5. Supplemental Methods

5.1 Preparation of mouse cortical homogenate

Preparation of mouse cortical membranes was carried out as previously described [14]. Briefly, young adult C57BL/6J male mice (8 weeks) were killed by cervical dislocation and then decapitated. Intact cortices were dissected and hand homogenized with a Teflon-coated pestle (Thomas Scientific, Swedesboro, NJ) in a buffer (2.5 ml per cortex) containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, pH 7.4, incubated at 4°C for 30 min and cleared by low speed centrifugation (1,000 *g*, 5 min, 4°C). The soluble material was collected and subjected to Bio-Rad protein assay (Bio-Rad, Hercules, CA).

For denaturing experiments, cortices were homogenized in a buffer containing 1% SDS and 5 mM EDTA, then incubated at 4°C with 10 mM 2-mercaptoethanol for 30 min.

5.2 Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously [7]. The soluble cortical extract (see above) was diluted to 0.5-0.7 mg protein ml^{-1} and a 1.4 ml aliquot was transferred to a clean microcentrifuge tube. Bovine serum albumin (BSA) was added to a final concentration of 1 mg ml^{-1}, and the samples were then pre-cleared by addition of 30 µl of a 50% slurry (v/v) of rehydrated protein A-Sepharose (Zymed, San Francisco, CA) or A/G Agarose (Thermo Scientific, Waltham, MA) beads, followed by rotation at 4°C for 2 h. Samples were centrifuged for 30 sec at 2,000 *g* to pellet the beads, and the soluble material was transferred to a clean tube. Pre-cleared supernatants were then incubated overnight at 4°C with specific antibodies (Supplemental Table 1) followed by further incubation for 2 h with 30 µl of fresh beads (50% slurry). The beads were pelleted by centrifugation at 2,000 *g* for 0.5 min, then washed twice by resuspension in 0.5 ml of wash buffer containing 20 mM Tris HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mM EGTA, 1 mM PMSF, 0.1% (v/v) Triton X-100, and a protease inhibitor cocktail (Roche, Basel, Switzerland), followed by a final wash in the same buffer minus Triton X-100. The beads with the bound immunoprecipitated proteins were then resuspended in 40 µl of sample buffer containing 100 mM 2N-morphelino ethanesulfonic acid, 10 mM Na EDTA, 15% (v/v)

glycerol, 10% SDS, 0.3 % Tritin X, 25 mM tris-2-carboxyethyl phosphine HCl, 7.5 mM DTT, 0.0025 % bromophenol blue, heated to 70°C for 20 min, and then centrifuged at 2,000 *g* for 0.5 min. The collected soluble proteins were then resolved by SDS-PAGE and analyzed by Western blotting or mass spectrometry.

5.3 Immunoblotting

The soluble proteins collected after immunoprecipitation assays were resolved by SDS-PAGE and electrotransferred to polyvinylidene fluoride membrane in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol for 1 h at 18 V. Membranes were briefly rinsed in a buffer containing 10 mM Tris HCl (pH 8.0), 150 mM NaCl and 0.01% (v/v) Tween-20 (TBS-T), and then incubated at room temperature for 1 h in TBS-T containing 5% (w/v) skimmed milk powder to block non-specific binding of antibodies. Incubation of membranes with specific primary antibodies (Supplemental Table 1) was performed in TBS-T containing 1% skimmed milk and 1% BSA powders (w/v) for 2 h at room temperature, followed by three 10 min washes with TBS-T alone. Membranes were then incubated for 1 h at room temperature with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP), diluted in TBS-T /1% (w/v) skimmed milk and BSA powders, followed by three 5 min washes with TBS-T. After the final wash, blots were immediately developed by applying the Enhanced Chemi-Luminescence (ECL) reagent (Pierce Chemical Co., Rockford, IL) for 2 min, then images were acquired using a Kodak Image Station 2000MM (Eastman Kodak, Rochester, NY).

