Supporting Information

PI(4,5)P_2-dependent regulation of exocytosis by amisyn, the vertebrate-specific competitor of synaptobrevin 2

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Material and Methods

Plasmids and material

Full length cDNA coding for amisyn (GenBank: BC009499.2, aa 1-210) was amplified by PCR using 5´-CCGGAATTCATGAGTGCCAAATCTGCTATCAG-3´ and 5´-CGCGGATCCCCACATTTGTGCTTCATGGCAAGCT-3´ primers (construct kindly provided by Dr Thomas Weimbs, University of Santa Barbara), and sub-cloned in the pEGFP-N1 vector using EcoRI and BamHI restriction sites. Subsequently cDNA was amplified using 5'-CGGGATCCATGAGTGCCAAATCTGCTATCAG-3' and 5'-CGGAATTCCGTTATTAACATTTGTGCTTCATGGCAAGCT-3' primers and sub-cloned in the pGEX-6p1 vector using BamHI and EcoRI restriction sites. The SNARE motif of amisyn (aa 162-210) was amplified using 5´-CCGGAATTCATGATCACAAAGGTCAAACAGTTT-3´ and 5´-CGCGGATCCCCACATTTGTGCTTCATGGCAAGCT-3´ primers and sub-cloned in the pEGFP-N1 vector using the EcoRI and BamHI resriction sites. It was then amplified using 5'- CGGGATCCATGATCACAAAGGTCAAACAGTTT-3' and 5'-CGGAATTCCGTTATTAACATTTGTGCTTCATGGCAAGCT-3' primers and sub-cloned in the pGEX-6p1 vector using BamHI and EcoRI restriction sites. The amisyn AADD/PH-domain mutant was generated by introducing 4-point mutations in the PH domain (aa 2-131): K30A, K32A, K64D and K66D by site directed mutagenesis (QuikChange II XL; Agilent, Ratingen, Germany) in the respective plasmids. Primer pairs designed to generate the mutations were:

K30A (5'-GTCCAAGTCAAGAGGAGGACAAAGAAAAAGATTCCTTTCTTGGC-3' and 5'-GCCAAGAAAGGAATCTTTTTCTTTGTCCTCCTCTTGACTTGGAC-3'),

K32A (5'-GTCAAGAGGAGGACAAAGAAAAAGATTCCTTTCTTGGCAACTGG-3' and 5'-CCAGTTGCCAAGAAAGGAATCTTTTTCTTTGTCCTCCTCTTGAC-3'),

K64D (5'-CACACAGGCGTCCATCACAAAGGTCAAACAGTTTGAAGGCT-3' and 5'-AGCCTTCAAACTGTTTGACCTTTGTGATGGACGCCTGTGTG-3') and

K66D (5'-GCGTCCATCACAAAGGTCAAACAGTTTGAAGGCTCCACA-3' and 5'-TGTGGAGCCTTCAAACTGTTTGACCTTTGTGATGGACGC-3').

The PH domain of amisyn, both with and without point mutations, was amplified using 5'-CCGGAATTCATGAGTGCCAAATCTGCTATCAG-3' and 5'-CGCGGATCCGCCTGGCAGGTATGGTGGAG-3' primers and subsequently sub-cloned in the pEGFP-N1 vector using the EcoRI and BamHI restriction sites. They were then amplified using 5'-CGCGGATCCATGAGTGCCAAATCTGCTATCAG-3' and 5'-CCGGAATTCTTATCACTGGCAGGTATGGTGGAG-3' primers and subsequently subcloned in the pGEX-6p1 vector using BamHI and EcoRI restriction sites. All constructs were checked by both control restriction enzyme digestions and by sequencing.

All material, reagents and software used in this study is listed in the Dataset S1.

Cell culture, cell transfections and cell stimulations

The neuroendocrine PC12 (ATCC CRL-1651TM) cells were maintained and propagated in 75 cm^2 flasks coated with 0.1 mg/ml poly-L-lysine (PLL, Sigma, St Louis, MS) in growth medium DMEM with 4.5 g/l glucose, 10% fetal calf serum (FCS), 5%

horse serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37℃ in 5% CO_2. PC12 cells (until passage 14) were transfected with plasmids expressing amisyn-EGFP, mRFP-PI4P5KI and mRFP-IPP1-CAAX (1). In some experiments, EGFP expressed from pEGFP-N1 was used as a control. Cell transfection was achieved using 1 µg plasmid DNA mixed with 3 µl Lipofectamine in 200 µl Opti-MEM medium (Gibco, ThermoFisher Scientific, San Jose, CA, USA). Cultures were incubated for 6 hrs with the Lipofectamine/DNA mix, after which the cells were maintained in fresh growth medium for 18-30 hrs before analysis. PC12 cells were either imaged live, used for plasma membrane sheet preparations, or fixed in 3.7% paraformaldehyde (PFA) in Phosphate-Buffered Saline (PBS) for 30 min at room temperature and further processed for confocal microscopy. Stimulation of PC12 cells was achieved with 59 mM KCl (Sigma), nicotine (Merck, N0267), or ionomycin (Merck, I0634) in Tyrode's buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES pH 7.4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 6g/l glucose), as indicated in the figure legends.
Bovine chromaffin cells (BCCs; ~5×10^5 cells) were plated on ø18 mm glass coverslips that had been pre-treated with a 0.1 mg/ml poly-L-lysine (Sigma, St Louis, MS, USA) for 30 min and kept at 37°C in 8% CO2. Cells were used for experiments 24 h after plating.

Protein expression, purification and labelling

Unless otherwise stated, proteins from other expression constructs were expressed through the pET28a expression vector (Novagen) in *E. coli BL 21* (DE3) competent cells (Merck). 10 ml of overnight culture was used to inoculate 1L Terrific Broth medium (TB: yeast extract 24 g/L, tryptone 20 g/L; glycerol 4 mL/L; 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) containing appropriate antibiotic, and the cells were grown until an OD_{600} of ~ 0.8. The recombinant protein expression was induced by the addition of IPTG (60 mg/L) for 4 hours at 37°C. Subsequently, the cells were pelleted and resuspended in 20 ml resuspension buffer (20mM Tris pH 7.4, 1 mM EDTA), and lysed with the combination of lysozyme (Sigma) treatment and sonication in the presence of DNase (Sigma) and PMSF (Roth; 200mM). All the proteins were soluble and contained a cleavable His-tag for affinity purification. The cell lysate was centrifuged at 13,000g to remove the cell debris and the supernatant was used for the affinity purification on Ni^{2+}-nitrilotriacetic acid beads (Qiagen; 1ml beads per 5 mg of recombinant protein was used). The beads were washed with 20mM Tris pH 7.4, 500 mM NaCl, and 8 mM imidazole, and the protein was eluted using three bead volumes of elution buffer (20mM Tris pH 7.4, 500 mM NaCl, 400 mM imidazole). The affinity purification was followed by ion exchange chromatography on an ÄKTA system (Amersham Biosciences). The proteins were quantified using Pierce™ BCA Protein Assay Kit (ThermoScientific, #23225).
The proteins were labelled following manufacturer's instructions using the sulfhydryl-reactive fluorophores Oregon Green 488 iodoacetamide, Texas Red C5-bromoacetamide or Alexa Fluor™ 488 C5 maleimide (Molecular Probes, Invitrogen). The labelled protein was separated from the dye by size exclusion chromatography on

a sephadex G-25 column (Amersham Biosciences). The labelling efficiency was later calculated as per the manufacturer's instructions.

Antibodies

The following antibodies were used: anti-amisyn (custom-made Aminchen, #172, rabbit, Western blot 1:500), anti-amisyn (Sigma HPA003552, rabbit, Western blot 1:500), anti-GAPDH (Sigma, #G9545, rabbit, Western blot 1:1000), anti-Na^+/K^+ ATPase (SYSY, #130930, mouse, Western blot 1:1000), anti-GST (Millipore, #ABN116, rabbit, Western blot 1:1000); anti-actin (Millipore, #C4, mouse, Western blot 1:10000), anti-mouse IRDye800CW and anti-rabbit IRDye680LT (LI-COR, 1:5000).
Custom-made anti-amisyn antibody (Aminchen #172) was affinity purified using the immobilized (in the column) recombinant amisyn protein. The column was washed with 0.1% Tween in Tris-buffered saline (TBS; 50 mM Tris-Cl pH 7.3, 150 mM NaCl) and TBS alone, before elution with 0.1 M glycine. The eluate was immediately neutralized with 1.5 M Tris (pH 7.3). The titer and specificity of the amisyn antibodies was verified using both native, expressed and recombinant amisyn blotted onto nitrocellulose membranes, as detailed in Results and SI Appendix, Fig S3.

