FUNCTIONAL STUDIES OF SNAP-25 USING A KNOCK-OUT AND RESCUE APPROACH

M.D.-Ph.D. Thesis

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Herewith I declare that I prepared the thesis "Functional studies of SNAP-25 using a knock-out and rescue approach" on my own and with no other sources and aids than quoted.

Göttingen, September 18th, 2006

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(Ignacio Delgado Martínez)

I. CONTENTS

II. PREFACE

"Understanding life through the studying of the human mind"

Nobody can solve the question 'Why' but everybody has answered it. The solution implies the understanding of life.

The conception of life starts when Philosophy, using reason as argument, opens the initial path by which knowledge walks. Science places the problem in an objective system and succeeds in explaining reality. The fabric of the world is taken down to the smallest pieces and so it is mainly understood by Physics. However, comprehension of life still remains unchanged. Life is enclosed in a vault, where observation is hard and complicated. Medicine aims at understanding human life. It states that the biological processes that sustain life are subordinated to the mental functions and, consequently, research should be redirected to the understanding of mind. Mind and self-consciousness arises from the tangled arrangement of the about 10^{12} neurons that conforms the human brain. Neurons are grouped in numerous networks, which build circuits of increasing complexity and function so that information can be efficiently processed. Understanding how neurons communicate is only the first step in order to understand mind. This thesis project sought to understand the function of one of the main proteins involved in neuronal transmission, SNAP-25, but the aim was to bring the own contribution to the understanding of life.

III. ACKNOWLEDGMENTS

This doctoral thesis could not be possible without the grateful collaboration of many people. Starting by Prof. Schild encouraged me to choose the Neuroscience program in Göttingen, becoming a great lighthouse from the top, and finishing by Prof. Erwin Neher, equally admirable as scientist and as person. The magnificent enthusiasm of Prof. Detlef Doenecke was also very encouragingin the GK521, which granted me.

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However, Dragana Jančić was there whenever I needed her and she gave meaning to my life.

To Draganita 

IV. SUMMARY

The neuronal SNARE complex, consisting of SNAP-25, synaptobrevin and syntaxin, is required for presynaptic exocytosis of neurotransmitter-filled vesicles during synaptic transmission. However, the SNARE complex undertakes neuronal developmental roles as well. SNAP-25 has been involved in vesicular fusion during axonal outgrowth, synaptic formation and trafficking of glutamate receptors. Its expression is developmentally regulated by alternative splicing from SNAP-25a to SNAP-25b. In GABAergic neurons, SNAP-25 seems to be replaced by SNAP-23, a ubiquitous SNAP-25 homologue, after synaptogenesis. Deletion of SNAP-25 compromises neuronal survival in culture, impeding detailed functional studies. Here, I overcame this difficulty by reintroducing SNAP-25a, SNAP-25b or SNAP-23 using the long-term expression lentiviral system in culture neurons from *Snap25* null mice and I was able to dissect the main functions of SNAP-25. I found that that SNAP-25 deficient cultured neurons presented impaired arborization and severe reduction in viability as well as complete arrest of evoked release and reduction in the amplitude and frequency of the spontaneous events. Expression of the SNAP-25 homologues restored neuronal survival, arborization and the properties of spontaneous release. In addition, it rescued evoked release, in both glutamatergic and GABAergic neurons, although SNAP-23 was found to support exclusively asynchronous release. SNAP-25b was superior to SNAP-25a in vesicle priming, which would produce larger releasable pools after synaptic maturation. My results revealed SNAP-25 as key component for neuronal survival and outgrowth, regulation of the synchronous and asynchronous release and spontaneous activity and demonstrate a hierarchical ability of the SNAP-25 homologues to support neuronal function.

V. INTRODUCTION

A. Overview

Neurons communicate with one another through specialized contact zones called synapses. At the synapse, the action potential (AP) that arrives from the soma along the axon is converted into a chemical signal at the presynaptic terminal, when presynaptic membrane depolarizations induce the following sequence of events (Zigmond, 1999; Kandel, 2000). First, voltage-gated calcium (Ca^{2+}) channels open at elevated membrane potentials, and as a consequence, Ca^{2+} flows into the cell due to a large concentration gradient with a low intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). After entering the cell, Ca^{2+} binds to Ca^{2+} sensors attached to synaptic vesicles. This in turn induces a lowering of the energy barrier for vesicles to fuse with the cell membrane, and therefore, the probability of vesicle fusion is increased markedly. When fusion occurs, neurotransmitters stored within the vesicles are released into the extracellular space, and then diffuse across the synaptic cleft to the postsynaptic neuron. Having arrived at the postsynaptic cell, the neurotransmitter molecules bind to specific receptors, and thus exert certain effects on the postsynaptic neuron. In the case of excitatory ionotropic receptors, neurotransmitter molecules will open pores that allow for cation flux into the cell, so that the postsynaptic membrane potential will depolarize. In this way, the chemical signal is again converted into an electrical signal, and the AP, generated in the postsynaptic cell by temporal and spatial summation, can travel through the neuron to arrive finally at the next synaptic connection.

B. Synapses are highly dynamic connections

Every neuron receives synapses from numerous other neurons and each of these synaptic contacts contributes differently to the final response. The generation of the action potential is the result of the spatial and temporal integration of small changes in the post-synaptic membrane potential, which are mainly caused by the opening of post-synaptic receptors upon activation by the neurotransmitter released at the presynaptic terminal. The contribution of each synapse to the final response, known as synaptic

strength, is not a static property of each contact, but it can be modulated to adapt to the physiological requirements (Bliss and Collingridge, 1993).

Synaptic plasticity is the general process by which synaptic strength is modified. It enables the storage and use of vast amounts of information in the form of learnt behaviors and conscious memories, therefore, representing the basis of information storage in the brain. Phenomena of synaptic plasticity can regulate synaptic strength either presynaptically, by altering the release of neurotransmitter, or post-synaptically, by changing the receptor availability. However, synaptic contacts are not static structures. New synapses are constantly formed and retracted, depending on the need of the connection (Luscher et al., 2000), adjusting continuously the connections between neurons and the develop of new networks.

Post-synaptic receptors are well-known to participate in the synaptic plasticity. For example, glutamate receptors are required for the induction of some forms of plasticity like long-term potentiation and long-term depression. Similarly, GABA (γ-aminobutyric acid) receptors, as well as many metabotropic receptors, are involved in the modulation of the synaptic strength (Collingridge et al., 2004). Moreover, the receptor population is highly dynamic and hardly constant. These receptors are rapidly mobilized from and to the post-synaptic membrane so that its number can be carefully modified. Post-synaptic receptors are constantly inserted into and removed from the plasma membrane by exocytosis and endocytosis, respectively, and they can, in addition, diffuse laterally within the membrane (Malinow and Malenka, 2002; Roberto, 2003; Collingridge et al., 2004; Pérez-Otaño and Ehlers, 2005). In this way, the post-synaptic response can be modulated by altering the number and composition of receptors available to respond to released neurotransmitter.

Changes in the release of neurotransmitter can modify, presynaptically, synaptic strength. Neurotransmitter release is a tight regulated process since it represents the outcome of the neuron. Neurotransmission is an extent topic of research in neuroscience, with more than half a century in its back. Synaptic transmission was firstly examined in the bullfrog neuromuscular junction (Fatt and Katz, 1952; Del Castillo and Katz, 1954; Dodge and Rahamimoff, 1967; Katz, 1969) establishing the basis of the quantal and

calcium hypotheses. According to this generally accepted view, neurotransmitter is released in quantal units, which correspond to synaptic vesicles. The fusion of these vesicles is a probabilistic event that depends on the intracellular calcium concentration, $[Ca^{2+}]_i$. The arrival of an action potential to the presynaptic membrane produces an increase in $[Ca^{2+}]_i$ and, thus, in vesicular fusion and neurotransmitter release. The ratio between the number of vesicles released and the total number of vesicles ready to be released (readily releasable pool, *RRP*) is called release probability, R_p. The release of neurotransmitter can be modified, for example, by altering the release probability. In most of the cases, an increase of the release probability is due to an increase in $[Ca^{2+}]_i$. Since vesicle fusion is a calcium dependent process, this increase will elevate the number of vesicles released and the final amount of neurotransmitter that arrives to the post-synaptic receptors. Additionally, the recruitment of a major number of vesicles in the *RRP* will also increase the overall vesicular fusion and the final neurotransmitter concentration (Zucker and Regehr, 2002).

C. Synaptic vesicle exocytosis is disclosed by the SNARE hypothesis

The comprehension of how the release of neurotransmitter can be modified requires the understanding of the processes that mediate vesicle fusion. However, not until recently the underlying physiological and molecular mechanisms were described. It was originally proposed that specific integral proteins in the vesicle membrane (vesicle-SNAREs, or v-SNAREs) bind to specific receptor proteins in the target membrane (target membrane or t-SNAREs) (Sollner et al., 1993). The SNARE family members contains a conserved stretch of 60-70 amino acids, referred to as the SNARE motif (Terrian and White, 1997; Weimbs et al., 1998). Four SNARE motifs assemble spontaneously into a thermostable, sodium dodecyl sulfate and protease resistant coiled-coil bundle, called the SNARE complex (Fasshauer et al., 1998; Sutton et al., 1998; Antonin et al., 2002). The center of the complex contains 16 highly conserved layers of interacting amino acid side chains. The amino acids in central ionic layer (denominated as 'layer 0') contain three glutamine residues ('Q') and one arginine residue ('R') (Fasshauer et al., 1998 and Figure 1B). Up to now, more than 30 SNARE proteins have been identified in humans, falling

into four subfamilies: Q_a-, Q_b-, Q_c- and R-SNAREs. Thus, all analyzed SNARE complexes that contain three or four SNARE proteins have a $Q_aQ_bQ_cR$ composition (Fasshauer et al., 1998; Bock et al., 2001). The association of these four motifs is supposed to approach the two membranes together and promote, in this way, fusion (Figure 1).

The exocytosis of synaptic vesicles is dependent on three SNARE proteins: one v-

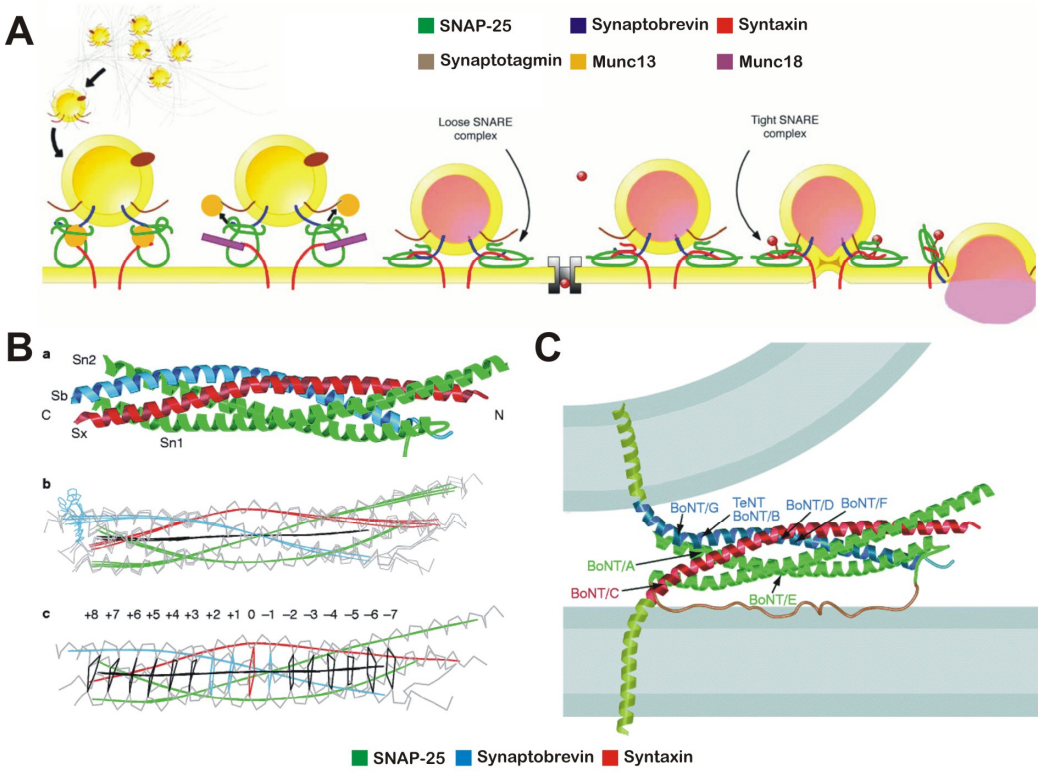


Figure 1
SNAREs in synaptic vesicle exocytosis

(A) Synaptic vesicles are recruited from the reserve pool that corresponds to vesicles attached to the actin cytoskeleton via synapsin. These vesicles then translocate to the vicinity of the plasma membrane. They undergo priming through an unknown mechanism in which Munc18 and Munc13, two syntaxin-binding proteins, could participate. This step could lead to the formation of a complex between synaptobrevin and SNAP-25. Formation of loose SNARE complexes between synaptobrevin, SNAP-25 and syntaxin 1 reduces the distance between the synaptic vesicle and the plasma membrane. At this point, the synaptic vesicle would be docked to the plasma membrane. Entry of calcium could trigger a conformational change in synaptotagmin 1 that would allow further zippering of the SNARE complex into a tight state, which would lead to lipid bilayer fusion. Modified from Galli and Haucke, 2001. **(B) a**, Backbone ribbon drawing of the synaptic fusion complex **b-c**, Organization of the synaptic fusion complex. C traces (grey), local helical axes for SNAREs, the superhelical axis (black), and layers (0, red; −1, +1 and +2, blue; all others black) are shown for one of the three complexes in the asymmetric unit. Layers are indicated by virtual bonds between corresponding C positions (Sutton et al., 1998) **(C)** Hypothetical model of the synaptic fusion complex as it joins two membranes, and location of neurotoxin-mediated cleavage sites (Sutton et al., 1998).

SNARE, synaptobrevin 2/vesicle associated membrane protein 2 (VAMP-2) (Trimble et al., 1988; Baumert et al., 1989) and two t-SNAREs, syntaxin 1 (Bennett et al., 1992) and synaptosome-associated protein of 25 kDa or SNAP-25 (Oyler et al., 1989). The association of this SNARE complex with synaptic transmission came from studies with neurotoxins that selectively cleave SNAREs, potently inhibiting exocytosis (Blasi et al., 1993b; Blasi et al., 1993a; Niemann et al., 1994). It is known that tetanus toxin (TeNT), botulinum neurotoxin (BoNT) B and BoNT/D cleaves synaptobrevin, BoNT/A, BoNT/E cleaves SNAP-25 and BoNT/C1 cleaves both SNAP-25 and syntaxin (Niemann et al., 1994; Rossetto et al., 2001, see Table 1 and Figure 1C). These three SNARE proteins, synaptobrevin 2, syntaxin 1 and SNAP-25, are sometimes considered the "minimal fusion machinery" since they are sufficient to fuse liposomes incorporating the purified proteins (Weber et al., 1998) as well as plasma membrane of living cells (Hu et al., 2003). However, for this fusion to be fast enough to allow neurotransmitter release, it requires the presence of a preformed syntaxin/SNAP-25 acceptor complex (Pobbati et al., 2006).

Synaptobrevin 2 is a 13-kDa vesicle protein that acts as R-SNARE. It has a central SNARE motif, a C-terminal transmembrane region and a proline-rich N-terminus (Trimble et al., 1988; Elferink et al., 1989). Syntaxin is a 35-kDa plasma membrane protein engaged as Q_a-SNARE. It consists of a C-terminal transmembrane domain, a central SNARE motif and a N-terminal H_{abc}-domain (Bennett et al., 1992), for binding to regulatory proteins. SNAP-25 is a Q_{bc}-SNARE and contributes with two of the four α-helices to the neuronal SNARE complex. The SNARE motifs are present at the N- and C-termini and separated by a central linker domain containing four cysteins (Sutton et al., 1998). SNAP-25 is efficiently targeted to the plasma membrane due to the palmitoylation of these cysteins (Hess et al., 1992a; Bark and Wilson, 1994).

The function of the neuronal SNARE complex was originally proposed to be the docking of synaptic vesicles at the active zone (Sollner et al., 1993). However, after neurotoxin treatment, this docking was normal whereas fusion was blocked (Schiavo et al., 2000), suggesting, instead, a downstream role for SNAREs. Later studies indicated that SNAREs are closely involved in the last steps of exocytosis (Xu et al., 1998; Xu et

al., 1999; Sørensen et al., 2003; Borisovska et al., 2005; Sakaba et al., 2005). The 'zippering' hypothesis proposes that the neuronal SNARE complex is partly assembled from distal to proximal end in a priming state and then progressively 'zips up' for vesicle fusion (Hanson et al., 1997; Xu et al., 1999; Sørensen et al., 2006). The energy liberated during the assembling of the complex can, then, overcome the energy barrier for membrane fusion (Chen and Scheller, 2001; Rizo and Sudhof, 2002). Therefore, vesicles primed by means of the SNARE proteins require less energy for undergoing fusion. These are, consequently, the first vesicles to be released with the action potential.

According to a 'three vesicle model', synaptic vesicles can be separated into three groups according to the kinetics of release (Rizzoli and Betz, 2005). The first pool represents those vesicles that are immediately available on stimulation, conforming the *readily-releasable pool* (*RRP*) (Rosenmund and Stevens, 1996). The second pool, which is called '*recycling pool*', is constituted by those vesicles that maintain release on moderate (physiological) stimulation. Finally, the third pool (*reserve pool*) is thought to be a depot of synaptic vesicles, from which release is only triggered during intense stimulation (Harata et al., 2001; de Lange et al., 2003). In a linear model, these three pools would represent different stages of vesicular fusion competence. From the depot pool, vesicles would need first to position next to the plasma membrane in the active zone and later to prime by means of the SNARE proteins (Voets et al., 1999).

However, at several CNS synapses, two kinetically distinct components of release have been observed, a fast and a slow one (Goda and Stevens, 1994; Murthy et al., 1997; Sakaba and Neher, 2001b, 2001a). The mechanisms that underlie the slow component remain unclear but those involved in the fast release are known in more detail. It has been proposed that fast-releasing vesicles are located nearby release-triggering calcium channels. The fast, transient calcium influx caused by opening of the voltage-gated calcium channels triggered by the action potential would cause, thus, the synchronous fusion of primed synaptic vesicles. The slow vesicles would be distant from these channels, sensing, as consequence, attenuated residual calcium wave (Meinrenken et al., 2003; Trommershauser et al., 2003; Schneggenburger and Neher, 2005). However, an alternative model proposes that fast and slow components differ in the way that they are coupled to the calcium signal. Shortly after its isolation, synaptotagmin-1 was found to be

able to bind calcium (Brose et al., 1992). Later, it was shown that knock-out neurons for synaptotagmin-1 presented a selectively loss of the fast component of release, which led to consider synaptotagmin-1 as the calcium sensor responsible for this component (Goda and Stevens, 1994). Synaptotagmin-1 is a vesicular protein of 65 kDa that contains two C_2-domains able to bind Ca^{2+}, SNAP-25 and SNARE complexes, and phospholipids (Gerona et al., 2000; Littleton et al., 2001; Sudhof, 2002). During priming, the synaptic vesicle is forced to be close to the plasma membrane. Synaptotagmin-1 is, at this point, associated to the SNARE complex. The binding of calcium to syntaptotagmin-1 partially insert the C_2-domains into the plasma membrane phospholipids, causing a mechanical perturbation in the membrane and promoting fusion (Sudhof, 2004, Figure 1A). Apart from synaptotagmin-1, some other isoforms, from an extended family of 16 members, are able to bind calcium to at least one of the two C_2 domains, showing higher affinity than synaptotagmin-1. These isoforms have been proposed to participate in the triggering of the slow, asynchronous, component of release, by sensing the residual calcium following the action potential (Hagler and Goda, 2001; Sudhof, 2002).

D. Neurons dispose SNAREs for constitutive vesicle fusion

Fusion of vesicles with the plasmalemma is essential not only for neurotransmitter release but also for many neuron-specific functions; for example, membrane expansion that supports axonal elongation as well as morphological and functional changes in mature synapses. This fact raises the question of whether common membrane fusion machineries are present in all these processes. Since the SNARE-mediated fusion of vesicles is considered as a universal mechanism for all type of eukaryotic membrane fusion events (Jahn and Sudhof, 1999), it could be licit to consider similar apparatus for many other processes requiring vesicle fusion.

Several studies have focused attention on the expression of SNARE proteins in the growth cone, which is responsible for axon guidance and elongation (Jessell and Kandel, 1993). The transition of the growth cone into a fully functional presynaptic terminal after reaching and recognizing its target cell is accompanied by the development of the complete SNARE machinery for synaptic transmission. One feature that distinguishes the growth cone from the synapse is its lack of synaptic vesicles. Instead, so-called "growth

cone vesicles" are present, which are thought to participate in the elongation of the axon by incorporation of their membranes within the growth cone itself (Futerman and Banker, 1996). Thus, studying the transition of the growth cone into a mature synapse has helped to elucidate which SNARE proteins participate in the different phases of this process. SNARE proteins are present in both growth cones and synaptic terminals, but several regulatory elements within synaptic terminals are missing from growth cones (Igarashi et al., 1997). This suggests that the SNARE mechanism operating during this stage may be sufficient for axonal growth, but not for regulated exocytosis; this regulatory system appears and becomes functional during synaptogenesis (Igarashi et al., 1996; Igarashi et al., 1997).

Several lines of evidence showed that SNAP-25, syntaxin and synaptobrevin are present in developing neurons and they are involved in neurite outgrowth, both in the central and peripheral nervous system (Osen-Sand et al., 1993; Miya et al., 1996; Shirasu et al., 2000). Axonal growth was found to be inhibited by SNAP-25 antisense oligonucleotides, both in vivo and in vitro (Osen-Sand et al., 1993). Similar results were obtained by treating neurons with botulinum neurotoxins, which specifically cleave SNAP-25 (Osen-Sand et al., 1996). Further experiments, in which SNAP-25 proteins were overexpressed in staurosporin-differentiated pheochromocytoma (PC12) cells, showed that SNAP-25 promotes the number of neurite per cell (Shirasu et al., 2000) and the neurite length (Zhou et al., 2000). However, not all SNAREs are involved in axonal growth. The cleavage of synaptobrevin by tetanus toxin impairs neurotransmission, but appears to have no effect on axonal growth (Osen-Sand et al., 1996). After the isolation of a tetanus-toxin-resistant synaptobrevin, synaptobrevin 7 or TI-VAMP (Galli et al., 1998), this was proposed as the substitute of tetanus-toxin-sensitive synaptobrevins in neurite outgrowth, since it was shown to be required for vesicular transport, mediating neurite outgrowth in PC12 cells (Martinez-Arca et al., 2000). Similarly, when over-expression of syntaxin-1 appeared not to affect neurite extension in PC12 cells (Shirasu et al., 2000; Zhou et al., 2000; Darios and Davletov, 2006), other family members were immediately proposed as substitutes. Syntaxin-3 was found to be present in growth cones and involved in axonal outgrowth (Darios and Davletov, 2006). Furthermore, syntaxin-13, an isoform implicated in endosomal trafficking, was reported to enhanced neurite

outgrowth when over-expressed in PC12 cells, while having no effect on secretion (Hirling et al., 2000). Although there are very few experiments performed in neurons to confirm these findings, it seems clear that SNAP-25 and some homologues of synaptobrevin and syntaxin are implicated in neurite outgrowth. However, the role of each individualform of these SNARE proteins has not yet been precisely defined.

As a consequence of these findings, a SNARE-mediated mechanism should be also considered in the membrane-carrying vesicle fusion processes involved in the budding of new processes and the morphological changes that occur during learning and memory. Furthermore, vesicle fusion is also required for many long-lasting adaptations in the potency of synaptic transmission, i.e. experience-dependent plasticity. The best-characterized forms of such synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), observed at excitatory synapses in the CA1 region of the hippocampus. It is now well accepted that the trafficking of AMPA receptors to and away from the post-synaptic plasma membrane plays an essential role in the post-synaptic forms of LTP and LTD, respectively. This could be due to various processes such as the slowing

	VAMP	TI-VAMP	Syntaxin	SNAP-25	SNAP-23	SNAP-29	SNAP-47
BoNT/A	×	×	×	✓	✓*	×	×
BoNT/B or TeNT	✓	×!	×	×	×	×	×
BoNT/C_1	×	×	✓	✓	×	×	×
BoNT/D	✓	×	×	×	×	×	×
BoNT/E	×	×	×	✓	✓*	×	×

Table 1
Effect of the different botulinum toxins in SNARE proteins

* Botulinum toxins can presumably cleave only murine but not human SNAP-23

			Receptor Trafficking			
	synaptic fusion	neurite outgrowth	*α7nAchR*	*AMPAR*	*NMDAR*	*mGluR1α*
R-SNARE	VAMP-2	× (TI-VAMP)	*BoNT/D*	*TeNT* *BoNT/B*		
Q_{ab}-SNARE	SNAP-25	SNAP-25	*BoNT/C*		SNAP-25	SNAP-25
Q_c-SNARE	syntaxin 1	syntaxin 3/13	*BoNT/C*			

Table 2
Involvement of SNARE proteins in different vesicle fusion processes involved in neuronal function

Summary of the conclusions obtained from studies showing SNARE participation in neuronal processes (see in text). When known, the molecular candidate is shown. Otherwise, impairment of the process by action of botulinum toxins is shown with the name of the corresponding neurotoxin. SNAP-25 has been involved in most of these processes.

down of endocytosis, redistribution of the receptors from extrasynaptic to synaptic plasma-membrane regions, and exocytosis of AMPA-receptor-rich vesicles. From all of them, the last process seems to be the most important. The fusion of vesicles containing the GluR1 subunit of the AMPA receptor is a process driven by calcium, in which synaptotagmin could be involved (Maher et al., 2005), and modulated by calmodulin-dependent protein kinase-II (Maletic-Savatic et al., 1998). Interestingly, this process is blocked by the clostridial toxins BoNT/B and TeNT, which cleave synaptobrevin (Luscher et al., 1999; Lu et al., 2001). In fact, other receptors seem to be controlled by a SNARE mechanism. The delivery of new NMDA receptors by potentiation is inhibited by BoNT/A as well as by a dominant-negative SNAP-25 mutant (Lan et al., 2001b; Lan et al., 2001a). Furthermore, treatment with either BoNT/C1 or BoNT/D prevents the recruitment of nicotinic receptors (α7-nAChR) in chick ciliary ganglion neurons (Liu et al., 2005). All these recent findings indicate that a SNARE-mediated mechanism, similar to the one involved in neurotransmitter release, serves in the post-synaptic receptor trafficking involved in synaptic plasticity.

When all these experiments from the last 10 years are considered together, it appears that the SNARE machinery involved in neurite extension and synaptic plasticity is similar to that involved in neurotransmitter release in neurons and neuroendocrine cells (Table 2). It also seems that, although different SNARE proteins associate in each complex, only SNAP-25 appears to have constant presence in all of them. SNAP-25 is a member of a very small family, when comparing to synaptobrevin or syntaxin, and it is highly conserved during evolution (Figure 2). This stability in SNAP-25 structure may indicate that SNAP-25 is precisely calibrated for neuronal function and difficult to be substituted.

E. SNAP-25 is a main character in the brain theater

SNAP-25 has a restricted expression pattern, being most abundant in neuronal and neuroendocrine cells (Bark et al., 1995; Boschert et al., 1996). Developmental studies noted a shift in localization from cell bodies to cell extensions and presynaptic terminals when developing axons approach their target (Oyler et al., 1991; Bark et al., 1995). A similar developmental regulation was found in the chick retina and in the spinal cord,

where this was shown to coincide with synaptogenesis (Catsicas et al., 1991). In addition, the relative levels of the two SNAP-25 isoforms, 'a' and 'b', undergo major changes during brain embryogenesis. SNAP-25a was found to be the major isoform expressed early during brain development while SNAP-25b predominates in the adult nervous system of the rat (Bark et al., 1995; Boschert et al., 1996). However, this switch in the expression does not happen in neuronal-related cells such neuroendocrine cells (Bark et al., 1995; Grant et al., 1999). Interestingly, the developmental profile of SNAP-25 differs from data on other SNARE proteins. Thus, in the developing brain, syntaxin-1 is strongly expressed up to 4 postnatal weeks and then decreases, while synaptobrevin was found to be present at a low level before birth, to increase after birth and then to decrease gradually with aging (Shimohama et al., 1998). Both the large increase in SNAP-25 expression and the switch between the two isoforms occur during the period when synapses form, thus suggesting that SNAP-25a is likely to participate at earlier stage, during neurite expansion, whereas SNAP-25b is more relevant later, when release of neurotransmitter from synaptic vesicles becomes important. Supporting an hypothetical function of SNAP-25a in membrane remodeling, it has been also shown that certain regions of the adult brain, which are known for their plasticity (synaptic remodeling, sprouting, etc.), continue to express this isoform in adults, e.g. olfactory bulb and the hippocampus (Boschert et al., 1996). The two isoforms, produced by the alternative

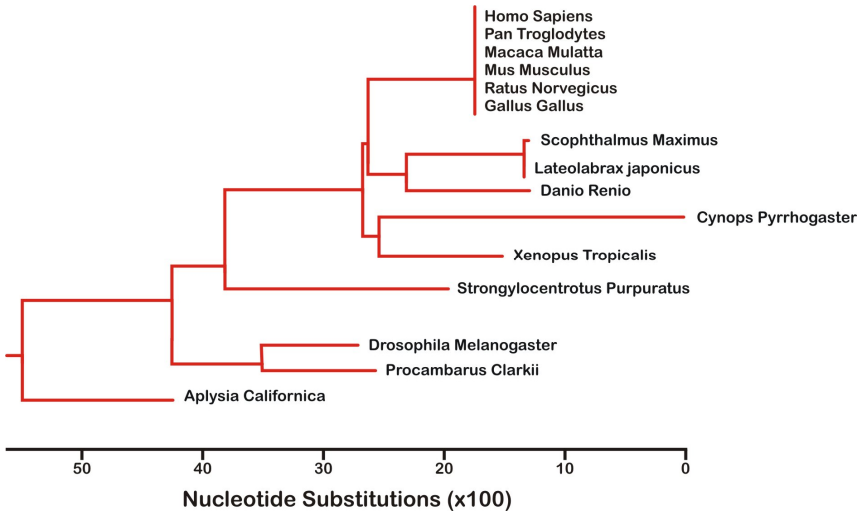


Figure 2
Phylogenetic tree for SNAP-25

Sequences corresponding to the SNAP-25 homologue in each specie were obtained from the National Center for Biotechnology Information (NCBI) and aligned using MegAlign software (DNAstar Inc., Madison, WI, USA).

splicing of the exon 5, differ in only 9 amino acids of the N-terminal region of the first SNARE domain and the linker between both domains (Bark, 1993). The reason of this differential splicing is still not well know. It is interesting to notice, in this respect, that duplicate exon 5 sequences have been found in vertebrates from zebrafish to human, but not in *Drosophila* (Risinger et al., 1997), which would suggest that alternative splicing contributes to increased functional plasticity and organization of higher-ordered neural systems. From the analysis of the structural differences between the isoforms, it has been proposed that the position of one of the four cysteine residues in the linker domain could target the two isoforms to different sites on the plasma membrane (Bark and Wilson, 1994). The functional differences between both isoforms have been examined recently in chromaffin cells, showing that the size of the readily-releasable pool of large-dense core vesicles was increased when SNAP-25b replaced SNAP-25a (Sørensen et al., 2003). In fact, only two amino acid substitution in the SNARE domain could account for the difference (Nagy et al., 2005). In neurons, analysis of a knock-in mouse in which the splicing mechanism was impaired led to the conclusion that SNAP-25a, rather than SNAP-25b, could contribute to short-term plasticity involved in strengthening selective synaptic contacts during activity-dependent synapse elimination (Bark et al., 2004).

