Supplementary Information to "**The dysbindin-containing complex (BLOC-1) in brain: developmental regulation, interaction with SNARE proteins, and role in neurite outgrowth**" by CA Ghiani, M Starcevic, IA Rodriguez-Fernandez, R Nazarian, VT Cheli, LN Chan, JS Malvar, J de Vellis, C Sabatti, and EC Dell'Angelica

Supplementary materials and methods

Recombinant proteins

For bacterial expression of glutathione-*S*-transferase (GST)-fusion proteins, the complete open-reading frames (ORFs) of the following human proteins were engineered by polymerase-chain reaction (PCR) and subsequently cloned "in frame" into the Eco*RI*–Sal*I* sites of the pGEX-5X-1 vector (GE Healthcare, Piscataway, NJ, USA): SNAP-23 isoform a (GenBank accession no. NM_003825), SNAP-25a (GenBank accession no. NM_003081), SNAP-25b (GenBank accession no. NM_130811), SNAP-29 (GenBank accession no. NM_004782), CK1BP (also known as DBNDD2; GenBank accession no. NM_001048221) and cypin (also known as guanine deaminidase; GenBank accession no. NM_004293). The ORF of human RGS4 (GenBank accession no. NM_005613) and a segment encoding residues 1-69 of human RGS7 (GenBank accession no. NM_002924) were PCR-engineered and cloned in frame into the Bam*HI*–Sal*I* sites of the same vector. The following ORF fragments of human proteins (amino acid numbers indicated between parentheses) were engineered by PCR and cloned in frame into the Eco*RI*–Sal*I* sites of pGEX-5X-1: SNAP-25b fragments "SN1" (1-83), "SN2" (120-206), "SN1+L" (1-119) and "L+SN2" (84-206), cytoplasmic regions "cyt" of Vti1a (1-217; GenBank accession no. NM_145206), Vti1b (1-204; GenBank accession no. NM_006370), VAMP2 (1-93; GenBank accession no. NM_014232), VAMP7 (1-182; GenBank accession no. NM_005638) and syntaxin 13 (1-249; GenBank accession no. NM_177424), SNAP-47 fragment containing Qb and Qc domains and lacking an extended *N*-terminal region[1] (160-464; GenBank accession no. NM_053052), and cytoplasmic region fragments "SN" lacking the Habc domain of

syntaxins 1 (156-266; GenBank accession no. NM_004603), 4 (166-271; GenBank accession no. NM_004604), 7 (130-237; GenBank accession no. NM_003569), 13 (138-249), 16 (199-301; GenBank accession no. NM_001001433) and 18 (171-311; GenBank accession no. NM_016930). The full-length ORFs of CK1BP, the cytoplasmic region of VAMP2 and the SN fragments of syntaxins 1, 4 and 13 were also cloned into the Eco*RI*–Sal*I* sites of the pET-30a(+) vector (EMD Biosciences Novagen, Madison, WI, USA) for bacterial expression of the corresponding His_6-S-tag-fusion proteins. All constructs were verified by DNA sequencing. Recombinant protein expression, purification and dialysis were carried out as described previously.[2]

Antibodies

A rabbit polyclonal antibody against dysbindin[2] and a mouse monoclonal antibody (mAb) against pallidin[3] (clone 2G6) were described in previous papers. A polyclonal antibody against CK1BP was raised in rabbits by immunization with purified GST-CK1BP and subsequently purified on an affinity column containing the corresponding His_6-S-tag-fusion protein immobilized onto Affi-Gel 15 beads (Bio-Rad, Hercules, CA, USA); the specificity of recognition of CK1BP and lack of cross-reactivity with dysbindin were verified by immunoprecitpitation and immunoblotting. A hybridoma supernatant containing mAb against O4 was a kind gift of Drs Steven Pfeiffer and Rashmi Bansal (University of Connecticut Health Center, Farmington, CT, USA). The following mAbs were purchased from the vendors indicated between parentheses: against α-tubulin, β-actin, AP-1 γ-adaptin, Hsp70 and MAP2 (Sigma-Aldrich, St. Louis, MO, USA), against syntaxin 1 (Synaptic Systems, Göttingen, Germany), against, CNPase (Chemicon International, Danvers, MA, USA), against GFAP (NeoMarkers/Thermo Fisher Scientific, Fremont, CA, USA), against βIII-tubulin (Covance Research Products, Princeton, NJ, USA) and against SNAP-25 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In some experiments, rabbit polyclonal antibodies against βIII-tubulin (Covance Research Products) and GFAP (Dako, Carpinteria, CA, USA), and a goat polyclonal antibody against SNAP-25 (ProteinTech Group, Chicago, IL, USA), were used in place of the corresponding mAbs. A rabbit polyclonal against

GAP-43 was purchased from Chemicon International. The sources of secondary antibodies were described elsewhere.[4]

Animals

BLOC-1-deficient pallid mutant mice, B6.Cg-*Pldn*^{pa}/J, and the "wild-type" control strain, C57BL/6J, were from our breeding colony maintained at the University of California, Los Angeles (UCLA). The pallid strain carries a non-sense mutation in the *Pldn* gene encoding pallidin,[5] an essential component of BLOC-1,[6,7] and no detectable mutations in the *Dtnbp1* gene encoding dysbindin. Timed pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA, USA). Euthanasia prior to tissue dissection was performed in accordance to procedures approved by the UCLA Chancellor's Animal Research Committee.

