult (P60) *Snap-25*$^{+/-}$ and control brains. No major structural alterations were observed in the brains of P2 (A,B) or P60 (G,H) mice. High magnification of developing cortices reveal comparable cortical lamination (D, F); the thickness and layering of the cortices are similar (I, J).

Neurogenesis and the cortical lamination occurring in the brains of wild-type and heterozygous adult mice were investigated through immunostaining for non-phosphorylated neurofilaments or for the protein doublecortin (DCX). We used the antibody SMI311, which labels a subpopulation of pyramidal cells mainly located in layers II–III and V, to evaluate the degree of cortical maturation, and an antibody directed against DCX to mark migrating neuronal precursor cells that eventually integrate into hippocampal circuitry. The expression of both non-phospho-neurofilaments and DCX in *Snap-25*$^{+/-}$ adult cortices and hippocampi relative to controls was comparable in amount and distribution, suggesting that no major alterations of neuronal migration and differentiation occurr when SNAP-25 is 50% reduced (Fig. 28).

Figure 28. Immunostaining for non-phosphorilated neurofilaments and doublecortin (DCX) in P60 *Snap-25*$^{+/-}$ and control mice. Staining for non-phospho-neurofilaments with SMI311 antibody and for DCX reveals no major alterations in neuronal migration or differentiation in the cortices (SMI311) and hippocampi (DCX) of *Snap-25*$^{+/-}$ mice relative to controls.

Since SNAP-25 is involved in the modulation of calcium currents, we investigated whether reduction of the protein expression may impact on the expression pattern of the calcium-binding proteins involved in calcium signaling. In particular, immunostaining for the calcium binding proteins calbindin-D28k (CB) and calretinin (CR) were performed. As shown in Fig. 29, no alterations in CB or CR expression or localization in the hippocampus of *Snap-25*$^{+/-}$ P2 and P60 mice were observed, suggesting that SNAP-25 reduction has no secondary effects on calcium regulatory proteins.

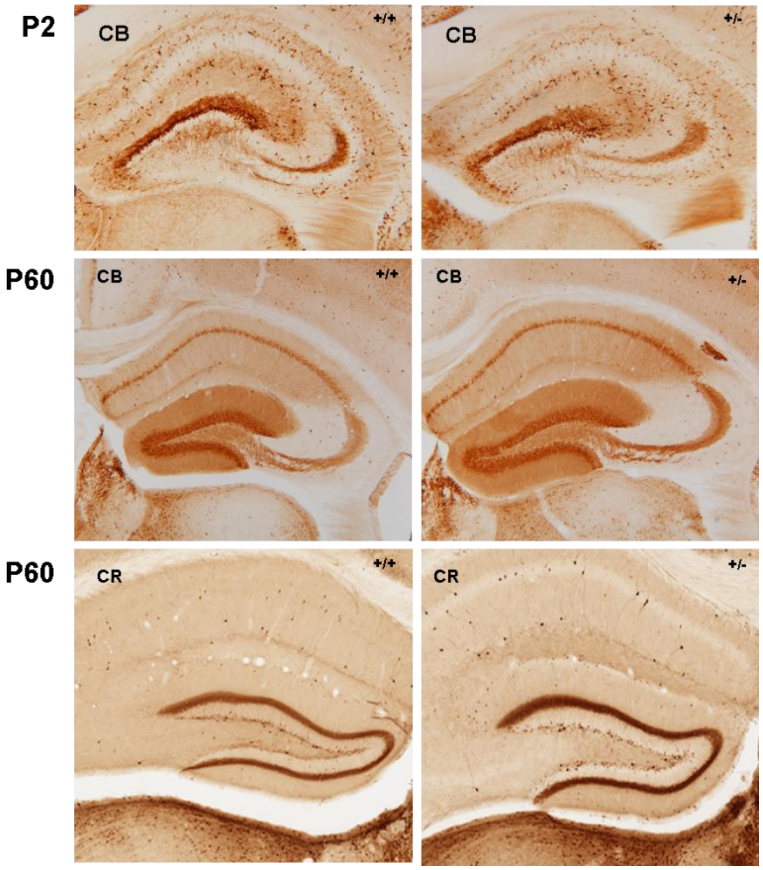


Figure 29. Evaluation of calcium-binding proteins calbindin (CB) and calretinin (CR) expression and localization in the hippocampi of pup (P2) and adult (P60) mice revealed no major changes in *Snap-25*$^{+/-}$ hippocampi at both ages compared with controls.

The distribution of excitatory and inhibitory terminals in CA1 hippocampal region, revealed by immunostaining for vesicular glutamate transporter 1 (vGlut1) and vesicular GABA transporter (vGAT), showed no differences between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice (Fig. 30 K,T). Analysis of vGAT and vGlut1 positive puncta, expressed as either a fraction of VAMP2 positive puncta or a reciprocal ratio, was almost equivalent between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice, at least in the CA1 region of the hippocampus. Also, no

major difference was observed in vGlut2 distribution in the dentate gyrus of *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice.

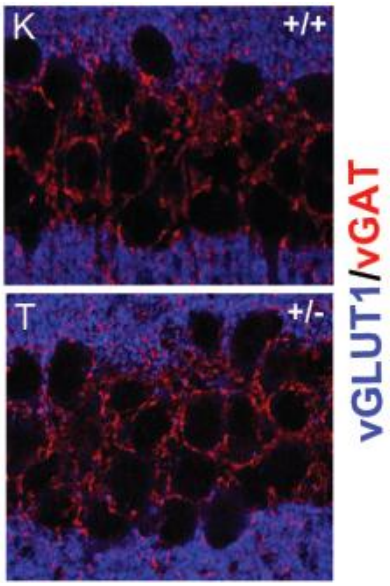


Figure 30. No gross difference in synaptic excitatory (vGlut1, blue) and inhibitory (vGAT, red) pattern is detectable in CA1 hippocampal regions (K,T).

Biochemical characterization of *Snap-25*$^{+/-}$ mouse brains

In the adult *Snap-25*$^{+/-}$ brain the expression of SNAP-25 was about 20% lower than the control. To investigate whether reduced levels of SNAP-25 may affect the expression of other synaptic proteins, we used a biochemical approach to evaluate the expression of several proteins of the presynaptic and postsynaptic compartments in homogenates obtained from the cortices of *Snap-25*$^{+/-}$ and control mice. The expression of the alternative isoforms of SNAP-25, namely SNAP-23, SNAP-29 and SNAP-47, did not change in the cortices of heterozygous mice compared with controls. The expression of the presynaptic markers SV2 and vesicular glutamate transporter vGlut1 was equal in the cortices of heterozygous and control mice. Regarding the postsynaptic compartment, we detected no alterations in the expression of NMDA receptor subunits 1 and 2A (NR1 and NR2A, respectively), and the post-synaptic density protein PSD95. Moreover, western blotting analysis did not show any variation in the expression of the calcium-binding protein calbindin (CB), in agreement with the immunohystochemistry data (Fig. 31).

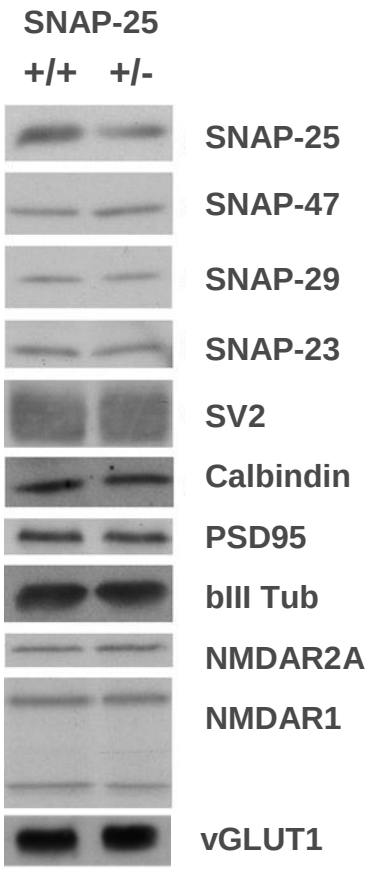


Figure 31. Biochemical analysis of presynaptic and postsynaptic proteins in the homogenates of adult *Snap-25*$^{+/-}$ and control mice. A similar expression of the alternative isoforms of SNAP-25 (SNAP-23, SNAP-47, SNAP-29), the presynaptic marker SV2, the postsynaptic markers NMDA receptor 1 (NMDAR1), NMDA receptor 2A (NMDAR2A), vGLUT1 and PSD95 was observed. Likewise, no alteration in the expression of the calcium-binding protein calbindin was observed. β_{III}tubulin (bIII Tub) was used as loading control.