Although all these studies offer important hints about the properties of each isoform, there are still important questions to solve about neuronal function of SNAP-25. It has shown that a certain amount of release persists even when SNAP-25 is missing. Ablation of the SNAP-25 gene in mice resulted in perinatal lethality. The SNAP-25 *null* embryos were morphological abnormal but the major brain structures were unchanged (Washbourne et al., 2002). Stimulus-driven release was absent in cortical brain slices and in chromaffin cells but, even so, remaining spontaneous activity was detected (Washbourne et al., 2002; Sørensen et al., 2003) . In fact, such an alternative exocytic pathway has been proposed to constitute the main source of neurotransmission in inhibitory, GABAergic, neurons. It was reported that SNAP-25 is expressed in inhibitory neurons during the early stages of neuronal development and then it decreases by the time that synapses are mature (Verderio et al., 2004; Frassoni et al., 2005). The most important candidate to substitute SNAP-25 is SNAP-23. SNAP-23/syndet is a ubiquitous expressed homologue, which shares 59% identity on the amino acid level with SNAP-25

(Ravichandran et al., 1996; Wang et al., 1997). It is found in glial cells (Hepp et al., 1999) and in glutamatergic and GABAergic hippocampal rat neurons as well (Verderio et al., 2004). Similarly to SNAP-25, it is targeted to the plasma membrane by palmitoylation of five cysteine residues (Wang et al., 1997) and it forms a SNARE complex with synaptobrevin 2 and several syntaxin isoforms (Ravichandran et al., 1996). It has been implicated in both constitutive and regulated exocytosis in non-neuronal cells (Leung et al., 1998; Rea et al., 1998; Vaidyanathan et al., 2001). In chromaffin cells, this homologue can partially substitute SNAP-25 function (Sørensen et al., 2003), in agreement with an alternative SNAP-23 pathway. Although there is still some controversy at this respect, the rodent SNAP-23, not the human, though, seems to be cleaved by BoNT/A and BoNT/E, albeit with reduced efficiency (Vaidyanathan et al., 1999). Although SNAP-23, for these reasons, seems to be the best candidate for an alternative release mechanism, the other two members of the SNAP-25 family, SNAP-29 and SNAP-47, could be also involved. SNAP-29 is a ubiquitous SNARE that shares 17% identity to SNAP-25 (Steegmaier et al., 1998) and is BoNT/A and –E resistant (Holt et al., 2006). It has been mainly involved in intra-Golgi trafficking steps (Hohenstein and Roche, 2001) although it has been also detected in synapses (Su et al., 2001), possibly modulating neurotransmission (Pan et al., 2005), which supports the possible implication of SNAP-29 in synaptic vesicle fusion. SNAP-47 is a still not well characterized novel homologue, ubiquitously expressed and BoNT/A and –E resistant (Holt et al., 2006).

The multifunctional action of SNAP-25 in neurons seems to be important, in addition, for some neuropsychiatric disorders. For example, mice hemizygous for the deletion coloboma (*Cm*/+), which encompasses the *Snap25* gene, were reported to exhibit significant behavioral deviations, including hyperactivity, maternal neglect of offspring and delayed developmental milestones (Hess et al., 1992b; Heyser et al., 1995). These symptoms are similar to those characteristic of the attention deficit-hyperactivity disorder (ADHD), which is believed to affect about 7% of school-aged children (American Psychiatric Association, 1995), and, therefore, *Cm*/+ mice were proposed as a model for the disease (Wilson, 2000). Apart from this, reduced SNAP-25 function has been associated to other abnormalities of behavioral development, cognition and neurotransmission, such as schizophrenia (Young et al., 1998; Fatemi et al., 2001;

Thompson et al., 2003), emphasizing the importance of SNAP-25 in the global brain function.

F. Aim of the work

The comprehension of the function of SNAP-25 has presented several difficulties up to now. SNAP-25 has been implied in functions that extend to the release of neurotransmitter. However, mostly due to the better general comprehension of this process, the study of SNAP-25 has been for long focused in calcium-triggered exocytosis. The rest of the functions, such as neurite outgrowth or receptor trafficking, did not receive extensive examination. The complete understanding of how SNAP-25 is used by the neuron requires a global study in which as many possible actions are taking into consideration. Most of the actual systems used for studying SNAP-25 are over-simplified models, e.g. PC-12 cells or chromaffin cells. These models are very potent because experimental conditions are under tight control; making results in these experiments are very solid and precise. However, since SNAP-25 performs most of its actions in neurons, where conditions might be very different from those in the models, the conclusions obtained in such models cannot be generalized. Studies in neurons are still insufficient. The analysis of SNAP-25 in neurons has been basically based in the inactivation of SNAP-25 by using botulinum toxins (Williamson and Neale, 1998; Grosse et al., 1999; Lan et al., 2001b; Verderio et al., 2004; Sakaba et al., 2005). However the potency by which these toxins act significantly differs depending on its serotype and its incorporation by the neuron (Purkiss et al., 2001), which has caused conflicting findings. Other advanced approaches used, instead, antisense oligonucleotides (Osen-Sand et al., 1993) or small interference RNAs (Sieburth et al., 2005) with successful results. However, all these techniques fail in producing the complete and exhaustive removal of SNAP-25 from the neuron, confusing the conclusions. The development of a *Snap25* knock-out mouse line has considerably expanded the possibilities, since it provides a genetically clean background in which SNAP-25 can be studied (Washbourne et al., 2002). However, this approach has an additional impediment in the fact that neither can these animals survive after birth nor can neurons survive in culture (Washbourne et al., 2002). This drawback has irremissibly limited the available techniques to exclusively those capable of examining

the embryonic brain or immature neurons.

This doctoral thesis was initially intended to procure a technique to examine *Snap25* null neurons in primary cultures and reintroduce SNAP-25 into them, as a way of understanding the importance of SNAP-25 in the neuronal function. The initial goal of this doctoral thesis constituted, for that reason, the establishment of a method that would allow the rescue of SNAP-25 deficient neurons in culture. This technique finally resulted on the long-term expression of SNAP-25 by means of the lentivirus system (Naldini et al., 1996a; Naldini et al., 1996b; Blomer et al., 1997). When this new methodological approach was finally established, it enabled us to study the role of SNAP-25 in neuronal survival, arborization and neurotransmitter release by examining the differences among the two splice-variants, SNAP-25a and SNAP-25b, and the closest homologue, SNAP-23. The final outcome demonstratedthe complexity and polyvalence of SNAP-25 in neurons. My findings made evident that SNAP-25 is crucial for synaptic vesicle exocytosis as well as for neuronal survival and neurite outgrowth. Supplementary to this, I observed a possible post-synaptic effect, consistent with down-regulation of AMPA receptors. The thesis firmly demonstrates, furthermore, that the synchronous release is exclusively driven by the SNAP-25 isoforms, excluding SNAP-23 for this process, not only in glutamatergic neurons and but in GABAergic ones as well, which refutes some previous experiments. I showed that, SNAP-23 is, in fact, able to produce release although asynchronously, which would resemble a lack of interaction with synaptotagmin-I. The probably reason for the alternative splicing of SNAP-25 appeared to be the different regulation of the priming of synaptic vesicles, since SNAP-25a supported a smaller readily releasable pool than SNAP-25b. This would suggest that the promotion of the expression of SNAP-25b over SNAP-25a, which occurs by the time that mature contacts are formed, provides a more efficient synaptic transmission by enhancing the priming properties of neurotransmitter release. Such a switch in the expression would be, in that case, important for the processing of the large amount of information received from the moment of birth.

VI. EXPERIMENTAL PROCEDURES

A. Experimental Approach

The participation of SNAP-25 in different processes in the neuron, such as synaptic transmission and arborization, makes necessary the use of specific experimental techniques for each of them. In this doctoral work, I used a combination of morphometric and functional methods to analyze the role of SNAP-25 in neuronal function. I performed the experiments in primary neuronal cultures, from hippocampus and from striatum, to investigate possible differences in excitatory and inhibitory neurons. As a prerequisite for that, I established a system that allowed us to induce an exogenous protein expression so that differences among the main SNAP-25 family members could be determined. This fact was essential since the expression of SNAP-25 was found to be critical for survival of neurons in culture. Several methods were tried but the lentivirus system, based on the ability of HIV-1 to integrate into the host genome (Naldini et al., 1996a; Naldini et al., 1996b), appeared to be the most convenient method to induce the expression of the different homologues and produce rescue. This system, which infects both dividing and non-dividing cells, produced a steady, long-term expression of the homologues in the neurons, allowing the recovery of survival and the development of mature neurons in cultures.

The use of cultures makes available an extended number of genetic manipulations, such the study of mutations that are lethal in mice or the expression of exogenous proteins or its mutants. But also, it provides availability to a wide range of techniques, which are important for the detailed examination of neuronal properties. By growing a constant number of cells on the plates, it is possible to compare differences in survival among different conditions. Morphological differences can be identified in neurons growing in cultures by the labeling with antibodies against specific proteins of interest or the expression of reporter genes by genetic transfer. The use of a high-resolution detection system in cultures, like confocal microscopy, is indispensable for visualizing small structures such as narrow branches and synapses. Furthermore, staining of lipid membranes by styryl dyes, which allows the staining of lipid membranes, is useful for

examining the functionality of the synapses and the recycling of vesicles. The combination of these techniques makes possible an accurate and complete examination of the neuronal properties.

However, for understanding utilization of SNAP-25 by the neuron, it is important not only the analysis of neuronal populations but isolated neurons as well. The use of autaptic cultures allows growing the neuron on an isolated micro-island formed by astrocytes. The neuron is excluded from other neurons by a space without astrocytes and forced to expand within the island, establishing synaptic contacts with itself. These autaptic contacts are completely functional and share similar properties to conventional synaptic connections in brain (Bekkers and Stevens, 1991; Clements, 1992). This kind of preparation offers many of the advantages and possibilities of brain slices and exocytotic model cells. It is possible to use electrophysiological approaches to investigate release kinetics, pharmacological properties, plasticity changes, spontaneous events, etc. The main electrophysiological tool is the patch-clamp technique (Neher and Sakmann, 1976; Hamill et al., 1981). This method allows the measurement of cell ionic current with an extraordinarily favorable signal-to-noise ratio. Its application to autaptic neurons permits the measurementfrom thousand of synapses, all of them sharing the same propertiessince they proceed from the same neuron, with a single electrode (Figure 5). This fact presents a great advantage over other preparations like brain slices, for example. Unfortunately, this preparation is limited in the production of long-term plasticity as well as in the control of some presynaptic mechanisms, which are possible in other kind of systems. Despite these limitations, the system has been proved powerful during this doctoral work, providing interesting results.

B. Miscellaneous methods used for cloning

To understand the function of the thousand of genes involved in the variety of brain functions, it is essential to be able to introduce and express DNA in neurons. Over the past two decades, the number of gene transfer methods developed has largely expanded and countless studies have taken advantage from this technique. However, there is still not a perfect technique that covers every application. The recombinant based viral techniques offers high-efficiency of transfection since they benefit from the innate ability

of virus for infecting host cells and express their genome. However, the non-viral methods are generally easier to use, less toxic, and not limited in the size of the plasmid that they deliver. In this doctoral work, I used both alternatives in order to find a method that allowed the rescue of *Snap25* null neurons.

For a gene being expressed in a neuron, it is necessary this gene to be incorporated into the cell by means of a vector. For that, DNA coding the gene (so-called insert) and the vector has to be cleaved by compatible restriction enzymes so that they can be ligated together. In some cases, it is necessary to include some new restriction sites to create compatible sites. This can be done by using PCR to amplify a fragment containing those sites. To obtain enough genetic material for the transfection, the ligated DNA vector needs to be amplified. For that, the vector is transformed into competent bacterial cells, which will duplicate the exogenous vector together with its own genomic vector. DNA is extracted from the bacteria and purified, usually obtaining ~1-2 µg/µl of product.

Cloning was performed according to standard protocols (Sambrook and Russell, 2001). Enzymes for DNA manipulations were obtained from New England Biolabs (Beverly, MA, USA). Unless indicated otherwise, all other reagents were purchased from Sigma (Deisenhofen, Germany).

The methods used for the cloning of the constructs will be explained in this section. The non-viral approaches utilized will be described in the next section and the lentiviral system in the following one.

1. Restriction Digests

Restriction digestion is the process of cleaving double stranded DNA molecules into discrete fragments with restriction endonucleases, which recognize specific sequences in the DNA molecule. Companies also deliver 10x concentrated buffer along with the enzymes for optimal reaction conditions. For digestion, 1µg of plasmid DNA is incubated with the restriction mixture for at least 1.5 to 2 hours and the enzyme specific temperature.

2. Amplification by Polymerase Chain Reaction (PCR)

PCR is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA (Saiki et al., 1988). The required materials include: the segment of double-stranded DNA to be amplified, two single stranded oligonucleotide primers flanking it (added in vast excess compared to the DNA to be amplified), high fidelity Pfu-polymerase, deoxyribonucleosides (dNTPs), buffer and salt. *Taq*-Polymerase is used for PCR for genotyping mice.

The reaction mixture for cloning is as follows (for genotyping, see Genotyping PCR):

PCR with Pfu-Polymerase:	
100 ng	diluted Template DNA
0.2 µl	5´ Oligo-nucleotide (5pmol)
0.2 µl	3´-Oligo-nucleotide (5pmol)
2 µl	dNTP-Mix (2.5mM each)
5 µl	10x Polymerase-Puffer
1 µl	Pfu-Polymerase
Final volume to 50µl with H2O	

The PCR product to be used for cloning reactions is purified with the *QIAquick PCR Purification Kit* from Qiagen.

3. Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate, identify and purify negatively charged DNA based on their size. In agarose gel electrophoresis, the DNA is forced to move through a sieve of molecular proportions that is made of agarose. The positions of the bands are made visible and can be photographed in UV-light with ethidium bromide (254 or 314nm). Usually 0.7 to 2% gels are used. The agarose is dissolved by heating in 100ml of the TAE-buffer (per liter, 4.84 g Tris Base, 1.14 ml Acetic Acid, 2 ml 0.5M EDTA pH 8.0) , and 0.5µg/ml Ethidium Bromide is added after cooling to approx. 50°C, then poured into the gel chamber. DNA is then separated at constant voltage (80-120V) in the buffer.

4. Isolation of DNA-Fragments from Agarose Gels

The product QIAquick PCR purification Kit from the Qiagen firm was used. The

protocol, outlined in the QIAquick Spin Handbook for purifying PCR fragments is provided by the producers.

5. Dephosphorylation of the 5´end with Alkaline Phosphatase

Alkaline phosphatases catalyze the hydrolysis of 5´-phosphate residue from DNA, RNA and ribo- and deoxyribonucleoside triphosphates. The digested vector is treated with alkaline phosphatase before ligation with the desired insert to prevent autoligation of the vector. The dephosphorylation protocol is provided by the producer.

6. Ligation

DNA ligases catalyze the formation of phosphodiester bonds between juxtaposed 5´phosphate and a 3´-hydroxyl terminus in duplex DNA. T4 ligase, which uses ATP as a source of energy, can ligate both sticky and blunt ends.

For that, 20-100ng digested vector DNA and approximately double or triple the amount of "insert" DNA are mixed, in a final volume of 20µl, together with 2µl of 10x T4 ligation buffer and 1µl T4 ligase. Incubate at 16°C for 4-16 hours.

7. Competent bacteria production

50 ml LB-Medium was transfected with a single colony of the bacterial strain and incubated while shaking at 37°C until an OD_{600} of 0.5 to 0.7 is reached. The cells were centrifuged for 10 minutes at 4200 x gmax, 4°C, and then washed in ice cold 15% glycin TFBI solution containing (in mM; sodium acetate, 300; $MnCl_2$, 50; NaCl, 100, $CaCl_2$, 10). Cells were centrifugated again, resuspended in ice cold 15% glycin TFBII (in mM, MOPS, 10; NaCl, 10, $CaCl_2$, 75), aliquoted and flash frozen in liquid Nitrogen and stored at –80°C.

8. DNA extraction from bacteria

DNA extraction and purification was made according to the QIAGEN's and protocols. Depending on the quantity and purity of the DNA we wish to obtain, two basic protocols are available. QIAGEN's miniprep is faster although little DNA material is obtained. It is mainly used for screening and verification. When DNA will be used for

transfection or sequencing, QIAGEN's maxiprep is preferred since it obtains a larger amount of clean DNA.

9. Determination of DNA Concentration

Due to their physical and chemical properties, DNA molecules in solution can absorb UV-light and this absorption can be measured by a spectrophotometer (GeneQuant RNA/DNA Calculator from Pharmacia, Uppsala). The relationship exists between the optical density (OD) and the DNA concentration is known (1 OD_{260} = 50 mg/ml DNA), so that the higher the concentration, the greater the optical density at 260nm.

C. Non-viral Transfection methods

1. Description of the construct for non-viral transfection

The cytomegalovirus promoter of the original pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) was removed using *Ase*I and *Eco47*III restriction enzymes and replaced by a neuron-specific human synapsin-I gene promoter, obtained from an adeno-associated virus vector from S. Kügler, University of Göttingen, Germany (Kugler et al., 2001) using *Pst*I and *Eco*RI restriction sites. This vector was named *pSynEGFP*. DNA fragments encoding for SNAP-25a, SNAP-25b and SNAP-23 were cleaved from Semliki forest virus vectors, pSFV1 (Sørensen et al., 2003) using *Bss*HII and *Age*I restriction sites and inserted between the *Bam*HI/*Age*I sites of pSynEGFP. Constructs were verified by sequencing and transformedin DH5α compentent bacteria for amplification.

2. Calcium phosphate transfection in neurons

Neurons were transfected by a previously described calcium phosphate transfection procedure (Wienisch and Klingauf, 2006), based on protocols developed by Threadgill, Dudek and collaborators (Xia et al., 1996; Threadgill et al., 1997) and optimized according to the cell culture conditions. At 4-6 days *in vitro* the neuronal growth medium was removed, saved and replaced with serum-free Neurobasal A Medium (NBA, Invitrogen, Carlsbad, California, USA) 30 min prior to transfection. A calcium

phosphate/DNA precipitate was formed at room temperature and in darkness for 15–20 min in a 100 µl water solution comprising of 5–30 µg/ml plasmid DNA and 5 µl of 2.5M $CaCl_2$ stock solution and 50 µl of a buffered saline consisting of 50 mM BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 2H_2O$ at pH 7.00. The precipitate was added drop-wise to 900 µl NBA while gently vortexing. 500 µl of the diluted calcium phosphate/DNA precipitate solution were added to the cells in each well. Following 5-30 min incubation at 37°C and 5% CO_2, during which the appearance of a fine sandy precipitate covering the cells was observed, the cultures were washed in Hank's Balanced Salt Solution (HBSS, Gibco) and returned to the original conditioned culture media. The transfection efficiency was typically between 5% and 20%, and there was no apparent toxicity to the cells. The product of a transfected gene coding for green fluorescence protein (eGFP) could be detected by epi-fluorescent microscopy as early as 6 hours post-transfection and expression was stable for at least two weeks. In all cases, the DNA was prepared with Qiagen (Hilden, Germany) endotoxin-free maxi-prep plasmid DNA purificationkits.

3. Magnetic assisted transfection (MAT)

Magnetic assisted transfection is based in the use of magnetic particles, MAT-A, (IBA GmbH, Göttingen, Germany) that are able to associate to plasmid DNA, oligonucleotides or siRNA. Exploiting magnetic force the plasmid is then rapidly drawn towards and delivered into the target cells leading to transfection.

1µl of MAT-A was initially mixed with 1 µg of plasmid in 200 µl of Neurobasal A and incubated for 30 minutes at room temperature. Mix at different dilutions (ranging from 1:1 to 1:8) was added to conventional hippocampal neuronal cultures at day *in vitro* 1, incubating for 20 minutes at 37°C over a magnetic plate (IBA GmbH). The day after, medium containing magnetic associates was replaced by fresh NeurobasalA medium.

D. Lentivirus

1. Overview

The retroviral vectors have been considered a potentially ideal system due to their

ability to integrate into the genome of target cells, allowing for long-term gene expression, the nonexistent immunological response and the large cloning capacity (up to 10Kb). However, retroviral vectors have a major drawback in that they fail to infect non-mitotic cells (Roe et al., 1993; Lewis and Emerman, 1994), which contravenes an efficient use, for example, in neurons. This disadvantage could be overcome by the discovery that human immunodeficiency virus-type 1 (HIV-1) can infect both mitotic and non-mitotic cells (Bukrinsky et al., 1992; Bukrinsky and Haffar, 1999), leading to the development of a new class of retroviral vectors suitable for neuronal studies. Since these vectors presented a slow and persistent rate of infection, they were termed lentiviral vectors, with '*lenti*' being the Latin term for slow. Most of the experimental vectors are based on HIV-1 because, among all the different lentiviruses described, this is the best understood.

2. The lentiviral cycle

Lentiviruses are enveloped viruses of the retrovirus family. The viral particle contains two copies of the viral RNA genome, which contains three essential genes: *gag*, *pol* and *env*. *Gag* encodes for the core proteins capsid, matrix and nucleocapsid. *Pol* codes for the viral enzymes protease, reverse transcriptase and integrase. The *env* gene encodes for the envelope proteins, which mediate virus entry. In addition, another six proteins are present (*tat*, *rev*, *vif*, *vpr*, *nef* and *vpu*). *Tat* and *rev* mediate, respectively, transactivation of viral transcription and nuclear export of unspliced viral RNA.

The lentiviral life cycle (Figure 3 and Figure 4; for review, Trono, 2002) is known in detail mainly from studies of HIV-1. After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion. The viral RNA genome is subsequently reverse transcribed into linear double-stranded DNA by the virion reverse transcriptase in the cytoplasm. Reverse transcription involves two jumps of the transcriptase enzyme from the 5' end to the 3' end of the viral template, causing a duplication of the sequences located at the ends of the viral RNA, which therefore are termed long terminal repeats (LTRs). The proviral DNA is heavily associated with viral proteins like nucleocapsid, reverse transcriptase and integrase forming the preintegration complex, and translocates to the nucleus where the viral enzyme integrase mediates

integration of the provirus into the host cell genome. The DNA intermediate stage uses its own *cis*-acting elements to control the host transcriptional machinery. These elements are situated within the proviral LTRs. Other additional proteins, like *tat*, serve as activators of RNA transcription. Host cell transcription factors initiate transcription from

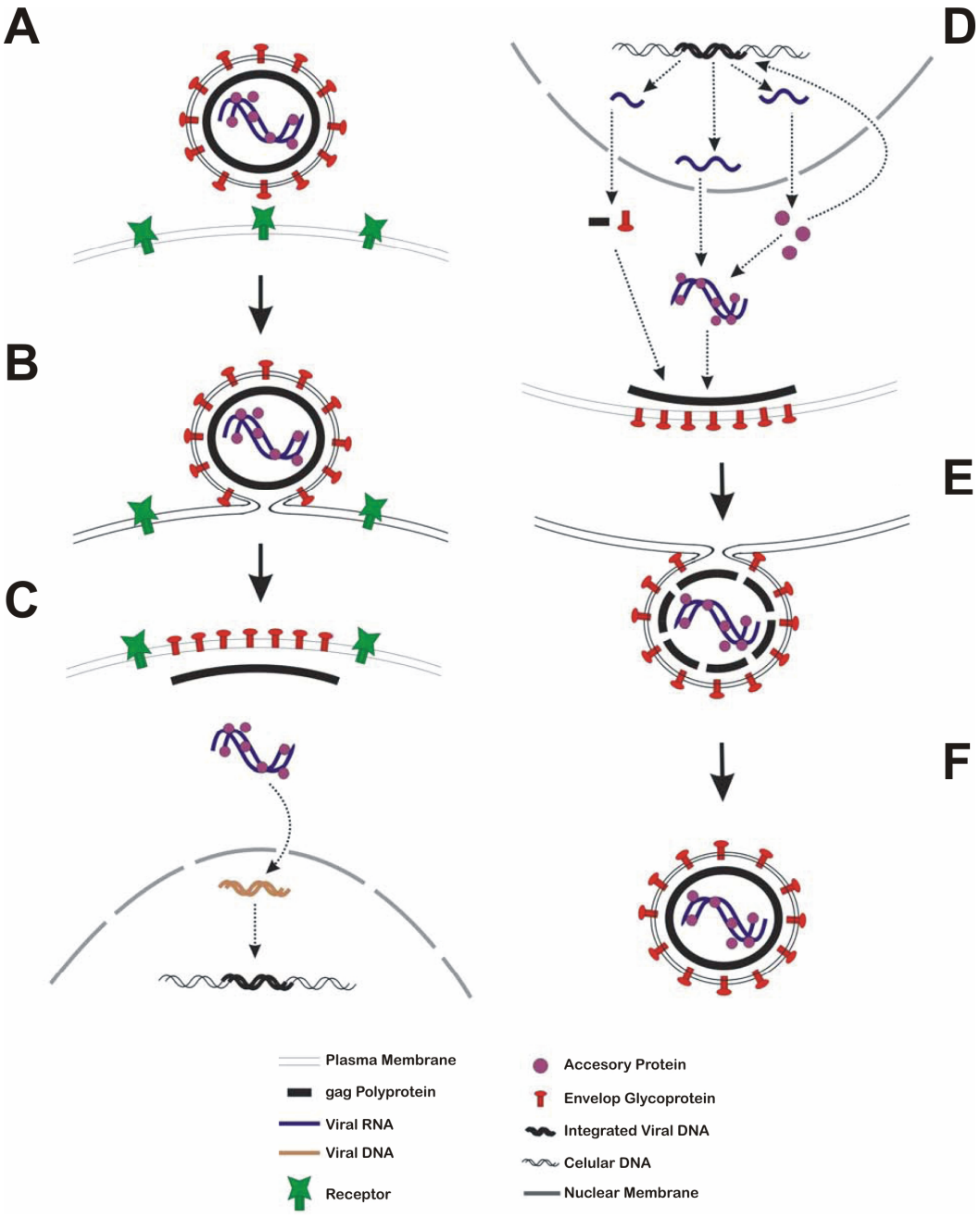


Figure 3
Life cycle of lentiviral vectors

(A) Binding of viral particle to surface receptor of target cell via envelope glycoprotein. **(B)** Fusion of the viral envelope with target cell membrane. **(C)** Uncoating of viral capsid, Viral RNA is reverse transcribed to form double stranded proviral DNA, which is translocated into nucleus and integrated into target cell genome. **(D)** Production of viral transcripts followed by translation of *cis*-acting regulatory factors. **(E)** Assembly of new viral particles and budding from cell membrane of target cell **(F)** Mature viral particle capable of infecting other cells.

the LTR, and new viral particles are formed at the plasma membrane. The LTRs are divided in to the U3, R, and U5 regions. The R region is defined as the transcription start site in the 5' LTR. The U3 region is found upstream to that and contains the majority of the *cis*-acting elements that regulates transcriptional initiation by the cellular RNA polymerase II. In addition, the immediate 5' end of the U3 region contains the so-called *att* site, which is necessary for integration. The retroviral RNAs are subject to the same processing events as cellular RNAs: cap addition at the 5' end, cleavage and polyadenylation of the 3' end, and splicing. This process is regulated by the viral proteins. For example, *rev* promotes the efficient transport of unspliced RNAs that contain *rev* response elements (RRE) from the nucleus to the cytoplasm. The *gag-pol* and *gag* precursors assemble together with two copies of viral RNA to form the viral particle. Finally, *env* glycoproteins are incorporated into the viral membrane during the budding process. In a newly formed virion, *gag* and *gag-pol* precursors are subjected to processing by the viral enzyme protease, which results in maturationof the virion.

