Article

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

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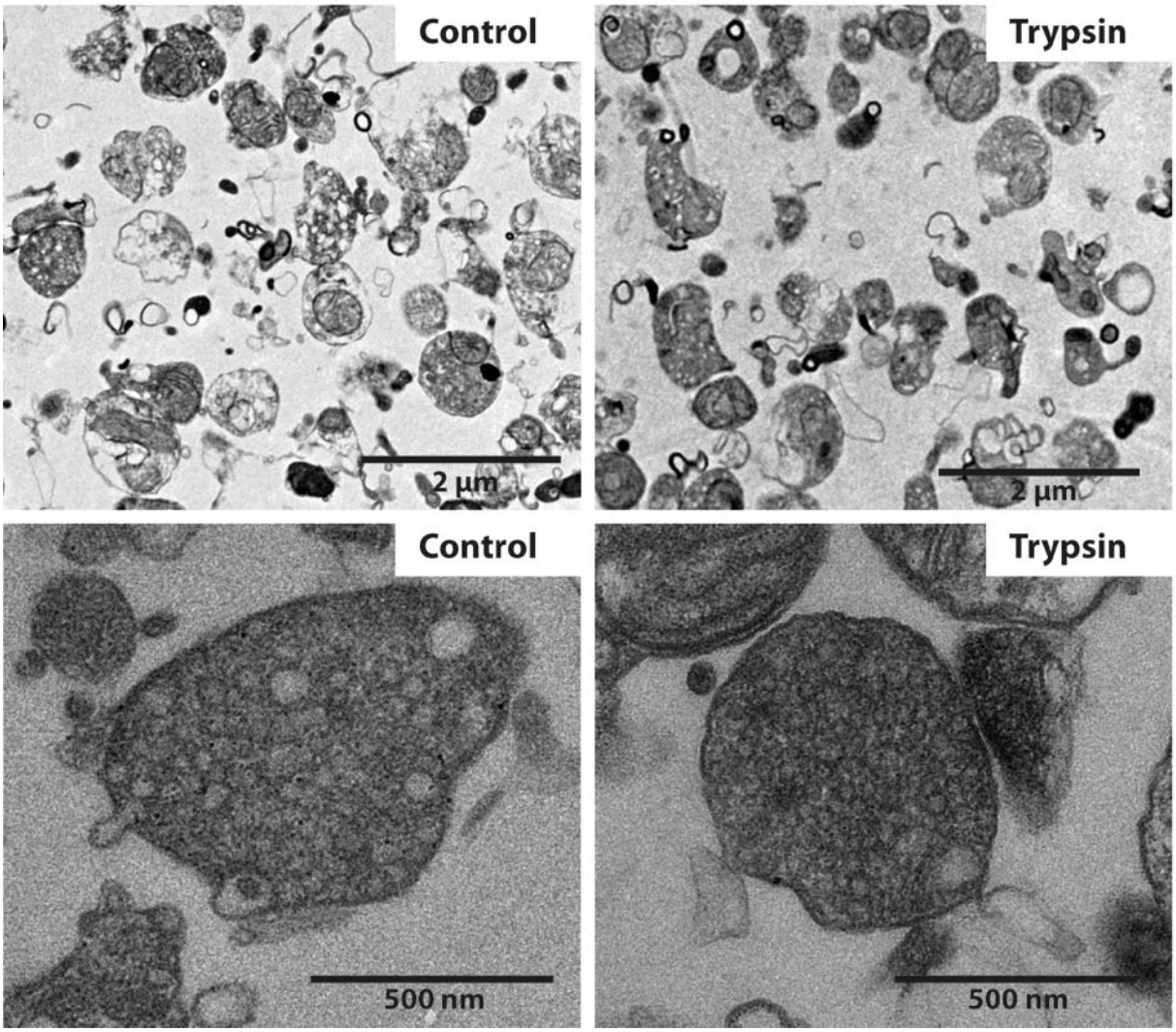

Figure S1: Trypsin-treatment does not affect the overall morphology of the synaptosome interior, related to Figure 2. Fractions of control and trypsin-treated synaptosomes sedimenting at 1.2 M sucrose after continuous gradient centrifugation were diluted 1:5 with 5 mM Hepes pH 7.4 and pelleted. Synaptosomes were subsequently fixed and processed for electron microscopic analyses. Ultrastructurally, no significant changes in morphology between treated and control synaptosomes were observed.

