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On the structure of the 'synaptosecretosome'

Evidence for a neurexin/synaptotagmin/syntaxin/Ca^{2+} channel complex

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Recent experiments have identified interactions between presynaptic and synaptic vesicle membrane proteins, that might be important in organizing the components of the fast neurotransmitter release mechanism to ensure that the process follows a rapid time course. Here we extend previous investigations to show that in addition to the α-latrotoxin receptor (neurexin) and synaptotagmin another presynaptic protein, syntaxin, co-purifies on a α-latrotoxin affinity column. This implies that syntaxin is associated with these two molecules in a complex; a conclusion supported by the immunoprecipitation of [^{125}I]latrotoxin binding by syntaxin antibodies. In addition, antibodies against syntaxin and the α-latrotoxin receptor immunoprecipitate [^{125}I]ω-conotoxin binding sites, indicating that calcium channels are associated with this complex. Thus, neurexin, synaptotagmin, syntaxin, and calcium channels can be found in a structure we propose to call the 'synaptosecretosome'. The components of the synaptosecretosome, in association with additional proteins, are postulated to organize the process of neurotransmitter release.

α-Latrotoxin; Neurexin; Synaptotagmin; Syntaxin; Ca^{2+} channel; Synaptosecretosome

1. INTRODUCTION

Fast neurotransmitters are released within 200 μs of presynaptic stimulation, suggesting that the molecular components that underlie this specialized stimulation/secretion coupling are highly organized [1]. This supposition has an ultrastructural correlate in the active zone. Here, the small synaptic vesicles (ssvs), the storage and release organelles of the neuron's fast transmitters, are clustered at specialized regions of the presynaptic membrane [2]. Proteins from both vesicle and other presynaptic compartments are thought to participate in this clustering and ensure the organized arrangement that enables rapid release. Initial studies focussed on the role of the synapsins, ssv-associated proteins which by virtue of their phosphorylation-dependent affinity for both ssvs and cytoskeletal elements have been implicated in vesicle mobilization [3]. Recent experiments also indicate that interactions exist between ssvs and presynaptic membrane proteins which might play an important role in vesicle–plasma membrane association.

The demonstration that the ssv protein synaptophysin binds to the presynaptic plasma membrane protein physophilin provided the first indication that protein/protein interactions might exist between the two membrane compartments that fuse during transmitter release [4]. More recently, another ssv protein synaptotagmin has been shown to bind directly to the α-Ltx receptor [5], a presynaptic protein of unknown physiological function which represents the target site of the presynaptically acting α-Ltx [6,7]. The interaction with synaptotagmin is mediated by the intracellular C-terminus of the α-Ltx receptor and the related neurexins, which are predicted from cDNA to constitute a highly polymorphic family of proteins [8,9]. It has also recently been shown that synaptotagmin, along with several other molecularly characterized ssv proteins, can exist as a multimeric complex [10]. This complex includes a previously uncharacterized presynaptic membrane protein which was subsequently cloned and called syntaxin [11]. Both syntaxin and synaptotagmin have also been identified as the epitopes for a series of independently produced antibodies that efficiently immunoprecipitate the calcium channels that bind ω-Ctx [11,12,13,14,15]. These ω-Ctx-sensitive channels are thought at least in part, to be responsible for the calcium influx that triggers transmitter release [16]. In this study we extend this

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Abbreviations: ω-Ctx, ω-conotoxin; α-Ltx, α-latrotoxin; NSF, *N*-ethylmaleimide-sensitive fusion protein; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; SNAPs, soluble NSF attachment proteins; TCA, trichloroacetic acid.

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line of investigation and show that syntaxin is associated with the α-Ltx receptor/synaptotagmin complex. We also provide evidence that these three proteins associate in a complex with the calcium channel. The very recent notion that syntaxin may be a neuronal receptor involved in the targeting of fusion-competent ssvs to active zones [17] indicates that this multimeric protein complex, which we propose to term 'synaptosecretosome', may be fundamental in adapting an ubiquitous fusion mechanism into one suited to the requirements of point to point communication in the nervous system.

