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N-Terminal Acetylation of the Neuronal Protein SNAP-25 Is Revealed by the SMI81 Monoclonal Antibody

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ABSTRACT: The monoclonal antibody SMI81 binds SNAP-25, a major player in neurotransmitter release, with high affinity and has previously been used to follow changes in the levels of this protein in neuropsychiatric disorders. We report here that the SMI81 epitope is present at the extreme N-terminus of SNAP-25 and, unusually, cannot be recognized when present as an internal sequence. Although it is known that SNAP-25 can be palmitoylated and phosphorylated in brain, we now reveal the existence of a third modification, acetylation of the N-terminus. This acetylation event greatly increases the efficiency of SMI81 antibody binding. We show that this highly specific antibody can be used for studying brain function in many vertebrate organisms.

The synaptosomal-associated protein of 25 kDa (SNAP-25)[1] is a highly conserved synaptic protein and a key component of the synaptic vesicle fusion machinery (*1*, *2*). It was initially identified as the most abundant methionine-rich protein to be rapidly transported along axons, accumulating at synaptic termini (*3*, *4*). Levels of SNAP-25 increase during development, and SNAP-25 expression coincides with the onset of synaptogenesis and neuronal maturation (*5*, *6*). SNAP-25 is a hydrophilic protein but is tightly associated with synaptic membranes because of the attachment of hydrophobic palmitate lipids to four clustered cysteines in the center of the molecule; indeed, SNAP-25 is the major palmitoylation target in brain (*3*, *4*, *7*, *8*).

Key to our understanding of SNAP-25 protein function was the finding that it forms a stoichiometric complex with syntaxin and synaptobrevin in brain (*9*, *10*). The central role of these three soluble NSF-attachment protein receptor (SNARE) proteins, including SNAP-25, in synaptic vesicle release was confirmed upon their identification as the targets of clostridial neurotoxin action (*11*). Botulinum neurotoxins A and E cleave SNAP-25 at specific points in the amino acid sequence of the C-terminus, rendering the cleaved protein unable to form the SNARE complex which in turn leads to neuromuscular paralysis and death (*12*, *13*).

In light of the importance of SNAP-25 for neurotransmission, several antibodies have been developed for recognition of this protein. One such commonly used antibody is mouse monoclonal SMI81, initially generated by Sternberger Monoclonals among a wide panel of antibodies raised against human brain extract (hence the abbreviation SMI81 for Sternberger Monoclonals Inc., clone 81). The SMI81 antibody was later shown to specifically recognize a single 25 kDa protein band by Western immunoblotting in wild-type neurons, but not in neurons from a SNAP-25 null mutant background (*14*). It recognizes botulinum toxin-truncated protein (*15*−*17*) and both alternatively spliced SNAP-25A and -B isoforms, which differ by only nine amino acids. It has been used to detect SNAP-25 in hippocampal slices from mice at various stages of embryonic and postnatal development (*18*). SMI81 immunoblotting of brain regions of mice suffering from hyperkinesis showed changes in the SNAP-25 expression pattern, reflecting presynaptic dysfunction and hyperactivity (*19*). Importantly, SMI81 immunoblotting allowed detection of alterations in brain SNAP-25 levels in schizophrenia (*20*). The SMI81 antibody has also been used to probe calcium-dependent release of glutamate from isolated synaptic termini (*21*). Finally, SMI81 has been used to detect SNAP-25 protein in non-neuronal contexts, including mast cells, neutrophils, and chromaffin cells (*16*, *22*, *23*).

Given that SMI81 is used to follow SNAP-25 protein in brain development and pathology, we decided to precisely map the antibody epitope. Our data show that the antibody recognizes a sequence at the extreme N-terminus of SNAP-25 in brain with high efficiency, and this sequence must be present at a free N-terminal end for recognition. Mass spectrometric characterization of the N-terminus of brain-purified SNAP-25 reveals a novel acetylation modification, with removal of the initiator methionine residue and acetylation of the following alanine. This cotranslational acetylation event, occurring only in eukaryotes (*24*), dramatically strengthens SMI81 binding, thus defining the antibody as a useful tool for studying N-terminal acetylation events. Finally, we show that the SMI81 epitope is conserved in vertebrates, first appearing in bony fish, and that the antibody can recognize SNAP-25 protein in the zebrafish model organism.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies. Full-length human SNAP-25A (amino acids 1−206) was inserted into the pGEX-KG vector (*25*) using BamHI and NcoI restriction sites, for expression of

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[1]Abbreviations: SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble NSF-attachment protein receptor; GST, glutathione *S*-transferase; GFP, green fluorescent protein; PC12, pheochromocytoma 12; BCA, bicinchoninic acid; NTA, nitrilotriacetic acid; RU, relative units.

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glutathione *S*-transferase (GST)-tagged protein in *Escherichia coli*. The same sequence was inserted into pEGFP-C1 (BD Biosciences) via XhoI and KpnI sites, for HeLa cell expression of green fluorescent protein (GFP)-tagged SNAP-25. For HeLa expression of untagged protein, the same sequence was inserted into pcDNA3.1(−) (Invitrogen) via XhoI and KpnI sites. All nucleotide sequences were verified by DNA sequencing. Mouse monoclonal antibody SMI81 against SNAP-25 was purchased from Covance; rabbit polyclonal antibody against GST-tagged SNAP-25 has been described previously (*26*), and the rabbit polyclonal anti-SNAP-23 antibody was from Synaptic Systems.

Preparation of Tissue Extracts. Extracts were prepared by homogenizing 2 g of adult tissue from rat, mouse, cow, zebrafish, or electric ray in 12 mL of 200 mM NaCl, 20 mM HEPES (pH 7.3), 1 mM EDTA, and 2% Triton X-100, with complete protease inhibitor cocktail (Roche) added according to the manufacturer's instructions. After solubilization of membrane proteins (1 h at 4 °C), the homogenate was centrifuged to remove insoluble debris (100000*g* for 1 h at 4 °C). The protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) assay (*27*). Immunoprecipitations of SMI81 from brain extract were conducted as described previously (*28*).