Circular Dichroism spectroscopy

Proteins were diluted in the recording buffer (50 mM KCl, 25mM Tris/HCl pH 7.5) to a final concentration of 0.3 mg/ml. Data were collected on a Chirascan CD spectrometer (Applied Photophysics). Buffer without protein was used as a control.

Anisotropy measurements

Anisotropy measurements were performed on a Fluorolog 3 spectrometer with magnetic stirrer and built-in T-configuration equipped for polarization (Model FL322, Jobin Yvon). The interaction of amisyn and syntaxin-1 was examined using 200 nM syntaxin-1 (aa 1-288) labelled with Oregon Green™ 488 (ThermoFischer, O-10241). For competition assays, different concentrations of synaptobrevin-2 (aa 1-96) labelled with Oregon Green™ 488 at C28 were used. The concentrations of amisyn are indicated in the Figure legends, the concentration of the syntaxin-1:SNAP-25 complex was 400 nM. Anisotropy (r) was calculated using the formula $r = (IVV\ G \times IVH)/(IVV + 2 \times G \times IVH)$, where "I" denotes the fluorescence intensity, and the first and second subscript letters indicate the polarization of the exciting light and the emitting light, respectively. For Oregon Green-labelled synaptobrevin-2 or syntaxin-1, the excitation wavelength was set to 490 nm and the emission wavelength was set to 520 nm. All experiments were performed at 37°C and in a reaction volume of 600 µl.

Preparation of Giant Unilamellar Vesicles (GUVs)

GUVs were prepared from 1 µl drops of the indicated lipids applied to indium/tin oxide-covered glass slides: 49,5 mol% L-α-phosphatidylcholine/PC, 30 mol% L-α-phosphatidylethanolamine/PE, 20 mol% L-α-phosphatidylserine/PS and 0,5 mol%Atto647N PE, or 44,5 mol% L-α-phosphatidylcholine/PC, 30 mol% L-α-phosphatidylethanolamine/PE, 20 mol% L-α-phosphatidylserine/PS, 5 mol% L-α-

phosphatidylinositol-4,5-bisphosphate/PI(4,5)P_2 and 0,5 mol% Atto647N PE, using a commercially available GUV generation setup (Vesicle Prep Pro, Nanion). Lipids are purchased from Avanti Polar Lipids (Alabaster, AL, USA). Homogenous GUVs were prepared freshly for each experiment and used immediately.

Liposome sedimentation assay

Liposomes were prepared from 45 mol% phosphatidylcholine (PC), 30 mol% phosphatidylethanolamine (PE), 20 mol% phosphatidylserine (PS) and indicated amounts of PI(4,5)P_2 (PC concentrations were adjusted to accommodate the changing PI(4,5)P_2 concentrations). Lipids were mixed and dried under a stream of nitrogen, with remaining solvents depleted by desiccation of the lipid mix for 2 h. Buffer was added to adjust final concentrations of liposomes to 1 mg/ml. Liposomes were hydrated in 150 mM NaCl and 20 mM Hepes pH 7.4 for 1 hr, followed by seven freeze-thaw cycles using liquid nitrogen. Subsequently, liposomes were extruded at least 20 times through a 100-nm filter (Nucleopore, Track-Etch, Whatman) and used immediately by incubation with 10 µM recombinant amisyn for 20 min at 37°C. The liposomes were then centrifuged for 1 hr at 70,000 rpm (Sorvall RC-M120, rotor S120-AT3). Supernatant and pellet were separated, analysed by SDS-PAGE, and the gel was scanned (EPSON Perfection V700 Photo).

Proteoliposome preparation

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), cholesterol, 1-oleoyl-2-{12-[(7-nitro-2-1,3- benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphoserine (NBD-PS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Proteoliposome preparation was performed as described (2, 3) at molar (%) ratio: 50% DOPC : 20% DOPE : 20% DOPS :10% cholesterol. For the preparation of labelled liposomes, the ratio of lipids was 50% DOPC: 18.5% DOPE: 1.5% Rhodamine-PE: 18.5% DOPS: 1.5% NBD-PS: 10% cholesterol. After drying, the lipid film was hydrated in 150 mM KCl, 20 mM HEPES pH 7.4, 5% (w/v) sodium cholate, and vortexed thoroughly. The respective protein(s) were added in 150 mM KCl, 20 mM HEPES pH 7.4, 5% (w/v) sodium cholat. Proteoliposomes were prepared by detergent removal using gel-filtration chromatography (Sephadex G-50) as in (4). The final proteoliposome preparation buffer was 150 mM KCl, 20 mM HEPES, 1 mM DTT, pH 7.4 with a final protein: lipid (n/n) ratio of 1:1000.

Liposome fusion assays

The ΔN ternary complex, which contains the SNARE motif and transmembrane domain of syntaxin-1 (residues 183-288), SNAP-25 and Synaptobrevin-2 (Syb) (49-96) was incorporated into a population of liposomes, termed the acceptor liposomes. The full-length Syb protein (1-116) was incorporated into another population of liposomes, termed donor liposomes. The donor liposomes contained quenched populations of two

fluorophores 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) and Rhodamine (ThermoFischer) coupled to lipids. Fusion with acceptor liposomes increases the total surface area and therefore the average distance between the fluorophores, resulting in an increase in donor (NBD) fluorescence. Liposome fusion reactions were performed at 30°C with 15 µl labelled and unlabelled liposomes mixed in a total volume of 1.2 ml, resulting in final protein concentrations of 200 nM for both liposome populations. Fluorescence de-quenching was measured at 460 nm excitation and 538 nm emission wavelengths. Fluorescence intensities were normalized to the initial fluorescence intensity.

Amisyn knock-down in primary neurons

The primary cultures of mouse cortical neurons were prepared as described in Murdoch et al (5). The siRNAs against mouse amisyn were bought as a pool of two siRNAs targeting the same gene in different sites (mm.Ri.Stxbp6.13.1 and mm.Ri.Stxbp6.13.2; 150 nM per transfection) from Integrated DNA Technologies. Negative control siRNA was designed to have minimal targeting of known genes in mouse cells (51-01-19-08; Integrated DNA Technologies). The siRNAs were diluted in in RNase-free water according to the manufacturer's instructions, aliquoted and stored at − 20°C. For delivery of siRNA into neuronal cells, primary cortical cells in suspension (P3 Primary Cell 4D-Nucleofector™ X Solution; Lonza) were electroporated using Amaxa™ 4D-Nucleofector™ (Lonza) just before plating. Cells were harvested for protein analysis 7-9 days after transfection.

Protein electrophoresis and immunoblotting

Plasma membranes isolated from PC12 cells as detailed above were resuspended and boiled in SDS-PAGE buffer. Proteins were separated on 12% gels. Samples were transferred to PVDF membranes (Invitrogen) that were subsequently washed in TBS, 0.1% Tween 20 (TBS-T), before non-specific binding sites were blocked with 5% milk in TBS-T. Primary antibodies were incubated with membranes in TBS-T and 5% milk. The appropriate secondary antibody coupled to IRDye 800W or 680W (diluted 1:10,000) were incubated with the PVDF membranes for 1h in dark at room temperature. After extensive washing with TBS-T, the resulting Western blots were imaged for quantitative analysis (Odyssey Imaging System; LI-COR). Digital images were analysed by Image Studio Lite (a software package from LI-COR Biosciences) or ImageJ. Both software were used to compare the density (i.e. intensity) of bands on a digital image of the Western blot after subtracting the background signal from the adjacent area.