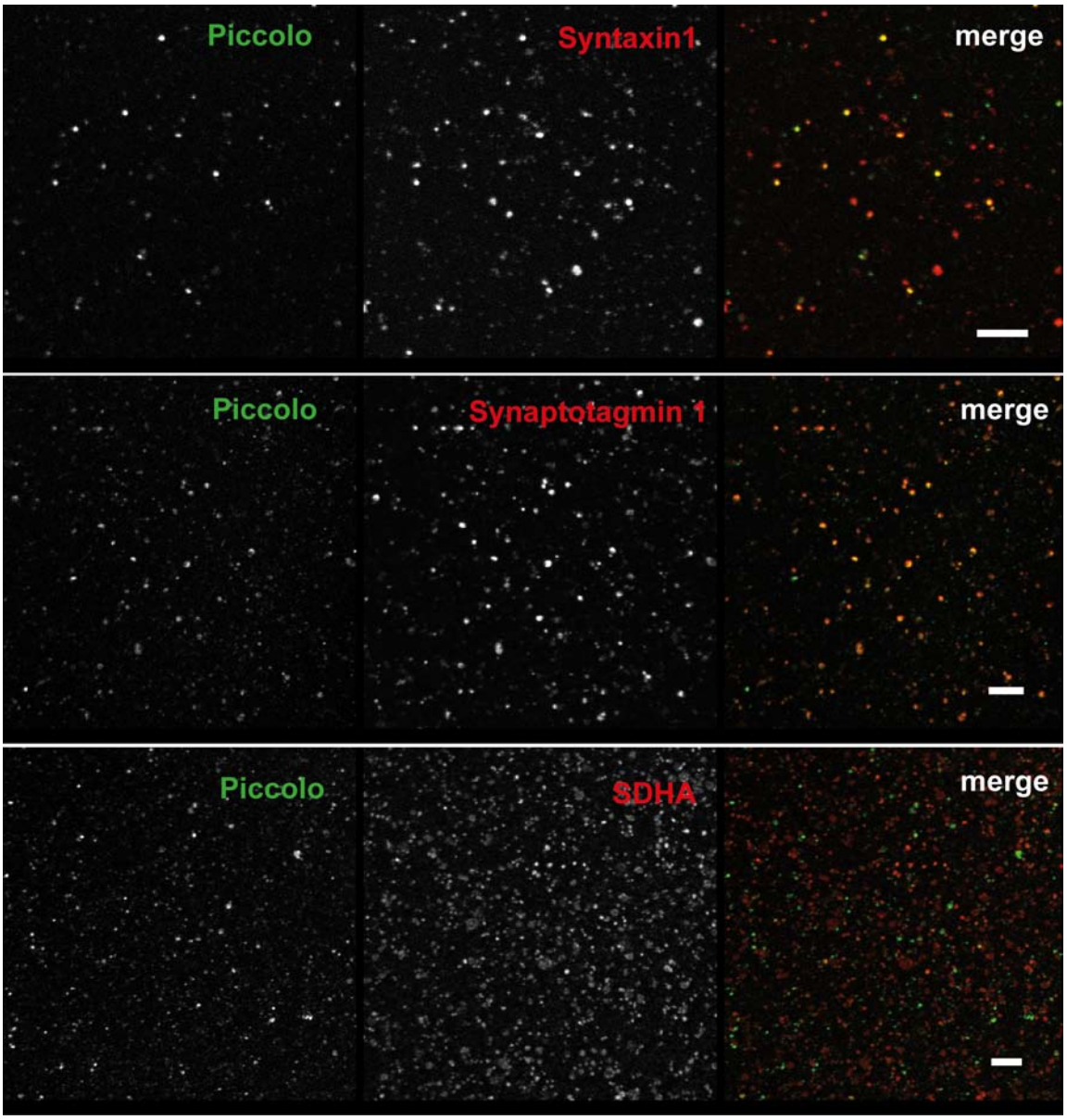

Figure S2: Immunofluorescent staining of docked synaptic vesicle fractions, related to Figure 3. Docked synaptic vesicle fractions from the continuous sucrose gradient were diluted in PBS, immobilized on coverslips and stained for the antigens indicated. The active zone protein Piccolo co-localizes well with the synaptic vesicle protein synaptotagmin 1 and partially overlaps with the plasma membrane SNARE syntaxin 1. Mitochondrial SDHA was present in high amounts, but little co-localization with Piccolo was observed. Scale bars, 5 µm.