Cell Culture, Transfection, and Immunostaining. HeLa cells (European Collection of Cell Cultures) were transfected with SNAP-25 when they were approximately 70% confluent, using Lipofectamine/Plus reagent (Invitrogen), according to the manufacturer's instructions. They were harvested 24 h post-transfection. For immunostaining, cells were washed twice with PBS to remove medium and fixed with 4% paraformaldehyde (30 min at 25 °C). They were then washed three times with PBS, and the paraformaldehyde was quenched with 10 mM NH_4Cl (5 min at 25 °C). After three further PBS washes, cells were treated with 0.1% Triton X-100 in PBS for 2 min, washed twice, and then incubated for 30 min in blocking buffer (PBS with 2% bovine serum albumin). Cells were then incubated with primary antibody (SMI81 at a 1:400 dilution and anti-SNAP-23 at a 1:50 dilution; 90 min at 25 °C), washed 2 × 5 min with blocking buffer, and incubated with Alexa fluor-conjugated secondary antibody at a dilution of 1:1000 (Invitrogen; 30 min at 25 °C). After 3 × 5 min washes with PBS, the immunostained cell coverslips were briefly dipped in water and mounted in Mowiol 4-88 (Calbiochem). Bound immunofluorescence was observed on a Radiance 2100 Confocal system (Zeiss/Bio-Rad) linked to a Nikon Eclipse E800 fluorescence microscope equipped with an oil-immersion objective (100×, 1.4 numerical aperture). Culture and immunostaining of rat pheochromocytoma cells (PC12s) were as described previously (*29*).

Preparation of HeLa Cell Extracts. We prepared extracts 24 h post-transfection by resuspending the HeLa cells in 100 mM NaCl, 20 mM HEPES (pH 7.3), 2% Triton X-100, 1 mM EDTA, and complete protease inhibitor cocktail (Roche). After a 10 min incubation on ice to allow cell lysis to proceed, insoluble material was removed by centrifugation (20800*g* for 20 min at 4 °C), and the protein concentration of the supernatant was determined using the BCA assay.

Western Immunoblotting and Dot Blot. For Western immunoblotting, samples were normalized by protein content and loaded onto a 10% NuPAGE Bis-Tris Mini Gel (Invitrogen) and then transferred to a PVDF membrane (Millipore). Membranes were incubated in blocking buffer (5% nonfat dried milk in PBS and 0.2% Tween 20) before addition of primary antibody (1:4000) for 1 h. After 3 × 5 min washes (PBS and 0.2% Tween 20), horseradish peroxidase-conjugated secondary antibody (Pierce) was added at a 1:6000 dilution in blocking buffer (30 min). After further washes (3 × 5 min), bound antibody was detected by enhanced chemiluminescence using the West Dura kit (Thermo Scientific). For the dot blot, peptides were spotted onto a PVDF membrane which had been prewetted in methanol. The membrane was left to dry before being wetted again in methanol and incubation in blocking buffer. Immunoblotting and detection of bound antibody were then conducted as described above.

Protein Preparation and Peptide Synthesis. Isolation of recombinant SNAP-25 has been described previously (*29*). Briefly, GST-tagged SNAP-25 was expressed in *E. coli* BL21 and purified on glutathione Sepharose beads (GE Healthcare). Bead-bound protein was washed with 100 mM NaCl and 20 mM HEPES (pH 7.3) and eluted via thrombin cleavage. SNAP-25 was further purified by gel filtration on a Superdex 200 column (GE Healthcare) equilibrated in 100 mM NaCl and 20 mM HEPES (pH 7.3). Brain-purified SNAP-25 was prepared as described previously (*30*). Peptides were synthesized by Peptide Protein Research.

Mass Spectrometry Analysis. A solution sample of brain-purified SNAP-25 (5 μM) in 20 mM NH_4HCO_3 was digested with trypsin overnight. A portion of this peptide mixture was separated by nanoscale liquid chromatography (LC Packings) on a reversed-phase C18 column (150 mm × 0.075 mm internal diameter, flow rate of 0.25 μL/min). The eluate was introduced directly into a Q-STAR pulsar *i* hybrid tandem mass spectrometer (MDS Sciex). The spectra were searched against an "NCBInr" nonredundant database with MASCOT MS/MS Ions Search (www.matrixscience.com). A doubly charged ion with a mass-to-charge ratio of 940 matched the acetylated N-terminal peptide. The acetylation site was confirmed by manual inspection of the fragmentation series.

Surface Plasmon Resonance. Real-time binding of SMI81 to the 11-amino acid epitope peptides was performed using a Biacore 2000 system. After an initial wash of the nitrilotriacetic acid (NTA) chip surface (GE Healthcare) with 100 mM NaOH and 1% SDS for 5 min and 100 mM phosphoric acid and 1% SDS for 5 min, the chip was charged with Ni^{2+}. Two channels of the chip were repeatedly washed and regenerated using 0.35 M EDTA for 3 min, followed by a 3 min incubation with 3 mM $NiCl_2$, using a flow rate of 5 μL/min. Approximately 50 relative units (RU) of Ni^{2+} was loaded on each channel at each cycle. His-tagged peptides were then reversibly immobilized on different channels of the Ni^{2+} chelate chip (*31*, *32*). Approximately 300 RU of nonacetylated peptide and 500 RU of acetylated peptide were loaded. For kinetic measurements, different concentrations of SMI81 in 100 mM NaCl and 20 mM HEPES (pH 7.3) were injected for 30 s at a flow rate of 30 μL/min using the "KINJECT" function of the instrument. Binding of SMI81 to both peptides was recorded simultaneously. For background subtraction, all dilutions of SMI81 were also injected, as part of the same program, but without peptides on the Ni^{2+}-NTA chip. To control for background drifts, the chip loaded with peptides was also incubated with buffer only (no SMI81), as part of the same program and using the same flow rates. All measurements were performed at 25 °C. Kinetic parameters were estimated using linear regression analysis of initial binding rates (*33*).