Plasma membrane sheet preparation and experiments

PC12 cells (2×10^5) were plated on Ø18 mm glass coverslips pre-treated with a 0.1 mg/ml PLL for 30 min, and maintained at 37°C in 5% CO_2 and 100% humidity. Transfection of PC12 cells with WT or mutated amisyn-EGFP, mRFP-PI4P5KI, or mRFP-IPP1-CAAX was performed as described above. PC12 plasma membrane sheets were generated by placing the coverslip in a round glass beaker with 150 ml ice-cold sonication buffer (120 mM potassium glutamate, 20 mM HEPES pH 7.3, 20

mM potassium acetate, 4 mm $MgCl_2$, 2 mM EGTA, 310 mOsm/kg; bubbled with N_2 for 30 min before use). The coverslip with attached cells was centred 10 mm under the sonication tip (2.5 mm), and the cells were disrupted applying a single ultrasound pulse: amplitude 25%, duty cycle 250 ms (Sonifier UP200S, Hielscher Ultrasound Technology).
In experiments with recombinant WT/mutant amisyn-EGFP, the freshly prepared membrane sheets from PC12 cells were incubated for 10 min at RT with 5 µM amisyn-EGFP in K-Glu buffer with 3% BSA. Subsequently, the sheets were washed for 100 s in K-Glu buffer and fixed for 30 min in 4% PFA in PBS at room temperature. The plasma membrane sheets were visualized with 1-(4-trimethyl-amoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Molecular Probes, Eugene, OR, USA).

Fluorescence microscopy of plasma membrane sheets of PC12 cells
Coverslips with freshly prepared membrane sheets from PC12 cells expressing wt/mutant Amisyn-EGFP, mRFP-PI4P5KI, or mRFP-IPP1-CAAX were placed in an imaging chamber in Tyrods buffer. Samples were examined by spinning-disc confocal microscopy (UltraVIEW VoX, Perkin Elmer, Waltham, MA, USA) with spinning disk confocal scan head (CSU-X1, Yokogawa, japan) on an inverted microscope (Nikon Eclipse Ti-E, Japan) with a Plan APO ʌ 60x / 1.40 Oil DIC N2 ∞ / 0,17 WD 0.13 OFN 25 objective, operated by Volocity software (Improvision). Images were recorded digitally (EMCCD iXon 897 camera, Andor Technologies). The samples were illuminated using a diode pumped solid state (DPSS) laser 488 nm/50 mW diode and cobalt solid state 561 nm/50 mW diode laser.
Digital image analysis of data shown in Figures 2I, 3C, 4D, 4H, 6D, 6H, 6J and 6J were performed using ImageJ (6). To perform unbiased comparative quantitation of fluorescence intensity and verify the integrity of plasma membrane patches, the plasma membranes were identified in the TMA-DPH images (blue channel). Regions of interest (ROI) were defined randomly on the membranes and transferred to the other channels. In each area, the average fluorescence intensity was quantitated. The local background was measured in the area outside the plasma membrane sheets, and subtracted. For each condition, at least 50 plasma membrane sheets from 3 independent experiments were analysed. Fluorescence intensity values are reported as mean±SEM.

Live imaging of PC12 cells
Live imaging of PC12 cells was performed using the spinning-disk setup detailed above with temperature control unit (kept at 37°C) and with a custom-built imaging chamber. For live recordings, cells were kept in imaging buffer (136 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4). PC12 cells were stimulated by addition of 59 mM KCl (77 mM NaCl, 59 mM KCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4). The digital images were recorded at 120 frames/min for 8 min. All fluorophores were excited at low laser power (3-8%) and short exposure times (50-100 ms). For post-acquisition analysis (ImageJ), ROI was marked near the cell edge, in each stimulated cell, and the change in intensity with time was measured.

The fluorescence at any given time point was normalized to the average of first 10 frames.

Electrophysiology and electrochemistry

Bovine adrenal chromaffin cells were maintained in extracellular solution (in mM: 145 NaCl, 2.8 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES pH 7.25, 2 mg/ml D-glucose, ~303 mOsM/kg) during the electrophysiological and electrochemistry recordings, which were performed at room temperature (22-24˚C). Capacitance and amperometric measurements were performed concurrently, and as described in (1) with minor modifications. Here, Zeiss Observer.D1 microscope equipped with Polychrome V monochromator (Till Photonics), an EPC-10 amplifier (HEKA Elektronik) for patch-clamp capacitance measurements and an EPC-9 (HEKA Elektronik) for amperometry were used. Catecholamine release was triggered by UV-flash photolysis (using a JML-C2, Rapp Optoelektronik) of a caged calcium compound nitrophenyl-EGTA, which was injected into each cell through a patch pipette. Amperometric recordings were done using Ø5 µm carbon fibers (Thornel P-650/42, Cytec) insulated by the polyethylene method. Carbon fibers were clamped to +700 mV. Currents were acquired at 25 kHz and filtered off-line using a Gaussian filter with a cut-off set at 1 kHz. Before electrophysiological and electrochemistry experiments were started, the setup was calibrated for calcium ratiometric measurements with two calcium dyes with different calcium binding affinities (Fura4F and Furaptra, ThermoFischer Scientific) by infusion of 8 solutions of known calcium concentrations into bovine chromaffin cells (4-5 cells/solution). The excitation light (originating from Polychrome V) was alternated between 350 nm and 380 nm to perform the ratiometric measurements of $[Ca^{2+}]_i$. The emitted fluorescence was detected with a photodiode (Till Photonics) and sampled using Pulse software (HEKA). The same software was used to control the voltage in the pipette and perform capacitance measurements.

Recombinant amisyn (5 µM), or SNARE-amisyn mutant (5 µM), were added to the intracellular pipette solution (in mM: 100 Cs-glutamate, 8 NaCl, 4 $CaCl_2$, 32 Cs-HEPES pH 7.25, 2 Mg-ATP, 0.3 GTP, 0.4 Fura4F and 0.4 Furaptra, 5 NPE, osmolarity ~295 mOsm/kg), and kept cold until injection into cells through the custom-made glass patch pipette (NPI Electronic, GB150TF-8P, prepared with P-97 Flaming/Brown Micropipette Puller, Sutter Instruments). Electrophysiological and amperometry experiments were performed in such way that conditions could be compared side-by-side: e.g., the WT or mutant proteins were injected in chromaffin cells from the same cell preparation, and the same number of cells for each condition is recorded on each experimental day. Number of cells indicated in Figure legends represent biological replicates; chromaffin cells are isolated from several animals, and five sets of recordings from five independent cell preparations were performed. Analysis of amperometric recordings were performed by a custom-written macro (IGOR Pro, Wave Metrics) as in (7). The kinetic analysis of the capacitance measurements was performed by fitting individual capacitance traces with a triple-exponential function using a custom-written macro (IGOR Pro, Wave Metrics). The amplitudes and time constants of the two faster exponentials as used as the size and release kinetics of the slowly releasable pool

(SRP) and the readily releasable pool (RRP), respectively. Because the time constant of the sustained component is too slow to be measured accurately in this way, this third exponential was not used directly. Instead, we subtracted the amplitudes of the fast and slow burst from the total amount of secretion during 5 s and calculated the linear rate of sustained release. Data are represented as mean±SEM, and analysed statistically by the Kruskal-Wallis test with Dunn's multiple comparison test. Statistical differences are indicated with the results (ns – non significant, *$p<0.05$, **$p<0.01$, and ***$p<0.001$).

REFERENCES

1. I. Milosevic, J. B. Sørensen, T. Lang, M. Krauss, G. Nagy, V. Haucke, R. Jahn, E. Neher, Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. J. Neurosci 25 (10), 2557-65 (2005).

2. D. Fasshauer, W. K. Eliason, A. T. Brunger, and R. Jahn, Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry 37, 10354–10362 (1998).

3. D. Fasshauer, W. Antonin, M. Margittai, S. Pabst, R. Jahn, Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. J. Biol. Chem. 274(22):15440-6 (1999).

