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Differential Abilities of SNAP-25 Homologs to Support Neuronal Function

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The SNAP receptor (SNARE) complex, consisting of synaptosome-associated protein of 25 kDa (SNAP-25), synaptobrevin-2, and syntaxin-1, is involved in synaptic vesicles exocytosis. In addition, SNAP-25 has been implicated in constitutive exocytosis processes required for neurite outgrowth. However, at least three isoforms of SNAP-25 have been reported from neurons: SNAP-23, which is also present in non-neuronal cells, and the two alternative splice variants SNAP-25a and SNAP-25b. Here, we studied the differential ability of these isoforms to support the functions previously broadly ascribed to "SNAP-25." We studied the rescue of *snap-25* null neurons in culture with different SNAP-25 homologs. We find that deletion of SNAP-25 leads to strongly reduced neuron survival, and, in the few surviving cells, impaired arborization, reduced spontaneous release, and complete arrest of evoked release. Lentiviral expression of SNAP-25a, SNAP-25b, or SNAP-23 rescued neuronal survival, arborization, amplitude, and frequency of spontaneous events. Also evoked release was rescued by all isoforms, but synchronous release required SNAP-25a/b in both glutamatergic and GABAergic neurons. SNAP-23 supported asynchronous release only, reminiscent of *synaptotagmin-1* null neurons. SNAP-25b was superior to SNAP-25a in vesicle priming, resembling the shift to larger releasable vesicle pools that accompanies synaptic maturation. These data demonstrate a differential ability of SNAP-25b, SNAP-25a, and SNAP-23 to support neuronal function.

Key words: **SNAP-25; SNAP-23; neurite outgrowth; asynchronous release; hippocampal neurons; striatal neurons**

Introduction

The neuronal SNAP receptor (SNARE) complex, consisting of synaptobrevin-2 [vesicle-associated membrane protein (VAMP-2)], syntaxin-1, and synaptosome-associated protein of 25 kDa (SNAP-25), is required for synaptic vesicle exocytosis (Jahn et al., 2003; Sudhof, 2004). However, SNARE functions extend to constitutive exocytosis required for branching, receptor trafficking, and housekeeping recycling of membrane components. The three neuronal SNAREs differentially participate in these events. The majority of data neither imply synaptobrevin-2 nor syntaxin-1 in outgrowth (Osen-Sand et al., 1996; Shirasu et al., 2000; Zhou et al., 2000; Schoch et al., 2001; Darios and Davletov, 2006), indicating the involvement of other SNAREs (Hepp and Langley, 2001), including tetanus neurotoxin insensitive VAMP/VAMP-7 (Martinez-Arca et al., 2000; Martinez-Arca et al., 2001), syntaxin-3 (Darios and Davletov, 2006), and syntaxin-13 (Hirling et al., 2000). In contrast, botulinum neurotoxin A (BoNT/A), which cleaves SNAP-25, and antisense oligonucleotides against SNAP-25 inhibit axonal and dendritic outgrowth (Osen-Sand et al., 1993, 1996; Grosse et al., 1999; Morihara et al., 1999), and SNAP-25 is involved in NMDA receptor trafficking (Lan et al., 2001). SNAP-25 might therefore be a shared component between different SNARE complexes responsible for fast exocytosis and constitutive membrane cycling, respectively.

However, the picture is not clear, because most studies were performed in pheochromocytoma PC12 cells rather than in neurons. In addition, at least three different homologs of SNAP-25 might coexist in neurons (or PC12 cells). Alternative splicing of exon five creates two different splice variants, SNAP-25a and SNAP-25b (Bark, 1993). Splicing is developmentally regulated, so that the expression switches from the "a" to the "b" splice variant after birth in neurons (Bark et al., 1995) but not in adrenal chromaffin cells (Bark et al., 1995; Grant et al., 1999). SNAP-25b supports a larger primed vesicle pool than SNAP-25a in chromaffin cells (Sorensen et al., 2003). In apparent contrast to this finding, a transgenic mouse with a higher SNAP-25a/b ratio displayed a difference in short-term synaptic plasticity in hippocampal neurons (Bark et al., 2004). Another puzzling fact is that embryonic axonal outgrowth is intact (Molnar et al., 2002), and spontaneous activity persists (Washbourne et al., 2002) in the *snap-25* knock-out mouse. In fact, a SNAP-25-independent exocytotic pathway was proposed to support neurotransmission in GABAergic neurons (Verderio et al., 2004; Frassoni et al., 2005), and the possibility was raised that the ubiquitously expressed homolog SNAP-23/syndet (Ravichandran et al., 1996; Wang et al., 1997) might substitute for SNAP-25 (Verderio et al., 2004). Most experiments performed until now do not allow the assign-

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ment of the functions broadly attributed to "SNAP-25" to a specific variant. For instance, botulinum neurotoxin A also cleaves rodent (but not human) SNAP-23 (Vaidyanathan et al., 1999), albeit with reduced efficiency, and neurons expressing only one of the two SNAP-25 splice variants have not been studied.

Here, using neurons isolated from *snap-25*$^{-/-}$ embryos, we investigated how these three SNAP-25 isoforms support neuronal function. We find that, whereas all isoforms support survival and outgrowth, only the SNAP-25 variants are able to support synchronous release in glutamatergic and GABAergic neurons and that SNAP-25b is superior to SNAP-25a in vesicle priming.

Materials and Methods

Cell culture. Astrocyte feeder cells were prepared as described previously (Pyott and Rosenmund, 2002). *snap-25*$^{-/-}$ embryos and control (+/+; +/−) littermates were obtained from heterozygous crossings at embryonic day 18 (E18). Brains were dissected out and cleaned for meninges and vascular tissue, and either the hippocampus or the striatum was dissected. Excised tissues were collected in HBSS (Sigma, St. Louis, MO), buffered with 7 mM HEPES and incubated for 30 min in 0.25% trypsinized HBSS at 37°C. After washing, neurons were triturated with fire-polished Pasteur pipettes, counted with a hemacytometer, and plated on astrocyte feeder cell layers in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen), 17.3 mM HEPES, 1% GlutaMax-I (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 25 μM β-mercapto-ethanol, and 100 nM insulin (Heeroma et al., 2004). Neurons were allowed to mature for 10–14 d before they were used for experiments. For electrophysiological experiments, cells were plated on astrocyte microislands (Bekkers and Stevens, 1991), and only islands containing single neurons were examined. Cell culture solutions were purchased from Invitrogen.

Lentivirus construction. The lentivirus plasmid corresponding to the SNAP-25 a and b isoforms and SNAP-23 were generated by subcloning the inserts of pSFV1 vectors (described by Sorensen et al., 2003) into the pRRLsin.cPPT.CMV.WPRE lentiviral transfer vector (Follenzi et al., 2002), which is a so-called "advanced" generation construct containing the cPPT sequence from the *pol* gene and the posttranscriptional regulatory element of woodchuck hepatitis virus (Follenzi et al., 2000). The cloned gene was placed under the control of the cytomegalovirus (CMV) promotor. Three different constructs were cloned for each homolog. In the first one, an internal ribosomal entry site was interposed between the SNAP-25 homolog and the downstream expression marker enhanced green fluorescent protein (eGFP). To produce neuron-specific expression of the reporter gene, a second construct was built in which the SNAP-25 isoform was placed under control of the CMV promotor and followed by the simian virus 40 (SV40) polyadenylation signal. Expression of eGFP was driven by a synapsin-I promoter placed downstream of SV40. The final set of constructs were made by fusing GFP N terminally to SNAP-23/25 separated by a 25 amino acid linker. These constructs were also under control of the CMV promotor. All constructs were verified by sequencing. The first set of constructs was used for functional electrophysiological studies, whereas the synapsin-driven eGFP constructs were used for morphometric studies. The GFP fusion constructs were used to estimate relative expression levels of the three isoforms and to investigate the localization of the constructs.

Lentiviral production. The transfer vector plasmid and the helper plasmids were transfected into human embryonic kidney HEK293T cell line using Lipofectamine 2000 according the standard protocol from Invitrogen. On the next day, the transfection medium was replaced by IMEM (Sigma) containing 2% FCS, 1% penicillin/streptomycin, 1% nonessential amino acids (Invitrogen), and 1% GlutaMax-I. Twenty-four hours later, the lentivirus was harvested and concentrated using a centrifugal filter device (100,000 molecular weight cutoff; Amicon Ultra-15; Millipore, Bedford, MA). Final volume was adjusted to 2 ml with 10 mM Tris-HCl and 150 mM NaCl, pH 7.4, and 250,000 infectious units were added per neuronal culture at 1 d *in vitro* (1 DIV).

