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Differential interaction patterns in binding assays between recombinant syntaxin 1 and synaptobrevin isoforms

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Abstract Syntaxin 1 and synaptobrevin play an essential role in synaptic vesicle exocytosis. Two isoforms for each of these proteins, syntaxin 1A and 1B and synaptobrevin 1 and 2, have been found in nerve endings. Previous morphological studies have revealed a characteristic co-localization of syntaxin 1 and synaptobrevin isoforms in nervous and endocrine systems; however, the physiological significance of differential distribution is not known. In the present study an in vitro assay has been used to study a possible isoform specific interaction between syntaxin and synaptobrevin isoforms. The results show that although both syntaxin 1A and 1B may interact with synaptobrevin 1 and 2, this interaction is not uniform, showing different affinity patterns depending on the syntaxin 1/synaptobrevin isoform combination. The addition of SNAP-25 increased the binding capacity of syntaxin and synaptobrevin isoforms without affecting specific interactions.

Key words: SNARE protein; Syntaxin; SNAP-25; Synaptobrevin; Regulated exocytosis

1. Introduction

It is well established that regulated exocytosis is a process mediated by a series of protein-protein interactions. Soluble factors such as *N*-ethylmaleimide sensitive factor (NSF) and α,β-SNAPs (soluble NSF attachment proteins) interact with complexes composed of proteins located at the vesicle membrane (v-SNARE, vesicle-SNAP receptors) and at the target membrane (t-SNARE, target-SNAP receptors) in the process of membrane targeting, fusion and/or retrieval [1,2]. Although the exact sequence of events that take place between soluble factors and SNARE proteins is still a matter for discussion [3], it has been demonstrated that the direct binding between v- and t-SNARE is fundamental for the fusion of membrane compartments (i.e. synaptic vesicles and presynaptic plasma membrane) and that it might also be required for proper membrane compartment recognition and docking [1,4]. Syntaxin 1 and synaptosomal associated protein of 25 kDa (SNAP-25) are two pre-synaptic membrane proteins capable of binding specific vesicle membrane proteins [5–9]. It is well established that syntaxin 1, SNAP-25 and synaptobrevin (also known as vesicle associated membrane protein, VAMP) form an SDS-resistant protein complex of 7s that acts in the later steps of synaptic vesicle fusion with the plasma membrane [1,2,10]. In addition, the fact that these SNARE proteins are the molecular targets of clostridial neurotoxins, which selectively block neurotransmitter release, highlights their functional importance in synaptic transmission [11]. At least two isoforms for each of these SNARE proteins have been found in nerve terminals (SNAP-25 a and b; syntaxin 1A and 1B; synaptobrevin 1 and 2) [12,13]. However, the functional significance of the existence of more than one SNARE isoform in nerve terminals is still unknown. Morphological studies have shown a differential distribution of syntaxin and synaptobrevin isoforms in some areas of the central and peripheral nervous system. Interestingly, syntaxin 1A and 1B are usually co-localized with synaptobrevin 2 and synaptobrevin 1, respectively [14–16]. Moreover, endocrine cells express syntaxin 1A and synaptobrevin 2 but not syntaxin 1B nor synaptobrevin 1 [17,18]. To date, no functional significance has been assigned to specific syntaxin and synaptobrevin isoform associations and the role that SNAP-25 may play in these interactions.

In vitro protein-protein interaction assays have been useful to study the functional aspects of SNARE protein interactions [6,19,20]. These assays have been outstanding in defining the minimum domains involved in the SNARE complex [4,20] and elucidating the three-dimensional structure of the complex core [21]. In the present work, an in vitro assay using recombinant SNARE proteins was used to study specific isoform interactions. We show relevant differences in affinities of the interaction between syntaxin and synaptobrevin isoforms, even though both syntaxin isoforms can bind to each synaptobrevin isoform. The addition of recombinant SNAP-25 to the assay increases the affinity between syntaxin and synaptobrevin isoforms.

The present results agree with the differential syntaxin and synaptobrevin isoform distributions observed in the endocrine system and some areas of the nervous system [14–16,18]. In addition, the effect of the recombinant SNAP-25 on the syntaxin and synaptobrevin isoform bindings supports a possible in vivo facilitative function for this protein in the assembly of the 7s complex core [4,10,21].