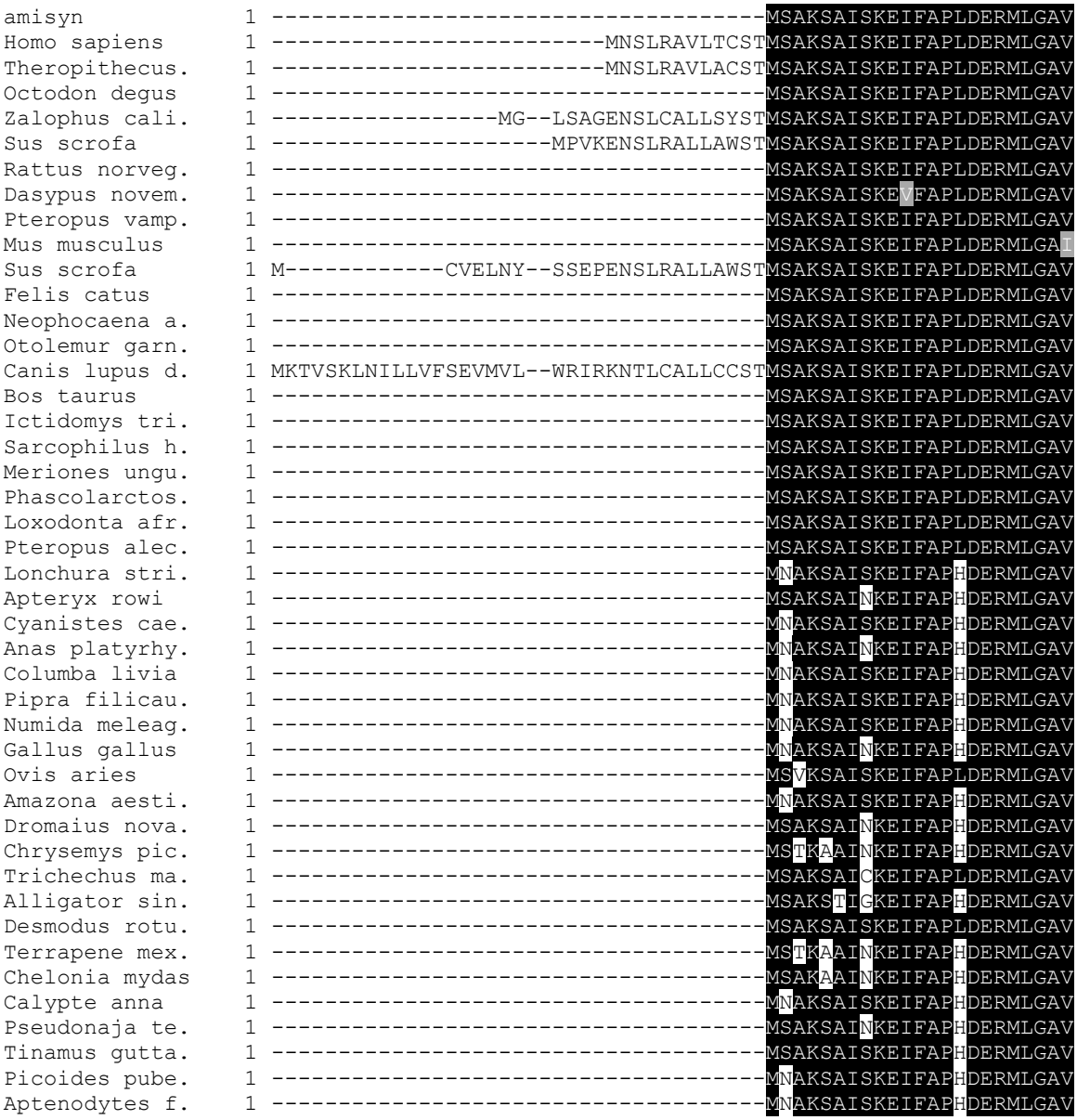
4. A. V. Pobbati, A. Stein, D. Fasshauer, N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. Science. 4;313(5787):673-6 (2006).

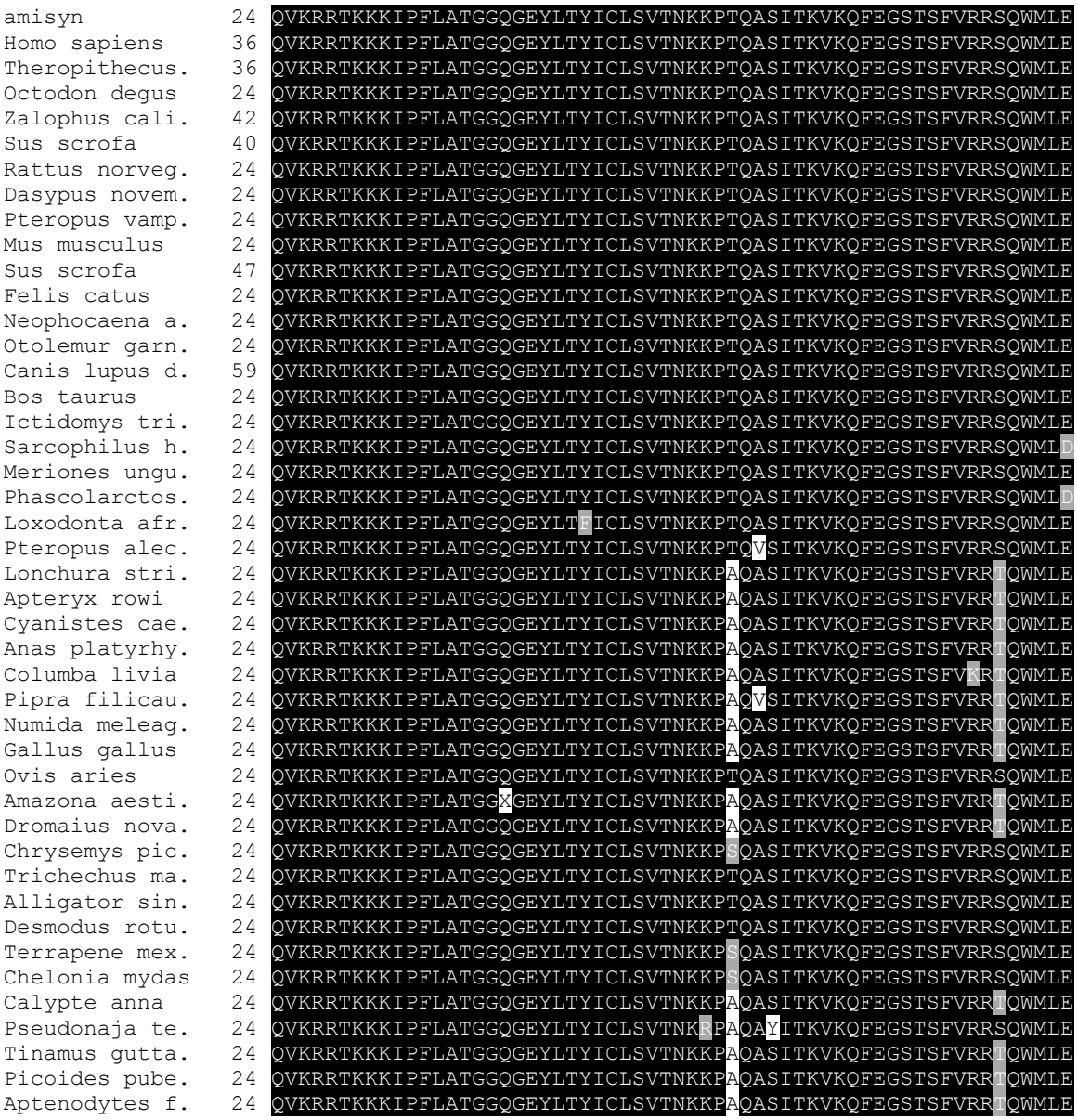
5. J. D. Murdoch et al. Endophilin-A Deficiency Induces the Foxo3a-Fbxo32 Network in the Brain and Causes Dysregulation of Autophagy and the Ubiquitin-Proteasome System. Cell Rep 17(4):1071-1086. (2016).

6. W. S. Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/ (1997-2018).

7. E. V. Mosharov, D. Sulzer, Analysis of exocytotic events recorded by amperometry. Nat. Methods. 2(9):651-8 (2005).

Supplementary Table 1.