FM 5-95 staining. Coverslips with hippocampal cultures growing on continuous astrocyte layers (10–14 DIV) were mounted in a perfusion chamber on a movable stage of an inverted microscope. Cells were perfused at room temperature in standard extracellular solution (140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM $CaCl_2$, and 4 mM $MgCl_2$, 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 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 at ~15 mm) in saline containing 10 μM FM 5-95 [*N*-(3-trimethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide], followed by an additional 60 s of dye exposure to ensure complete labeling of all recycling vesicles. Individual boutons were imaged after 10 min perfusion with dye-free external solution. Destaining of hippocampal terminals was achieved by three electrical trains of 400 stimulations at 10 Hz using the same stimulation parameters as for loading. Images were taken using a cooled slow-scan CCD camera (PCO SensiCam, Kelheim, Germany) on an Axiovert 135 TV inverted microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany) with a 63×, 1.2 numerical aperture (NA) water-immersion objective (Zeiss) and a modified filter set [dichroic long pass 495 nm and bandpass (BP) 525/50 nm for eGFP; dichroic long pass 565 nm and long pass (LP) 620 nm for FM 5-95]. FM 5-95 was excited at 515 nm by repetitive xenon arc lamp illumination (Polychrom II; T.I.L.L. Photonics, Martinsried, Germany). Imaging data were digitized, and synaptic boutons were identified using an automated spot detection algorithm (Bergsman et al., 2006). Additional analysis was performed with custom-written macros in IgorPro (WaveMetrics, Lake Oswego, OR).

Immunostaining. Hippocampal neuronal cultures on astrocyte layers (see Fig. 1) or on poly-L-lysine/collagen-coated coverslips (see Fig. 2) were fixed for 1 h at room temperature in PBS containing 4% paraformaldehyde. They were washed twice in PBS, incubated for 10 min with 50 mM NH_4Cl in PBS, and washed again. Cultures were incubated for 1 h with primary antibodies in the presence of goat serum (10%) and BSA (3%). The primary antibodies used were anti-SNAP-25 (1:400, rabbit polyclonal; recognizing both SNAP-25a and SNAP-25b; Synaptic Systems, Göttingen, Germany), anti-SNAP-23 (1:400; Synaptic Systems), synaptophysin (1:200, mouse monoclonal; Synaptic Systems), and anti-GFP (1:200, rabbit polyclonal; Synaptic Systems). The cells were washed four times for 10 min with PBS and then incubated for 1 h with secondary antibodies diluted 1:1000 to 1:200. We used Alexa 546-coupled goat-anti-rabbit (Invitrogen) against the anti-SNAP-23 or anti-SNAP-25, Alexa 647-coupled goat anti-mouse for detection of synaptophysin and Alexa488-coupled goat anti-rabbit (in experiments with anti-GFP). Cultures were finally washed four times in PBS and imaged immediately or kept at 4°C overnight .

Immunofluorescence images in Figure 1 were taken with a confocal microscope (LSM 410 controlled by LSM 3.98 software attached to an Axiovert 135TV; Zeiss). Argon lasers were used for exciting at 488 and 543 nm and a helium–neon laser for 633 nm excitation. Emitted light was filtered with a 510–525 nm BP filter, a 570 nm LP filter, and a 665 nm LP filter, respectively. Images were taken using a 63× oil immersion (1.4 NA) objective at 1024 × 1024 pixels. Images were imported into IgorPro (WaveMetrics) and analyzed with custom-written IgorPro functions. The number of neuronal branches was calculated from the hybrid median filtered maximal projection of a confocal stack of images. An IgorPro function was used to detect the number of eGFP-positive processes crossing a circumference (radius of 0–50 μm) centered in the cell soma. Crosses were distinguished by detecting changes over threshold of the first derivative of the fluorescence intensity level. The number of synapses was calculated from synaptophysin-specific staining using an automated spot detection algorithm (Bergsman et al., 2006).

The relative expression levels (see Fig. 2*B*) were estimated from the GFP fluorescence of live (unfixed) neurons growing on poly-L-lysine/collagen-coated coverslips and expressing GFP-tagged versions of the three isoforms. The fluorescence was measured with an Imago QE camera on an Axiovert-200 driven by TILLVision. The light source was a Polychrome V (T.I.L.L. Photonics), and appropriate filters were used (excitation filter, 470/40 nm bandpass; beam splitter, 495 nm; emission filter, 525/50 nm bandpass). The fluorescence was quantified using an area-of-interest placed over the cell body in TILLVision. After fixation (see above), the neurons were stained against GFP (to intensify the fluo-

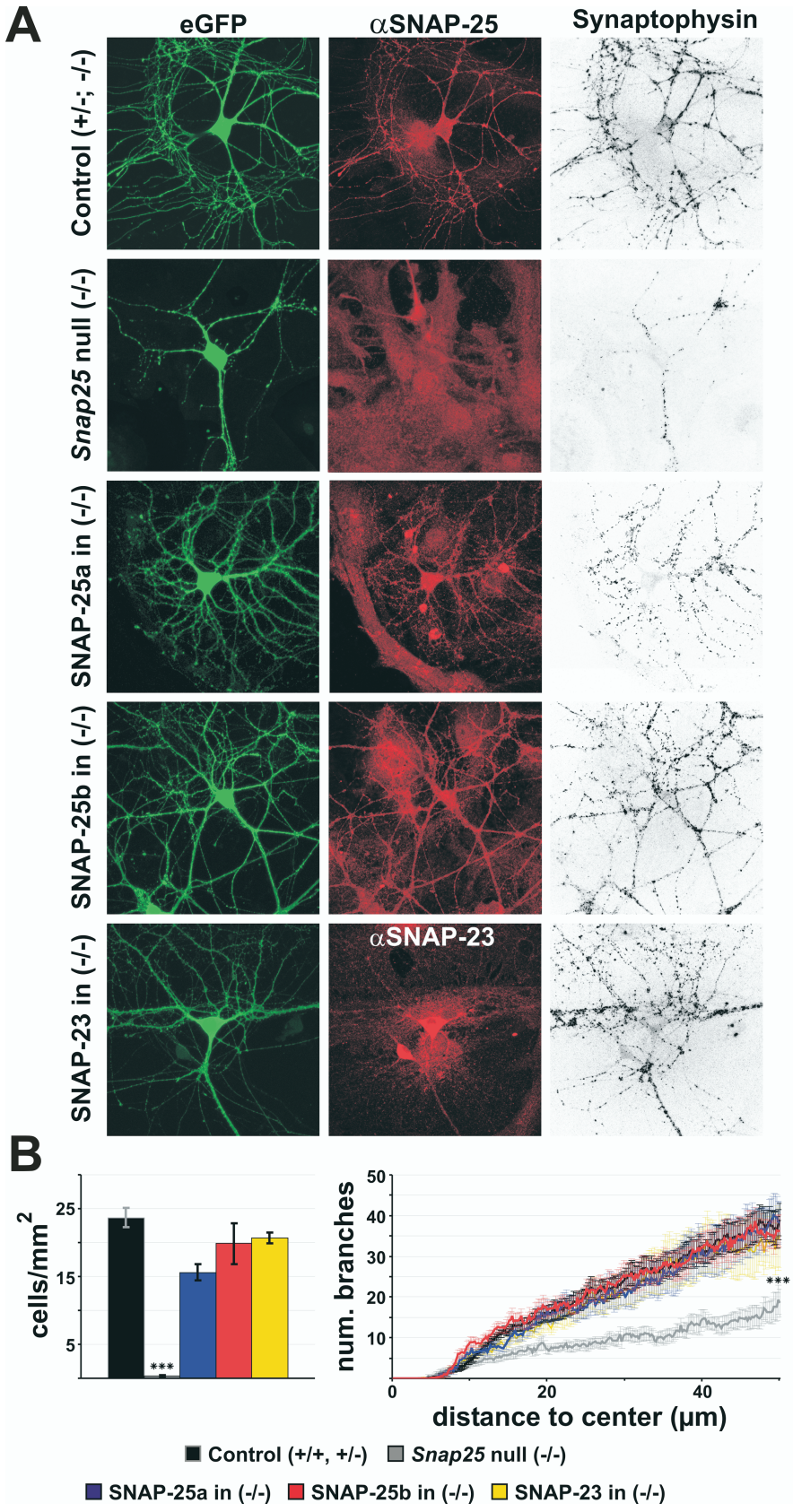

Figure 1. Elimination of SNAP-25 leads to impaired neuronal survival and outgrowth. ***A***, Double staining for SNAP-25 (or SNAP-23) and synaptophysin as a synaptic marker of primary cultured hippocampal neurons infected with recombinant lentiviruses. A synapsin promotor was used to restrict eGFP expression to neurons and enable morphological analysis (left column). This revealed inferior outgrowth/branching in *Snap-25* null (−/−) neurons compared with control (+/+; +/−). Nevertheless, neurons lacking SNAP-25 still formed synaptophysin-positive synapses. Expression of SNAP-25a, SNAP-25b, or SNAP-23 in *Snap-25* null neurons recovered the morphology. ***B***, Left, The number of cells (mean ± SEM) present after 10–14 d in culture. Survival of null neurons was dramatically reduced but rescued with SNAP-25a, SNAP-25b, or SNAP-23 expression. Right, The number of branches (mean ± SEM) as a function of distance to the soma. Neurite extension in surviving *Snap-25* null neurons was significantly depressed (***$p = 0.001$, Student's *t* test) compared with control neurons or null neurons rescued with SNAP-25a, SNAP-25b, or SNAP-23.

rescence) and synaptophysin, and the cells were imaged on the same setup to visualize the localization of expressed protein (see Fig. 2*A*).