RESULTS

SMI81 Efficiently Recognizes Native SNAP-25. We first confirmed that the SMI81 monoclonal antibody can specifically

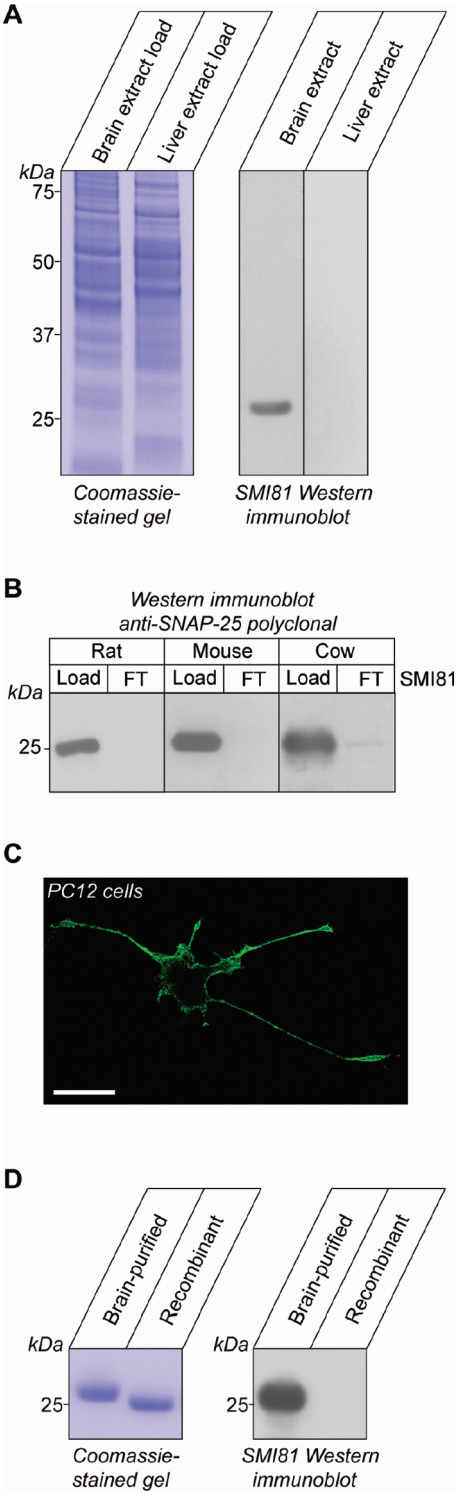

FIGURE 1: Specific recognition of all brain SNAP-25 by SMI81. (A) Total protein load (left) and SMI81 immunoblot (right) of Triton X-100 extracts from rat brain and liver. SMI81 specifically recognizes SNAP-25, a single band of 25 kDa from brain. Liver does not contain SNAP-25. (B) Samples of brain detergent extract (load) and flow-through (FT) after SMI81 immunoprecipitation were immunoblotted with a rabbit polyclonal antibody to detect the presence of all SNAP-25 protein. SMI81 recognizes all SNAP-25 from rat, mouse, and cow brain. (C) SMI81 immunostaining of a neuroendocrine PC12 cell containing endogenous SNAP-25 at the plasma membrane. The scale bar is 20 μm. (D) Total protein load (left) and SMI81 immunoblot (60 s exposure, right) of SNAP-25 purified from cow brain or recombinantly expressed in *E. coli*. Recombinant protein cannot be recognized by SMI81.

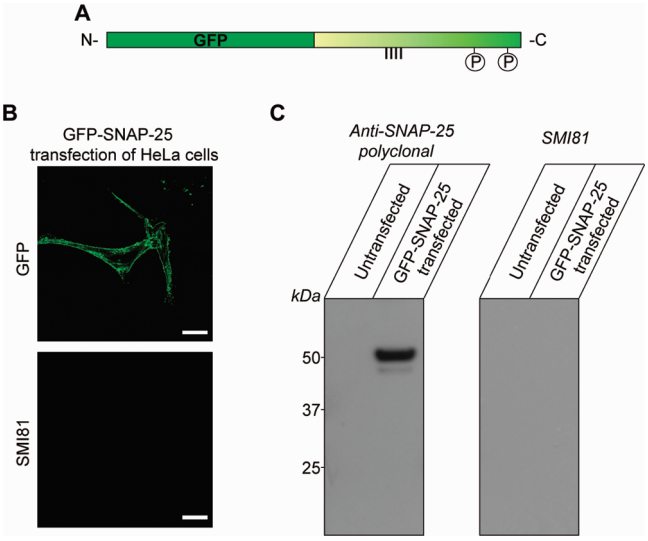

FIGURE 2: GFP−SNAP-25 fusion protein expressed in eukaryotic cells cannot be recognized by SMI81. (A) Schematic representation of the SNAP-25 construct fused with an N-terminal GFP, to follow localization of transfected protein. Known post-translational modifications are depicted as follows: palmitoylation sites with short black vertical lines and phosphorylation with black encircled P. (B) Confocal microscopy of GFP−SNAP-25 fusion protein-transfected HeLa cells. In the top panel, the GFP−SNAP-25 protein is localized at the plasma membrane. In the bottom panel, SMI81 immunostaining cannot detect the GFP−SNAP-25 protein. The scale bar is 20 μm. (C) Western immunoblotting of HeLa cell Triton X-100 extracts with or without GFP−SNAP-25 protein transfection. In the left panel, anti-SNAP-25 rabbit polyclonal antibody detects transfected GFP−SNAP-25 protein. In the right panel, SMI81 cannot detect GFP−SNAP-25 protein.

recognize SNAP-25 present in rat brain cortex Triton X-100 extract. Figure 1A shows an SMI81 Western immunoblot with a single detected band of 25 kDa, corresponding to the correct molecular mass for SNAP-25. As a further control for antibody specificity, we also tested rat liver extract, which does not contain SNAP-25. Indeed, no SMI81 immunoreactive band was observed here. Next, SMI81 immunoprecipitations were conducted in brain detergent extract from rat, mouse, and cow and the load and unbound material probed with a polyclonal anti-SNAP-25 antibody. Figure 1B shows that virtually all brain SNAP-25 is recognized by highly efficient SMI81. We then confirmed SMI81 recognition of endogenous SNAP-25 in neuroendocrine pheochromocytoma (PC12) cells. Staining was observed at the plasma membrane (Figure 1C), corresponding to the expected localization of SNAP-25 (*34*).