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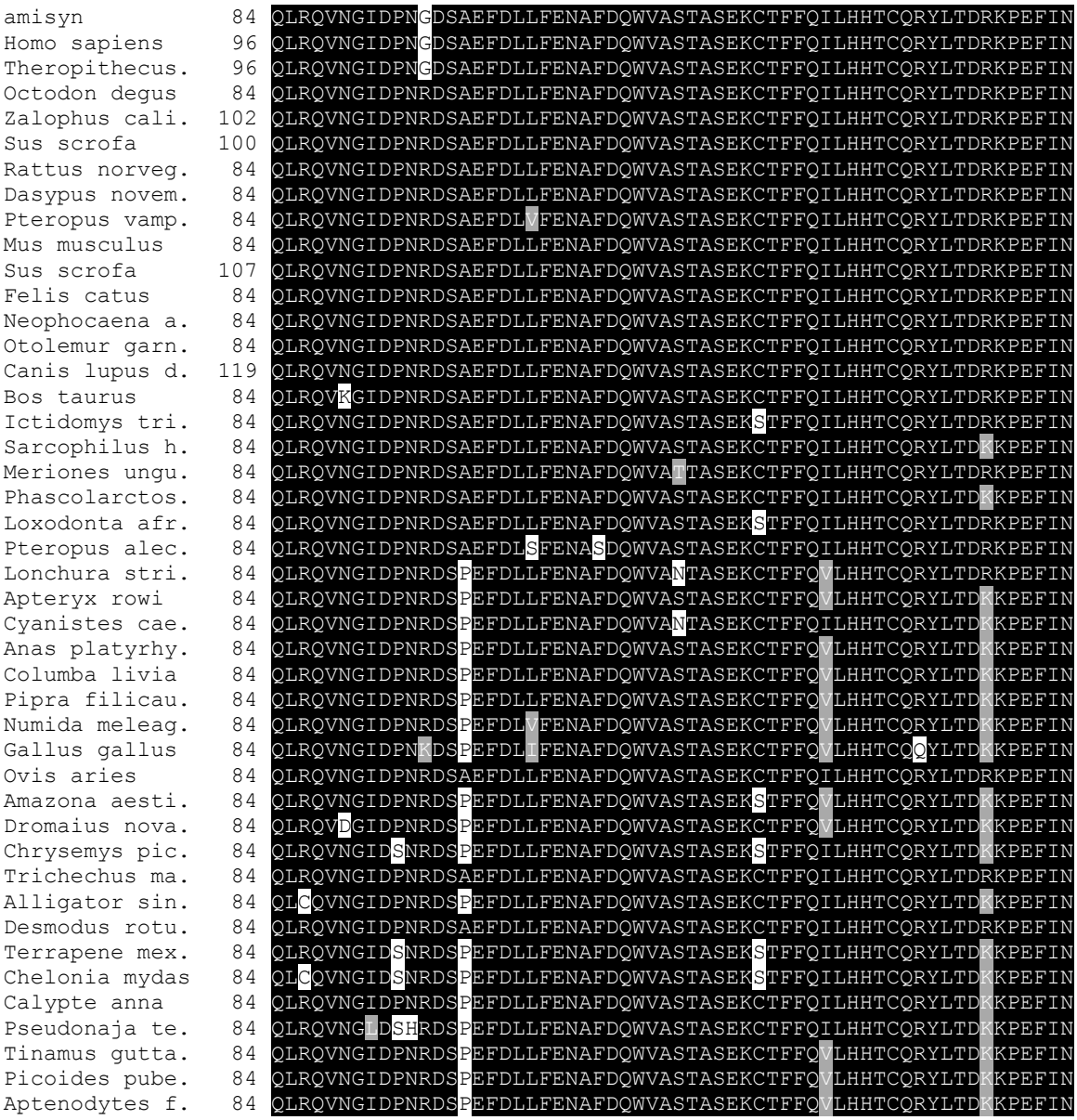
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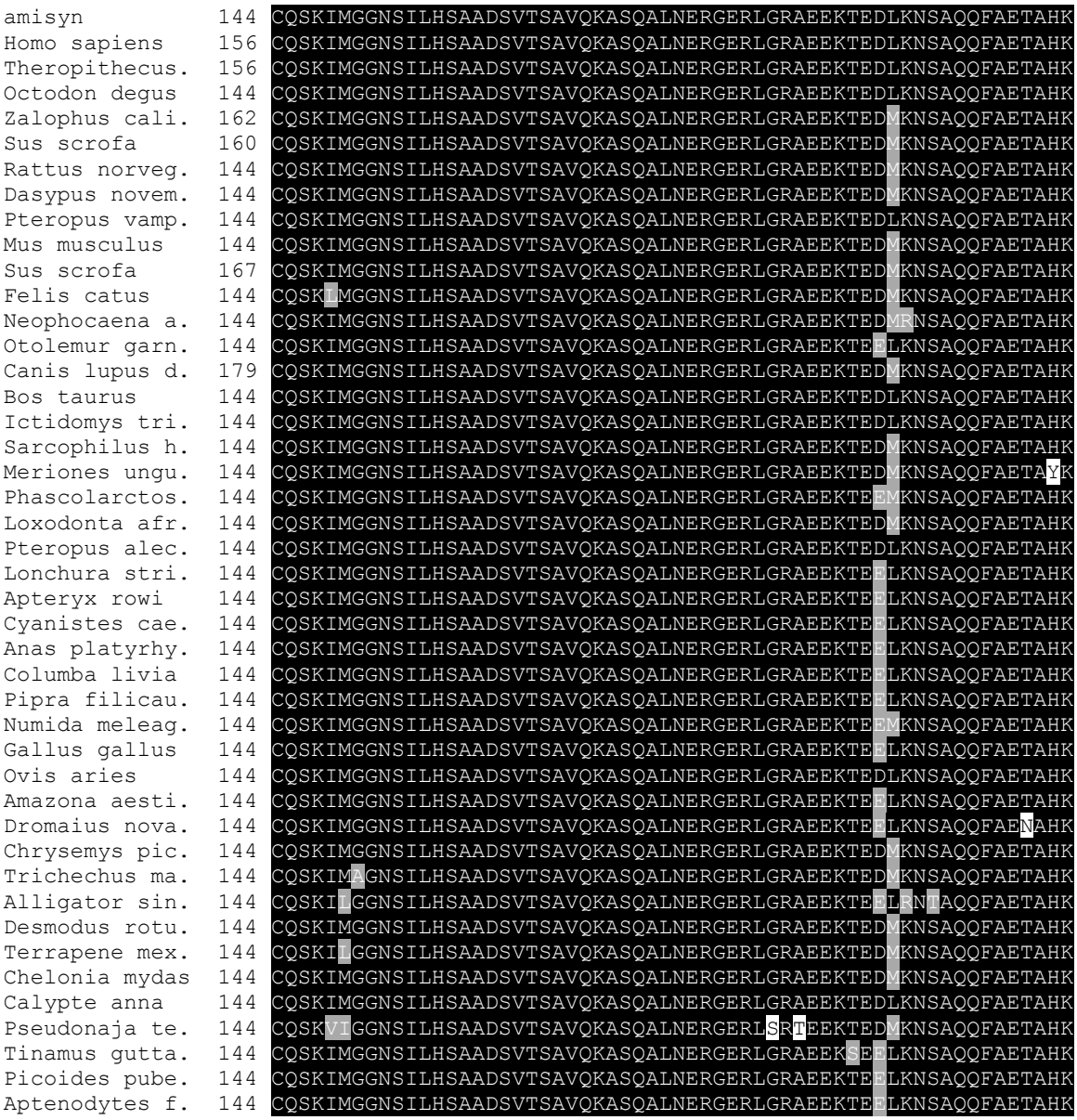
