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Molecular Profiling of Synaptic Vesicle Docking Sites Reveals Novel Proteins but Few Differences between Glutamatergic and GABAergic Synapses

Janina Boyken,[1] Mads Grønborg,[1,2,5] Dietmar Riedel,[3] Henning Urlaub,[2,4] Reinhard Jahn,[1,*] and John Jia En Chua[1]
[1]Department of Neurobiology
[2]Bioanalytical Mass Spectrometry Group
[3]Electron Microscopy Group
Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany
[4]Bioanalytics, Department of Clinical Chemistry, University Medical Center Göttingen, 37075 Göttingen, Germany
[5]Hagedorn Research Institute, Niels Steensensvej 1, DK-2820 Gentofte, Denmark
*Correspondence: rjahn@gwdg.de
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SUMMARY

Neurotransmission involves calcium-triggered fusion of docked synaptic vesicles at specialized presynaptic release sites. While many of the participating proteins have been identified, the molecular composition of these sites has not been characterized comprehensively. Here, we report a procedure to biochemically isolate fractions highly enriched in docked synaptic vesicles. The fraction is largely free of postsynaptic proteins and most other organelles while containing most known synaptic vesicle and active zone proteins. Numerous presynaptic transmembrane proteins were also identified, together with over 30 uncharacterized proteins, many of which are evolutionarily conserved. Quantitative proteomic comparison of glutamate- and GABA-specific docking complexes revealed that, except of neurotransmitter-specific enzymes and transporters, only few proteins were selectively enriched in either fraction. We conclude that the core machinery involved in vesicle docking and exocytosis does not show compositional differences between the two types of synapses.

INTRODUCTION

Synaptic transmission initiates when an intracellular influx of Ca^{2+} triggers release of neurotransmitters from presynaptic nerve terminals, which is mediated by exocytosis of synaptic vesicles (Chua et al., 2010). Prior to exocytosis, a fraction of SVs is specifically docked to an electron dense region of the presynaptic plasma membrane, termed active zone, and activated by additional steps referred to as priming (Gray, 1963).

During the past decade, major progress was made in unraveling the molecular composition of the exocytotic apparatus. Synaptic vesicles are among its best characterized components. They contain a set of conserved membrane proteins functioning both in vesicle membrane trafficking and neurotransmitter uptake and storage (Burré et al., 2006; Takamori et al., 2006). In addition, several protein constituents of active zones have been characterized including evolutionarily conserved proteins such as Rab3-interacting molecule (RIM) proteins, RIM-binding proteins, Munc13, Liprin-α, CASK, and ELKS/Rab6-interacting/CAST (ERC) members, and less well-conserved proteins such as Bassoon and Piccolo/Aczonin. These proteins are thought to form a cytoplasmic scaffold that organizes exocytotic sites and connects synaptic vesicles with the presynaptic plasma membrane, with RIM probably functioning as a central organizer (Südhof, 2012).

The presynaptic plasma membrane at the active zone contains the core components of the exocytotic fusion machinery including the SNAREs syntaxin 1 and SNAP25, and probably the accessory proteins Munc18 and complexin. Furthermore, both functional and structural studies have shown that the voltage-gated Ca^{2+}-channels responsible for triggering neurotransmitter release by gating calcium entry are concentrated at the docking sites (Catterall and Few, 2008). In addition, the presynaptic plasma membrane contains a diverse array of membrane proteins that govern presynaptic function including ion pumps, neurotransmitter transporters, ion channels, receptors and signaling complexes but it is not known whether these proteins are also concentrated in the vicinity of active zones. Finally, the presynaptic plasma membrane contains neuronal cell adhesion molecules such as neurexins, N-CAM, ephrins and SynCAMs that connect the presynaptic with the postsynaptic membrane and thus are expected to be concentrated at these contact areas (Bukalo and Dityatev, 2012).

Despite such progress, we still have only incomplete knowledge about the molecular composition of SV docking complexes containing the active zone and the associated areas of the presynaptic plasma membrane. Presently, it cannot be excluded that, in spite of dedicated searches, major functionally relevant components are still missing. The main reason for this deficiency is that, in contrast to synaptic vesicles, it has been very difficult to isolate such docking complexes at purity sufficient for identifying specific components and discriminating them from copurifying

contaminants. Protocols for the purification of postsynaptic densities (PSD) date back to the late sixties and early seventies of the last century and usually involve detergent treatment that removes all membrane proteins not directly connected to the protein scaffolds (Carlin et al., 1980; Davis and Bloom, 1973; Fiszer and Robertis, 1967). These preparations were instrumental in the identification of major PSD components, but it was only appreciated later that these detergent-insoluble "PSD"-fractions also contain most of the components of the presynaptic active zone (Langnaese et al., 1996). Indeed, the tight connection between the pre- and postsynaptic membrane and the adjacent protein scaffolds (active zone on the presynaptic side, postsynaptic density on the postsynaptic side) has proven to be a major obstacle in purifying presynaptic membranes.

In recent years, the availability of sensitive mass spectrometry proteomics has prompted a renewed interest in unraveling pre- and postsynaptic proteomes. Several groups used high-resolution proteomics to analyze the protein composition of PSD fractions. As result, a comprehensive, and in part quantitative, description of the PSD and the associated postsynaptic membrane receptors was achieved, which contains several hundred different proteins (Li et al., 2004; Peng et al., 2004). A comparable analysis of the presynaptic active zone and the plasma membrane has proven to be more difficult. Several attempts were made to separate pre- from postsynaptic membranes. The protocols include differential extraction at different pH values in the presence of detergent (Abul-Husn et al., 2009; Phillips et al., 2005; Phillips et al., 2001) and treatment of isolated nerve terminals (synaptosomes) with urea (Berninghausen et al., 2007) to dissociate transsynaptic adhesion complexes. However, neither procedure achieved an efficient separation. Furthermore, contaminating organelles were not removed by conventional subcellular fractionation. To overcome the latter problem, Morciano and colleagues introduced an immunoisolation step using antibodies specific for synaptic vesicle proteins (Morciano et al., 2005, 2009). Together, many previously known synaptic vesicle or presynaptic membrane proteins were identified in these studies, together with a few hitherto unknown proteins. However, none of the proteomic studies is likely to be comprehensive since most of the known constituents of active zones were not detected. Most likely this is due to sample complexity, highlighting the need for improving the purity of the presynaptic docking sites.

In the present study, we developed a procedure for the isolation of a subcellular fraction highly enriched in vesicles docked to active zones, henceforth referred to as docked synaptic vesicle fraction. The key step involves mild proteolysis of synaptosomes, resulting in the dissociation of the pre- and postsynaptic membranes. During this step, all proteins are accessible to the protease except those protected by an intact membrane such as the interior of synaptosomes containing synaptic vesicles and docking complexes. After hypotonic lysis, free synaptic vesicles are separated from docking complexes by gradient centrifugation followed by immunoisolation, i.e., a procedure similar to that employed by Morciano and colleagues. Our results show that an almost complete removal of postsynaptic components is achieved. Furthermore, with the exception of mitochondria, proteins from other organelles were largely absent indicating that the degree of contamination is low. In this fraction, we identified not only virtually all presently known proteins of the active zone but also numerous ion channels and transporters expected to be present in the presynaptic plasma membrane. In addition, more than two dozen hitherto uncharacterized proteins were identified. By slightly modifying the procedure, we were able to compare docking complexes specific for glutamatergic and GABAergic synapses, respectively. Surprisingly, this revealed only few differences in their protein composition, suggesting that the machinery responsible for docking and fusion is largely identical in glutamatergic versus GABAergic synapses.