Supplemental Tables

Table S1: Complete list of proteins identified in the iTRAQ comparison of docked and free synaptic vesicles, related to Figures 4, 5, and 6. iTRAQ ratios were calculated as docked SV/SV. Proteins that were identified at least twice and found to be solely present in the docked synaptic vesicle fraction in at least half of the experiments are denoted as “docked SV” in the table. Proteins were manually annotated accordingly to protein localization and function as annotated by Gene Ontology. Lowest and highest quantified ratios within the 3 experiments are depicted as negative and positive range.

Table S2: List of biological functions significantly enriched in the docked synaptic vesicle fraction as determined by IPA analyses ($p < e^{-06}$), related to Figure 4.

Functional Annotation	#	% of proteins identified	Proteins
Synaptic Transmission	34	12.88	BSN, CACNB3, CAMK2A, CPLX1, CTNNB1, DLG4, DNAJC5, DPP6, GNAI1, GNAQ, ITPR1, KCNMA1, KIF1B, MYO5A, NSF, PI4KA, PRKACA, PRKCG, RAB3A, Rims1, SLC12A5, SLC17A7, SLC1A2, SLC1A3, SNAP25, STX1A, STXBP1, SV2A, SV2B, SYN1, SYN2, SYP, SYT1 (includes EG:20979), SYT5
Neurotransmission	37	14.02	BSN, CACNB3, CAMK2A, CD47, CPLX1, CTNNB1, DLG4, DNAJC5, DPP6, GNAI1, GNAQ, ITPR1, KALRN, KCNMA1, KIF1B, MYO5A, NSF, PI4KA, PRKACA, PRKCG, RAB3A, Rims1, SLC12A5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, STX1A, STXBP1, SV2A, SV2B, SYN1, SYN2, SYP, SYT1 (includes EG:20979), SYT5
Exocytosis	25	9.47	ATP2A2, CAMK2A, CPLX1, DNAJC5, MYH10, MYO5A, NCAM1, NSF, PCLO, RAB27B, RAB3A, RAB3B, RALA, SCAMP1, SCAMP5, SEPT5, SNAP25, SRCIN1, STX1A, STX1B, STXBP1, SV2A, SYT1 (includes EG:20979), SYT2, VAMP2
Long-Term Potentiation	22	8.33	CACNB3, CAMK2A, CAMK2B, CD47, DLG4, DPP6, GNAI1, GNAQ, ITPR1, KALRN, NCAM1, NPTN, PRKACA, PRKCG, RAB3A, Rims1, SLC17A7, STX1A, SYNGAP1, SYNGR1, SYP, THY1
Synaptic Depression	15	5.68	CAMK2A, CAMK2B, DLG4, GNAQ, ITPR1, KCNMA1, MYO5A, PCLO, RAB3A, Rims1, SV2A, SV2B, SYN1, SYN2, SYN3
Quantity Of Synaptic Vesicles	10	3.79	CAMK2A, ERC2, KIF1B, MYO5A, PCLO, PTPRN2, Rims1, SLC17A6, SLC17A7, SYN1
Long-Term Potentiation Of Synapse	16	6.06	CACNB3, CAMK2A, CAMK2B, DLG4, DPP6, GNAI1, KALRN, PRKCG, RAB3A, Rims1, SLC17A7, STX1A, SYNGAP1, SYNGR1, SYP, THY1
Release Of Neurotransmitter	16	6.06	GNAQ, NSF, PCLO, PRKCG, RAB3A, RAB3B, SEPT5, SLC32A1, SNAP25, STX1A, SYN1, SYN2, SYN3, SYT1 (includes EG:20979), THY1, VAMP2
Secretion Of Neurotransmitter	15	5.68	CAMK2A, CPLX1, NSF, PTPRN2, RAB27B, RTN4 (includes EG:57142), SIRPA, SNAP25, STX1A, STXBP1, SYN1, SYN2, SYN3, SYT1 (includes EG:20979), VAMP2
Quantity Of Vesicles	11	4.17	CAMK2A, ERC2, KIF1B, MYO5A, PCLO, PTPRN2,

