Reduced SNAP-25 Increases PSD-95 Mobility and Impairs Spine Morphogenesis

Abbreviated title: **SNAP-25 in spine formation and function**

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ABSTRACT

Impairment of synaptic function can lead to neuropsychiatric disorders collectively referred to as synaptopathies. The SNARE protein SNAP-25 is implicated in several brain pathologies and, indeed, brain areas of psychiatric patients often display reduced SNAP-25 expression. It has been recently found that acute downregulation of SNAP-25 in brain slices impairs long-term potentiation, however the processes through which this occurs are still poorly defined. We show that in vivo acute downregulation of SNAP-25 in CA1 hippocampal region affects spine number. Consistently, hippocampal neurons from SNAP-25 heterozygous mice show reduced densities of dendritic spines and defective PSD-95 dynamics. Finally, we show that, in brain, SNAP-25 is part of a molecular complex including PSD-95 and p140Cap, with p140Cap being capable to bind to both SNAP-25 and PSD-95. These data demonstrate an unexpected role of SNAP-25 in controlling PSD-95 clustering and open the possibility that genetic reductions of the protein levels -as occurring in schizophrenia- may contribute to the pathology through an effect on postsynaptic function and plasticity.

Keywords: SNAP-25/PSD-95/p140Cap/dendritic spines/hippocampus

INTRODUCTION

Synapses are complex cellular junctions specialized for communication between neurons. Epidemiological and genetic studies demonstrated that deficiencies in synapse function (1) are implicated in a wide range of brain disorders, including neurodegenerative (2) and psychiatric diseases such as schizophrenia (3, 4) and autism (5, 6). A hallmark of synaptic specializations is their

dependence on highly organized complexes of proteins that interact with each other. Therefore the loss or modification of key synaptic proteins might directly affect the properties of such networks and, ultimately, synaptic function.

SNAP-25 is a component of the SNARE complex, which is central to synaptic vesicle exocytosis (7, 8) and which plays a role in the regulation of voltage-gated calcium channels (9, 10). The *SNAP25* gene has been associated with Attention Deficit Hyperactivity Disorder (ADHD) (11, 12) and with schizophrenia (13, 14). Consistently, SNAP-25 levels are lower in the hippocampus (15) and in the frontal lobe (16) of patients with schizophrenia. DNA variants of the *SNAP25* gene that associate with ADHD are also associated with reduced expression level of the transcript in prefrontal cortex (17). Finally, reduction of SNAP-25 levels has been found to cause neurodegeneration in mice lacking the synaptic vesicle protein Cysteine-string protein-α, possibly due to the impaired SNARE-complex assembly produced by the decreased SNAP-25 (18). The mechanisms by which reduced SNAP-25 expression may result in a psychiatric disease are still undefined, although alterations in neurotransmitter release have been indicated as potential causative processes (19, 20).

Recently an unexpected postsynaptic role of SNAP-25 has been described, with the protein controlling NMDA and kainate receptor trafficking (21). Furthermore, it has been lately found that acute SNAP-25 downregulation results in LTP impairment (21, 22) and that acute reduction of the protein affects spine morphogenesis in vitro, through a process involving the protein p140Cap (23). These data, together with the evidence of a role for SNAP-25 in human intellectual disability (24), open the possibility that besides a presynaptic impact, chronic reductions of SNAP-25 levels, as described in the hippocampus of schizophrenic patients, may impair the structure and/or function of the postsynaptic compartment. If confirmed, this possibility, which has never been addressed before, would provide a logical frame for the protein involvement in different psychiatric diseases, characterized by defective formation and function of the postsynaptic compartment.

In this study, we demonstrate that a genetic, partial reduction of the protein levels, as occurring in psychiatric diseases, results in a flawed maturation of postsynaptic specializations, reduced densities of dendritic spines, impaired synaptic potentiation, and PSD-95 destabilization.

RESULTS

ACUTE REDUCTION OF SNAP-25 EXPRESSION IN MICE HIPPOCAMPUS ALTERS SPINE MORPHOLOGY

We used a lentiviral approach to induce *in vivo* silencing of *SNAP-25* by expressing short hairpin RNA specific for SNAP-25 (SNAP-25-shRNA) or scrambled shRNA together with eGFP (Fig. S1A). We validated SNAP-25 knock-down in mouse primary neuronal cultures by real time-PCR analysis (Fig. S1B). To functionally confirm *in vivo* the SNAP-25 shRNA efficacy, we took advantage of the previous demonstration **that SNAP-25 heterozygous mice show defective conditioned test aversion (CTA) memory** (25) and that SNAP-25 immunoreactivity increases in the gustatory cortices in response to food and chemical stimulation (26). We then used CTA as a paradigm test to provide the functional demonstration that acute down-regulation of SNAP-25 is effective and induces the same behavioral defects already described in transgenic mice (25). We therefore injected the indicated vectors into the gustatory cortex, which resides within the insular cortex, and CTA memory was tested 7 days after. The scrambled-injected rats displayed normal CTA (27) while SNAP-25 shRNA-injected rats were impaired (Fig. S1C), thus indicating that acute reduction of the protein recapitulates the functional defects already shown in SNAP-25 heterozygous mice.

It has been recently shown that postsynaptic SNAP-25 regulates surface NMDAR levels and plays a role in LTP induction in the CA1 hippocampal neurons (22). Given that activity-driven changes of synaptic efficacy underlying hippocampal LTP require proper dendritic spine morphogenesis, we quantified by confocal microscopy spine density and morphology of CA1 neurons positive for the fluorescent reporter eGFP upon injection of the SNAP-25 shRNA or the scramble vectors. Mice were

perfused 15 days after lentiviral vector injection and the brains were sectioned and immunostained for GFP (Fig. 1A low magnification images). Spine density and morphology was analyzed on secondary and tertiary branches of apical dendrites in the stratum radiatum (SR; Fig. 1A high magnification images). SNAP-25 knockdown causes a significant decrease in spine density on CA1 dendrites with respect to controls (Fig. 1B). Such a decrease was accompanied by a reduction in the number of mushroom spines and an increase in the number of thin spines (Fig. 1C). Adult Het mice show similar but minor defects in spine density and morphology (Fig. S1D-E), probably due to the higher protein level (25) in the adult Het animals with respect to the SNAP-25 shRNA injected hippocampi.