RESULTS

Isolation of Synaptic Vesicle Docking Complexes

To isolate docking complexes from rat brain, we first prepared synaptosomes and subjected them to limited proteolysis to dissociate pre- and postsynaptic membranes (Figure 1A). Synaptosomes represent isolated nerve terminals that resealed during homogenization. Thus, the presynaptic compartment (including the release apparatus) should remain protected from proteolysis, with only external proteins and protein domains being degraded. Indeed, presynaptic components including both active zone and synaptic vesicle proteins remained intact after limited proteolysis whereas cell adhesion molecules and plasma membrane resident neurotransmitter receptors were cleaved (Figure 1B). Intriguingly, PSD95/SAP90 and Homer1, two PSD scaffolding proteins, were not measurably degraded, suggesting at least partial resistance of the PSD network to proteolytic degradation (Figure 1B).

To investigate whether the pre- and postsynaptic compartment were dissociated following proteolysis, both untreated and trypsin-treated synaptosomes were separated by continuous sucrose density gradient centrifugation, followed by immunoblot analysis of the gradient fractions. In untreated samples, pre- and postsynaptic marker proteins comigrated as expected. In contrast, PSD95 was shifted toward a position of higher density in the trypsin-treated samples demonstrating an at least partial separation of the PSD from the presynaptic compartment (Figure 2A). To confirm that an effective dissociation of pre- and postsynaptic protein complexes is achieved, we analyzed the distribution of pre- and postsynaptic markers by immunofluorescence microscopy after immobilizing the purified synaptosomes on glass surfaces. Whereas untreated samples exhibit a very high degree of colocalization between synaptophysin and PSD95, very little colocalization was observable in protease-treated samples (Figure 2B). Additionally, PSD95 intensities of the treated samples also significantly decreased. To confirm that the interior of the nerve terminals was structurally intact following trypsinization, we analyzed protease-treated samples by electron microscopy, revealing an intact morphology (see Figure S1).

Next, protease-treated synaptosomes were lysed by osmotic shock to release their cytoplasmic constituents, including non-docked synaptic vesicles. The sample was then fractionated on a 0.4–1.4 M continuous sucrose gradient. Synaptic vesicle markers exhibited two distinct peaks at positions of significantly lower and higher buoyant densities that likely represent free

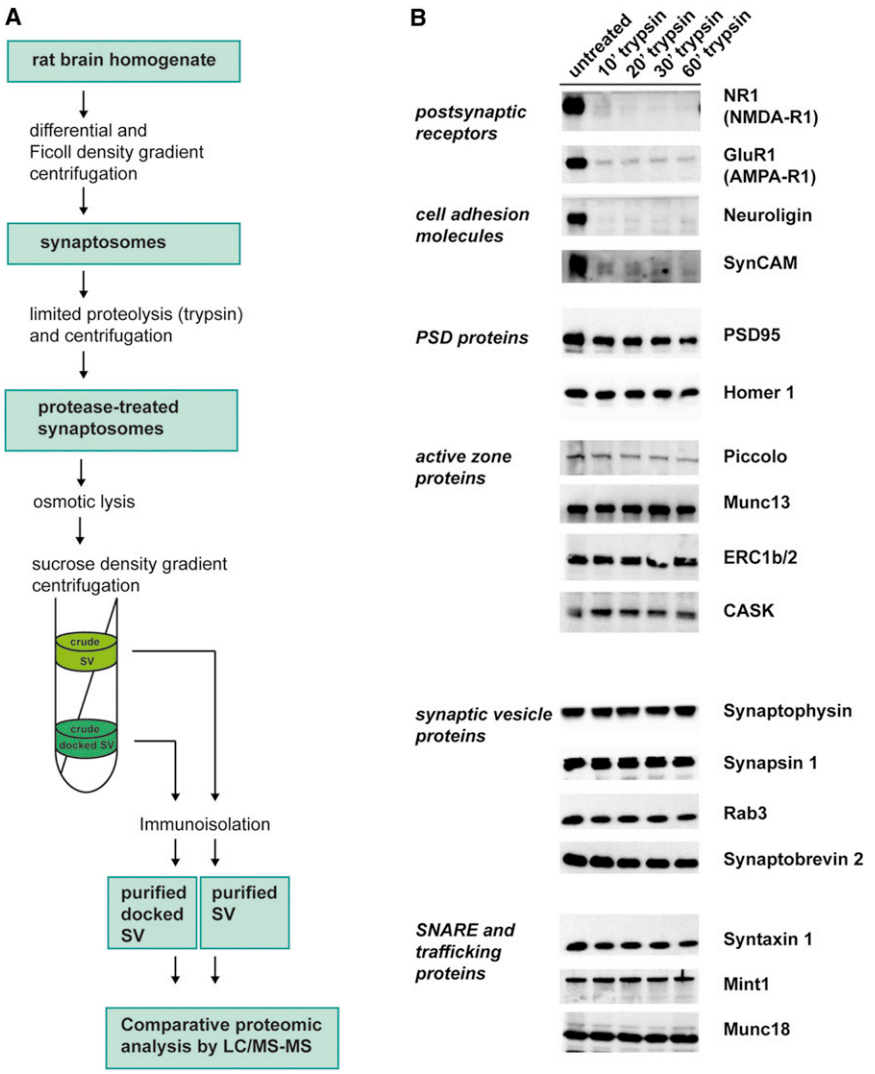


Figure 1. Limited Proteolysis Removes Postsynaptic but Not Presynaptic Proteins from Purified Synaptosomes

(A) Overview of the isolation protocol for vesicle docking complexes from nerve terminals. See text for details.

(B) Proteins inside nerve terminals are protected whereas extracellular and postsynaptic proteins are proteolysed during trypsinization of synaptosomes. Synaptosomes were incubated with trypsin at 30°C for the times indicated (untreated: 30°C for 60 min without trypsin) and analyzed by immunoblotting for selected pre- and postsynaptic proteins. Note that the PSD proteins Homer 1 and PSD95 are largely resistant to trypsinization.

(non-docked) and docked synaptic vesicles, respectively (Figure 3A; see also, e.g., Morciano et al., 2005). Indeed, Munc13, a component of the active zone, was only found in the docked synaptic vesicle fraction (Figure 3A).

For further purification of docked synaptic vesicles, we carried out immunoisolation using nonporous microbeads covalently coated with antibodies against the synaptic vesicle protein synaptophysin. For comparison, a parallel immunoisolation step was carried out using the fraction containing free synaptic vesicles. Analysis of the immunoisolates for marker proteins revealed that the docked synaptic vesicle fraction contains both components of the active zone (Munc13) and of the presynaptic plasma membrane (Na^+/K^+-ATPase), both of which were absent from the free synaptic vesicle fraction and from immunoisolates using control IgG (Figure 3B). Mitochondrial proteins such as the succinate dehydrogenase complex subunit A (SDHA) that comigrated with docked synaptic vesicles on the gradient were not removed during immunoisolation raising the possibility that some mitochondria might be physically attached to the presynaptic plasma membrane and/or the active zone. To check for this possibility, aliquots of the gradient fractions enriched in docked synaptic vesicles were immobilized on coverslips and immunostained for mitochondria and presynaptic markers. No significant overlap was detected (Figure S2).

Quantitative Proteomic Comparison of Free Synaptic Vesicles and Docking Complexes

To identify proteins specifically associated with docking complexes, we carried out a quantitative proteomic comparison of the immunoisolated free and docked synaptic vesicle fraction using isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) in combination with tandem mass spectrometry (LC-MS/MS). In this case, free and docked synaptic vesicle immunoisolates were digested by trypsin, followed by labeling of the resulting peptides with isobaric tags of *m/z 116* and *m/z 117*, respectively. These tags are chemically identical but give rise to reporter ions of different mass during fragmentation in the MS/MS analysis, allowing for direct quantitative comparison. Both samples were combined after the labeling; the resulting peptides mixture prefractionated by SCX chromatography and analyzed by LC-MS/MS (see Figure 1B and Grønborg et al., 2010). A complete list of all proteins quantified/identified is shown in Table S1.