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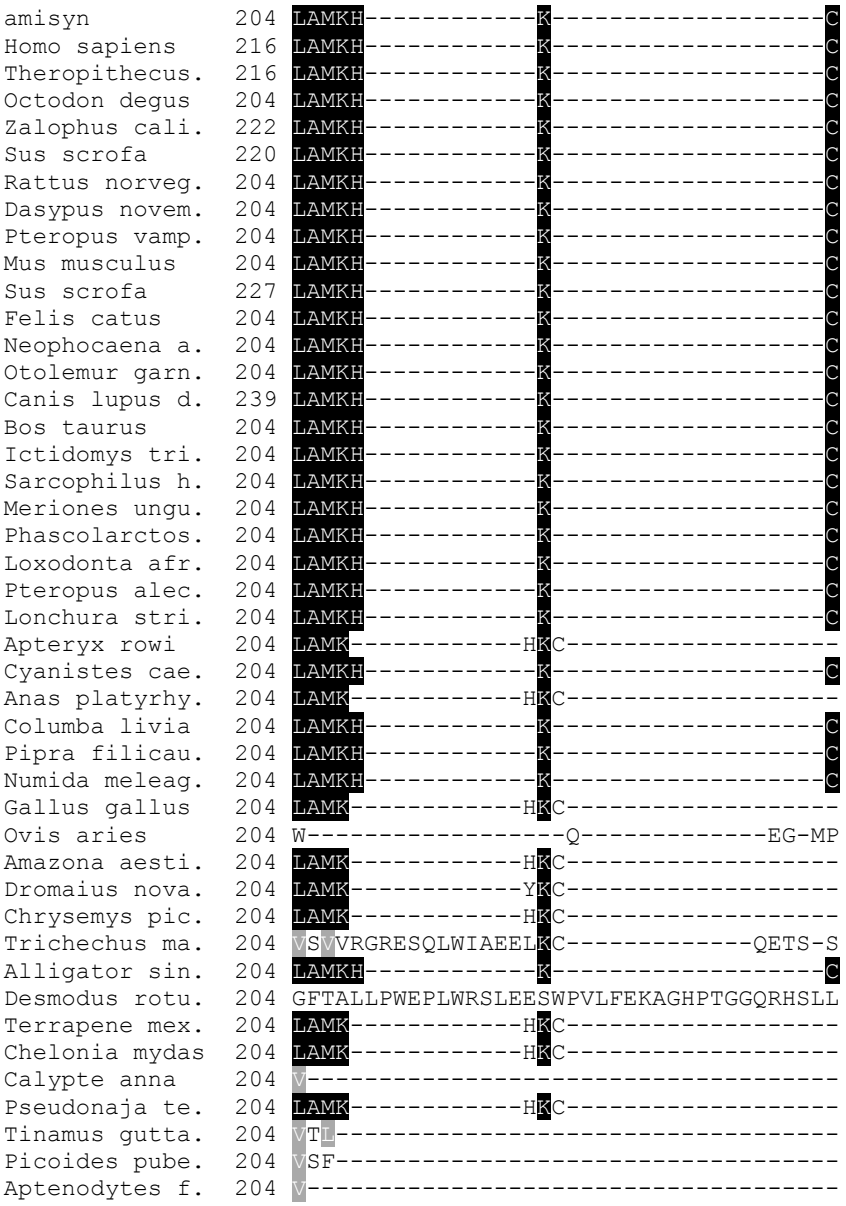
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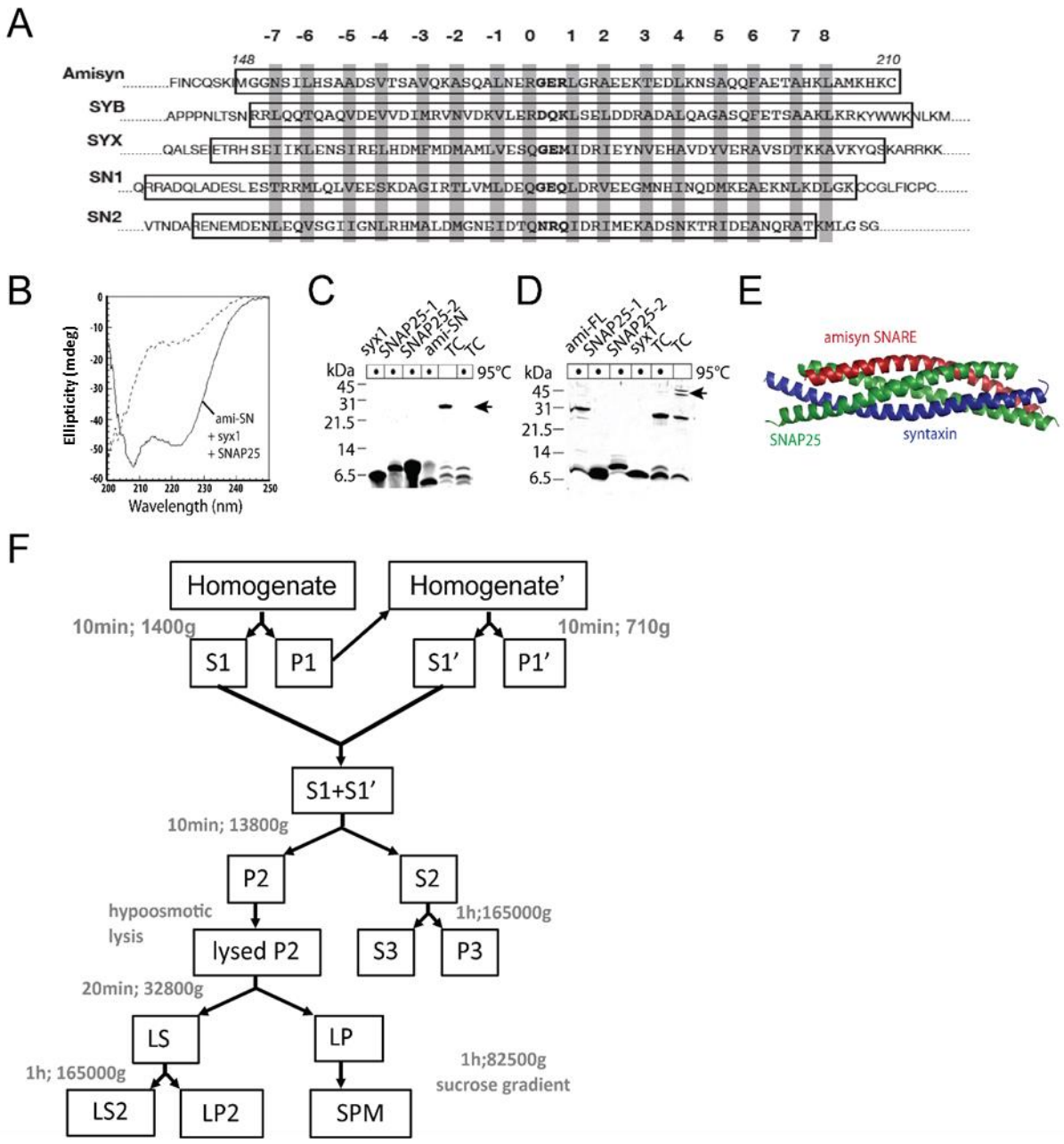
Supplementary Figures