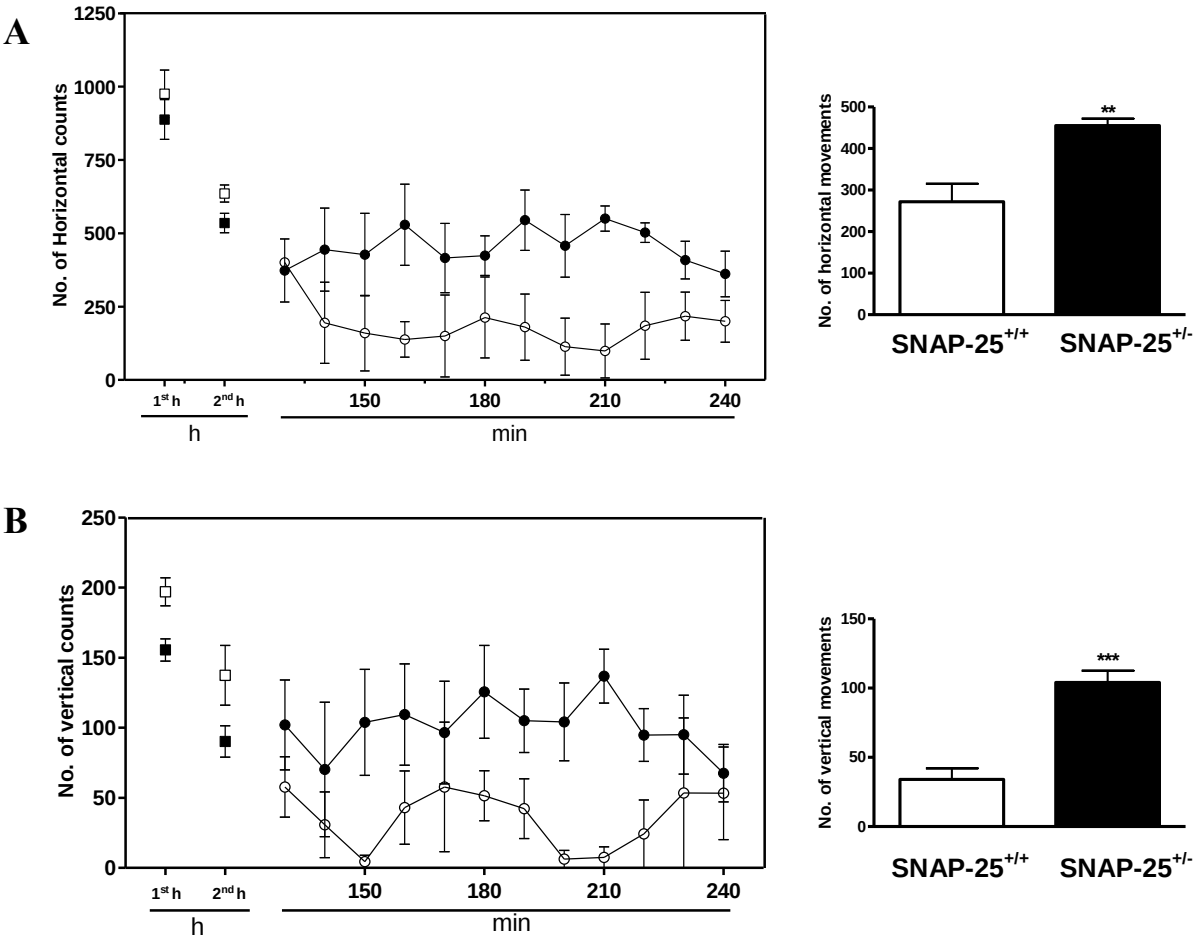
Behavioural characterization of *Snap-25*$^{+/-}$ mice

The evidence that reduction of SNAP-25 expression may directly impact the behavioural phenotype derives from the analysis of the *Coloboma* mouse, which was identified as an animal model for attention-deficit hyperactivity disorder (ADHD). This mouse exhibits spontaneous locomotor hyperactivity and this phenotype could be reduced by the expression of the *Snap-25* transgene (Hess et al. 1996). During the last decade, evidence accumulated suggesting a role for alterations in SNAP-25 expression in the onset of neuropsychiatric disorders such as schizophrenia, ADHD and epilepsy (Gabriel et al., 1997; Faraone et al. 2005; Corradini et al. 2009). Concurrently, the same kind of disorders have been associated to alterations in calcium homeostasis (Corradini et al., 2009). Previous data from our lab demonstrated that SNAP-25 negatively regulates neuronal calcium responsiveness to stimuli (Verderio et al., 2004; Pozzi et al., 2008; Condliffe et al., 2010) by modulating VGCCs activity. We could hypothesized that the reduction of SNAP-25 levels and alteration in calcium homeostasis are associated in the occurence of these neuropsychiatric disorders. For this reason, in collaboration with the group of M. Sala

(University of Milano) we aimed to analyze the behaviour of *Snap-25*$^{+/-}$ mice, which express lower levels of the protein.

Spontaneous motor activity

Spontaneous motor activity, estimated as horizontal and vertical movement counts, was monitored using an activity cage (43 x 43 x 32 cm), placed in a sound-attenuated room, in *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice at 7 weeks of age and in the adult. Horizontal and vertical activity was recorded every 10 min. As shown in Fig. 32, during the first 2-h recording both genotypes, at 7 weeks of age, showed a similar horizontal and vertical activity; however, during the following 2 h (120–240 min) *Snap-25*$^{+/-}$ mice failed to habituate, thus resulting more active than wildtype littermates. The increased number of counts is indicative of hyperactivity and higher excitability, possibly associated with the reduction of SNAP-25 expression. A normal locomotor activity was found in *Snap-25*$^{+/-}$ adult mice.



C

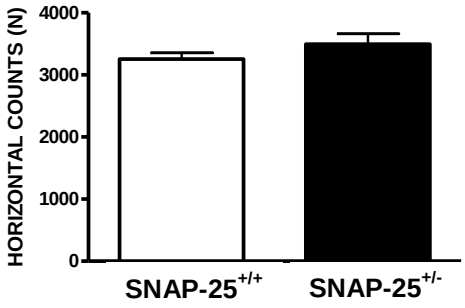
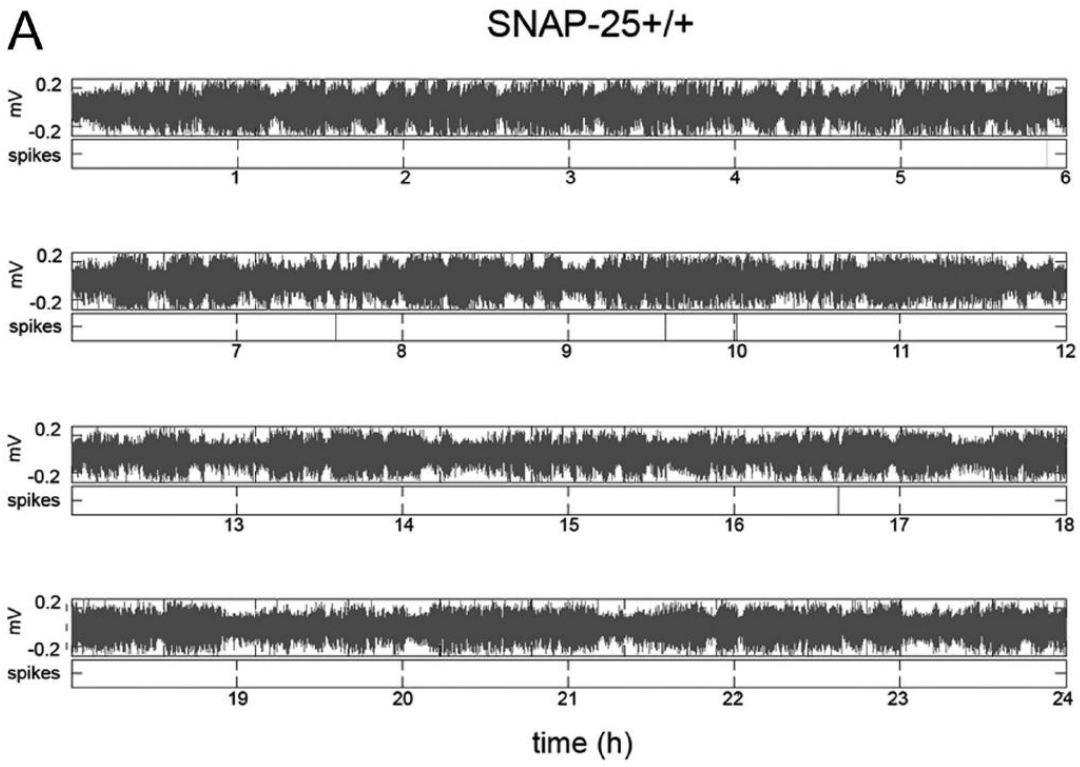


Figure 32. Activity cage. (**A,B**)Time-course of locomotor activity evaluated in 4 hours. After 2h abituation *Snap-25*$^{+/-}$ mice exhibited an increased horizontal and vertical activity. (**C**) Normal locomotor activity in *Snap-25*$^{+/-}$ adult animals.

Epileptiform activity in cortices and hippocampi of *Snap-25*$^{+/-}$ mice

As SNAP-25 is involved in the release of neurotransmitter and controls VGCC activity, we performed electroencephalographic recordings (EEG) of wild-type and *Snap-25*$^{+/-}$ mice in order to evaluate possible effects of the reduction of SNAP-25 on brain electrical activity. Freely moving animals were recorded for cortical and hippocampal EEG for 24 hours. The analysis included abnormal synchronous discharges with a minimum amplitude of twice the background EEG voltage and a minimum duration of 1 sec, and spike bursts separated by <1 sec were counted as a single discharge (Zhang et al., 2004). Recordings revealed that heterozygous mice displayed frequent spontaneous spikes of high amplitude both in the cortices and in the hippocampi, as show in the representative traces in Fig. 33. The increased spike frequency was observed in all tested *Snap-25*$^{+/-}$ mice, however, did not lead to spontaneous seizures. In only one case (a het mouse displaying 365 spikes/24 h), we could observe occurrence of generalized seizures following handling. The percentage of *Snap-25*$^{+/-}$ mice, showing abnormal EEG pattern, was significantly larger than of age-matched control mice. Thus, reduction of SNAP-25 expression in heterozygous mice is associated with spontaneous epileptiform activity extended both at the cortex and at the hippocampus.