Since SMI81 is a highly efficient antibody that is used to follow neuronal changes in normal and pathological conditions, we decided to map the antibody epitope. Full-length recombinant SNAP-25 was expressed in *E. coli* from a plasmid containing the entire human SNAP-25A sequence, preceded by an N-terminal glutathione *S*-transferase tag for purification on glutathione Sepharose beads. After removal of the tag with thrombin, a protein of the expected molecular mass was produced (Figure 1D, left). This protein was able to form the SNARE complex and could be cleaved by botulinum neurotoxins A and E, as expected (data not shown). Paradoxically, it was not recognized by SMI81 (Figure 1D, right). Note that brain-purified SNAP-25 appears at a slightly higher molecular mass than the recombinant protein on SDS−PAGE; this is because it carries post-translational modifications such as palmitoylation (*4*).

The GFP−SNAP-25 Fusion Protein Expressed in Mammalian Cells Is Not Recognized by SMI81. The inability of the SMI81 antibody to recognize bacterially expressed protein with the correct primary amino acid sequence suggested that the antibody might recognize a region of SNAP-25 that is co- or post-translationally modified, only when present in a eukaryotic cellular context. SNAP-25 is the major palmitoylation target in brain (*4*), with four palmitoylated cysteines in the center of the molecule. In addition, it has been reported that both protein kinases A and C phosphorylate SNAP-25 at residues in the C-terminal SNARE helix (*35*−*37*).

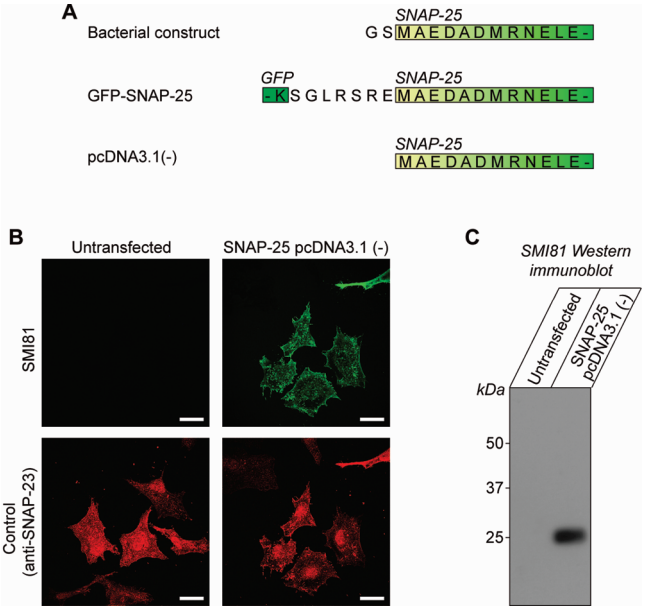

FIGURE 3: Free N-terminus of SNAP-25 recognized by SMI81. (A) Amino acid sequences of the SNAP-25 N-terminal region in the protein expression constructs used. (B) Confocal microscopy images of HeLa cells transfected with untagged SNAP-25 in pcDNA3.1(−) vector and immunostained with SMI81 (green) or with an antibody against endogenous SNAP-23 (red), as a positive staining control. SNAP-25 without an N-terminal tag can be recognized by SMI81. The scale bar is 20 μm. (C) Western immunoblot of HeLa cell Triton X-100 extracts. SMI81 detects transfected untagged SNAP-25 protein.

To determine whether modifications to the primary amino acid sequence might play a role in SMI81 antibody binding, SNAP-25 with a GFP tag at the N-terminus (Figure 2A) was expressed in HeLa cells, which do not contain SNAP-25 but do contain eukaryotic palmitoylation and phosphorylation machinery. The GFP tag allowed protein expression levels and localization to be followed. The GFP−SNAP-25 fusion protein was localized predominantly at the plasma membrane of HeLa cells (Figure 2B, top). However, when cells were immunostained with the SMI81 antibody, this protein also could not be recognized (Figure 2B, bottom). To confirm that the expressed protein contained the intact SNAP-25 amino acid sequence, we performed Western immunoblotting of cell extracts using a polyclonal anti-SNAP-25 antibody (Figure 2C, left). This antibody recognized a single 50 kDa band in the transfected cell lysate, corresponding to the expected molecular mass of the GFP−SNAP-25 fusion protein. Again, SMI81 could not recognize this GFP fusion protein (Figure 2C, right).

SMI81 Recognizes the Free SNAP-25 N-Terminus. Since SMI81 was capable of efficient recognition of SNAP-25 in rat brain extract but not of bacterially expressed SNAP-25 or GFP−SNAP-25 fusion protein in HeLa cells, we sought an explanation for this difference. It was noted that in both the bacterial and GFP−SNAP-25 constructs the expressed SNAP-25 protein would not have a free N-terminus. In the bacterial case, two additional residues forming part of the thrombin cleavage site upstream of the initiator methionine remained attached at the N-terminus of SNAP-25 (Figure 3A). In the GFP−SNAP-25 construct, the N-terminus was blocked by the presence of GFP and a short linker sequence. We therefore cloned the SNAP-25 sequence into the pcDNA3.1(−) vector, for mammalian expression of the untagged protein. We expressed this construct in HeLa cells and then fixed and immunostained it with SMI81 and with an antibody against endogenous SNAP-23 protein. SMI81 was able to recognize the untagged transfected SNAP-25 protein, which was correctly localized to the plasma membrane of the cells (Figure 3B). Western immunoblotting of Triton X-100 cell extracts confirmed that SMI81 specifically recognizes a single