Primary cell culture

Primary hippocampal neurons were isolated from postnatal day (P)0 or P1 mice, and primary cortical neurons from embryonic day (E)16 rats, as previously described.[8] Cells were plated onto dishes that had been coated with poly-*D*-lysine, and cultured in a chemically defined serum-free medium, named Tii, supplemented with 10 ng/ml bFGF (Invitrogen, Carlsbad, CA, USA), 2% (v/v) B27 (Invitrogen), and 2 mg/ml creatine (Sigma-Aldrich).[9] In some experiments, cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum and 2 mM glutamine. In both culture conditions, the majority of cells were immunopositive for neuronal markers, such as microtubule-associated protein 2 (MAP2) and βIII-tubulin, with a small percentage of cells expressing the astrocytic marker, glial fibrillary acidic protein (GFAP). No cells immunopositive for markers of oligodendrocytes or microglial cells were detected. Primary cultures of astrocytes and oligodendrocytes were prepared as described.[10,11]

Preparation and fractionation of brain tissue extracts

All extracts were prepared at 0-4ºC, using buffers containing a protease inhibitor mixture consisting of 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 mg/l leupeptin, 5 mg/l aprotinin and 1 mg/l pepstatin A. Whole brain and selected brain areas (cortex, hippocampus and

cerebellum) were dissected from mice of various developmental ages. For the analysis of steady-state protein levels, “whole-tissue” detergent extracts were prepared in 50 mM Tris-HCl (pH 7.5), 1% (w/v) Triton X-100, 0.25% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1 mM EDTA and protease inhibitor mixture, first by homogenizing minced tissue with the aid of a plastic pestle and then by subjecting the sample to brief (20 s) sonication. The crude extracts were subsequently cleared by centrifugation for 5 min at 15 000 $\times$ *g*. The total protein concentration in the cleared extracts prepared from dissected brain regions was estimated by Bradford’s method using a commercial reagent (Bio-Rad) and fatty-acid-free bovine serum albumin (Sigma-Aldrich) as a standard. Total protein concentration in cleared extracts prepared from primary hippocampal neurons was estimated using Pierce’s BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific) and bovine serum albumin as a standard. Based on the resulting values of total protein concentration, appropriate amounts of homogenization buffer were added in order to match the total protein concentration of all samples. For the analysis of native molecular size of dysbindin, mouse brains were homogenized in detergent-free buffer containing 0.3 M Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$ and protease inhibitor mixture, and the crude homogenate subjected to ultracentrifugation for 90 min at 120 000 $\times$ *g* to yield a membrane pellet and a soluble fraction. Subsequently, 0.2-ml aliquots of the soluble fractions prepared from P7 brain (1.5 mg of total protein) and adult brain (0.6 mg of total protein) were fractionated by high-resolution size-exclusion chromatography on a Superose 6 column (GE Healthcare), as previously described.[2] For the analysis of steady-state membrane-association and complex assembly of SNAP-25, freshly dissected brains from wild-type and pallid mice were homogenized in Hepes buffer [20 mM Hepes, pH 7.4, 50 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$ and protease inhibitor mixture], and the crude extracts subjected to ultracentrifugation for 90 min at 120 000 $\times$ *g* to yield soluble (cytosolic) and membrane fractions. The membrane fraction was resuspended in a volume of SDS-PAGE sample buffer [50 mM Tris/HCl, pH 6.8, 0.1 M dithiothreitol, 12% (w/v) glycerol, 2% (w/v) SDS and 0.05% (w/v) bromophenol blue] equivalent to twice the volume of the original crude extract. The cytosolic fraction was diluted with an equal volume of 2$\times$ SDS-PAGE sample buffer. In order to detect the presence of SDS-resistant

SNARE complexes,[12] both cytosolic and membrane fractions were divided into two aliquots; one of then was kept at room temperature ("not boiled") while the other was heated at 65ºC for 5 min and then at 95ºC for 5 min ("boiled") prior to SDS-PAGE. For *in vitro* binding experiments, frozen bovine brains were purchased from Pel-Freez Biologicals (Rogers, AR, USA), quickly thawed, and homogenized in 10 mM Hepes (pH 7.4), 0.25 M sucrose, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$ and protease inhibitor mixture, using a tissue blender (3 × 15 s). The homogenate was subjected to two cycles of centrifugation at 13 000 × *g* for 15 min followed by filtering through gauze, and the resulting supernatant was subjected to ultracentrifugation at 120 000 × *g* for 90 min to obtain a cytosolic extract, which was divided into aliquots, frozen in liquid nitrogen, and stored at -80ºC until further use.