			RAB27B, Rims1, SLC17A6, SLC17A7, SYN1
Morphology Of Nervous Tissue	31	11.74	ANK2, ATP2B2, CACNB3, CD47, CKB, CLCN6, CNTNAP1, CTNNA2, DNAJC5, ERC2, GAP43, ITPR1, KALRN, KIF1B, KIF2A, MYH10, NCAM1, PLEC, PRKACA, Rims1, RTN4 (includes EG:57142), SHH, SLC12A5, SLC17A7, SLC1A3, SNTB2, SYN1, SYN2, SYNGAP1, UTRN, VPS13A
Morphology Of Nervous System	38	14.39	ANK2, APOB, ATP2B2, CACNB3, CANX, CD47, CEP290, CKB, CLCN6, CNTNAP1, CTNNA2, CTNNB1, DNAJC5, ERC2, GAP43, ITPR1, KALRN, KIF1B, KIF2A, MYH10, NCAM1, PLEC, PRKACA, Rims1, RTN4 (includes EG:57142), SHH, SLC12A5, SLC17A6, SLC17A7, SLC1A2, SLC1A3, SNAP25, SNTB2, SYN1, SYN2, SYNGAP1, UTRN, VPS13A
Morphology Of Neurites	15	5.68	ANK2, CACNB3, CD47, CKB, CLCN6, CNTNAP1, GAP43, ITPR1, KALRN, KIF2A, MYH10, NCAM1, PLEC, RTN4 (includes EG:57142), SLC17A7
Transport Of Synaptic Vesicles	9	3.41	CPLX1, CTNNB1, HSPA8, PCLO, RAB3A, STX1A, SYT1 (includes EG:20979), SYT2, VAMP2
Plasticity Of Synapse	12	4.55	ATP2B2, CAMK2A, CAMK2B, CAMK2D, CAMK2G, CPLX1, CTNND2, DLG4, Rims1, SYNGAP1, SYNGR1, SYP
Microtubule Dynamics	40	15.15	ACTB, ATP2B1, BSN, CANX, CD47, CDK5RAP2, CNTNAP1, CTNNA2, CTNND1, CTNND2, DLG4, ERN1, FLOT1, GAP43, GAPDH, Gnas (rat), GPM6A, HSP90AA1, ITPR1, KALRN, KIF2A, MYH10, MYH9, MYO5A, NCAM1, PRKACA, RAB3A, RALA, RP1, RTN4 (includes EG:57142), SEPT7, SEPT9, SHH, SLC9A1, SYN1, SYNGAP1, SYT1 (includes EG:20979), THY1, TUBB, TUBB3
Abnormal Morphology Of Neurites	13	4.92	ANK2, CACNB3, CD47, CKB, CLCN6, CNTNAP1, GAP43, KALRN, KIF2A, MYH10, NCAM1, PLEC, SLC17A7
Abnormal Morphology Of Axons	10	3.79	ANK2, CD47, CKB, CLCN6, CNTNAP1, GAP43, KIF2A, NCAM1, PLEC, SLC17A7
Action Potential Of Neurons	11	4.17	CAMK2A, CD47, CPLX1, KALRN, KCNMA1, Rims1, SLC12A5, SLC17A7, SLC32A1, STX1A, SYN1
Excitatory Postsynaptic Potential	11	4.17	CAMK2A, CD47, CPLX1, KALRN, Rims1, SLC17A7, SNAP25, STX1A, SYN1, SYT1 (includes EG:20979), VAMP2
Paired-Pulse Facilitation Of Synapse	7	2.65	CAMK2A, RAB3A, RAB3B, Rims1, SYN1, SYNGR1, SYP
Growth Of Neurites	21	7.95	CD47, CTNND2, DNAJA3, GAP43, GNAO1, GNAQ, Gnas (rat), KALRN, KIF2A, MYH10, MYH9, NCAM1,