Electrophysiology. Autaptic cells between 10 and 14 d *in vitro* were used for experiments. The patch-pipette solution included 135 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 4.6 mM $MgCl_2$, 4 mM Na^+-ATP, 15 mM creatine phosphate, and 50 U/ml phosphocreatine kinase, 300 mOsm, pH 7.3. The standard extracellular medium was described above. Cells were whole-cell voltage clamped at −70 mV with an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) under control of Pulse 8.70 program (HEKA Elektronik). 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 pipette tip diameter was kept at ~2 μm; the resistance ranged from 2.5 to 3.5 MΩ.

All recordings were made at room temperature. EPSCs were evoked by depolarizing the cell from −70 to 0 mV for 2 ms every 5–10 s (0.1–0.2 Hz). The readily releasable pool (RRP) was determined by a 3.5 s application of an external saline solution made hypertonic by the addition of 500 mM sucrose. Solutions were applied using a fast-flow system that provides reliable and precise solution exchanges with time constants of ~20–30 ms (Rosenmund et al., 1995). The patch pipettes were made of borosilicate glass and pulled using a multi-step puller (P-87; Sutter Instruments, Novato, CA). Recording of miniature EPSCs (mEPSCs) were performed in the presence of 200 nM tetrodotoxin. Spontaneous events were detected using an event detection algorithm (Clements and Bekkers, 1997) on 2 kHz digitally filtered traces. Only events with amplitudes 3.5 times greater than the SD of the baseline noise were detected. After computer detection, events were individually examined to verify that they satisfied the detection criteria.

Statistics. Results are shown as average ± SEM, with *n* referring to the number of cells from each group. Because we experienced significant between-preparation variability in the electrophysiological parameters examined, we usually tested significance by two-way ANOVA, in which the genetic background (knock-out or wild type) and/or the expressed isoforms were defined as a fixed factor, and the culture used was defined as an orthogonal "random" factor. In the case of more than two levels of the fixed

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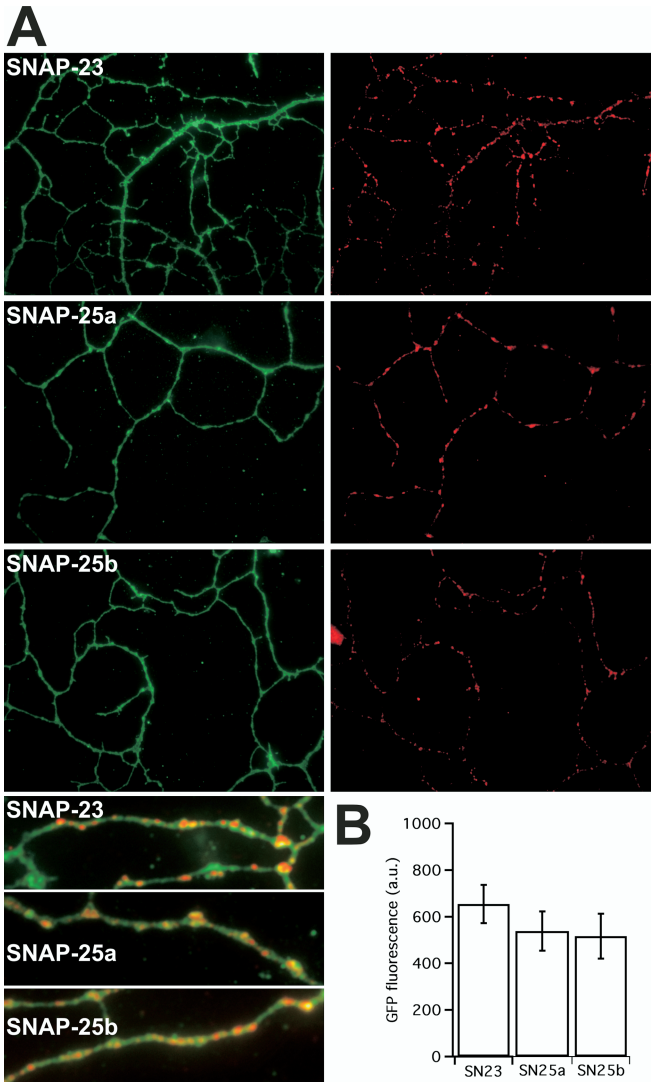

Figure 2. Localization of SNAP-25 isoforms. ***A***, Neurons (from wild-type animals) expressing GFP–SNAP-25a/b or GFP–SNAP-23 were fixed and stained with anti-GFP and Alexa-488 to increase the fluorescence in the green channel. The neurons were costained with anti-synaptophluorin, which was imaged in the red channel (right column). All SNAP-25 isoforms were present throughout the neuritic tree and overlapped with the synaptic marker. ***B***, GFP fluorescence (± SEM) in live (unfixed) neurons expressing GFP–SNAP-23 (SN23; $n = 55$ cells), GFP–SNAP-25a (SN25a; $n = 50$ cells), and GFP–SNAP-25b (SN25b; $n = 55$ cells). The fluorescence was measured in the cell body and indicates similar expression levels.

factor, appropriate *post hoc* tests were used. Statistical testing was done using JMP version 5 (SAS Institute, Cary, NC).

Results

Long-term expression of SNAP-25 homologs rescues survival and branching of −/− neurons

To study the different functionality of SNAP-25 variants, we used *Snap-25* null mice (Washbourne et al., 2002). Hippocampi were obtained from embryos at E18 fetal stage because homozygous mutants die perinatally. Dissociated neurons from *Snap-25*$^{-/-}$ mutants and heterozygous or wild-type embryos from the same litter were cultured on a layer of astrocytes prepared from normal (NMRI) mice and studied in parallel. Mutant (−/−) neurons first developed normally but then underwent degeneration, as reported previously (Washbourne et al., 2002). Cultures from +/− or +/+ littermates survived normally. Quantification of the number of neurons remaining after 10–14 d in culture showed that the density in −/− cultures was 1.3 ± 0.7% ($n = 5$ cultures; $p < 0.001$ Student's t test) of control +/− or +/+ cultures (Fig. 1*B*, left).

To be able to rescue *snap-25*$^{-/-}$ neurons, we searched for an expression method that would supply stable expression of moderate amounts of SNAP-25, whereas the onset of expression should be fast enough to rescue survival. We used the lentiviral system, which relies on replication-incompetent human immunodeficiency virus-1 (HIV-1)-derived virus (Naldini et al., 1996). Viral particles expressing SNAP-25a, SNAP-25b, or SNAP-23 driven by the CMV promoter were created as described in Materials and Methods.

Neurons were infected with recombinant lentivirus during 1 DIV and examined 10–14 d later. The infection rate was estimated to be 80–100%. Using this system to reexpress SNAP-25 isoforms, *snap-25*$^{-/-}$ neurons survived almost normally in culture. Expression of SNAP-25b in −/− cells increased the neuron density to 83 ± 12.7% ($n = 6$ cultures) of the control value, i.e., the number of neurons surviving in cultures made in parallel from wild-type or heterozygous littermates. The same level of survival was achieved by SNAP-23 (87.3 ± 3.3%; $n = 5$ cultures), whereas the survival induced by SNAP-25a was on average slightly lower (65.9 ± 5.0%; $n = 7$ cultures). The expression of each homolog in the rescued neurons was verified by immunostaining with specific antibodies against SNAP-25 (an antibody recognizing both isoforms were used) and SNAP-23 (Fig. 1*A*, middle).

Next, we examined the morphological differences observed in the cultures of neurons. Two parameters were analyzed: the number of branches and the number of synapses. For these studies, we used a virus, which in addition to CMV-driven SNAP-25 isoform expression, also expressed eGFP under control of a synapsin-I promoter (see Materials and Methods). We used the GFP fluorescence for morphological measurements. The branching was calculated as the number of neurites crossing a circumference centered in the soma in a confocal projection image. In control neurons, we counted 38.2 ± 5.1 branches ($n = 13$) in a 50 μm radius (Fig. 1*A*, left, *B*, right). However, in surviving −/− neurons, only 18.5 ± 3.4 branches were detected ($n = 8$; $p < 0.001$ Student's t test). When we compared −/− neurons rescued with the different homologs, we found that arborization was completely restored by all three homologs. The number of branches within a 50 μm circle 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 (Fig. 1*B*, right). This finding indicates that the number of branch points of the neurites is dependent on the expression of a SNAP-25 isoform. Because these neurons were kept for 14 d in culture, the dendritic tree extended (far) beyond the 50 μm circle, so that the entire dendritic length was not measurable. However, this finding also implies that the dendritic length within the 50 μm circle was changed by approximately a factor 2.