Preparation of recombinant SNARE complexes

SNARE complexes were assembled *in vitro* using a modification of published procedures.[12,13] Briefly, a purified His_6-S-tag-fusion protein containing the SN fragment of a syntaxin (1, 4 or 13) was incubated overnight at 4ºC in phosphate-buffered saline (PBS) with a 2-molar excess of purified GST-SNAP-25b and a 5-molar excess of a His_6-S-tag-fusion containing the cytoplasmic region of VAMP2. The resulting mixture was centrifuged at 15 000 × *g* for 10 min at 4ºC to remove any protein aggregates, and the supernatant incubated with Glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) to isolate GST-SNAP-25b-containing complexes (and any unassembled GST-SNAP-25b) from unassembled His_6-S-tag-fusion proteins. Following extensive washing with PBS, bound proteins were eluted from the beads with 20 mM reduced glutathione in PBS, and then directly incubated with Protein-S-agarose beads (EMD Biosciences Novagen) to isolate SNARE complexes from any unassembled GST-SNAP-25b. Following extensive washing with PBS, beads were either incubated with 2× SDS-PAGE sample buffer (with or without heating at 95ºC, to verify the assembly of SDS-resistant SNARE complexes) or used directly for *in vitro* binding experiments.

Protein electrophoresis and immunoblotting

SDS-PAGE and immunoblotting analyses were performed as described previously.[14] For quantitative analysis of protein expression levels, enhanced chemiluminescence signals within the linear range were captured as images on Kodak X-ray films, scanned at a resolution of 720 dots per inch and 8 bits per pixel, and digitally integrated using NIH Image 1.62 software. Background-corrected values were normalized as follows: for the comparison of relative protein levels in cerebral cortex through the developmental period spanning E14 through P45 (Figure 1b), each value was divided by the sum of values derived from the same immunoblot image for one sample each of P1, P7, P14 and P21. For the comparison of relative protein levels in cerebral cortex and hippocampus of P1 versus P45 mice, as well as in cerebellum of P7 versus P45 mice (Figure 2), each background-corrected value was divided by the sum of values calculated from the same immunoblot image for one sample each of P45 cortex, hippocampus and cerebellum (in immunoblots containing more than one sample per P45 brain region, averages values per brain region were used for normalization). In both sets of experiments, equal protein loading was first verified by Coomassie Brilliant Blue staining of duplicate SDS-PAGE gels, and each extract was also analyzed for relative protein levels of α-tubulin and β-actin, often by stripping the membranes that had been probed with anti-dysbindin and re-probing them with mAbs to these control proteins. While Figures 1b and 2 show the values obtained for each protein separately, similar results were obtained by further normalization according to the relative β-actin levels of each sample (*e.g.*, dysbindin/β-actin ratios; data not shown).

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Table S1 Summary of experiments performed to test for effects of BLOC-1 deficiency on neurite outgrowth using primary hippocampal neuronal cultures

Independent primary culture preparation	*Culture medium*	*1 day in culture*		*3 days in culture*	
		Wild type	*Pallid*	*Wild type*	*Pallid*
1	Serum free (Tii)	32	26	17	17
2	Serum free (Tii)	63	64	17	15
3	Serum free (Tii)	–	–	25	25
4	Serum free (Tii)	–	–	15	34[a]
5	DMEM + serum	–	–	53[a]	52[a]
All		95	90	127	143

Values indicate the numbers of randomly selected, microscopic field images that were analyzed using a blinded approach. An average of 6.2 neurons per image were analyzed. Calculated means per image were used for statistical analyses.

[a] Data obtained for two primary cultures, prepared from pups born to different female mice and processed in parallel, were pooled.