3. Lentiviral vectors

In the generation of the initial lentiviral vectors, the transgene of interest was replacing the *env* gene, which was provided in *trans* in a separate vector (Page et al., 1990; Landau et al., 1991). In a first improvement, all the dispensable protein-encoding sequences were separated as well. The essential *cis*-acting sequences, required for encapsidation of the vector RNA, such as the packaging signal sequences (Ψ), and the vital sequences necessary for reverse transcription and integration, the LTRs, the transfer RNA-primer binding site, the *rev* response elements (RRE) and the polypurine tract (PPT) had to be present in the vector construct with the transgene (Parolin et al., 1994). This system originally resulted in low viral tires and was limited to the transductionof the natural target cells of HIV-1, mediated by *env*. However, replacement of the U3 region in the 5' LTR with the immediate early region of the human cytomegalovirus (CMV) enhancer-promoter resulted in a massive increase in viral titers and reduced the sequence homology between the vector and packaging construct by deleting *tat* (Kim et al., 1998). In addition, the substitution of the viral *env* protein by the G protein of the vesicular stomatitis virus (VSV-G) enormously broadened the host range of the virus and

permitted, in addition, the concentration of particles by ultracentrifugation (Burns et al., 1993; Bartz and Vodicka, 1997). The separation of the viral genome onto three separate constructs, encoding the vector construct, the packaging vector and the VSV-G protein, made expression dependent on the presence of the *trans*-acting proteins and reduced the

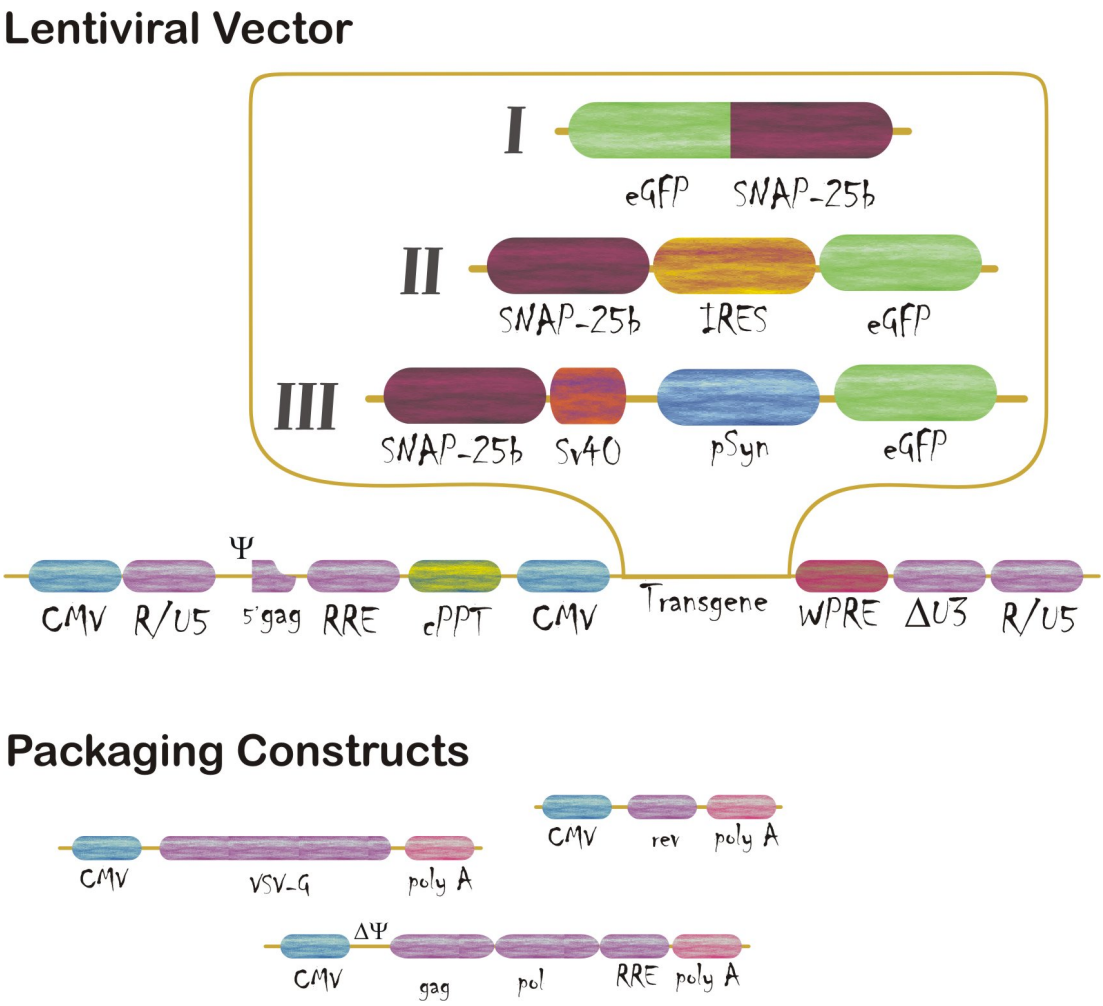


Figure 4
Lentivirus design

In the lentiviral vector, the viral genes *gag*, *pol*, and *env* have been replaced by promotor and transgene sequences and flanked by the viral LTRs. Packaging of the viral RNA genome is ensured by the presence of the packaging signal (Ψ), comprised of the 5' untranslated region and the 5' sequence of the *gag* open reading frame. In addition, the vector contains two additional *cis*-acting sequences, the RRE, which is essential for nuclear export of unspliced viral RNA in the presence of *rev*, and the cPPT, which supports nuclear import of the proviral DNA in the transduced cell. The 3' LTR contains a large deletion in the U3 region (depicted as ΔU3) to prevent transcription from the LTR. The lentiviral packaging system consists of three constructs encoding for *gag/pol*, VSV-G, and efficient nuclear export similar to the vector construct. Transgenes were designed according to three different strategies:

I) eGFP is fused to the N-terminal of the SNAP-25 homologue.

II) An internal ribosomal entry site (IRES) is placed between SNAP-25 homologues and eGFP, so that both proteins are produced separately.

III) eGFP expression is driven by a neuron-specific promoter, synapsin-I (pSyn) to exclude expression in the astrocytic layer.

homology between the sequences. The matrix protein and *vpr*, which are involved in the nuclear importation, were shown to be provided by the packaging cells and they could be removed. Other proteins, such *vpu*, *vif* and *nef*, were also unnecessary. In addition, viral titers were improved by the introduction of a central copy of the PPT (cPPT), which enhanced nuclear import, and other *cis*-acting transcriptional regulatory elements, such as the WPRE, which enhanced transgene expression in the target cells (Hu and Temin, 1990; Naldini et al., 1996a; Naldini et al., 1996b; Dull et al., 1998). Thus, this third generation of vectors was expressing structural and regulatory proteins but no longer contained sequences for *tat* and for *rev*, which was delivered on a separate construct. Furthermore, since the U3 region of the 5' LTR of the provirus is derived from the U3 region of the 3' end of the vector RNA, deletions of the promoter/enhancer sequences of the U3 region of the 3' LTR are carried over to the 5' LTR during reverse transcription, producing the self-inactivation of the vector. By using these self-inactivating vectors (SIN), a possible unwanted expression of genes proximal to the site of integration was prevented, which increased the biosafety of the system (Swain and Coffin, 1989, , 1992; Miyoshi et al., 1998; Zufferey et al., 1998; Follenzi et al., 2000).

4. Production of the Lentiviral constructs

The lentivirus plasmid corresponding to the murine SNAP-25 isoforms and SNAP-23 were generated from the inserts of pSFV1 vectors (described in (Sørensen et al., 2003) and cloned into the multiple cloning site of the pRRLsin.cPPT.CMV.WPRE (referred as pRRL from now) lentiviral transfer vector (Follenzi et al., 2000; Follenzi et al., 2002). Expression of SNAP-25 homologues was under the control of a cytomegalovirus (CMV) promoter. Three different constructs were cloned for each homologue (Figure 4). In the first one, full-length eGFP was fused to the N-terminal of SNAP-25 homologue through a 24-amino acid linker. To avoid a possible interfere of the eGFP molecule in the SNAP-25 function, an internal ribosomal entry site (IRES) was interposed, in a second construct, between the homologue and the downstream eGFP. In a third construct, expression of eGFP was driven by a synapin-I promoter in order to produce neuron-specific expression of the reporter gene, whereas expression of the SNAP-25 homologues was still under control of the CMV promoter.

(1) eGFP fusion construct

DNA fragments encoding for SNAP-25a, SNAP-25b and SNAP-23 were cleaved from semliki forest virus vectors, pSFV1 (Sørensen et al., 2003) using *Bam*HI and *Bss*HII. eGFP fragment was obtained from a pEGFP-N1 vector using the *Nhe*I and *Bam*HI restriction sites. Both fragments were ligated and inserted between the *Xba*I and *Mlu*I restrictionsites of the pRRL lentivirus vector.

(2) IRES-eGFP construct

Fragments containing the SNAP-25 homologues followed by the IRES sequence and eGFP were cleaved from semliki forest virus vectors (Sørensen et al., 2003) using *Bam*HI and *Xho*I and inserted in between the *Bam*HI/*Sal*I sites of the pRRL vector.

(3) Synapsin promoter construct

A fragment containing synapsin-I promoter together with eGFP was obtained from the pSynEGFP-N1 vector (see Non-viral Transfection methods) by PCR using the primers detailed below (see Primers used), so that new restriction sites were added (*Nhe*I upstream and *Msc*I downstream). The pSyn-eGFP fragment was afterwards inserted into the pRRL backbone. The SNAP-25 homologue genes were obtained by PCR, adding new *Spe*I and *Age*I restriction sites up- and downstream (see Primers used), respectively. In addition, an SV40 origin for replication expressing the SV40 T-antigen was cloned by PCR, adding new *Age*I and *Mlu*I restriction sites up- and downstream, respectively (see Primers used). Finally, both SNAP-25 and SV40 fragments were inserted separately into the lentivirus vector containingsynapsin-I promoter.

(4) Primers used

Cloning of the Synapsin promoter construct required the addition of new restriction sites in the respective fragments so they could be inserted in the pRRL vector. This was made by PCR amplification, designing the following primers so that they included the necessary restrictionsites (highlighted in *italic* in the below sequences).

(a) pSyn-eGFP

Forward: TAT *GCT AGC* ATT AGA GGG CCC TGC GTA TGA GTG

Reverse: GCA *TGG CCA* TTA CTT ATG CAG CTC GTC CAT GCC

(b) SNAP-25a

Forward: TAT *ACT AGT* ATG GCC GAA AGA CGC AGA CAT GCG

Reverse: GCA *ACC GGT* TTA ACC ACT TCC CAG CAT CTT TGT T

(c) SNAP-25b

Forward: TAT *ACT AGT* ATG GCC GAG GAC GCA GAC ATG

Reverse: GCA *ACC GGT* TTA ACC ACT TCC CAG CAT CTT TGT T

(D) SNAP-23

Forward: TAT *ACT AGT* ATG GAT AAC CTG TCC CCA GAG G

Reverse: GCA *ACC GGT* TTA ACT ATC AAT GAG TTT CTT TGC

(e) SV40

Forward: TAT *ACC GGT* TAA TCA GCC ATA CCA CAT TTG TAG AGG

Reverse: GCA *ACG CGT* GTT AAG ATA CAT TGA TGA GTT TGG A

(5) Sequencing

Constructs were verified by sequencing (SEQLAB GmbH, Göttingen, Germany) using the following primers:

pRRL forward: AGT GAA CCG TCA GAT CGC CTG GAG

psyn-mid forward: AAC AGG ATG CGG CGA GGC GCG TGC

psyn-mid reverse: GGG CGA AGG CAC TGT CCG CGG TGC

p156RRL-IDM reverse: CGT AAA AGG AGC AAC ATA GTT AAG

5. Transformation of plasmids in Stbl2/Stbl3 bacteria

Lentivirus constructs were heat-transformed in Stbl2 or Stbl3 competent bacteria for amplifications. For that, a 30 second heat shock at 30°C was applied at a mix containing 1 µg of plasmid DNA and 50 µl of competent bacteria. After resting on ice, bacteria were incubated with 250 µl of S.O.C. medium (Invitrogen) for 45 minutes to

recover from shock and finally plated on LB plates and incubated overnight at 30°C.

6. Production of Lentiviral particles

The production of functional lentiviral particles is performed by cotransfection of a packaging cell line with four different vectors as described above (Figure 4). As *gag* and *pol* proteins are not assembled accordingly in murine cells, this procedure is performed in human cell line (Mariani et al., 2001).Many common laboratory human cell lines, e.g. HeLa, may be successfully transfected and produce large amount viral protein but, however, secrete few viral particles (Haselhorst et al., 1998). For this reason, the cell line of choice is normally based on 293 cells, a human embryonic kidney cell line. The stable expression of the SV40 large T antigen of the 293T cell line variant allows the replication of plasmids containing the SV40 origin of replication, which is present on the transfer vector used. Viral particles are secreted into the culture medium, from which they are collected. Secretion of viral particles is maximal 24h following transfection and decreases two-fold in the second 24h period. Low viral titers may be dealt with by altering culture conditions, e.g. decreasing the temperature to 32°C as well as using low serum concentration, since VSV-G pseudotyped particles inactivate upon contact with serum (DePolo et al., 2000).

a) 293FT cell culture

To re-culture deep frozen cells, the cells were removed from the liquid nitrogen tank transported on dry ice and thawed by gentle shaking in a 37°C water-bath. All cell culture work was done under the sterile hood. The thawed cells were then transferred to a 75cm^2-flask and the culture grown in a steam saturated, 37°C, 5% CO_2 incubator. The culture medium was: D-MEM containing 10% fetal calf serum (FCS), 1% Penicilin/Streptomicin and 500µg/ml G418 antibiotic (Geneticin®, Invitrogen). When the cells grew to a confluent layer in the dish, they were split after first washing with D-PBS, then adding 0.25% Trypsin. When the cells detached from the flask they were diluted 1:10 with medium and transferred to another cell culture container. For transfection experiments, the cell density was calculated through microscopy using a haematocytometer and plated according to each protocol.

b) 293FT cell freezing

Some determined culture cells can be frozen for storage with minor damage. This method allows the long-term preservation of the cell line for later use. To this end, cells are deattached from the culture dish with 0.25% Trypsin and, next, centrifuged at 1200 r.p.m. for 2 minutes to remove the medium. Then, cells are resuspended in sterile DMSO (Invitrogen) and aliquoted (~$3 \cdot 10^6$ cells/ml). Freezing is done progressively, initially at -20°C for several hours, then at -80°C over night and finally in liquid nitrogen.

c) Transfection

The lentiviral plasmids were transfected into 293FT according to two different protocols in order to determine which gave the highest efficiency.

(1) Calcium Phosphate transfection in 293FT cells

The day before transfection, 293FT were plated at a density of ~10.000 cells/cm^2 on poly-D-lysine coated tissue dishes in D-MEM medium containing 10% fetal calf serum (FCS, Gibco-BRL GmBH, Eggenstein-Leopoldshafen, Germany), so that cells attain 90-95% confluence. On the day of transfection, 10 µg of the lentivirus plasmid was mixed with 3.5 µg pMD.G2 (packaging vector) and with 6.5 µg pCMVARP2 (envelop vector) and combined with 0.25 M $CaCl_2$ in 10mM Tris solution (pH 7.00). The mix was added drop-wise to 2xHBS buffered saline solution while gently vortexing and incubating for 30 minutes to produce a calcium phosphate/DNA precitate, which was, then, added to the culture dishes. After over-night incubation, medium was replaced by fresh 10% FCS D-MEM medium. The medium from the culture dishes was collected after 48-72 hours and centrifugated for 2 minutes at 2000 r.p.m. to remove cellular detritus and later filtered through a sterile, 0.45µm low protein binding filter. To enhance viral tittering, lentivirus was harvested by ultracentrifugation (2 hours at 30.000 r.p.m.) and resuspended in 200 µl of D-MEM.

(2) Lipofectamine transfection

Plasmids were transfected into 293FT by a modified procedure based on the standard ViraPower™ Lentiviral Expression System from Invitrogen (Carlsbad, CA, USA). This method consists on the use of an optimized mix of the three packaging

plasmids (pLP1, pLP2, and pLP/VSVG) denominated ViraPower™ Packaging Mix. Briefly, the day before transfection, 293FT cells were plated on 10 cm tissue culture dishes at a density of ~20.000 cells/cm^2 such that they will be 90-95% confluent on the next day. At the day of transfection, the culture medium was removed and replaced with 5 ml of OptiMEM® I medium containing 10% FCS. Then 3 µg of the expression plasmid was mixed with 9 µg of the ViraPower™ Packaging Mix in 1.5 ml of OptiMEM® I without serum. This mix was added to a separated tube containing 36 µl of Lipofectamine™ 2000 in 1.5 ml of OptiMEM® I without serum. After 20 minutes of incubation, the solution was added dropwise to the culture plate. The next day, the medium was replaced by 11 ml I-MEM (Sigma) containing 2% FCS, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% Glutamay™-I. 24 hours later, lentivirus was harvested under security level 2 conditions and concentrated using a centrifugal filter device (100K NMWL; Amicon Ultra-15, Millipore, USA). Final volume was adjusted to 2 ml with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) and 30-60 µl of purified virus was added to neuronal cultures at day *in vitro* 1.

E. Hippocampal cultures

1. Glass coverslip preparation

Glass coverslips were sterilized first with 1N HCl and then with 1N NaOH and extensively washed with distilled H_2O. Clean coverslips were coated with 0.15% agarose (Type II-A, Sigma, Steinheim, Germany), a substrate upon which cells fails to attach, and afterwards covered by a solution containing 0.5% Collagen (Type I, rat-tail, BD Bioscience, San José, CA , USA) and 10µM Poly-L-Lysin (Sigma) in 10 mM acetic acid. For autaptic cultures, a rubber stamp with protruding pins was applied on the top of the agarose cover to form small 200 µM diameter microdots. In the substrate forming microdot, astroglial as well as neuronal processes grow within the borders of the coated island but cannot reach outside because of the agarose (Figure 5).

2. Astrocytic supporting cultures

Astrocytic supporting cultures were prepared from normal wild-type (NMRI)

mice. After excision of hippocampus (see Neuronal SNAP-25 knock-out mice preparation), the rest of the hemisphere was collected and digested for 45-60 minutes in Dulbecco's modified Eagle's medium (D-MEM; Gibco, Grand Island, NY, USA) containing 20-25 units/ml papain (Worthington Biochemical Corp., Lakewood, NJ, USA), supplemented with 200 mg/l L-cystein, $CaCl_2$ 1mM, EDTA (ethyldiaminetetraacetic acid) 20 mM, and equilibrated with bubbling 5%/95% CO_2/O_2 for 20 minutes. Papain activity was afterwards inactivated by incubation for 10 minutes in a D-MEM-based solution containing 2.5 g/l trypsin inhibitor (Sigma, St. Louis, MO, USA) and supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 2.5 g/l bovine serum albumin (Sigma). Inactivating medium was carefully removed and replaced by pre-warmed D-MEM medium for astrocyte culture, containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% MITO$^+$ Serum Extender (Collaborative Biomedical Products, Bedford, MA, USA). Digested tissues were homogenized with a pipette and plated on 75-cm^2 flask in 10 ml astrocytic medium, and allowed to grow for approximately one week at 5% CO_2, 95% humidity, and 37°C until reaching confluence. At this point, the medium was substituted by 10 ml trypsin/EDTA (Biochrom Co.) and incubating for 10 minutes at 37%. The culture flask was vigorously vortexed and buoyant cells, which are mainly comprised of microglia, were discarded to enrich the astroglial population. De-attached astrocytes were then resuspended and transferred to a 15ml Falcon's tube and centrifuged at 1.600 r.p.m. for 5 minutes. The supernatant was poured off and the pellet was resuspended in 10 ml astrocytic medium. Cells were examined under microscope and counted using a haemacytometer. Astrocytes were plated on previously collagen-coated coverslips (see Glass coverslip preparation) at 10 cells/mm^2, for autaptic cultures, and 35 cells/mm^2, for conventional cultures and grew at 5% CO_2, 95% humidity, and 37°C. When 80-90% confluence was obtained, growing was stopped by addition of 0.04 mM 5-Fluoro-2'-deoxyuridine (FUDR, Sigma).

3. Neuronal SNAP-25 knock-out mice preparation

The SNAP-25 null mouse line was generated by M. C. Wilson (Washbourne et al., 2002). Homozygous mutant animals die at birth as a consequence of respiratory failure so embryos were obtained at embryonic day (E) 18 by caesarian section of the mother.

Mutant embryos were readily distinguished by their characteristic tucked position, smaller size and external blotchy appearance and failed to exhibit either spontaneous movement or sensorimotor reflexes in response to mechanical stimuli. The genotype was confirmed by genomic DNA extraction followed by PCR.

Neuronal cultures were prepared from littermates -/- and control (+/+; +/-) animals as following. Brain was dissected from skin and skull and bathed in cold Hank's balanced solution (HBSS, Sigma), buffered with 7mM HEPES (Gibco-Invitrogen, Karlsruhe, Germany). Under binocular preparation microscope, meninges were removed and both brain hemispheres were pulled apart from the diencephalon. The hippocampi appeared as an antero-posterior oriented half-moon structure, dorsally convex, on the inner side of the hemisphere. Striatum was recognized as a rounded body, on the anterior-ventral part of the brain, between the cortex and the foremost extreme of the hippocampus. Tissues were excised with a scalpel and collected in HBSS-HEPES. To avoid cellular damage caused by mechanical tractions between cells during trituration, mass intercellular connections were enzymatically digested by incubating into 0.25% trypsinated HBSS at 37°C for 20-40 minutes. After washing in HBSS-HEPES, the tissue was triturated using fire-polished Pasteur glass pipettes. The quality of the dissociated neurons was controlled by microscope and healthy cells were counted using a haemacytometer. Neurons were plated on a layer of astrocytes prepared as detailed above

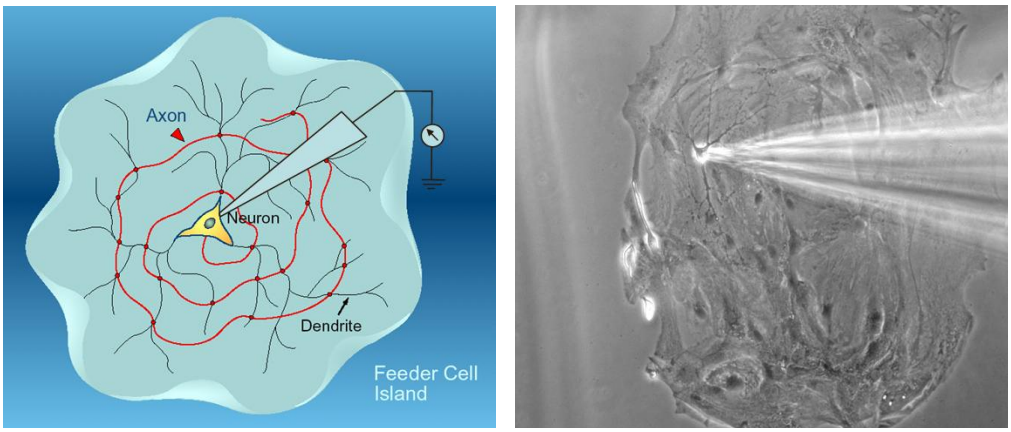


Figure 5
Schematic diagram (left) and example (right) of autaptic neuron.

On top of astroglial island, a single neuron was grown to form all of synapses with its own dendrites. This model synaptic system is ideally suited to determine quantitatively the most important parameters underlying synaptic transmission. Using only one electrode, this system allows to measure evoked release and spontaneous release as well as the action of a wide range of drugs and substances from thousand of synapses at once.

(see Astrocytic supporting cultures), at 1 cell/mm^2 for autaptic cultures and at 15 cells/mm^2 for conventional cultures, containing Neurobasal medium (Invitrogen) supplemented for neuronal survival with 2% B-27 (Invitrogen), 1.8% HEPES, 0.5 mM glutamax (Invitrogen), 100 µg/ml Penicilin/Streptomicine (Invitrogen), 25 µM ß-mercaptoethanol and 100 mM Insulin (Heeroma et al., 2004). Neurons were growing for 10-14 days at 5% CO_2, 95% humidity, and 37°C.

4. Quantifying survival

For quantification of neuronal survival, conventional cultures at equal density (~15 cells/cm^2) were prepared. At 10-14 DIV, cultures were examined using a Eclipse TS100 microscope (Nikon, Melville, NY, USA), with a 10x 1.2NA objective. At least 10 different pictures from random fields were taken using a CCD camera (DS-5Mc, Nikon). Only those neurons exhibiting healthy features were manually selected and counted.

F. Genotyping

1. Genomic DNA purification

The template DNA was obtained from the tail biopsy from embryos and extracted using a standard phenol/chloroform protocol. Tissues were incubated at 55°C, with 600 µl SNET buffers (20 mM Tris-HCl (pH 8.0), 5 mM Na_2 EDTA (pH 8.0), 400 mM NaCl and 1% sodium dodecyl sulfate (SDS)) for lysis of cells, along with 0.17 mg/ml Proteinase K (Roche Diagnostics, Mannheim, Germany), which breaks down polypeptides for better dissolution in phenol. Cell lysates were mixed with the same volume of a 1:1 mixture of phenol and chloroform (USB chemicals, Cleveland, USA). These organic solvents precipitate proteins but leave nucleic acid in the aqueous phase. After 15 min of centrifugation at 13.000 rpm, protein molecules are left as a white coagulated mass at the interface between the aqueous and organic phases. The upper aqueous phase containing nucleic acids was transferred into a clean tube. The DNA in the aqueous phase was precipitated with 0.6 volume of isopropanol and pellets were washed twice with 70% ethanol, and then dried out in a speed vacuum drier (Eppendorf, Hamburg, Germany). Purified DNA was resuspended in 300 µl Tris-EDTA (10 mM

Tris-HCl, 1 mM Na_2-EDTA; TE) buffer (pH 8.0) before PCR was performed.

2. Genotyping PCR

Two separate polymerase chain reactions (PCRs), one for wild-type and other for mutant, were designed for detecting the genotype. The reactions were performed according to Table 3 using a T-gradient® thermal cycler (Biometra, Göttingen, Germany). The amplified fragments were isolated by means of electrophoresis at 120 - 200 V in a 1.8% agarose gel in Tris-Borate-EDTA buffer (TBE: 100 mM Tris-Cl (pH 8.0), 1 mM Na_2EDTA, 90 mM borate). Ethidiumbromide (EtBr) was added in the gel and the PCR products were visualized under UV light. All chemicals used for gel electrophoresis were purchased from Life Technologies (Carlsbad, CA, USA), Gibco BRL (Grand Island, NY, USA), Roche (Indianapolis, IN, USA), Invitrogen (Grand Island, NY, USA) or Sigma (Steinheim,Germany).

- Wild-type reaction

  Forward (SNAP25 Ex. 5A1) CGA AGA AGG CAT GAA CCA TAT CAA C

  Reverse (SNAP25 Ex. 5C1) GCC CGC AGA ATT TTC CTA GTT CCG

- SNAP-25 KO reaction:

  This reaction includes a forward primer in the end of the *neo* gene and a reverse primer in the intron between exon 5a/5b and 6 in SNAP-25. This reaction is therefore specific for a *neo* insertion in the SNAP-25 (SNAP-25 KO).

  Forward (B1for): GCC GCT CCC GAT TCG CAG CG

  Reverse (B1 rev): ACT ATC TGA GAC ACT GAA ATG TCC

WT reaction 5A1/5C1

	94°C	10:00
30 cycles	**94°C**	01:00
	60°C	01:00
	72°C	01:00
	72°C	07:00
	Temperature	*mm:ss*

SNAP-25 KO reaction B1for/B1rev

	94°C	10:00
34 cycles	**94°C**	01:00
	62°C	00:30
	72°C	01:00
	72°C	05:00
	Temperature	*mm:ss*

Per 25 µl reaction

10xPCR Buffer	2.5µl
dNTPs	5 nmol
Primer forward	12.5 pmol
Primer reverse	12.5 pmol
RedTaq polymerase	1 µl
DNA template	100 ng

Table 3
Genotyping PCR reactions

G. FM staining

1. Overview

For the understanding of synaptic function, it is important to visualize the processes occurring at the synapse. However, as we gain detailed structural information, we lose detailed information about functional aspects, and viceversa. For example, the time resolution of conventional light microscopic techniques allows fine dynamic measurement in a multitude of systems, however the optical resolution limit of conventional light microscopic is only slightly larger the diameter of hippocampal boutons, which limits the resolvable details to the whole-synapse level. To overcome this problem, specific markers can be used to detect events related to synaptic function using these techniques.

Lichtman and colleagues first described the activity-dependent uptake and release of sulforhodamine and other fluorescent dyes in reptilian preparations (Lichtman et al., 1985). However, the development of styryl dyes that stain synaptic vesicles in an activity-dependent manner made possible the visualization of synaptic function in a variety of preparations (Betz et al., 1996; Cochilla et al., 1999). Styryl dyes are molecules consisting of a hydrophilic head group with a double positively charge pyridinium residue and a lipophilic tail group of variable length (Figure 6B). Whereas the lipophilic part can

easily penetrate in the bilipid layer, the positively charged headgroup prevents the dye from passively diffuse across the membrane. This structure permits the molecule to insert

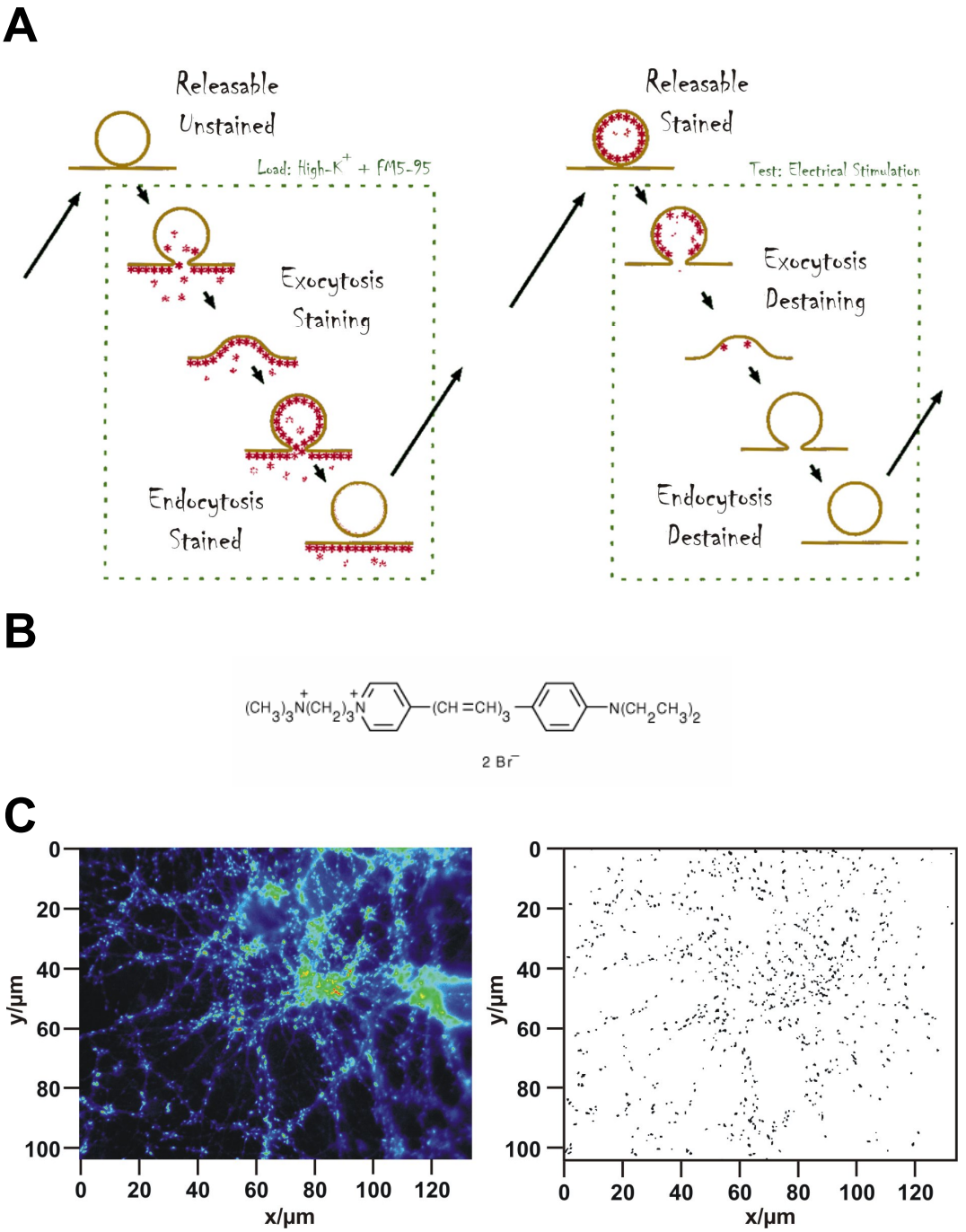


Figure 6
Use of FM dyes in the detection of vesicle recycling.