DEFECTIVE POSTSYNAPTIC MATURATION OF GLUTAMATERGIC SYNAPSES IN SNAP-25 HETEROZYGOUS CULTURES

To investigate if reduced SNAP-25 expression impairs the proper postsynaptic maturation of glutamatergic synapses, we used primary hippocampal cultures prepared from Het embryos. In these neurons, a 50% reduction of SNAP-25 levels was detected by quantitative immunocytochemistry in the somato-dendritic region and around 30% at axonal varicosities (Fig. S2A-B). Immunocytochemical staining for the synaptic vesicle protein SV2A, for the active zone component Bassoon and for the postsynaptic scaffold protein PSD-95 were performed at 14 and 21 days in vitro (DIV) and percentages of co-localization among these proteins were quantified. Since the levels of SV2A do not differ between wt and Het cultures (20), we used this protein as a reference marker. The percentage of juxtaposed pre- and postsynaptic terminals relative to the total presynaptic sites (SV2A&PSD-95/SV2A), the percentage of synapses showing immunoreactivity for the all three markers (SV2A&PSD-95&Bsn/SV2A) and the percentage of mature presynaptic terminals (SV2A&Bsn/SV2A) were quantified (Fig. 2A-C). The increase in the first two parameters occurring in wt cultures between 14 and 21 DIV (Fig. 2B) is consistent with the physiological maturation of synaptic contacts, while the lack of increase in the percentage of SV2A&Bsn/SV2A colocalizing

puncta is in line with the earlier maturation of presynaptic relative to postsynaptic terminals, which is completed already at 14DIV (28). A similar analysis, carried out in Het cultures, revealed a lack of postsynaptic maturation between 14 and 21DIV (Fig. 2C).

In order to exclude the possibility that the alteration of PSD-95 density might result from a progressive decline of neuronal viability in culture, cell mortality was assessed by propidium iodide (PI) and calcein staining. While calcein emits green fluorescence signal in viable cells, PI reaches nuclei of dead cells only. Quantification of PI-positive/calcein-negative cells relative to the overall nuclei (labeled with Hoechst) indicated no difference in the extent of cell death between wt and Het cultures, at both 14DIV and 21DIV (Fig. S2C-D).

Consistently the fine morphological analysis of dendritic spines, performed in wt and Het GFP-transfected neurons at 21DIV, revealed a significant reduction in the number of mushroom spines and a parallel increase in the density of thin, filopodia-like protrusions (Fig. 2D and 2E). A significant reduction in the number of PSD-95 puncta per unit length (Fig. 2F and G) and a smaller PSD-95 cluster size (Fig. 2F and H) were also detected in Het cultures relative to wt. Finally, miniature excitatory postsynaptic current (mEPSC) analysis in SNAP-25 Het networks revealed a significant reduction in both the frequency and the amplitude relative to controls (Fig. 2I). Notably, no difference in mEPSC frequency and amplitude occurs in primary hippocampal cultures at earlier developmental stages (18, 20), thus confirming that morphological and functional postsynaptic defects become evident only at later stages during neuronal development.

A POSTSYNAPTIC DEFECT IS RESPONSIBLE FOR THE LACK OF PLASTICITY OCCURRING IN NETWORKS CONSTITUTIVELY DEVELOPING IN THE PRESENCE OF REDUCED SNAP-25 LEVELS

Since SNAP-25 plays crucial roles in the presynapse, the possibility exists that the postsynaptic defects might have resulted from a functional alteration at the presynaptic level, in turn leading to altered postsynaptic development. To address this possibility, we prepared mixed cultures of wt

hippocampal neurons, from genetically modified mice constitutively expressing GFP under the beta-actin promoter (29), and neurons heterozygous for SNAP-25, originating from the same strain of mice, but not expressing GFP. Cultures were then maintained for three weeks before functional and morphological analysis. We generated two complementary experimental conditions. By plating wt-GFP and SNAP-25 Het neurons in a 1:10 ratio (wt-GFP/Het), the formation of networks in which several Het neurons impinged on a postsynaptic wt-GFP cell (Fig. 3A) was favored. Conversely, plating SNAP-25 Het and wt-GFP neurons at an opposite ratio (Het/wt-GFP), favored the formation of networks in which Het neurons received most synaptic inputs from wt-GFP cells (Fig. 3A'). Given the large excess of presynaptic inputs from Het neurons in the former setting, it would be reasonable to conclude that postsynaptic alterations in the wt-GFP neurons would be the result of defective presynaptic function of SNAP-25 Het neurons. In contrast, in the latter setting, given the large excess of presynaptic inputs from wt neurons, it would be reasonable to conclude that a postsynaptic defect in SNAP-25 Het neurons results from a cell-autonomous, postsynaptic failure.

Interestingly, no difference in PSD-95 density was detected in wt-GFP neurons surrounded by Het neurons (Fig. 3B), whereas RFP transfected SNAP-25 Het neurons receiving synaptic inputs from wt-GFP neurons (Het-RFP/wt-GFP) showed a significant reduction of PSD-95 puncta density relative to RFP-transfected wt neurons receiving from wt-GFP cells (wt-RFP/wt-GFP) (Fig. 3B'). Consistently, electrophysiological analysis showed that mEPSC frequency (Fig. 3C) and amplitude (Fig. 3D) of wt-GFP neurons grown together with a majority of Het neurons (wt-GFP/Het) did not differ with respect to control cultures of wt neurons. However, mEPSC frequency and amplitude of Het neurons cultured together with a majority of wt-GFP neurons (Het/wt-GFP) were significantly reduced with respect to control cultures. These data fully recapitulate the postsynaptic functional defects of SNAP-25 heterozygous cultures and rule out the possibility that functional defects in spines are secondary to presynaptic alterations.

It has been recently reported that the process of synaptic plasticity, i.e. the ability of neurons to modify synaptic function depending on external stimuli, is defective in neurons where the expression of

SNAP-25 is acutely down-regulated, both in vitro and in vivo (22). In particular, Jurado and colleagues have found that acute down regulation of SNAP-25 by lentiviral injection of acute hippocampal slices strongly impaired the generation of LTP in the CA1 region (22). We then asked whether neuronal networks constitutively developing in the presence of reduced SNAP-25, as occurring in psychiatric diseases, are able to compensate for this defect or display defective plasticity as well and, in the latter case, whether this could be univocally ascribed to a postsynaptic failure. Neuronal cultures were subjected to a chemical LTP protocol (30) consisting of an application of high doses of glycine for 3min, which was followed by washout and immunolabeling for PSD-95, v-Glut1 and βIII-tubulin after a 1 hour recovery period. It has been shown that this procedure results in significant increase in the size of PSD-95 positive puncta and in a higher extent of colocalization of PSD-95 and v-Glut1 staining, in line with a potentiation of synaptic connections (31). Notably, in neurons heterozygous for SNAP-25, this protocol did not induce increases in either the size of PSD-95 positive puncta or the colocalization of pre and postsynaptic markers, whereas unmistakable increases were observed in wt neurons (Fig. 3E and F).

The lack of potentiation in SNAP-25 Het cultures was also confirmed by electrophysiological recordings of mEPSCs. Indeed, while both the frequency (Fig. 3G and H) and amplitude (Fig. 3G and I) of mEPSCs significantly increased 35 minutes after glycine application in SNAP-25 wt neurons, no potentiation occurred in SNAP-25 Het neurons (Fig. 3G-I).