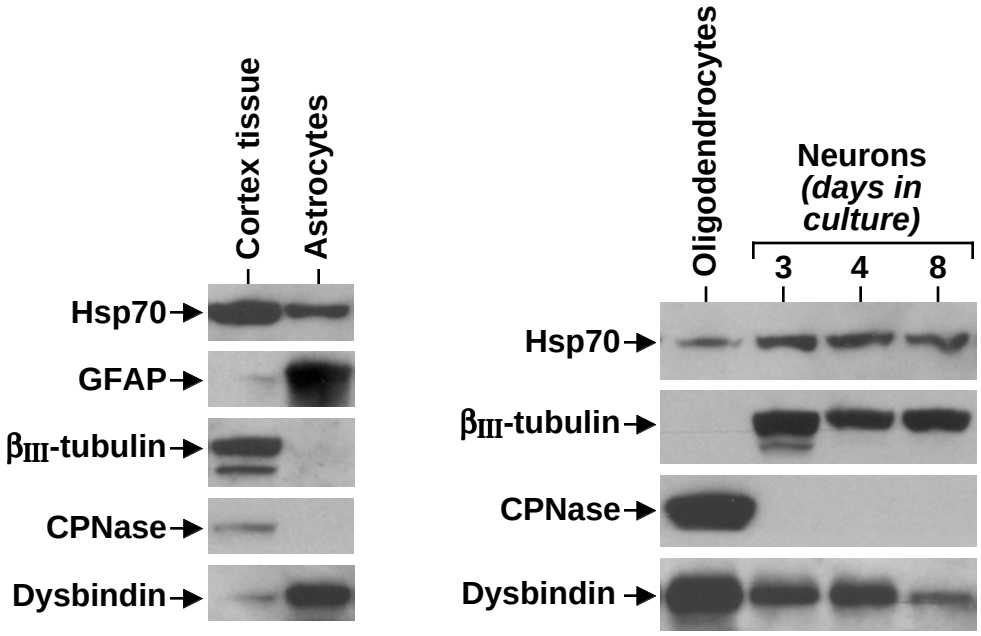

Figure S1 Endogenous expression of dysbindin protein in primary cortical cultures of mouse astrocytes and of rat oligodendrocytes and neurons. Cell lysates, and whole-tissue extract prepared from adult mouse cerebral cortex (~18 μg total protein), were analyzed by immunoblotting using antibodies against dysbindin, heat-shock protein 70 (Hsp70; a housekeeping gene product), glial fibrillary acidic protein (GFAP; an astrocytic marker), class III β-tubulin (βIII-tubulin, a neuronal marker) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, an oligodendrocytic marker).