In total, 493 proteins were identified from both fractions. As expected, a substantial portion (224 proteins) is of mitochondrial origin as classified by the MitoCarta and NCBI protein databases (Pagliarini et al., 2008). Of the remaining proteins, the largest fraction includes constituents of synaptic vesicles and proteins involved in exocytosis and recycling of synaptic vesicles (Figure 4). In addition, components of the active zone and the presynaptic plasma membrane, neuronal cell adhesion molecules, and components of the cytoskeleton were identified. Noteworthy, only three exclusively postsynaptic proteins

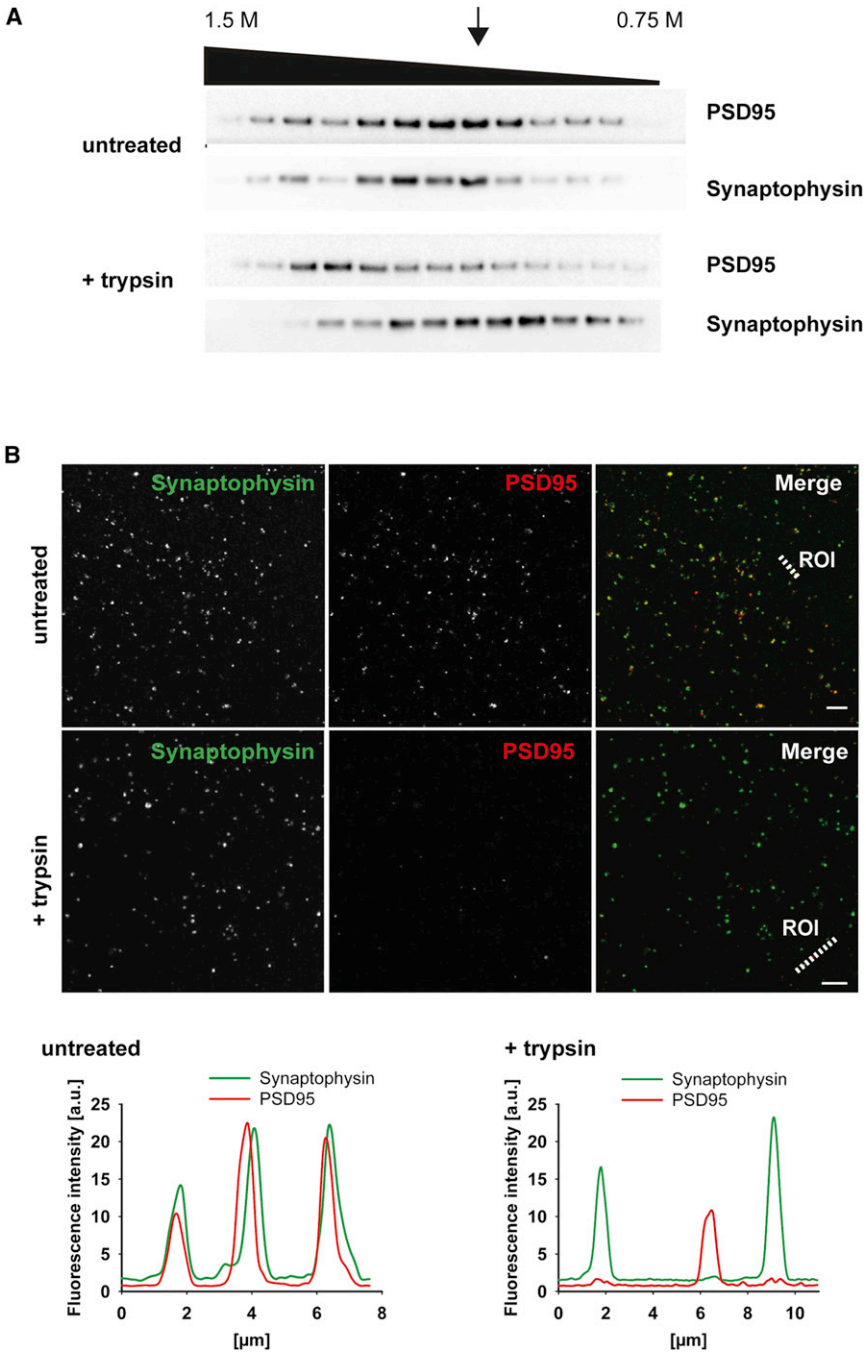


Figure 2. Limited Proteolysis of Synaptosomes Results in an Efficient Dissociation of Pre- and Postsynaptic Membranes

(A) Untreated and protease-treated synaptosomes were fractionated on a continuous sucrose gradient. The gradient fractions were probed by immunoblotting for the presynaptic marker synaptophysin and the postsynaptic marker PSD95. Whereas both proteins tightly co-migrate in untreated synaptosomes, PSD95 and synaptophysin peak fractions distinctively shift to heavier and lighter fractions, respectively, after protease treatment indicative of physical separation.

(B) Gradient fractions of both treated and untreated synaptosomes corresponding to the fraction marked by an arrow in (A) (approx. 1.2 M sucrose) were pelleted on coverslips, fixed and immunostained for synaptophysin and PSD95 or analyzed by electron microscopy (see Figure S1). Synaptophysin and PSD95 puncta strongly colocalize in untreated samples. In agreement with the immunoblot analysis of the fractions in (A), immunoreactivity for PSD95 was markedly reduced in the treated sample. Importantly, the remaining PSD95-positive puncta showed almost no overlap with synaptophysin confirming the efficient separation of the pre- and postsynaptic membranes. Scale bars, 5 μM.

(PSD95, SynGAP1, kalirin) were detected among the 493 proteins identified along with a few proteins from other organelles (see Table S1). We also performed functional and disease association analyses using the Ingenuity Pathways Analyses (IPA) software (Ingenuity Systems; www.ingenuity.com) to determine if synaptically relevant clusters of proteins were enriched in our preparation. Using a cutoff of $p < 0.01$ and a minimum protein cluster size of 7, we indeed observed that a significant number of proteins were associated with key synaptic neurotransmission processes (Table S2). In addition, many of the proteins identified were linked to neurological disorders (Table S3).

Unsurprisingly, synaptic vesicle proteins (Takamori et al., 2006) constituted the largest group of proteins in the docked synaptic vesicle fraction (Figure 4). We reasoned that the amount of integral synaptic proteins (which are present in both fractions) can be used as an internal reference standard to normalize the iTRAQ ratios and thus standardize between different experiments (see Supplemental Experimental Procedures for details). Proteins varying from this normal ratio can then be identified as being enriched or absent in one fraction as compared to the other. As predicted, all synaptic vesicle proteins showed approximately the same ratio between the free and docked synaptic vesicle fractions (close to 1:1 ratio of the reporter ions *m/z 117* and *m/z 116*), thus documenting the accuracy of our iTRAQ quantification (Figure 5). Most other proteins were either not detectable in the free synaptic vesicle fraction or at least highly enriched in the docked synaptic vesicle fraction. These include the major known proteins of the active zone such as Piccolo, Liprin-α, Bassoon, RIM1, CASK, and ERC2, and a large group of presynaptic ion channels, transporters, and signaling molecules. For instance, various subunits of voltage-gated calcium channels, the BK channel KCNMA1 which localizes at presynaptic terminals (Hu et al., 2001; Knaus et al., 1996), the

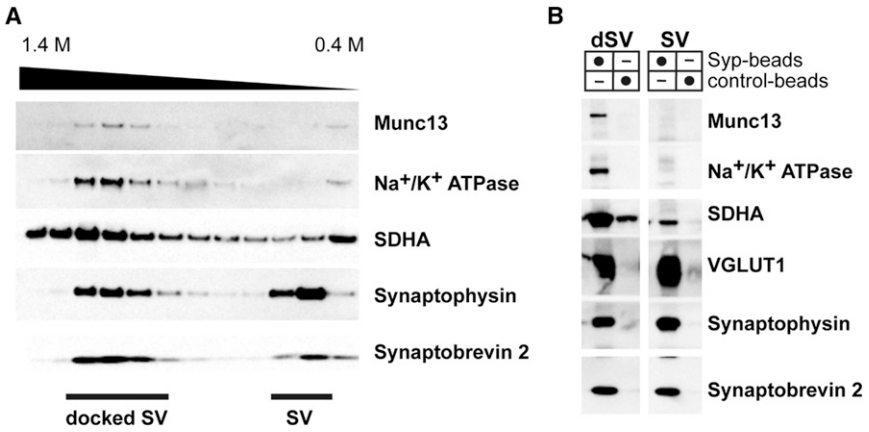


Figure 3. Immunoisolation from Gradient Purified Samples of Protease-Treated Synaptosomes Lysed by Osmotic Shock Enables the Enrichment of Highly Purified Docked and Free Synaptic Vesicle Fractions

(A) Immunoblot analysis of sucrose density gradient fractions from protease-treated osmotically lysed synaptosomes yields two peaks of synaptic vesicle proteins. The peak at higher density containing plasma membrane (Na^+/K^+-ATPase) and active zone (Munc13) components corresponds to the docked synaptic vesicle fraction. Mitochondria, monitored here using succinate dehydrogenase complex subunit A (SDHA) as a marker, also co-migrate in this fraction (see also Figure S2). The second lighter peak containing mainly synaptic vesicle proteins corresponds to the free synaptic vesicle fraction.