2. MATERIALS AND METHODS

2.1. *α-Ltx purification, iodination and affinity-column synthesis*

α-Ltx was purified from lyophilized Central Asia Black Widow spider venom as described previously [18]. Purified toxin (200 pmol) was labelled with ^{125}I (Amersham) using the lactoperoxidase method under conditions described elsewhere for nerve growth factor [19]. This yielded [^{125}I]α-Ltx with a specific activity of 2500–4400 Ci/mmol. α-Ltx-Sepharose was prepared by reacting the toxin with CNBr-Sepharose (0.3–0.5 μg/ml matrix) under conditions recommended by the manufacturer (Pharmacia).

2.2. *Purification of the α-Ltx receptor*

Crude rat brain membranes were prepared and solubilized in Triton X-100 as described previously for bovine brain [20]. Prior to loading onto the α-Ltx column (1 μl), the solubilized extract was diluted to reduce the Triton X-100 concentration to 0.5% (w/v). The column was exhaustively washed with 200 column volumes of Tris-buffer (20 mM Tris-HCl, pH 7.7) containing 2.5 mM $CaCl_2$, 130 mM KCl, 0.1% (w/v) Triton and 0.3 mM PMSF. The columns were subsequently eluted by either Tris buffer containg 1 M KCl/10 mM EDTA/0.1% (w/v) Lubrol-PX or, sequentially, by first a 0.13–0.6 M KCl gradient in Tris-buffer that contained 2.5 mM $CaCl_2$/0.1% (w/v) Lubrol-PX followed by the 1 M KCl buffer used above. Fractions of 1.5 ml were collected at selected points of the load and washing and throughout the elution profiles. Initial experiments used the solubilized α-Ltx receptor binding assay [20] to detect the receptor eluted from the column.

2.3. *Sucrose gradient centrifugation*

The initial fraction from a one step elution of the α-Ltx affinity column was dialyzed overnight against 800 volumes of Tris-buffer containing 200 mM KCl, 2.5 mM $CaCl_2$, 0.1% (w/v) Triton X-100 and 0.3 mM PMSF. Aliquots (500 μl) were loaded onto a 11.2 μl 5–20% (w/v) sucrose gradient over a 0.3 μl 50% (w/v) sucrose cushion. All sucrose mixtures were prepared in Tris-buffer that contained 200 mM KCl, 2 mM $CaCl_2$ and 0.1% (w/v) Triton X-100. The gradients were centrifuged at 33,000 rpm in a SW-41 Beckman rotor at 4°C for 16 h and divided into 17 fractions of equal volume. Parallel gradients were used to determine the position of marker proteins as described previously [4].

2.4. *Gel electrophoresis and Western blotting*

Protein samples from receptor purification and sucrose gradients were concentrated by TCA precipitation [21] and subjected to SDS-PAGE on 5–18% linear gradient gels according to Laemmli [22]. Gels were either silver-stained [23] or transferred to nitrocellulose according to the method of Burnette [24]. The nitrocellulose was divided into three strips that contained peptides of apparent molecular weight > 97 kDa, those between 97 and 45 kDa, and those < 45 kDa, and probed with antibodies generated against the C-terminal of neurexins I, a fusion protein of synaptotagmin [25], and syntaxin [14], respectively. Immunoreactivity was detected using horseradish peroxidase-coupled secondary antibody in combination with the ECL detection system (Amersham).

2.5. *Generation of neurexin-specific antibodies*

Antisera were raised in New Zealand White rabbits against the synthetic peptide CAKSANKNKKNKDKEYYV (Nrx-1) which excluding the N-terminal cysteine corresponds to the extreme C-terminal of both the 1α and 1β neurexins as predicted from their cDNA sequences [8]. The peptide was coupled to keyhole limpet haemocyanin through the N-terminal cysteine [26]. After one boost, the sera gave a specific response against the neurexins detected in purified fractions, as determined by peptide competition. These sera and subsequent bleeds were affinity-purified on a Nrx-1 peptide column before use [26].