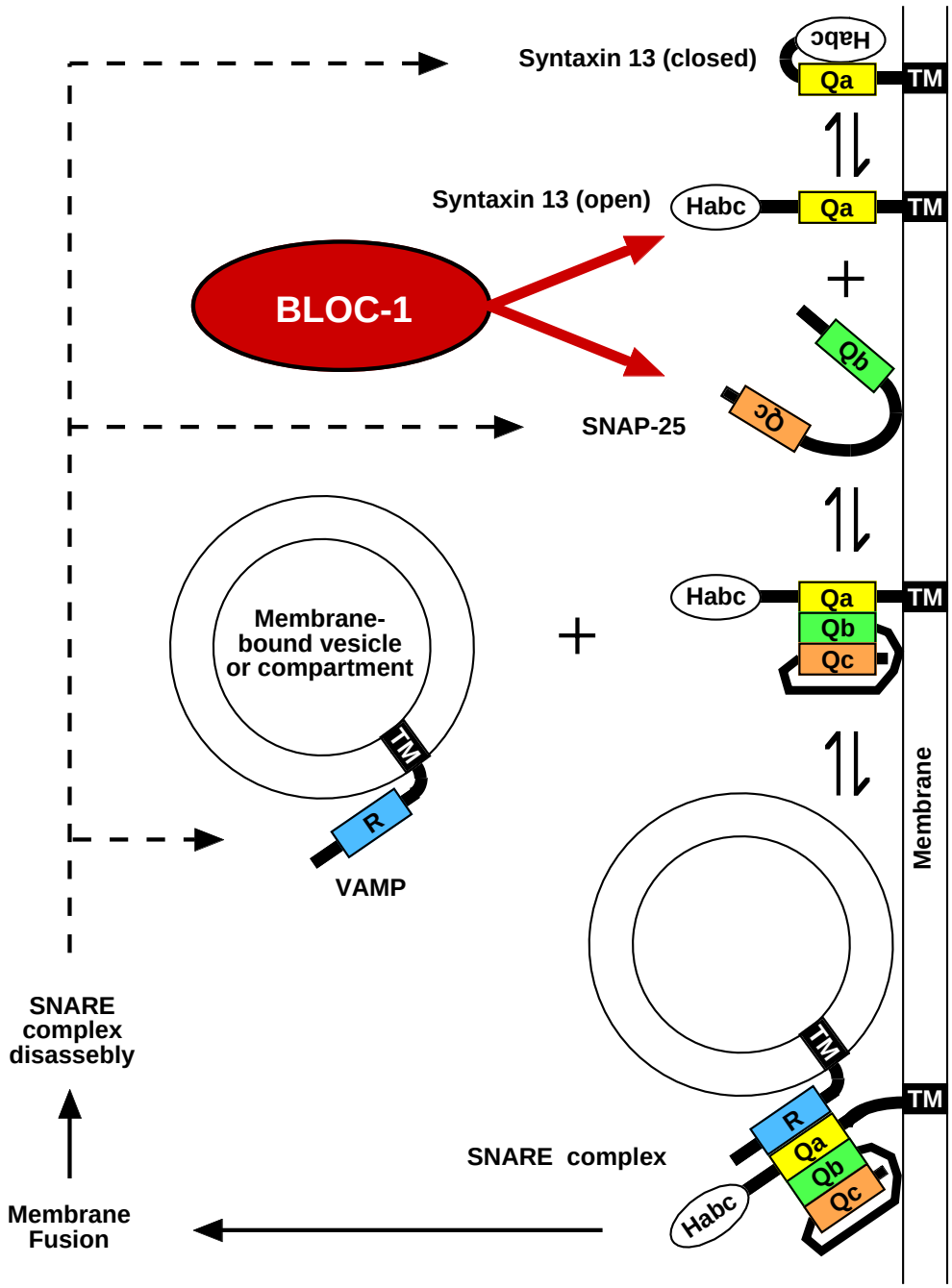

Figure S2 Cartoon representation of the SNARE complex assembly/disassembly cycle during membrane docking and fusion, indicating conformational states to which BLOC-1 may bind. The SNARE domains are classified into R, Qa, Qb and Qc types. Syntaxins contain an autoinhibitory domain (Habc), and both syntaxins and VAMPs typically contain a transmembrane domain (TM). For the sake of clarity, proteins and their functional domains are depicted out of scale.