			PRKACA, RTN4 (includes EG:57142), SLC9A1, SYN1, SYN3, SYNGAP1, SYNGR1, TUBA1A, YWHAZ
Transport Of K+	8	3.03	ATP1A1, ATP1A3 (includes EG:232975), ATP1B1, DPP6, KCNMA1, NSF, SLC12A5, SLC1A2
Outgrowth Of Neurites	19	7.20	CD47, DNAJA3, GAP43, GNAO1, GNAQ, Gnas (rat), KIF2A, MYH10, MYH9, NCAM1, PRKACA, RTN4 (includes EG:57142), SLC9A1, SYN1, SYN3, SYNGAP1, SYNGR1, TUBA1A, YWHAZ
Transport Of Ca2+	10	3.79	ATP2A2, ATP2B1, ATP2B2, CACNA2D1, CACNB3, CAMK2A, CAMK2B, CAMK2D, CAMK2G, ITPR1
Morphology Of Brain	22	8.33	ANK2, ATP2B2, CEP290, CKB, CTNNA2, CTNNB1, GAP43, KALRN, KIF1B, KIF2A, MYH10, NCAM1, Rims1, SHH, SLC17A6, SLC1A2, SLC1A3, SNAP25, SYN1, SYN2, SYNGAP1, VPS13A

Table S3: List of neurological diseases significantly associated with proteins identified from the docked synaptic vesicle fraction as determined by IPA analyses ($p < e^{-03}$), related to Figure 4.

Disease	#	% of proteins identified	Proteins
Huntington's Disease	38	14.39	ACTB, ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, CACNB3, CAMK2A, CAMK2B, CKB, CTNNB1, EEF1A2, GAPDH, GNAL, GNAO1, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, MAL2, MYO5A, PFKM, PRKCG, RAB3A, SCAMP5, SEPT5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, SYNPR, TUBA1A, VAMP1, VAMP2, YWHAZ
Schizophrenia	33	12.50	APOB, ATP1A1, ATP1A3 (includes EG:232975), ATP2B2, ATP6V1B2, CADM3, CNTNAP1, CPLX1, DLG4, DNAH10, GAP43, GNAI1, HPCAL1, KIF2A, NCAM1, NPTN, NSF, PCLO, PI4KA, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SLC12A5, SLC17A7, SLC1A2, SLC1A3, SLC6A1, SNAP25, STX1A, SYN2, SYN3, SYNGR1, SYP, SYT1 (includes EG:20979)
Ataxia	13	4.92	ATP2B2, CAMK2B, CANX, CNTNAP1, CPLX1, DNAJC5, GNAQ, ITPR1, KCNMA1, SLC1A2, SLC1A3, SLC6A1, SLC9A1
Epilepsy	11	4.17	ALDH7A1, BSN, CACNA2D1, KCNMA1, SLC12A5, SLC32A1, SLC6A1, SLC9A1, SPTAN1, STXBP1, SYN1
Parkinson's Disease	12	4.55	ATP6V1E1, FLOT1, GAP43, KIF1B, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SNAP25, SYN1, SYN2, THY1, TUBA1A, TUBA1B
Dementia	19	7.20	ACTB, APOB, ATP6V1G2, CAMK2A, CAMK2B, CANX, GAP43, GAPDH, PRKACA, RAB14, SLC1A2, SLC1A3, SLC2A3, SLC30A3, SV2A, SYP, THY1, TUBB, YWHAZ
Alzheimer's Disease	18	6.82	ACTB, ATP6V1G2, CAMK2A, CAMK2B, CANX, GAP43, GAPDH, PRKACA, RAB14, SLC1A2, SLC1A3, SLC2A3, SLC30A3, SV2A, SYP, THY1, TUBB, YWHAZ
Neurodegenerative Disorder	19	7.20	ACTB, ATP6V1G2, CAMK2A, CAMK2B, CANX, FLOT2, GAP43, GAPDH, PRKACA, RAB14, SLC1A2, SLC1A3, SLC2A3, SLC30A3, SV2A, SYP, THY1, TUBB, YWHAZ
Mood Disorder	15	5.68	ATP1A3 (includes EG:232975), CACNA2D1, CAMK2A, CPLX1, DLG4, MYH9, NCAM1, PDE2A,