The number of synapses was quantified after staining with a synaptophysin antibody (Fig. 1*A*, right). We found 217 ± 27 synaptophysin-positive synapses in *Snap-25* null neurons ($n = 16$; $p < 0.002$, Student's t test), whereas 390 ± 44 synapses were detectable in control neurons ($n = 20$). This loss of synaptic density was fully recovered after expression of any of the SNAP-25 homologs: 536 ± 93 synaptophysin-positive synapses were detected when rescued with SNAP-25b ($n = 10$), 607 ± 152 synapses with SNAP-25a ($n = 8$), and 443 ± 78 with SNAP-23 ($n = 7$).

Because our lentiviral particles also expressed in the underly-

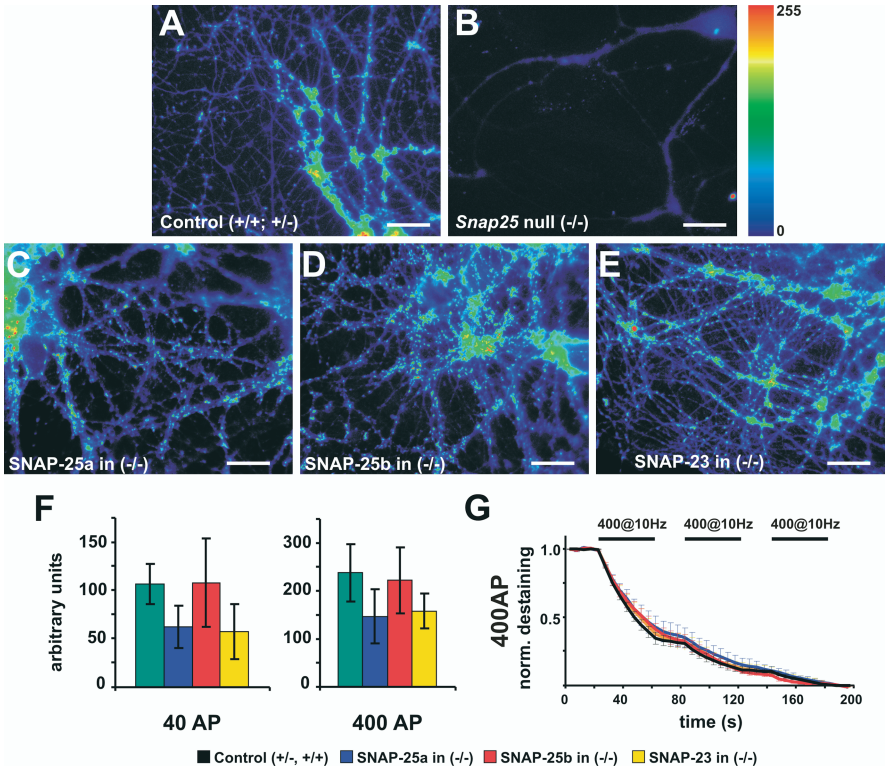

Figure 3. Stimulus-dependent recycling of synaptic vesicles requires a SNAP-25 isoform. ***A–E***, Examples of control hippocampal neurons stained with 400 APs (***A***), *Snap-25* null neurons (***B***), and null neurons rescued with SNAP-25a (***C***), SNAP-25b (***D***), and SNAP-23 (***E***). Staining was not possible in the absence of SNAP-25, indicating a lack of synaptic vesicle recycling. Scale bars, 10 μm. ***F***, The intensity of FM 5-95 staining by 40 AP and 400 AP loading. The background intensity after full destaining was subtracted. ***G***, Destaining (mean ± SEM) of synaptic boutons under strong electrical stimulation (3 pulses of 400 AP at 10 Hz) after loading with 400 AP. Color coding as above. The destaining kinetics was indistinguishable between groups.

ing glial cell layer, we turned to conventional cultures to investigate neuronal expression levels and the localization of expressed protein. We expressed N-terminally GFP-tagged versions of the three SNAP-25 isoforms in conventional cultures. N-terminally tagged SNAP-25b rescues synaptic transmission when expressed in *snap-25*$^{-/-}$ neurons (data not shown). Fluorescence levels in the soma of live expressing neurons were quite variable from cell to cell but comparable between isoforms [SNAP-23, 654 ± 82 arbitrary units (a.u.), $n = 55$ cells; SNAP-25a, 538 ± 85 a.u., $n = 55$; SNAP-25b, 516 ± 97 a.u., $n = 50$], indicating no major differences in expression levels (Fig. 2*B*). Note that this estimation was done using the GFP fluorescence of nonfixed cells. After fixation, costaining against GFP and synaptophysin showed that all three isoforms are expressed along all neuronal processes and in synapses (Fig. 2*A*). This localization corresponds well to previous findings for both endogenous and overexpressed SNAP-25 (Bark et al., 1995; Grosse et al., 1999; Morihara et al., 1999; Shirasu et al., 2000; Verderio et al., 2004; Frassoni et al., 2005; Tafoya et al., 2006).

These results show that the lack of SNAP-25 results in a parallel reduction in arborization and the number of synapses, indicating that SNAP-25 is necessary for neurite branching rather than for synaptic formation per se. These defects can be restored during viral expression of SNAP-25a, SNAP-25b, or SNAP-23.

Functional synaptic vesicle cycling in the presence of SNAP-25 isoforms

We continued by investigating whether synapses retained functionality in the *snap-25*$^{-/-}$ neurons and after rescue. To that end, we assayed uptake and release of FM 5-95 as a measure of exo-endocytosis function in the cultured neurons (Cochilla et al., 1999). Uptake of the dye was induced by electrical field stimulation. After a train of 400 stimuli in the presence of the dye, staining was absent in cultured *snap-25*$^{-/-}$ neurons, indicating a lack of synaptic vesicle cycling (Fig. 3*B*), in agreement with Tafoya et al. (2006). In contrast, staining of rescued neurons was successful (Fig. 3*C–E*). Quantification of the staining intensity of identified synapses revealed that staining in the presence of SNAP-25a and SNAP-23 was slightly depressed (staining intensity: +/+ neurons, 238 ± 60 a.u., $n = 5$ fields of views; −/− neurons expressing SNAP-25b, 222 ± 69 a.u., $n = 5$; −/− neurons expressing SNAP-25a, 147 ± 56.8 a.u., $n = 3$; SNAP-23, 158 ± 36 a.u., $n = 4$), but the depression did not reach statistical significance. In each field of view, 100–400 synapses were analyzed.

The trend was similar when only 40 action potentials (APs) were used (Fig. 3*F*), which indicates a reduction in the number of functional recycling vesicles in the synapse when SNAP-25a or SNAP-23 are replacing SNAP-25b. This tendency was also not statistically significant when using the very conservative method of comparing mean bouton intensities between fields of views but would be significant if comparing between all boutons. The latter method, however, does not take into account variability between fields of views.

The kinetics of destaining by electrical field stimulation was complete with similar kinetics in rescue and control neurons (Fig. 3*G*), indicating that, although the pool of functionally recycling vesicles might be decreased with SNAP-25a or SNAP-23, the dynamics of release under strong stimulation was identical.

Glutamatergic transmission is asynchronous in the presence of SNAP-23

To detect more subtle differences in synaptic transmission between SNAP-25 isoforms, we next performed whole-cell patch clamp on autaptic neurons expressing each of the homologs. We began by ensuring that lentivirus infection is innocuous to neurons. We observed no change in the EPSC between uninfected (2.99 ± 0.46 nA; $n = 40$) and eGFP-expressing wild-type neurons (3.10 ± 0.38 nA; $n = 30$; $p = 1.00$, two-way ANOVA). For the following considerations, we therefore pooled eGFP-expressing and nonexpressing wild-type neurons (3.04 ± 0.31 nA; $n = 70$). In addition, no differences were found between heterozygous and homozygous wild-type neurons (data not shown). We next examined the effect of overexpression of the SNAP-25 homologs in wild-type neurons. The results indicated a small and statistically nonsignificant reduction of the EPSC amplitude in all cases (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) (Fig. 4*A*,*B*). Previous data using SNAP-25 overexpression with Semliki Forest virus, which induces much stronger expression, led to a strong reduction of EPSC size (Owe-Larsson et al., 1999), a

result that we confirmed (data not shown). These data show that lentivirus expression can be used to rescue *snap-25*$^{-/-}$ neurons without deleterious effects to synaptic transmission.

In agreement with the results of the FM analysis, none of the 35 *Snap-25* null neurons or the 41 null neurons expressing eGFP examined displayed detectable postsynaptic currents (estimated as 0.019 ± 0.003 and 0.024 ± 0.004 nA, respectively) (Fig. 4). However, knock-out cells were able to generate normal action potentials when stimulated in current-clamp mode (data not shown). Because no difference was found between these two groups, they were considered as the null group (0.022 ± 0.002 nA; $n = 76$) for negative comparison. The expression of SNAP-25 restored synaptic transmission in knock-out neurons (Fig. 4*B*, right). Neurons rescued with SNAP-25b displayed similar EPSC size as control neurons (2.88 ± 0.30 nA; $n = 53$; $p > 0.05$, two-way ANOVA). However, in neurons rescued with SNAP-25a, the EPSC size reached only 65–70% of this value (1.94 ± 0.22; $n = 42$; $p < 0.02$ 2-way ANOVA compared with wild type or $p < 0.01$ when compared with SNAP-25b rescue) (Fig. 4*B*). This indicates a difference between SNAP-25a and SNAP-25b in governing neurotransmission.