5.4 Mass spectrometry

Tryptic peptides from proteins present in excised gel bands were loaded onto a C18 trap column (0.3 X 5.0mm) and washed for 15 min in mobile phase A containing: 2% acetonitrile (AcN), 0.1% formic acid (FA), 0.01% trifluoroacetic acid (TCA) to remove all reagents from the previous steps. An analytical RP-C18 column, 15 cm X 75 µm, (Dionex Corporation, Sunnyvale, CA) was then incorporated into the flow path, and peptides were separated with a 60 min linear gradient of 5-60% solvent B (90% AcN, 0.1% FA, 0.01% TFA) at a flow rate of 240 nl/min (Dionex ULTIMATE, Sunnyvale, CA). Eluted peptides were spotted directly onto a 576 well MALDI plate (Applied Biosystems, Foster City, CA) at a rate of 15 sec

per spot for a total of 192 spots (3 samples/plate). α-cyano-4-hydroxycinnamic acid (5 mg/mL) was then spotted on each peptide-containing spot at a rate of 10 sec per spot (~1.0 μl/mm flow rate). The spotted peptides were analyzed using the 4700 TOF-TOF proteomics analyzer (Applied Biosystems, Foster City, CA). A job-wide interpretation method was used that collected up to 10 tandem mass spectrometry (MS/MS) spectra of the top 10 most abundant peaks in the original MS spectrum of each spot.

5.5 Strategy for protein identification

The MS/MS data were searched against the SwissProt mouse database using the GPS Explorer v.3.6 software suite (Applied Biosystems, Foster City, CA) and the MASCOT v.2.2 search engine [15], as previously described [12,16]. Briefly, the search engine theoretically digests proteins in the database and generates theoretical fragment ions for each possible tryptic peptide from the digest. Then it compares the best matches between experimental and theoretical fragment ion patterns to generate a peptide identification; each match is assigned a statistical weight based on theoretically determined factors. Eventually, it generates a protein identification based on the peptide sequences identified; the MASCOT total score (S) is based on the probability (P) that peptide mass matches are non-random events [S = -10*Log(P)] [15].

Searches were conducted with a mass tolerance of 50 or 75 ppm for MS and 0.2 Da for MS/MS. The following variable modifications were applied to the search: carbamidomethyl (C) and oxidation (M). LCMS search and standard-scoring output parameters were used; thus, sequence information only (MS/MS) was considered without peptide fingerprint (MS). A non-redundant database was searched without applying any constraint on molecular weight; the molecular weight of the band as determined by gel electrophoresis agreed with the protein identified.

If the protein score is equal to or greater than the MASCOT significance level calculated for the search, the protein match is considered to be statistically non-random at the 95% confidence interval [15]. However, to control for potential erroneous identification of proteins, we also determined if the sum of marginal peptide scores resulted in a significant protein score [15].

Supplemental Figures

Supplemental Table 1.

Antibody	***Clone***	***Host***	***Epitope***
BK_{Ca} α subunit		rpAb	aa 1184-1200
Clathrin, HC	C-20	gpAb	
Dynamin-1	D5	mmAb	aa 1-750
FLAG*		mmAb	FLAG sequence
FLAG*		rpAb	FLAG sequence
Munc-18	31	mmAb	
Synaptophysin	SVP38	mmAb	
SNAP-25		rpAb	aa 195-206
Syntaxin-1A	HPC-1	mmAb	
VAMP-2		rpAb	aa 1-18

*referred as *NsAb*, non-specific antibody, in the text.

Supplemental Table 1.

Antibodies used in this study (see Materials and Methods).

Reciprocal immunoprecipitations followed by immunoblots were performed using the different antibodies listed to evaluate the interacting partners of synaptic proteins. For each antibody, either the clone number or epitope information are provided, as well as the host species. *gpAb*, goat polyclonal antibody; *mmAb*, mouse monoclonal antibody; *rpAb*, rabbit polyclonal antibody.

Supplemental Table 2

A. *MS analysis of SNAP-25 interacting proteins*

Protein name	*Gene*	*Accession*	*Mr (kDa)*	*PM*	*MOWSE*
Nck-associated protein1	NCKAP1	P28660	129	7	187
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	Q8VDN2	113	6	159
Kinesin heavy chain isoform 5C	KIF5C	P28738	109	5	107
Kinesin-1 heavy chain	KIF5B	Q61768	109	3	48
AP-2 complex subunit alpha-1	AP2A1	P17426	108	6	158
Phosphofurin acidic cluster sortin protein 1	PACS1	Q8K212	105	10	347
AP-2 complex subunit beta-1	AP2B1	Q9DBG3	105	4	63
Microtubule-associated protein 6	MTAP6	Q7TSJ2	96	8	148
Tubulin alpha-1A chain	TUBA1A	P68369	50	5	101
Tubulin alpha-4A chain	TUBA4A	P68368	50	4	54
Tubulin beta-2A chain	TUBB2A	Q7TMM9	50	3	114
Tubulin beta-5 chain	TUBB5	P99024	50	3	98
Zinc finger homeobox protein 3	ZFHX3	Q61329	41	2	43
V-type proton ATPase subunit d 1	ATP6V0D1	P51863	40	3	155
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	Q05793	37	2	48
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P16858	36	4	99
Synaptophysin	SYP	Q62277	34	3	72