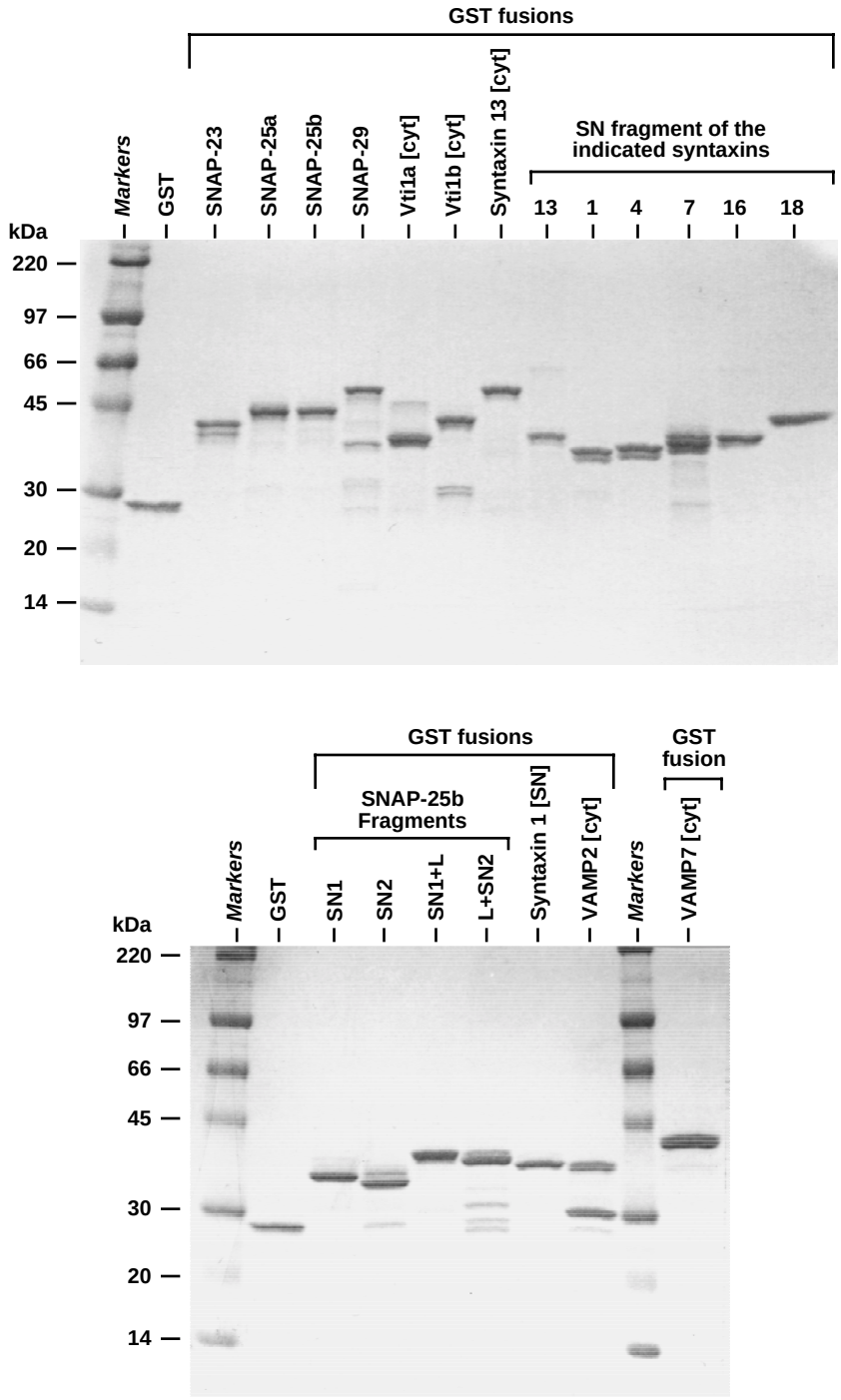

Figure S3 Recombinant proteins used for the experiments shown in Figure 4. Proteins were analyzed by 4-20% SDS-PAGE (Laemmli system) and stained with Coomassie Brilliant Blue. The amount of each protein corresponds to one sixth of the amount used for the pulldown experiments shown in Figure 4, except for the GST-fusion protein containing the SN domain of syntaxin 13, which corresponds to one ninth.