A
SNAP-25+/+
mV
0.2
-0.2
spikes
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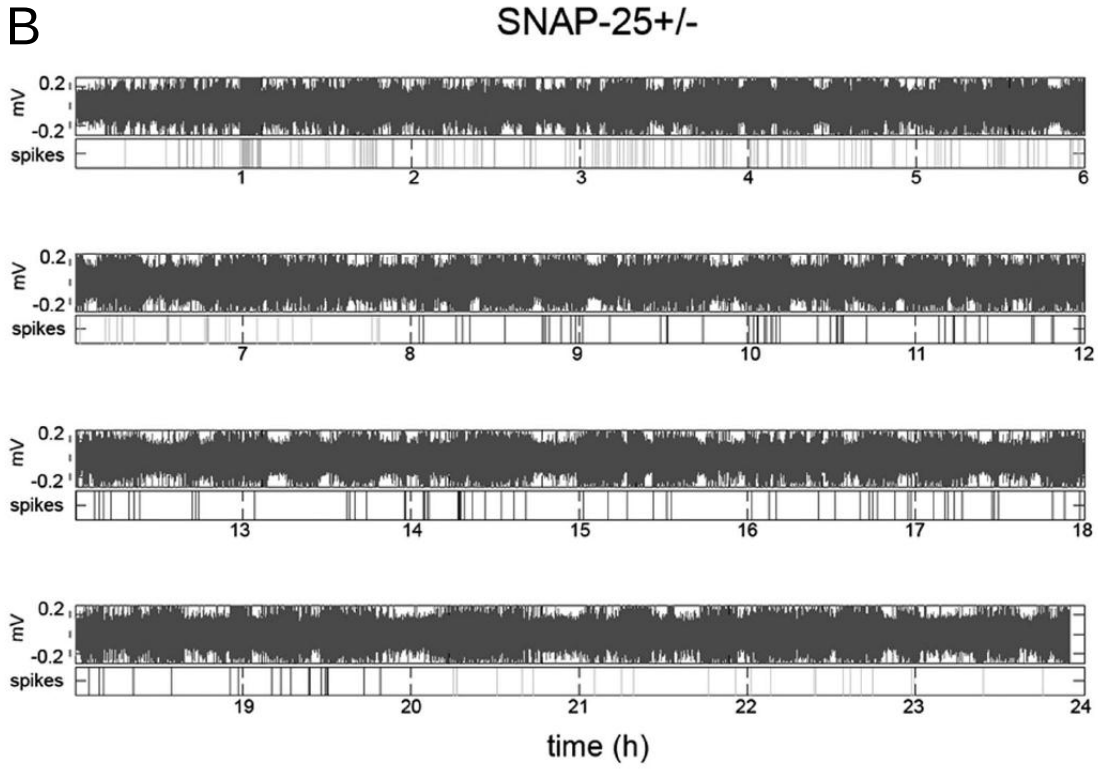
time (h)


B
SNAP-25+/-
mV
0.2
-0.2
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1
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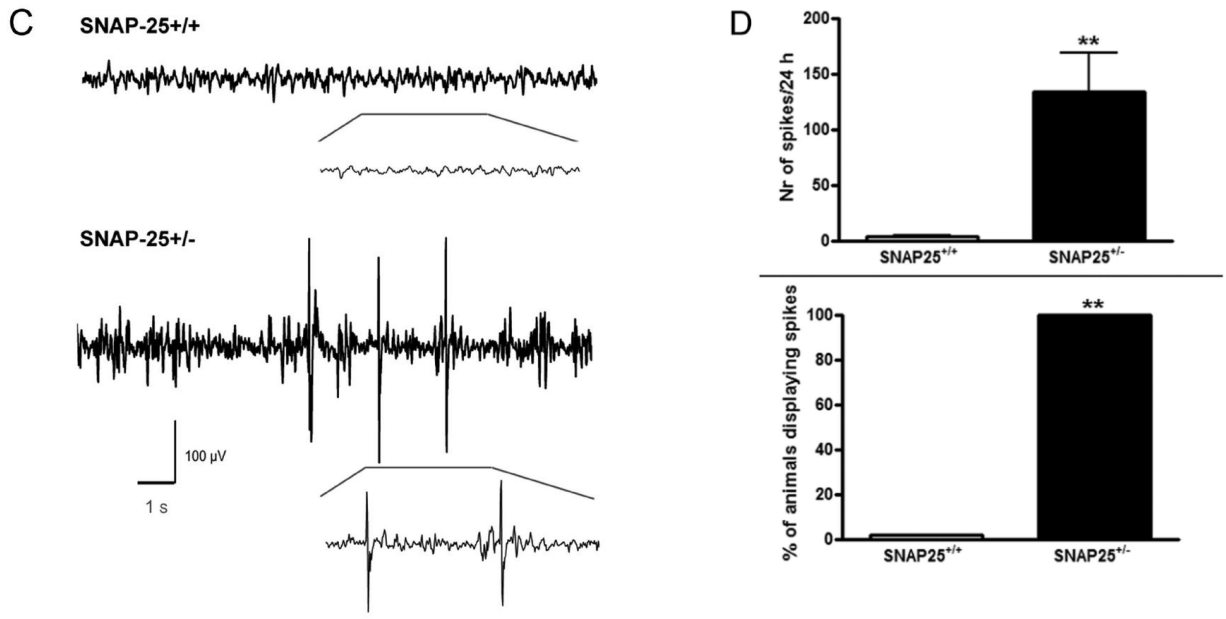

Figure 33. Electroencephalographic recordings from *Snap-25*$^{+/-}$ and wild-type mice. (**A,B**) Representative cortical EEG trace (24 hr recording). (**C**) Magnification of representative EEG traces (30-s) from one *Snap-25*$^{+/+}$ and one *Snap-25*$^{+/-}$ mouse. (**D**) The average spike number is significantly higher in heterozygous respect to wild-type, as well as the percentage of mice displaying spikes.

Increased susceptibility to kainate-induced seizures in *Snap-25*$^{+/-}$ mice

The time-course of the behavioral response of wild-type and *Snap-25*$^{+/-}$ mice to an intraperitoneal administration of kainate (KA, 35 mg/kg) was evaluated over a period of 3 hours. In all mice, this dose of KA resulted within the first 10 min in immobility and staring, followed by head bobbing and isolated limbic motor (Stage 4) seizures, characterized by forelimb clonus and rearing. Latency to the first stage 4 seizure did not differ from heterozygous or wild-type mice. In contrast with control animals that only displayed isolated limbic motor seizures, *Snap-25*$^{+/-}$ mice rapidly progressed to Stage 5 (status epilepticus), showing continuous generalized activity lasting for about 2 h. The average time of Stage 5 seizure onset in *Snap-25*$^{+/-}$ mice was 45.7 ± 6.5 min. Mortality rate following KA administration was comparable between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice (Fig.34). Statistical analysis performed by two-way repeated measures ANOVA followed by Holm-Sidak post-hoc-test revealed that *Snap-25*$^{+/-}$ mice had significantly higher behavioral scores than *Snap-25*$^{+/+}$ mice starting from 40 min after KA. Thus, the injection of KA lead to a dramatic difference of response in *Snap-25*$^{+/+}$ and mutant animals,

revealed by clinical signs. Saline-injected animals of both genotypes showed no behavioral seizures during the whole period of observation.

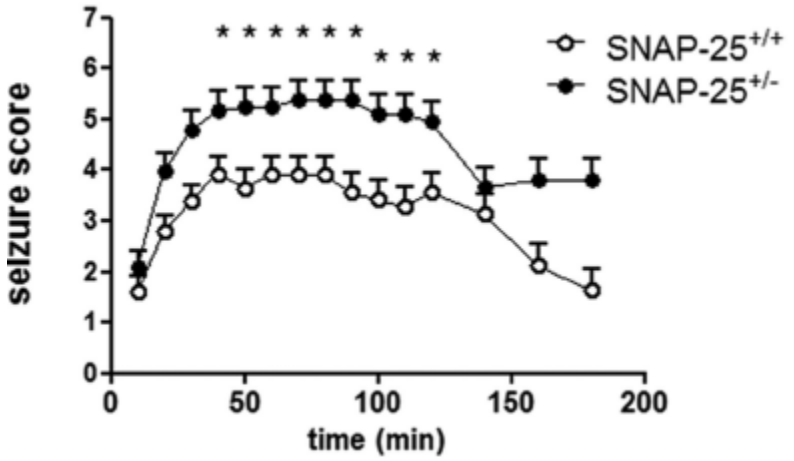

Figure 34. Increased susceptibility to KA-induced seizures in *Snap-25*$^{+/-}$ mice. Progression of behavioral changes after systemic KA administration (35 mg/kg, i.p.) in *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice over a 3- h observation period. *Snap-25*$^{+/-}$ mice showed clear signs of focal epilepsy (head bobbing), culminating in continuous generalized seizures (stage 5, status epilepticus) that last for about 2 hours. Two Way Repeated Measures ANOVA followed by Holm-Sidak test, $p<0.05$

Neuronal loss and mossy fiber sprouting in the hippocampus of *Snap-25*$^{+/-}$ mice following seizures.

14 days after KA administration all mice were killed to evaluate histopathological changes in the hippocampus. We observed an extensive loss of interneurons with NPY immunostaining in *Snap-25*$^{+/-}$ mice respect to controls, indicating that the higher seizure scores of *Snap-25*$^{+/-}$ mice are paralleled by loss of hilar neurons. Moreover, we evaluated sprouting of mossy fibers using NPY immunohistochemistry and we found a strong upregulation of NPY immunoreactivity in the mossy fiber pathway of all *Snap-25*$^{+/-}$ mice treated with KA (Fig.35A). This result was confermed by a quantitative analysis indicating that NPY labeling in the CA3 stratum radiatum of KA-treated het mice was significantly upregulated with respect to KA-treated wt animals (Fig. 35B); on the contrary, no alteration in any of the KA-injected wild type animals was observed. The absence of NPY increased immunoreactivity in *Snap-25*$^{+/-}$ untreated mice indicate that such upregulation may be a consequence of KA-induced seizures and does not depend on the spontaneous epileptiform activity recorded by EEG. Examination of individual animals revealed a significant correlation between NPY staining values and the maximum KA-induced seizure score (Fig. 35C).