To univocally ascribe the lack of potentiation to postsynaptic defects, we tested the ability of wt-GFP neurons cultured together with Het neurons (wt-GFP/Het) or Het neurons cultured together with wt-GFP neurons (Het/wt-GFP) (Fig. 3L and M) to undergo synaptic potentiation. These experiments indicated that wt-GFP neurons receiving synaptic inputs from SNAP-25 Het neurons, when subjected to a chemical LTP protocol, displayed potentiation levels that were similar to their control counterparts. By contrast, SNAP-25 Het neurons receiving synapses from wt neurons did not exhibit such potentiation (Fig. 3L and M). These results indicate that genetic, moderate reductions of SNAP-

25 levels impair synaptic plasticity and that a cell autonomous, postsynaptic defect is responsible for this defect.

PSD-95-DEPENDENT SPINE FORMATION REQUIRES POSTSYNAPTIC SNAP-25

PSD-95 is a major organizer of the postsynaptic density, playing a crucial role in determining spine size and morphology (32"reviewed in", 33). Our data so far suggest that SNAP-25 reductions affect the localization and/or stabilization of PSD-95 in dendritic protrusions, influencing synapse organization and strength during development and plasticity. Consistent with this possibility, acute downregulation of SNAP-25 expression by siRNA (10), a treatment which reduces the protein expression by about 60% (34) (Fig. S2E) resulted in a significant reduction of PSD-95 area (Fig. 4A and B). Notably, also the NR1 density and size was reduced upon acute reduction of SNAP-25, further confirming a postsynaptic defect (Fig. S4). The density ("not shown," (23)) and the size (Fig. 4A and B) of PSD-95 puncta were fully rescued by the overexpression, in the same neurons, of a mouse SNAP-25-GFP which is insensitive to the rat siRNA.

Overexpression of PSD-95, a procedure which results in the marked increase in spine volume and the enlargement of the postsynaptic densities (35"and Fig 4C compare the first and the third bars"), induced the formation of significantly smaller PSD-95 positive puncta in SNAP-25 siRNA-treated neurons relative to scramble-transfected neurons (Fig. 4C and D). Therefore postsynaptic SNAP-25 is required for proper PSD-95 accumulation and spine morphogenesis.

To investigate whether SNAP-25 controls the latter processes via its SNARE function, neurons were transfected with a cDNA codifying for the BonT/E light chain, which cleaves SNAP-25 at its N-terminal side and prevents the protein entering the fusion complex (36). Supplementary figure 3 shows that cleavage of SNAP-25, assessed by the specific staining for the BonT/E-cleaved SNAP-25 fragment, does not significantly reduce spine density or PSD-95 size ("Fig. Suppl. 2B-C, see also"23). Since PSD-95 continuously migrates between synaptic and extra synaptic pools (37, 38), we then reasoned that SNAP-25 could operate at the spine by restricting, either directly or indirectly, PSD-95

mobility. We therefore measured the dynamics of synaptic PSD-95 upon acute reduction of SNAP-25 expression by siRNA, using fluorescence recovery after photobleaching (FRAP) analysis. To carry out PSD-95 FRAP analysis, we selected spines with similar morphologies in siRNA- or scramble-treated cultures, four to five days after transfection. PSD-95-GFP bleaching, followed by temporal analysis of fluorescence recovery over the next 40 seconds (Fig. 4D), revealed a significantly larger mobile fraction of PSD-95 in acutely SNAP25-downregulated cells compared to scramble control cells (Fig. 4E). These data indicate that SNAP-25 affects PSD-95 localization and mobility through a mechanism, which does not involve its fusion activity, but rather implicates a scaffolding, protein-protein interaction role.

SNAP-25 IS PART OF THE POSTSYNAPTIC PROTEIN COMPLEX CONTAINING PSD-95

To assess whether SNAP-25 controls PSD-95 mobility through direct participation to the PSD complex, we performed Proximity Ligation Assay (PLA) in cultured neurons to evaluate an in-situ complex formation. The results revealed a significantly higher signal for SNAP-25 and PSD-95 compared to PSD-95 and the merely presynaptic protein Piccolo (Fig. 5A-B) suggesting that PSD-95 and SNAP-25 can be part of the same complex in their natural cellular environment. To further probe the possible existence of a postsynaptic protein network including SNAP-25, we applied a LUminescence-based Mammalian intERactome (LUMIER) assay (39, 40) to test the following protein:protein interactions: SNAP-25 and PSD-95; PSD-95 and p140Cap (SRCIN1). The rationale for including p140Cap in the assay results from our previous demonstration that acute down-regulation of SNAP-25 impairs spines morphogenesis through p140Cap (23), a synaptic protein involved in Src-kinase regulation and microtubules organization inside the spine (41). The well characterized protein:protein interaction Bcl2-BAD (42) served as a positive control. The bait protein fused to a protein-A tag and linked to a Renilla Luciferase and the prey protein fused to a Firefly Luciferase were co-expressed in HEK293 cells for 48h. After cell lysis, protein complex formation

was assessed by Firefly Luciferase activity of the co-immunoprecipitated prey fusion-protein (Fig. 5C). Results indicated that SNAP-25 and PSD-95 do not interact directly, whereas p140Cap specifically interacts with PSD-95 (Fig. 5C). Given that p140Cap ineterct with SNAP-25 (41), an indirect association of SNAP-25 and PSD-95 potentially mediated by p140Cap may occur at the postsynaptic level. To test this hypothesis we performed immune-precipitation of PSD-95 from wt and p140Cap null brain lysates. Fig. 5D shows that in wt brain the spine protein p140Cap (23, 41) and SNAP-25 co-immunoprecipitated with PSD-95, whereas in p140Cap null brain a significantly less amount of SNAP-25 co-immunoprecipitated with PSD-95, suggesting the existence, in the spine, of a novel postsynaptic network of proteins formed by SNAP-25, p140Cap and PSD-95 .

DISCUSSION

Variations in dendritic spine number and morphology occur both during development and in adulthood, allowing the establishment and remodeling of neuronal circuits. Spine structural plasticity is tightly coordinated with synaptic function, with spine enlargement occurring during long-term potentiation and spine shrinkage during long-term depression (43). In the present study, we demonstrate an unexpected defect in dendritic spine morphology in SNAP-25 Het neurons after 3 weeks in culture. At this time, SNAP-25 Het neurons display a lower density of spines, which appear longer than controls. Consistently, SNAP-25 Het cultures display a reduction in both frequency and amplitude of mEPSCs and are unable to undergo a common form of experimentally induced potentiation. Moreover, rats injected with viral vector harboring shRNA to SNAP-25 into the gustatory cortex display an impaired taste memory similarly to rats injected with viral vector expressing shRNA to PSD-95 (44).