(A) Experimental procedure to selectively stain and destain synaptic vesicles. Insertion of the FM dyes (red) into the plasma membrane (brown) causes a fluorescent increase of the dye. (*left panel*) Protocol for loading the dye (*right panel*) Destaining of a successfully stained vesicle. Modified from Ryan et al., 1993 **(B)** Chemical structure of the red fluorescent N-(3-trimethyl-ammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM 5-95) **(C)** Automated detection of functional boutons by a wavelet-generated mask. (*left panel*) Image of axonal arborizations during electrical stimulation. (*right panel*) Corresponding wavelet mask identifying active synapses as black spots.

reversibly into the outer leaflet of exposed membranes. In addition, the presence of the dye in the membrane produces a dramatic increase in quantum yield (about 350 times), which allows the imaging of stained membranes while the dye is present in the external solution. In this study, the red-shifted styryl dye FM5-95 (Molecular Probes) was used to stain synaptic vesicles since its far-red emission does not interfere with the imaging of the green fluorescence of the reporter gene, eGFP. FM5-95 can be internalized by endocytosis and then, after washing off non-internalized dye, released by subsequent rounds of exocytosis (Figure 6A). Uptake of the dye reflects endocytosis, while the extent of exocytosis is reflected by the loss of fluorescence from labeled vesicles during departitioning from the vesicle's luminal membrane into the extracellular solution.

2. Epifluorescence FM 5-95

Coverslips from conventional hippocampal cultures of 10-14 days *in vitro* were mounted in a perfusion chamber on a movable stage of an inverted microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany). Cells were perfused at room temperature in standard extracellular solution (in mM: NaCl, 140; KCl, 2.4; HEPES, 10; glucose, 10; $CaCl_2$, 4; $MgCl_2$, 4; 300 mOsm, pH 7.3). To prevent recurrent activity, 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 µM $_{D,L}$-2-amino-5-phosphonovaleric acid (APV) were added to the medium. Synaptic boutons were labeled by electric field stimulation (1 ms current pulses of 40 mA and alternating polarity delivered by platinum electrodes spaced ~15 mm) in saline containing 10 µM FM 5-95, following by additional 60 seconds of dye exposure to ensure complete labeling of all recycling vesicles. Individual boutons were imaged after 10 minutes perfusion with dye-free external solution. Destaining of hippocampal terminals was achieved by three electrical trains of 400 electrical stimulations at 10 Hz using the same stimulation as for loading. Images were taken using a cooled slow-scan CCD camera (PCO SensiCam, Kelheim, Germany) on an Axiovert 135 TV inverted microscope with a 63x 1.2 numerical aperture water-immersion objective (both Zeiss, Oberkochen, Germany) and a modified filter set (DCLP 495, BP 525/50 for eGFP and DCLP 565, LP 620 for FM 5-95). FM 5-95 was excited at 475 nm by repetitive Xe-arc lamp illumination (Polychrom II; T.I.L.L. Photonics, Germany).

To avoid the bias introduced by manual selection of functional boutons, an automated detection algorithm was used (Bergsman et al., 2006), kindly provided by M. Wienisch. The average image from the baseline sequence was subjected to an à-trous wavelet transformation with the level k=4 and detection level l_d=1.0 (Olivo-Marin, 2002), resulting in a segmented mask image (Figure 6C). Spots on mask images, each representing putative functional boutons, were identified, and only masks with areas between 4 and 20 pixels were accepted for calculating single bouton fluorescence transients. Time courses of all automatically identified spots were visually inspected for correspondence to individualbouton destaining.

H. Immunocytochemistry

1. Immunostaining

Immunostaining is a technique used to reveal a specific protein on the cell. A primary antibody is used to recognize a specific epitope on the protein of interest. Unspecific binding is inhibited additionally by the use of blocking solution containing either protein or detergent blocking agents. To detect the antigen-antibody binding reaction, a fluorescence-labeled secondary antibody is used, which binds to the first unlabelledantibody (indirect staining method).

Hippocampal neuronal cultures were fixed for 1h at room temperature in PBS containing 4% paraformaldehyde. They were washed twice in PBS, incubated for 10 minutes with 50 mM NH_4Cl in PBS to block free aldehyde groups, and washed again. Cultures were incubated for 1h with primary antibodies raised against SNAP-25 (rabbit polyclonal; recognizing both SNAP-25a and b) and synaptophysin (mouse monoclonal; Synaptic Systems, Göttingen, Germany, kindly gift of Prof. Dr. R. Jahn) diluted 1:400 and 1:200, respectively, in PBS containing 1% BSA fraction V (PBS-BSA). They were washed four times 10 min with PBS and then incubated for 1h with secondary antibodies diluted 1:1000 in PBS-BSA (Alexa 546-coupled goat-anti-rabbit and Alexa 647-coupled goat-anti-mouse, Molecular Probes, Eugene, OR). Cultures were finally washed four times in PBS and kept at 4°C overnight for the posterior analysis in a confocal microscope.

2. Confocal scanning microscopy principles

The study of the morphological characteristics of the neurons requires the visualization of hippocampal boutons and other neuronal structures that are at the optical resolution limit of the standard fluorescence microscopy. Due to the wave property of light, the image formed by a point light at the focal plane in a conventional microscope, is not a single point but it is distributed in an Airy pattern in a possible area of the image plane. The width at half amplitude of the first lobe of the Airy pattern is defined as the full width at half-maximum (FWHM) and it is often used as an estimate of the resolution power of the lens

$$FWHM_{lateral} = \frac{0.51}{NA} \cdot \lambda$$

Equation 1

with λ being the wavelength in free space and NA, the numerical aperture of the lens.

Confocal scanning microscopy increases resolution by producing the optical sectioning of the sample. This effect is achieved by placing a small pinhole in front of the detector, in a plane conjugated to the focal plane. Regions of the sample that are not in focus will appear defocused, and light rays originating in these regions will be projected off-center onto the ”pinhole” wall, thus being filtered out. Only in-focus light can pass through the pinhole. The pinhole in a confocal microscope makes possible to influence the optical performance of the microscope. At large pinhole sizes (greater than the diameter of the central lobe of the Airy pattern), the lateral and axial in-focus resolutions are not much different from a standard microscope, although the remaining out-of-focus fluorescence is still blocked by the pinhole. At infinitely small pinhole sizes, the axial resolution is critically dependent on the pinhole size. According to Equation 2 and 3, the optical lateral resolution scales with the first order of NA whereas the axial resolution scales with the second order, as calculated by

$$FWHM_{lateral} \approx \frac{0.37}{NA} \cdot \lambda_{em}$$

Equation 2

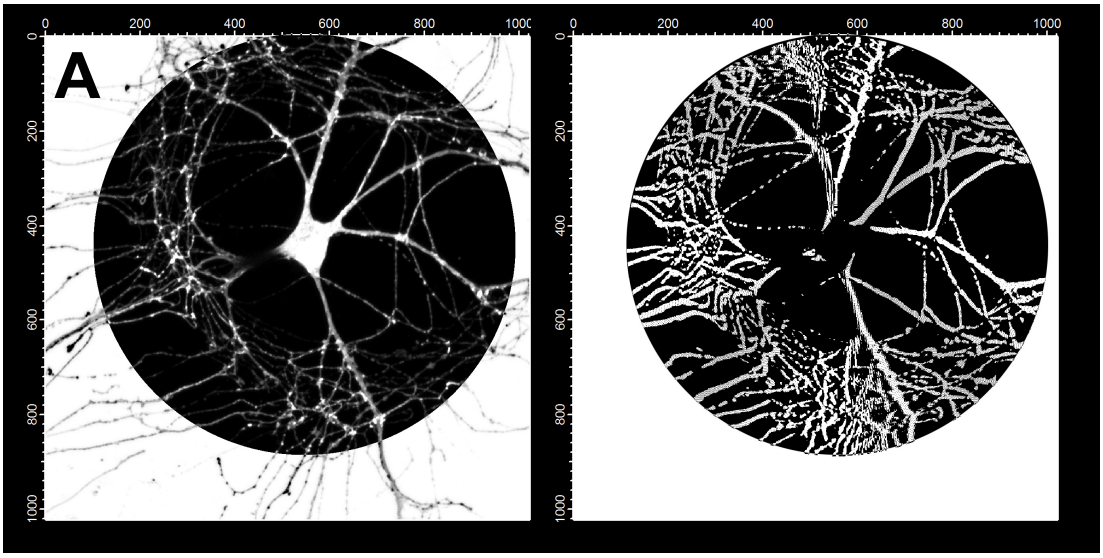
$$FWHM_{axial} \approx \frac{0.64}{n - \sqrt{n^2 - NA^2}} \cdot \lambda_{em}$$

Equation 3

where λ_{em} is the fluorescence emission wavelength and n is the refractive index of the medium. However, the resolution advantage is often compromised by the vulnerability to noise due to its reduced detecting volume and general weaker signal.

3. Data acquisition and analysis

Immunofluorescence images were taken with a confocal microscope (LSM 410 controlled by LSM 3.98 software attached to an Axiovert 135TV, Zeiss, Oberkochen, Germany). Argon lasers were used for exciting at 488 nm and at 543 nm and a helium-neon laser for 633 nm excitation. Emission wavelength was filtered at 510nm, 570 nm and 665 nm, respectively. Images were taken using a 63X oil immersion (1.4 NA) objective at 1024 X 1024 pixels. Contrast and brightness were adjusted to standard values for sections intended to quantify SNAP-25/SNAP-23 expression. Images were imported into IgorPro (WaveMetrics Inc.) and analyzed with custom-written IgorPro-functions.



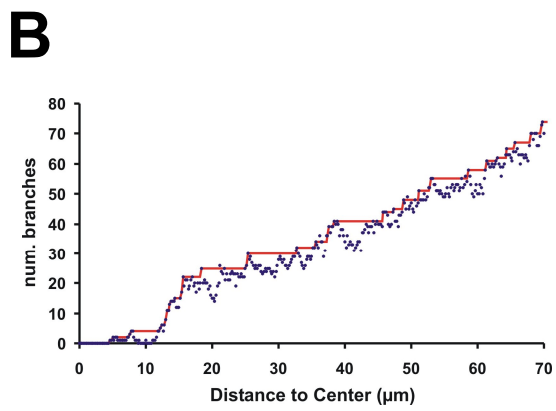


Figure 7
Quantification of neuronal outgrowth

(A) *(left panel)* Example of hybrid median filtered maximal projection of a confocal stack. Inner black circle represents the area considered for analysis. *(right panel)* Mask imaged generated from the left picture obtained by detecting the changes over threshold on the first derivative of the fluorescence intensity. **(B)** Example of a plot showing number of branches detected as a function of distance to the soma (blue dots). Considering that each branch divides into two daughter branches, data can be reduced to single steps involved in the expansion(red trace).

The number of neuronal branches was calculated from the number of eGFP-positive processes that crossed a circumference centered in the soma from a hybrid median filtered maximal projection of a confocal stack. Crosses were distinguished by detecting changes over threshold on the first derivative of the fluorescence intensity level (Figure 7). The number of synapses was calculated from synaptophysin-positive individualregions using same procedure as for FM detection (Figure 6C).

I. Electrophysiology

1. Overview

Neuronal function derives mainly from the electrical properties of its membrane. The impermeable lipid bilayer is an insulator element that separate two different ionic solutions, however, it is permeated by the presence of ionic channels, producing a potential difference. According to Goldman-Hodgkin-Katz's equation, this potential is

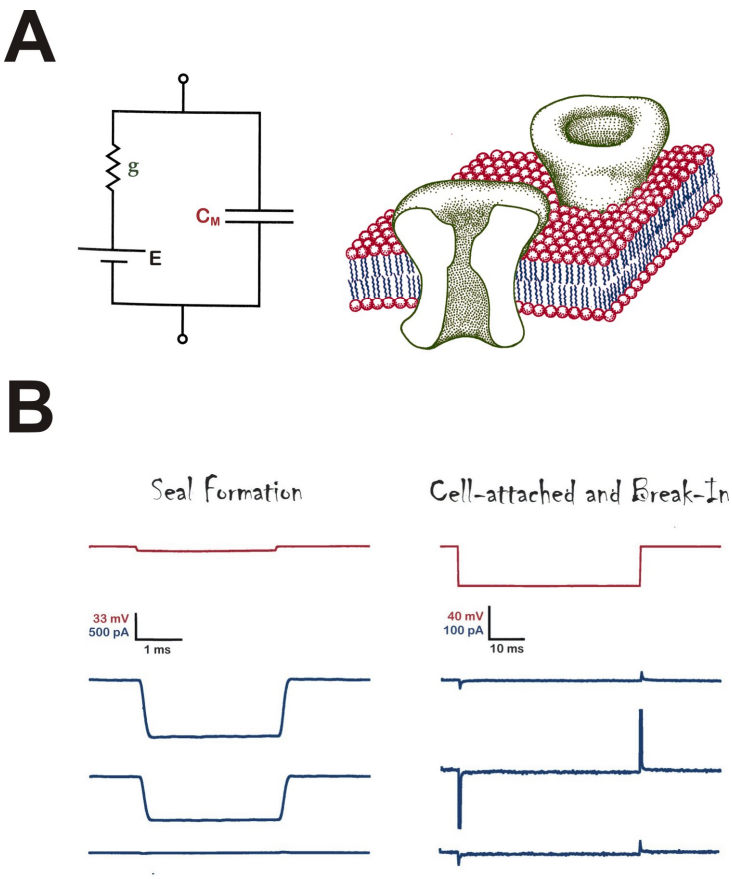


Figure 8
Basis for electrophysiological measurements

(A) In electrical experiments, the membrane acts like an equivalent circuit with two branches, in which specific ionic channels are represented as conductive elements (green) and the cell membrane, as capacitors (red). The driving force of the circuit is provided by the equilibrium potential of the ionic population. Modified from Hille, 2001 **(B)** Example signals observed during sealing and break-in. The top voltage traces (red) show the test pulse applied. The resulting current traces are shown below (blue). (*left column*) During sealing, from top to bottom, the pipette is initially in the water bath. The amplitude of the current proportional too the pipette resistance according to Ohm's law. When the pipette touches the membrane of the cell, the current amplitude decreases. After applying suction, the current signal virtually disappers, indicating the formation of the 'giga-seal'. (*right column*, from top to bottom) During cell-attached configuration, capacitive transients are visible and they should be compensated. Subsequently, a pulse of suction breaks the membrane and produces an increase in the current amplitude. Finally, transients corresponding to the cell are cancelled and series resistance corrected. Modified from Rudy and Iverson, 1992.

controlled by the change in membrane permeability through the opening and closure of ionic channels. The whole membrane-channels system of the neuron can be reduced to an equivalent electrical circuit, in which channels would act as conductive elements and membrane as a capacitor that accumulates charges (Figure 8A). This mathematical model allows the calculation of the passive electrical properties of the system as if it was an electrical circuit.

The ionic movement can be transformed to a flow of electrons using an electrode. The most common electrode type used is a silver/silver chloride (Ag/AgCl) electrode, consisting on a silver wire, coated with silver chloride. When a chloride ion (Cl^-) combines with a silver atom (Ag) produces the formation of silver chloride (AgCl), liberating an electron (e^-) from the oxidation of Ag. This reaction is reversible so that the flow of an electron to the electrode AgCl pellet produces free Cl^- ions that become hydrated and enter in the solution. Two electrodes are needed, therefore, to close the circuit; one producing the Cl^- ions and other accepting them and generating the electron outflow. With the development of electrophysiological measurements, in special the patch-clamp technique (Neher and Sakmann, 1976), one of these electrodes, the measuring electrode, could be approached to close contact with the cell surface. This allowed, for first time, the measurement of minuscule single channel currents but made also possible the access to the intracellular compartment of small neurons. This technique encloses the measuring electrode within a small glass pipette, which contains similar ionic concentration as the intracellular one. Using a sequence of heatings and mechanical pulls, it is possible to break the glass pipette in such a way that the tip of the pipette is as small as 1 μm. As the pipette holding the electrode is advanced toward the cell in the external bath, the electrical resistance of the tip aperture can be monitored by applying a small voltage step to the pipette (Figure 8B). When the pipette touches the cell surface, there is an increase in resistance. Applicationof suction in the interior of the pipette brings a small patch of membrane into the pipette, raising the resistance until virtually no current can pass between the pipette and the electrode. In this situation, called "giga-seal" due to the resistance over than 1 GΩ, a brief strong suction pulse provokes the rupture of the patch of membrane trapped inside the pipette, resulting in continuity between the pipette and the cytoplasm. This step is observed by a sudden increase in capacitative current in

response to a test voltage step. According to the equivalent circuit, if the access resistance is negligible comparing to the membrane resistance, the membrane potential corresponds in this situation to the potential between both electrodes supplied by the measuring system. In this situation, called whole cell voltage clamp, current is proportional to membrane conductance and can be measured therefore in a low background noise. However, in most of the cases, there is a potential drop across the micropipette due to a non-zero resistance and it needs to be corrected using a signal proportional to the measured current.

2. Experimental condition

Autaptic cells between 10 to 14 days in vitro were used for experiments. The patch-pipette solution included (in mM): K-Gluconate, 135; HEPES, 10, EGTA, 1; $MgCl_2$, 4.6; Na^+-ATP, 4; creatine phosphate, 15; phosphocreatine kinase, 50 U·ml^{-1}; 300 mOsm, pH 7.3. The standard extracellular medium consisted of (in mM) NaCl, 140; KCl, 2.4; HEPES, 10; glucose, 10; $CaCl_2$, 4; $MgCl_2$, 4; 300 mOsm, pH 7.3. Cells were whole-cell voltage-clamped at -70 mV with an EPC-9 amplifier (HEKA, Germany) under control of Pulse 8.70 program (HEKA, Germany). Currents were low-pass filtered at 2.87 kHz and stored at either 10 or 20 kHz. The series resistance was compensated 75%. Only cells with series resistances below 15 MΩ were analyzed. The patch-pipettes were made of borosilicate glass and pulled using a multi-step puller (P-87, Sutter Instr., Novato, CA, USA). The pipette tip diameter was kept at around 2 µm; the resistance ranged from 2.5 to 3.5 MΩ. Solutions were applied using a fast-flow system that provides reliable and precise solution exchanges with time constants of approximately 20-30 ms (Rosenmundet al., 1995).

3. Stimulation Protocols and Electrophysiological parameters

a) Evoked response

Neurons were somatically voltage-clamped in the whole cell patch clamp configuration at a holding membrane potential of −70 mV. The cells were depolarized to 0 mV for 2 ms to evoke action potentials, leading to an immediate Na^+ inward current followed by K^+ outward current. Depolarization of membrane and subsequent action

potential propagation induces Ca^{2+} influx in the presynaptic terminal which triggers a cascade of events evoking neurotransmitter release which can be detected as a post-synaptic inward transient of quantal events normally with a 2-3 ms synaptic delay (EPSC in case of excitatory cells; Figure 9A). As standard procedure, excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs, respectively) were recorded every 10 seconds to monitor the quality of voltage clamping and evaluate non-specific changes in EPSC amplitude such as the influence of time-dependent run-down, leaky seals, etc. The height of the peak was measured for EPSC amplitude. The postsynaptic response over a second was integrated, yielding the charge, and fitted to a double exponentialfunction as follows:

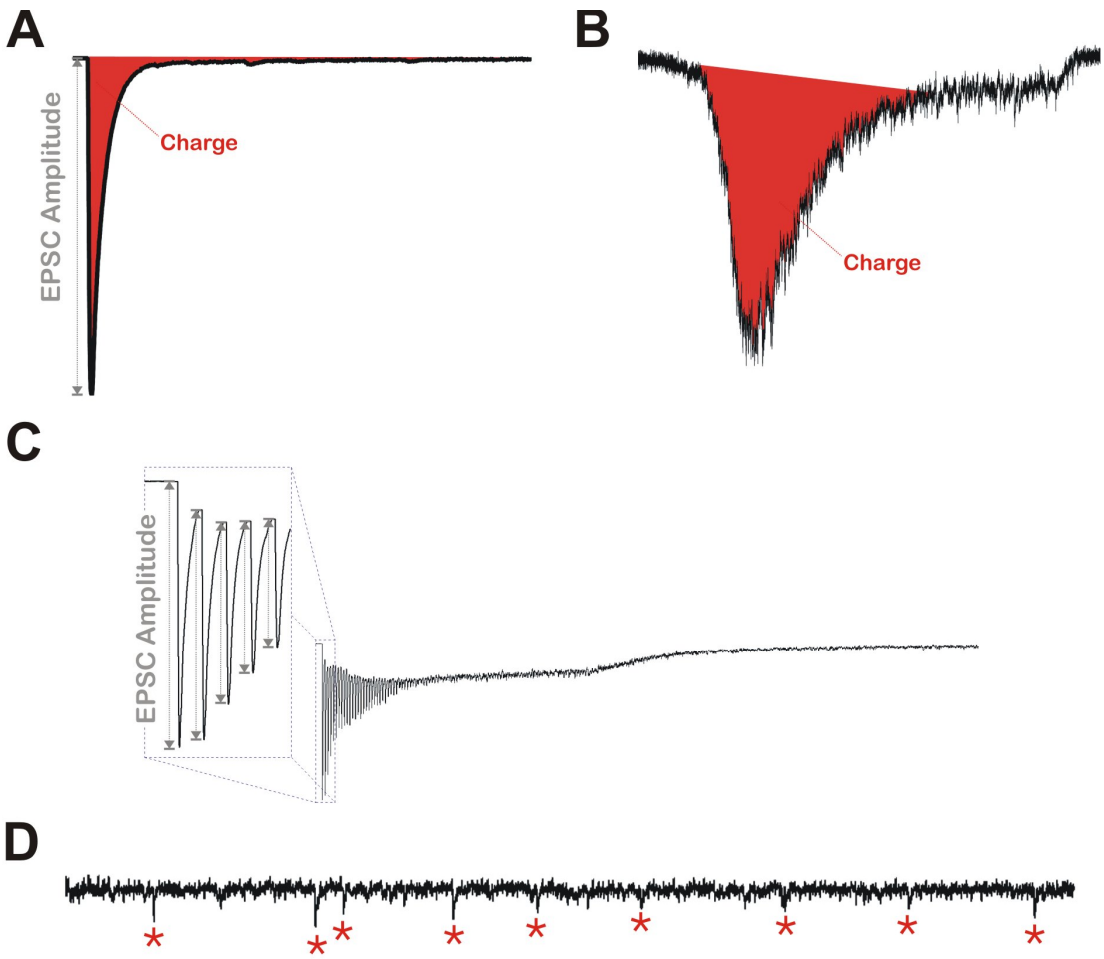


Figure 9
Measuring protocols in autaptic cultures

(A) Example of an EPSC evoked after a single stimulation. ESPC amplitude is measured as the peak amplitude after baseline substraction (grey). Charge is calculated by integrating the EPSC over time (red). **(B)** Example of a trace evoked by 500 mM sucrose application for 3.5 seconds. Readily releasable pool is estimated by measuring the charge of the transient component (red) **(C)** Example trace of 100 stimulations at 40Hz. The EPSC amplitude for each stimulation is calculated from the peak to the baseline of actual stimulus (inset) **(D)** Example of recording of spontaneuos release. Single fusion events (red asteriks) are detected using an automated detection algorithm.

$$y(t) = y_0 + A_0 \cdot e^{\frac{-t}{\tau_0}} + A_1 \cdot e^{\frac{-t}{\tau_1}} \qquad (2 \cdot \tau_0 \leq \tau_1)$$

Equation 4

with A_0 and A_1 being the charge of the synchronous and asynchronous component, correspondingly, and t_0 and t_1, the time constant of the kinetics for synchronous and asynchronous release, respectively.

b) Determining the size of readily releasable pool and vesicular release probability

As a manner of determining the total charge of readily releasable pool, the charge induced by hypertonic solution was measured as described in Rosenmund and Stevens, 1996. 500 mM sucrose was extracellularly applied for more than 3 s using the fast flow application system. This application induces a calcium-independent release of vesicles, here denominated 'sucrose pool', which leads to a transient followed by a steady state inward current (Figure 9B). Since the transient part consists of a burst-like release of all fusion-competent, primed vesicles, the integral of the transient component, after subtraction of steady state component, approximates the total charge of the readily-releasable pool (Figure 9B). The sustained component induced by sucrose application is believed to represent the release of vesicles that have just been primed into the readily-releasable pool (Reim et al., 2001).

To determine the vesicular release probability, 500 mM sucrose were applied for 3.5 sec, 3 sec after evoking the action potential. The vesicular release probability was calculated as the charge released by an action potential divided by total charge of the readily releasable pool.

c) High frequency train stimulation

To explore the different short-term properties at different inter-pulse intervals, 10Hz, 40Hz or 50Hz train were applied. The peak amplitude of each EPSC during the train was measured as shown in Figure 9C. The readily releasable pool and the replenishment rate were calculated from the linear fit to the sustained phase in the cumulative trace from the evoked amplitudes measured during 40Hz stimulation, as described in Differential control of the releasable vesicle pools by SNAP-25a and SNAP-

25band Figure 15.

d) Spontaneous release

Recording of miniature EPSC (mEPSC) was performed at -70 mV for at least 60 seconds (Figure 9D) in the presence of 200 nM tetradotoxin (TTX) to completely block the possibility of undesired action potentials. Spontaneous events were detected using an event detection algorithm (Clements and Bekkers, 1997) on 2 kHz digitally filtered traces, kindly provided by H. Taschenberger. A standard mEPSC template with variable amplitude is first defined for this algorithm according to the next equation:.

$$Template(t) = \left(1 - e^{-\frac{t-t_0}{\tau_{rise}}}\right) \cdot \left(FractionFast \cdot e^{-\frac{t-t_0}{\tau_{DecayFast}}} + (1 - FractionFast) \cdot e^{-\frac{t-t_0}{\tau_{DecaySlow}}}\right)$$

Equation 5

with τ_{rise} being the time constant of the rising phase of the template; $\tau_{DecayFast}$ and $\tau_{DecaySlow}$ being the time constant of the fast and slow falling phase of the template, respectively; *FractionFast* being the ratio between the fast and slow falling phase of the template; and t_0 being the onset of the template. The detection criterion is calculated from the template scaling factor (*scale*) and from the goodness-of-fit (*SSE*, sum of squares error) between the scaled template and the data. The standard error (*StdError*) is derived between the data points and fitted template:

$$criterion = \frac{scale}{StdError}; \quad StdError = \frac{1}{\sqrt{\frac{SSE}{N-1}}}$$

Equation 6

where N is the number of points in the template. When the template is aligned with and accurately fitted to a synaptic event in the data, the numerator, *scale*, approximates the peak amplitude of the event and the denominator approximates the noise standard deviation. Thus, the detection criterion is related to the signal-to-noise ration for the detected events. Only events which criterion was greater than 3.5 times were accepted, since this value allows the detection of most of the events with a low false-positive rate (Clements and Bekkers, 1997). After computer detection, events were individually verified before they were considered mEPSC. The mean mEPSC of a cell was

calculated by averaging all detected events.

J. Analysis program

The only available analysis program for neuronal studies, Axograph 4 (Axon Instruments, Union City, CA), is arduous and wearisome when mass analysis is required. Furthermore, the variety of data collected in this study required a program with high degree of flexibility. For that reason, a completely new set of procedures, called Neurignacio©, had to be developed from scratch. For this purpose, IgorPro 5.0.3 (WaveMetrics, Inc.) was preferred since it offers a wide range of built-in operations and function that facilitates the programming work. The latest revision, Neurignacio© 0.1b, comprised more than 150 functions in about 6000 lines of code and covered the analysis of electrophysiological recordings as well as FM and confocal images, allowing for batch-processing of the data. Due to time restriction, this version, although completely functional, was not totally optimized.

K. Statistics

Results are shown as mean±S.E.M, with *n* referring to the number of cells from each group. Since a significant between-preparation variability was found in the electrophysiological parameters examined, significance was tested by two-way ANOVA, where the genetic background (knock-out or wildtype) and the expressed isoforms were defined as a fixed factor, and the culture used defined as an orthogonal 'random' factor. In the case of more than one level of the fixed factor, appropriatepost-tests were used.

VII. RESULTS

A. Long term expression of SNAP-25 homologues recovers survival of Snap25 null neurons

The only study on function of SNAP-25 in hippocampal cultures used a Semliki Forest Virus (SFV) vector to over-express the chick homologue into rat neurons, resulting in an impairment of neurotransmission (Owe-Larsson et al., 1999). This work, which main purpose was the test of SFV as a tool for the study synaptic transmission, did not consider whether the effect observed was independent from the isoform used. In order to investigate this question, I reproduced that experiment, over-expressing each SNAP-25 isoform in wild-type mouse hippocampal. The result showed a reduction in the excitatory post-synaptic current (EPSC) by over-expression of SNAP-25a (SNAP-25a SFV-overexpression: 2.55±0.96 nA, n=11; control: 9.04±1.96 nA, n=10, $p<0.001$; Mann-Whitney U-test) or SNAP-25b (SNAP-25b SFV-overexpression: 2.33±0.88 nA, n=21; control: 4.95±0.790 nA, n=27, $p<0.001$). The precise mechanism underlying this inhibition is unknown yet. As explanation, it has been suggested that the supra-physiological levels of SNAP-25 after SFV overexpression would cause an overload of the palmitoylation machinery, leading to a collapse of the exocytic machinery. This side-effect, generated by an excess of protein, has hitherto prevented mutagenetic analysis of SNAP-25 in neurons. For this reason, among others, it has been for long proposed that the appropriate strategy should consist on the use of a genetically clean background, such the one offered by the *Snap25* null mouse (Washbourne et al., 2002).