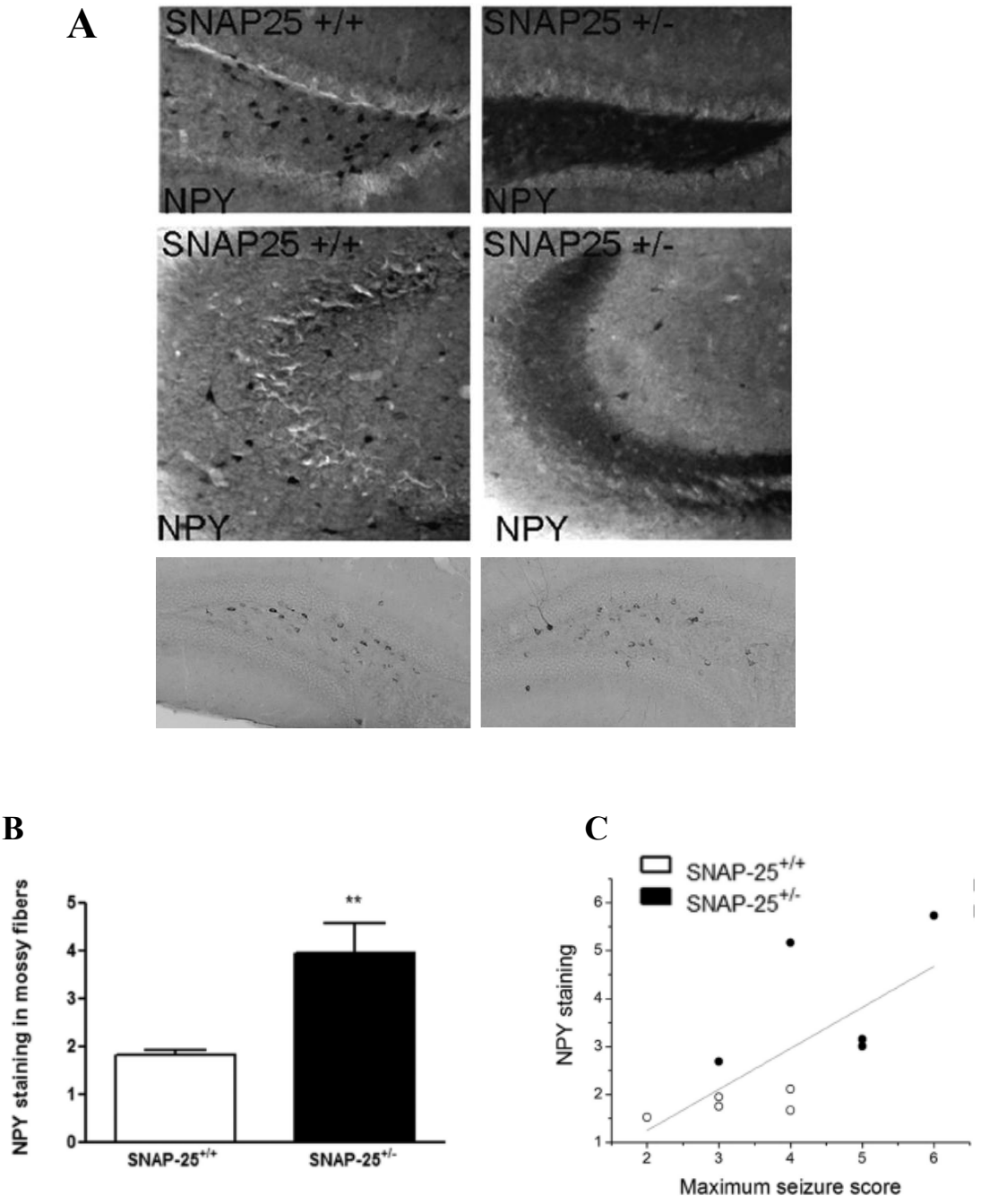

Figure 35. Immunohistochemistry was performed on brain slices obtained from *Snap-25*$^{+/-}$ and control mice 14 days after kainate injection. (**A**) Upregulation of NPY following seizures in *Snap-25*$^{+/-}$ mice. NPY staining in the dentate gyrus (first panels) and CA3 region (second panels); untreated animals (third panels). (**B**) Quantification of NPY staining intensity in KA-treated mice. (**C**) Scatter plot showing NPY staining values and maximum KA-induced seizure score for each individual mouse. There is a significant correlation between the 2 variables (Pearson correlation coefficient=0.69, P=0.025).

Conditioned taste aversion

Snap-25$^{+/+}$ and *Snap-25*$^{+/-}$ mice were tested for implicit associative learning.

Animals are equipped with learning mechanisms, such that their ingestive behavior can be modified by experience. The most important of these learning mechanisms is responsible for conditioned taste aversion (CTA). When ingestion of a novel taste (termed the conditioned stimulus or CS) is followed by transient visceral illness (termed the unconditioned stimulus or US) a CTA develops, demonstrated by a diminished intake of the CS upon subsequent encounters (Garcia and Ervin, 1968, Garcia et al., 1955 and Revusky and Garcia, 1970). First of all we measured in wild-type and heterozygous mice their ability to discriminate different tastes, by the the 2-bottle preference test. We found a strong preference for water over quinine and for saccharin over plain water, suggesting a normal taste sensitivity. There was no significant difference in HCl (0.008 M) intake between *Snap-25*$^{+/+}$ and *Snap-25*$^{+/-}$ mice.

We investigated the mice on CTA as an associative learning and memory paradigm. During three choice sessions 1, 2, and 3 days after aversive conditioning, *Snap-25*$^{+/-}$ mice drank significantly more saccharin, as expressed in percentage of total fluid intake, than *Snap-25*$^{+/+}$, indicating the lack of CTA and therefore an impairment in associative learning (Fig.36).

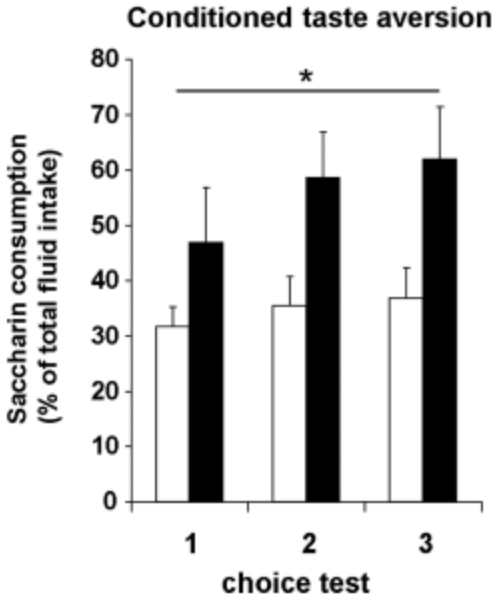

Figure 36. Conditioned taste aversion. *Snap-25*$^{+/-}$ mice consumed more saccharine solution showing a significantly weaker taste aversion than *Snap-25*$^{+/+}$ mice.

Novel object recognition

The Novel Object Recognition (NOR) task is used to evaluate cognition, particularly recognition memory, in rodent models of CNS disorders. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one and it is useful for assessing impaired cognitive ability. The choice to explore the novel object reflects the use of learning and recognition memory.

At first the animals were checked for sensorial parameters, and all mice appeared in health, displaying normal motor coordination, sensory abilities and were not aggressive.

The task procedure consists of three phases: habituation, familiarization, and test phase. The results showed that during the familiarization (T1) phase both genotypes spent a similar amount of time to explore the 2 objects, indicating that wild-type and *Snap-25*$^{+/-}$ mice had the same motivation to explore the object. However, during the test phase *Snap-25*$^{+/-}$ mice spent significantly less time exploring the novel object compared with the familiar one, as shown in Fig. 37 by a significant decrease of the discrimination index, thus indicating an impairment in recognition memory.

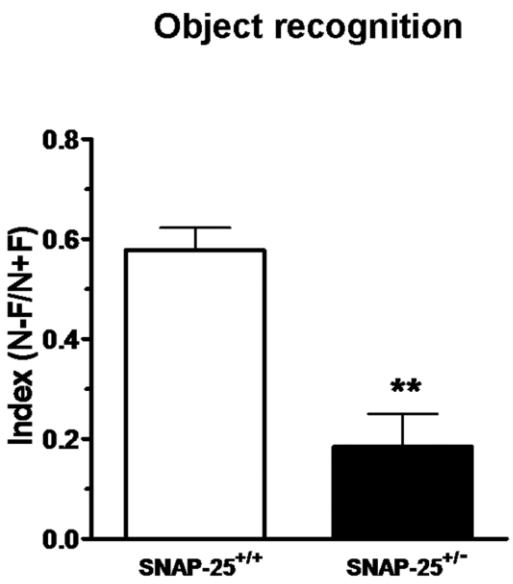

Figure 37. Object recognition test. Mice were allowed to explore an identical pair of objects and after 120 min they are presented with a familiar and a new object. *Snap-25*$^{+/-}$ mice show no net preference between novel and familiar objects, as shown by the reduced discrimination index (**P<0.0001, unpaired t-test).