During development, synapse formation is directed by reciprocal signaling between pre- and postsynaptic neurons. Synaptic organizing proteins stabilize early synaptic contacts via recruitment of synaptic vesicles to the presynaptic active zone and NMDA receptors to the postsynaptic density.

PSD-95 thereby acts as a major organizer of signaling complexes at the postsynaptic membranes and serves as scaffold for the recruitment of synaptic components required for synapse development. PSD-95 appears very early at nascent synapses (37) where it clusters NMDA receptors (45, 46) and controls synaptic AMPA receptors number, thus determining synaptic strength (47, 48). Our data indicate that PSD-95 has the ability to bind p140Cap, thus forming a complex with SNAP-25, and that acute down-regulation of SNAP-25 leads to PSD95 destabilization, probably affecting the stability of the post-synapse and impairing synaptic plasticity.

Although different models concerning the dynamic aspects of synaptogenesis have been proposed, presynaptic differentiation appears to occur slightly faster than postsynaptic development (49, 50). Cycling of synaptic vesicle protein transport vesicles at predefined sites along the axon appears to selectively attract dendritic filopodia and initiate synapse formation (51). Furthermore, although vesicular glutamate release is not required for excitatory synapse formation (52, 53), the number of synapses formed in the absence of neurotransmitter release is dramatically decreased (54), suggesting that glutamate release could influence excitatory synaptogenesis (55). Given that SNAP-25 plays a critical role at the presynaptic terminal during synaptic vesicle fusion and considering the possible role of presynaptic function in the maturation of the postsynaptic compartment, the postsynaptic effects observed here might have been the result of SNAP-25 related alterations in presynaptic release (7), even if electron microscopy studies showed that reduced levels of SNAP-25 do not alter the presynaptic terminal structure and vesicles content (56). By taking advantage of mixed cultures of wt and Het neurons, we demonstrated that postsynaptic defects in SNAP-25 Het neurons do not stem from alterations in the presynaptic terminal, but are instead related to a cell-autonomous impairment at the postsynaptic compartment.

In recent years SNARE/SM proteins have been shown to be involved in activity-dependent AMPA receptor exocytosis during LTP (22) and SNAP-25, in particular, has been reported to play a role in the removal of kainate receptors from the postsynaptic membrane (57), in the insertion of NMDA

receptors in neuronal plasma membrane (21) and of P/Q and L type-voltage gated calcium channels (10, 58). Also, acute regulation of SNAP-25 expression has been found to impair synaptic plasticity probably affecting NMDAR trafficking (22). Our data indicate that SNAP-25 plays also a structural role in the postsynaptic compartment by interacting with the postsynaptic protein p140Cap, which in turn binds to PSD-95. The integrity of this macromolecular complex appears to be fundamental for both structural and functional synaptic plasticity. Indeed, p140Cap$^{-/-}$ mice display similar defects in episodic memory and are impaired in LTP and in LTD (59). These data provide new mechanistic insights as to SNAP-25 involvement in synaptopathies that go beyond the protein's known roles in presynaptic function, indicating a protein role as a postsynaptic structural hub. Indeed, the activity-driven spine remodeling is defective in neuronal networks constitutively developing in the presence of reduced levels of SNAP-25, as it might presumably occur in human pathologies, such as schizophrenia, where both a reduction of SNAP-25 expression (15) and reduction in dendritic spine density (60) have been described.

MATERIALS AND METHODS

Animals

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. SNAP-25 wild type and SNAP-25 heterozygous male mice (61) were housed in cages with free access to food and water at 22°C and with a 12-h alternating light/dark cycle. Genotyping was performed by PCR as described in (61). p140Cap wild-type and knock out mice strain is described in (59).

Cell cultures

Mouse hippocampal or rat hippocampal and cortical neurons were prepared from E18 fetal SNAP-25 heterozygous (Het) or wild type (wt) littermates C57BL/6 mice as described by Banker & Cowan (1977) and (62) with slight modifications. Briefly, hippocampi were dissociated by treatment with trypsin (0.125% for 15 min at 37°C), followed by trituration with a polished Pasteur pipette. The dissociated cells were plated onto glass coverslips coated with poly-L-lysine at density of 400 cells/mm^2. The cells were maintained in Neurobasal (Invitrogen, San Diego, CA) with B27 supplement and antibiotics, 2mM glutamine, and 12.5μM glutamate (neuronal medium).

Mixed cell cultures

Primary hippocampal GFP-positive neuronal cultures were prepared from the hippocampi of E18 fetal C57BL/6 GFP transgenic mice (29), with the GFP gene controlled by the actin promoter. To isolate the neuronal type of interest among wild type or SNAP-25 heterozygous neurons GFP-positive neurons were co-cultured with SNAP-25 Het or wt neurons in a ratio of 1 to 10 or 10 to 1.

DNA constructs and expression.

Neuronal cultures were transfected at 17DIV with pEGFP-C1 (Clontech, Palo Alto, CA) or pSUPER-DsRed plasmid (obtained from pSUPER-GFP, Oligoengine, Seattle, USA) and FU(PSD95:EGFP)W (63). Silencing of SNAP-25 was achieved via transfection of a pSUPER construct (10, 58). A nonspecific siRNA duplex of the same nucleotides but in an irregular sequence (scrambled SNAP-25 siRNA) was prepared using oligonucleotides 5-GATCCCCGAGGAGTTATGCGATAGTATTCAAGAGAATGATAGCGTATTGAGGAGTTTTTGGAAA-3 and 5-AGCTTTTCCAAAAACTCCTCAATACGCTATCATTCTCTTGAATACTATCGCATAACTCCTCGGG- 3 that were annealed and ligated into the pSuper vector as described previously (10, 58).

For LUMIER assay, mouse Srcin1 was PCR amplified using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) and the following primers: 5-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGAACGCTCCGTCCCAAG-3 and 5-GGGACCACTTTGTACAAGAAAGCTGGGTCGGAAGGAGATGGAAGAATTCCTTGC-3. The PCR product was inserted into pDONR221 using the BP clonase, (Invitrogen), amplified and further shuttled into the LUMIER prey vector (FireV5DM)**.** Human full-length cDNA clones of SNAP25, PSD95 and CTTN where available as entry clones and shuttled into the respective LUMIER bait (PAReniDM) and prey (FireV5DM) vectors.

Lentiviral constructs

A short hairpin RNA construct directed against SNAP-25 was generated by PCR and subcloned into a lentiviral vector plasmid (pLKO.1-puro-CMV-tGFP^{TM}, Sigma-Aldrich, Israel). A scrambled shRNA sequence was generated in a similar way and served as control. The SNAP-25 or scrambled U6-shRNA expression cassettes were co-expressed with eGFP driven by CMV promoter. High titer lentiviral vectors were produced by a transient transfection of the third generation transfer, packaging and envelope plasmid set into 293FT cell line (Invitrogen, Carlsbad, CA, USA), and allowed to express and form viral particles for 48 h. Then the medium was collected; the viral particles were purified and concentrated by multiple centrifugation steps, dissolved in sterile PBS, aliquot and stored at -80 °C until further use. Viral titer was determined using the FACS analysis. A titer higher than 10^8 TU/ML was used for the experiments.