2. Materials and methods

2.1. Materials

Synaptobrevin 1 and 2 cDNAs were obtained by reverse transcription-polymerase chain reaction (RT-PCR, Pharmacia Biotech) from rat brain according to the manufacturer's protocol. 5′-TCATTACAGATTTAGGAGG-3′ and 5′-TTTAGGAACCCTCAACC-3′ were used as sense and antisense primers, respectively, for synaptobrevin

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Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; IPTG, isopropyl β-D-thiogalactopyranoside; GST, glutathione *S*-transferase; NSF, *N*-ethylmaleimide sensitive factor; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SNAP, soluble NSF attachment proteins; SNAP-25, synaptosomal associated protein of 25 kDa; v- and t-SNARE, vesicle- and target-SNAP receptor

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1, while 5′-GGTTGAGGGTTCTAAAG-3′ and 5′-CTTGAGGTTT-TTCCACCA-3′ were used for synaptobrevin 2. *Bam*HI and *Eco*RI restriction sites were introduced in the sense and antisense primers, respectively, to facilitate subcloning of the amplified DNA sequences. SNAP-25b (S25b) cDNA was obtained as previously described [22]. DNA with the sequences encoding the cytosolic regions of syntaxin (Stx) 1A and 1B were introduced into pTRCHis $(His)_6$ fusion protein expression vector (Invitrogen) as previously described [14].

2.2. Fusion proteins

Glutathione *S*-transferase (GST-)-synaptobrevin isoforms (1 and 2) and SNAP-25b cDNAs encoding the respective full protein sequences were cloned into pGEX 4T-1 GST fusion protein expression vector (Pharmacia). GST and the GST fusion proteins were expressed in the Bl 21 DE3 pLys *Escherichia coli* strain after isopropyl β-D-thiogalactopyranoside (IPTG) induction (2 mM). The cells were centrifuged at $6000\times g$ for 20 min and the pellet was resuspended in ice-cold phosphate buffered saline (PBS) with 1% Triton X-100, sonicated and centrifuged at $12\,000\times g$ for 30 min. The resulting supernatants were incubated at 4°C for 1 h with glutathione Sepharose beads (Pharmacia) previously equilibrated in PBS buffer. After incubation, the beads were washed three times with PBS and kept in the same buffer at 4°C until use. SNAP-25 was further purified by incubating the glutathione Sepharose beads containing GST-SNAP-25 with thrombin (0.02 U/μl) in buffer A (PBS, with 1 mM Cl_2Ca). The cytosolic fragments of $(His)_6$-Stx 1A and 1B were expressed in TOP 10 *E. coli* strain, after induction with IPTG (2 mM). The cells were centrifuged, resuspended, disrupted and centrifuged as above. Supernatants were applied to Ni-NTA agarose (Qiagen) containing columns and the attached proteins were eluted with a 50–300 mM imidazole gradient. To eliminate imidazole, the fractions containing the recombinant proteins were applied to desalting columns (Bio-Rad) previously equilibrated with PBS buffer. The concentration of recombinant proteins was defined after protein quantification by the bicinchoninic acid (BCA) method (Pierce). The purity and the amount of the recombinant proteins were further analyzed by SDS-PAGE after staining of the gels with Coomassie blue R-250.

2.3. In vitro binding assay

GST, GST-synaptobrevin 1 and 2 and GST-SNAP-25 fusion proteins (1 mM) previously bound to glutathione-agarose beads were incubated with bovine serum albumin (BSA, 10 mg/ml) for 60 min at 4°C in PBS. After washing with PBS, the beads with GST-synaptobrevin 1 or GST-synaptobrevin 2 were incubated overnight at 4°C with syntaxin 1A and 1B at different concentrations (ranging from 4 μM to 139 μM), and in the presence or absence of soluble SNAP-25 (20 μM), as specified. Each condition point was washed at least three times with 1 ml of phosphate buffer, 20 mM pH 7.4, 300 mM NaCl, 10% glycerol and 0.5% Triton X-100. After washing, SDS-sample buffer was added to the beads, and the proteins were analyzed by SDS-PAGE and Western blotting. GST coupled to glutathione-agarose beads was used in parallel to determine the non-specific binding.