Sociability and social novelty

Since reduced levels of SNAP-25 are found in the brain of schizophrenic and bipolar patient, we investigate the possibility that a reduction of SNAP-25 expression may impact the social interaction. To quantitate sociability tendencies in mice, we developed two tasks that measure (a) the animal's interest in a social stimulus (a conspecific in a wired cage placed in one of the outer chambers), versus a neutral stimulus (an empty wired cage placed in the other outer chamber) and (b) preference for a novel vs. a familiar conspecific. When tested for sociability, *Snap-25*$^{+/+}$ mice behaved normally, spending longer time to explore the compartment with the stranger mouse than the empty cage. Conversely, *Snap-25*$^{+/-}$ mice spent the same amount of time in the 2 compartments. 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 (Fig. 38).

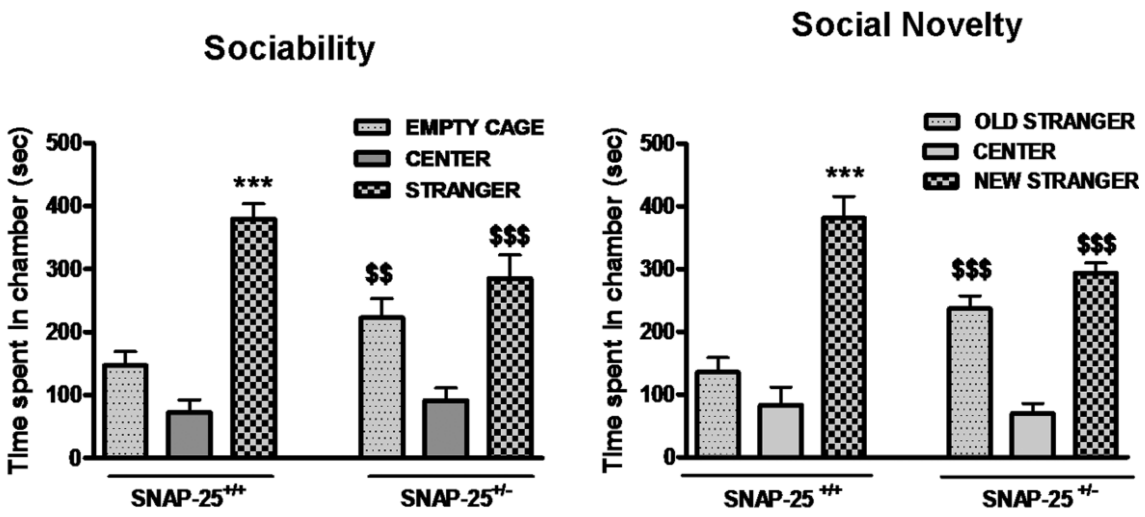

Figure 38. Sociability and social novelty test. *Snap-25*$^{+/-}$ mice show impaired social interaction, as they spend the same amount of time exploring a stranger mouse or an empty cage in a social choice paradigm. Social novelty (right). *Snap-25*$^{+/-}$ mice fail to show any preference for a novel mouse in a social recognition task. In contrast, *Snap-25*$^{+/+}$ mice spend more time exploring the new stranger versus old stranger and center.

Pharmacological treatment with antiepileptic drugs normalize the EEG profile and improve the cognitive defects in *Snap-25*$^{+/-}$ mice

To better study the abnormal EEG pattern of *Snap-25*$^{+/-}$ mice, we treated the animals with vehicle (saline) and with different conventional antiepileptic drugs, namely valproate

sodium salt VLP (250 mg/kg, i.p.), ethosuximide ETO (200 mg/kg, i.p.) and carbamazepine CBZ (50 mg/kg, i.p.); as SNAP-25 modulates VGCC activity, animals were also treated with the calcium antagonist nimodipine NIMO (10mg/kg, i.p.). To investigate whether these different treatments were able to normalize the altered EEG profile, we recorded basal EEG cortical activity 30 minutes before treatment, and then over 2 hours. All treatments with different antiepileptic drugs significantly reduced spike activity as shown in Fig. 39A. We obtained the maximal effect after treatment with VLP (95% reduction) and ETO (80% reduction), whereas a partial but significant reduction was obtained with CBZ (60% reduction). Treatment with nimodipine also reduced the epileptiform activity, although the effect was slighter (35% reduction) than with other drugs. The mean number of spikes was significantly reduced in *Snap-25*$^{+/-}$ mice treated with VLP (Fig. 39C).

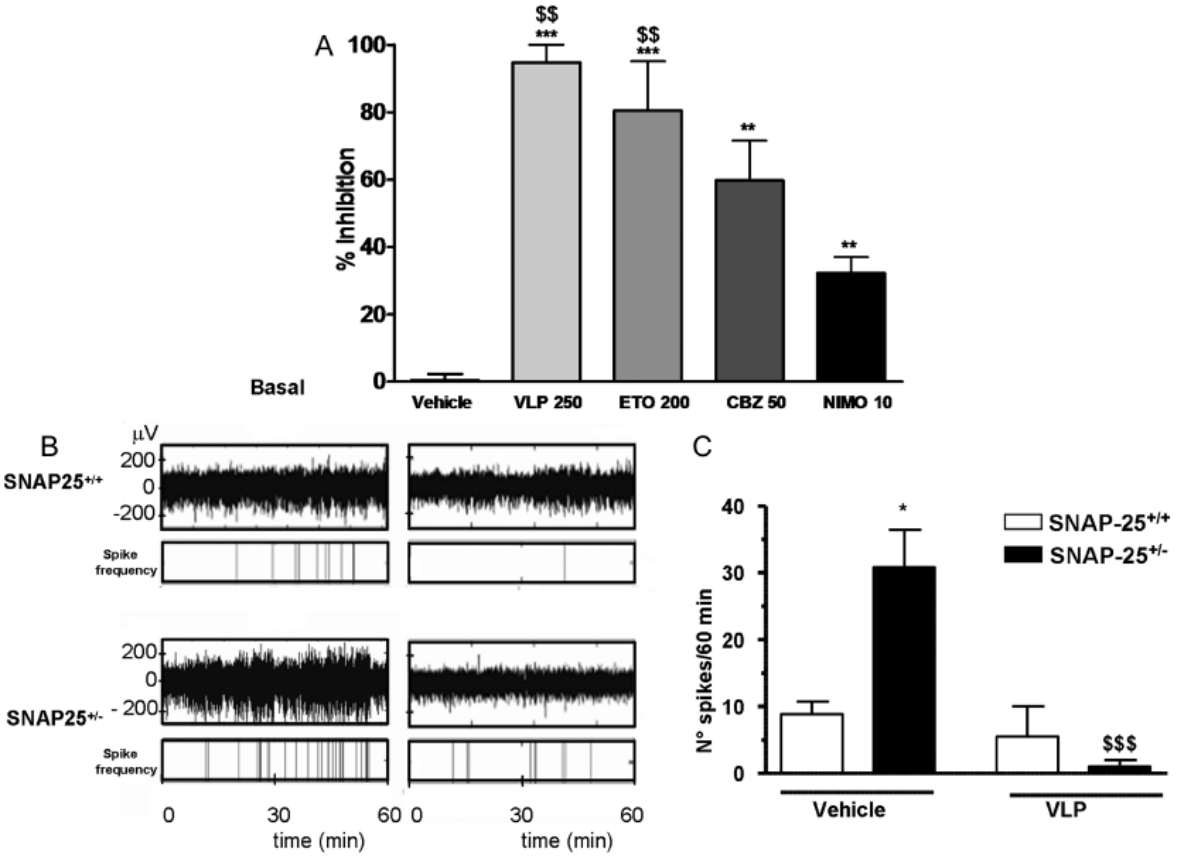

Figure 39. Antiepileptic drugs normalize the EEG profile. (**A**) Treatment with VLP (250 mg/kg), ethosuximide (ETO) (200 mg/kg), carbamazepine (CBZ) (50 mg/kg), and nimodipine (NIMO) (10 mg/kg) to *Snap-25*$^{+/-}$ mice significantly reduces spike activity. (**B**) EEG recording of one representative *Snap-25*$^{+/-}$ mouse treated with VLP showing a decreased spike activity in comparison to one *Snap-25*$^{+/+}$ mouse. (**C**) The mean number of spikes recorded for 60 min in *Snap-25*$^{+/-}$ mice is significantly reduced by VLP compared with vehicle.

Given the beneficial consequence produced by VLP in the EEG profile, we also analyzed the effects of this treatment on the behavioural tests.

VLP, given 2 h before test performance, reversed the cognitive deficit of *Snap-25*$^{+/-}$ mice in the object recognition task. CTA impairment, noted in heterozygous mice, was fully reversed by pretreatment with VLP, given immediately before HCl exposure; moreover this antiepileptic drug normalized the defects in social memory, although it was ineffective in restoring sociability (Fig. 40).

It has been reported that VPL can interfere with learning and memory processes both in experimental models (Wu et al., 2002; Sgobio et al., 2010) and patients (Mula and Trimble, 2009); in agreement with these evidence we observed a slight, but not significant, worsening of cognitive abilities in *Snap-25*$^{+/+}$ mice treated with VLP (not shown).