Since *Snap25*$^{-/-}$ homozygous mice die perinatally, hippocampi were obtained from embryos at E18 fetal stage. Cultures from -/- mutants (knock-out, KO) and control mice, heterozygous and wild-type embryos, were prepared from the same litter and studied in parallel. In agreement with previous observations when characterizing the mutant mouse (Washbourne et al., 2002), most -/- neurons died after several days in culture. The survival was only 1.3±0.7% (n=5, $p<0.001$ Student's t-test, Figure 10B, left panel), when compared to the cultures from control neurons (set equal to 100% survival), by the time that neurons have already established mature connections (after 10-

14 days *in vitro*, DIV). Wild-type and heterozygous neurons, on the other hand, survived normally for the whole duration of the culture. The few *null* neurons that survived seemed to present severe developing disturbances, such smaller body and defective arborization. Previous results showed that degeneration of the neurons was progressive after 5-7 DIV and it was not prevented neither by promotion of synaptic activity with 25 µM glutamate or 0.25 µM AMPA nor by prolongation of depolarized state using 25 mM KCl (Washbourneet al., 2002).

The decreased viability of the SNAP-25 knock-out neurons recommended the use of a stable lasting expression system for rescue, which would enable neurons to develop properly and which on-set of the expression is fast enough to reach a sufficient level of SNAP-25 before neurons start to die. To produce such a long and steady early expression of SNAP-25, a new long-term transfection approach had to be adopted. Table 4 shows the summary of the methods available and the success of the transfection in different cellular systems. The classical approach consists on the coprecipitation of a plasmid DNA containing a transfer gene with calcium phosphate so that it can be incorporated into the neuron (Kohrmann et al., 1999). This method produced an adequate expression level of the reporter gene, eGFP, in hippocampal neurons; however, the transfection efficiency (~5-20%) was too reduced for an efficient use in autaptic cultures. Other chemical methods, such lipofectamin transfection, may cause citotoxicity (Washbourne and McAllister, 2002), which would have been impossible to differentiate from the SNAP-25 intrinsic effect on survival, and were not considered. A novel magnetic-assisted transfection method has been recently developed. In this system, DNA is associated to

Table 4
Comparison of gene transfer methods in different cell systems

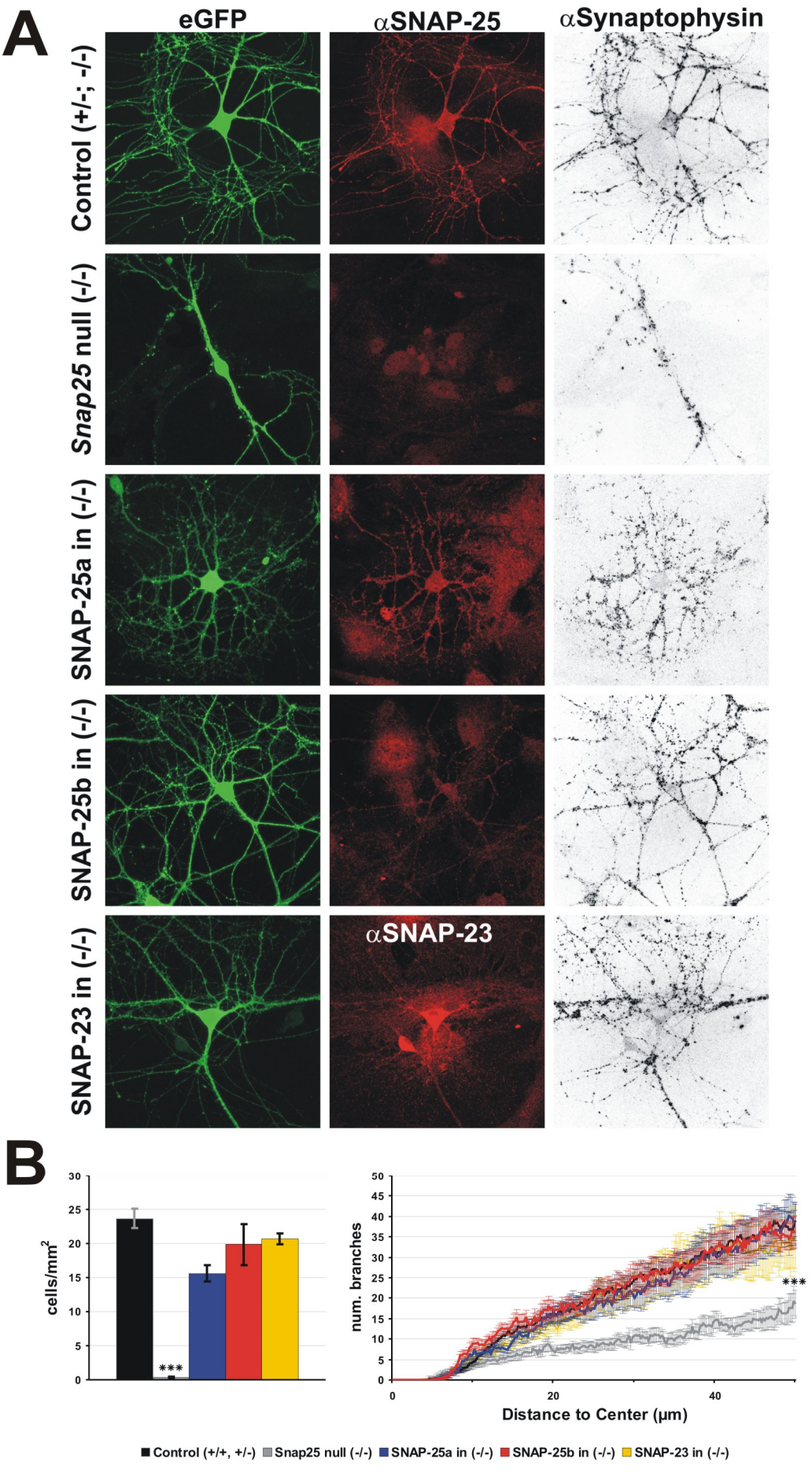
	Human Embrionic Kidney cells	Chromaffin cells	Hippocampal neurons culture
Lentivirus	✓	✓	✓
Adeno-associated virus	☑	×	☑
Adenovirus	☑	☑	☑
Calcium Phosphate	✓	?	✓
Magnetic Assisted Transfection	×	×	×

✓ Transfection was succesful
☑ Success was previously reported
× Transfection was unsuccesful
? No information

magnetic nanoparticles which, then, by application of a magnetic, can be incorporated into the cell leading to the expression of the transgene (Bertram, 2006). However, my attempts in different cell systems did not produce a successful transfection. To increase the transfection efficiency, viral methods had to be adopted instead. Although different viruses have been successfully tried for the delivery of plasmid DNA into neurons (Hermening et al., 2006; and Table 4), the lentivirus system appeared to be the most suitable for my purposes. This system relies on a replication-incompetent HIV-1-based virus to produce a stable, long-term expression of the gene of interest in either dividing or non-dividing mammalian cells (Naldini et al., 1996a; Naldini et al., 1996b; Blomer et al., 1997); and see above, Lentivirus, for an overview). The main advantages that this system offered over others were high-infection rate, moderate expression levels and simplicity of use.

Constructs from the splice-variants SNAP-25a and SNAP-25b were cloned using three different strategies to exclude a possible negative effect of eGFP in the SNARE complex formation (see Figure 4). I also wanted to differentiate the effect of the SNAP-25 isoforms from the one of SNAP-23, a homologue that supports constitutive secretion and might substitute SNAP-25 in GABAergic neurons (Verderio et al., 2004) and, thus, it was cloned in the same way. Results obtained by the alternative constructs were indistinguishable. In addition, vectors expressing only eGFP, with either promoter, were also produced as control.

Neurons were infected with these constructs during the first day in vitro and examined 10-14 days later. The working infection rate was estimated to be 90-100%. Using this system, the survival of knock-out neurons in culture raised to approximately normal levels. The culture cell density increased to 83±12.7% (n=6) of the control value by expression of SNAP-25b. A similar survival was observed by SNAP-23 over-expression (87.3±3.3%, n=5) and approximately equal values happened when SNAP-25a was used (65.9±5.0, n=7), Figure 10B, left panel. These results show that SNAP-25 function is critical for neuronal viability and it cannot be substituted by endogenous expression of the other homologues. However, over-expression of SNAP-23 overcomes the absence of SNAP-25 and it succeeds in the recovery of the survival.


A
eGFP
αSNAP-25
αSynaptophysin
Control (+/-; -/-)
Snap25 null (-/-)
SNAP-25a in (-/-)
SNAP-25b in (-/-)
SNAP-23 in (-/-)
αSNAP-23
B
cells/mm²
***
num. branches
***
Distance to Center (µm)
Control (+/+, +/-)
Snap25 null (-/-)
SNAP-25a in (-/-)
SNAP-25b in (-/-)
SNAP-23 in (-/-)

B. SNAP-25 has an active role in neuronal morphogenesis

The expression of each homologue in the rescued neurons was verified by immunostaining with a specific antibody against SNAP-25 (which recognized both isoforms) or SNAP-23. Expression level was determined from a confocal section of the soma, with identical scanning conditions in all the cases, and compared to wild-type neurons. Such measurements confirmed that the levels of SNAP-25 and SNAP-23 in *null* neurons reached by lentiviral expression were similar to those in control neurons (data not shown).

The function of SNAP-25 in neurite outgrowth was assessed by examining the morphometric differences observed between the knock-out and the rescued cultures. Two parameters were analyzed: the extension of the neurite tree and the number of synapses. To avoid the expression of the reporter gene in the underlying astrocytic layer, I designed a lentivirus in which eGFP expression was driven by a synapsin-I promoter (see Figure 4). The extension of the neurite outgrowth was calculated as the number of branches that crossed a circumference centered in the soma in a confocal projection image. In control neurons, I counted 38.2±5.1 branches (n=13) in a 50 µm radius circumference (Figure 10A and B, right panel). However, only 18.5±3.4 branches were detected in surviving *Snap25*$^{-/-}$ neurons (n=8, $p<0.001$ Student's t-test). This finding demonstrates the involvement of SNAP-25 in final neuronal arborization. When I next compared neurons rescued with the different homologues, I found that arborization was completely restored by all of them. The number of branches at 50 µm was 36.3±3.8 (n=7) in SNAP-25b rescued neurons, 38.2±5.1 (n=6) in SNAP-25a rescued and 37.5±7.8 (n=6) in SNAP-23 rescued (Figure 10A and B, right panel).

Figure 10
Elimination of SNAP-25 leads to impaired neuronal survival and outgrowth

(A) Double staining for SNAP-25 or SNAP-23 and synaptophysin as a synaptic marker of primary cultured hippocampal neurons infected with recombinant lentiviruses. A synapsin-promotor was used to restrict eGFP expression to neurons and enable morphologycal analysis. This revealed inferior outgrowth/branching in *Snap25*$^{-/-}$ neurons compared to control (+/+; +/-). Nevertheless, neurons lacking SNAP-25 still formed synaptophysin-positive synapses. Expression of SNAP-25a, SNAP-25b or SNAP-23 in *Snap25* null neurons recovered the morphology. **(B)** *(left panel)* The number of cells (mean±SEM) remaining after 10-14 days in culture. Survival of null neurons was dramatically reduced, but rescued by SNAP-25a, SNAP-25b or SNAP-23 expression. *(right panel)* The number of branches (mean±SEM) as a function of the distance to the soma was significantly depressed in *Snap25* null neurons ($p<0.001$, Student's t-test) compared to control neurons or null neurons rescued with SNAP-25a, SNAP-25b or SNAP-23

In addition, I observed that the number of branches detected with this method increased with the distance (Figure 10B, right panel), which would correspond to the expansion of the dendritic tree. If we consider that each branch dichotomizes into two daughter branches, we could reduce the trace obtained by plotting the number of branches over distance to the single steps involved in the expansion and, therefore, being able to calculate the number of segments and bifurcations that shape the tree (Figure 7B). As expected, the total number of branches within 50 µm was significantly reduced in the *Snap25*$^{-/-}$ neurons (183±41 branches, $p<0.001$ Student's t-test) comparing to control neurons (529±76 branches). Recovery was produced by each homologues and indistinguishable among them (509±102 branches for SNAP-25a, 470±66 for SNAP-25b and 464±112 branches for SNAP-23). The number of bifurcations in the neurite tree estimated here was also different between the *null* neurons (22±3 divisions, $p<0.001$ Student's t-test) and the rest of the groups (42±4 divisions for control neurons, 43±6 for SNAP-25a rescue, 42±4 divisions for SNAP-25b rescue, and 39±7 divisions for SNAP-23 rescue).

Additionally, I used an antibody against a synaptic-specific protein, in this case, synaptophysin, to estimate the number of synapses (Figure 10A, right column). I detected 390±44 ($n=20$) synaptophysin-positive synapses in control neurons but only 217±27 in Snap25 null neurons ($n=16$, $p<0.002$, Student's t-test). The expression of any of the SNAP-25 increased the number of synapses to control values. 536±93 ($n=10$) synaptophysin-positive synapses were detected when rescued with SNAP-25b, 607±152 ($n=8$) synapses with SNAP-25a and 443±78 ($n=9$) with SNAP-23. No significant differences amongst them were found. At this point, it is important to notice that the number of synapses in knock-out neurons was decreased in a parallel way as the total number of branches, which would suggest that SNAP-25 is, in fact, not required for synaptic formation but for neuronal morphogenesis.

C. Synaptic vesicles are functionally incompetent for stimulation-dependent recycling in absence of SNAP-25

I continued by investigating whether synapses retained functionality in the *Snap25* null neurons and following rescue. For that, I assayed stimulation-induced uptake of FM 5-95 as a measure of exo-endocytosis function in the cultured neurons (Cochilla et al., 1999; see FM staining). Following a train of 400 action potentials, staining was absent in

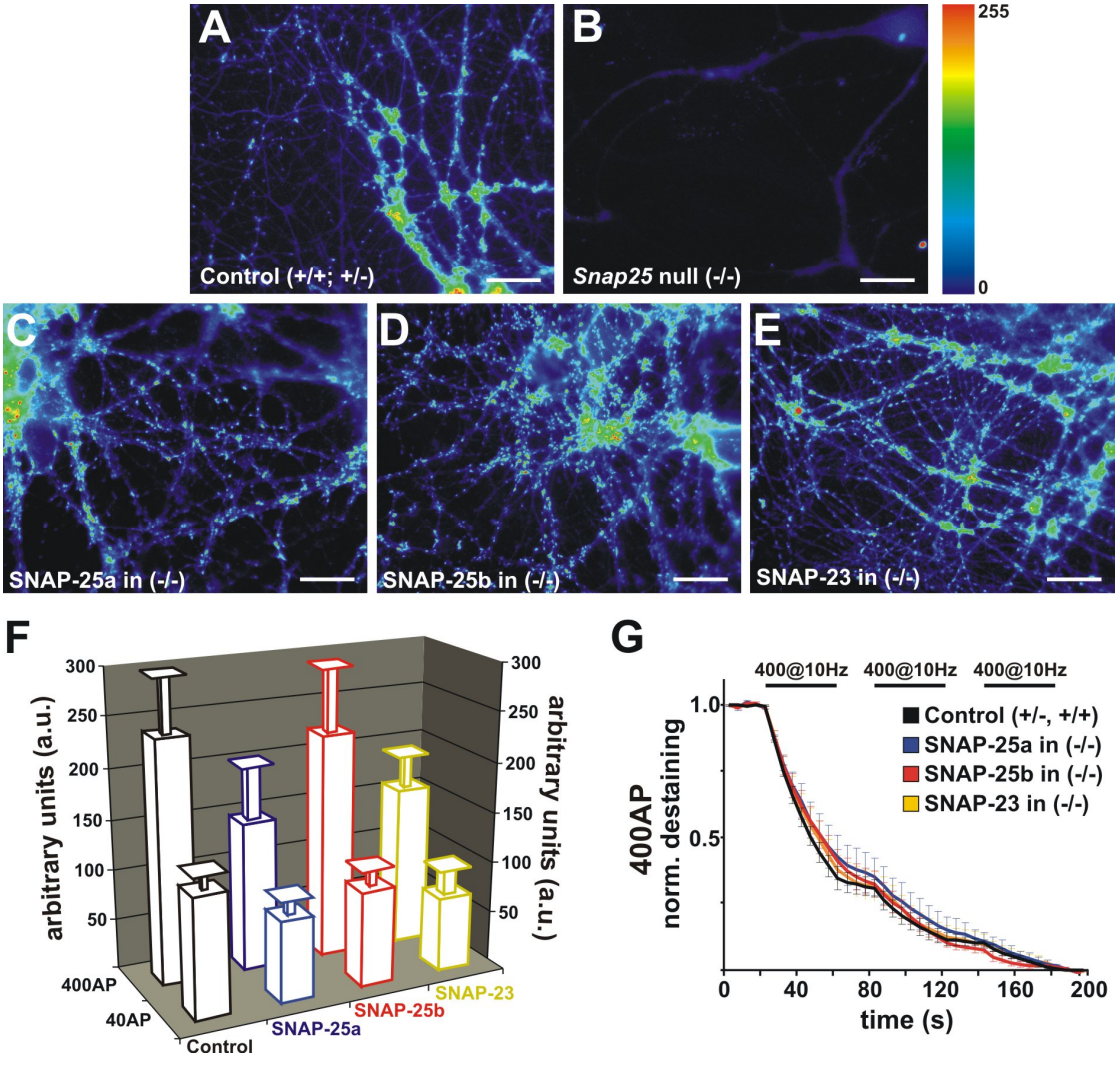


Figure 11
Stimulus-dependent recycling of synaptic vesicles requires a SNAP-25 homologue.

(A-E) Examples of control hippocampal neurons (A), *Snap25* null neurons (B) and null neurons rescued with SNAP-25a (C), SNAP-25b (D) and SNAP-23 (E) stained with 400 action potentials (APs). Staining was not possible in the absence of SNAP-25, indicating a lack of synaptic vesicle recycling. Scale bar is 10 µm. **(F)** The intensity of FM5-95 staining by 40 AP and 400 AP loading. The background intensity following full destaining was substracted. **(G)** Destaining (mean±SEM) of synaptic boutons under strong electrical stimulation (3 pulses of 400 AP at 10Hz) after loading with 400 AP. Color-coding as above. The destaining kinetics was indistinguishable among groups.

cultured *Snap25* null neurons (Figure 11B), indicating that the synaptophysin-positive synapses that I had been detected previously were lacking vesicle recycling, in agreement with recent data (Tafoya et al., 2006). In contrast, staining was successful in rescued neurons (Figure 11C-E). Since styryl dyes specifically stains functional synaptic vesicles, the intensity of the staining will be determined by the number of vesicles that undergo exo-endocytosis during the stimulation. For this reason, by varying the strength in the stimulation during loading of the dye, we can investigate differences in the recycling pools mobilized. Using a train of 400 action potentials at 20 Hz, I observed similar levels of staining intensity for the rescue with each homologue. However, it is interesting to notice that the intensity of the loading for SNAP-25a (147.0±56.8 a.u., N=3) and SNAP-23 (158.0 a.u.±36.1, N=4) was 30-35% reduced when comparing to control (237.9 a.u.±59.5, N=5) and SNAP-25b rescued neurons (222.4 a.u.±68.8, N=5), Figure 11F. Comparable result was obtained when 40 action potentials, which are believed to produce the release of the readily releasable pool, were used for staining. Although the staining intensity reached similar values in all the cases, the loading mediated by SNAP-25b (93.64±20.52 a.u., N=7) or control neurons (117.77±18.73 a.u., N=7) was still slightly higher than the one by SNAP-25a (79.80±18.63 a.u., N=8) and SNAP-23 (72.86±27.98 a.u., N=5), Figure 11F. This tendency would suggest that vesicle fusion mediated by SNAP-25a and SNAP-23 is less efficient. The destaining of the synaptic boutons by three trains of 400 action potentials at 10 Hz was complete for each homologue and no differences were found in the kinetics (Figure 11G).

D. Synchronous release of vesicles is mediated by SNAP-25

In order to analyze in detail synaptic transmission mediated by the SNAP-25 homologues, I proceed to examine, by whole-cell patch-clamp, autaptic neurons expressing each of them. Since this present work represented the one of the first attempts of using the lentiviral system in this preparation, I began by assuring that lentivirus infection is innocuous to neurons. Synaptic amplitudes were relatively constant for each neuron but differed between neurons, presumably because they reflect a variable number of simultaneous synaptic inputs. In spite of this, the observed excitatory post-synaptic current (EPSC) was indistinguishable in uninfected wild-type neurons (2.99±0.46 nA,

n=40) an eGFP-expressing wild-type neurons (3.10±0.31, n=30, p=1.00 2 way ANOVA), indicating that lentivirus is not intrinsically harming neuronal function and, therefore, it can be used on my purpose. For the following considerations, I assumed that uninfected ad eGFP-expressing wild-type neurons were interchangeable and they together were considered the control group (3.04±0.31 nA, n=70). In addition, no differences were found between heterozygous and homozygous wild-type neurons (data not shown).

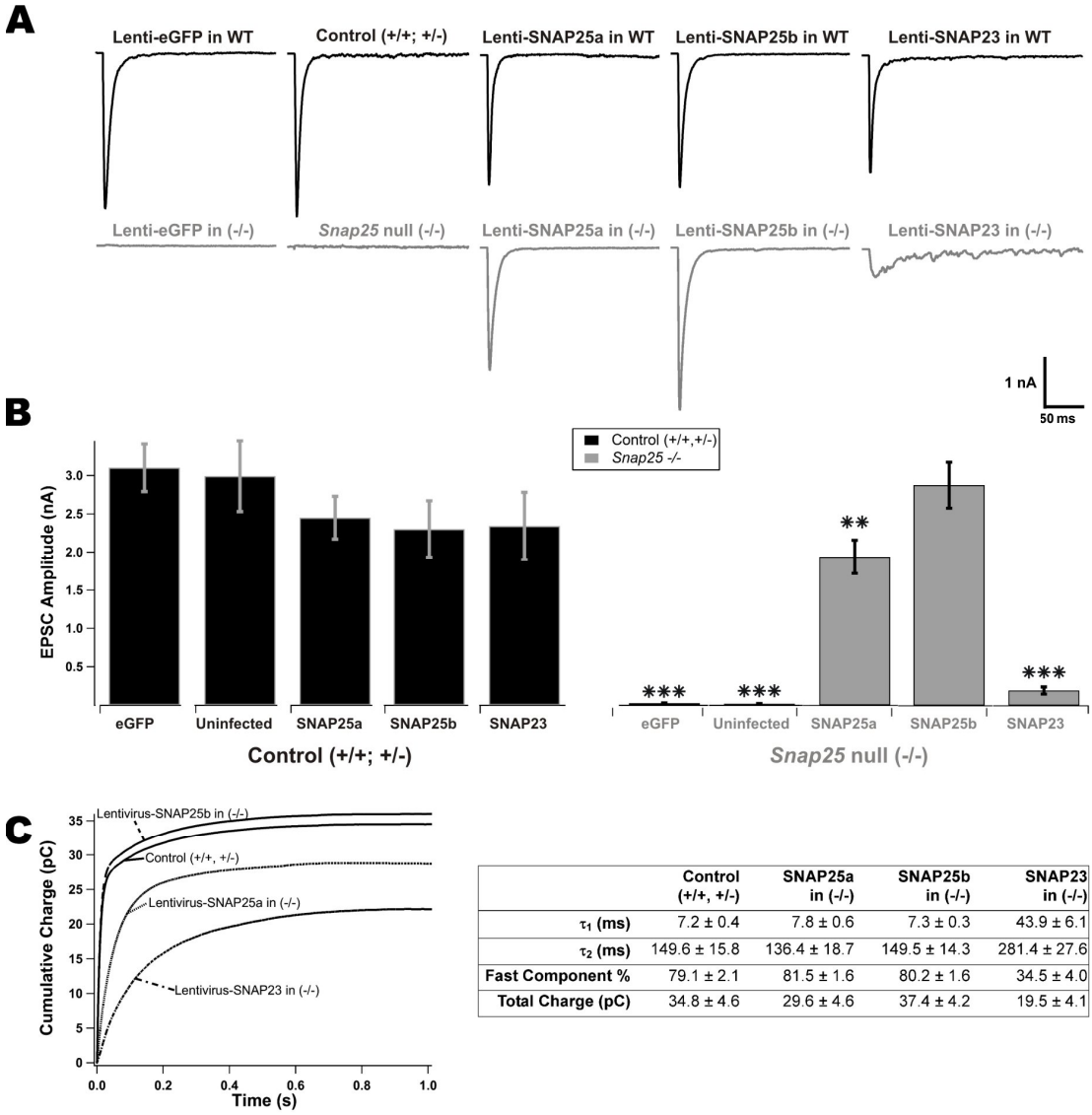
I observed a reduction in the EPSC amplitude by over-expression of SNAP-25a and SNAP-25b in wild-type neurons using the SFV. For that reason, I next wanted to examined the effect that lentivirus over-expression has in wild-type neurons. Responses evoked using the SNAP-25 isoforms as well as SNAP-23 consisted of a fast EPSC, identical to the one of control neurons. However, in all the cases, the EPSC amplitude was slightly reduced when comparing to control neurons (2.45±0.28 nA, n=35 for SNAP-25a; 2.30±0.37 nA, n=28 for SNAP-25b; and 2.34±0.44 nA, n=33 for SNAP-23), Figure 12A and B. Although these differences were not significant, they would warn about the delicate equilibrium of SNAP-25 in the exocytic machinery, as concluded from first experiments using SFV (Owe-Larsson et al., 1999). For this reason, I proceed to work only in the knock-out background.

In agreement with the results obtained by FM staining, none of the 35 Snap25 null neurons or the 41 null neurons expressing only eGFP examined displayed any detectable EPSC (roughly estimated as 0.019±0.003 nA and 0.024±0.004 nA respectively, Figure 12A and B). However they were able to generate normal action potentials when activated in current-clamp mode (data not shown), indicating that these neurons were alive although incapable of evoking a response. Since no differences was found between these two groups, they were considered as the null group (0.022±0.002, n=76, $p<0.0001$; compared to control neurons) for negative comparison.

The expression of SNAP-25 recovered synaptic transmission in knock-out neurons. However, significant differences were found between both splice-variants. Evoked responses of neurons rescued with SNAP-25b were similar to those of control neurons (2.88±0.30 nA, n=53) but, interestingly, those EPSCs driven by SNAP-25a reached only

65-70% of this value. (1.94±0.22, n=43; $p<0.02$ two-way ANOVA compared to wildtype, or $p<0.01$ when compared to SNAP-25b rescue, Figure 12A and B).

Strikingly, *Snap25*$^{-/-}$ neurons expressing SNAP-23 produced a slower and attenuated EPSC, which missed the fast component of release and presented additionally



	Control (+/+, +/-)	SNAP25a in (-/-)	SNAP25b in (-/-)	SNAP23 in (-/-)
τ_1 (ms)	7.2 ± 0.4	7.8 ± 0.6	7.3 ± 0.3	43.9 ± 6.1
τ_2 (ms)	149.6 ± 15.8	136.4 ± 18.7	149.5 ± 14.3	281.4 ± 27.6
Fast Component %	79.1 ± 2.1	81.5 ± 1.6	80.2 ± 1.6	34.5 ± 4.0
Total Charge (pC)	34.8 ± 4.6	29.6 ± 4.6	37.4 ± 4.2	19.5 ± 4.1

Figure 12

Synchronous release of vesicles is produced by SNAP-25 but not SNAP-23

(A) Autaptic EPSCs in neurons expressing SNAP-25a, SNAP-25b or SNAP-23 compared to eGFP-expressing and uninfected neurons. Control (+/+; +/-) neurons are shown in black and *Snap25*$^{-/-}$ neurons in grey. No evoked responsed were found in the absence of SNAP-25. Strikingly, *Snap25*$^{-/-}$ neurons expressing SNAP-23 produced evoked responses lacking teh fast synchronous component. **(B)** Mean±SEM EPSC amplitudes for the groups described above. SNAP-25a rescue led to smaller EPSC amplitudes than SNAP-25b rescue or control. **(C)** *(left panel)* Integrated EPSCs for control and rescued neurons. SNAP-23 rescued neurons presented slower release of vesicles. *(right)* Summary table containing mean±SEM values for a two-exponential fit to the EPSC 1 second integral for control and rescued neurons. Time constants for the fast (t1) and slow (t2) components as well as the fractional contribution of the fast component were significantly reduced in SNAP-23 rescued cells when comparing to the rest of the groups ($p<0.001$, Student's t-test)

abundant asynchronous release for about 1 second after stimulation (Figure 12A). This was an astonishing result since over-expression of SNAP-23 in wild-type neurons did not change the characteristics of transmitter release.

This unusual evoked response presented an amplitude calculated of 0.198±0.046 nA (n=52; $p<0.001$ compared to SNAP-25b rescue, two-way ANOVA). When I analyzed the kinetics of neurotransmitter release by integrating EPSCs over one second, I observed that the charge liberated by SNAP-23 rescue (19.5±4.1 pC, $p<0.05$, two-way ANOVA comparing to SNAP-25b rescue) was only 50-55% of the one of control neurons (34.8±4.6 pC) and SNAP-25b rescue (37.4±4.2 pC), whereas SNAP-25a rescued neurons displayed an intermediate phenotype (29.6±4.6 pC). The charge transfer could be described by a sum of two exponential functions, showing the kinetics of release (fig. 3C). The contribution of the fast component to the total release was remarkably reduced in SNAP-23 rescue null neurons (34.5±4.0%) when compared to the rest of the groups (see table in Figure 12C). The time constant corresponding to the first component of release was found to be 6-fold slower in the neurons rescued by SNAP-23 (43.9±6.1 ms; $p<0.001$; two-way ANOVA) than in control neurons or in each of the SNAP-25 rescue (see table in Figure 12C) and also the time constant for the slow, asynchronous component of release was slower for SNAP-23 (281.4±27.6 ms, $p<0.001$; two-way ANOVA) than that for the rest of the groups (see table in Figure 12C).