Lentiviral injections

C57BL6N mice (Janvier labs) were deeply anaesthetized (100 mg/kg Ketamine, (KETAVET, INTERVET), 10 mg/kg Xilazina (Rompun, BAYER) in physiologic solution) and placed on a stereotaxic frame (Kopf). The viral suspension was delivered via glass needle attached to a 10µl Hamilton syringe. Bilateral injection (2µl/each) was performed in the CA1 area of dorsal hippocampus (coordinates of injection: anteroposterior -2.0, lateral ±1.5, dorsoventral-1.1 from duramadre surface) at a rate of 1µl/min. Coordinates of injection were calculated from the Bregma

level according to Paxinos and Franklin Mouse Brain Atlas (64) under microscope guide (L-0940SD, INAMI). The needle remained in place for an additional 5 minutes to facilitate the controlled delivery of the virus. The titer of the SNAP-25-shRNA virus is 2.3*10^9tu/ml and for the scramble 5*10^8 tu/ml. Stereotaxic injections into the gustatory cortex were performed as described (44).

Immunocytochemical staining

Neuronal cultures were fixed with 4% paraformaldehyde + 4% sucrose, or with 100% cold methanol, depending on the markers. The following antibodies were used: rabbit anti-SV2A (1:1000; Synaptic Systems, Goettingen, Germany), guinea pig anti-Bassoon (1:300; Synaptic Systems, Goettingen, Germany), guinea pig anti-vGLUT1 (1:1000; Synaptic Systems, Goettingen, Germany), mouse anti-PSD95 (1:400; UC Davis/NIH NeuroMab Facility, CA), rabbit anti-GFP (1:400; Invitrogen, San Diego, CA), mouse anti-beta III tubulin (1:400; Promega corporation, Madison, USA), rabbit anti-tubulin (1:80; Sigma-Aldrich, Milan, Italy), rabbit anti-MAP2 (Millipore, Billerica, MA, USA), mouse anti-SNAP-25 (1:1000; SMI81 Sternberger Monoclonals, Baltimore MD). Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633fluorophores (Invitrogen, San Diego, CA). Images were acquired using a Leica SPE confocal microscope equipped with an ACS APO 63X/1.30 Oil objective. Image analysis was performed using ImageJ software (NIH, Bethesda, Maryland, USA). Dendritic spines were classified according the following parameters: mushroom (length ≤1.2µm, width ≥0.5µm); filopodia (length >1.2µm, width <0.5µm), in line with (65). Colocalization of two or three selected markers was measured using the boolean function "AND" for the selected channels. The resulting image was binarized and used as a colocalization mask to be subtracted to single channels. The number of the puncta resulting from colocalization mask subtraction were measured for each marker. A colocalization ratio was set as colocalizing puncta / total puncta number.

Immunohistochemical staining

Experiments were performed on C57BL/6 mice. Animals were anaesthetized with chloral hydrate (4%; 1 ml/100 g body weight, i.p.) and perfused with 4% paraformaldehyde. The brain was postfixed and coronally cut with a Vibratome in 50µm thick serial sections. Immunofluorescence staining was carried out on free-floating sections as described in (66). Free-floating sections were processed for rabbit anti-GFP (1:400, Invitrogen, San Diego, CA) followed by incubation with secondary antibody Alexa-488 fluorophore (Invitrogen, San Diego, CA) and mounted in Fluorsave (Calbiochem, San Diego, CA, USA). Sections were examined by means of a Zeiss LSM 510 META confocal microscope (Leica Microsystems, Germany). The images were acquired using a 40X oil immersion lens (numerical aperture 1.0) with additional electronic zoom factor up to 4. Up to 10 different neurons were acquired and analysed for each animal.

Co-immunoprecipitation

Brain tissues were frozen in liquid nitrogen. Proteins were extracted from mice brain with lysis buffer (1% Triton X-100, 150mM sodium chloride, 50mM Tris–HCl pH 7.5, protease inhibitors (Roche, Basel, Switzerland), 1 mM phenylmethylsulphonyl fluoride, 1mM Sodium Vanadate, 1mM Sodium Fluoride, 1mM DTT). For Immunoprecipitation (IP) assays 5mg of total extract were immunoprecipitated for 2 hours at 4°C with specific or unrelated antibodies in presence of Dynabeads® Protein G (Invitrogen).The proteins were resolved by reducing SDS–polyacrylamide gel electrophoresis and transferred to nitro- cellulose filters, which were incubated with the indicated antibodies and developed with ECL system.The following antibodies were used: PSD-95 (1:4000, monoclonal; UC Davis/NIH NeuroMab Facility, CA); SNAP-25 (1:10000, Synaptic System, Gottingen, Germany); mouse monoclonal to p140Cap for western blot was homemade produced as described in (67).

LUMIER assay

The Lumier assay was performed as described previously (40). Briefly, PSD-95, Srcin1and SNAP-25, were cloned into the bait PA-Renilla luciferase vector and/or into the prey firefly luciferase vector using the gateway technology (Invitrogen). Vectors were co-transfected into HEK293 cells using jetPEI transfection reagent (Polyplus) and 48h after transfection lysed in HEPES-lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerin, 1% NP-40, 20 mM NaF, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1x Benzonase, 1x Protease Inhibitor Cocktail –EDTA (Roche), 1 mM PMSF). 384-well high-binding white microplates (Greiner) were coated with sheep-gamma globulin (Dianova) and rabbit anti-sheep IgG (Dianova). Bait and prey protein expression was confirmed by measuring Renilla and Firefly luciferease activity of the crude lysates. Baits were immunoprecipitated from cell extracts via the PA-tag and immunoprecipitation and co-immunoprecipitation evaluated by renilla and firefly activity, respectively. Luciferase activies were determined using the Dual-Glo Luciferase Kit (Promega) in a luminescence plate reader (TECAN Infinite M1000). To determine background protein binding, bait protein binding to empty prey vectors, as well as prey protein binding to empty bait vector were determined as well.