2.4. Immunoblots

After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes. The membranes were blocked using 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS), and incubated overnight at 4°C with anti-syntaxin monoclonal antibody (clone HPC-1, Sigma) in the same buffer. After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase, the blots were developed using the enhanced chemiluminescence (ECL) method (Amersham Life Science). The films were scanned and then subjected to band densitometry and quantification (Phoretix System Software).

3. Results and discussion

3.1. Binding between recombinant SNARE isoforms

An in vitro assay has been performed to study the interactions between recombinant synaptobrevin and syntaxin isoforms and the effect of SNAP-25 on such interactions. Similar approaches have been used in other studies to analyze protein-protein interactions between SNARE proteins [6,19,23]. In the first series of experiments, the cytosolic fragments of syntaxin 1A and 1B (18 μM) were incubated separately with GST-synaptobrevin 1 or 2 bound to glutathione-Sepharose beads. In these experiments the concentration of GST-synaptobrevin 1 or 2 was in excess with respect to syntaxin. The binding of syntaxins was analyzed by Western blotting using a monoclonal antibody (HPC-1 clone), which recognized both syntaxin 1A and 1B isoforms (Fig. 1). Syntaxin 1A and 1B were specifically bound to GST-synaptobrevin isoforms (no significant amount of syntaxins was found when incubated with GST conjugated to glutathione-Sepharose beads). However, the amount of syntaxin bound to GST-synaptobrevin was different depending on the combination of syntaxin and synaptobrevin isoforms analyzed. Thus, under these conditions, syntaxin isoforms showed a greater affinity for synaptobrevin 2 than for synaptobrevin 1. Similar results were obtained with the inverse experiments: incubating synaptobrevin 1 or 2 with $(His)_6$-syntaxin 1A or 1B bound to Ni-agarose beads and analyzing the amount of synaptobrevin bound to $(His)_6$-syntaxin-Ni-agarose beads (data not shown).

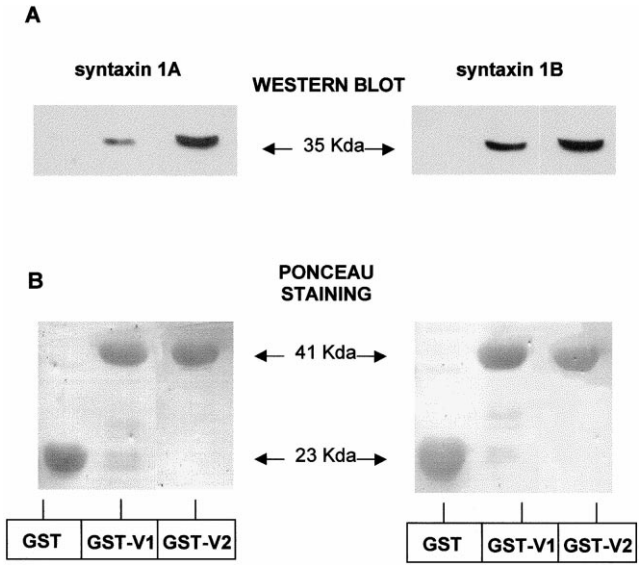

Fig. 1. GST and GST-synaptobrevin 1 and 2 (GST-V1 and GST-V2) were bound to glutathione-Sepharose beads and incubated with 18 μM $(His)_6$-syntaxin 1A and 1B. A: The amount of syntaxin bound to the beads was analyzed by SDS-PAGE and Western blotting using the monoclonal HPC-1 antibody (1:1000) and ECL method. B: Ponceau staining of the nitrocellulose membranes containing GST (∼23 kDa) and GST-synaptobrevin 1 and 2 (∼41 kDa).

These results suggest that although all four isoforms may coexist in the same areas of the nervous systems (and at the same nerve terminals), a specific combination of isoforms may improve efficiency in the formation of protein core complex (7s) essential for vesicle exocytosis.