			PI4KA, SLC30A3, STX1A, SYN1, SYN2, SYN3, SYNGR1
Movement Disorder	61	23.11	ACTB, ATP1A3 (includes EG:232975), ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, ATP6V1E1, CACNA2D1, CACNB3, CAMK2A, CAMK2B, CANX, CKB, CNTNAP1, CPLX1, CTNNB1, DNAJC5, EEF1A2, FLOT1, GAP43, GAPDH, GNAL, GNAO1, GNAQ, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, KCNMA1, KIF1B, MAL2, MYO5A, PFKM, PRKCG, RAB3A, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SCAMP5, SEPT5, SLC12A5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SLC6A1, SLC9A1, SNAP25, SYN1, SYN2, SYNGAP1, SYNPR, THY1, TUBA1A, TUBA1B, VAMP1, VAMP2, VPS13A, YWHAZ
Disorder Of Basal Ganglia	49	18.56	ACTB, ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, ATP6V1E1, CACNB3, CAMK2A, CAMK2B, CKB, CTNNB1, EEF1A2, FLOT1, GAP43, GAPDH, GNAL, GNAO1, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, KIF1B, MAL2, MYO5A, PFKM, PRKCG, RAB3A, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SCAMP5, SEPT5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, SYN1, SYN2, SYNPR, THY1, TUBA1A, TUBA1B, VAMP1, VAMP2, VPS13A, YWHAZ
Neuromuscular Disease	51	19.32	ACTB, ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, ATP6V1E1, CACNB3, CAMK2A, CAMK2B, CD47, CKB, CTNNB1, EEF1A2, FLOT1, GAP43, GAPDH, GNAL, GNAO1, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, KCNMA1, KIF1B, MAL2, MYO5A, PFKM, PRKCG, RAB3A, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SCAMP5, SEPT5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, SYN1, SYN2, SYNPR, THY1, TOP2A, TUBA1A, TUBA1B, VAMP1, VAMP2, YWHAZ
Dyskinesia	40	19.32	ACTB, ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, CACNB3, CAMK2A, CAMK2B, CKB, CTNNB1, EEF1A2, GAPDH, GNAL, GNAO1, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, KCNMA1, MAL2, MYO5A, PFKM, PRKCG, RAB3A, SCAMP5, SEPT5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, SYNPR, TUBA1A, VAMP1, VAMP2, VPS13A, YWHAZ
Chorea	39	15.15	ACTB, ATP2A2, ATP2B1, ATP2B2, ATP6V1A, ATP6V1B2, CACNB3, CAMK2A, CAMK2B, CKB, CTNNB1, EEF1A2, GAPDH, GNAL, GNAO1, GNB1, GPI, HPCA, HSP90AA1, HSPA8, ITPR1, MAL2,

			MYO5A, PFKM, PRKCG, RAB3A, SCAMP5, SEPT5, SLC17A7, SLC1A2, SLC1A3, SLC32A1, SNAP25, SYNPR, TUBA1A, VAMP1, VAMP2, VPS13A, YWHAZ
Seizures	21	14.77	BSN, CACNA2D1, CKB, CPLX1, GAP43, GNAO1, GNAQ, HCN1, ITPR1, PTPRN2, SLC12A5, SLC1A2, SLC1A3, SLC32A1, SLC6A1, SLC9A1, SV2A, SV2B, SYN1, SYN2, TUBB
Progressive Motor Neuropathy	19	7.95	ACTB, ATP6V1E1, CD47, DPP6, FLOT1, GAP43, KCNMA1, KIF1B, RTN1 (includes EG:104001), RTN4 (includes EG:57142), SLC1A2, SLC1A3, SNAP25, SYN1, SYN2, THY1, TOP2A, TUBA1A, TUBA1B

Table S4: Features of evolutionary conserved novel presynaptic candidates, related to Figure 6. Sequences were analysed for the presence of putative transmembrane domains by Dense Alignment Surface method (DAS, http://www.sbc.su.se/~miklos/DAS/), HMMTOP (http://www.enzim.hu/hmmtop/); TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Transmembrane domains predicted with low confidence are indicated in parentheses. Data for mRNA expression in the mammalian brain were obtained from Allen Brain Atlas (http://mouse.brain-map.org). Abbreviations of brain regions: HPF: Hippocampal formation; CTXsp: Cortical subplate; CB: Cerebellum; OLF: Olfactory areas; MY: Medulla; N/A: no information available.Orthologs were identified using HomoloGene from the NCBI server.

Table S5: Complete list of proteins indentified in the iTRAQ comparison of VGLUT1 and VGAT docked vesicle immunoisolates, related to Figure 7. iTRAQ ratios were calculated as indicated (VGLUT1/VGAT, VGAT/VGLUT1). Lowest and highest ratios within the 3 experiments are depicted as negative and positive range.