Proximity Ligation Assay

Proximity Ligation Assay was performed according to the manufactures protocol using custom blocking solutions during antibody incubations (Olink, Bioscience). Briefly, rat cortical neurons were grown at low density (34000 cells/cm^2) on coverslips for 2DIV before AraC (5µM, Sigma) was added to inhibit glial growth and enable signal quantification per neuronal cell. Cells were grown further till 14-DIV and then fixed with 4% PFA, washed with PBS and permeabilized with 0.2% TX-100 in PBS for 2 minutes. Afterwards, cells were washed with PBS-T (0.05% Tween-20) and blocked with 1% BSA PBS-T for 30 min. Primary antibodies were diluted in 1% BSA PBS-T and incubated for 60 min at 37°C before the cells were washed three times with PBS-T. PLA probes PLUS (anti-rabbit) and MINUS (anti-mouse) were diluted 1:5 in 1% BSA PBS-T and incubated for another 60 min at 37°C

before the Ligation and Amplification Reactions were performed as described in the PLA assay protocol. PLA signals were detected using the 20x or 40x objective of a Zeiss Imager Z1 fluorescence microscope and quantified using the "find maxima" function of ImageJ. Cell numbers were determined from intact DAPI signal and fragmented nuclear signals resulting from dying glia cells excluded. To exclude an effect of AraC on protein interactions, PLA was performed initially on neuronal culture grown without the addition of AraC (not shown). The following antibodies were used for PLA: rabbit anti-PCLO (1:500, Synaptic Systems, Goettingen, Germany), mouse anti-PSD95 (1:500, Synaptic Systems, Goettingen, Germany) and rabbit anti-SNAP25 (1:100, Synaptic Systems, Goettingen, Germany).

Cell culture electrophysiology

Whole-cell patch-clamp recordings of EPSCs were obtained from 20/21 DIV neurons using a Multiclamp700A amplifier (Molecular Devices) and pClamp-10 software (Axon Instruments, Foster City, CA). Recordings were performed in the voltage-clamp mode. Currents were sampled at 5 kHz and filtered at 2-5 kHz. External solution [Krebs' Ringer's-HEPES (KRH)] had the following composition (in mM): 125 NaCl, 5 KCl,1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 6 glucose, and 25 HEPES-NaOH, pH 7.4. Only cells obtained from wt and Het embryos that had resting membrane potentials < -50 mV were considered for experiments. Resting membrane potentials were measured immediately upon breaking into whole-cell mode by setting the current to 0 pA. No differences were found between neuronal genotypes. Cells were then voltage-clamped at a holding potential of -70 mV, unless otherwise noted. Recordings of mEPSCs were obtained in presence of Tetrodotoxin (TTX, l µM, Tocris, Bristol, UK) to block spontaneous action potentials propagation. Recording pipettes, tip resistances of 3-5MΩ were filled with the intracellular solution of the following composition (in mM): 130 potassium gluconate, 10 KCl, 1 EGTA, 10 Hepes, 2 $MgCl_2$, 4 MgATP,

0.3 Tris-GTP. Off-line analysis of miniature events was performed by the use of Clampfit- pClamp-10 software and events had to exceed a threshold of 10 pA to be taken into account.

For chemical LTP experiments, recordings of mEPSCs were performed using the same intracellular solution of miniature events while glycine (100 µM, Sigma-Aldrich, Milan, Italy) was applied for 3 minutes at room temperature in Mg^{2+}-free KRH also containing TTX (0.5 µM), bicuculline (20 µM, Tocris, Bristol, UK) and strychnine (1 µM, Sigma-Aldrich, Milan, Italy).

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed by maintaining coverslips in a 37°C heated chamber with 5% CO_2 in their own growth medium. Live imaging was performed with a confocal microscopy Leica SP5 using a HCX PL APO 63X/ 1.4 OIL objective. Photobleaching was obtained using a 488nm laser light at 100%. Images were collected every 500ms. The region of interest (ROI) placed over the spine was used for both photobleaching and fluorescence recovery analysis. Analysis was performed on the first 40sec of acquisition. Each image at each time point was corrected for the background and for the ongoing bleaching and normalized according to this formula: $((F_t-F_b)/(F_r-F_b))/(F_a-F_b)$, where F_t is the fluorescence of a ROI at time t; F_b is the fluorescence of the background; F_r is the fluorescence of the reference ROI at time t and F_a is the fluorescence of the ROI immediately before photobleaching. Data were fitted with a single exponential using the Leica SP5 software and the relative mobile fraction values have been obtained.

Statistical analysis

Statistical analysis was performed using SigmaStat 3.5 (Jandel Scientific), with statistical tests used based on initial testing distribution normality. Data are presented as mean±SEM. Differences were

considered to be significant if $p<0.05$ and are indicated by an asterisk; those at $p<0.01$ are indicated by double asterisks; those at $p<0.001$ are indicated by triple asterisks.

Authors contribution: GF designed and performed experiments and analysed data; RM, IC, FA, DP, PT, EE, VS, ET and AP performed experiments and analysed data; JM, PDF and provided reagents; NEZ, KR, RB and EW discussed the data and contribute writing the paper; MM and EM designed the research and wrote the paper.

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FIGURE LEGENDS

Fig.1 Altered spine morphology and density upon in vivo downregulation of SNAP-25.

A) Representative images of coronal slices showing an hippocampus injected with the lentiviral vector in the CA1 area (at different degrees of magnifications) and the secondary branches of apical dendrites of scramble and SNAP-25-shRNA treated mice (high magnification). Scale bars 400µm, 200µm, 20µm, 10µm respectively. **The white dotted line defines hippocampal structure**. B) Quantification of spine density in the CA1 field of scramble and SNAP-25-shRNA treated mice shows significant reduction of spine densities upon SNAP-25 downregulation (*left*, number of spines per µm (considering cells): scramble=0.89±0.03; SNAP-25-shRNA =0.60±0.02; Student t-test, p= <0.001; *right*, number of spines per µm (considering animals) scramble: 0.88±0.04, n=7; shSNAP-25:0.59±0.04, n=6; Student t-test, p<0.001). C) Quantitative analysis of mushroom-type and thin filopodia-like spines percentage. Of note, SNAP-25-shRNA neurons show a decrease in mature spine number (mushroom-type) which is accompanied by an increase in thin spine number (filopodia-like) (percentage of spines: mushroom: scramble=71.9±1.4; SNAP-25-shRNA=53±1.7; Student t-test, p=<0.001. Filopodia: scramble=28.1±1.4; SNAP-25-shRNA=47±1.7; Student t-test, p= <0.001).

Number of examined cells: scramble=38, SNAP-25-shRNA= 36. 7 scramble and 6 SNAP-25-shRNA treated animals were analyzed and all data are expressed as mean ± SEM.