3.2. Differential binding affinity between syntaxin 1A and 1B, and synaptobrevin 1 and 2

To determine if the differential patterns of syntaxin isoforms recovered from the binding to GST-synaptobrevin 1 or 2 were dependent on protein concentration, we performed the same experiment using different syntaxin concentrations (ranging from 4 to 32 μM). Densitometric analysis of Western blots for syntaxin corroborated the previous qualitative data showing the differential binding patterns in the syntaxin 1/

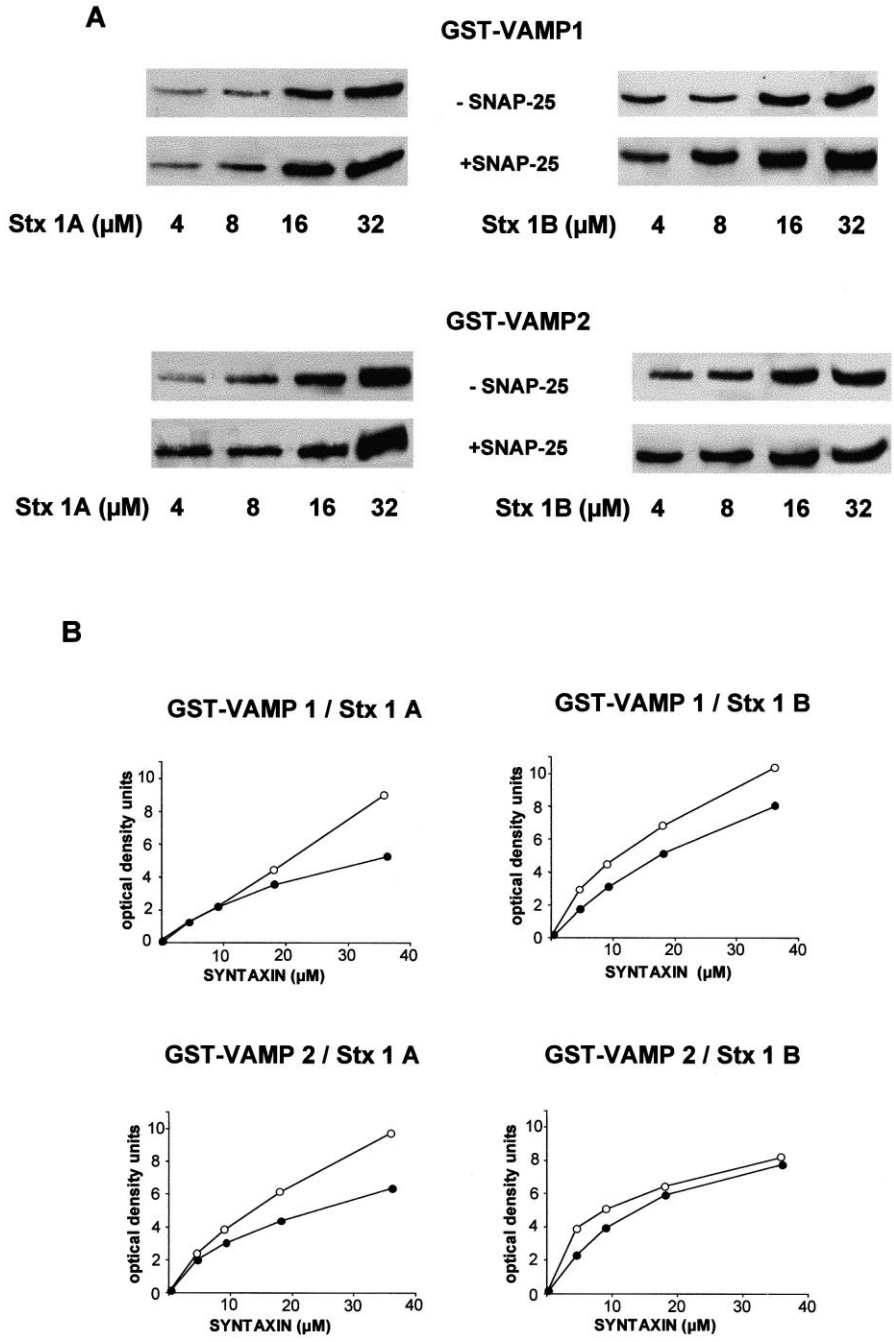

Fig. 2. GST-synaptobrevin 1 and 2 (GST-VAMP 1-2) bound to glutathione-Sepharose beads were incubated with increasing concentrations of $(His)_6$-syntaxin 1A and 1B (Stx 1A/1B) in the presence or absence of SNAP-25 (20 μM). A: The amount of syntaxin bound to the beads was detected by SDS-PAGE and Western blotting using the monoclonal antibody HPC-1 (1:1000) and the ECL method. B: Densitometric analysis from a representative experiment of the amount of syntaxin 1 bound to glutathione-Sepharose-GST-synaptobrevin in the presence (open circles) or absence (filled circles) of SNAP-25.