Supplementary Figure S1. Amisyn is a conserved, vertebrate-specific protein that forms SNARE complex with syntaxin-1 and SNAP-25

(A) Sequence alignment of amisyn SNARE motif with those in synaptobrevin-2 (SYB), syntaxin-1a (SYX) and SNAP-25 (SN1 & SN2). The conserved positions of the heptad repeat layers (-7 to +8) are shaded in grey, with the neuronal core SNARE motifs boxed.

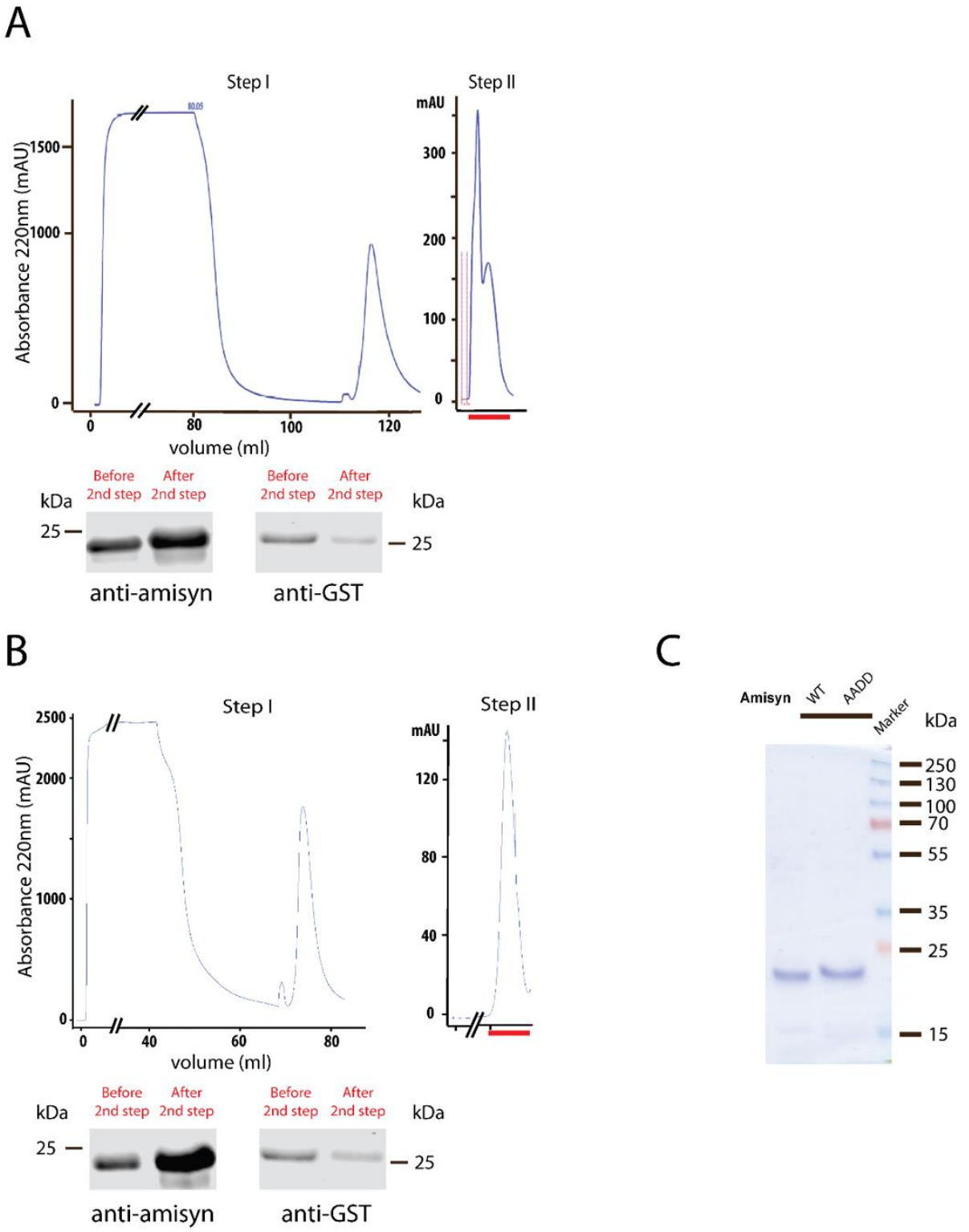
(B) CD spectra reveal structural changes by formation of the amisyn-SNARE complex. The SNARE motif of amisyn (ami-SN) interacts with SNAP-25a and the H3-syntaxin-1a (syx1), and forms the α-helical SNARE complex (solid tracing). The dotted line represents the theoretical non-interacting spectrum of the monomers.

(C) The SNARE motif of amisyn (ami-SN) interacts with the Q-SNARE syntaxin-1a (syx1), the first helix of SNAP-25 (SNAP25-1) and the second helix of SNAP-25 (SNAP25-2) to form SDS-resistant ternary complex (TC) (arrowheads) that dissociates by heating at 95°C.

(D) Full-length amisyn (ami-FL) also formed a ternary complex with Q-SNAREs. We note a presence of two bands: we are not fully aware of the biochemical composition of the second band, but both bands are SDS-resistant and dissociate by heating at 95°C.

(E) The amisyn SNARE complex is depicted as a parallel four helical coiled-coil bundle, based on the synaptobrevin-2 SNARE complex

(F) The schematic representation of the brain fractionation protocol used to isolate synaptic plasma membranes.

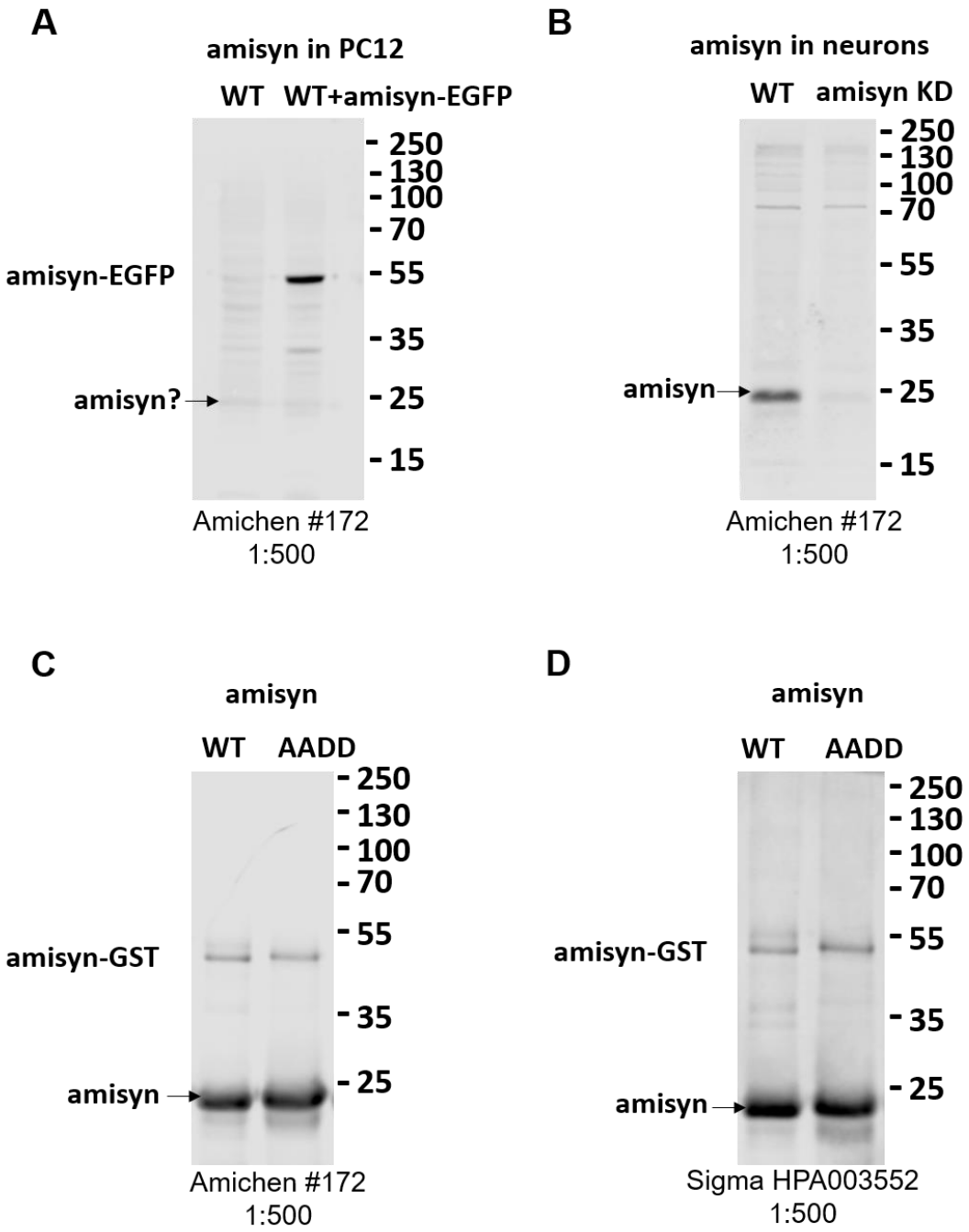


Suppl. Figure S2. Purification of recombinant amisyn (WT and AADD mutant) proteins.

(A) Purification of WT amisyn-GST by 2-step affinity purification of amisyn-GST on a GSTrap column before (left panel) and after (right panel) proteolytic cleavage (PreCission Protease).
Lower panels: immunoblotting with antibodies against amisyn (left panel) and GST (right panel).

(B) Purification of AADD amisyn-GST by 2-step affinity purification of amisyn-GST on a GSTrap column. Details as in panel (A).