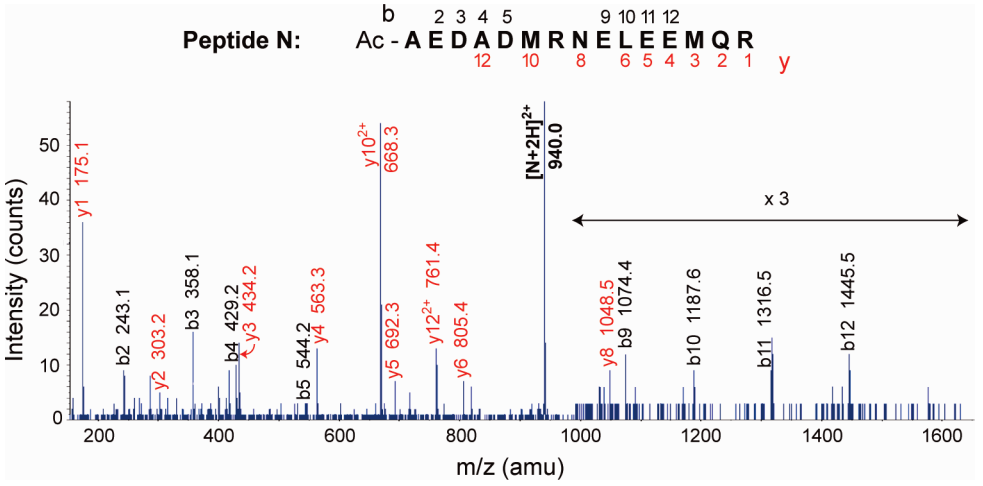

FIGURE 4: Mass spectrometric analysis of the N-terminus of brain-purified SNAP-25. The N-terminal 15-amino acid tryptic peptide (Peptide N) was fragmented, and b and y peptides, corresponding to ions fragmented from the N- and C-termini, respectively, were identified (colored black and red). All identified b peptides carry acetylation of an N-terminal alanine residue. The molecular masses of the fragments are shown above the bars. m/z is the mass-to-charge ratio (in atomic mass units). All fragments shown are singly protonated ($z = 1$), unless otherwise indicated. × 3 indicates a 3-fold zoom of the intensity signal.

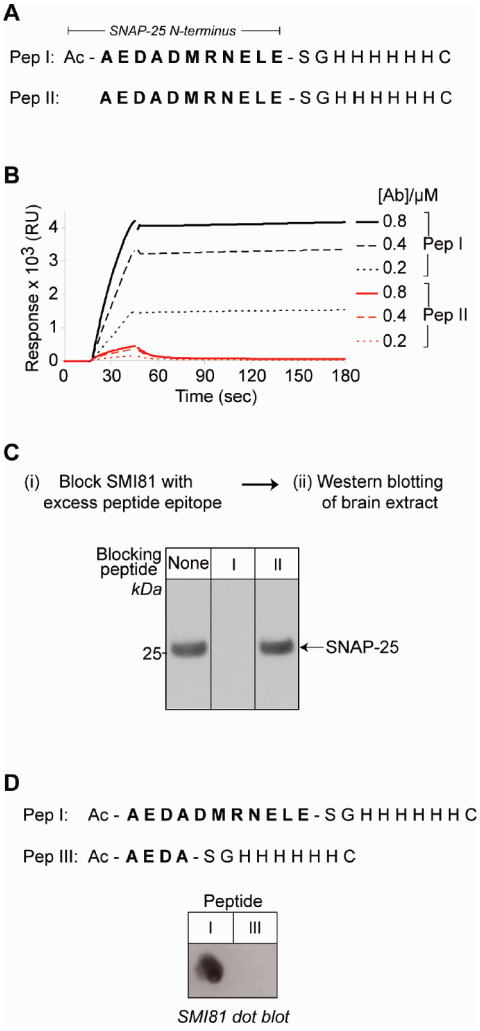

FIGURE 5: Acetylation of a peptide corresponding to the N-terminus of SNAP-25 promotes SMI81 recognition. (A) Amino acid sequences of the peptides used to investigate the effect of acetylation on SMI81 binding. Both peptides contained the 11 N-terminal residues of brain SNAP-25, and a His tag for immobilization purposes. Ac is an acetate group attached to the N-terminal amine. (B) Surface plasmon resonance traces of the SMI81 antibody binding to His-tagged peptides. Peptides were immobilized on individual channels of a Ni^{2+} chelate affinity sensor chip. SMI81 binds faster to the acetylated N-terminal peptide (Pep I), compared with the nonacetylated peptide (Pep II) (6-fold difference in association rate). Dissociation of the antibody from the acetylated peptide was not detected over the time course of the experiment. In contrast, SMI81 rapidly dissociates from the nonacetylated peptide. [Ab] is the concentration of SMI81 (in micromolar). (C) Western immunoblot blocking experiment. SMI81 was blocked with addition of excess peptide, before incubation of blocked antibody with brain extract on a PVDF membrane. Only an acetylated peptide can block binding of SMI81 to native SNAP-25. (D) Dot blot of N-terminal peptides. The top panel shows amino acid sequences of peptides tested. The bottom panel shows an SMI81 dot blot showing that the four acetylated N-terminal amino acids are insufficient for SMI81 recognition, compared with the 11-amino acid epitope peptide.

protein band of 25 kDa, present only upon transfection of untagged SNAP-25 (Figure 3C).

Brain SNAP-25 Undergoes N-Terminal Methionine Excision and Acetylation of the Following Alanine. Having determined that the free N-terminus of SNAP-25 is critical for SMI81 binding, we analyzed the N-terminus of brain-purified SNAP-25 by mass spectrometry, to check for modifications of the amino acid sequence here that could affect antibody association. Brain-purified protein was digested with trypsin, producing several peptides, including one containing the 15 N-terminal amino acids. Following fragmentation of this peptide in a tandem mass spectrometer, we found that the initiator methionine was not present and that the N-terminal alanine residue was acetylated (Figure 4). This result is in accordance with the known preference of methionine aminopeptidase enzymes, which remove N-terminal methionines if the next residue is a small amino acid such as alanine (*38*, *39*). In eukaryotes, the newly exposed N-terminal alanine is usually cotranslationally acetylated by an *N*-acetyltransferase (*38*, *40*), as is the case for SNAP-25.