Supplemental Experimental Procedures

List of antibodies used in this study

Antibody	Species	Source
Synaptophysin clone 7.2	mouse monoclonal, affinity purified	(Jahn et al., 1985)
Synaptophysin clone G96	rabbit polyclonal, serum	(Jahn et al., 1985)
Synaptobrevin 2 clone 69.1	mouse monoclonal, ascites	(Edelmann et al., 1995)
Synapsin 1	mouse monoclonal	Synaptic Systems
Munc18	rabbit polyclonal, serum	in house
Munc13	mouse monoclonal, affinity purified	Synaptic Systems
Piccolo	rabbit polyclonal, affinity purified	Synaptic Systems
Bassoon	rabbit polyclonal, serum	Synaptic Systems
	guinea pig polyclonal, serum	Synaptic Systems
Synaptotagmin 1 clone 41.1	mouse monoclonal, ascites	(Brose et al., 1992)
PSD95	mouse monoclonal, affinity purified	NeuroMab
Homer 1	rabbit polyclonal, affinity purified	Synaptic Systems
Syntaxin 1A clone 78.2	mouse monoclonal, ascites	(Chapman et al., 1995)
NMDA receptor 1	mouse monoclonal, ascites	(Brose et al., 1994)
AMPA receptor 1	rabbit polyclonal, affinity purified	Synaptic Systems
Na^+/K^+ ATPase 1	mouse monoclonal, ascites	Abcam
SDHA	mouse monoclonal, affinity purified	Abcam
Neuroligin 1	rabbit polyclonal, affinity purified	Synaptic Systems
VGLUT1	rabbit polyclonal, serum	(Takamori et al., 2001)
	guinea pig polyclonal, serum	Synaptic Systems
VGAT	rabbit polyclonal, affinity purified	(Takamori et al., 2000)
	mouse monoclonal, affinity purified	Synaptic Systems
	guinea pig polyclonal, serum	Synaptic Systems
Mint	rabbit polyclonal, affinity purified	Synaptic Systems
CASK	mouse monoclonal, affinity purified	NeuroMab
ERC1b/2	rabbit polyclonal, affinity purified	Synaptic Systems
SynCAM	rabbit polyclonal, affinity purified	Synaptic Systems
Rab3a	mouse monoclonal, ascites	(Matteoli et al., 1991)
GAD2	rabbit polyclonal, serum	Synaptic Systems
SNAP25	rabbit polyclonal, serum	in house
SNAP23	rabbit polyclonal, serum	Synaptic Systems
GAP43	rabbit polyclonal, affinity purified	Abgent
CAMK2 alpha subunit	mouse monoclonal [clone 6G9]	Abcam
ZnT3	rabbit polyclonal, serum	Synaptic Systems
SV2B	rabbit polyclonal, serum	Synaptic Systems

Data normalization

Docked and free synaptic vesicle immunoisolates were first quantified by immunoblotting. Input amounts of docked and free synaptic vesicle fractions were optimized so that comparable amounts of immunoisolated SVs were present in both samples used for iTRAQ labeling. As a result, during iTRAQ comparison of docked and free vesicles, the amount of immunoisolated vesicle proteins within the docked and free vesicle fractions was comparable in two of the biological replicates without requiring additional normalization. In the third biological replicate, analysis of the statistical distribution of the docked/free synaptic vesicles peptide and protein ratios showed a slightly higher amount of synaptic vesicles compared to the docked vesicle fraction. Normalization between the two fractions was performed at the peptide level as previously described using iTRAQ ratios obtained for the multi-subunit vATPase (Gronborg et al., 2010). The vATPase is considered an integral component of all synaptic vesicles and thus serve as an internal loading control. In the example shown here, box plots of raw docked SV/SV ratios for two vATPase subunits (V1 E and Vo a, Figure S3 A, left) indicate that docked synaptic vesicle fractions appear to contain more V1E and Vo compared to the free synaptic vesicle fraction. Raw peptide ratios were divided by the average of both median values (median V1 E: 1.59, median Vo a: 1.38) resulting in a normalized distribution of the peptide ratio of around 1:1 (Figure S3A, right). At the protein level, dividing all protein ratios by this normalization factor (1.49) also yielded in an approximately 1:1 ratio for the various vATPase subunits (Figure S3B).