(B) Immunoisolation using synaptophysin (Syp) antibody-conjugated microbeads of the pooled peak fractions obtained from density gradients in (A) yields docked synaptic vesicle (dSV) and free synaptic vesicle (SV) fractions of high purity. An appreciable amount of mitochondria, monitored here using succinate dehydrogenase complex subunit A (SDHA) as marker, co-purifies with docked synaptic vesicle fractions even after the immunoisolation step.

hyperpolarization-activated cyclic nucleotide-gated potassium channel (HCN1) known to be present at active zones (Huang et al., 2011) were identified. Furthermore, the docked synaptic vesicle fraction contains the plasma membrane Ca^{2+}-ATPases (PMCA1 and 2) and the Na^+/Ca^{2+} exchanger NCX2 that together maintain synaptic calcium homeostasis and an array of neurotransmitter transporters such as the glutamate transporters EAAT1, EAAT2 and the GABA transporter GAT1. While the former are known to be mainly present in glia cells, the notable absence of most other abundant glial proteins suggests that the proteins are also localized to the presynaptic plasma membrane, in agreement with previous reports (Chaudhry et al., 1995; Rose et al., 2009).

Interestingly, the plasma membrane Q-SNAREs syntaxin 1 and SNAP25 were only enriched 3- to 6-fold in the docked synaptic vesicle fraction. This reflects the presence of a sizeable pool of these SNAREs in the membrane of synaptic vesicles (Walch-Solimena et al., 1995; Takamori et al., 2006). In addition, many trafficking proteins were identified that shuttle between the cytoplasm and the membrane during the synaptic vesicle cycle such as complexin, Munc18, N-ethylmaleimide-sensitive factor (NSF), Rab-GTPases, and other endocytosis-related proteins. These proteins were detected in both free and docked synaptic vesicles at variable ratios. It cannot be excluded that the levels of these proteins are altered due to adsorption or dissociation during isolation of the fractions (see e.g., Pavlos et al., 2010). The same applies to cytoskeletal components identified in our fractions. Among these are components of the actin and microtubule cytoskeleton, of the spectrin-based membrane skeleton, and septins (Figure 6). Septins have been previously localized to presynaptic membranes and suggested to be involved in positioning SVs at the active zone (Beites et al., 2005; Xue et al., 2004).

Finally, 30 hitherto uncharacterized proteins were detected (Table S4). Of these, many contain predicted transmembrane domains and thus probably are integral membrane proteins. Considering that the majority of the characterized proteins (particularly the membrane proteins) are bona fide synaptic components, it is likely that many of the unknown proteins are associated with the presynaptic membrane. Several of these appear to be conserved during evolution and preliminary characterization of few selected proteins indeed suggests enrichment in synapses.

Proteomic Comparison of Glutamatergic and GABAergic Docking Complexes

We previously showed that glutamatergic and GABAergic synaptic vesicles exhibit only few differences in their protein composition (Grønborg et al., 2010). On the other hand, the postsynaptic signaling complex is profoundly different between glutamatergic and GABAergic synapses involving distinct receptors, scaffolding proteins and even transsynaptic adhesion molecules (Craig et al., 1996; Varoqueaux et al., 2004). Since only scant information is available about transmitter-specific presynaptic proteins except of those involved in transmitter synthesis and transport, we have employed our protocol to obtain docked synaptic vesicle fractions from glutamatergic and GABAergic synaptosomes, respectively, in order to compare their protein composition.

For immunoisolation of glutamatergic and GABAergic docked synaptic vesicle fractions, we have taken advantage of the fact that the two vesicular transporters VGLUT1 and VGAT are specifically associated with glutamatergic and GABAergic nerve terminals in the brain, with virtually no overlap (Takamori et al., 2000a, 2001). For confirmation, we immunostained our protease-treated synaptosomes for VGLUT1 and VGAT. As expected, no significant overlap was detectable (Figures 7A and 7B). Next, we immunoisolated docked synaptic vesicle fractions as described above but using beads coated with antibodies specific for VGLUT1 and VGAT, respectively. Immunoblot analysis confirmed that the fractions were highly enriched for VGLUT1 or VGAT, respectively, with only a low degree of cross-contamination (Figure 7C).

Next, we compared the proteomes of glutamatergic and GABAergic docking complexes using iTRAQ labeling as described above. The recovery of proteins suitable for quantification was lower than in the experiments described above

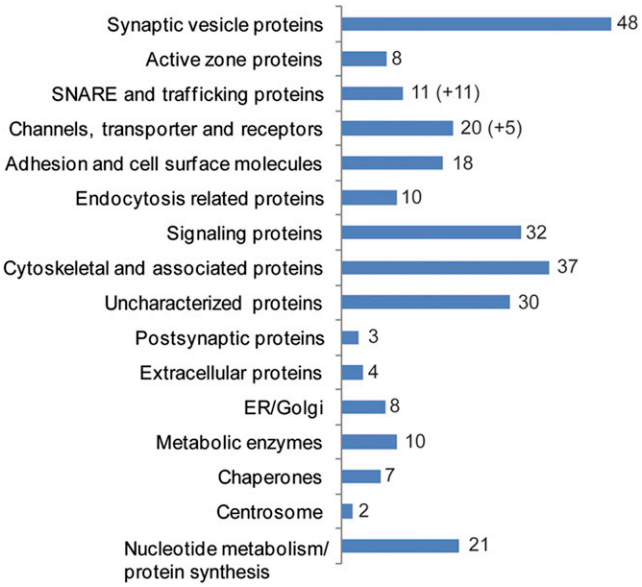


Figure 4. Classification of Proteins in the Docked Synaptic Vesicle Fraction Identified by iTRAQ Analysis According to Function

The number in parentheses indicates proteins that were assigned to more than one category (synaptic vesicle specific transporters and trafficking proteins). Note that proteins assigned as originating from the mitochondria according to the NCBI and MitoCarta databases are not included here (see text). See also Tables S1, S2, and S3.

(probably due to lower yields): 307 proteins were quantified, with 161 of them originating from mitochondria (Table S5). Here, we only included proteins that were identified in at least two of three independent experiments. Of these, 260 proteins were identical to those identified in the docked synaptic vesicle fraction described above (85%). Most of the remaining 47 proteins appear to be contaminants except of 7 that mostly include new subunits or isoforms of synaptic proteins already identified above (not shown). Due to a higher variability in the ratios we only counted proteins as specifically enriched in glutamatergic and GABAergic docking complexes if the ratio was $\geq$3, which still gives a sufficient safety margin when considering that the ratios of VGLUT1/VGAT and VGAT/VGLUT1 in the corresponding immunoisolates were 9.3 and 8.2, respectively.