synaptobrevin combinations tested (Fig. 2). Thus, syntaxin 1A has a higher binding capacity to synaptobrevin 2 than synaptobrevin 1. Syntaxin 1B interaction with synaptobrevin isoforms seems to be different. The affinity of recombinant syntaxin 1B to GST-synaptobrevins could vary in a concentration-dependent manner. At the lower concentration tested, syntaxin 1B binding was more effective to synaptobrevin 2 than synaptobrevin 1 (similar to syntaxin 1A, see above). Nevertheless, this situation changed when the syntaxin 1B concentration was increased. At the higher concentrations, the binding of syntaxin 1B to synaptobrevin 2 decreased compared to the syntaxin 1B/synaptobrevin 1 combination. Therefore, at higher syntaxin 1B concentrations, there was a higher affinity to synaptobrevin 1 than to synaptobrevin 2.

These results suggest specific functional interaction for the isoforms of syntaxin 1 and synaptobrevin as well as a possible explanation for previous morphological observations. Actually, the fact that recombinant syntaxin 1A has a higher binding capacity for synaptobrevin 2 than for synaptobrevin 1 agrees with the co-localization of syntaxin 1A and synapto-

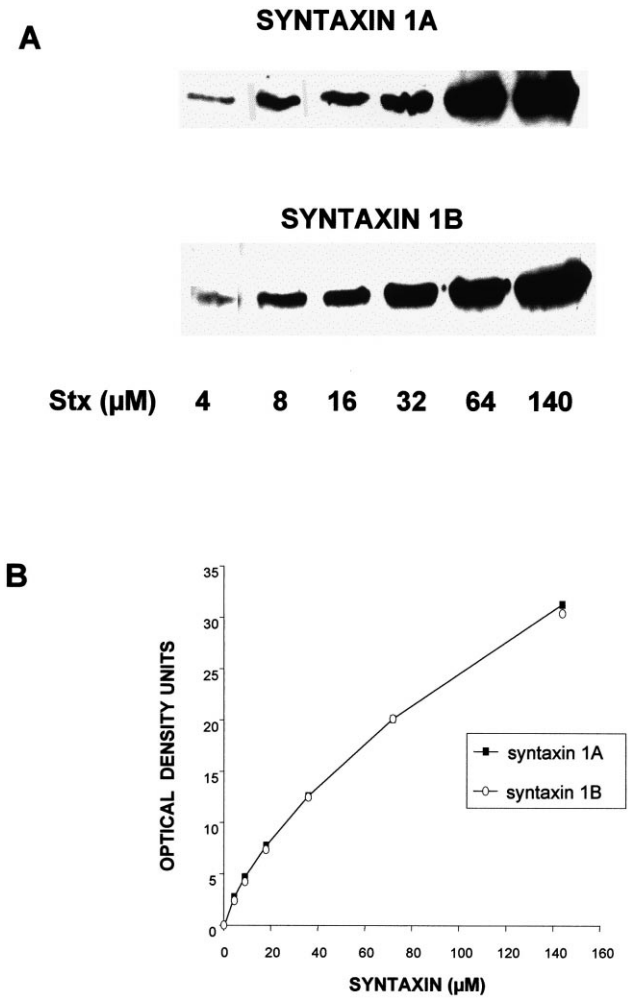

Fig. 3. GST-SNAP-25 bound to glutathione-Sepharose beads was incubated with increasing concentrations (from 4 to 140 μM) of $(His)_6$-syntaxin 1 A and 1B. A: Syntaxin 1 bound to the beads was detected by Western blotting using monoclonal HPC-1 antibody (1:1000) and the ECL method. B: Densitometric analysis from a representative experiment of syntaxin 1A (filled squares) or syntaxin 1B (open circles) bound to the glutathione-Sepharose-GST-SNAP-25.

brevin 2 in some areas of the nervous and the endocrine systems [14–18].