Acetylation of the SNAP-25 N-Terminus Promotes Antibody Binding. Since the free N-terminus of SNAP-25 forms the epitope for SMI81, and this region is acetylated in brain-purified protein, it was important to measure the effect of acetylation on SMI81 binding. Linear monoclonal antibody epitopes are usually 10−15 amino acids long, so we designed two peptides within this length range, corresponding to the N-terminus of native SNAP-25 in brain with and without N-terminal acetylation (Figure 5A). To characterize the kinetics of SMI81 binding to these peptides, real-time binding experiments were performed using the Biacore surface plasmon resonance technique. The peptides were immobilized on Ni^{2+} chelate affinity surfaces of an NTA chip via their C-terminal hexahistidine tags. The SMI81 antibody was then injected onto each chip, and binding was observed over 3 min. Figure 5B shows binding of various concentrations of the SMI81 antibody to acetylated and nonacetylated peptide. The curves have been normalized for the amount of Ni^{2+} and peptide loading onto each chip. To compensate for the background drift (because of the slow leaching of Ni^{2+} from the chip surface), equivalent data obtained in the absence of SMI81 antibody were subtracted from the antibody traces. The acetylated peptide (peptide I) binds with a faster association rate than the nonacetylated peptide (peptide II). The apparent binding constants (k_{on}) of both peptides were estimated using an initial rate approach (*33*), yielding a value of 0.98×10^5 M^{-1} s^{-1} for the acetylated peptide and a value of 0.16×10^5 M^{-1} s^{-1} for the nonacetylated peptide. These results show that acetylation of the N-terminal alanine of SNAP-25 results in 6-fold increase in the association rate of SMI81 antibody binding. Furthermore, the SMI81−acetylated peptide interaction shows no apparent dissociation (even upon extension of the measurement period to 10 min), whereas SMI81 dissociates rapidly from the nonacetylated peptide.

To confirm that the interaction of SMI81 with acetylated peptide is specific, we compared the ability of peptides I and II (with and without acetylation, respectively) to compete with native SNAP-25 for SMI81 binding. First, the SMI81 antibody was blocked by preincubation with an excess of either peptide I or II. Brain detergent extract samples were then loaded onto a SDS−PAGE gel and subsequently transferred to a PVDF membrane. The membrane was cut into strips, and each lane with the extract was incubated with SMI81 antibody, either free or preblocked with excess peptide I or II. Blocking with the acetylated peptide (I) was able to completely prevent SMI81 recognition of SNAP-25 from brain extract, whereas the nonacetylated peptide (II) was ineffective (Figure 5C). This indicates that interaction of SMI81 with acetylated peptide is specific, occurring via the regions of the antibody that would otherwise interact with native SNAP-25 on the membrane. In addition, since this acetylated 11-amino acid peptide completely blocked

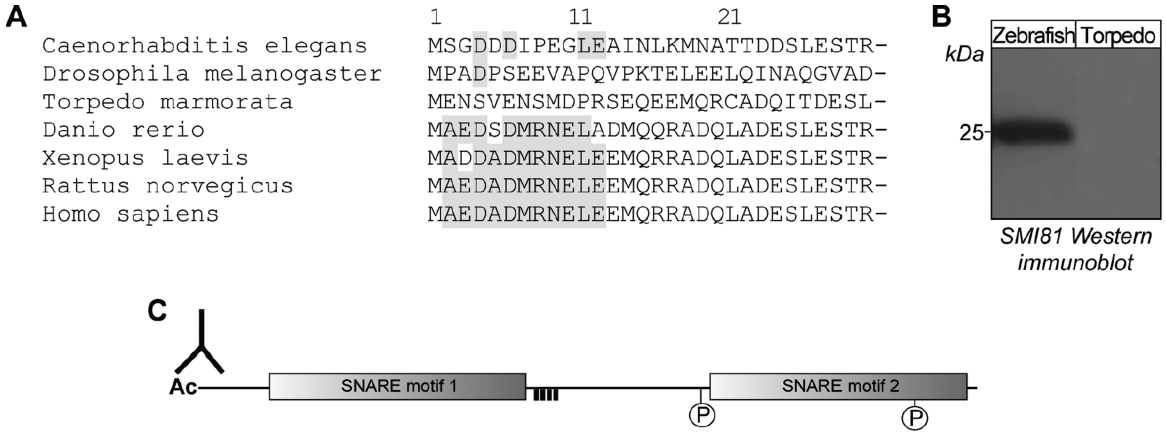

FIGURE 6: SMI81 can recognize zebrafish SNAP-25. (A) Sequence alignment of the 30 N-terminal amino acids of SNAP-25A homologues throughout evolution. Sequences of worm, fruit fly, electric ray fish, zebrafish, frog, rat, and human are shown (from top to bottom). Gray shading indicates residues within the group of 11 N-terminal amino acids that are identical to the human SNAP-25 sequence. (B) Western immunoblot showing SMI81 recognition of SNAP-25 in the Triton X-100 extract from zebrafish brain but not *Torpedo* electric organ. (C) Schematic of the SNAP-25 protein showing SMI81 binding at the free acetylated N-terminus.

recognition of brain-purified protein, it contains the full SMI81 epitope.

To date, our results show that acetylated alanine at the free N-terminus of SNAP-25 is necessary for SMI81 binding. An acetylated alanine residue is found at the N-terminus of many abundant proteins with long half-lives (*41*, *42*). In addition, a Blast search of the human proteome indicated that there are 25 other proteins also beginning with the MAED amino acid sequence (data not shown). However, since SMI81 recognizes only one protein from brain (Figure 1A), and the MAED**A** N-terminal sequence is unique to SNAP-25, this could represent the minimal epitope. To check whether this sequence is sufficient, a third peptide was synthesized corresponding to these amino acids, without methionine and with N-terminal alanine acetylation, as for native SNAP-25 [peptide III (Figure 5D, top)]. Dot blot analysis of peptides I and III indicates that the four N-terminal amino acids alone, even when acetylated, are insufficient for SMI81 recognition (Figure 5D, bottom).

The SMI81 Epitope Is Conserved in Most Vertebrates. Having identified the SMI81 antibody N-terminal epitope, we investigated the degree of conservation of this region by aligning SNAP-25 amino acid sequences from several model organisms (Figure 6A). The first 12 amino acids are almost identical from human (*Homo sapiens*) to bony fish [e.g., zebrafish (*Danio rerio*)]. However, cartilaginous fish have divergent SNAP-25 N-terminal sequence [e.g., electric ray (*Torpedo marmorata*)]. Accordingly, Western immunoblotting of detergent extracts from zebrafish brain and *Torpedo* electric organ (both containing many synaptic junctions) indicated that zebrafish SNAP-25, but not *Torpedo*, can be recognized by SMI81 (Figure 6B).