Surprisingly, only few proteins were found to be specifically enriched in either of the fractions (Figure 7D). In glutamatergic docking complexes these include the SV proteins SV2B, SV31, ZnT3, and MAL2, which is in agreement with our previous study (Grønborg et al., 2010) and provides a positive control for the method. Two additionally enriched proteins, Ca^{2+}-calmodulin-dependent protein kinase II alpha subunit (CAMKIIα) and the glycoprotein M6a, were previously reported to be specific for excitatory neurons (Benson et al., 1992; Cooper et al., 2008; Jones et al., 1994). Furthermore, significant enrichment was also observed for the active zone protein Bassoon and for GAP43, a well-characterized membrane protein associated with neuronal growth cones (Skene et al., 1986). Bassoon was previously shown to be present in both excitatory and inhibitory synapses (Richter et al., 1999). Finally, the list includes proteins where the significance of the enrichment is unclear including components of the complement system and a mitochondrial calcium transporter. Intriguingly, EAAT2, the major transporter responsible for the re-uptake of glutamate from the synaptic cleft, was not significantly enriched in glutamatergic docking complexes, suggesting that this transporter is present in both types of nerve terminals.

Less is known about the few proteins specifically enriched in GABAergic docking complexes except of those involved in GABA transport (VGAT) and GABA metabolism (ABAT). Slc35F5 is an orphan transporter that is related to a family of transporters specific for nucleotide-activated sugars. Most of the other proteins have hitherto not been well characterized. QN1 homolog appears to have a widespread distribution while LRRCC1 was reported to operate in spindle pole organization during mitosis (Muto et al., 2008). More information is required to assess whether these proteins are specifically localized to GABAergic synapses. Unfortunately, reliable peptide quantification of the GABA transporter GAT1 was not possible since it was only detected only in one of the three replicates with few peptides.

To verify the preferential localization of some of these proteins by an independent approach, we analyzed their association with glutamatergic and GABAergic synaptosomes using immunocytochemistry (Figure 8A). As before, we used synaptosomes pretreated with trypsin (see above) to exclude any postsynaptic contribution. Colocalization with either VGLUT1 or VGAT was considered when the center of intensity in the two channels was within a distance of 200 nm (see Experimental Procedures for details). Exemplary images and line scans are shown in Figure 8A. Synaptobrevin 2, the ubiquitous R-SNARE of all synaptic vesicles, colocalizes equally well with both vesicular transporters, serving as positive control. In contrast, GAP43 is preferentially associated with VGLUT1-positive synaptosomes. Quantification of several additional proteins yielded results that were in very good agreement with the results obtained by iTRAQ quantification, thus confirming the enrichment of GAP43 and CAMKIIα in glutamatergic synapses (Figure 8B). We also included glutamate decarboxylase 2 (GAD2), the GABA-synthesizing enzyme that was not detected in the MS analysis (probably washed out during isolation of the docking complexes). As expected, GAD shows a strong preference for VGAT-containing synaptosomes although a significant fraction of VGLUT1-positive synaptosomes also contained this enzyme. Intriguingly, the active zone proteins Piccolo and Munc13 did not show significant differences in selecting for either synapse types (Figure 8B). For the Piccolo-related scaffolding protein Bassoon, we observed a smaller but significant increase in the extent of colocalization with VGLUT1 versus VGAT (74% versus 46%), again confirming the data obtained with the iTRAQ quantification.

DISCUSSION

Docking, priming, and exocytosis of synaptic vesicles are governed by molecular machines containing multiple proteins and occur at specialized release sites at the presynaptic membrane. Using a purification protocol, we have characterized the protein composition of these release sites, resulting in a comprehensive

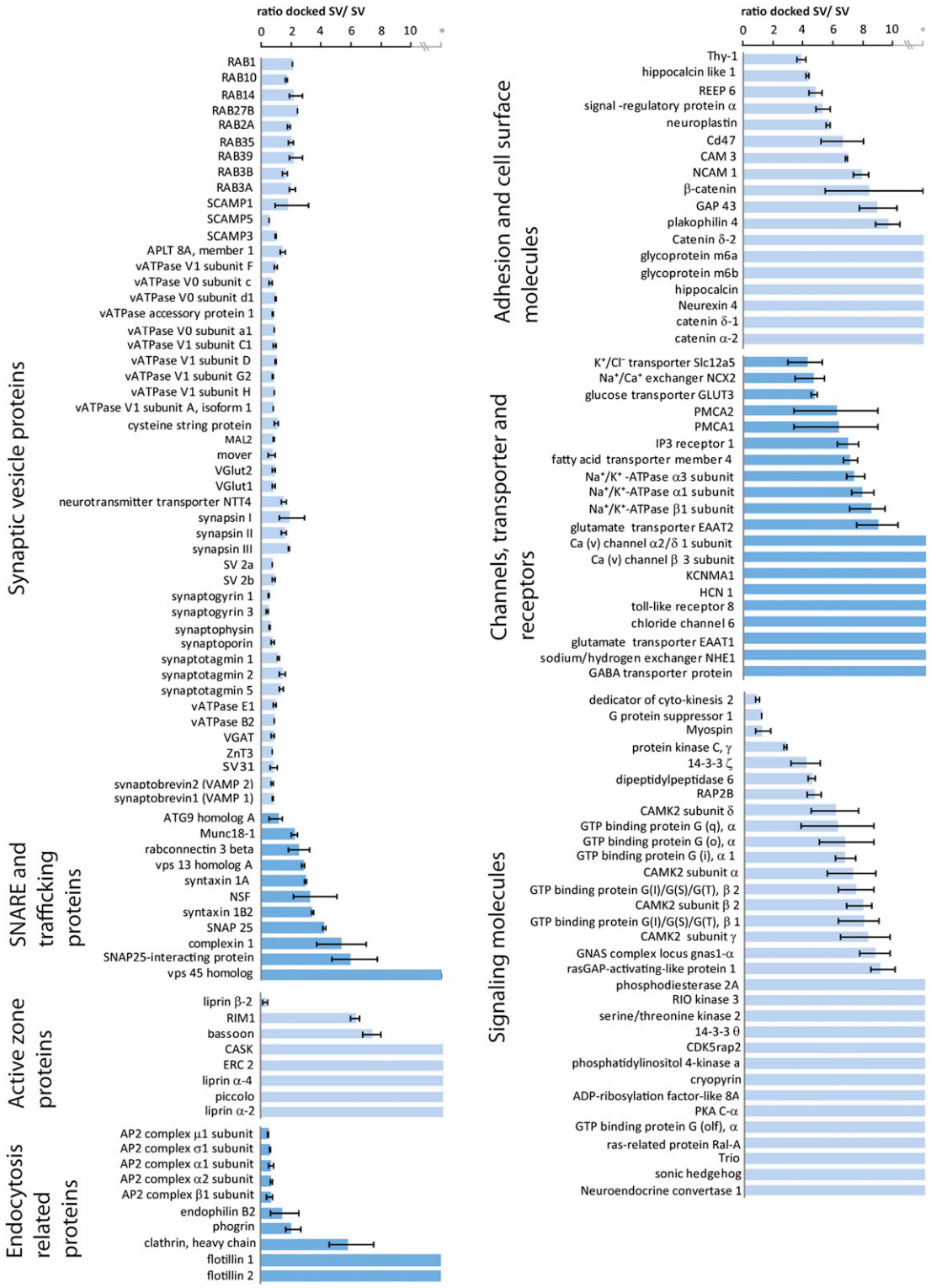


Figure 5. iTRAQ Quantification of Proteins Identified from Free and Docked Synaptic Vesicle Fractions by Mass Spectrometry

Data here shows the average of three independent biological replicates. Only proteins identified in at least two of the independent experiments are listed. Ratios of proteins that were only detected in the docked SV fraction are depicted with an "*". The error bars indicate the range of data points (lowest-highest). Details concerning data normalization are described in supplemental experimental procedures and Figure S3. Proteins with an iTRAQ ratio of more than 2.5 are considered to be significantly enriched in the docked synaptic vesicle fraction.