E. GABAergic neurons use SNAP-25 but not SNAP-23 for synaptic transmission

Very remarkable was the absence of inhibitory post-synaptic currents (IPSCs) in any of the 76 neurons examined in the null group, even though they represented 11.9% of the responses in wild-type hippocampal neurons. Immunofluorescence and neurotoxin data led to the suggestion the GABAergic neurons preferentially use SNAP-23, rather than SNAP-25, for synaptic release (Verderio et al., 2004; Frassoni et al., 2005). However, a recent study carried out in parallel with ours demonstrated SNAP-25 immunofluorescence in GABAergic neurons and the absence of transmission in *Snap25*$^{-/-}$ neurons. In addition, I found, in SNAP-25 knock-out hippocampal neurons rescued with

SNAP-25a and SNAP-25b, a similar ratio of GABAergic responses (7.8% and 6.7%, respectively) as in control wild-type neurons (11.9%). In order to find a definitive answer, I examined SNAP-25/SNAP-23 rescue in striatal cultures, where GABAergic neurons are more abundant. I found that survival of *Snap25* null neurons was as reduced as in hippocampal cultures (1.7±0.5% of control values, n=3, p<0.001 Student's t-test) and increased when SNAP-25a (72.3±8.2%, n=3), SNAP-25b (77.4±9.6%, n=3) or SNAP-23 (75.2±8.5%, n=3) were expressed. IPSCs were missing in all the Snap25 null neurons examined (n=14). However typical GABAergic evoked responses were present

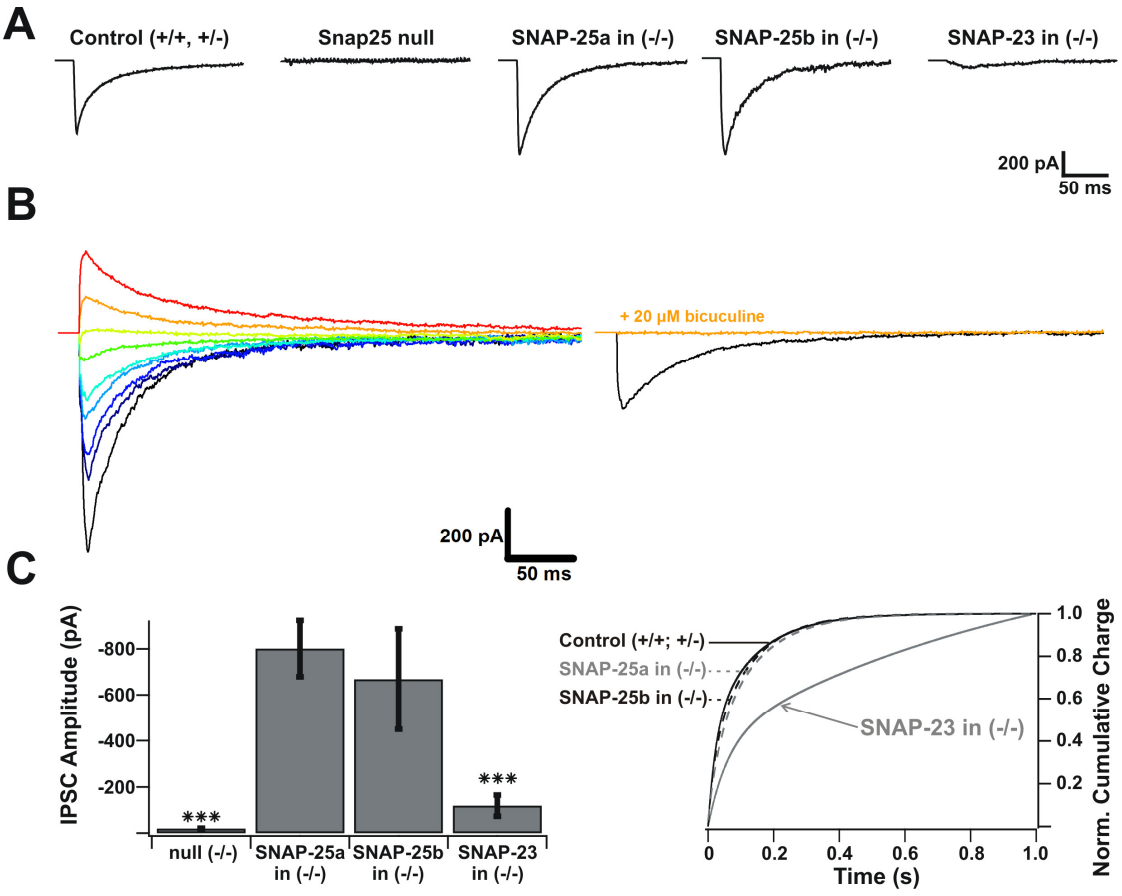


Figure 13

SNAP-25 is essential for fast release in GABAergic neurons.

(A) Example traces of typical GABAergic responses in striatal neurons from control (+/+; +/-), *Snap25* null neurons and null neurons rescued with SNAP-25a, SNAP-25b and SNAP-23. No inhibitory post-synaptic currents (IPSC) were found in null, suggesting that inhibitory neurons need SNAP-25 for synaptic transmission. Null neurons rescued by SNAP-25a or SNAP-25b evoked normal IPSCs. However, responses in SNAP-23 rescue neurons were smaller and the fast component was abolished, mimicking the situation in glutamatergic neurons. **(B)** Exampled of *Snap25* null neuron rescued with SNAP-25b showing *(left panel)* reversal of the IPSC after changing teh holding potential from -100 mV (black) to -20 mV (red) in 10 mV steps; and *(right panel)* blockage by the $GABA_A$ antagonist, bicuculine. **(C)** *(left panel)* Mean±SEM values of the IPSC amplitudes. *(right panel)* Example of the integrated charge released by the IPSC for each of the groups. The kinetics of evoked release for SNAP-23 rescue neurons was slower than for the rest of the groups.

when striatum -/- neurons were rescued with SNAP-25a (n=11) or SNAP-25b (n=14), with no significant differences in the IPSC amplitude (Figure 13A and C). Post-synaptic responses showing a reversal potential that followed the Nernst potential for chloride and was susceptible to blockage by 20µM of the GABAA receptor antagonist bicuculline were considered GABAergic (Figure 13B). On the other hand, responses blocked by 7µM of CNQX were considered glutamatergic. In addition, GABAergic responses were found in striatum knock-out neurons expressing SNAP-23 (n=9). However, these responses lacked the fast component of release (Figure 13B), similarly as observed in glutamatergic rescue. These data show that neuronal survival in GABAergic neurons as well as normal synchronized GABAergic transmissionis strictly SNAP-25 dependent.

F. Differential control of the releasable vesicle pools by SNAP-25a and SNAP-25b

The differences observed in the evoked responses among the homologues in the hippocampal cultures could be initially attributed, since the number of synapses was similar, to changes in the release probability, *Rp*, or in the readily releasable pool (*RRP*), according to the following relation:

$$EPSC = R_p \cdot RRP \cdot quantal\ size$$

Equation 7

The application of a solution made hypertonic by the addition of 500 mM sucrose is supposed to release all those vesicles that are already primed (Rosenmund and Stevens, 1996), designated here as 'sucrose pool'. With this method, I provoked a small but detectable current in the *Snap25* null neurons. Integrating over time I estimated the sucrose pool in 34.8±6.2 pC (n=39, Figure 14A) in this case. This indicates the existence of a small ,SNAP-25-independent, reluctant vesicle pool, which can be elicited by sucrose application but not at all by Ca2+-dependent stimulation. This pool could possibly provide vesicles for spontaneous events in the null neurons (see below). Responses elicited by sucrose in the SNAP-25a rescue group (287.2±54.7 pC, n=27) were 20-30% smaller than those in the SNAP-25b rescue group (441.0±69.1 pC, n=31), though this was not statically significant (p=0.16, Student's t-test, Figure 14B). Similar tendency towards a reduction was observed in SNAP-23 rescued neurons (246.5±68.7 pC, n=39;

p>0.05). The release probability for the sucrose pool, calculated by dividing the charge released in one EPSC during one second over the sucrose pool of the same neuron, was similar in presence of SNAP-25a (0.11±0.02, n=22) or SNAP-25b (0.13±0.01, n=22). Interestingly, the release probability was very similar in SNAP-23 rescue group (0.12±0.03), which indicates that the fraction of the sucrose pool released in the presence of SNAP-23, although highly asynchronous, is similar to the one release in the presence of SNAP-25.

Recent experiments suggest that the size of the pool of vesicles released by sucrose differs from the one released by stimulation trains in glutamatergic neurons (Moulder and Mennerick, 2005). To complement the results, therefore, I used a stimulation train to determine the readily releasable pool size by evoked release (RRP_{er}), as previously described (Schneggenburger et al., 1999; Otsu et al., 2004). The cumulative EPSC amplitude was plotted versus time and a linear fit to a steady-state phase was extrapolated to zero time in order to determine the RRP_{er} size in absence of refilling. In addition, assuming that the release is much faster than the priming process, then, the slope of the

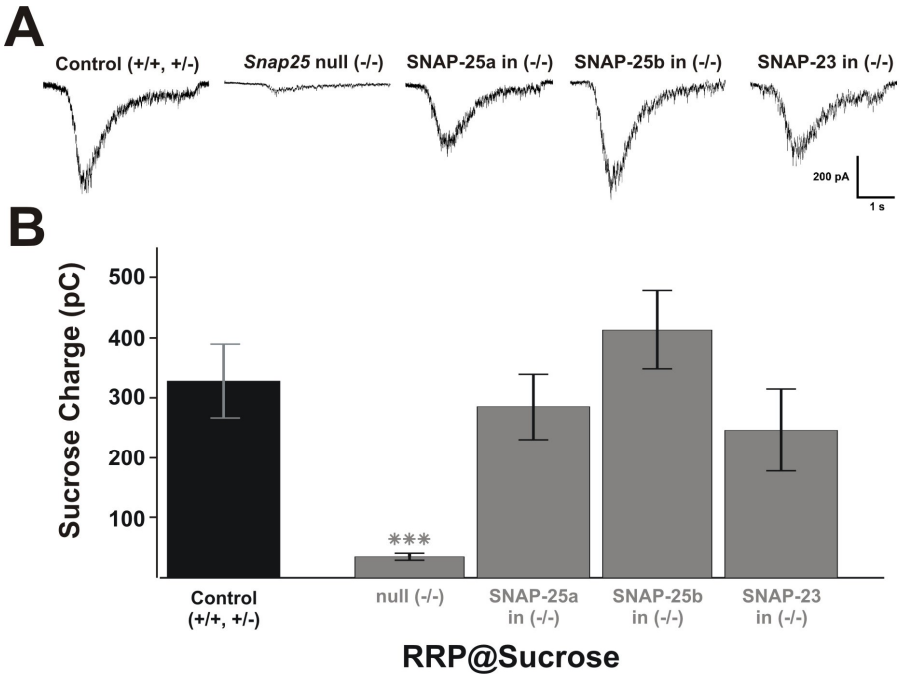
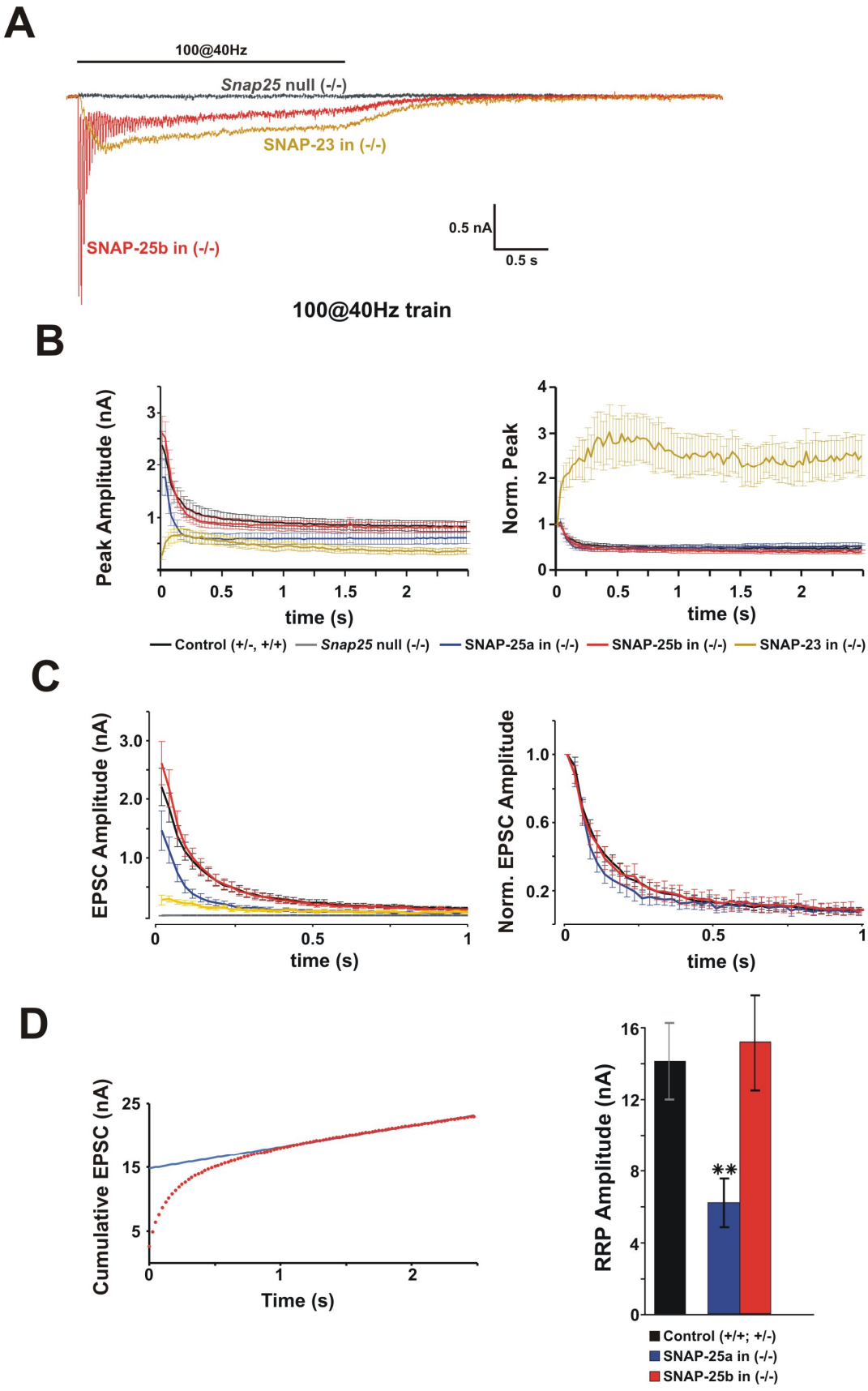


Figure 14
Sucrose pool is recovered by expression of any of the SNAP-25 homologues

(A) Example traces of 500 mM sucrose application in control hippocampal neurons (+/+; +/-), *Snap25* null neurons (-/-) and after rescue with SNAP-25a, SNAP-25b and SNAP-23. A small vesicle pool was released by sucrose in knock-out neurons as well. **(B)** Mean±SEM values of the 'sucrose pool' for each of the groups described above.

fitted line would indicate the replenishment rate. Such stimulation trains also allow examining the short-term properties of rescued neurons. This is important since a higher degree of facilitation was seen in hippocampal slices derived from a transgenic mouse with a higher SNAP-25a/b ratio (Bark et al., 2004).

The stimulation by 100 action potentials at 40Hz caused evoked no release in the *Snap25* null neurons (Figure 15A and C). In knock-out neurons rescued with SNAP-23, the stimulation protocol produced a large facilitation of the charge released (Figure 15A and B), mainly due to an increase in the asynchronous component. In the rest of the conditions, repetitive stimulation produced a rapid decrease of the EPSC amplitude until a final steady current. The amplitudes during the train followed a double exponential time course of comparable decay (data not shown). Similar values of RRP_{er} for control neurons and *Snap25* null neurons rescued with SNAP-25b were estimated using this method (14.04±2.13nA, n=45 and 15.12±2.22nA, n=35, respectively). However, the pool size estimated for the rescue with SNAP-25a (6.15±1.35 nA, n=24, $p<0.001$, two-way ANOVA, Tukey-Kramer test) was 55-60% reduced (Figure 15B). The replenishment rate was also significantly reduced in SNAP-25a rescue (1.92±0.37 nA/s, $p<0.01$, for SNAP-25a, two-way ANOVA, Tukey-Kramer test) when compared to SNAP-25b rescue (3.21±0.43 nA/s) and control neurons (3.63±0.49 nA/s). Additionally, the release probability, calculated as the ratio between the first stimulus in the train and RRP_{er}, was similar between neurons rescued by the SNAP-25 isoforms (0.29±0.03, for SNAP-25a; and 0.24±0.03, for SNAP-25b) and control neurons (0.23±0.03). The release probability estimated by depletion of the readily releasable pool with high-frequency action potentials differed from the one estimated using sucrose pulse, indicating that the 'sucrose pool' is greater than the RRP_{er}, as previously reported (Moulder and Mennerick, 2005). The significant decrease in RRP_{er}, considering them together with the tendency to a decrease in the sucrose pool size (Figure 14), the decrease in the intensity of FM5-95-stained boutons (Figure 11) and the equal number of synaptophysin-positive synapses demonstratethat priming under SNAP-25a and SNAP-23 action is slower than under SNAP-25b.


A
100@40Hz
Snap25 null (-/-)
SNAP-23 in (-/-)
SNAP-25b in (-/-)
0.5 nA
0.5 s
100@40Hz train
B
Peak Amplitude (nA)
time (s)
Norm. Peak
time (s)
Control (+/-, +/+)
Snap25 null (-/-)
SNAP-25a in (-/-)
SNAP-25b in (-/-)
SNAP-23 in (-/-)
C
EPSC Amplitude (nA)
time (s)
Norm. EPSC Amplitude
time (s)
D
Cumulative EPSC (nA)
Time (s)
RRP Amplitude (nA)
**
Control (+/+; +/-)
SNAP-25a in (-/-)
SNAP-25b in (-/-)

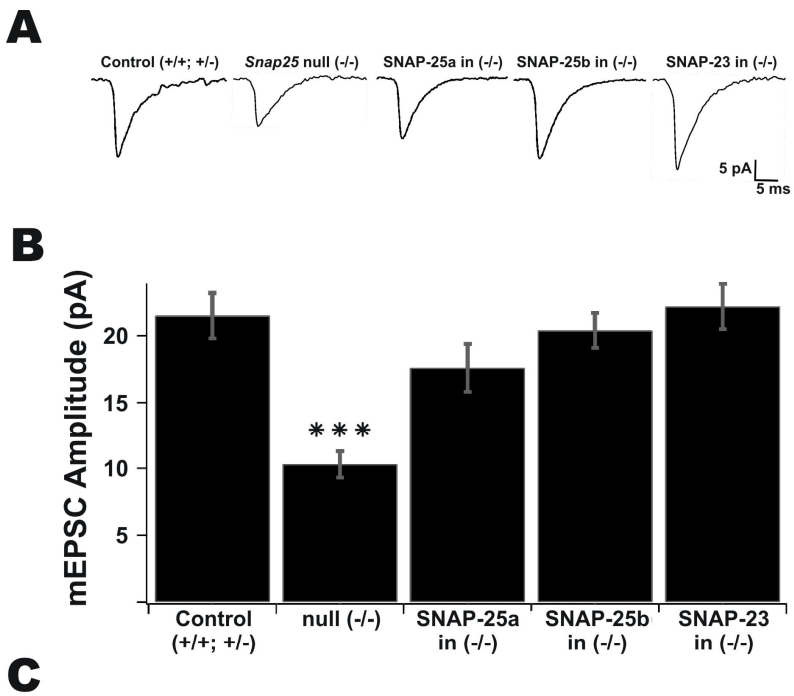
These results, likewise, indicate that priming, rather than release-probability, is decreased in SNAP-25a. Release probability has a high relevance for short-term properties of neurotransmission. A neuron will preferentially exhibit depression when the release probability is high and facilitationwhen is low. Experimentsmeasuring hippocampal slices from a knock-in mouse in which the developmental switch from SNAP-25a to SNAP-25b was partly interrupted presented a higher degree of facilitation than normal wild-type (Bark et al., 2004). For this reason, I also examined the pair-pulse behavior at 40Hz in the Snap25 null autaptic cultures when rescued with each isoform. The pair-pulse ratio, defined as the ration between the amplitude of the second EPSC and the first one in a train, was very similar among the SNAP-25 rescued neurons (0.85±0.08, n=22, for SNAP-25a; and 0.86±0.05, n=34, for SNAP-25b). Around two thirds of the cells showed paired-pulse depression (PPD), with no differences observed between both groups (0.70±0.05, n=16, for SNAP-25a; 0.72±0.04, n=24, for SNAP-25b). Pair-pulse facilitation (PPF) mediated by SNAP-25a (1.26±0.15, n=6) was also very similar to SNAP-25b (1.20±0.05, n=10). In contrast, pair-pulse ratio in SNAP-23 rescue neurons showed significantly higher values (1.11±0.10, two-way ANOVA comparing to SNAP-25b). Results were qualitatively equivalent when stimulation frequencies of 10Hz and 50Hz were used (data not shown). All these data indicates that the main consequence of the developmental change from SNAP-25a to SNAP-25b expression is the increase of the readily-releasable pool by the time that synaptic maturationis necessary.

Figure 15
High frequency stimulation (100@40Hz) reveals differences between SNAP-25a, SNAP-25b and SNAP-23

(A) Example traces of high-frequency train stimulation (100 action potentials at 40Hz) of *snap25* null neurons (grey), SNAP-25b rescued neurons (red) and SNAP-23 (yellow). Stimulation artifacts have been removed. **(B)** (*left panel*) Mean±SEM of amplitudes from baseline to peak during high-frequency stimulation, which approximates to the charge released during stimulation (*right panel*) Mean±SEM of normalized peak amplitudes from left panel **(C)** *(left panel)* Mean±SEM values of EPSC amplitudes during high-frequency stimulation *(right panel)* Mean±SEM values of normalized amplitudes from left panel. Only SNAP-25a and SNAP-25b are displayed. Kinetics of depression did not show significant differences between both. **(C)** *(left panel)* Example cumulative trace of the EPSC amplitudes during the 40Hz train stimulation (red). The steady component was fitted with a straight line (blue) and back-extrapolated to 0-seconds to calculated the readiliy releasable pool (RRP_{er}) *(right panel)* Mean±SEM of the RRP_{er} values calculated as shown in the upper panel. RRP_{er} was significantly smaller when SNAP-25a was used for rescuing null neurons instead of SNAP-25b.

G. Spontaneous release in the presence and absence of SNAP-25 homologues

The reduction of the readily-releasable pool observed in null neurons rescued with SNAP-25a can also be explained by a reduction of the vesicular quantal size (Equation 7). Therefore, I examined the spontaneous release and miniature single events in the hippocampal cultures. Miniature EPSCs (mEPSC) were detected in -/- neurons (Washbourne et al., 2002), whose source could be the small sucrose pool previously measured. Spontaneous activity without involvement of a conventional SNARE complex has been already described (Schoch et al., 2001) and it could imply an independent mechanism for spontaneous release (Sara et al., 2005). However, I found, surprisingly, that the size of the events in the Snap25 null neurons (10.4±1.0 pA and 54.2±5.6 fC,



C

	Control (+/+, +/-)	Snap25 *null* (-/-)	SNAP25a in (-/-)	SNAP25b in (-/-)	SNAP23 in (-/-)
Peak (pA)	21.5 ± 1.7	10.3 ± 1.0	17.6 ± 1.8	20.4 ± 1.3	22.2 ± 1.7
Charge (fC)	107.3 ± 7.0	54.2 ± 5.6	91.8 ± 9.0	102.4 ± 5.9	109.5 ± 6.9
Frequency (Hz)	1.76 ± 0.3	0.63 ± 0.20	2.69 ± 0.65	1.80 ± 0.34	3.11 ± 0.57

Figure 16

Smaller and fewer spontaneous events in the *Snap25* null neurons are rescued by SNAP-25 and SNAP-23.

(A) Example averaged mEPSC from single cells. Events were indistinguishable in shape; however they were smaller in *Snap25* null neurons. **(B)** Mean±SEM values of the mEPSC amplitudes. The mEPSC size was significantly reduced in *Snap25* null neurons. **(C)** Summary table with the corresponding mean±SEM values of peak amplitudes and charge of the mEPSC and frequency. The mEPSC rate was rescued by SNAP-25b and seemed slightly higher for SNAP-25a and SNAP-23.

n=11, p<0.001, two-way ANOVA) was 50% smaller than in the control neurons (21.5±1.7 pA and 107.3±7.0 fC, n=23), fig. 6. The frequency was also lower in null neurons (0.63±0.20 Hz, p<0.02, two-way ANOVA) than in control neurons (1.76±0.30 Hz), which can probably be explained by the reduced arborization and synaptic contacts in the *Snap25*$^{-/-}$ neurons (Figure 16). The reintroduction of the SNAP-25 homologues in the knock-out neurons led to a fully recovery of the mEPSC size and frequency (17.6±1.8 pA, 2.69±0.63 Hz, n=19, for SNAP-25a; 20.4±1.3 pA, 1.80±0.34 Hz, n=25, for SNAP-25b; and 22.2±1.7 pA, n=23, for SNAP-23). The mEPSC frequency appeared to be higher in the case of SNAP-25a and SNAP-23 than in SNAP-25b, however this was not quite significant (p=0.0827, two-way ANOVA). There are several possible explanations for the reduction in mEPSC size: first, the synaptic vesicles might be smaller in absence of SNAP-25; second, the glutamate content might be lower; and third, the number of post-synaptic receptors might be decreased or not correctly clustered to the postsynaptic density, so the same concentration of neurotransmitter produces a lower depolarization. The results cannot distinguish among these possibilities. However, since SNAP-25 have been already implicated in the trafficking of glutamate receptors (Lan et al., 2001b; Lan et al., 2001a; Washbourne et al., 2004), a post-synaptic effect of SNAP-25 seems to be the best hypothesis to explain the reduction in the quantal size.

VIII. DISCUSSION

Neuronal communication fundamentally depends on two factors: the number of synaptic contacts and the strength of each contact. For a neuron to expand and establish new contacts, it needs the fusion of membrane containing vesicles at the growth cone. Similarly, neurons use vesicular fusion to produce the release the neurotransmitter and regulate the number of post-synaptic receptors. Over the last years, a common SNARE mechanism has been proposed to mediate these vesicular fusion processes. It seems likely that different sets of SNARE proteins participate in each of these processes. However, only SNAP-25 appears to be involved in each of them. Different studies implicated SNAP-25 in the priming of synaptic vesicles as well as in neurite outgrowth and, recently, in trafficking of NMDA receptors (see SNAP-25 is a main character in the brain theater, page 17). Despite the evident importance of SNAP-25 in neuronal function, studies of this aspect are insufficient and incomplete. Except a few exceptions, most of the studies have been performed in PC12 cells rather than in neurons and they covered only a particular fusion process. One of the major drawbacks for a complete analysis of SNAP-25 in neurons has been the lack of viability of Snap25 null neurons in primary cultures, which has considerably limited the study. These difficulties have made impossible to ascribe SNAP-25 isoforms to different neuronal functions.

This doctoral work confronts this gap in knowledge by producing long-term expression of SNAP-25 in culture null neurons, offering, as a result, new insights into how SNAP-25 is used by the neuron. Using a knock-out and rescue approach, I was able to analyze the function of SNAP-25 in survival, neurite outgrowth and synaptic transmission. With my results, SNAP-25 appears then as a multivalent protein, essential for neuronal function. I demonstrate that SNAP-25 participates in the control of arborization, through a mechanism that is vital for the neuron. I showed that the closest homologue, SNAP-23, can substitute SNAP-25 in neurite outgrowth and survival and that, in addition, can support synaptic vesicle release. However, this release lacks synchronization, in both glutamatergic and GABAergic neurons, producing inefficient synaptic transmission. I demonstrate that alternative splicing of SNAP-25 is used to

enhance the priming properties of the synaptic vesicles through the raise in SNAP-25b expression levels. In addition, I observe a decrease in the quantal content in absence of SNAP-25, suggesting,thus, a post-synaptic function of SNAP-25.

A. Neuronal survival and maintenance of arborization is dependent of SNAP-25 action

Generally speaking, absence of presynaptic proteins has little consequences in mouse and neuron development, as shown in the case of, for example, synaptotagmin I (Geppert et al., 1994), complexin 1 (Reim et al., 2001), basson (Altrock et al., 2003) and RIMα knock-out (Schoch et al., 2002). In some other cases, the absence is occasionally fatal for the mutant mouse, although neuronal development is not compromised. For instance, ablation of synaptobrevin-2, the vesicular SNARE partner of SNAP-25, caused mutant mice to die after birth. Resembling SNAP-25 knock-out neurons, no evoked responses could be measured but a certain spontaneous activity persisted. However neuronal cultures preserved viability and arborization in this case (Schoch et al., 2001). Similarly, double ablation of Munc13-1 and Munc13-2, which are priming factors for exocytosis (Ashery et al., 2000; Rosenmund et al., 2002), produced death of mutant mice upon birth. However, although there was no detectable evidence of synaptic activity or vesicle fusion, neurons grew and expanded normally in cultures (Varoqueaux et al., 2002). It is well known that there exist an extensive selective pressure on those cells that are not able to establish coherent connections during a critical stage in development (Herrmann and Shatz, 1995). This led to suppose that lack of synaptic activity could cause reduction in the survival. However, these experiments demonstrated that neuronal survival and outgrowth in vitro is unrelated to fusion of synaptic vesicles.