2.6. *Immunoprecipitation of [^{125}I]α-Ltx and [^{125}I]ω-Ctx receptors*

Protein A-Sepharose (Pharmacia) was washed and incubated with antibodies as described [10] although the buffers used varied to ensure conditions optimized in each case for [^{125}I]α-Ltx [20] or [^{125}I]ω-Ctx binding [14]. Solubilized crude brain membranes equivalent to the load material or purified receptor dialysed overnight as described above, were incubated with [^{125}I]α-Ltx for 4 h at 4°C. To assess immunoprecipitation of [^{125}I]ω-Ctx binding, membranes were prelabelled prior to solubilization in Triton X-100 essentially as described previously [13]. The labelled fractions were incubated with the antibody–protein A–Sepharose conjugate overnight. The Sepharose was pelleted, washed 3 times, and the pellets were counted for radioactivity.

3. RESULTS AND DISCUSSION

Affinity purification on a α-Ltx column of crude brain membrane extracts has been reported to result in specific [^{125}I]α-Ltx binding fractions containing a series of polypeptides with the following apparent molecular weights, 200–160, 120, 79, 65, 43, 36 and 29 kDa [5,20,27]. Here, a similar purification protocol led to the elution of specific [^{125}I-α]Ltx binding sites and a similar complex polypeptide pattern (Fig. 1). The major bands at 200–160, 120, 72, 65 and 45 kDa (Fig. 1a) probably correspond to previously described bands, and minor differences in apparent molecular weight relate to the different gel conditions used. The bands at 72 and 45 kDa are likely to represent the ubiquitous proteins which can be purified from tissues that lack [^{125}I-α]Ltx binding sites and are retained on the column by a direct interaction with the immobilized toxin [27]. The band at 120 kDa has been described as α-Ltx leaching from the column [5,27], and we have confirmed this by immunoblotting with toxin-specific sera (result not shown). The group of proteins running at 160–200 kDa represent the components of the eluted fractions that bind α-Ltx and have been identified as members of the neurexin family [7,20,27]. Using an antibody generated against the C-terminus of neurexin 1 we confirmed that the high molecular weight bands in our preparation are neurexins (Fig. 1b). Similarly, immunoblotting identified the band at 65 kDa as the ssv protein synaptotagmin. Synaptotagmin has been shown to interact with the cytoplasmic C-terminal tail of neurexins [9]. Thus, the co-purification of synaptotagmin on the column is due to an interaction with the neurexin isoform that represents the α-Ltx receptor [9]. Among the series of bands in the eluate that ran at approximately 35–38 kDa [5,27] we identified syntaxin, a molecule of presynaptic origin