Fig.2 Structural and functional alterations in SNAP-25 heterozygous cultures. A) Representative images of 14DIV and 21DIV wt cultures stained for the presynaptic markers SV2A (red), Bassoon (blue) and the postsynaptic protein PSD-95 (green). Scale bar: 5µm. B-C) Quantification of the percentage of colocalizing puncta of juxtaposed pre- and postsynaptic terminals relative to the total presynaptic sites (SV2A&PSD-95/SV2A), the percentage of synapses showing immunoreactivity for the all three markers (SV2A&PSD-95&Bsn/SV2A) and the percentage of mature presynaptic terminals (SV2A&Bsn/SV2A) during development, in wt (B) and Het (C) cultures at 14 and 21 DIV. Postsynaptic maturation occurs in wt cultures between 14 DIV and 21DIV (wt 14DIV= SV2A&PSD-95/SV2A: 34.1±3.0; SV2A&PSD-95&Bsn/SV2A: 12.9±1.4; SV2A&Bsn/SV2A: 49±4.0, number of fields analyzed: 31; 21DIV= SV2A&PSD-95/SV2A: 58.8±2.7; SV2A&PSD-95&Bsn/SV2A: 23.4±1.5; SV2A&Bsn/SV2A: 51.4±3.2; number of fields analyzed: 27; Mann-Whitney rank sum test p= <0.001; 3 independent experiments). A lack of postsynaptic maturation occurs in Het cultures during development (Het 14DIV= SV2A&PSD-95/SV2A: 28.5±3.0; SV2A&PSD-95&Bsn/SV2A:13.5±1.8; SV2A&Bsn/SV2A: 44.3±4.5, number of fields analyzed: 20; 21DIV= SV2A&PSD-95/SV2A: 32.2±2.7, SV2A&PSD-95&Bsn/SV2A: 13.3±2.3; SV2A&Bsn/SV2A: 47.7±6.0, number of fields analyzed: 15; Mann-Whitney rank sum test p=0.397, p=0.881, p=0.726 respectively; 3 independent experiments). D) left, 3D reconstruction of dendritic shaft of neurons from 21DIV wt or Het cultures transfected with GFP; right, exemplary mushroom-type or filopodia-like spine. Scale bar 2µm. E) Quantification shows a reduction of the number of total protrusions in Het neurons with respect to wt (number of protrusions per micron, wt=0.58±0.02, number of examined dendrites: 83, number of neurons: 37; Het=0.43±0.02, number of examined dendrites: 87; number of neurons: 45; Mann-Whitney rank sum test p= <0.001; 3 independent experiments). The decrease in mature spine number (mushroom-type) is accompanied by an increase in thin spine

number (filopodia-like) (number of mushroom spines per micron, wt: 0.31±0.02, number of examined dendrites: 63; number of neurons: 37; Het: 0.16±0.01, number of examined dendrites: 84; number of neurons: 45; number of filopodia-like spines per micron, wt: 0.06±0.01; Het: 0.11 ±0.01; Mann-Whitney rank sum testp= <0.001; 3 independent experiments). F) Representative images of 21 DIV wt and Het cultures transfected with GFP and immunostained for PSD-95 (red). G) Quantification of PSD-95 positive puncta per unit length of parent dendrite (µm) (wt= 0.64±0.04, number of examined dendrites: 88, number of neurons: 34; Het= 0.44±0.02, number of examined dendrites: 68, number of neurons: 33; Mann-Whitney rank sum test p<0.001; 3 independent experiments). H) Quantification of PSD-95 puncta size reveals a significant reduction in Het neurons (in μm^2, wt=0.101±0.004, number of puncta: 433; number of analyzed neurons: 10; Het=0.086±0.005, number of puncta: 354, number of analyzed neurons: 7; Mann-Whitney rank sum test p=0.003; 3 independent experiments). Data are expressed as mean ± SEM. Scale bar 2µm. I) Representative traces of mEPSCs from 21DIV wt or Het neurons followed by the corresponding quantitative analysis of miniature frequency (frequency (Hz) wt=7.23±0.78; Het=2.56±0.32; Student t-test, p= <0.001) and amplitude (Amplitude (pA) wt=37.67±2.3; Het=29.61±0.99, cumulative probability; Kolmogorov-Smirnov test, p<0.05;number of neurons: wt=17, Het=10; 2 independent experiments).

Fig. 3 A postsynaptic defect impairs plasticity in SNAP-25 heterozygous cultures

A-A') Schematic representation and representative images of the two experimental settings used for mixed wt-GFP/Het neurons. Scale bar: 20µm. See also (20), fig. 3E. B-B') Left, representative images of wt-GFP/Het mixed cultures transfected with GFP or RFP as indicated and stained for PSD-95. Right, analysis of density of PSD-95 puncta in wt-GFP/Het mixed cultures. Results show no difference in PSD-95 density in wt neurons surrounded by either Het or wt neurons (B, PSD-95 puncta per unit length: wt-GFP/wt=1.00±0.08, number of examined dendrites: 45, number of neurons: 16; wt-GFP/Het=1.13±0.08, number of examined dendrites: 63, number of neurons: 27; Mann-Whitney rank sum test p=0.481) whereas a significant decrease of the density of PSD-95 puncta is observed

in Het neurons surrounded by either wt or Het neurons (B', PSD-95 puncta per unit length: wt-RFP/wt-GFP=1.00±0.07, number of examined dendrites: 9, number of neurons n=4; Het-RFP/wt-GFP=0.6±0.07, number of examined dendrites: 21, number of neurons: 8; normalized values; Mann-Whitney rank sum test p= <0.001). Data are expressed as mean±SEM (normalized values). Scale bar: 2µm. C) mEPSC recordings of wt-GFP surrounded by Het neurons or of Het neurons surrounded by wt-GFP neurons reveal a significant reduction in mEPSC frequency in the latter condition. D) A reduction of mEPSC amplitude, is also evident (frequency (Hz) control=6.22±0.67, number of cells: 13; wt-GFP/Het=6.53±0.92, number of cells: 10; Het-/wt-GFP=4.14±0.64, number of cells: 9; control vs Het/wt-GFP p= 0.02, wt-GFP/Het vs Het/wt-GFP p=0.03, Mann-Whitney rank sum test; amplitude (pA), control=19.78±1.01; wt-GFP/Het=19.04±1.6; Het/wt-GFP=17.01±1.08; control vs Het/wt-GFP p= 0.005; wt-GFP/Het vs Het/wt-GFP p=0.005; Kolmogorov Smirnov test; 3 independent experiments). E) Representative images of wt and Het cultures before and after performing a chemical LTP procedure. Cultures were stained for βIIItubulin (red), PSD-95 (green) and v-Glut1 (blue). Scale bar depicts 2µm. F) Extent of potentiation is represented by the quantification of the mean size of PSD-95 positive clusters and by the PSD-95&vGlut1 colocalization area. Potentiation occurs in wt but not Het cultures (PSD-95 size: wt ctr:1.00±0.05, wt LTP: 1,26±0.04, Het ctr: 1.00±0.03, Het LTP: 0.99±0.03. PSD-95&v-Glut1 size: wt ctr:1.00±0.03, wt LTP: 1,23±0.04, Het ctr: 1.00±0.03, Het LTP: 1.01±0.03. Number of analyzed fields: wt ctr: 46, wt LTP: 50, Het ctr: 62, Het LTP: 75. Mann-Whitney rank sum test, p= <0.001; 3 independent experiments). Data are expressed as mean±SEM; normalized values. G-I). Electrophysiological recordings of mEPSCs before and after chemical LTP induction in wt and Het neurons (5 min before LTP application, 15, 30 and 45 min after LTP application). G) Representative mEPSC traces. Differently from wt, Het neurons are unable to undergo LTP. H) mEPSC frequency (normalized values): wt pre gly: 1± 0.06 (n=29), wt post 5': 1.4±0.17 (n=20); wt post 30': 1.59±0.20 (n=17); wt post 45': 1.73±0.27 (n=10); Het pre gly: 1±0.06 (n=15); Het post 5': 1.00±0.13 (n=14); Het post 30': 1.11±0.20 (n=10); Het post 45': 1.10±0.19 (n=10).wt pre gly vs wt post 5': p<0.021, wt pre gly vs wt post 30':