Nonetheless, it seems that both processes are critically dependent on SNAP-25. The absence of SNAP-25 in culture neurons reduced the survival probability of the cell. The reason for this fact is unknown. Previous studies showed that brain development proceeds normally in *Snap25*$^{-/-}$ embryos, with no loss in cell density (Washbourne et al., 2002). This would indicate the presence of some undetermined factor that would prevent

cell degeneration in the brain. The co-culture of *Snap25* null neurons with astrocytes from wild-type NMRI mice, which would presumably provide neurotrophic or other factors, did not avoid the degeneration in my study. Furthermore, promotion of synaptic activity, using glutamate agonists or producing a persistent depolarized state with high potassium, can not prevent decrease of viability in *Snap25* null cultured neurons, as previously shown (Washbourne et al., 2002). Interestingly, these findings are comparable to those obtained from Munc18-1 knock-out neurons. Munc18-1 is a SM-protein that binds, preferably, Qa-SNAREs such as syntaxin-1 and prevents the formation of SNARE complexes (Dulubova et al., 1999; Misura et al., 2000; Yang et al., 2002). Ablation of Munc18-1 abolished synaptic activity, similarly as Munc13-1/2. But interestingly, as in the case of SNAP-25, neurons cultured from *munc18-1* knock-out mice developed normally for several days in vitro but they degenerated and died afterwards (Heeroma et al., 2004). The reduced viability of *Snap25* and *munc18-1* null neurons indicates that, although survival is independent on synaptic activity, the role of specific pre-synaptic proteins is necessary for its maintenance. In this way, the fact that *Snap25*$^{-/-}$ neurons exhibited a 50% decrease in arborization may suggest a relation between neurite outgrowth and survival. The reduction of neuronal branching observed in absence of SNAP-25 agrees, as well, with previous studies, which shown a participation of SNAP-25 on neuronal branching (Igarashi et al., 1996; Osen-Sand et al., 1996; Grosse et al., 1999). It is known, furthermore, that neurite outgrowth shares similar molecular machinery to synaptic vesicle exocytosis (Martinez-Arca et al., 2001). It is interesting to note that SNAP-25 as well as Munc18-1 can interact with syntaxin 1, which participates in the fusion of synaptic vesicles, but also with syntaxin 3 and, at least for SNAP-25, syntaxin 13 (Hata and Sudhof, 1995; Morgans et al., 1996; Hirling et al., 2000), possible players in the fusion of growth cone vesicles (see Neurons dispose SNAREs for constitutive vesicle fusion). Therefore, an attractive hypothesis to explain the reduced survival in *Snap25* and Munc18-1 null neurons could be the contribution of both proteins in a complex involved in the membrane transport implicated in arborization. The relatively conserved branching and survival of neurons in embryonic brain and in young cultures (within few days *in vitro*) may suggest that arborization proceeds in two different stages. In an initial stage, neuron would start massive sprouting and extension so it can

reach soon other neurons. Later, this process would be substituted by a local, more controlled, expansion, probably aimed at the fine-tuning of connections. The mechanisms involved in each phase are undisclosed, although, according to above discussed, SNAP-25 and Munc18-1 may participate by forming a complex that would mediate vesicle fusion during the second stage. In this way, the malfunction of this 'blossoming' SNARE complex would produce a deficit membrane cycling at this period, which would cause neurites to retract, leading to apoptosis.

B. Presence of spontaneous release in *Snap25* null neurons reveals more about SNAP-25 functions

Absence of SNAP-25 completely abolished evoked Ca2+-dependent release although it did not eliminate spontaneous release. Indeed, the decrease in the mEPSC frequency detected was approximately similar to the decrease in synaptophysin-positive synapses counted. Furthermore, the number of synapses labeled was reduced in the same proportion as the number of branches, when comparing to control neurons. This fact brings two remarkable conclusions. First of all, it indicates that although SNAP-25 is involved in neurite outgrowth, it has not relevant role in synaptogenesis per se, since the number of synapses per branch was not altered. Secondly, it suggests that spontaneous release can proceed normally without SNAP-25. Classical studies evidenced that all synapses manifest spontaneous release in absence of action potential (Katz, 1969) and this is required for signaling leading to maturation and stability of neuronal networks (McKinney et al., 1999; Verhage et al., 2000). This release is possibly due to a low probability fusion of single vesicles, which source is supposed to be identical as the one for evoked release (Del Castillo and Katz, 1954; Murthy and Stevens, 1999). My results indicate, however, differences between the processes governing spontaneous and evoked release. Whereas Ca^{2+}-triggered fusion of synaptic vesicles is completely absent in neurons lacking SNAP-25, spontaneous fusion seems normal. This would suggest that the mechanism for spontaneous release differs from that for evoked release. Recent experiments supports that both kind of release are sustained by different pool of vesicles (Sara et al., 2005; Deak et al., 2006). Interestingly, neurons lacking synaptobrevin-2 present a 10-fold reduction of the frequency of mEPSC (Schoch et al., 2001), which

would imply that this protein participates in both types of fusion. From my results, it seems that SNAP-25 is mostly involved in triggered but not in spontaneous fusion. This indicates that rather other SNAP-25 homologue, probably SNAP-29 or SNAP-47, contributesto spontaneousfusion. However, SNAP-29 expression in *Snap25* null neurons might be insufficient for evoked release although still some vesicle recycling could be present (work in progress). Yet, another possibility would be that a different set of Qb-, Qc-SNAREs is used in spontaneous release.

In addition, the presence of spontaneous release in *Snap25*$^{-/-}$ neurons revealed a reduction in mEPSC size. My study cannot discern the possible reason for that. Up to now, deletion of any of the proteins involved in synaptic vesicle exocytosis did not affect the quantal size. This would indicate that SNAP-25 has a singular function in the regulation of the quantal size. It would be interesting to know whether Munc18-1 shares this property with SNAP-25. However, since these neurons do not present spontaneous release, the determination of quantal size in *Munc18-1*$^{-/-}$ neurons has been impossible. It is known, interestingly, that SNAP-25 is involved in the trafficking of glutamate receptors (Lan et al., 2001b; Lan et al., 2001a). Therefore, SNAP-25 could act, hypothetically, through the priming and fusion of vesicles transporting the receptors to the membrane of the dendritic spines. This action would probably be possible by the formation of a SNARE complex specific for this fusion. Alternative explanations to the involvement of SNAP-25 in the regulation of quantal size include the decrease in the synaptic vesicle size or neurotransmitter content. However, until further experiment can corroborate any of these, or other, hypotheses, the mechanism by which SNAP-25 is acting is still speculative.

C. SNAP-25 action is common to glutamatergic and GABAergic neurons

The presence of spontaneous release in the *Snap25*$^{-/-}$ neurons led to the suggestion that other homologues might be responsible for some of the functions attributed to SNAP-25. Such a SNAP-25 independent mechanism of release was proposed in inhibitory neurons recently when (Verderio et al., 2004) observed increased resistance to BoNT/A and BoNT/E in those neurons. Later, (Frassoni et al., 2005) reported a gradual decrease

in SNAP-25 specific staining at later developmental stages in GABAergic neurons. The use of SNAP-25 for these neurons was then proposed to be uniquely the initial step of outgrowth and synaptic consolidation. (Verderio et al., 2004) showed, in addition, that inhibitory neurons, despite lacking SNAP-25, were SNAP-23 immunopositive. Therefore, SNAP-23 was proposed as substitute for the function of SNAP-25 in GABAergic transmitter release.

Our results demonstrate, on the contrary, that both glutamatergic and GABAergic transmission is abolished in absence of SNAP-25. Furthermore, as presented in this doctoral work, the endogenous expression level of SNAP-23 is not able to compensate for the lack of SNAP-25 in the null neurons. Thus, *Snap25* null neurons present reduced survival, defective neurite outgrowth and void of evoked release. Identical result was obtained in striatal null neurons, which are predominately GABAergic, indicating that those neurons require SNAP-25 as well. A parallel work with ours has recently reported a co-localization of SNAP-25 and the GABAergic specific marker, VGAT, as well as a lack of release in *Snap25* null GABAergic neurons (Tafoya et al., 2006), which supports my findings. In addition, I showed that rescue of GABAergic *Snap25*$^{-/-}$ neurons is complete only when the SNAP-25 splice-variants are used, which is a definitive proof for a universal SNAP-25 mechanism in both kind of neurons. There are many possibilities to explain the discrepancy observed between *Snap25*$^{-/-}$ and treatment with BoNT. As discussed in (Tafoya et al., 2006), a possibly lower susceptibility of GABAergic neurons surface to the binding and incorporation of the neurotoxins could explain the disagreement. Verderio et al., 2004 also reported that, after exposure to KCl, GABAergic neurons presented an increased intra-cellular calcium concentration when compared to glutamatergic neurons. Since BoNT/A treatment can be overcome with high calcium concentrations (Capogna et al., 1997; Trudeau et al., 1998; Sakaba et al., 2005), the higher calcium increase in GABAergic neurons after KCl stimulation could, therefore, cancel the effect of the neurotoxins. In the same study, Verderio et al., 2004 observed a decrease in calcium responsiveness to depolarizing stimuli when SNAP-25b was over-expressed in wild-type neurons, which would alternatively explain the reduction in EPSC amplitude by SNAP-25a or SNAP-25b over-expression in control neurons observed in my work. However, they also reported that over-expression of SNAP-23 does not modify the calcium

responsiveness after KCl exposure. However, in the view of my results, neurons seem to use preferentially SNAP-25 over SNAP-23, even when that one is over-expressed and, thus, the possible effect of SNAP-23 in calcium responsiveness could have been covered by the endogenous SNAP-25 expression. It would be then interesting to clarify whether SNAP-23 is involved in the regulation of calcium dynamics.

D. SNAP-25 and synaptotagmin homologues are coupled in synchronous and asynchronous release

The expression of SNAP-23 produced the recovery of Ca2+-trigger responses in *Snap25*$^{-/-}$ neurons, indicating that this homologue is able to couple exocytosis to a calcium-sensor for release, as previously observed for SNAP-25 (Sørensen et al., 2002). However, SNAP-23 was incompetent in producing synchronized release. It is generally accepted that synaptotagmin-I synchronizes synaptic vesicle fusion by acting as calcium-sensor for fast release (Fernandez-Chacon et al., 2001; Nishiki and Augustine, 2004). In fact, the phenotype of null neurons rescued by SNAP-23 surprisingly resembled that of synaptotagmin-I knock-out neurons (Geppert et al., 1994; Nishiki and Augustine, 2004; Maximov and Sudhof, 2005). It is interestingto notice that whereas other members of the family, such as synaptotagmin-III and synaptotagmin-VII, can bind to both homologues, synaptotagmin-I binds only to SNAP-25 but not to SNAP-23 (Chieregatti et al., 2004). Thus, when SNAP-23 is expressed in *Snap25* null neurons, it would fail in the binding with synaptotagmin-I and therefore release could not be synchronized, similarly as for synaptotagmin-I knock-out neurons. Yet, since SNAP-23 can bind to some other synaptotagmin members, calcium-triggered exocytosis is still possible. This finding, furthermore, supports the idea that binding to SNAP-25 couples synaptotagmin-I to release. The putative binding sites of this interaction have been ascribed to the C-terminal (Zhang et al., 2002) and the N-terminal of the SNARE-domain (Rickman et al., 2006), which, remarkably, are highly conserved in SNAP-23. Therefore, the possible molecular basis of this binding remains undetermined. As well as this, it is also not clear what possible synaptotagmin would couple to SNAP-23 dependent exocytosis. Synaptotagmin-III and synaptotagmin-VII are intrinsically slower calcium-sensors than synaptotagmin-I, which agrees with a role in asynchronous release (Hui et al., 2005). They are relatively

abundant synaptotagmins, possibly localized to the synaptic plasma membrane instead to the vesicle (Butz et al., 1999; Sugita et al., 2001).

Synaptotagmin-VII (*syt7*) is a highly probable candidate since it was found to function as a plasma membrane calcium-sensor (Sugita et al., 2001). In addition it was involved in calcium-dependent lysosomal exocytosis for reparation of the plasma membrane in fibroblasts (Martinez et al., 2000; Reddy et al., 2001), insulin-containing vesicle exocytosis in pancreatic β-cells (Gao et al., 2000) and secretion in PC12 cells by over-expression (Fukuda et al., 2004; Bhalla et al., 2005). The over-expression of SNAP-23 in *Snap25/Syt7* double knock-out neurons still produced an asynchronous evoked response. However, the kinetics of this response were even more slower to those from *Snap25*$^{-/-}$ neurons over-expressing SNAP-23. Although these data are still preliminary, they suggest that synaptotagmin-VII triggers at least one part of the asynchronous release (Figure 17).

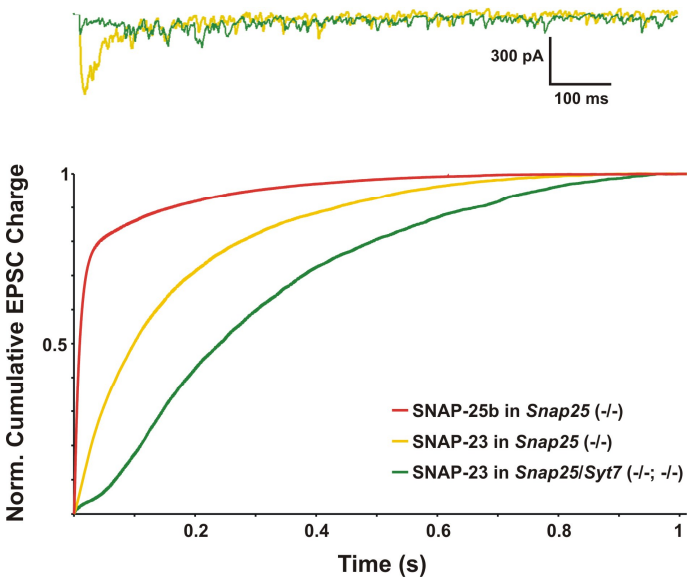


Figure 17
Synaptotagmin-VII partially drives SNAP-23 exocytosis

(*Upper panel*) Example traces of autaptic EPSCs caused by SNAP-23 over-expression in *Snap25* knock-out (yellow trace) and in *Snap25/Syt7* double-knockout neurons (red trace). (*Lower panel*) Example traces of integrated EPSCs for SNAP-25 over-expression in *Snap25* knock-out neurons (red trace) and for SNAP-23 over-expression in *Snap25* knock-out (yellow trace) and in *Snap25/Syt7* double-knockout neurons (red trace). EPSCs caused by SNAP-25b over-expression in double-knockout and *Snap25* knock-out neurons was comparable (data not shown)

E. The alternative splicing in SNAP-25 provides supports enhanced neurotransmission in adult synapses

The alternative expression of SNAP-25 isoforms during development and between neurons and neuroendocrine cells would suggest that the isoforms might be involved in differential developmental and functional roles. The regulated expression of the two splice-variants might provide a molecular framework for differential use of the fusion machinery during specific stages of neuronal maturation. Similarly, differential isoform expression in adult synapses may reflect their capacity to undergo structural plasticity. According to this view (Bark et al., 1995; Boschert et al., 1996), SNAP-25a is likely to participate in axonal outgrowth by directing regulated fusion of 'construction' vesicles that supply general membrane components needed for elongation throughout the neurite, as well as for plasmalemma expansion at growth cones. On the other hand, the predominance of SNAP-25b in adult nervous system would likely indicate that this isoform is most important for release of classical neurotransmitters from synaptic vesicles.

With this doctoral work, it has been finally possible to study the alternative expression of SNAP-25 variants in *Snap25* null neurons, permitting the study of the morphological and functional properties of each splice-variant in isolation. The performance of both isoforms in developmental aspect was found, however, analogous. No major differences between them were found in neuronal survival, neurite outgrowth and synaptogenesis. By generating a mutation that perturbs alternative splicing, limiting the expression of SNAP-25b in mice, Bark et al., 2004 reported no neuroanatomical differences in these animals, suggesting that brain development and morphogenesis were not greatly impaired. My findings show that, although neuronal development in vitro is markedly dependent on SNAP-25, it is presumably splice-variant unspecific. As discussed above, the loss in plasma membrane-carrying vesicles at the growth sites would lead to developmental deficits in cultures. The recovery of exocytosis by reintroduction of SNAP-25 or SNAP-23 would repair the loss. In this work, I noticed identical kinetics of destaining after FM loading, which would indicate that the exocytotic capabilities of these isoforms were identical. In a developing neuron, constitutive vesicle fusion occurs slowly in comparison to neurotransmitter release. It seems then reasonable to assume that

outgrowth is not necessary affected by the rapidity of the fusion.

Therefore, the alternative splicing of SNAP-25 would rather provide a refinement of the exocytic machinery towards different modes of transmitter release during development. My results support this hypothesis. I found a decrease in the EPSC size mediated by SNAP-25a when compared to SNAP-25b. Detailed analysis of the pool of vesicles stainable by FM dyes, sucrose pool and readily-releasable pool evoked by action potentials showed a consistent decrease, although moderate, in the priming process. These results are, moreover, comparable to those obtained by the analysis of SNAP-25 function in chromaffin cells, which constitute one of the most potent models for the accurate study of calcium-triggered exocytosis (Sørensen et al., 2003; Nagy et al., 2005). SNAP-25 expression switches from 'a' to 'b' isoform after birth and SNAP-25b isoform becomes the predominant species within the first seven days. Interestingly, the most dramatic increase in SNAP-25b expression is observed between 3 and 8 weeks after birth, a period when many final cortical synapses attain their mature morphology (Bark et al., 1995). The alternative splicing would necessary, therefore, to provide a more efficient neurotransmission by enhanced priming properties, which would be essential for processing the large amount of information received from now on.

IX. OUTLOOK AND PERSPECTIVES

A. Limitations and improvements of the method

1. Use of Lentivirus over other systems

This work represents the first time that the role of SNAP-25 in neuronal function is studied extensively. This was possible thanks to the use of a knock-out and rescue approach, which consists on the long-term expression of the different SNAP-25 homologues in *Snap25*$^{-/-}$ neurons. The use of the lentiviral system was fundamental for this purpose. Expression mediated by this virus was permanent and started early enough to supply sufficient SNAP-25 to the neuron and prevent death. Furthermore, the expression level seemed to be adequate to produce rescue without affecting synaptic transmission, which occurs with Semliki Forest Virus. These characteristics of the lentiviral system confer certain advantage over other systems, like, for example, adeno-associated viruses (AAV). The AAV type 2 is a non-enveloped, non pathogenic virus of the parvovirus family, containing a linear, single-stranded DNA genome. AAV requires coinfection with a helper virus for productive replication; otherwise the AAV genome integrates into the host cell genome to establish latent infection (Bueler, 1999). Recombinant AAV particles have been successfully used for gene transfer into culture neurons, allowing for long-term transgene expression. However the onset of expression after AAV infection is late (Ehrengruber et al., 2001), which would represent a handicap when rescuing survival of *Snap25* null neurons.

On the other hand, the adenovirus system is a system that produces a fast onset in expression, within few days. This virus is a non-enveloped, double stranded DNA virus, which, upon infection, persists in the nucleus as a linear, extrachromosomal molecule producing a long-term sustained expression (Kugler et al., 2001). This technique represents an advantage over the lentivirus system since it produces earlier expression. For the cases where a fast expression of the protein is essential, like, for example, when examining the initial axonal sprouting, this method should constitute an alternative. However, the adenovirus expression is generally higher than the lentivirus one, which

would represent a problem to consider, since overload of SNAP-25 impairs the exocytotic machinery.

2. The study of SNAP-25 is continued in the brain

The analysis of the SNAP-25 function in adult brain is not possible because of the perinatal death of *Snap25*$^{-/-}$ mice. Therefore, the possibilities for study SNAP-25 are limited to exclusively the use of neuronal cultures. This doctoral thesis successfully uses this alternative to gain further knowledge on SNAP-25. Studies on primary cultures present a wide range of advantages, such as the great accessibility for genetical and pharmacological manipulations as well as the enormous variety of analytical tools. However, this is an in vitro system and, therefore, it does not reproduce faithfully what is happening in the brain. For example, the reduction in survival of *Snap25*$^{-/-}$ neurons in culture does not correspond with *in vivo* observations (Washbourne et al., 2002). The reason for that could probably be the existence of compensatory mechanisms in the brain that counteract the decrease in viability of these neurons and that are not present in cultures. Furthermore, some of the mechanisms that enhance facilitation in brain neurons expressing SNAP-25a are missing in cultures. Moreover, alternative splicing of SNAP-25 starts to be prominent after birth. In this doctoral work, I observe differences between SNAP-25a and SNAP-25b in the regulation of the readily releasable pool. I also demonstrate that, after 10-14 days in culture, neurons are mostly expressing SNAP-25b, since the level of neurotransmission in SNAP-25b rescued null neurons matches the one of wild-type neurons. However, some of the mechanisms that occur in adult brain could still have strong influence in SNAP-25 and they could be unseen by the *in vitro* system.

For the study of the function of SNAP-25 in the brain, the ideal approach would be the construction of a SNAP-25 conditional mouse. This genetic manipulation consists on the alteration of a gene function under the activity of regulatory proteins, such as the tetracycline transactivator (tTA) or Cre recombinase that either alter gene transcription or inactivate genes by making deletions. The condition can be created in a defined population of cells and at defined points in time, making the modification spatially and temporally restricted (Nagy, 2000; Mills, 2001; for review, Lewandoski, 2001). By this, SNAP-25 could be selectively inactivated in one region of the brain, such as the

hippocampus, without affecting the expression in the rest of the body. This would propitiate the analysis of SNAP-25 at later stages in maturation and in the adult animal. Furthermore, and since SNAP-25 has been linked to psychiatrical diseases, such as the attention deficit hyperactivity disorder (Barr et al., 2000; Wilson, 2000), a conditional SNAP-25 mouse would allow to investigate the function of SNAP-25 in behavior or learning and memory.

However, since the production of a conditional mouse is difficult and problematic, a possible alternative approach consists on the use of organotypic hippocampal slice cultures (Stoppini et al., 1991; Bahr, 1995). This technique offers a preparation that mimics very closely the situation in vivo since cultured slices retain their morphology and much of their local connections. They are also able to mature physiologically during culturing in a way comparable to what takes place in vivo (Muller et al., 1993). In this way, slice cultures could be prepared from E18 embryonic brains and preserved for several weeks. In addition, since this preparation offers possibility for viral transfection (Ehrengruber et al., 2001), this would allow the introduction of the different SNAP-25 homologues in the same way as presented in this study. However, the preparation of hippocampal slice cultures from small brain is still very challenging.

B. Continuation Projects

1. Functional analysis of SNAP-25 structure

a) SNAP-25/SNAP-23 chimeras

During this doctoral work, I show that SNAP-25 is involved in several processes in the neuron, like arborization, survival and synaptic transmission. I observed, in addition, that the closest SNAP-25 homologue, SNAP-23, shares the same functions as SNAP-25, with the only exception of synchronization of release. This would indicate that SNAP-25 contains in its structure a part that is able to regulate synchronous release but that is missing in SNAP-23. The side-by-side comparison of the structures of SNAP-25 and SNAP-23 can, probably, bring some new clues about which part of the molecule is participating in synchronizing the release and how it does. By using the appropriate cloning techniques (described in Miscellaneous methods used for cloning), it is possible to

design SNAP-25/SNAP-23 chimeras by combining different parts of each homologues. These chimeras can be introduced into *Snap25*$^{-/-}$ neurons and analyze its effect. In this way, the region corresponding to the structure that mediates synchronous release could be, then, dissected.

b) SNAP-25 point mutations

As I presented in this thesis, I found no significant differences in the pair-pulse ratio between SNAP-25a and SNAP-25b rescued neurons. However, Bark et al., 2004 proposed that SNAP-25a enhances facilitation of synaptic transmissionin brain slices. This discrepancy would mean that the facilitation mechanism that is present in the brain is missing in cultures. This facilitation would contribute to the selective strengthening of synaptic contacts in the central nervous system during activity-dependent synapse elimination (Lichtman et al., 1985). In a simplified model for short-term plasticity, the amplitude of each EPSC in a train of action potentials depends on the size of the vesicle pool available for release, *RRP*. This pool is replenished according to a steady 'replenishment rate'. In addition, the release probability, *Rp*, is not constant, since it mainly depends on the residual intracellular calcium concentration (Zucker and Regehr, 2002):

$$EPSC_0 = RRP \cdot Rp_0$$

$$EPSC_1 = (RRP - EPSC_0 + replenishment\ rate) \cdot Rp_1$$

...

$$EPSC_i = (RRP - \sum_{j=0}^{i} EPSC_j + (i-1) \cdot replenishment\ rate) \cdot Rp_i$$

$$with\ Rp_i = Rp_{basal} \cdot f(Ca^{2+})$$

Equation 8
Simplify model for short-term plasticity

According to this model, facilitation ($EPSC_1/EPSC_0$) can be enlarged, mainly, by a decrease in Rp_{basal} or by an increase in the calcium-dependency of Rp. I present in this doctoral thesis that substitution of SNAP-25 by SNAP-23 has severe consequences in the fast triggering of release mediated by synaptotagmin-I, although no significant differences in the release probability exist between SNAP-25a and SNAP-25b rescued neurons. Bark

et al., 2004 proposed that the mechanism by which SNAP-25a enhances facilitation in brain slices is an improved calcium affinity of the exocytotic machinery. Previous studies have suggested that SNAP-25 can modify the calcium-exocytosis coupling, which would possibly modify the release probability. For example, experiments using botulinum toxins have suggested a relation between SNAP-25 and the calcium signal (Chen et al., 1999; Gerona et al., 2000). The structure revealed by X-ray crystallography showed several divalent cation-binding sites on the SNARE domain (Sutton et al., 1998). Verderio et al., 2004 observed a modulation of calcium dynamics by SNAP-25. In addition, overexpression of a mutant SNAP-25 bearing a double residue substitution (E170A/Q177A) decreases the calcium cooperativity of secretion in chromaffin cells (Sørensen et al., 2002). These experiments indicate that SNAP-25 is involved in the transductionof the calcium signal by a very precise mechanism.

The functional analysis of the structure of SNAP-25 would help to understand how SNAP-25 is involved in calcium dynamics. This can be done by the introduction of specific point mutations in SNAP-25 and the expression in the *Snap25* null mouse. This approach could be used on the study of other functions of SNAP-25 as well. For example, a mutation in the layer 5 of SNAP-25, which absolutely abolishes secretion in chromaffin cells (Sørensen et al., 2006), produced a reduction in the survival of both wild-type and *Snap25*$^{-/-}$ null neurons (preliminaryresults).

2. Mechanisms of spontaneous and synchronous release

The presence of spontaneous release in *Snap25*$^{-/-}$ neurons raised the question whether other SNAP-25 homologues could drive this release. From all the possible candidates, SNAP-29 seems to be the most likely since it is present at the synapses, regulating synaptic transmission to some extent (Su et al., 2001; Pan et al., 2005). Using the knock-out and rescue approach, it is possible to express SNAP-29 in *Snap25* null neurons and examine neuronal function under these conditions. SNAP-47, although it seems to participate in endosomal transport (Holt et al., 2006), could be also involved in spontaneous release and it should be examined in parallel.

This doctoral thesis describes how the rescue of SNAP-23 in *Snap25* null is very similar to the phenotype observed in absence of synaptotagmin-I. I concluded that this

could be due to a lack of binding between SNAP-23 and synaptotagmin-I. However, the mechanisms by which SNAP-23 produces this effect are obscure. As discussed above, other members of the synaptotagmin family could trigger the SNAP-23 release. Therefore, it would be interesting to examine the function of SNAP-23 in absence of synaptotagmins. This could be possible by disrupting the binding site between SNAP-23 and synaptotagmin members using point-mutations or by deleting synaptotagmins using knock-out mice. For example, the expression of SNAP-23 into SNAP-25/synaptotagmin-VII double knock-out neurons caused slower kinetics in the evoked release than its expression in SNAP-25 knock-out neurons (Figure 17).

3. Modulation of quantal size by SNAP-25

In this study, I detect a reduction in the mEPSC size in absence of SNAP-25, compatible with a decrease in the AMPA receptor trafficking. This kind of trafficking has been involved in several mechanisms of long-term plasticity (Malinow and Malenka, 2002). Production of long-term properties in hippocampal cultures is difficult and it has not been possible until recently, using very specific protocols (Carroll et al., 1999; Lu et al., 2001). Interestingly, these studies observed that long-term depression and potentiation depends on the exo- and endocytosis of AMPA receptors, respectively. Therefore, it seems important to examine whether SNAP-25 is involved in AMPA receptor trafficking. In the same way, since it is likely that other receptors share a common SNAP-25-dependent trafficking mechanism, like NMDA receptors (Lan et al., 2001b; Lan et al., 2001a), the study should be extended to other families of receptors.

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XI. PUBLICATIONS

Ruder C, Reimer T, Delgado-Martinez I, Hermosilla R, Engelsberg A, Nehring R, Dorken B, Rehm A. EBAG9 adds a new layer of control on large dense-core vesicle exocytosis via interactionwith Snapin. Mol Biol Cell. 2005 Mar;16(3):1245-57.

Delgado-Martinez I, Nehring RB, Sørensen JB. Hierarchical ability of SNAP-25 homologuesto support neuronal function. To be submitted

XII. REFERENCES

Altrock WD, tom Dieck S, Sokolov M, Meyer AC, Sigler A, Brakebusch C, Fassler R, Richter K, Boeckers TM, Potschka H, Brandt C, Loscher W, Grimberg D, Dresbach T, Hempelmann A, Hassan H, Balschun D, Frey JU, Brandstatter JH, Garner CC, Rosenmund C, GundelfingerED (2003) Functional inactivation of a fraction of excitatory synapses in mice deficient for the active zone protein bassoon. Neuron 37:787-800.

American Psychiatric Association (1995) Diagnostic and statistical manual of mental disorders, fourth edition. Washington,DC: American Psychiatric Association.

Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR (2002) Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nat Struct Biol 9:107-111.

Ashery U, Varoqueaux F, Voets T, Betz A, Thakur P, Koch H, Neher E, Brose N, Rettig J (2000) Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. Embo J 19:3586-3596.

Bahr BA (1995) Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 42:294-305.

Bark C, Bellinger FP, Kaushal A, Mathews JR, Partridge LD, Wilson MC (2004) Developmentally regulated switch in alternatively spliced SNAP-25 isoforms alters facilitation of synaptic transmission. J Neurosci 24:8796-8805.

Bark IC (1993) Structure of the chicken gene for SNAP-25 reveals duplicated exon encoding distinct isoforms of the protein. J Mol Biol 233:67-76.

Bark IC, Wilson MC (1994) Human cDNA clones encoding two different isoforms of the nerve terminal protein SNAP-25. Gene 139:291-292.

Bark IC, Hahn KM, Ryabinin AE, Wilson MC (1995) Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. Proc Natl Acad Sci U S A 92:1510-1514.

Barr CL, Feng Y, Wigg K, Bloom S, Roberts W, Malone M, Schachar R, Tannock R, Kennedy JL (2000) Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. Mol Psychiatry 5:405-409.