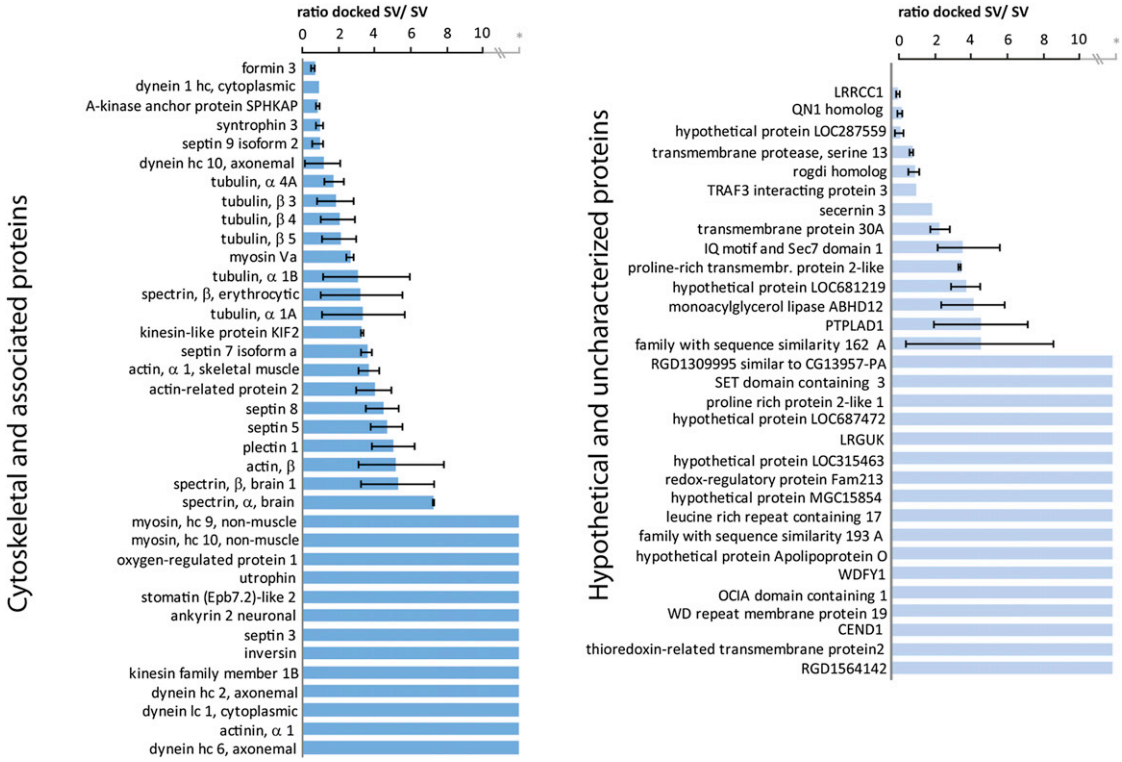


Figure 6. iTRAQ Quantification of Additional Proteins Identified from Free and Docked Synaptic Vesicle Fractions by Mass Spectrometry
Refer to Figure 5 for details. A description of novel candidates can be found in Table S4.

list of protein constituents. In addition to most known synaptic vesicle and active zone proteins, we have identified many transporters and ion channels known to operate in presynaptic function and a large number of hitherto uncharacterized proteins. Further, we have compared the proteomes of glutamatergic and GABAergic docking complexes showing that, rather intriguingly, only few quantitative differences seem to exist between glutamatergic and GABAergic docking complexes. Apparently, the molecular mechanisms underlying docking and release are largely identical between different types of synapses.

While we cannot exclude that major proteins have remained undetected, several lines of evidence suggest that we have achieved a high coverage of the docking site proteome. Foremost, all known active zone proteins (with the exception of Munc13) were identified in our mass spectrometric approach. Second, all proteins of the exocytotic machinery were recovered including the SNAREs, Munc18, complexin, and synaptotagmins. Moreover, a high coverage of the protein inventory is supported when comparing the proteins identified here with those found in the previous studies. For instance, the list of the presynaptic proteome reported by Morciano and colleagues (Morciano et al., 2009) contained 135 proteins (excluding mitochondrial proteins), 62 of which were also identified by us. Of the remaining 73 proteins, only few can be assigned to a specific presynaptic function (such as additional isoforms of membrane transporters) whereas most others are soluble proteins with general cellular functions. Similarly, the proteins that were identified by Abul-Husn and colleagues but not in our study (52 of 99 proteins) are also mostly general cellular proteins, with the exception of a group of proteins involved in clathrin-mediated endocytosis (Abul-Husn et al., 2009). We assume that soluble or only loosely membrane-associated proteins were washed off during our isolation procedure.

It needs to be borne in mind that the mild proteolysis required for separating pre- from postsynaptic membranes constitutes an inherent limitation for proteomic analysis. Thus it is not surprising that our recovery of cell adhesion molecules is somewhat lower than in the other studies (Abul-Husn et al., 2009; Morciano et al., 2009). These proteins possess only small intracellular but large extracellular domains that are expected to be degraded during the protease treatment of the synaptosomes. On the other hand, we identified a large number of plasma membrane residents documenting that the remaining intracellular regions are generally sufficient for protein identification. In this context it is notable that in neither our nor in any of the previous studies were receptors for neurotransmitters or neuromodulators found. While the function of such receptors in regulating presynaptic function is well established, many of these receptors likely function only in subsets of synapses and others may be expressed in low copy numbers, explaining why they may have escaped detection.

Intriguingly, substantial overlap was also found with the proteome of protein complexes associated with presynaptic calcium channels that were isolated by immunoprecipitation of Cav2

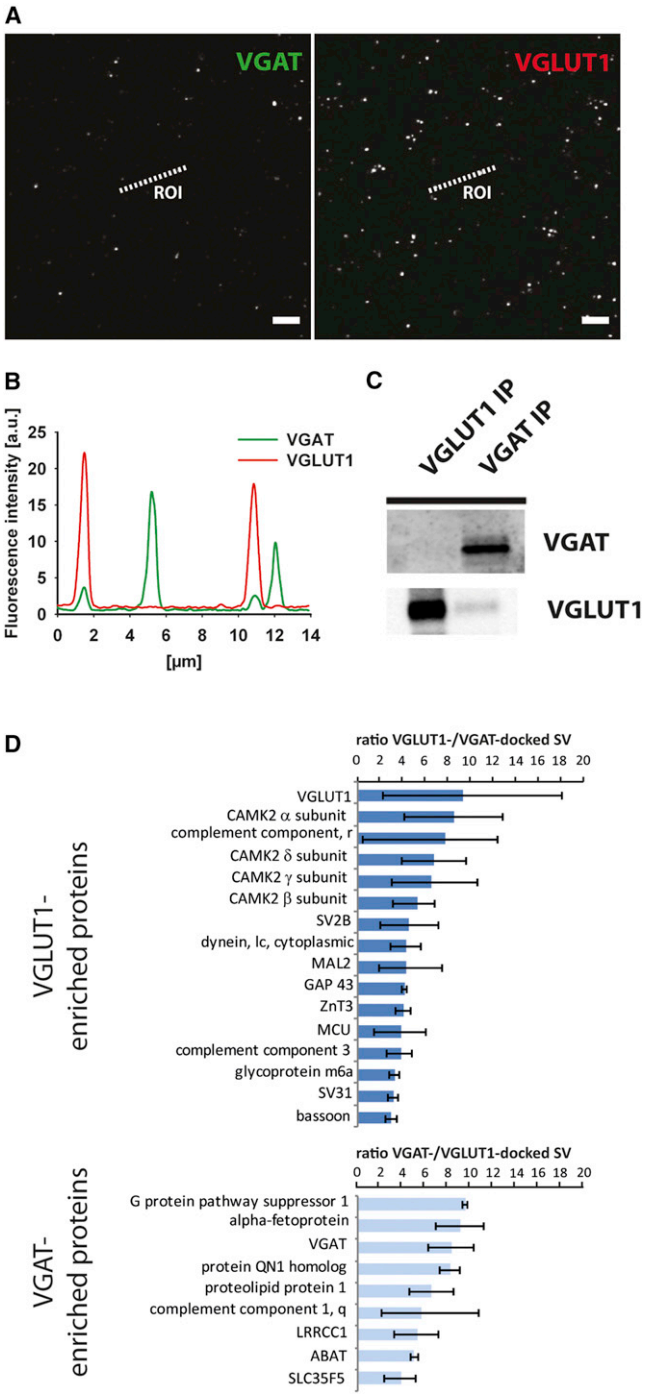


Figure 7. GABAergic and Glutamatergic Vesicle Docking Complexes Show Only Few Differences in Their Protein Composition

(A) Protease-treated synaptosomes were immunostained for the vesicular glutamate transporter 1 (VGLUT1) and the vesicular GABA transporter (VGAT). The distribution of both proteins is largely nonoverlapping (see also line scan in B), confirming that these two transporters reside in different synapse populations. Scale bars, 5 µm.