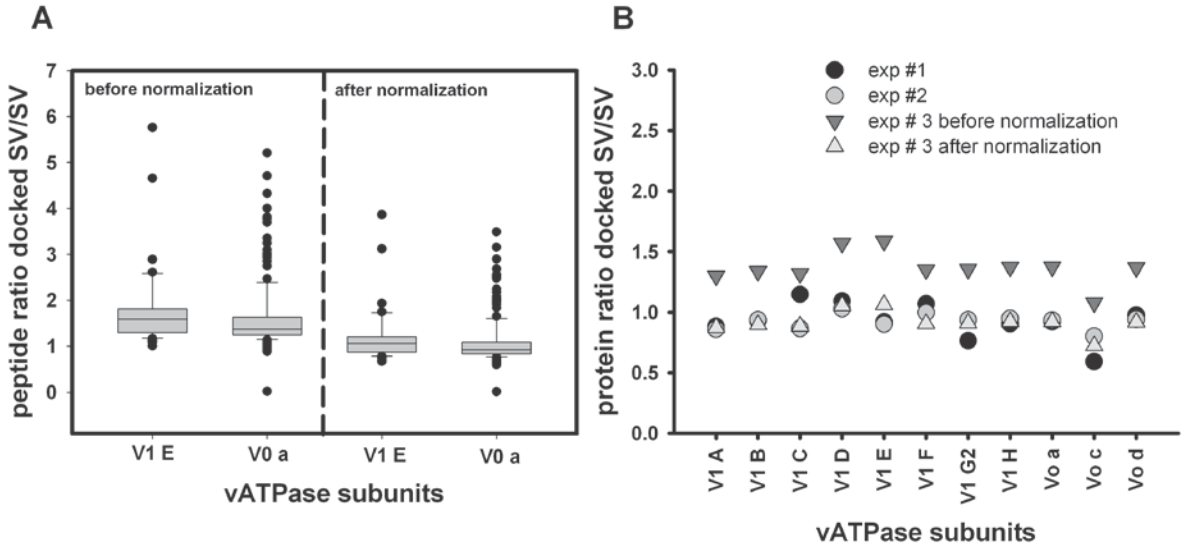

Figure S3: Normalization of iTRAQ data for the comparison of free and docked vesicles. (A) Normalization on peptide level. Box plots of raw and normalized docked SV/SV ratios. (B) Normalization on protein level. Protein ratios of vATPase subunits are shown before and after normalization (experiment 3). The vATPase ratios from biological replicates 1 and 2 are also shown here for comparison.

In the analyses of VGLUT1- and VGAT-docked synaptic vesicle fractions, 4 times as many immunoisolations were used for VGAT as for VGLUT1 to partially compensate for the lower amount of GABAergic synapses available in the sample. In the iTRAQ analysis, raw docked VGLUT1/VGAT peptide ratios were significantly above one for all three biological replicates indicating a persistent disparity in protein amounts (Figure S2, left). Therefore, iTRAQ data for all biological replicates for these analyses were additionally normalized as described above. Raw peptide ratios from each replicate were divided by the their median value of all peptides resulting in a balanced distribution of around 1:1 (Figure S2, right). The box plots represent the interquartile range (IQR) of 25-75%, the center line the median and the whiskers (horizontal lines) extend up to 1.5-fold of the IQR range.

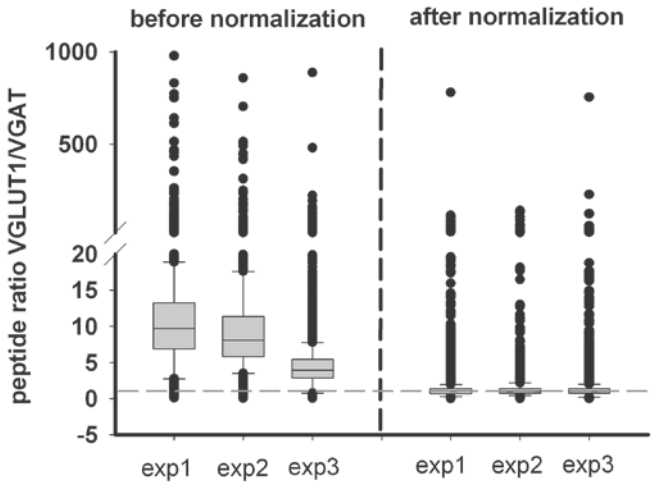

Figure S4: Normalization of iTRAQ data for the comparison of VGLUT1- and VGAT-docked vesicle immunoisolates. Box plots of VGLUT1/VGAT peptide ratios are illustrated before and after normalization by peptide median.

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