Strikingly, *snap-25*$^{-/-}$ neurons expressing SNAP-23 produced an attenuated and highly asynchronous EPSC, which lasted for at least 1 s (Fig. 4*A*). The peak amplitude was 0.198 ± 0.046 nA ($n = 52$; $p < 0.001$ compared with SNAP-25b rescue, two-way ANOVA). We analyzed the kinetics of neurotransmitter release by integrating the EPSCs over 1 s and found that the charge liberated by SNAP-23 rescued neurons (19.5 ± 4.1 pC) amounted to 50–55% 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 (Fig. 4*C*, Table 1). The contribution of the fast component to the total release was reduced in SNAP-23 rescued −/− neurons (34.5% instead of 79.1 ± 2.1%; $p < 0.001$, two-way ANOVA), and the time constant was sixfold slower (43.9 ± 6.1 ms; $p < 0.001$) than in the case of SNAP-25b rescue (Table 1), whereas all kinetic parameters were statistically identical between the two SNAP-25 isoforms.

It is interesting that overexpression of SNAP-23 in wild-type neurons did not modify neurotransmission (see above), whereas expression in *snap-25*$^{-/-}$ neurons produced a strong phenotype. This could be attributable to a low level of SNAP-23 expression compared with endogenous SNAP-25, but our lentivirus obviously expressed sufficient amounts of SNAP-23 to fully rescue neuronal survival and branching. Thus, when present together with SNAP-23 in moderate amounts, SNAP-25 seems to be used preferentially for synaptic transmission.

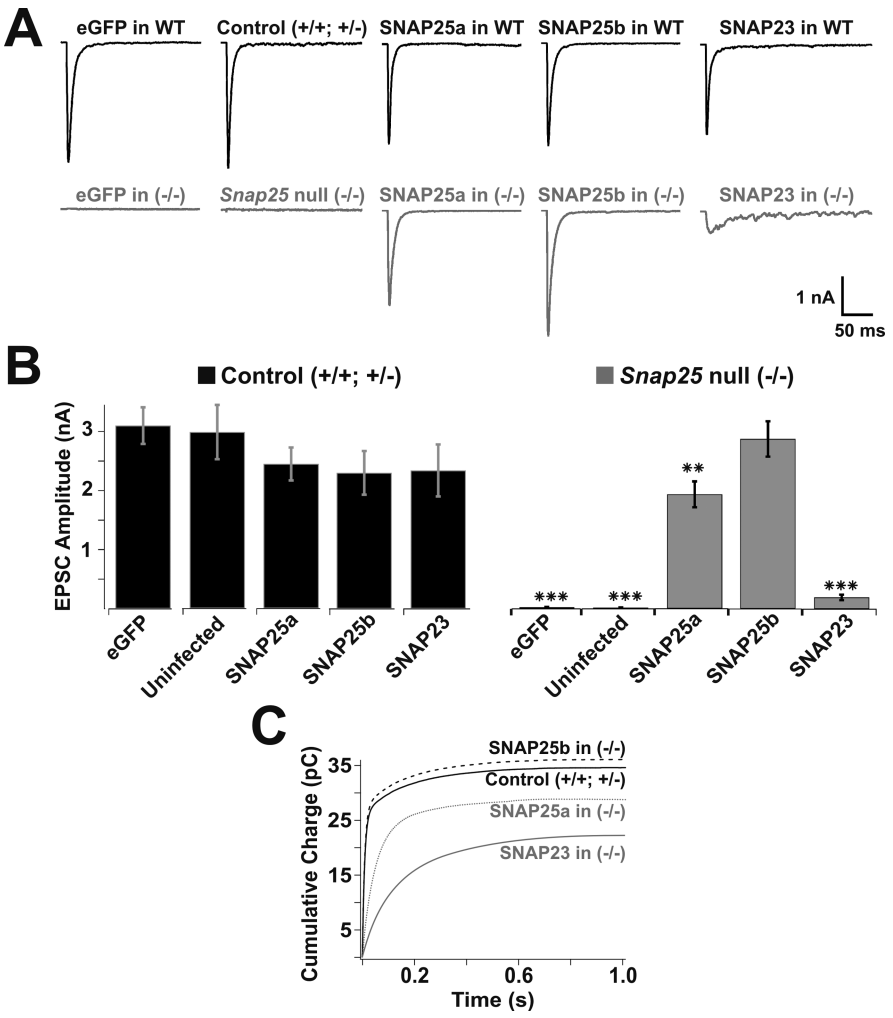

Figure 4. SNAP-25 supports synchronous and SNAP-23 asynchronous release. ***A***, Autaptic EPSCs in neurons expressing SNAP-25a, SNAP-25b, or SNAP-23 compared with eGFP-expressing and uninfected neurons. Control (+/+; +/−) neurons are shown in black, and *snap-25* null neurons are shown in gray. No evoked responses were found in the absence of SNAP-25. Strikingly, *snap-25* null neurons expressing SNAP-23 produced evoked responses lacking the 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. **$p < 0.01$; ***$p < 0.001$. ***C***, Integrated EPSCs for control and rescued neurons. SNAP-23 rescued neurons presented slower release of vesicles. For kinetic parameters, see Table 1. WT, Wild type.

Table 1. EPSC properties

	Control (+/+, +/−)	SNAP-25a in (−/−)	SNAP-25b in (−/−)	SNAP-23 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

Summary table containing mean ± SEM values for a two-exponential fit to the EPSC integral for control and rescued neurons. Time constants for the fast (τ_1) and slow (τ_2) components were significantly increased and the fractional contribution of the fast component was significantly reduced in SNAP-23 rescued cells when compared with the rest of the groups ($p < 0.001$, Student's *t* test).

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

The differences observed in evoked responses between SNAP-25 homologs could be attributed to changes in the release probabil-

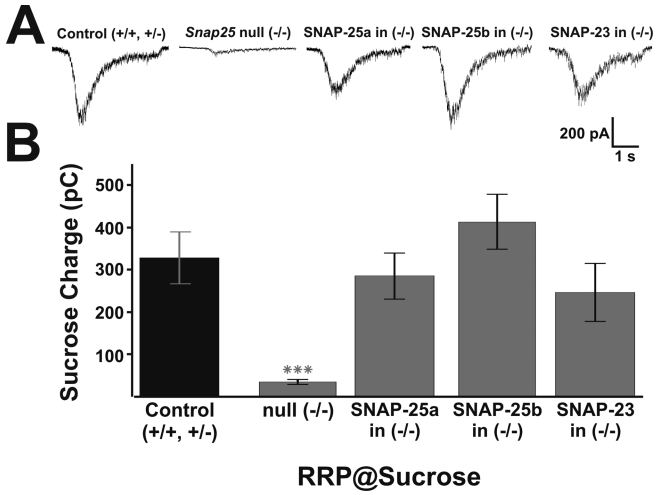

Figure 5. All SNAP-25 isoforms rescue the sucrose pool. ***A***, Example recordings of 500 mM sucrose application in control hippocampal neurons (+/+; +/−), *Snap-25* null neurons (−/−), and after rescue with SNAP-25a, SNAP-25b, and SNAP-23. A small vesicle pool was released by sucrose even in knock-out neurons. ***B***, Mean ± SEM values of the "sucrose pool" for each of the groups described above. ***$p < 0.001$.

ity or in the RRP size, because the number of functional synapses was equal (see above). The application of a solution made hypertonic by the addition of 500 mM sucrose releases a pool of primed vesicles, here referred to as the "sucrose pool" (Rosenmund and Stevens, 1996). With this method, we provoked a small but detectable current even in *snap-25*$^{-/-}$ neurons. Integrating over time, we estimated the sucrose pool to 34.8 ± 6.2 pC ($n = 39$) (Fig. 5*A*) in this case. This indicates the existence of a small vesicle pool, which might provide vesicles for spontaneous events in the absence of SNAP-25 (see below) but which cannot be released at all by evoked stimulation. Responses elicited by sucrose in the SNAP-25a rescue group (287.2 ± 54.7 pC; $n = 27$) were on average 20–30% smaller than those in the SNAP-25b group (441.0 ± 69.1 pC; $n = 31$), although this was not statistically significant ($p = 0.16$, Student's *t* test). The release probability for the sucrose pool is calculated by dividing the charge released during one EPSC by the sucrose pool of the same neuron. The mean size of the sucrose pool of the three isoforms (SNAP-23, SNAP-25a, and SNAP-25b) varied in size in the same sequence (SNAP-23 < SNAP-25a < SNAP-25b) (Fig. 5*B*) as the EPSC charge (SNAP-23 < SNAP-25a < SNAP-25b) (Fig. 4*C*), although the differences in the latter case were more significant because of a lower variability of those measurements. Consequently, the release probabilities were similar in the presence of SNAP-25a (0.11 ± 0.02; $n = 22$), SNAP-25b (0.13 ± 0.01; $n = 22$), or the SNAP-23 rescue group (0.12 ± 0.03; $n = 39$). These findings show that a single stimulation releases the same fraction of the sucrose pool in the presence of SNAP-23 and SNAP-25, although release in the first case is strongly asynchronous.