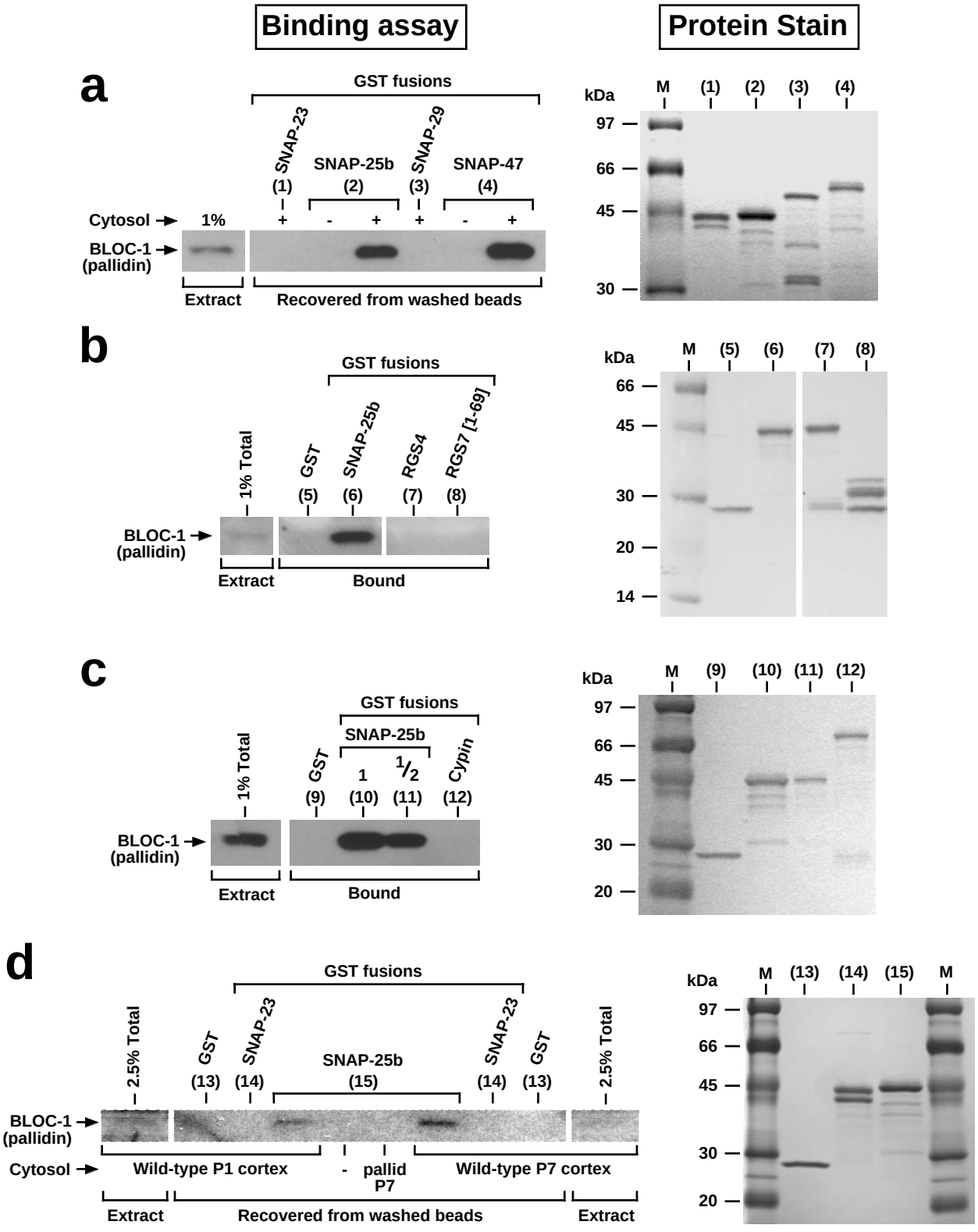

Figure S4 *In vitro* pulldown binding assays using cytosol from bovine brain (**a**-**c**) or mouse cerebral cortex (**d**) as a source of native BLOC-1. Recombinant GST-fusion proteins were bound to gluthathione-Sepharose beads and incubated with cytosol (except for samples labeled with the negative sign "-"). Proteins in the washed beads were analyzed by immunoblotting using a mAb against the pallidin subunit of BLOC-1, which is absent from pallid mutant mice. The images on the *right* show the SDS-PAGE analysis of one fifth (**a, c**), one sixth (**b**), or one fourth (**d**) of the amounts of GST-fusion proteins used for binding.