DISCUSSION

The major neuronal protein SNAP-25 is critical for action potential-triggered neurotransmitter release (*1*), and its deregulation has been implicated in the development of several neuropsychiatric disorders (*43*). Here we have characterized an unusual epitope of the anti-SNAP-25 monoclonal antibody, SMI81. This antibody is relatively inexpensive and is capable of binding all SNAP-25 from brain. It has previously been extensively used to detect SNAP-25 protein and can specifically bind both SNAP-25A and -B isoforms, which have different abilities to facilitate neurotransmitter release (*44*). In addition, it recognizes SNAP-25 truncated at the C-terminus by botulinum neurotoxins A and E (*15*−*17*). This makes it a useful tool for investigating the mode of action of these toxins and also indicates that the antibody epitope does not lie in the C-terminal part of the protein. The precise SMI81 epitope has not previously been elucidated.

We now present evidence that SMI81 binds the extreme N-terminus of SNAP-25 (Figure 6C). For antibody association, the epitope must be present at the N-terminal end of the protein, and not as part of an internal sequence, implying that N-terminal fusions to SNAP-25 must be avoided if SMI81 is to be used for further analysis. We determine for the first time that the N-terminus of SNAP-25 in brain is acetylated, after removal of the initiator methionine residue. Acetylation of the N-terminus strengthens SMI81 binding, with a dramatic effect on both antibody association and dissociation rates. Efficient N-terminal acetylation occurs only in eukaryotes (*24*); therefore, recombinant bacterially expressed SNAP-25, even with a free N-terminus, may not be well recognized by SMI81. Since an N-terminally acetylated peptide corresponding to the first 11 amino acids of brain SNAP-25 is sufficient to block SMI81 recognition of native protein, we conclude that this stretch of sequence contains the full antibody epitope. Finally, we show that the SMI81 binding epitope is conserved from bony fish through the vertebrate lineage to humans. Indeed, SMI81 can recognize SNAP-25 in zebrafish brain, providing a tool for researchers studying SNARE proteins and synaptic function in bony fish, amphibians, reptiles, birds, and mammals.

The finding that brain SNAP-25 is modified by methionine excision and acetylation represents the first description of alteration to this protein's primary amino acid sequence at the N-terminus. Excision of the initiator methionine occurs cotranslationally in both prokaryotes and eukaryotes only when the second residue is small (e.g., alanine in SNAP-25), because of steric constraints in the active site of the methionine aminopeptidase enzymes (*38*, *45*). According to the "N-end" rule, proteins with a small amino acid such as alanine exposed at the N-terminus are predicted to have a long half-life (*41*). Further, in the case of eukaryotes, a small exposed N-terminal amino acid is likely to be cotranslationally acetylated at the α-amine functional group (*24*, *46*). Proteins such as SNAP-25 that undergo N-terminal methionine excision and acetylation of the following

residue are overrepresented among the most abundant proteins in a cell (*42*), suggesting that either or both of these modifications may be important for protein stability and/or function. Although this report focuses on characterization of the SMI81 antibody epitope, future studies might ascribe a functional role for the N-terminal SNAP-25 modifications described herein. Indeed, it will be worth investigating how the acetylated N-terminus of SNAP-25 could be processed further in response to physiological stimuli and whether cell types different from cortical neurons could have alternative modifications of this protein. The identification of SMI81 as a tool for efficient recognition of the acetylated SNAP-25 N-terminus may also help advance our understanding of the role of *N*-acetyltransferase enzymes in the cell.