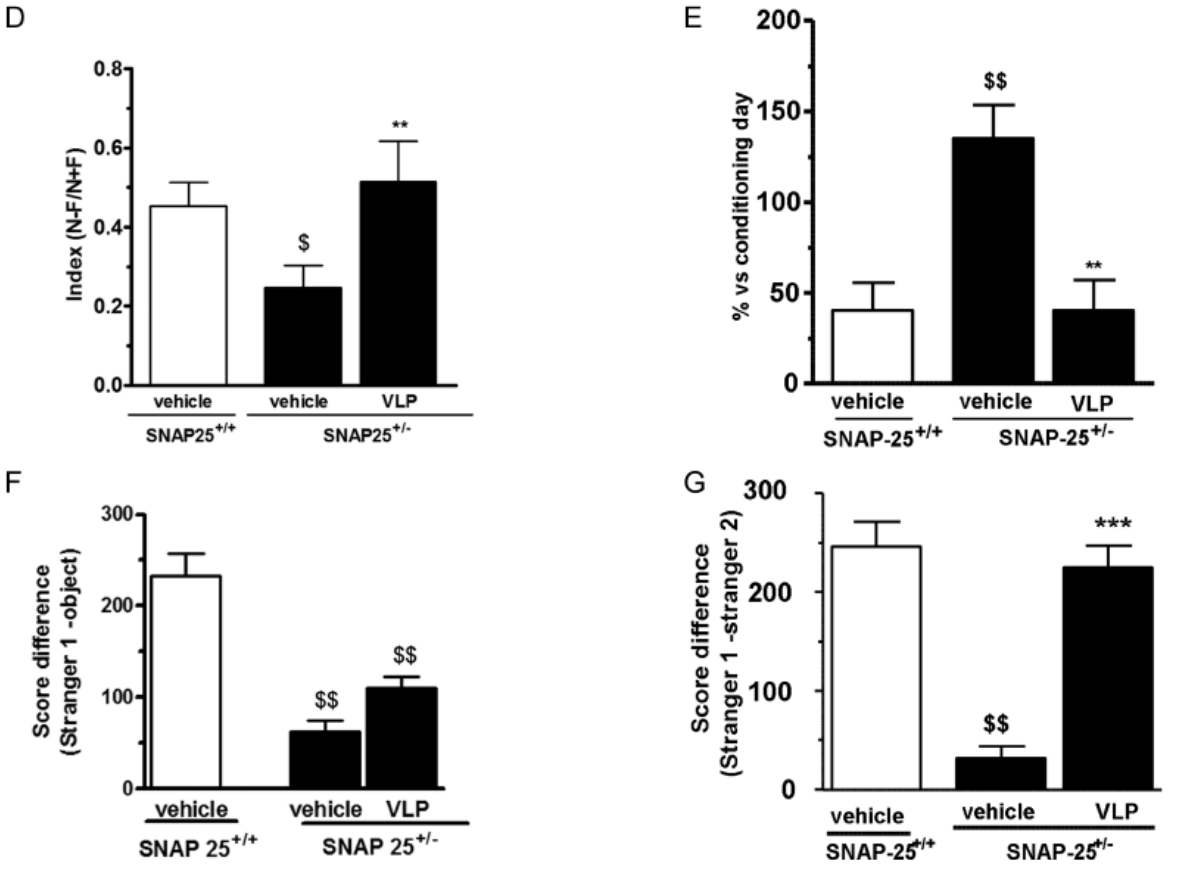

Figure 40. Antiepileptic drugs reverse learning defects in *Snap-25*$^{+/-}$ mice. (**D**)VLP reverses the defect in object recognition test. The reduced discrimination index, found in *Snap-25*$^{+/-}$ mice, is reversed by pretreatment with VLP given i.p. at the dose of 250 mg/kg, 20 min before T1 trial. (**E**) VLP reverses CTA impairment. (**F**) Effect of VLP in sociability in *Snap-25*$^{+/-}$ mice. VLP do not reverse the impaired social interaction. (**G**) VLP is effective in improving putative social memory.

FUNCTIONAL STUDY OF A POLYMORPHISM IN THE GENE OF *SNAP-25*

Cognitive ability is currently considered as a polygenic trait influenced by many genes that, in turn, may interact with each other and with environmental factors.

Previous studies have reported a strong association between three SNPs in the *Snap-25* gene and intelligence. These SNPs, namely rs363039, rs363050 and rs363043, all located on intron 1, showed association in the same direction and the same order of magnitude, although strongest evidence was found for SNP rs363050. SNPs located in regulatory region (promoters, UTRs, introns) may have a significant influence on the expression level of a gene.

Aiming to define whether these polymorphisms play a role in *Snap-25* gene expression, we conducted an analysis of the functional effects of the polymorphism (rs363050) on transcriptional activity, by means of luciferase reporter gene. A series of plasmids, in which the cassette containing the SNP (parental or minor allele) was inserted upstream or downstream the TK promoter in pGL4 basic vector, was originated. Plasmids were transiently transfected in human neuroblastoma SH-SY5Y cell line, and the activity of the constructs was compared with that of the plasmid lacking the cassette. The presence of the parental allele (A), when cloned upstream the TK promoter (construct rs363050A-TK-luc), did not increase the promoter activity, while the presence of the minor allele (G; construct rs363050G-TK-luc) significantly reduced the transcription of luciferase by 2-fold. When the cassette was inserted downstream the luciferase reporter gene, both constructs (TK-luc-rs363050A and TK-luc-rs363050G) showed a dramatic decrease in the capability of driving the expression of the reporter gene with respect to the construct with the cassette cloned upstream the TK promoter. However, the reduced transcription capability of the minor allele compared with the parental allele was confirmed (Fig. 41).

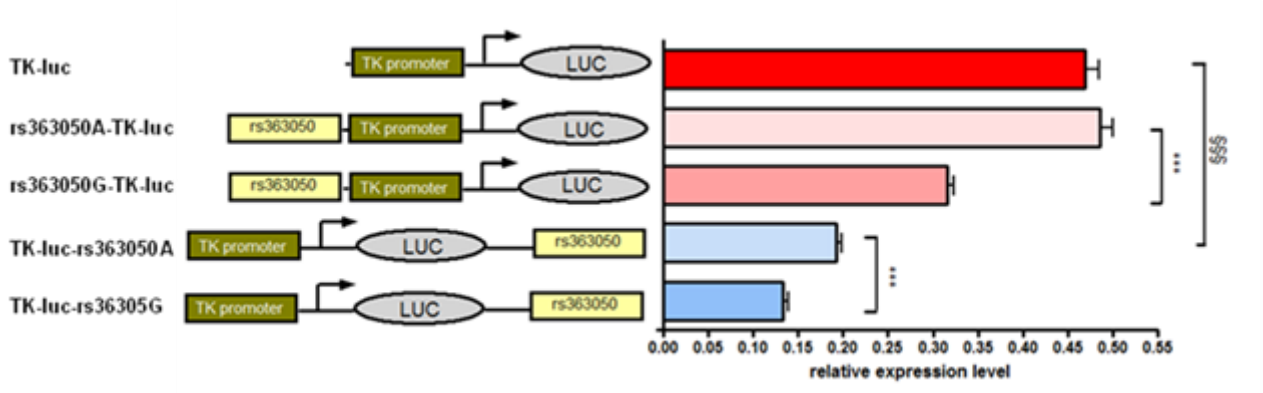

Figure 41. Schematic representation of the reporter constructs used in the luciferase assay. SY5Y cells were transiently transfected with the constructs indicated on the left, and the luciferase assays were performed 48 hr later. The bars represent the relative expression levels luc/ren (firefly luciferase/renilla luciferase). Data represent means ± SD. The presence of the minor allele (G), upstream or downstream the TK promoter, significantly decreases the luciferase transcription.

These findings suggest that the region encompassing the rs363050 SNP contains a regulatory element whose function is dependent on its position; furthermore, the presence of the minor allele may influence the transcription of the *Snap-25* gene.

In order to study more in detail the regulatory activity of the rs363050 SNP, a small oligonucleotide encompassing the SNP was cloned in multiple copies upstream the TK promoter. As shown in Fig. 42, the presence of 4 copies of rs363050 SNP cassette (4X A-TK-luc) resulted in a slight increase in the promoter activity, whereas 4 copies of the minor allele (4X G-TK-luc) reduced the promoter activity in a manner equivalent to that of rs363050-G-TK-luc construct. Taken together, these results support the evidence that the rs363050 polymorphism might have a role in modulating *Snap-25* gene expression levels.

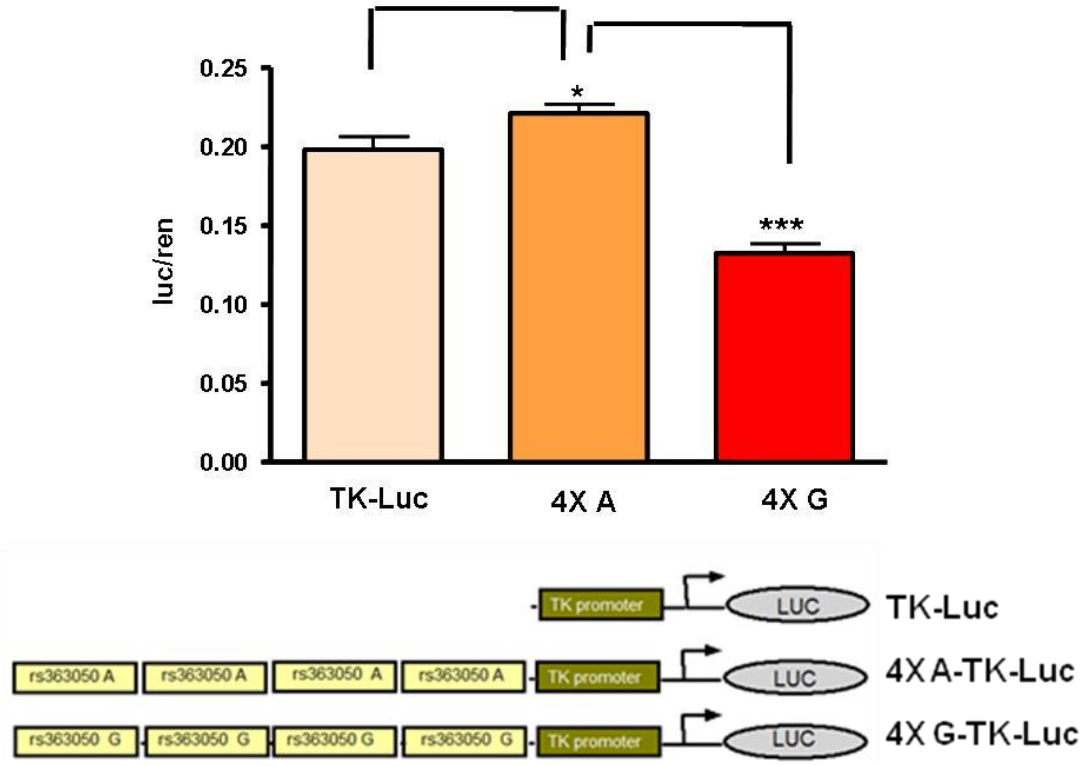

Figure 42. Effects of four copies of rs363050 SNP cassette (parental or minor allele) on promoter activity in SY5Y cells. Data represent means ± SD of luc/ren ratio.