(B) Line scan of regions indicated in (A).

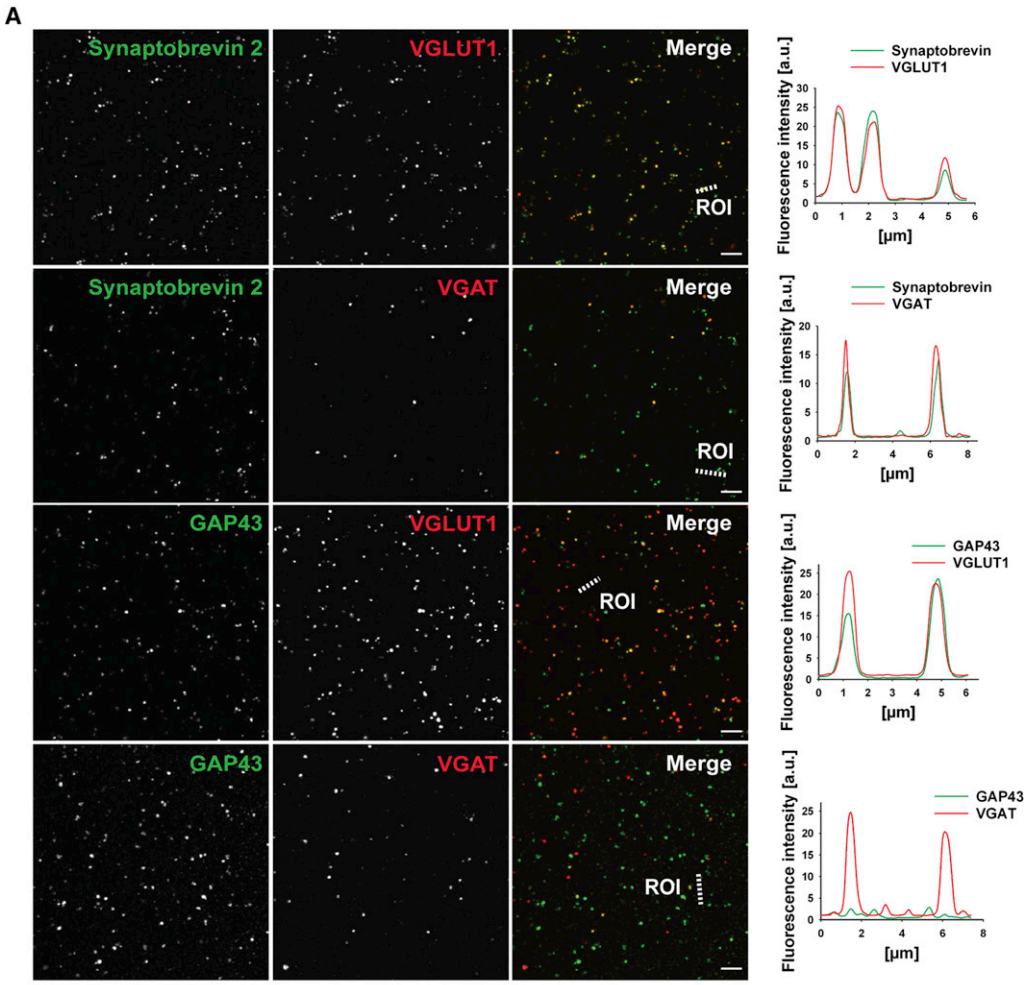
(C) Docking complexes were immunoisolated from lysed protease-treated synaptosomes using antibodies specific for VGLUT1 or VGAT, respectively, as in Figure 3B. Immunoblot analysis shows high enrichment for the relevant transporter with only a very low degree of cross-contamination between both transporters.

(D) iTRAQ quantitative comparison of the protein composition of VGLUT1- and VGAT-containing vesicle docking complexes purified from (C). Only proteins displaying more than a three-fold difference are shown. The error bars indicate the range of data points (lowest–highest). For a complete list of proteins see Table S5. Details concerning data normalization are described in supplemental experimental procedures and Figure S4.

after detergent extraction (Müller et al., 2010). In this study, 207 Cav2 channel associated proteins were identified, 48 of which are identical to those found in our docked synaptic vesicle fraction. This overlap is easily explained by the fact that Cav2 channels are physically and functionally tightly associated with exocytotic sites. Not surprisingly, the Cav2 proteome also contains many PSD proteins since in that study no separation of pre- and postsynaptic compartments was attempted.

Considering the high purity of our docked synaptic vesicle fraction, with proteins from other organelles (except mitochondria) being virtually absent, the identification of 30 hitherto uncharacterized proteins suggests that many of them are indeed constituents of the presynaptic active zone and adjacent areas. While further work will be needed to clarify which of them is involved in presynaptic function, we have used in silico-based analyses for a preliminary characterization (Table S4). Accordingly, 16 proteins possess one or more predicted transmembrane domains. Twenty-six proteins appear to be conserved between vertebrates, among these 12 are also conserved in invertebrates. Noteworthy, 18 proteins appear to be well expressed in the mammalian brain based on standardized in situ hybridization (Allen Mouse Brain Atlas, http://mouse.brain-map.org). Thus, we consider it highly probable that at least some of these proteins will turn out to be constituents of the presynaptic membrane and/or the vesicular release apparatus.

We extended our study to investigate the differences between glutamatergic versus GABAergic docking complexes by a slight modification of our original protocol. We find that, except of the transmitter-specific transporters and enzymes, only very few proteins are selectively enriched in glutamatergic and GABAergic docking complexes. These results confirm and extend our previous observation that glutamatergic and GABAergic synaptic vesicles have a largely identical protein composition. Two major conclusions can be drawn from these findings. First, the release machineries of glutamatergic and GABAergic synapses are very similar if not identical. In particular, we did not detect any major difference between the expression levels of SNAP25 and SNAP23 in the two types of synapses, arguing against a specialization of these SNAREs for glutamatergic versus GABAergic release as suggested previously (Garbelli et al., 2008; Verderio et al., 2004). Obviously, the overall similarity between the populations does not exclude major variations in the composition of the docking and release apparatus between individual synapses. However, such variations do not appear to correlate with the neurotransmitter phenotype. Second, it is only the biosynthetic enzymes and the transmitter transporters (particularly the vesicular transporters) that define the neurotransmitter phenotype of glutamatergic and GABAergic synapses.



(legend on next page)

Taken together, we have made significant progress toward the aim of establishing a "parts list" of the presynaptic docking and release machinery and of the presynaptic membrane. It is inherent to the approach that it is limited to the identification of core constituents, with less efficient coverage of proteins interacting transiently or being present in low copy numbers. Integrating the "parts" into a coherent picture explaining synaptic vesicle docking and release will be a major task for future work.

EXPERIMENTAL PROCEDURES

Antibodies

A list of antibodies used in this study can be found in the Supplemental Information.

Protease Treatment of Synaptosomes

All animal procedures used here fully comply with the guidelines as stipulated in the German Animal Welfare Act. Synaptosomes were isolated as previously described (Fischer von Mollard et al., 1991). To separate pre- and postsynaptic membranes, 3–5 mg of synaptosomes were carefully centrifuged for 3 min at 8,700 × g, 4°C. The resulting pellet was then resuspended in 20 ml of sucrose buffer (320 mM sucrose, 5 mM HEPES [pH 8]). To initiate proteolytic digestion, 300–500 μl of a trypsin stock solution (0.1 mg/ml, Roche) was added to the mixture to give a final protein-protease ratio of 100:1. Synaptosomes were incubated for 30 min at 30°C with occasional mixing. Afterward, synaptosomes were pelleted again for 3 min at 8,700 × g and protease activity was stopped by resuspending the pellet in sucrose buffer containing 400 μM Pefabloc (Roche).