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REFERENCES

1. Washbourne, P., Thompson, P. M., Carta, M., Costa, E. T., Mathews, J. R., Lopez-Bendito, G., Molnar, Z., Becher, M. W., Valenzuela, C. F., Partridge, L. D., and Wilson, M. C. (2002) Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat. Neurosci. 5*, 19–26.
2. Robinson, L. J., and Martin, T. F. (1998) Docking and fusion in neurosecretion. *Curr. Opin. Cell Biol. 10*, 483–492.
3. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J. Cell Biol. 109*, 3039–3052.
4. Hess, D. T., Slater, T. M., Wilson, M. C., and Skene, J. H. (1992) 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.
5. Oyler, G. A., Polli, J. W., Wilson, M. C., and Billingsley, M. L. (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.
6. Catsicas, S., Larhammar, D., Blomqvist, A., Sanna, P. P., Milner, R. J., and Wilson, M. C. (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.
7. Veit, M., Sollner, T. H., and Rothman, J. E. (1996) Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25. *FEBS Lett. 385*, 119–123.
8. Salaun, C., James, D. J., Greaves, J., and Chamberlain, L. H. (2004) Plasma membrane targeting of exocytic SNARE proteins. *Biochim. Biophys. Acta 1693*, 81–89.
9. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell 75*, 409–418.
10. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature 362*, 318–324.
11. Montecucco, C., and Schiavo, G. (1995) Structure and function of tetanus and botulinum neurotoxins. *Q. Rev. Biophys. 28*, 423–472.
12. Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T. C., Niemann, H., and Jahn, R. (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature 365*, 160–163.
13. Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F., and Montecucco, C. (1993) Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. *J. Biol. Chem. 268*, 23784–23787.
14. Tafoya, L. C., Mameli, M., Miyashita, T., Guzowski, J. F., Valenzuela, C. F., and Wilson, M. C. (2006) Expression and function of SNAP-25 as a universal SNARE component in GABAergic neurons. *J. Neurosci. 26*, 7826–7838.
15. Baldwin, M. R., and Barbieri, J. T. (2007) Association of botulinum neurotoxin serotypes A and B with synaptic vesicle protein complexes. *Biochemistry 46*, 3200–3210.
16. Lawrence, G. W., Foran, P., Mohammed, N., DasGupta, B. R., and Dolly, J. O. (1997) Importance of two adjacent C-terminal sequences of SNAP-25 in exocytosis from intact and permeabilized chromaffin cells revealed by inhibition with botulinum neurotoxins A and E. *Biochemistry 36*, 3061–3067.
17. Banerjee, A., Kowalchyk, J. A., DasGupta, B. R., and Martin, T. F. (1996) SNAP-25 is required for a late postdocking step in Ca^{2+}-dependent exocytosis. *J. Biol. Chem. 271*, 20227–20230.
18. Soriano, E., Del Rio, J. A., Martinez, A., and Super, H. (1994) Organization of the embryonic and early postnatal murine hippocampus. I. Immunocytochemical characterization of neuronal populations in the subplate and marginal zone. *J. Comp. Neurol. 342*, 571–595.
19. Hess, E. J., Collins, K. A., and Wilson, M. C. (1996) Mouse model of hyperkinesis implicates SNAP-25 in behavioral regulation. *J. Neurosci. 16*, 3104–3111.
20. Thompson, P. M., Sower, A. C., and Perrone-Bizzozero, N. I. (1998) Altered levels of the synaptosomal associated protein SNAP-25 in schizophrenia. *Biol. Psychiatry 43*, 239–243.
21. Mehta, P. P., Battenberg, E., and Wilson, M. C. (1996) SNAP-25 and synaptotagmin involvement in the final Ca^{2+}-dependent triggering of neurotransmitter exocytosis. *Proc. Natl. Acad. Sci. U.S.A. 93*, 10471–10476.
22. Nabokina, S., Egea, G., Blasi, J., and Mollinedo, F. (1997) Intracellular location of SNAP-25 in human neutrophils. *Biochem. Biophys. Res. Commun. 239*, 592–597.
23. Salinas, E., Ventura, J., Cordova, L. E., and Luis Quintanar, J. (2004) Presence of SNAP-25 in rat mast cells. *Immunol. Lett. 95*, 105–108.
24. Driessen, H. P., de Jong, W. W., Tesser, G. I., and Bloemendal, H. (1985) The mechanism of N-terminal acetylation of proteins. *CRC Crit. Rev. Biochem. 18*, 281–325.
25. Guan, K. L., and Dixon, J. E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem. 192*, 262–267.
26. Rickman, C., Meunier, F. A., Binz, T., and Davletov, B. (2004) High affinity interaction of syntaxin and SNAP-25 on the plasma membrane is abolished by botulinum toxin E. *J. Biol. Chem. 279*, 644–651.
27. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150*, 76–85.
28. Connell, E., Darios, F., Broersen, K., Gatsby, N., Peak-Chew, S. Y., Rickman, C., and Davletov, B. (2007) Mechanism of arachidonic acid action on syntaxin-Munc18. *EMBO Rep. 8*, 414–419.
29. Bajohrs, M., Darios, F., Peak-Chew, S. Y., and Davletov, B. (2005) Promiscuous interaction of SNAP-25 with all plasma membrane syntaxins in a neuroendocrine cell. *Biochem. J. 392*, 283–289.
30. Rickman, C., and Davletov, B. (2003) Mechanism of calcium-independent synaptotagmin binding to target SNAREs. *J. Biol. Chem. 278*, 5501–5504.
31. Gershon, P. D., and Khilko, S. (1995) Stable chelating linkage for reversible immobilization of oligohistidine tagged proteins in the BIAcore surface plasmon resonance detector. *J. Immunol. Methods 183*, 65–76.
32. Nieba, L., Nieba-Axmann, S. E., Persson, A., Hamalainen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A. F., and Pluckthun, A. (1997) BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal. Biochem. 252*, 217–228.
33. Edwards, P. R., and Leatherbarrow, R. J. (1997) Determination of association rate constants by an optical biosensor using initial rate analysis. *Anal. Biochem. 246*, 1–6.
34. Arunachalam, L., Han, L., Tassew, N. G., He, Y., Wang, L., Xie, L., Fujita, Y., Kwan, E., Davletov, B., Monnier, P. P., Gaisano, H. Y., and Sugita, S. (2008) Munc18-1 is critical for plasma membrane localization of syntaxin1 but not of SNAP-25 in PC12 cells. *Mol. Biol. Cell 19*, 722–734.
35. Genoud, S., Pralong, W., Riederer, B. M., Eder, L., Catsicas, S., and Muller, D. (1999) Activity-dependent phosphorylation of SNAP-25 in hippocampal organotypic cultures. *J. Neurochem. 72*, 1699–1706.
36. Risinger, C., and Bennett, M. K. (1999) Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. *J. Neurochem. 72*, 614–624.

37. Hepp, R., Cabaniols, J. P., and Roche, P. A. (2002) Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A. *FEBS Lett. 532*, 52–56.
38. Boissel, J. P., Kasper, T. J., and Bunn, H. F. (1988) Cotranslational amino-terminal processing of cytosolic proteins. Cell-free expression of site-directed mutants of human hemoglobin. *J. Biol. Chem. 263*, 8443–8449.
39. Giglione, C., Boularot, A., and Meinnel, T. (2004) Protein N-terminal methionine excision. *Cell. Mol. Life Sci. 61*, 1455–1474.
40. Polevoda, B., and Sherman, F. (2000) Nα-terminal acetylation of eukaryotic proteins. *J. Biol. Chem. 275*, 36479–36482.
41. Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science 234*, 179–186.
42. Martinez, A., Traverso, J. A., Valot, B., Ferro, M., Espagne, C., Ephritikhine, G., Zivy, M., Giglione, C., and Meinnel, T. (2008) Extent of N-terminal modifications in cytosolic proteins from eukaryotes. *Proteomics 8*, 2809–2831.
43. Corradini, I., Verderio, C., Sala, M., Wilson, M. C., and Matteoli, M. (2009) SNAP-25 in neuropsychiatric disorders. *Ann. N.Y. Acad. Sci. 1152*, 93–99.
44. Sorensen, J. B., Nagy, G., Varoqueaux, F., Nehring, R. B., Brose, N., Wilson, M. C., and Neher, E. (2003) Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell 114*, 75–86.
45. Lowther, W. T., Orville, A. M., Madden, D. T., Lim, S., Rich, D. H., and Matthews, B. W. (1999) *Escherichia coli* methionine aminopeptidase: Implications of crystallographic analyses of the native, mutant, and inhibited enzymes for the mechanism of catalysis. *Biochemistry 38*, 7678–7688.
46. Persson, B., Flinta, C., von Heijne, G., and Jornvall, H. (1985) Structures of N-terminally acetylated proteins. *Eur. J. Biochem. 152*, 523–527.