The polymorphic sequence was analysed in silico for putative transcription factor binding sites using the Genomatix program (Genomatix Software GmbH, Munich, Germany). The analysis revealed that the polymorphic site disrupts a putative binding site for the transcription factor AP-1. AP-1 is ubiquitously expressed and its activity is induced by physiological stimuli and environmental insults. It is constituted by Jun (c-Jun, JunB, JunD) homodimers, or Jun heterodimers with Fos (c-Fos, FosB, Fra-1, Fra-2) or ATF (activating transcription factor) proteins. AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation.

To assess the possible interaction between the polymorphic site and AP-1, a gelshift assay was performed. An oligonucleotide encompassing the region of *Snap-25* that included the putative binding site for AP-1 (parental allele, rs363050 A) was incubated in the presence of SH-SY5Y nuclear extract, resulting in the formation of three retarded complexes. The specificity of these complexes was tested by competition in the presence of a molar excess of the same cold oligonucleotide (100x and 300x), (lanes 2 and 3). A molar excess of a cold oligonucleotide bearing the minor allele (G instead of A, lanes 4 and 5) was unable to compete with any of the three complexes, suggesting that specific factors recognizing the sequence bearing the parental allele can bind this region.

To test whether these factors belong to the AP-1 family of transcription factors, the nuclear extract was pre-incubated with antibodies directed against Jun or Fos. As shown in fig. 43 (lanes 6 and 7), no ultra-retarded bands were observed, indicating that the transcription factor AP-1 is probably not involved in the binding of this region.

In conclusion, the presence of the cassette bearing the parental allele did not increase the basal activity of a heterologous promoter, whereas the minor allele resulted in a reduction of the transcription capability. This indicates that the presence of the minor versus the parental allele may result in the impairment of the binding of factors involved in the modulation of *Snap-25* gene expression, or in the binding of other factors, different from those recognizing the parental sequence, acting as repressors. Further characterization of these factors is required in order to analyse more in depth the effects of the presence of the minor polymorphic allele.

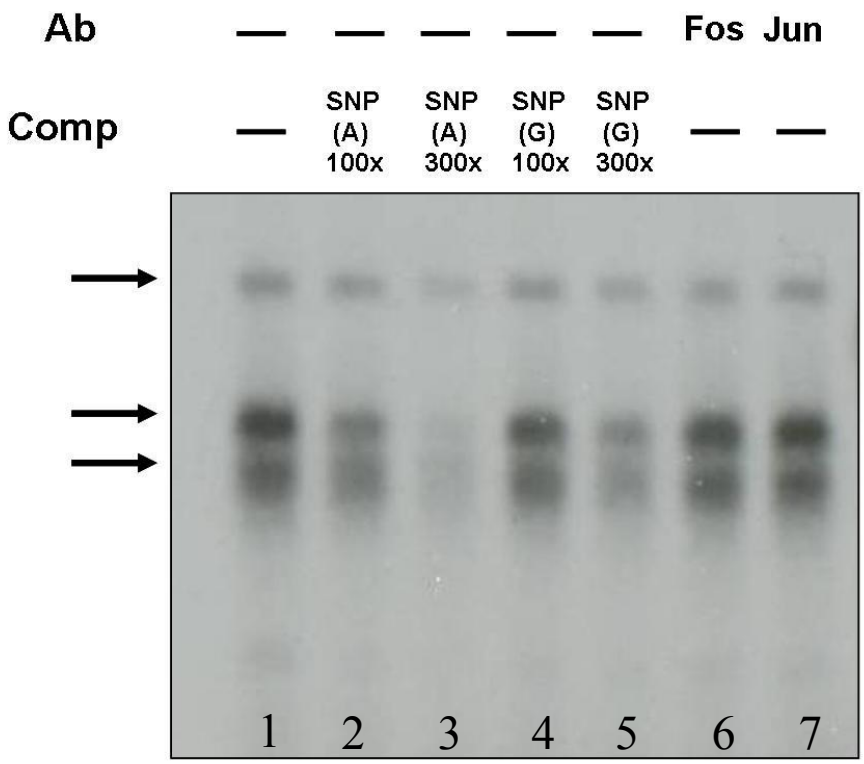

Figure 43. Molecular analysis by gelshift of the putative binding site for AP-1. On the *left*, the *arrows* indicate the retarded complexes formed upon incubation of the labeled probe with SY5Y nuclear extract (*lane 1*). Competition experiments were performed in the presence of a molar excess (100x or 300x) of the cold oligonucleotide bearing the parental allele (*lanes 2, 3*) or the minor allele (*lanes 4, 5*). No ultra-retarded bands were observed when the nuclear extract was pre-incubated with the anti-Fos and anti-Jun antibodies *(lanes 6, 7)*.

DISCUSSION

SNAP-25 is a fundamental component of the SNARE complex responsible for synaptic vesicle exocytosis. Besides participating in exocytosis, SNAP-25 also modulates various voltage-gated calcium channels (VGCCs), interacting through a region known as synaptic protein interaction (*synprint).* In the last years, evidence accumulated suggesting a differential expression of SNAP-25 in neuronal subpopulations; in particular, adult glutamatergic neurons express high levels of SNAP-25, while mature GABAergic terminals virtually lack immunoreactivity for the protein (Verderio et al., 2004; Frassoni et al., 2005; Garbelli et al., 2008; Bragina et al., 2007; Mandolesi et al., 2009). It has been recently demonstrated that SNAP-25 negatively controls neuronal calcium responsiveness to depolarization, through voltage-gated calcium channel inhibition upon phosphorylation on the residue Ser187 (Pozzi et al., 2008). Furthermore, a recent paper of our group (Condliffe et al., 2010) showed that endogenous SNAP-25 levels negatively regulate native VGCC properties in glutamatergic neurons, which displayed significantly lower VGCC current densities, enhanced inactivation rates and shifts in the voltage-dependence of activation and steady-state voltage-dependent inactivation curves relative to GABAergic neurons, which express lower levels of the protein. Moreover, silencing of endogenous SNAP-25 in glutamatergic neurons increased VGCC current density, while over-expressing SNAP-25 in GABAergic neurons reduced inward VGCC current and inactivation kinetics. Furthermore, VGCC current densities in glutamatergic neurons from *Snap-25*$^{+/-}$ mice were significantly elevated compared with wild type glutamatergic neurons, (Condliffe et al., 2010). Consistently, *Snap-25*$^{+/-}$ neurons displayed enhanced evoked glutamatergic neurotransmission, and shifted from paired pulse facilitation to paired pulse depression, thus indicating that reduced levels of SNAP-25 may induce an increase in presynaptic release probability (Antonucci et al., submitted).

In the last years, altered levels of SNAP-25 expression have been associated to different neurological and neuropsychiatric conditions. Case-control and family based studies associated SNAP-25 with Attention Deficit-Hyperactivity Disorder (ADHD; Barr et al., 2000; Kustanovich et al., 2003; Mill et al., 2004; Faraone et al., 2005; Feng et al., 2005); in line with this, single nucleotide polymorphisms (SNPs) in the *Snap-25* gene have been linked to inattentive hyperactivity in a group of ADHD children (Zhang et al., 2010), and

associated with hyperactivity in autism spectrum disorders (Guerini et al., 2011). Moreover, reduction of SNAP-25 expression has been described in psychiatric patients. In particular, reduced levels of SNAP-25 have been described in the hippocampus (Young et al., 1998; Thompson et al., 2003) and in the frontal lobe (Thompson et al., 1998) of patients with schizophrenia, while other modifications of SNAP-25 levels occur in the brain of bipolar patients (Fatemi et al., 2001; Scarr et al., 2006). The demonstration that SNAP-25 expression is altered in psychiatric patients suggests that variations in SNAP-25 levels may have a pathogenic effect, possibly affecting synaptic function and network activity. Up to now, the only evidence linking SNAP-25 reduction to an altered phenotype derives from the *Coloboma* mutant mouse, that is heterozygous (*Cm*/+) for a neutron irradiation induced, semi-dominant deletion spanning 4.6 Mb on mouse chromosome 2, that encompasses 10-12 genes including *Snap-25* (Hess et al., 1996), thus resulting in 50% reduction of SNAP-25 expression. The *Coloboma* mice is a model for ADHD (Wilson 2000; reviewed in Faraone et al., 2005; Russell 2007), and it is characterized by an hyperactive phenotype, which is reduced by the expression of the *Snap-25* transgene (Hess et al., 1996). Given that the deleted region in this mutant mouse comprises several genes, including genes for phospholipase C beta-1 (Plcb1), coloboma (cm), Plcb4, and Jag1, this model maynot be suited for investigating the neurophysiological and behavioural phenotype induced by reduction of SNAP-25 levels.