p<0.012, wt pre gly vs wt post 45': p<0.014. Mann-Withney rank sum test. I) mEPSC amplitude (normalized values): wt pre gly: 1±0.02; wt post 5': 1.03±0.03; wt post 30': 1.04 ±0.04; wt post 45': 1.15±0.05; Het pre gly: 1±0.02; Het post 5': 1.02±0.03; Het post 30': 0.96±0.04; Het post 45': 1±0.03. wt pre gly vs wt post 45': p=0.001. Student t-test. n is the number of cells. 4 independent experiments. L-M) mEPSC frequency and amplitude recorded from neurons in mixed cultures experimental settings were normalized to values obtained before glycine application. Note that wt-GFP neurons surrounded by Het neurons undergo LTP, whereas Het neurons surrounded by wt-GFP neurons fail to potentiate (frequency (Hz) pre-gly: control=0.99±0.09 (n=16), wt-GFP/Het=0.96±0.2 (n=5), Het/wt-GFP=1.06±0.11 (n=8); 20±5min: control=1.30±0.1 (n=15), wt-GFP/Het=1.19±0.07 (n=3), Het/wt-GFP=0.88±0.13 (n=8); 60±5min: control=1.27±0.1 (n=7), wt-GFP/Het=1.18±0.10 (n=2), Het/wt-GFP=0.83±0.13 (n=5); n is the number of cells; ctr vs ctr 20min: p=0.04, Mann-Withney rank sum test. Amplitude (pA) pre-gly: control=0.99±0.04, wt-GFP/Het=0.99±0.07, Het/wt-GFP=0.97±0.09; 20±5min: control=1.17±0.06, wt-GFP/Het=1.13±0.10, Het/wt-GFP=0.88±0.12; 60±5min: control=1.34±0.08,wt-GFP/Het=1.23±0.07, Het/wt-GFP=0.84±0.09; ctr vs ctr 20min: p=0.03, ctr vs ctr 60 min: p=0.2; Mann-Withney rank sum test; 3 independent experiments). Data are expressed as mean±SEM.

Fig. 4 PSD-95-dependent spine formation requires postsynaptic SNAP-25. A) Representative images of dendrite portions of neurons transfected with (left) RFP and a scramble construct, or (middle) RFP and a construct silencing SNAP-25 (siRNA), or (right) RFP and a rat construct silencing SNAP-25 plus a mouse **siRNA-insensitive** SNAP-25-GFP **(iSNAP-25-GFP)** construct. All neurons are stained for PSD-95 (blue). Right, high magnification images of scramble, siRNA or siRNA plus SNAP-25-GFP spines. Scale bar: 6µm for low mag images and 1.5µm for high mag images. B) Quantification of the size of PSD-95 puncta (in μm^2, scramble=0.16±0.01, number of analyzed puncta: 305, number of analyzed neurons: 45; siRNA SNAP-25=0.11±0.01, number of analyzed puncta: 180, number of analyzed neurons: 40; SNAP-25-GFP + siRNA SNAP-25=0.23±0.01, number of analyzed puncta:

117, number of analyzed neurons: 18. Kruskal-Wallis One Way Analysis of Variance on Ranks $p \leq 0.001$; 3 independent experiments for SNAP-25-GFP+siRNA SNAP-25 condition, 6 independent experiments for scramble and silencing conditions). Data are expressed as mean±SEM. C-D) Representative images of **proximal** dendrite shafts co-transfected with PSD-95-GFP and a scramble or siRNA construct for SNAP-25 respectively and the relative quantification of the size of PSD-95 puncta (in μm^2, scramble=0.17±0.01, number of analyzed puncta: 152, number of analyzed neurons: 21; scramble PSD-95-GFP=0.74±0.02, number of analyzed puncta: 203, number of analyzed neurons: 24; siRNA SNAP-25 PSD-95-GFP=0.45±0.02, number of analyzed puncta: 296, number of analyzed neurons: 28. Mann-Whitney rank sum test p= <0.001; 3 independent experiments). Data are expressed as mean±SEM. Scale bar: 2µm. E-F) FRAP measurements of PSD-95-GFP in spines of siRNA-treated or scramble-treated neurons. E) Representative images of the same area before photobleaching (Pre-bleaching), at t= 0sec and 40sec after photobleaching of scramble- and siRNA-treated neurons. Neurons were maintained at 37°C during the experiment. Scale bar: 1µm for each image. F) Left, FRAP curves of PSD-95-GFP in spines over a 40sec period. Number of analysed spines: 58 for scramble-treated neurons; 52 for siRNA-SNAP-25- treated neurons. Right, histogram showing the increase of mobile fraction of PSD-95-GFP in siRNA-treated spines with respect to scramble-treated spines (scramble=39.72±2.09; siRNA-SNAP-25=55.81±3.30; Mann-Whitney rank sum test p= <0.001; 4 independent experiments). Data are normalized and expressed as mean±SEM.

Fig.5 SNAP-25 and PSD-95 are part of the same molecular complex in the brain

A) Representative images obtained from Proximity Ligation experiments. Neurons where incubated with primary antibodies against PSD-95/PCLO or PSD-95/SNAP-25 after which PLA probes where added, ligated, amplified and detected with a Cy3 probe. B) Quantification of PLA signals per neuron. PLA signals: 21.1±0.9 per nucleus in PSD-95/PCLO stained cells (34 analysed cells); 88.3±6.3 per nucleus in PSD-95/SNAP-25 stained cells (32 analysed cells); p=< 0.0001, unpaired t-test. C) Left, scheme representing the LUMIER co-immunoprecipitation assay. Right, interactions between the

tested proteins in HEK293 cells as detected by LUMIER co-immunoprecipitation assay. The green bar indicates a specific interaction, evaluated in term of firefly luciferase activity. Red bars are controls. D) Brain extracts were prepared as in material and method and immunoprecipitated as indicated, and run on SDS PAGE. Western blot was performed with antibodies to p140Cap, PSD-95 and SNAP-25. E) Summary of the proteins' interactions according to LUMIER assay results and literature. F) Model of interaction in the postsynaptic compartment: SNAP-25 and PSD-95 interact through p140Cap.