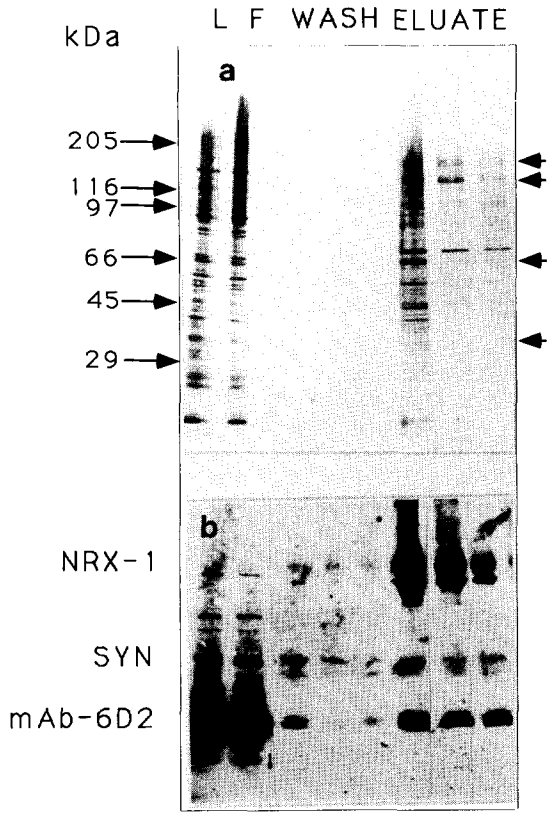

Fig. 1. Synaptotagmin and syntaxin copurify with neurexin on an affinity column. Solubilized rat brain membranes prepared from 15 g of brain tissue were loaded onto an α-Ltx column, which was subsequently eluted in one step. Fractions of 1.5 ml were collected, concentrated by TCA-precipitation and resuspended in 100 μl sample buffer. Fractions were run on parallel 5–18% gradient gels which were either silver stained (a) or transferred to nitrocellulose for immunoblotting (b) with neurexin (Nrx-1, 4 μg/ml), syntaxin (mAb 6D2, 1:1000) or synaptotagmin (Syn, 1:10,000) antibodies. Load (L), flowthrough (F), and fractions from the beginning, middle and end of the 200 ml wash are shown in addition to the eluted fractions. The position of molecular weight markers is shown on the left. Arrowheads mark the positions of neurexin, synaptotagmin and syntaxin.

that has been shown to interact with synaptotagmin. The syntaxin detected in the eluted fractions co-distributed with neurexin and synaptotagmin, indicating that formation of a complex between these molecules causes their co-purification. However, only a minority of the solubilized syntaxin loaded onto the column is retained within this complex. In contrast to the above, we were unable to detect either the ssv protein synaptophysin or its presynaptic partner protein, physophilin [4], in the eluted fractions (data not shown).

To further characterize the interaction between synaptotagmin, syntaxin, and neurexin, the α-Ltx column eluate was subjected to sucrose gradient centrifugation after extensive dialysis to remove EDTA and salts potentially interfering with neurexin/synaptotagmin [5,20,27] and synaptotagmin/syntaxin [11] interactions. Analysis of the gradient fractions (Fig. 2) showed that both neurexin and synaptotagmin immunoreactivities co-migrated on the gradient. In contrast, syntaxin migrated as a distinct peak of immunoreactivity, indicating that its association with the synaptotagmin/neurexin complex implied by its co-purification on the α-Ltx column was not recovered after dialysis.

Independent evidence for an association of neurexin, synaptotagmin, and syntaxin in a macromolecular complex was derived from immunoprecipitation experiments (Table I). [^{125}I]α-Ltx was incubated with purified receptor (dialysed against low salt and Ca^{2+}) or solubilized crude rat brain extract . These extracts were equivalent to those loaded onto the α-Ltx column, from which neurexin, synaptotagmin and syntaxin co-purify, and thus should contain these molecules in a complex. The Nrx-1 antibody efficiently precipitated specifically bound toxin from both purified receptor preparations and solubilized extracts. Control experiments showed that this immunoprecipitation of [^{125}I]α-Ltx binding sites was prevented by preincubation with excess unlabelled toxin; moreover, none of the antibodies used directly precipitated [^{125}I]α-Ltx (data not shown). The syntaxin antibody (mAb 10D2; 14) was able to precipitate specific binding sites from solubilized extracts, but not purified receptor fractions. This observation further supports the view that syntaxin is complexed with the

Table I

Immunoprecipitation of [^{125}I]α-Ltx and [^{125}I]ω-Ctx binding sites from membrane extracts and purified α-Ltx receptor preparations

Antibody	Sites precipitated		
	[^{125}I]α-LTX		[^{125}I]-ω-CTX
	Receptor	Extract	Extract
NRX-1	1250 ± 39*	614 ± 15	763 ± 12
Synaptotagmin	23 ± 1	35 ± 1	140 ± 5
Syntaxin (mAb10D2)	17 ± 1	254 ± 25	4004 ± 33