The pool of vesicles released by sucrose differs from the one released by short stimulation trains in glutamatergic neurons (Moulder and Mennerick, 2005). To complement our results, we therefore used a stimulation train to determine the RRP size by evoked release (RRP_{er}), as described previously (Schneggenburger et al., 1999; Otsu et al., 2004). The stimulation by 100 APs delivered at 40 Hz did not evoke release in *snap-25* null neurons (Fig. 6*A*). In neurons rescued with SNAP-25 isoforms, repetitive stimulation produced EPSCs, which depressed rapidly to a steady-state level (Fig. 6*A*,*B*). In contrast, in SNAP-23 rescued neurons, the peak current recorded after the stimulation displayed strong buildup during trains mainly attributable to the overlap of asynchronous release components (Fig. 6*A*,*B*). The difference between SNAP-25 and SNAP-23 driven release is seen most clearly after normalization (Fig. 6*B*, right). The synchronized part of the EPSC amplitude is shown in Figure 6*C*, measured as the difference between peak EPSC amplitude after stimulation and the steady current, which builds up during the train. This amplitude displayed depression in all cases. After normalization of these synchronous EPSC amplitudes, it can be appreciated that SNAP-25a and SNAP-25b resulted in similar depression time courses (Fig. 6*C*, right).

The cumulative EPSC charge was plotted versus time, and a linear fit to the steady-state phase was back extrapolated to zero to determine RRP_{er} size in the absence of refilling (Fig. 6*D*). Similar values of RRP_{er} for control neurons and *Snap-25* null neurons rescued with SNAP-25b were estimated using this method (128 ± 19 pC, $n = 61$ and 134 ± 18 pC, $n = 39$, respectively). However, the pool size estimated for the rescue with SNAP-25a was significantly reduced (80 ± 17 pC; $n = 25$; $p < 0.001$, two-way ANOVA, Tukey–Kramer test, $p < 0.001$) (Fig. 6*D*, right). Additionally, the release probability calculated as the ratio between the first stimulus in the train and the RRP_{er} were not different between SNAP-25 isoforms (SNAP-25a rescue, 0.29 ± 0.03; SNAP-25b rescue, 0.24 ± 0.03) and control neurons (0.23 ± 0.03).

We also examined the paired-pulse behavior during the first two stimulations in the 40 Hz train. In *snap-25*$^{-/-}$ cells rescued by SNAP-25b, the paired-pulse depression amounted to 0.86 ± 0.05 (second EPSC amplitude divided by the first), whereas for SNAP-25a the value was 0.85 ± 0.08. These values were not significantly different from control neurons (0.92 ± 0.05). SNAP-23 induced a paired-pulse ratio of 1.11 ± 0.10, indicating increased facilitation. However, it should be noticed that, although SNAP-23-expressing neurons displayed increased facilitation, the main reason for the strong buildup of current during trains (Fig. 6*A*,*B*) is overlap of highly asynchronous EPSCs. Results were qualitatively similar when trains of 10 or 50 Hz were used (data not shown). These findings indicate that the main consequence of the developmental change from SNAP-25a to SNAP-25b expression is an increase in RRP size by the time of synaptic maturation, whereas short-term synaptic plasticity remains unchanged.

Spontaneous release in the presence and absence of SNAP-25 homologs

Finally, we examined the spontaneous release and miniature single events in the hippocampal cultures. mEPSC were detected in −/− neurons, as reported previously (Washbourne et al., 2002). However, we found that the size of the events found in the *Snap-25* null neurons (10.4 ± 1.0 pA and 54.2 ± 5.6 fC; $n = 11$; two-way ANOVA on the charge, $p < 0.0001$) was 50% smaller than in the control neurons (21.5 ± 1.7 pA and 107.3 ± 7.0 fC; $n = 23$) (Fig. 7, Table 2). The frequency was also lower in null neurons (0.63 ± 0.20 Hz; two-way ANOVA, $p < 0.02$) than in control neurons (1.76 ± 0.30 Hz), which might be explained by the reduced arborization and number of synaptic contacts in *snap-25*$^{-/-}$ neurons (Fig. 1). The reintroduction of the SNAP-25 homologs in the knock-out neurons led to a full 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, 3.11 ± 0.57 Hz, $n = 23$ for SNAP-23). The mEPSC frequency appeared somewhat higher in the case of SNAP-25a and SNAP-23 than with SNAP-25b, but this was not quite significant ($p = 0.083$, two-way ANOVA). There are three

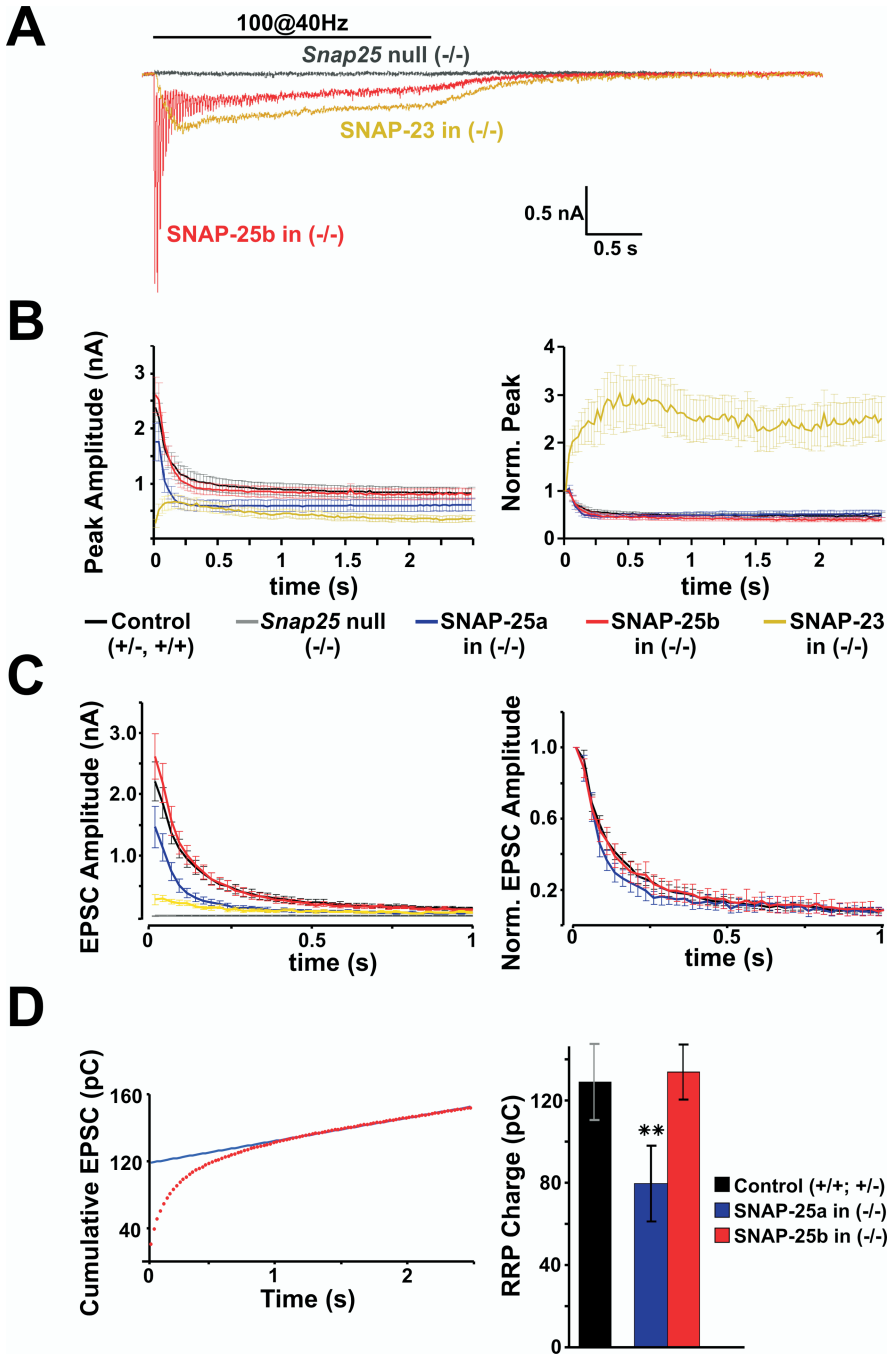

Figure 6. High-frequency stimulation reveals differences between SNAP-25a, SNAP-25b, and SNAP-23. ***A***, Example traces of high-frequency train stimulation (100 AP at 40 Hz) of *snap-25* null neurons (gray), SNAP-25b rescued neurons (red), and neurons rescued with SNAP-23 (yellow). Stimulation artifacts have been removed. ***B***, Mean ± SEM peak current amplitudes (left) and after normalization to the first stimulation (right) during high-frequency train stimulation. SNAP-23 expression in null neurons caused build-up of currents attributable to overlap of asynchronous release components during the train. ***C***, Mean ± SEM synchronized EPSC amplitude (left) and after normalization to the first stimulation (right) during high-frequency train stimulation. Depression during the train was indistinguishable between SNAP-25a and SNAP-25b. ***D***, Left, Example cumulative trace of the EPSC amplitudes during the 40 Hz train stimulation. The data between 1 and 2.5 s were fitted with a straight line (blue). The line was back-extrapolated to 0 to calculate the RRP size as determined by RRP_{er}. Right, Mean ± SEM of the RRP_{er} values calculated as shown to the left. RRP_{er} was significantly smaller when SNAP-25a was used for rescuing null neurons instead of SNAP-25b. $^{**}p < 0.01$.