B. *MS analysis of VAMP-2 interacting proteins*

Protein name	*Gene*	*Accession*	*Mr (kDa)*	*PM*	*MOWSE*
Potassium-transporting ATPase alpha chain 1	ATP4A	Q64436	114	5	181
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	Q8VDN2	113	12	383
Sodium/potassium-transporting ATPase subunit alpha-3	ATP1A3	Q6PIC6	112	15	477
Sodium/potassium-transporting ATPase subunit alpha-2	ATP1A2	Q6PIE5	112	13	423
Dynamin-1	DNM1	P39053	98	3	84
Zinc transporter 3	ZNT3	P97441	42	2	55
V-type proton ARPase subunit d 1	ATP6V0D1	P51863	40	6	195
Guanine nucleotide-binding proteinG subunit alpha	GNAO1	P18872	40	5	145
Guanine nucleotide-binding protein subunit beta1	GNB1	P62874	37	5	136
Guanine nucleotide-binding protein subunit beta3	GNB3	Q61011	37	4	58
Synaptophysin	SYP	Q62277	34	7	236
Beta-soluble NSF attachment protein	NAPB	P28663	34	2	59
Syntaxin 1B	STX1B	P61264	33	15	455
Syntaxin 1A	STX1A	O35526	33	9	239
Neuronal membrane glycoprotein M6-a	GPM6A	P35802	31	3	121
Syntaxin-7	STX7	O70439	30	4	102
Vesicle-associated membrane protein 2	VAMP2	P63044	13	12	291

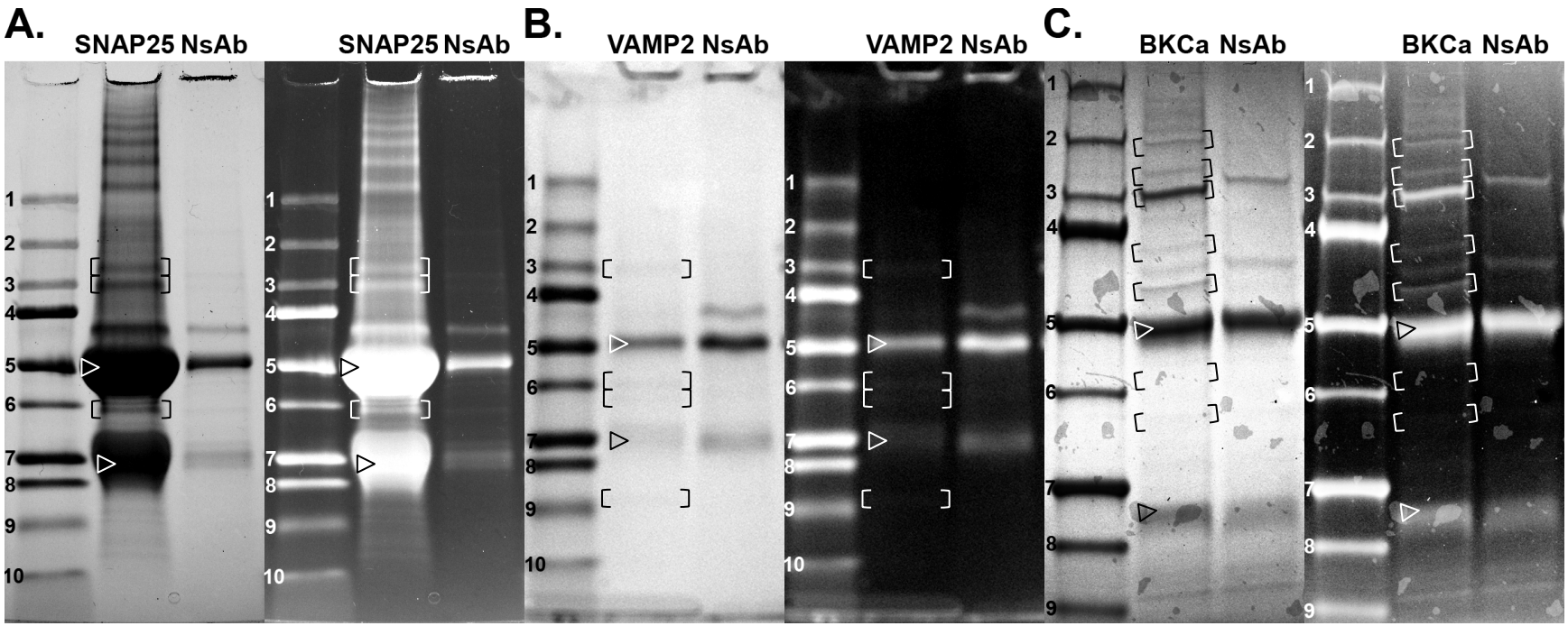
C. *MS analysis of BK_{Ca} interacting proteins*