For precipitation of [^{125}I]α-Ltx binding sites, the antibodies (250 μg or *15 μg) were incubated with 12.5 μl of protein A-Sepharose. Dialysed receptor or detergent extracts were incubated with [^{125}I]α-Ltx (0.2–0.5 nM) for 4 h at 4°C. 200 μl of these incubations containing 250,000 cpm were added to the antibody protein A–Sepharose conjugates. After overnight incubation and 3 washes, the bound radioactivity was measured. Crude brain membranes resuspended in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, were prelabelled with 0.3 nM [^{125}I]ω- Ctx for 4 h at 4°C (> 90% of the counts bound under these conditions could be blocked if labelling was performed in the presence of excess unlabelled toxin). The labelled binding sites were solubilized in 1% (w/v) Triton X-100, before this was diluted to 0.5% in a Tris/EDTA buffer containing 400 mM NaCl. 400 μl of this fraction containing 30,000 cpm were incubated overnight with 10 μl aliquots Protein A–Sepharose preabsorbed with 120 μg of the antibodies. These incubations were pelleted and washed 3 times before counting bound radioactivity. Values are the mean of duplicate or triplicate estimates determined in parallel and are expressed as % of the radioactivity precipitated by Protein A–Sepharose coated with an equivalent amount of preimmune IgG. Other experiments using separate membrane and iodinated toxin preparations gave similar results.

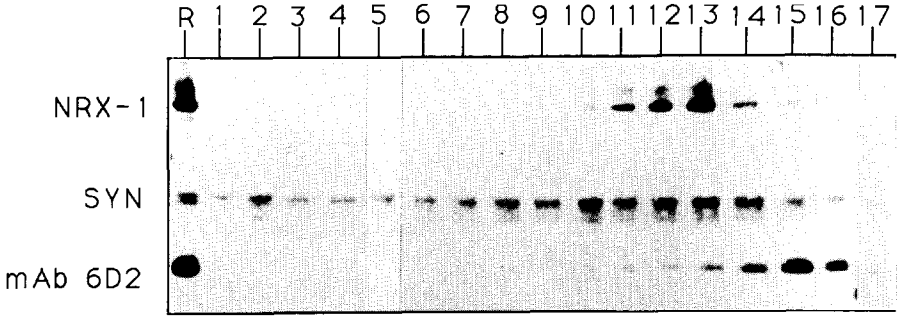

Fig. 2. Sucrose gradient centrifugation of affinity-purified α-Ltx receptor fractions. The first two fractions from a one step eluted α-Ltx column were pooled and dialysed before loading onto a sucrose gradient. The gradient was divided into 17 fractions which were TCA precipitated, dissolved in 40 μl sample buffer and subjected to PAGE on a 5–18% gradient gel. A TCA precipitated fraction of the purified receptor representing 1/3 of the fraction loaded onto the sucrose gradient was also analyzed (R). After transfer to nitrocellulose, strips were cut and immunoblotted with neurexin, synaptotamin or syntaxin antibodies as described in Fig. 1. Fraction 1 marks the bottom of the gradient. The position of marker enzymes measured in identically prepared gradients, run in parallel, were: β-galactosidase (fraction 3), catalase (fraction 5), lactate dehydrogenase (fraction 9), malate dehydrogenase (fraction 12) and cytochrome *c* (fraction 15).

α-Ltx receptor, but this interaction is irreversibly disrupted by the high salt used to elute the column. Relatively high syntaxin antibody concentrations were required to precipitate [^{125}I]α-Ltx binding. This is consistent with only a fraction of the solubilized syntaxin being complexed under the conditions used. Under none of the conditions tested were we able to immunoprecipitate α-Ltx binding with the synaptotagmin antibodies although this has been succesfully done using different synaptotagmin antibodies [9]. Thus, either the antibodies used in this study do not recognize native rat synaptotagmin, or the epitopes which they recognize are masked by protein/protein interactions.