Bartz SR, Vodicka MA (1997) Production of high-titer human immunodeficiency virus type 1 pseudotyped with vesicular stomatitis virus glycoprotein. Methods 12:337-342.

Baumert M, Maycox PR, Navone F, De Camilli P, Jahn R (1989) Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. Embo J 8:379-384.

Bekkers JM, Stevens CF (1991) Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. Proc Natl Acad Sci U S A 88:7834-7838.

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255-259.

Bergsman JB, Krueger SR, Fitzsimonds RM (2006) Automated criteria-based selection and analysis of fluorescent synaptic puncta. J Neurosci Methods 152:32-39.

Bertram J (2006) MATra - Magnet Assisted Transfection: Combining Nanotechnology and Magnetic Forces to Improve Intracellular Delivery of Nucleic Acids. Curr Pharm Biotechnol 7:277-285.

Betz WJ, Mao F, Smith CB (1996) Imaging exocytosis and endocytosis. Curr Opin Neurobiol 6:365-371.

Bhalla A, Tucker WC, Chapman ER (2005) Synaptotagmin isoforms couple distinct ranges of Ca2+, Ba2+, and Sr2+ concentration to SNARE-mediated membrane fusion. Mol Biol Cell 16:4755-4764.

Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R (1993a) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. Embo J 12:4821-4828.

Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R (1993b) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365:160-163.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.

Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. J Virol 71:6641-6649.

Bock JB, Matern HT, Peden AA, Scheller RH (2001) A genomic perspective on membrane compartment organization. Nature 409:839-841.

Borisovska M, Zhao Y, Tsytsyura Y, Glyvuk N, Takamori S, Matti U, Rettig J, Sudhof T, Bruns D (2005) v-SNAREs control exocytosis of vesicles from priming to fusion. Embo J 24:2114-2126.

Boschert U, O'Shaughnessy C, Dickinson R, Tessari M, Bendotti C, Catsicas S, Pich EM (1996) Developmental and plasticity-related differential expression of two SNAP-25 isoforms in the rat brain. J Comp Neurol 367:177-193.

Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science 256:1021-1025.

Bueler H (1999) Adeno-associated viral vectors for gene transfer and gene therapy. Biol Chem 380:613-622.

Bukrinsky MI, Haffar OK (1999) HIV-1 nuclear import: in search of a leader. Front Biosci 4:D772-781.

Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, Haggerty S, Stevenson M (1992) Active nuclear import of human immunodeficiency virus type 1

preintegration complexes. Proc Natl Acad Sci U S A 89:6580-6584.

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci U S A 90:8033-8037.

Butz S, Fernandez-Chacon R, Schmitz F, Jahn R, Sudhof TC (1999) The subcellular localizations of atypical synaptotagmins III and VI. Synaptotagmin III is enriched in synapses and synaptic plasma membranes but not in synaptic vesicles. J Biol Chem 274:18290-18296.

Capogna M, McKinney RA, O'Connor V, Gahwiler BH, Thompson SM (1997) Ca2+ or Sr2+ partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin. J Neurosci 17:7190-7202.

Carroll RC, Lissin DV, von Zastrow M, Nicoll RA, Malenka RC (1999) Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. Nat Neurosci 2:454-460.

Catsicas S, Larhammar D, Blomqvist A, Sanna PP, Milner RJ, Wilson MC (1991) Expression of a conserved cell-type-specific protein in nerve terminals coincides with synaptogenesis. Proc Natl Acad Sci U S A 88:785-789.

Chen YA, Scheller RH (2001) SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol 2:98-106.

Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH (1999) SNARE complex formation is triggered by Ca2+ and drives membrane fusion. Cell 97:165-174.

Chieregatti E, Chicka MC, Chapman ER, Baldini G (2004) SNAP-23 functions in docking/fusion of granules at low Ca2+. Mol Biol Cell 15:1918-1930.

Clements JD, Bekkers JM (1997) Detection of spontaneous synaptic events with an optimally scaled template. Biophys J 73:220-229.

Clements JV (1992) Sympathomimetics, inotropics, and vasodilators. AACN Clin Issues Crit Care Nurs 3:395-408.

Cochilla AJ, Angleson JK, Betz WJ (1999) Monitoring secretory membrane with FM1-43 fluorescence. Annu Rev Neurosci 22:1-10.

Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5:952-962.

Darios F, Davletov B (2006) Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. Nature 440:813-817.

de Lange RP, de Roos AD, Borst JG (2003) Two modes of vesicle recycling in the rat calyx of Held. J Neurosci 23:10164-10173.

Deak F, Shin OH, Kavalali ET, Sudhof TC (2006) Structural determinants of synaptobrevin 2 function in synaptic vesicle fusion. J Neurosci 26:6668-6676.

Del Castillo J, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560-573.

DePolo NJ, Reed JD, Sheridan PL, Townsend K, Sauter SL, Jolly DJ, Dubensky TW, Jr. (2000) VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivatedby human serum. Mol Ther 2:218-222.

Dodge FA, Jr., Rahamimoff R (1967) Co-operative action a calcium ions in transmitter release at the neuromuscular junction. J Physiol 193:419-432.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L (1998) A third-generation lentivirus vector with a conditional packaging system. J Virol 72:8463-8471.

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC, Rizo J (1999) A conformational switch in syntaxin during exocytosis: role of munc18. Embo Journal 18:4372-4382.

Ehrengruber MU, Hennou S, Bueler H, Naim HY, Deglon N, Lundstrom K (2001) Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. Mol Cell Neurosci 17:855-871.

Elferink LA, Trimble WS, Scheller RH (1989) Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. J Biol Chem 264:11061-11064.

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci U S A 95:15781-15786.

Fatemi SH, Earle JA, Stary JM, Lee S, Sedgewick J (2001) Altered levels of the synaptosomal associated protein SNAP-25 in hippocampus of subjects with mood disorders and schizophrenia. Neuroreport 12:3257-3262.

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109-128.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature 410:41-49.

Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet 25:217-222.

Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L (2002) Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum Gene Ther 13:243-260.

Frassoni C, Inverardi F, Coco S, Ortino B, Grumelli C, Pozzi D, Verderio C, Matteoli M (2005) Analysis of SNAP-25 immunoreactivity in hippocampal inhibitory neurons during developmentin culture and in situ. Neuroscience 131:813-823.

Fukuda M, Kanno E, Satoh M, Saegusa C, Yamamoto A (2004) Synaptotagmin VII is targeted to dense-core vesicles and regulates their Ca2+ -dependent exocytosis in PC12 cells. J Biol Chem 279:52677-52684.

Futerman AH, Banker GA (1996) The economics of neurite outgrowth--the addition of new membrane to growing axons. Trends Neurosci 19:144-149.

Galli T, Haucke V (2001) Cycling of synaptic vesicles: how far? How fast! Sci STKE 2001:RE1.

Galli T, Zahraoui A, Vaidyanathan VV, Raposo G, Tian JM, Karin M, Niemann H, Louvard D (1998) A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. Mol Biol Cell 9:1437-1448.

Gao Z, Reavey-Cantwell J, Young RA, Jegier P, Wolf BA (2000) Synaptotagmin III/VII isoforms mediate Ca2+-induced insulin secretion in pancreatic islet beta -cells. J Biol Chem 275:36079-36085.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC (1994) Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. Cell 79:717-727.

Gerona RR, Larsen EC, Kowalchyk JA, Martin TF (2000) The C terminus of SNAP25 is essential for Ca(2+)-dependent binding of synaptotagmin to SNARE complexes. J Biol Chem 275:6328-6336.

Goda Y, Stevens CF (1994) Two components of transmitter release at a central synapse. Proc Natl Acad Sci U S A 91:12942-12946.

Grant NJ, Hepp R, Krause W, Aunis D, Oehme P, Langley K (1999) Differential expression of SNAP-25 isoforms and SNAP-23 in the adrenal gland. J Neurochem 72:363-372.

Grosse G, Grosse J, Tapp R, Kuchinke J, Gorsleben M, Fetter I, Hohne-Zell B, Gratzl M, Bergmann M (1999) SNAP-25 requirement for dendritic growth of hippocampal neurons. J Neurosci Res 56:539-546.

Hagler DJ, Jr., Goda Y (2001) Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. J Neurophysiol 85:2324-2334.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85-100.

Hanson PI, Heuser JE, Jahn R (1997) Neurotransmitter release - four years of SNARE complexes. Curr Opin Neurobiol 7:310-315.

Harata N, Ryan TA, Smith SJ, Buchanan J, Tsien RW (2001) Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1-43 photoconversion. Proc Natl Acad Sci U S A 98:12748-12753.

Haselhorst D, Kaye JF, Lever AM (1998) Development of cell lines stably expressing human immunodeficiency virus type 1 proteins for studies in encapsidation and gene transfer. J Gen Virol 79 (Pt 2):231-237.

Hata Y, Sudhof TC (1995) A novel ubiquitous form of Munc-18 interacts with multiple syntaxins. Use of the yeast two-hybrid system to study interactions between

proteins involved in membrane traffic. J Biol Chem 270:13022-13028.

Heeroma JH, Roelandse M, Wierda K, van Aerde KI, Toonen RF, Hensbroek RA, Brussaard A, Matus A, Verhage M (2004) Trophic support delays but does not prevent cell-intrinsic degeneration of neurons deficient for munc18-1. Eur J Neurosci 20:623-634.

Hepp R, Perraut M, Chasserot-Golaz S, Galli T, Aunis D, Langley K, Grant NJ (1999) Cultured glial cells express the SNAP-25 analogue SNAP-23. Glia 27:181-187.

Hermening S, Kugler S, Bahr M, Isenmann S (2006) Improved high-capacity adenoviral vectors for high-level neuron-restricted gene transfer to the CNS. J Virol Methods 136:30-37.

Herrmann K, Shatz CJ (1995) Blockade of action potential activity alters initial arborization of thalamic axons within cortical layer 4. Proc Natl Acad Sci U S A 92:11244-11248.

Hess DT, Slater TM, Wilson MC, Skene JH (1992a) The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. J Neurosci 12:4634-4641.

Hess EJ, Jinnah HA, Kozak CA, Wilson MC (1992b) Spontaneous locomotor hyperactivity in a mouse mutant with a deletion including the Snap gene on chromosome 2. J Neurosci 12:2865-2874.

Heyser CJ, Wilson MC, Gold LH (1995) Coloboma hyperactive mutant exhibits delayed neurobehavioral developmental milestones. Brain Res Dev Brain Res 89:264-269.

Hille B (2001) Ion channels of excitable membranes, 3rd ed. Edition. Sunderland, Mass. ; [Great Britain]: Sinauer.

Hirling H, Steiner P, Chaperon C, Marsault R, Regazzi R, Catsicas S (2000) Syntaxin 13 is a developmentally regulated SNARE involved in neurite outgrowth and endosomal trafficking.Eur J Neurosci 12:1913-1923.

Hohenstein AC, Roche PA (2001) SNAP-29 is a promiscuous syntaxin-binding SNARE. Biochem Biophys Res Commun 285:167-171.

Holt M, Varoqueaux F, Wiederhold K, Takamori S, Urlaub H, Fasshauer D, Jahn R (2006) Identification of SNAP-47, a novel Qbc-SNARE with ubiquitous expression. J Biol Chem 281:17076-17083.

Hu C, Ahmed M, Melia TJ, Sollner TH, Mayer T, Rothman JE (2003) Fusion of cells by flipped SNAREs. Science 300:1745-1749.

Hu WS, Temin HM (1990) Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. Proc Natl Acad Sci U S A 87:1556-1560.

Hui E, Bai J, Wang P, Sugimori M, Llinas RR, Chapman ER (2005) Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis. Proc Natl Acad Sci U S A 102:5210-5214.

Igarashi M, Tagaya M, Komiya Y (1997) The soluble N-ethylmaleimide-sensitive factor attached protein receptor complex in growth cones: molecular aspects of the axon terminal development. J Neurosci 17:1460-1470.

Igarashi M, Kozaki S, Terakawa S, Kawano S, Ide C, Komiya Y (1996) Growth cone collapse and inhibition of neurite growth by Botulinum neurotoxin C1: a t-SNARE is involved in axonal growth. J Cell Biol 134:205-215.

Jahn R, Sudhof TC (1999) Membrane fusion and exocytosis. Annu Rev Biochem 68:863-911.

Jessell TM, Kandel ER (1993) Synaptic transmission: a bidirectional and self-modifiable form of cell-cell communication. Cell 72 Suppl:1-30.

Kandel ER (2000) Principles of neural science, 4. Edition. New York, NY. [u.a.]: McGraw-Hill.

Katz B (1969) The release of neural transmitter substances. Liverpool: University Press. University of Liverpool.

Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. J Virol 72:811-816.

Kohrmann M, Haubensak W, Hemraj I, Kaether C, Lessmann VJ, Kiebler MA (1999) Fast, convenient, and effective method to transiently transfect primary hippocampal neurons. J Neurosci Res 58:831-835.

Kugler S, Meyn L, Holzmuller H, Gerhardt E, Isenmann S, Schulz JB, Bahr M (2001) Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinantadenoviral vectors. Mol Cell Neurosci 17:78-96.

Lan JY, Skeberdis VA, Jover T, Zheng X, Bennett MV, Zukin RS (2001a) Activation of metabotropic glutamate receptor 1 accelerates NMDA receptor trafficking. J Neurosci 21:6058-6068.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV, Zukin RS (2001b) Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 4:382-390.

Landau NR, Page KA, Littman DR (1991) Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J Virol 65:162-169.

Leung SM, Chen D, DasGupta BR, Whiteheart SW, Apodaca G (1998) SNAP-23 requirement for transferrin recycling in Streptolysin-O-permeabilized Madin-Darby canine kidney cells. J Biol Chem 273:17732-17741.

Lewandoski M (2001) Conditional control of gene expression in the mouse. Nat Rev Genet 2:743-755.

Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J Virol 68:510-516.

Lichtman JW, Wilkinson RS, Rich MM (1985) Multiple innervation of tonic

endplates revealed by activity-dependent uptake of fluorescent probes. Nature 314:357-359.

Littleton JT, Bai J, Vyas B, Desai R, Baltus AE, Garment MB, Carlson SD, Ganetzky B, Chapman ER (2001) synaptotagmin mutants reveal essential functions for the C2B domain in Ca2+-triggered fusion and recycling of synaptic vesicles in vivo. J Neurosci 21:1421-1433.

Liu Z, Tearle AW, Nai Q, Berg DK (2005) Rapid activity-driven SNARE-dependent trafficking of nicotinic receptors on somatic spines. J Neurosci 25:1159-1168.

Lu W, Man H, Ju W, Trimble WS, MacDonald JF, Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron 29:243-254.

Luscher C, Nicoll RA, Malenka RC, Muller D (2000) Synaptic plasticity and dynamic modulationof the postsynaptic membrane. Nat Neurosci 3:545-550.

Luscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC, Nicoll RA (1999) Role of AMPA receptor cycling in synaptic transmission and plasticity. Neuron 24:649-658.

Maher BJ, Mackinnon RL, 2nd, Bai J, Chapman ER, Kelly PT (2005) Activation of postsynaptic Ca(2+) stores modulates glutamate receptor cycling in hippocampal neurons. J Neurophysiol 93:178-188.

Maletic-Savatic M, Koothan T, Malinow R (1998) Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II. J Neurosci 18:6814-6821.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103-126.

Mariani R, Rasala BA, Rutter G, Wiegers K, Brandt SM, Krausslich HG, Landau NR (2001) Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. J Virol 75:3141-3151.

Martinez-Arca S, Alberts P, Zahraoui A, Louvard D, Galli T (2000) Role of tetanus neurotoxin insensitive vesicle-associated membrane protein (TI-VAMP) in vesicular transport mediating neurite outgrowth. J Cell Biol 149:889-900.

Martinez-Arca S, Coco S, Mainguy G, Schenk U, Alberts P, Bouille P, Mezzina M, Prochiantz A, Matteoli M, Louvard D, Galli T (2001) A common exocytotic mechanism mediates axonal and dendritic outgrowth. J Neurosci 21:3830-3838.

Martinez I, Chakrabarti S, Hellevik T, Morehead J, Fowler K, Andrews NW (2000) Synaptotagmin VII regulates Ca(2+)-dependent exocytosis of lysosomes in fibroblasts. J Cell Biol 148:1141-1149.

Maximov A, Sudhof TC (2005) Autonomous function of synaptotagmin 1 in triggering synchronous release independent of asynchronous release. Neuron 48:547-554.

McKinney RA, Capogna M, Durr R, Gahwiler BH, Thompson SM (1999) Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat

Neurosci 2:44-49.

Meinrenken CJ, Borst JG, Sakmann B (2003) Local routes revisited: the space and time dependence of the Ca2+ signal for phasic transmitter release at the rat calyx of Held. J Physiol 547:665-689.

Mills AA (2001) Changing colors in mice: an inducible system that delivers. Genes Dev 15:1461-1467.

Misura KM, Scheller RH, Weis WI (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404:355-362.

Miya F, Yamamoto A, Akagawa K, Kawamoto K, Tashiro Y (1996) Localization of HPC-1/syntaxin1 in developing rat cerebellar cortex. Cell Struct Funct 21:525-532.

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirusvector. J Virol 72:8150-8157.

Morgans CW, Brandstatter JH, Kellerman J, Betz H, Wassle H (1996) A SNARE complex containing syntaxin 3 is present in ribbon synapses of the retina. J Neurosci 16:6713-6721.

Moulder KL, Mennerick S (2005) Reluctant vesicles contribute to the total readily releasable pool in glutamatergic hippocampal neurons. J Neurosci 25:3842-3850.

Muller D, Buchs PA, Stoppini L (1993) Time course of synaptic development in hippocampal organotypiccultures. Brain Res Dev Brain Res 71:93-100.

Murthy VN, Stevens CF (1999) Reversal of synaptic vesicle docking at central synapses. Nat Neurosci 2:503-507.

Murthy VN, Sejnowski TJ, Stevens CF (1997) Heterogeneous release properties of visualized individualhippocampal synapses. Neuron 18:599-612.

Nagy A (2000) Cre recombinase: the universal reagent for genome tailoring. Genesis 26:99-109.

Nagy G, Milosevic I, Fasshauer D, Muller EM, de Groot BL, Lang T, Wilson MC, Sørensen JB (2005) Alternative Splicing of SNAP-25 Regulates Secretion through Nonconservative Substitutions in the SNARE Domain. Mol Biol Cell.

Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996a) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A 93:11382-11388.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996b) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267.

Neher E, Sakmann B (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature 260:799-802.

Niemann H, Blasi J, Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. Trends Cell Biol 4:179-185.

Nishiki T, Augustine GJ (2004) Synaptotagmin I synchronizes transmitter release in

mouse hippocampal neurons. J Neurosci 24:6127-6132.

Olivo-Marin J-C (2002) Extraction of spots in biological images using multiscale products. Pattern Recognition35:1989-1996.

Osen-Sand A, Catsicas M, Staple JK, Jones KA, Ayala G, Knowles J, Grenningloh G, Catsicas S (1993) Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature 364:445-448.

Osen-Sand A, Staple JK, Naldi E, Schiavo G, Rossetto O, Petitpierre S, Malgaroli A, Montecucco C, Catsicas S (1996) Common and distinct fusion proteins in axonal growth and transmitter release. J Comp Neurol 367:222-234.

Otsu Y, Shahrezaei V, Li B, Raymond LA, Delaney KR, Murphy TH (2004) Competition between phasic and asynchronous release for recovered synaptic vesicles at developing hippocampal autaptic synapses. J Neurosci 24:420-433.

Owe-Larsson B, Berglund M, Kristensson K, Garoff H, Larhammar D, Brodin L, Low P (1999) Perturbation of the synaptic release machinery in hippocampal neurons by overexpression of SNAP-25 with the Semliki Forest virus vector. Eur J Neurosci 11:1981-1987.

Oyler GA, Polli JW, Wilson MC, Billingsley ML (1991) Developmental expression of the 25-kDa synaptosomal-associated protein (SNAP-25) in rat brain. Proc Natl Acad Sci U S A 88:5247-5251.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 109:3039-3052.

Page KA, Landau NR, Littman DR (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J Virol 64:5270-5276.

Pan PY, Cai Q, Lin L, Lu PH, Duan S, Sheng ZH (2005) SNAP-29-mediated modulation of synaptic transmission in cultured hippocampal neurons. J Biol Chem 280:25769-25779.

Parolin C, Dorfman T, Palu G, Gottlinger H, Sodroski J (1994) Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. J Virol 68:3888-3895.

Pérez-Otaño I, Ehlers MD (2005) Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229-238.

Pobbati AV, Stein A, Fasshauer D (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. Science 313:673-676.

Purkiss JR, Friis LM, Doward S, Quinn CP (2001) Clostridium botulinum neurotoxins act with a wide range of potencies on SH-SY5Y human neuroblastoma cells. Neurotoxicology 22:447-453.

Ravichandran V, Chawla A, Roche PA (1996) Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. J Biol Chem 271:13300-13303.

Rea S, Martin LB, McIntosh S, Macaulay SL, Ramsdale T, Baldini G, James DE (1998) Syndet, an adipocyte target SNARE involved in the insulin-induced translocation of GLUT4 to the cell surface. J Biol Chem 273:18784-18792.

Reddy A, Caler EV, Andrews NW (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 106:157-169.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell 104:71-81.

Rickman C, Jimenez JL, Graham ME, Archer DA, Soloviev M, Burgoyne RD, Davletov B (2006) Conserved prefusion protein assembly in regulated exocytosis. Mol Biol Cell 17:283-294.

Risinger C, Deitcher DL, Lundell I, Schwarz TL, Larhammar D (1997) Complex gene organization of synaptic protein SNAP-25 in Drosophila melanogaster. Gene 194:169-177.

Rizo J, Sudhof TC (2002) Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci 3:641-653.

Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev Neurosci 6:57-69.

Roberto M (2003) AMPA receptor trafficking and long-term potentiation. Philosophical Transactions of the Royal Society B: BiologicalSciences 358:707-714.

Roe T, Reynolds TC, Yu G, Brown PO (1993) Integration of murine leukemia virus DNA depends on mitosis. Embo J 12:2099-2108.

Rosenmund C, Stevens CF (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16:1197-1207.

Rosenmund C, Feltz A, Westbrook GL (1995) Synaptic NMDA receptor channels have a low open probability.J Neurosci 15:2788-2795.

Rosenmund C, Sigler A, Augustin I, Reim K, Brose N, Rhee JS (2002) Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. Neuron 33:411-424.

Rossetto O, Seveso M, Caccin P, Schiavo G, Montecucco C (2001) Tetanus and botulinum neurotoxins: turning bad guys into good by research. Toxicon 39:27-41.

Rudy B, Iverson LE (1992) Ion channels. San Diego ; London: Academic.

Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ (1993) The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron 11:713-724.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA-Polymerase.Science 239:487-491.

Sakaba T, Neher E (2001a) Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse. Neuron 32:1119-1131.

Sakaba T, Neher E (2001b) Quantitative relationship between transmitter release and calcium current at the calyx of held synapse. J Neurosci 21:462-476.

Sakaba T, Stein A, Jahn R, Neher E (2005) Distinct kinetic changes in neurotransmitter release after SNARE protein cleavage. Science 309:491-494.

Sambrook J, Russell DW (2001) Molecular cloning : a laboratory manual, 3rd ed. Edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Sara Y, Virmani T, Deak F, Liu X, Kavalali ET (2005) An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron 45:563-573.

Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. Physiol Rev 80:717-766.

Schneggenburger R, Neher E (2005) Presynaptic calcium and control of vesicle fusion. Curr Opin Neurobiol 15:266-274.

Schneggenburger R, Meyer AC, Neher E (1999) Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse. Neuron 23:399-409.

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof TC, Kavalali ET (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294:1117-1122.

Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC, Sudhof TC (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. Nature 415:321-326.

Shimohama S, Fujimoto S, Sumida Y, Akagawa K, Shirao T, Matsuoka Y, Taniguchi T (1998) Differential expression of rat brain synaptic proteins in development and aging. Biochem Biophys Res Commun 251:394-398.

Shirasu M, Kimura K, Kataoka M, Takahashi M, Okajima S, Kawaguchi S, Hirasawa Y, Ide C, Mizoguchi A (2000) VAMP-2 promotes neurite elongation and SNAP-25A increases neurite sprouting in PC12 cells. Neurosci Res 37:265-275.

Sieburth D, Ch'ng Q, Dybbs M, Tavazoie M, Kennedy S, Wang D, Dupuy D, Rual JF, Hill DE, Vidal M, Ruvkun G, Kaplan JM (2005) Systematic analysis of genes required for synapse structure and function. Nature 436:510-517.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-324.

Sørensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, Neher E (2003) Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. Cell 114:75-86.

Sørensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, Grubmuller H, Fasshauer D (2006) Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. Embo J.

Sørensen JB, Matti U, Wei SH, Nehring RB, Voets T, Ashery U, Binz T, Neher E,

Rettig J (2002) The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. Proc Natl Acad Sci U S A 99:1627-1632.

Steegmaier M, Yang B, Yoo JS, Huang B, Shen M, Yu S, Luo Y, Scheller RH (1998) Three novel proteins of the syntaxin/SNAP-25 family. J Biol Chem 273:34171-34179.

Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173-182.

Su Q, Mochida S, Tian JH, Mehta R, Sheng ZH (2001) SNAP-29: a general SNARE protein that inhibits SNARE disassembly and is implicated in synaptic transmission. Proc Natl Acad Sci U S A 98:14038-14043.

Sudhof TC (2002) Synaptotagmins: why so many? J Biol Chem 277:7629-7632.

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.

Sugita S, Han W, Butz S, Liu X, Fernandez-Chacon R, Lao Y, Sudhof TC (2001) Synaptotagmin VII as a plasma membrane Ca(2+) sensor in exocytosis. Neuron 30:459-473.

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395:347-353.

Swain A, Coffin JM (1989) Polyadenylation at correct sites in genome RNA is not required for retrovirus replicationor genome encapsidation. J Virol 63:3301-3306.

Swain A, Coffin JM (1992) Mechanism of transduction by retroviruses. Science 255:841-845.

Tafoya LC, Mameli M, Miyashita T, Guzowski JF, Valenzuela CF, Wilson MC (2006) Expression and function of SNAP-25 as a universal SNARE component in GABAergic neurons. J Neurosci 26:7826-7838.

Terrian DM, White MK (1997) Phylogenetic analysis of membrane trafficking proteins: a family reunion and secondary structure predictions. Eur J Cell Biol 73:198-204.

Thompson PM, Kelley M, Yao J, Tsai G, van Kammen DP (2003) Elevated cerebrospinal fluid SNAP-25 in schizophrenia. Biol Psychiatry 53:1132-1137.

Threadgill R, Bobb K, Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. Neuron 19:625-634.

Trimble WS, Cowan DM, Scheller RH (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci U S A 85:4538-4542.

Trommershauser J, Schneggenburger R, Zippelius A, Neher E (2003) Heterogeneous presynaptic release probabilities: functional relevance for short-term plasticity. Biophys J 84:1563-1579.

Trono D (2002) Lentiviral vectors. Berlin ; London: Springer.

Trudeau LE, Fang Y, Haydon PG (1998) Modulation of an early step in the secretory machinery in hippocampal nerve terminals. Proc Natl Acad Sci U S A 95:7163-

7168.

Vaidyanathan VV, Puri N, Roche PA (2001) The last exon of SNAP-23 regulates granule exocytosis from mast cells. J Biol Chem 276:25101-25106.

Vaidyanathan VV, Yoshino K, Jahnz M, Dorries C, Bade S, Nauenburg S, Niemann H, Binz T (1999) Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains and amino acid residues controlling the formation of enzyme-substrate complexes and cleavage. J Neurochem 72:327-337.

Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C, Reim K, Rosenmund C (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A 99:9037-9042.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M (2004) SNAP-25 modulation of calcium dynamics underlies differencesin GABAergic and glutamatergic responsiveness to depolarization. Neuron 41:599-610.

Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M, Geuze HJ, Sudhof TC (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287:864-869.

Voets T, Neher E, Moser T (1999) Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. Neuron 23:607-615.

Wang G, Witkin JW, Hao G, Bankaitis VA, Scherer PE, Baldini G (1997) Syndet is a novel SNAP-25 related protein expressed in many tissues. J Cell Sci 110 (Pt 4):505-513.

Washbourne P, McAllister AK (2002) Techniques for gene transfer into neurons. Curr Opin Neurobiol 12:566-573.

Washbourne P, Liu XB, Jones EG, McAllister AK (2004) Cycling of NMDA receptors during trafficking in neurons before synapse formation. J Neurosci 24:8253-8264.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC (2002) Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci 5:19-26.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772.

Weimbs T, Mostov K, Low SH, Hofmann K (1998) A model for structural similarity between different SNARE complexes based on sequence relationships. Trends Cell Biol 8:260-262.

Wienisch M, Klingauf J (2006) Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. Nat Neurosci 9:1019-1027.

Williamson LC, Neale EA (1998) Syntaxin and 25-kDa synaptosomal-associated

protein: differential effects of botulinum neurotoxins C1 and A on neuronal survival. J Neurosci Res 52:569-583.

Wilson MC (2000) Coloboma mouse mutant as an animal model of hyperkinesis and attention deficit hyperactivity disorder. Neurosci Biobehav Rev 24:51-57.

Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 16:5425-5436.

Xu T, Binz T, Niemann H, Neher E (1998) Multiple kinetic components of exocytosis distinguished by neurotoxinsensitivity. Nat Neurosci 1:192-200.

Xu T, Rammner B, Margittai M, Artalejo AR, Neher E, Jahn R (1999) Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. Cell 99:713-722.

Yang Y, Udayasankar S, Dunning J, Chen P, Gillis KD (2002) A highly Ca2+-sensitive pool of vesicles is regulated by protein kinase C in adrenal chromaffin cells. Proc Natl Acad Sci U S A 99:17060-17065.

Young CE, Arima K, Xie J, Hu L, Beach TG, Falkai P, Honer WG (1998) SNAP-25 deficit and hippocampal connectivityin schizophrenia. Cereb Cortex 8:261-268.

Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA, Martin TF (2002) Ca2+-dependent synaptotagmin binding to SNAP-25 is essential for Ca2+-triggered exocytosis. Neuron 34:599-611.

Zhou Q, Xiao J, Liu Y (2000) Participation of syntaxin 1A in membrane trafficking involving neurite elongationand membrane expansion. J Neurosci Res 61:321-328.

Zigmond MJ (1999) Fundamental neuroscience. San Diego ; London: Academic.

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355-405.

Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72:9873-9880.

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