For this reason, here we aimed at evaluating the possible effects of SNAP-25 reduction in a mouse model in which only SNAP-25 expression is reduced. For this purpose, we performed morphological, biochemical and behavioural studies in the *Snap-25* heterozygous mouse. The *Snap-25* gene is a single gene spanning more than 80 kb and the polypeptide is encoded by eight different exons spaced by quite large introns (Bark, 1993). Differential splicing of exon 5 results in the expression of two transcripts, SNAP-25a or SNAP-25b; these two isoforms are developmentally regulated, with SNAP-25b being mostly expressed in the adult. In the *Snap-25*$^{+/-}$ mutant mouse, the region containing both exon 5a and 5b was disrupted by the insertion of a PGKneo cassette, thus reducing by 50% both alternatively spliced isoforms of the protein. Differently from the *Coloboma* mutant, in the *Snap-25*$^{+/-}$ mouse only the expression of SNAP-25 is affected. Up to now all the analysis for quantifying the protein expressionin *Snap-25*$^{+/-}$ mouse have been carried out at embryonic day (E)17.5, when a 50% reduction of the protein was demonstrated (Washbourne et al., 2000). In order to confirm SNAP-25 reduction during brain development, we analyzed the levels of the protein during different developmental stages,

from E18 embryo to adult mouse (30 days old, P30). We observed a progressive increase of the protein expression during development, which was not accompanied by a parallel increase in SNAP-25 mRNA levels, suggesting that *post-transcriptional* mechanisms are activated during postnatal development and are responsible for a compensation in protein expression. The molecular basis for SNAP-25 expression increase are not clarified yet. Since *Snap-25*$^{+/-}$ mice are characterized by an abnormal EEG profile, one could hypothesize that the increase in protein synthesis may derive from an activity-dependent regulation of the protein transcription. This possibility needs to be tested in detail. Whatever the mechanisms involved, it is however interesting that hyperactivity, which is associated to reduced protein expression, tends to disappear in adult *Snap-25*$^{+/-}$ mice (Corradini et al., 2012). This situation closely reminds what described for ADHD, where symptoms continue from childhood to adulthood only in a percentage of patients, disappearing in the vast majority of cases.

As some neuropsychiatric disorders may arise as consequence of malformations, tumours or inflammation, we evaluated the possible effects of SNAP-25 reduction on brain anatomy. No major morphological and structural alterations were observed in the pups (postnatal day 2, P2) or in the adult brain (postnatal day 60, P60), thus indicating that the reduction of SNAP-25 expression does not lead to altered brain morphology. Also, we did not detect defects in neurogenesis or neuronal maturation, evaluated by means of immunostaining for the non-phosphorylated neurofilaments and the neurogenesis marker doublecortin. As SNAP-25 is involved in modulation of calcium currents, we evaluated the expression of two calcium binding proteins, namely calbinin-D28k and calretinin, in the heterozygous and wild-type hippocampi. Calbindin and calretinin are highly expressed in most neuronal populations in the central nervous system and they participate in the control of calcium concentration at the synapse, thus representing a possible mechanism of control of release probability and synaptic plasticity. We did not observe alterations ofcalbindin orcalreti nin expression or localization in the hippocampi of P2 and P60 *Snap-25*$^{+/-}$ mice, compared with wild-type controls, thus excluding the possibility that reduced SNAP-25 expression may lead to altered calcium homeostasis through mechanisms other than the modulation of VGCCs.

Different homologous forms of SNAP-25 are present in neuronal and non-neuronal cells, namely SNAP-23, SNAP-29 and SNAP-47. Similarly to SNAP-25, these different isoforms can form a SNARE complex with different partners (Ravichandran et al., 1996; Hohenstein, 2001; Holt, 2006). Moreover, SNAP-29 was suggested to modulate synaptic

transmission (Su et al., 2001; Pan et al., 2005). In order to evaluate possible alterations of SNAP-23, SNAP-29 and SNAP-47 expression caused by reduction of SNAP-25, we performed biochemical experiments on cortical homogenates obtained from *Snap-25*$^{+/-}$ and wild-type mouse brains. We did not find altered expression for any of these proteins, which excludes the possibility that SNAP-23, -29 and -47 expression may vary upon reduction of SNAP-25 expression constituting a compensatory mechanism.

Snap-25$^{+/-}$ mice were then analysed for several behavioural tasks in order to characterize their phenotype. Like coloboma mutant mice (Hess et al., 1996), we found a hyperactive phenotype in the *Snap-25*$^{+/-}$ mice, measured in the activity cage. It has been described that SNAP-25 reductions lead to the onset of absence epilepsy in the coloboma mouse model, which were preceded by intracellular calcium elevations (Zhang et al., 2004). We then evaluated the possibility that *Snap-25*$^{+/-}$ mice display an epileptic phenotype. Analysis of the EEG recordings showed frequent spontaneous spikes both in the cortices and in the hippocampi of heterozygous mice. Such abnormal EEG was never observed in the wild-type control animals. However, the presence of spikes did not lead to an overt epileptic phenotype, except than in sporadic cases. Moreover, heterozygous mice showed a higher susceptibility to kainate-induced seizures compared to controls, which was accompanied, 14 days after kainate injection, by a strong sprouting of mossy fibers, that is a marker of epileptogenesis after kainate injection. Taken together, these data suggest that reduction of SNAP-25 expression affects network activity, leading to hyperexcitability. Interestingly, the incidence of epilepsy is 2.7 times higher in ADHD children (Davis et al., 2010), and patients with schizophrenia are 6 times more likely to develop epilepsy than the controls (Chang et al., 2011). Although the mechanismsby which SNAP-25 reduction impacts network excitability still remain unclear, enhanced calcium currents selectively occurring in glutamatergic but not GABAergic neurons (Condliffe et al., 2010) may generate an excitatory/inhibitory imbalance which can eventually result in altered EEG activity.

Besides electroencephalographic abnormalities, *Snap-25*$^{+/-}$ mice display an impairment in associative learning and recognition memory, indicated by the lack of conditioned taste aversion, and deficits in the novel object and social recognition tasks, respectively. Furthermore, reduction of SNAP-25 expression also impact social interaction; indeed our results revealed an altered social behavior in *Snap-25*$^{+/-}$ mice respect to controls as demonstrated by deficits in the test for social preference.

We reasoned that the abnormal EEG profile observed in SNAP-25$^{+/-}$ mice could contribute to the learning and memory defects. Indeed, the cognitive effects of epileptiform discharges are very well known and may be very similar to those observed upon short epileptic seizures (Aarts et al., 1984). Consistently, a decline in IQ scores was reported in patients with frequent episodes of epileptiform discharges (Aldenkamp et al., 2005; Aldenkamp et al., 2010).

Treatment with antiepileptic drugs, or with the calcium antagonist nimodipine, largely normalized the altered EEG profile of *Snap-25*$^{+/-}$ mice, the larger beneficial effects produced by valproate and ethosuximide, i.e. drugs effective at controlling absence seizures. Even more interestingly, we demonstrated that treatment with VLP rescued associative and recognition memory defects, while it did not revert the deficit observed in social interaction. Notably, carbamazepine has been reported to exert a positive effect on a child with ADHD and subclinical EEG discharges without seizures (Laporte et al, 2002), while treatment with VLP appeared to ameliorate ADHD symptoms in Fragile X Syndrome boys (Torrioli et al., 2008). These data therefore indicate that reductions in SNAP-25 expression may produce a hyperexcitable background, resulting in altered EEG profile and in the occurrence of cognitive deficits, both of which can be efficaciously treated by antiepileptic drugs.

A number of single nucleotide polymorphisms (SNPs) have been implicated in different neuropsychiatric and neurological disorders. In particular, recent evidence have suggested an association between SNPs in the *Snap-25* gene and ADHD (Barr et al., 2000; Mill et al., 2004; Feng et al., 2005), schizophrenia (Tachikawa et al., 2001; Spellmann et al., 2008), bipolar disorder (Eitain et al., 2010) and autism (Guerini et al., 2011). Two SNPs (rs363050 and rs363039) in the *Snap-25* gene have been associated with variations in intellectual abilities in two independent Dutch samples (Gosso et al., 2006 and 2008). SNPs in regulatory regions may result in functional changes in the gene product and may play a role in the regulation of gene expression. Based on the results we have obtained, showing the occurrence of cognitive disabilities in mice expressing reduced levels of SNAP-25, we conducted a functional analysis on one of these SNPs, rs363050, which associates with low performance IQ (Gosso et al., 2006) and is located on intron 1 of the *Snap-25* gene, to investigate whether the presence of the parental or minor allele may affect the expression of SNAP-25. Here we demonstrate that the presence of the minor allele, inserted either upstream or downstream the promoter, significantly decreases the

transcription capability, although we did not obtain an amplification of this effect in the promoter activity when we inserted 4 copies of the minor allele.

On the other hand, the presence of the cassette bearing the parental allele did not increase the basal activity of an heterologous promoter. The presence of the minor versus the parental allele may thus result in the impairment of the binding of factors involved in the modulation of the *Snap-25* gene expression level, or in the binding of other factors, unlike the ones that recognize the sequence of the parental allele, acting as repressors. Due to the importance that the presence of the minor allele can have on SNAP-25 expression level, a further characterization of these factors is mandatory. Interestingly, a higher frequency of the rs363050 polymorphism has been recently associated with intellectual disability (ID) cases compared to controls (Rizzi et al., 2012).

Our data offer a logical frame for these findings, by directly linking polymorphisms in the *Snap-25* gene, reduction of the protein expression, generation of subclinical epileptiform discharges and learning disabilities. They also suggest that human genetic variations, resulting in reduced protein expression, may create a background susceptible to functional failures, which in turn may lead to network hyperexcitability. Finally, we demonstrate the beneficial effect of antiepileptic drugs in ameliorating the abnormal excitability and the cognitive defects linked to reduction of SNAP-25 expression, thus offering the possibility of new therapeutic strategies.

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