possible explanations for the reduction in mEPSC size in the absence of SNAP-25: the synaptic vesicles might be smaller in the absence of SNAP-25; the glutamate content might be lower or the number of postsynaptic receptors might be decreased or not correctly clustered at the postsynaptic density. Our results cannot distinguish between these possibilities, but we note that a very recent paper reported unchanged synaptic vesicle size in the *snap-25*$^{-/-}$ neurons (Bronk et al., 2007) and that SNAP-25 has been implicated in the trafficking of glutamate receptors (Lan et al., 2001; Washbourne et al., 2004), which agrees with the latter possibility.

GABAergic neurons require SNAP-25 for survival and synchronous release

Immunofluorescence and neurotoxin data led to the suggestion that mature GABAergic neurons preferentially use SNAP-23, rather than SNAP-25, for synaptic release and/or outgrowth (Verderio et al., 2004; Frassoni et al., 2005). In conflict with this finding, a recent study demonstrated SNAP-25 immunofluorescence in GABAergic neurons and the absence of transmission in *snap-25*$^{-/-}$ GABAergic neurons (Tafoya et al., 2006). However, because the knock-out mouse dies before birth, that study could not be extended to mature synapses. Our strategy of expressing SNAP-25 variants in −/− neurons should be able to clarify the question which SNAP-25 isoform is required in GABAergic neurons.

Remarkable was the absence of IPSCs in any of the 76 hippocampal neurons examined in the −/− group, although they represented 11.9% (33 of 244) of the responses in wild-type hippocampal neurons. In contrast, we found IPSCs in *snap-25*$^{-/-}$ hippocampal cultures when rescued with SNAP-25a (7.8%) or SNAP-25b (6.7%). To find a definitive answer, we examined SNAP-25/SNAP-23 rescue in striatal cultures, in which GABAergic neurons are more abundant. We found that survival of *Snap-25*$^{-/-}$ striatum neurons in culture was reduced as in the case of hippocampal cultures (1.7 ± 0.5% of wild-type values; $n = 3$ cultures; $p < 0.001$ 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 *Snap-25*$^{-/-}$ neurons examined ($n = 14$). However, typical GABAergic evoked responses were present when striatal −/− neurons were rescued with SNAP-25a ($n = 11$) or SNAP-25b ($n = 14$), with no significant differences in the IPSC amplitude (Fig. 8*A*,*C*). The postsynaptic currents were identified as GABAergic, because they were blocked by 20 μM bicuculline and had a reversal potential at the Nernst potential for chloride (Fig. 8*B*). In addition, GABAergic responses were found in striatal −/− neurons expressing SNAP-23 ($n = 9$), but as in the case of glutamatergic neurons, the fast release component was missing (Fig. 8*A*). These data show that neuronal

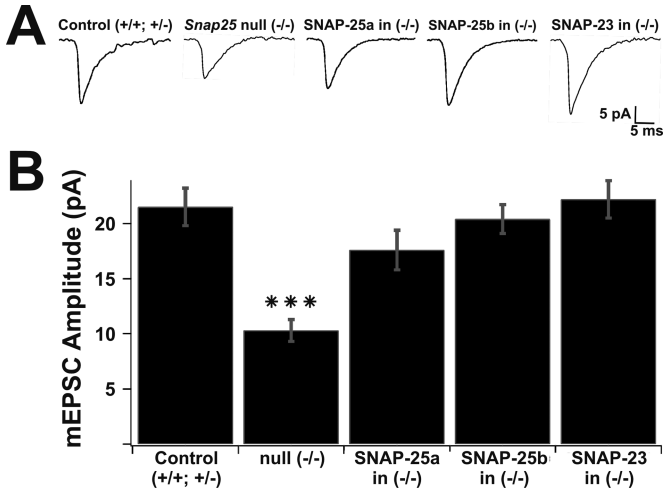

Figure 7. Smaller and fewer spontaneous events in the *Snap-25* null neurons are rescued by SNAP-25 and SNAP-23. ***A***, Example averaged mEPSCs from single cells. Events were indistinguishable in shape; however, they were smaller in *snap-25* null neurons. ***B***, Mean ± SEM values of mEPSC amplitudes. The mEPSC size was significantly reduced in *Snap-25* null neuron. ***$p < 0.001$.

survival as well as synchronized transmission in GABAergic neurons is SNAP-25 dependent.

Discussion

By expressing different SNAP-25 isoforms in *snap-25*$^{-/-}$ neurons, we studied their ability to rescue the defects encountered in knock-out neurons, which can be summarized as reduced survival in culture, inferior branching and less synapses of surviving cells, fewer and smaller miniature EPSCs, and abolished evoked release.

SNAP-23 expression rescued survival of −/− neurons *in vitro*, and such neurons developed the normal number of branches and synaptophysin-positive synapses. SNAP-23 restored the normal size and frequency of miniature EPSCs. Furthermore, SNAP-23 could restore evoked release, as measured electrophysiologically and by FM dye staining. However, evoked postsynaptic currents were strongly asynchronous. These data show that it is possible that SNAP-23 substitutes for SNAP-25, for instance during axonal outgrowth in the embryo. However, only 1–2% of *snap-25*$^{-/-}$ neurons survived for 10–14 d in culture, and the remaining −/− neurons did not display any evoked release, unlike SNAP-23 rescued cells. It therefore seems that, at least in cultured hippocampal and striatal neurons, endogenous SNAP-23 expression is negligible. It remains possible that SNAP-23 is involved in embryonic development and initial outgrowth and that expression is later downregulated.

Our data help resolve the conflicting results produced by immunostaining: Verderio et al. (2004) and Frassoni et al. (2005) reported that hippocampal GABAergic interneurons are virtually devoid of SNAP-25-specific staining during later developmental stages, whereas Tafoya et al. (2006) reported colocalization of SNAP-25 and GABAergic markers. In addition, Tafoya et al. (2006) demonstrated lack of release from cortical GABAergic neurons in *snap-25*$^{-/-}$ slices. However, because the *snap-25* knock-out mouse is not viable, the possibility remained that SNAP-25 could play an early role in GABAergic neurons and that another isoform would take over in mature synapses (Frassoni et al., 2005). Here, we show that the survival of hippocampal and striatal neurons, which are predominantly glutamatergic and GABAergic, respectively, is SNAP-25 dependent *in vitro* and that evoked release from the few surviving *snap-25*$^{-/-}$ neurons of both preparations is abolished. In addition, SNAP-25, but not SNAP-23, expression restores evoked synchronous GABAergic release both from hippocampal and striatal neurons after 14 DIV. Together with the data of Tafoya et al. (2006), we consider this conclusive evidence that most GABAergic neurons use SNAP-25 rather than SNAP-23. Tafoya et al. (2006) have discussed alternative reasons for the findings of Verderio et al. (2004), and Frassoni et al. (2005) have pointed out that low levels of SNAP-25 undetectable by immunostaining might drive exocytosis. In addition to these arguments, we note that the lower efficiency of BoNT/A in inhibiting GABAergic vesicle cycling might be attributable to the demonstrated higher calcium increase in GABAergic neurons (Verderio et al., 2004), because BoNT/A-inhibition can be overcome by high calcium concentrations (Capogna et al., 1997; Trudeau et al., 1998; Sakaba et al., 2005).

The high mortality of *snap-25*$^{-/-}$ neurons in culture is puzzling. We cultured these neurons on layers of astrocytes obtained from wild-type (NMRI) mice, ensuring a normal release of trophic factors from astrocytes. A very recent study showed that culturing *snap-25*$^{-/-}$ neurons at very high densities (Bronk et al., 2007) can overcome or at least delay degeneration, suggesting that a trophic factor from the neurons themselves might limit survival. The phenotype contrasts with results from knock-outs of most other presynaptic proteins, whose neurons develop normally in culture, even in the complete absence of evoked and spontaneous release (Varoqueaux et al., 2002). The phenotype of *snap-25* neurons *in vitro* resembles that of *munc18–1*$^{-/-}$ neurons, which also develop normally for several days and then degenerate (Heeroma et al., 2004). An attractive possibility is that SNAP-25 and Munc18–1 both participate in two exocytotic complexes: one that drives synaptic transmission, and one that is responsible for membrane cycling needed for outgrowth or secretion of trophic factors. However, *in vivo munc18–1,* but not *snap-25* mice display widespread neurodegeneration at E18 (Verhage et al., 2000; Molnar et al., 2002). This could be explained if both Munc18–1 and SNAP-25 are replaced by other isoforms at early embryonic stages, with the shift to Munc18–1 preceding the shift to SNAP-25.