Two of the components identified in the complex described above, namely synaptotagmin and syntaxin, have also been shown to associate with ω-Ctx binding

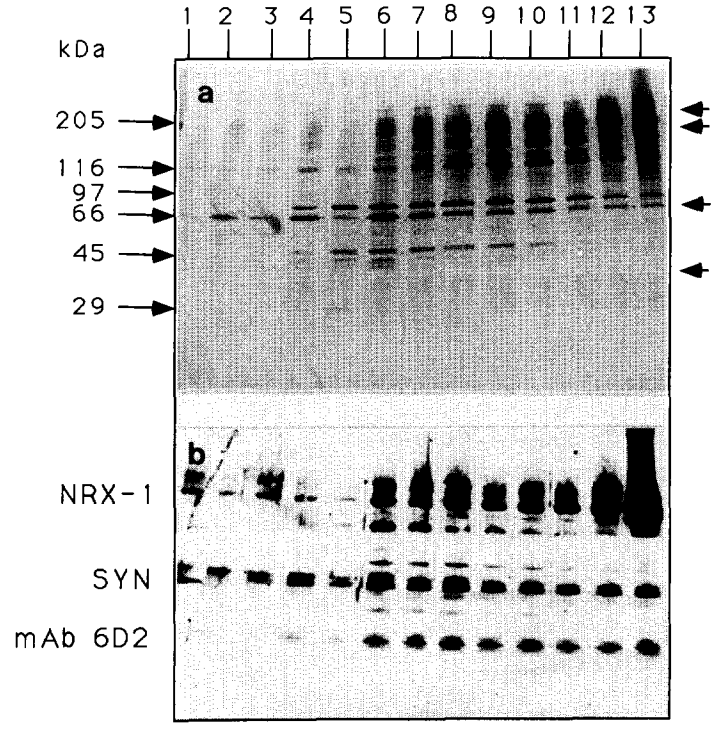

Fig. 3. Two-step elution of the α-Ltx affinity column. Membranes from 15 g of rat brains were solubilized and loaded onto the column, which was washed as described in section 2. The column was eluted with a gradient of KCl (0.13–0.6 M) containing 2.5 mM $CaCl_2$ and 0.1% (w/v) Lubrol-PX in Tris-buffer, (fractions 1–8). After washing with the 0.6 M KCl containing buffer (fractions 8, 9), the column was subjected to further elution with Tris/PMSF buffer containing 10 mM EDTA and 1 M KCl (fractions 11–13). Aliquots (1 ml) of the collected 1.5 ml fractions were concentrated by TCA precipitation and divided into two portions for analysis on 5–18% gradient gels. Two gels run in parallel were either silver stained (a) or transferred to nitrocellulose for immunoblotting (b) with antibodies against the neurexins (Nrx-1), synaptotagmin (Syn) or syntaxin (mAb-6D2).