Protein name	*Gene*	*Accession*	*Mr (kDa)*	*PM*	*MOWSE*
Phosphoinositide 3-kinase regulatory subunit 4	PIK3R4	Q8VD65	153	23	1031
Uncharacterized protein KIAA1107	KIAA1107	Q80TK0	149	7	206
Calcium-activated potassium channel subunit alpha-1	KCNMA1	Q08460	134	9	210
Activated CDC42 kinase 1	ACK1	O54967	117	2	44
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	Q8VDN2	113	7	184
Sodium/potassium-transporting ATPase subunit alpha-3	ATP1A3	Q6PIC6	112	8	203
Sodium/potassium-tranporting ATPase subunit alpha-2	ATP1A2	Q6PIE5	112	6	146
Kinesin heavy chain isoform 5C	KIF5C	P28738	109	9	178
Gamma-tubulin complex component 2	TUBGCP2	Q921G8	103	17	655
Gamma-tubulin complex component 3	TUBGCP3	P58854	103	9	227
Phosphatidylinositol 3-kinase catalytic subunit type 3	PIK3C3	Q6PF93	101	11	374
Dynamin-1	DNM1	P39053	98	58	2673
AMP deaminase 2	AMPD2	Q9DBT5	92	6	157
Gamma-tubulin complex component 4	TUBGCP4	Q9D4F8	76	2	44
Heat shock cognate 71 kDa protein	HSPA8	P63017	71	4	95
Syntaxin-binding protein 1	STXBP1	O08599	68	2	55
Kinesin light chain 2	KLC2	O88448	67	8	126
Tubulin gamma	TUBG1	P83887	51	3	79
Elongation factor 1-alfa 1	EEF1A1	P10126	50	5	92
KH domain-containing, RNA-binding, signal transduction-assoc. protein 1	KHDRBS1	Q60749	48	11	327
Alfa-centractin	ACTR1A	P61164	43	5	97
beta-centractin	ACTR1B	Q8R5C5	42	5	128

Supplemental Table 2.

Identification of synaptic protein complexes using mass spectrometry.

Immunoprecipitation (IP) experiments from mouse cortex membranes were performed by using *(A)* SNAP-25, *(B)* VAMP-2, *(C)* large-conductance calcium-activated potassium channel (BK_{Ca}), alpha subunit (or their respective non-specific control antibodies). Co-immunoprecipitated proteins were resolved by 4-20% SDS-PAGE and the resulting gels were subjected to Coomassie staining. Discrete specific bands ranging from (A) 37 to 120 kDa, (B) 15 to 100 kDa, and (C) 37 to 150 kDa, were excised, digested with trypsin, and subjected to LC-MS/MS. Identified proteins were sorted by molecular weight. For each protein, the gene name, the accession number, the molecular weight, the number of peptides detected, and the total MOWSE score [15] are reported; proteins were classified into high- (no background) and medium-probability (grey background) hits (see Methods for details). Basic information for the proteins and their corresponding genes are hyperlinked in the electronic version. *Mr*, relative molecular mass; *PM*, peptide matched (total number of matching peptides sequenced by MS/MS for a specific protein). Note that the number of peptides sequenced from each protein does not directly correlate to quantity of protein.



Supplemental Figure 1.
Representative gels used for mass spectrometric analysis.

Mass spectrometry of synaptic protein interacting partners. Immunoprecipitation of (A) SNAP-25, (B) VAMP-2, or (C) BK_{Ca} α-subunit associated proteins. The pictures show representative gels with specifically-associated or nonspecifically-bound (NsAb lanes) bands of proteins co-immunoprecipitated from mouse cortex. The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4-20%) and visualized with Coomassie staining. Brackets indicate excised regions on the gel. Arrows indicate immunoglobulin G (IgG) heavy and light chains. Position of molecular weight markers is indicated on the left lanes by a number: 1, 250; 2, 150; 3, 100; 4, 75; 5, 50; 6, 37; 7, 25; 8, 20; 9, 15; 10, 10 kDa. For non-specific co-IP of proteins, the same amount of protein as in the specific IP-lane was used in the NsAb-lane using a rabbit polyclonal anti-flag control antibody. For a better view, inverted filter pictures are provided; nevertheless, this low resolution figure is only intended to show the size of the cut bands. *NsAb*, non-specific antibody.