Snap-25$^{-/-}$ neurons display no evoked release, but this was restored by both SNAP-25 and SNAP-23 expression, indicating that both isoforms are able to couple exocytosis to a calcium sensor for release, as suggested previously for SNAP-25 (Sorensen et al., 2002). In this respect, it is interesting that both SNAP-25 and SNAP-23 have been described to bind to synaptotagmin 7, whereas SNAP-25, but not SNAP-23, binds to synaptotagmin 1 (Chieregatti et al., 2004). Indeed, the asynchronous release observed in the presence of SNAP-23 appears to be similar to release in the *synaptotagmin-1* knock-out mouse (Geppert et al., 1994; Nishiki and Augustine, 2004; Maximov and Sudhof, 2005), which supports the idea that binding to SNAP-25 couples synaptotagmin-1 to release. Synaptotagmin-1 is by most investigators considered to be the calcium sensor for fast release, whereas the function of neuronal synaptotagmin-7 is not understood, but it might play a role during neurite outgrowth (Arantes and Andrews, 2006). It will be important to investigate whether this or other neuronal functions of synaptotagmin-7 is related to its binding to SNAP-25 and SNAP-23.

Our analysis of neurons from the *snap-25*$^{-/-}$ mouse indicates that SNAP-25 is involved in branching, but not in synaptogenesis per se, because the number of synapses in surviving −/− cells was reduced in proportion to the number of branches. In addition, the rate of mEPSCs was reduced by approximately the same fac-

Table 2. mEPSC properties

	Control (+/+, +/−)	*Snap-25*$^{-/-}$	SNAP-25a in (−/−)	SNAP-25b in (−/−)	SNAP-23 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

Summary table containing mean ± SEM values of peak amplitude, charge, and frequency of mEPSCs. The mEPSC rate was rescued by SNAP-25b and seemed slightly higher for SNAP-25a and SNAP-23.

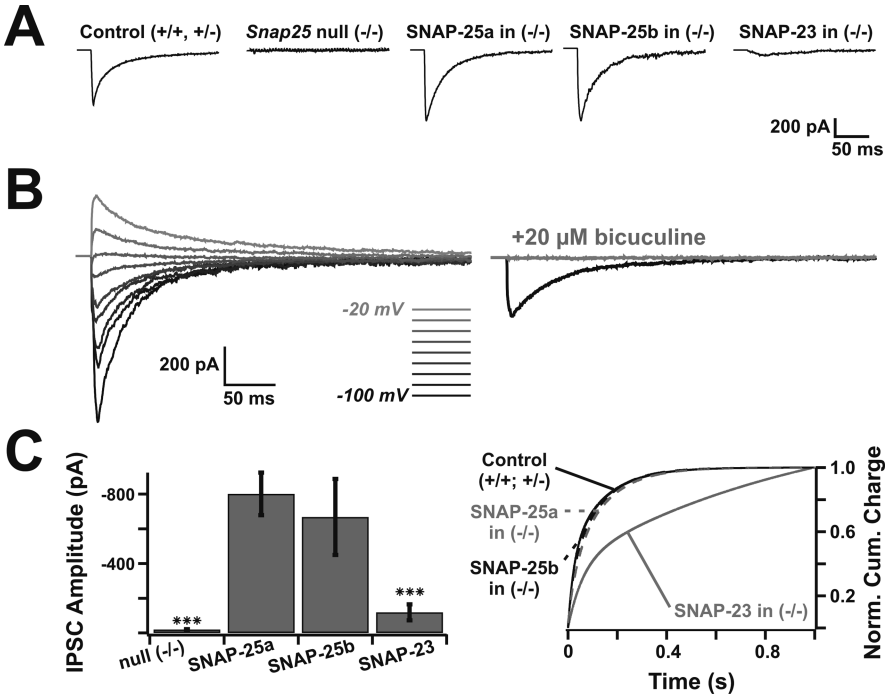

Figure 8. SNAP-25 is essential for fast release in GABAergic neurons. ***A***, Example recordings of GABAergic responses in striatal neurons from control (+/+; +/−), *snap-25* null neurons, and null neurons rescued with SNAP-25a, SNAP-25b, and SNAP-23. No IPSCs were found in null, suggesting that inhibitory neurons need SNAP-25 for synaptic transmission. Null neurons rescued by SNAP-25a or SNAP-25b evoked normal IPSCs. Responses in SNAP-23 rescued neurons were smaller and the fast component was abolished, mimicking the situation in glutamatergic neurons. ***B***, Example of a *Snap-25* null neuron rescued with SNAP-25b showing (left) reversal of the IPSC after changing the holding potential from −100 mV (black) to −20 mV (light gray) in 10 mV steps and blockage by the $GABA^A$ antagonist bicuculline (right). ***C***, Left, Mean ± SEM values of the IPSC amplitudes. Right, Example of the integrated charge released by the IPSC for each of the groups. The kinetics of evoked release for SNAP-23 rescued neurons was slower than for the other groups. ***$p < 0.001$.

tor as the number of synapses per cell (i.e., by a factor of two to three), suggesting that spontaneous release proceeds almost unimpeded in the absence of SNAP-25. Also in neurons from synaptobrevin-2 knock-out mice, mEPSC persisted at a lower rate, whereas evoked release was absent (Schoch et al., 2001), and it was suggested that different vesicle populations are involved in spontaneous and evoked release (Sara et al., 2005; Deak et al., 2006). However, recent experiments using FM dyes led to the opposite conclusion (Groemer and Klingauf, 2007). Our data could be taken to indicate that SNAP-25 plays no essential role in spontaneous release, which would support the suggestion of two release machineries specialized for evoked and spontaneous release. However, it is also possible that, although spontaneous release is driven by SNAP-25 in the normal case, another isoform or mechanism able only to support spontaneous release substitutes in its absence. This substitute cannot be SNAP-23, because we could show that it supports evoked release, but it might be a more distantly related isoform, for instance SNAP-29 (Steegmaier et al., 1998; Hohenstein and Roche, 2001; Su et al., 2001) or SNAP-47 (Holt et al., 2006).

The alternative expression of SNAP-25 splice variants in knock-out neurons made it possible to study the behavior of each splice variant in isolation. SNAP-25a-expressing neurons were on average inferior to −/− neurons expressing SNAP-25b in both the pool of vesicles stainable by FM dyes, the sucrose pool, the EPSC size, and the primed vesicle pool size. The latter two effects were statistically significant. In contrast, no differences were found in neuronal survival, branching, synaptogenesis, or spontaneous release. These data indicate that SNAP-25b supports a larger primed vesicle pool than SNAP-25a. SNAP-25b-expressing −/− neurons were undistinguishable from control neurons in all aspects tested, which agrees with the preferential expression of SNAP-25b in adult hippocampus (Bark et al., 1995, 2004). In contrast, in chromaffin cells, in which SNAP-25a is the native isoform, −/− cells overexpressing SNAP-25a mimicked the wild-type phenotype, with SNAP-25b causing over-rescue (Sorensen et al., 2003; Nagy et al., 2005). These data demonstrate that the alternative splicing of SNAP-25 in different cell types suffices to change the pool of release-ready vesicles, thereby modulating exocytotic strength. The shift in splicing from the SNAP-25a to the SNAP-25b isoform in the brain happens during the first 2 postnatal weeks (Bark et al., 1995), at the time of synaptic maturation. In this respect, it is interesting that, in both differentiating hippocampal neurons *in vitro* (Mozhayeva et al., 2002) and the calyx of Held (Taschenberger and von Gersdorff, 2000; Iwasaki and Takahashi, 2001), maturation of the synapse is associated with an increase in RRP size. We did not find evidence for a difference in release probability or short-term synaptic plasticity between SNAP-25b and SNAP-25a isoforms. Results from a transgenic mouse with a higher a/b ratio indicated a shift toward facilitation (Bark et al., 2004). A reason for the discrepancy might be that the expression of the "a" isoform *in vivo* leads to secondary changes to counteract a lower RRP size.

In conclusion, we studied cultured hippocampal neurons expressing only one SNAP-25 isoform. We show that, although SNAP-23 can rescue evoked release, EPSCs and IPSCs are strongly asynchronous, resembling results from the *synaptotagmin-1*$^{-/-}$. In addition, the two SNAP-25 splice variants differentially support vesicle priming, which might underlie the shift to larger RRP size during synapse maturation. Together, these data demonstrate a differential ability of SNAP-23, SNAP-25a, and SNAP-25b to support neuronal function.

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