Separation of Protease-Treated Synaptosomes from the Postsynaptic Density

Continuous sucrose gradients (25%–50% [w/v] sucrose in 5 mM HEPES [pH 8.0]) were generated using an automatic gradient mixer (Gradient Master, Biocomp) according to the manufacturer's instructions. Three milliliters of protease-treated synaptosomes as described previously were loaded onto each gradient and centrifuged at 180,000 × g_{max} (28,000 rpm) for 3 hr, 4°C in a SW28 swing-out rotor (Beckman). After centrifugation, 2 ml fractions were collected from the gradient from bottom to top using a pump system (Minipuls3, Abimed Gilson). Fractions containing digested synaptosomes, so called "shaved" synaptosomes, were either identified by measuring the refraction index of each fraction or by immunoblotting. Shaved synaptosomes were found in the fractions with a refraction index of 1.391–1.392, which corresponds to ~1.2 M sucrose.

Immunoisolation of Docked and Free Synaptic Vesicles

Protease-treated synaptosomes were resuspended in 300 μl sucrose buffer containing 400 μM Pefabloc (Roche). Synaptosomes were lysed by adding 2.7 ml ice-cold H_2O followed by rapid homogenization with a glass-Teflon homogenizer with three strokes at maximum speed. Fifteen microliters of 1 M HEPES [pH 8], 3 μl of 200 mM PMSF, and 3 μl of 2 mg/ml pepstatin were then immediately added to the solution. Docked and free synaptic vesicles were separated on a 15%–45% continuous sucrose gradient (w/v) by centrifugation at 100,000 × g_{max} for 1 hr, 4°C in a SW28 swing-out rotor (Beckman). Two ml fractions were collected from bottom to top.

To determine the migration of docked versus free synaptic vesicles, 2 μl from each fraction was spotted on a nitrocellulose membrane and allowed to dry for 5 min. The membrane was then blocked for 10 min at room temperature in blocking buffer (5% nonfat milk powder in TBST) and probed with monoclonal antibodies against synaptophysin (clone 7.2) for 15 min. After washing the membrane 3 times for 3 min each with blocking buffer, the blot was incubated with secondary HRP-conjugated antibody for 15 min. After another 3 washes (3 min each) with TBST, membranes were incubated with Western Lightening TM*Plus*-ECL (Perkin Elmer) and protein bands visualized by using chemiluminescence detection on a LumiImager (Boehringer Mannheim). Docked synaptic vesicles generally localized in fractions 4–7 and free synaptic vesicles in fractions 19–21. Fractions containing docked synaptic vesicles or free synaptic vesicles were respectively pooled.

For immunoisolation, immunobeads (Eupergit C1Z methacrylate microbeads; Röhm Pharmaceuticals) were coupled to monoclonal antibodies against synaptophysin (clone 7.2), VGLUT1 or VGAT as described previously (Burger et al., 1989; Takamori et al., 2000b, 2001). For each immunoisolation, 5 μl of antibody-conjugated immunobeads were washed with 1× IP buffer (1× PBS, 3 mg/ml BSA, 5 mM HEPES [pH 8.0]). For the isolation of docked synaptic vesicles, 600 μl docked SV fraction and 600 μl 2× IP buffer (2× PBS, 6 mg/ml BSA, 5 mM HEPES [pH 8.0]) were mixed and added to the immunobeads. For the isolation of free synaptic vesicles, 300 μl SV fractions were mixed with 900 μl 1× IP buffer and introduced to the immunobeads. Following overnight incubation at 4°C, beads were centrifuged for 3 min at 300 × g_{max} (2,000 rpm) in a tabletop centrifuge and then washed three times with PBS by vortexing, incubation on ice for 5 min, and centrifugation for 3 min at 300 × g_{max} (2,000 rpm). Samples were then eluted either by adding 2× LDS sample buffer and heated for 10 min at 70°C or were directly processed for mass spectrometric analysis according to the iTRAQ labeling method. For the iTRAQ comparison of docked and free synaptic vesicles, 10 immunoisolates each were pooled after the washing step and used for a single iTRAQ experiment.

Digestion, iTRAQ Labeling, Mass Spectrometry, and Data Analyses

Sample preparation, iTRAQ labeling, mass spectrometry and data analyses were performed as previously described (Grønborg et al., 2010) with the following modifications: proteins were solubilized in RapiGest SF (Waters) for 10 min at 70°C and then digested by trypsin in the presence of the beads. Beads were removed afterward by centrifugation for 20 min (4°C) at maximum speed in a tabletop centrifuge and the peptide containing supernatants transferred to fresh tubes. Tryptic peptides derived from the docked SVs were labeled with iTRAQ 117 and free SVs with iTRAQ 116, respectively. A detailed description of the data normalization procedure is available in the supplemental experimental procedures.

Functional Analysis using Ingenuity Pathway Analyses (IPA)

The Ingenuity Pathway Analyses software (build version 162830) was used to perform functional analysis on the docked synaptic vesicle proteome to identify biological functions and/or diseases that were most significant to the data set. Protein associated with biological functions and/or diseases with a minimum cluster size of 7 and having a p value < 0.01 were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

Figure 8. Differential Distribution of Proteins between Glutamatergic (VGLUT1) versus GABAergic (VGAT) Synaptosomes as Determined by Quantitative Immunocytochemistry

(A) Exemplary immunofluorescent images from four different protein pairs. Protease-treated synaptosomes were immobilized on coverslips, immunostained for the antigens indicated, and analyzed as described in Figures 2 and 7 (examples of line scans on the right). Scale bars, 5 μm.

(B) Quantitative immunofluorescent microscopic analyses of the extent of colocalization between VGLUT1 or VGAT and selected proteins identified in Figure 7 using purified protease-treated synaptosomes. The proportions of synaptosomes containing the counter-stained protein and either VGLUT1 or VGAT were determined and expressed as percent colocalization between the protein and the transporter (see Experimental Procedures for details on image acquisition and quantification by Matlab). The data show mean values of three independent experiments with error bars indicating the range of data observed (lowest–highest). At least 500 synaptosomes were analyzed for each given protein pair.

Immunofluorescent Staining of Synaptosomes

Two milliliters of shaved synaptosomes collected from sucrose gradients were diluted in 5 ml PBS and centrifuged for 30 min at 5,500 × g_{max}, 4°C, in a swing out rotor. The synaptosomal pellet was then resuspended in 2.4 ml PBS. One hundred microliters of this suspension were carefully aliquoted onto each poly-L-lysine precoated coverslip placed in a 12-well plate and incubated for 45 min at room temperature. Afterward, 1 ml PBS was added to each well and synaptosomes pelleted on the coverslip by centrifugation for 30 min at 5,580 × g_{max} (5,500 rpm) at 4°C in a HIGHplate rotor 75006444 (Sorvall Heraeus). Synaptosomes were then fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and stained with primary and secondary antibodies.

Image Acquisition and Processing

Synaptosomal images were acquired using an AOBS SP2 confocal microscope (Leica Microsystems) with a 63× oil-immersion objective, standard filter sets (Leica Microsystems), and Leica LCS software. Line scan analyses were performed using the LAS AF Lite software (Leica).

The extent of colocalization between different protein pairs in glutamatergic versus GABAergic synaptosomes were determined using a custom written Matlab algorithm (The Mathworks Inc.) kindly provided by Prof. Silvio Rizzoli. Colocalization with either VGLUT1 or VGAT was considered when the center of intensity in the two channels was within a distance of 200 nm. At least 500 synaptosomes were analyzed for each given protein pair in three independent biological replicates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.02.027.

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