(C) Coomassie staining of SDS-PAGE gel with equal concentrations of WT amisyn and AADD amisyn. The Western blots of the same samples are shown in Suppl. Fig S3.

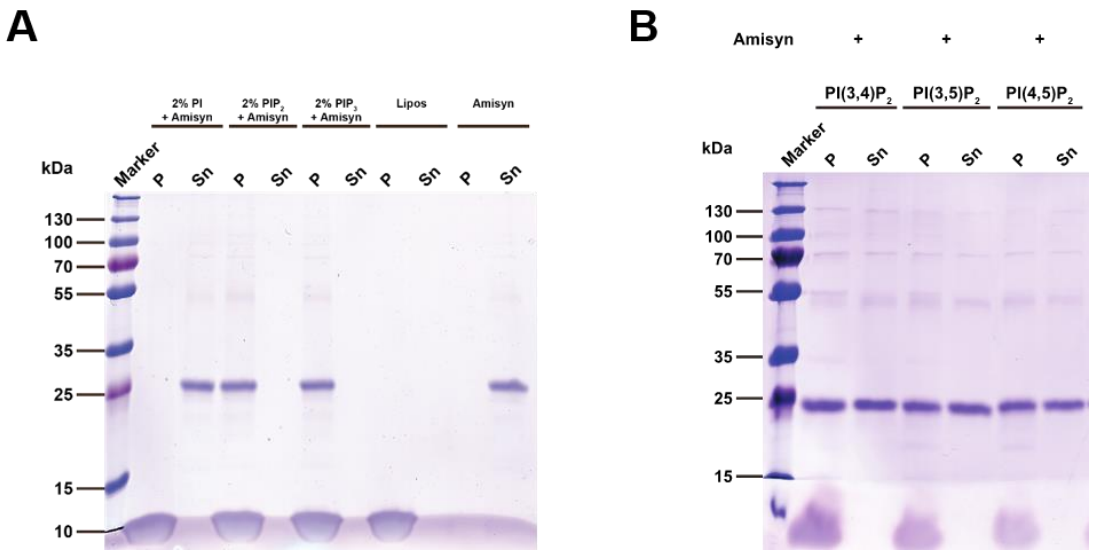


Suppl. Figure S3. Specificity of custom-made anti-amisyn polyclonal rabbit antibody.

(A) Control and amisyn-EGFP transfected PC12 cells (expression for 24h) were collected and equal protein concentrations of the samples were subjected to 12% SDS-PAGE gel. Custom-made anti-amisyn polyclonal rabbit antibody (Aminchen #172, 1:500) was used for Western blot. Of note, no specific antibody against amisyn was commercially available when this project started.

(B) WT neurons and neurons with amisyn knock-down were collected and equal protein concentrations of the samples were subjected to 12% SDS-PAGE gel. Custom-made anti-amisyn polyclonal rabbit antibody (Aminchen #172, 1:500) was used for Western blot.

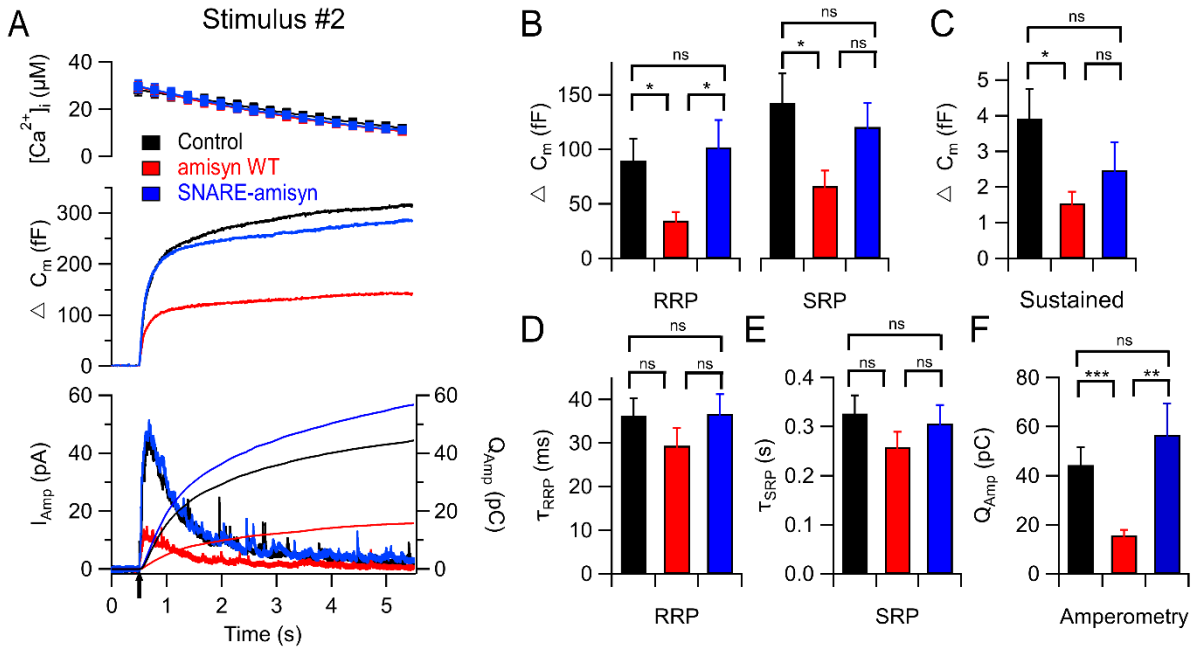
(C) Equal concentration of purified recombinant WT and AADD amisyn were subjected to 12% SDS-PAGE gel and blotted with custom-made anti-amisyn polyclonal rabbit antibody (Aminchen #172, 1:500)
(D) Equal concentration of purified recombinant WT and AADD amisyn were subjected to 12% SDS-PAGE gel and blotted with commercially available antibody from Sigma (Sigma #HPA003552, 1:500).



Suppl. Figure S4. Amisyn interaction with liposomes requires the presence of phosphatidylinositol bisphosphates or phosphatidylinositol trisphosphate.

(A) Representative SDS-PAGE (12%) gel with co-sedimentation assay samples showing that amisyn interaction with liposomes depends on the presence of PI(4,5)P_2 (2%) and PI(3,4,5)P_3 (2%), but not PI (2%). P – pellet, Sn – supernatant. Three independent experiments were performed.

(B) Representative SDS-PAGE (12%) gel with co-sedimentation assay samples showing that amisyn can bind to liposomes containing PI(4,5)P_2 (0.5%), PI(3,4)P_2 (0.5%) or PI(3,5)P_2 (0.5%). P – pellet, Sn – supernatant. Three independent experiments were performed.



Suppl. Figure S5. Amisyn, but not amisyn SNARE-domain, reduced number of released vesicles but did not alter rates of vesicle fusion in bovine chromaffin cells.

(A-F) Exocytosis induced by a second stimulus, elicited 100 s after the first stimulus. Similar phenotype for amisyn-loaded and amisyn-SNARE loaded cells was observed like during the first stimulus. Exocytosis induced by UV-flash photolysis of caged calcium ions (stimulus #2, arrow) was reduced in amisyn-loaded chromaffin cells (red trace) compared to control cells (black trace). Cells loaded with amisyn-SNARE protein (blue trace) did not differ significantly from control cells (with an exception of sustained release). Panels are arranged as detailed in Figure 7A-F. 32 control cells, (black); 31 amisyn-loaded cells (red); 33 amisyn-SNARE-loaded cells (blue); from 5 independent experiments. Kruskal-Wallis test with Dunn's multiple comparison test; ns – non significant,*p<0.05, **p<0.01, ***p<0.001.

(A) Top: intracellular calcium level increase induced by flash photolysis (at t=0.5 s). Middle: averaged traces of membrane capacitance changes upon Ca2+-induced exocytosis. Bottom: mean amperometric current (Iamp)(left axis) and cumulative charge (right axis).

(B-C) Exponential fitting of the capacitance traces revealed significant changes in RRP and SRP size, and sustained phase of release (C).

(D-E) Fusion kinetics of vesicles from RRP and SRP pools were not altered.

(F) Reduced detection of catecholamines in cells loaded with amisyn by amperometry: cumulative charge during 5s after stimulation.