Differences in syntaxin 1 and synaptobrevin distribution and concentration throughout the nervous system [14–16], together with the different affinity pattern of syntaxin 1A and 1B for synaptobrevin isoforms, point to a possible syntaxin 1B and 1A preponderant role in different neural networks or nerve terminals. Neurons secrete neurotransmitters and neuropeptides by exocytosis of small synaptic vesicles (SVs) and large dense core vesicles (LDCVs). SV exocytosis has been associated with fast neurosecretion of classical neurotransmitters (glutamate, acetylcholine), whereas LDCVs are responsible for substances with a neuromodulatory action (neuropeptides, norepinephrine, etc.). In spite of differences in content, localization in the nerve terminal, and sensitivity to calcium levels for exocytosis, both sets of secretory organelles contain the molecular machinery for regulated exocytosis, including SNARE proteins, synaptotagmin and Rab3a, among others. It has been suggested that the differences in sensitivity to calcium concentrations of SV and LDCV can be explained by the existence of different synaptotagmin isoforms [24], voltage gated calcium channel isoform diversity [25] and the differential distribution inside the nerve terminal of SVs and LDCVs. In any case, in addition to the presence of different protein isoforms related to the entry and detection of calcium inside nerve terminals, the presence of the precise combination of SNARE isoform proteins that form part of the exocytotic machinery would be required for a high efficiency of SV and/or LDCV exocytosis. According to these results, syntaxin 1B would be mainly involved in neuronal networks which need a very concrete amount of neurotransmitter liberated in a short time. Conversely, syntaxin 1A could be related to a more regulated but slower neurosecretion, for example in those systems involved in neuropeptide and hormone secretion. Supporting this suggestion, it has been found that neuromuscular junction, considered a fast synapsis, only contains syntaxin 1B and synaptobrevin 1, whereas cells of the endocrine system with peptide secretion only express syntaxin 1A and synaptobrevin 2 [16–18].

In addition to the existence of proteins that interact with syntaxin 1 and synaptobrevin and that may regulate their availability to form the 7s protein core complex required for exocytosis [26,27], the different binding capacity of syntaxin 1 isoforms to synaptobrevin isoforms suggests the presence of a differential self-regulated process for the core complex formation before calcium entry and neurotransmitter release.

3.3. SNAP-25 increases binding without changing the affinity pattern

In the next step, different concentrations of cytosolic fragments of recombinant syntaxin isoforms were incubated with both recombinant GST-synaptobrevin isoforms in the absence or the presence of recombinant SNAP-25 to determine the effect of SNAP-25 on syntaxin-binding isoforms to synaptobrevin isoforms. The amount of syntaxin bound was determined by Western blotting and densitometric analysis.

The addition of SNAP-25 to the assay increased the amount of syntaxin 1A and 1B bound to GST-synaptobrevin isoforms, but maintained the previously observed isoform specificity (Fig. 2). To further investigate the nature of the increase in the binding of syntaxin 1 to synaptobrevin isoforms in the presence of SNAP-25, we decided to incubate increasing concentrations of syntaxin isoforms with GST-SNAP-25 bound to glutathione-agarose beads and to analyze the amount of syntaxin bound by Western blotting. In this case, no significant difference in the binding of both recombinant syntaxin 1 isoforms to GST-SNAP-25 was found (Fig. 3). Thus, we could conclude that SNAP-25 does not affect the isoform-dependent differential formation of syntaxin 1/synaptobrevin complexes. These results also suggest that SNAP-25, besides its specific role in membrane fusion during exocytosis, enhances the binding of syntaxin 1 to synaptobrevin, and that this effect is not isoform-dependent.

Therefore, the present results agree with several works that suggest a possible facilitative role of SNAP-25 in SNARE complex formation [4,10,21]. So SNAP-25 could help to facilitate the proper binding between syntaxin 1 and synaptobrevin and to stabilize the 7s complex. In line with the location of SNAP-25 and syntaxin 1 at the plasma membrane, acting as t-SNAREs, the present results support a previous suggestion by Sutton et al. [21] concerning the sequence of the 7s complex

assembly, that would begin with the formation of an heterodimer complex composed of syntaxin 1 and SNAP-25 followed by the binding of synaptobrevin to the t-SNARE complex.

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