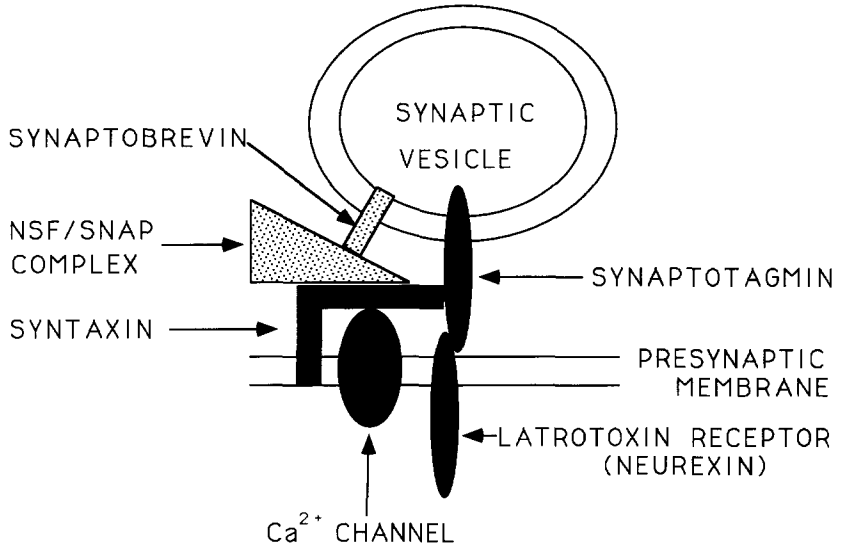

Fig. 4. Model of the interactions between presynaptic proteins of the synaptosecretosome. The components of the complex shown in black include the α-Ltx receptor (neurexin), synaptotagmin, syntaxin and the ω-conotoxin binding Ca^{2+} channel. Accordingly, we propose that binding of neurexin and syntaxin is mediated by synaptotagmin. The exact organization of the ternary complex depicted is likely oversimplified as both synaptotagmin and syntaxin have been proposed to exist as tetrameric structures [14,28]. Also shown is the ubiquitous 20S fusion complex containing NSF/SNAPs that is thought to contribute to the specificity of vesicle–plasma membrane interaction through binding to syntaxin and synaptobrevin, which act as the receptors for this complex in the presynaptic plasma and ssv membranes, respectively [17].

sites, i.e presynaptic Ca^{2+} channels [11–15]. This prompted us to investigate using immunoprecipitation whether neurexin could be found in a complex with ω-Ctx binding sites. In agreement with previous studies [14], the syntaxin antibody efficiently precipitated ω-Ctx binding sites from solubilized extracts. In addition, neurexin antibodies were able to precipitate a smaller but clearly significant portion of the ω-Ctx binding. This indicates that at least some Ca^{2+} channels are complexed with neurexin.

These data suggest that neurexin, synaptotagmin and syntaxin are organized in a complex that is associated with the Ca^{2+} channel. As indicated above both the synaptotagmin/neurexin and the synaptotagmin/syntaxin interaction are disrupted by high salt concentrations [5,11,20,27]; therefore we eluted an α-Ltx column loaded with crude rat brain extract with a gradient of increasing KCl concentration in the presence of Ca^{2+} before applying elution with a high salt/EDTA buffer. Apart from the neurexins the other proteins associated with the column began to elute at early stages of the salt gradient (Fig. 3a). The slightly earlier elution of synaptotagmin as compared to the 72 and 45 kDa proteins is consistent with previous results. Although the peak of neurexin was eluted by high salt/EDTA, some of the neurexin protein was found in the fractions of the salt gradient. This prevented the complete separation of neurexin and synaptotagmin that has been described previously using such a 2-step elution [5,27]. Protein bands of a molecular weight corresponding to syntaxin, while stained poorly with silver, co-eluted with synaptotagmin. This result was confirmed by immunoblotting, which shows that the peak syntaxin and synaptotagmin immunoreactivities elute together. This data does not rule out a direct interaction between syntaxin and neurexin, but along with previous results (see section 1) leads us to conclude that a proportion of neurexin, synaptotagmin, syntaxin and the ω-Ctx binding sites, by sequential associations, lie within a ternary complex, which we propose to call the 'synaptosecretosome' (Fig. 4).

Syntaxin has recently been implicated as a receptor for fusion competent ssvs [17]. Vesicle binding has been proposed to involve the ssv protein synaptobrevin and the fusion complex which contains NSF and SNAP proteins [17]. In this regard it is interesting to note that synaptobrevin immunoreactivity was not found in the syntaxin containing fractions that eluted from the α-Ltx column. However, we cannot exclude the possibility that the minor bands at approximately 36 kDa contain SNAP proteins, which have been shown to co-migrate with syntaxin on SDS-polyacrylamide gels [17].

In conclusion, this study extends the notion that components of the presynaptic plasma and ssv membranes can exist as a complex.Within this complex, the synaptosecretosome (Fig. 4), we find the calcium channel; synaptotagmin, a calcium sensor [28] which has been directly implicated in the control of fast transmitter release [25]; and both syntaxin and the neurexins, molecules which qualify as potential acceptor sites for synaptic vesicles [5,8,11,17]. The fast time course of transmitter release implies the predocking of ssvs at the site of the calcium influx that triggers the release process. Therefore, the in vitro interactions described here provide a seductive working model of potential compo-

nents involved in the organization of the active zone. Further approaches will be required to establish how these molecules and the interactions they show with each other control and/or modulate neurotransmitter release.

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