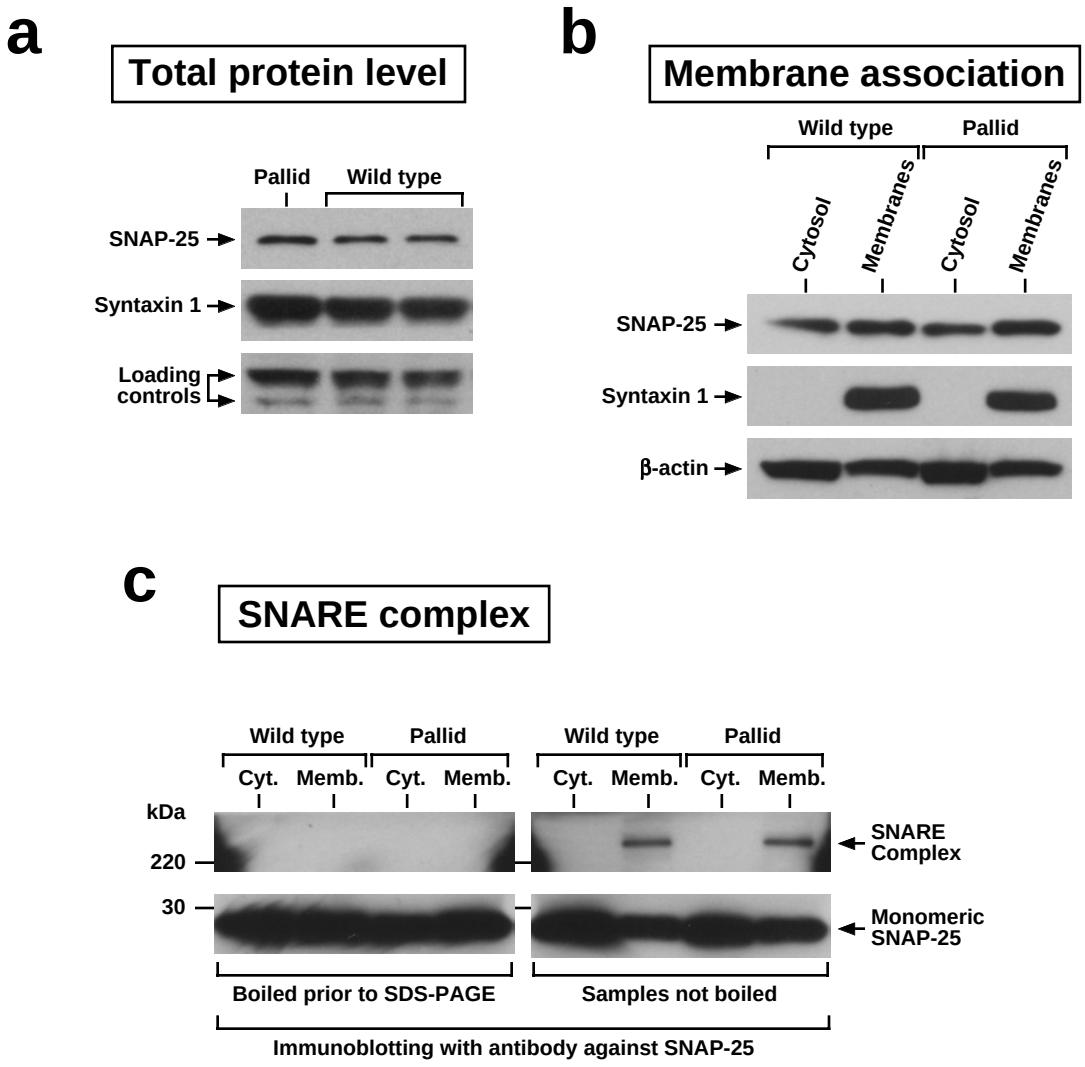

Figure S5 Analysis of steady-state levels of total protein (**a**), membrane association (**b**) and SNARE complex assembly (**c**) of SNAP-25 in the cortex of wild-type and BLOC-1-deficient pallid mutant mice. (**a**) Whole-tissue extracts (~20 μg total protein) were analyzed by immunoblotting using antibodies to SNAP-25 and syntaxin 1. (**b**) The soluble fraction (cytosol) and post-nuclear membrane pellet (membranes) prepared from equivalent wet-tissue amounts of cortex from wild-type and pallid mice were analyzed by immunoblotting using antibodies to the indicated proteins. (**c**) Cytosol and membranes prepared as in panel (**b**) were divided into two equivalent aliquots; one set of samples was boiled in the presence of SDS-PAGE sample buffer (*left*) while the other was kept at room temperature in the same buffer to reveal the presence of a SNAP-25-containing, SDS-resistant SNARE complex (*right*). All four images in (**c**) are portions of a single immunoblot and were acquired and processed under identical conditions.

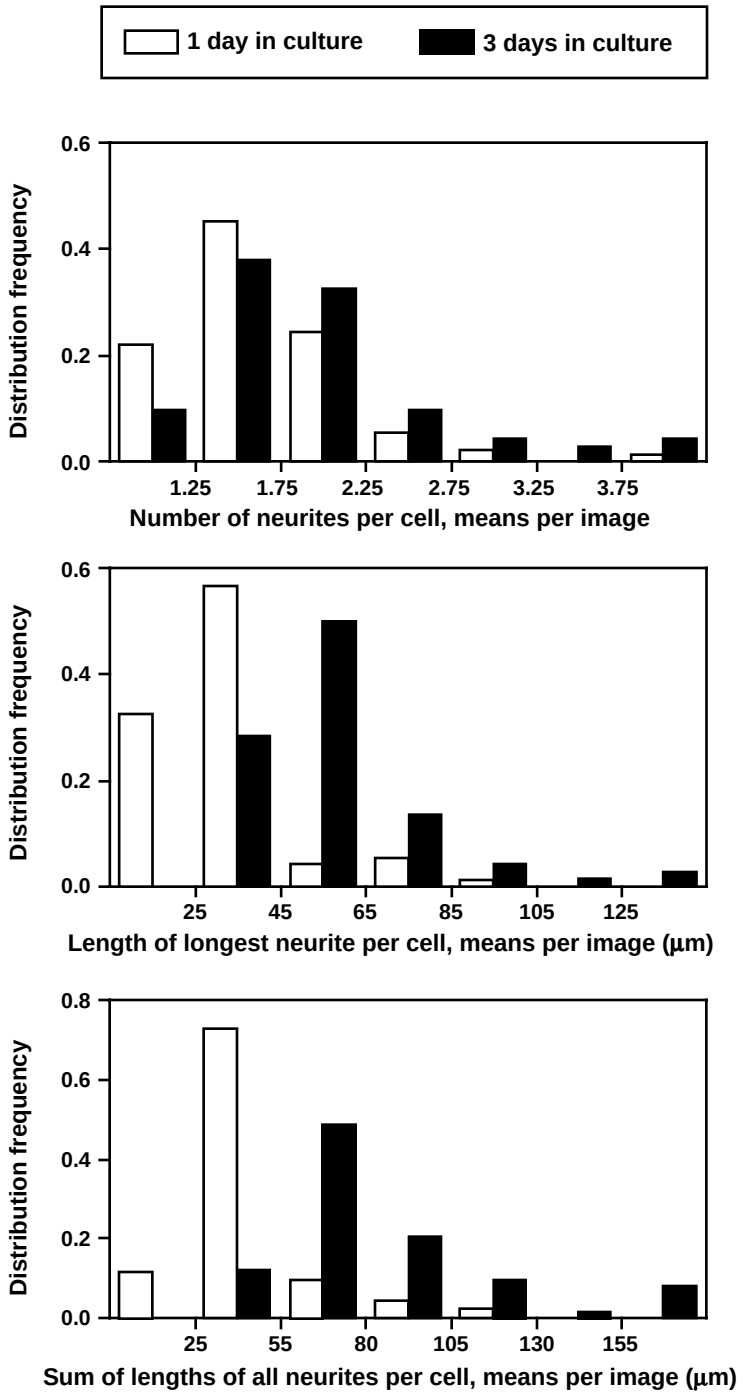

Figure S6 Frequency distributions of values obtained by averaging, for each randomly selected microscopic image, the number of neurites per neuron (*top panel*), the length of the longest neurite per neuron (*middle panel*) and the sums of lengths of all neurites per neuron (*bottom panel*). Image-based analyses were performed on wild-type cells cultured for one day (*empty bars*) or three days (*solid bars*) in Tii medium. Numbers on the *x*-axes denote bin limits; the first bin on the left includes all values smaller than the first limit, and the last bin on the right includes any value greater than the last limit.