Supporting Information

Davies et al. 10.1073/pnas.0908735107

SI Materials and Methods

Heterologous Expression of cDNAs. The calcium channel cDNAs used were rat $\alpha_2\delta$-1 (M86621), mouse $\alpha_2\delta$-2 (AF247139), mouse $\alpha_2\delta$-3 (AJ010949), rabbit $Ca_V2.2$ (D14157 without 3′ UTR) or GFP-$Ca_V2.2$ (1), rat β1b (2), and HA-tagged $\alpha_2\delta$-2 (3), and the glycosylphosphatidylinositol (GPI)-site mutants used were $\alpha_2\delta$-2 GAS (1108-1110)-WKW, $\alpha_2\delta$-3 CGG (1061-1063)-WKW, and $\alpha_2\delta$-3 GAS (1063-1065)-WKW. Human 5′nucleotidase (BC065937) was also used. The cDNAs were cloned into the pMT2 vector for expression, unless otherwise stated. All mutations were made by standard molecular biological techniques and verified by DNA sequencing. Mammalian cell lines (Cos-7 or tsA 201 cells) were transfected with the cDNA combinations stated. The cDNA for green fluorescent protein (mut3 GFP) (4) was also included to identify transfected cells from which electrophysiological recordings were made. The nicotinic receptor α7-V201-5HT3A receptor described previously (5) was a gift from Neil Millar(University College London). Transfection was performed as described previously (6). In control experiments where $\alpha_2\delta$ was omitted, the ratio was made up, as stated, with buffer or with pMT2 with equivalent results.

Antibodies. Ca channel antibodies used or mentioned in discussion were anti-mouse α_2-2(16-29), α_2-2(102-117) (7), δ-2(1062-1079), δ-2(1080-1094), and δ-2 (C terminus, CT; 1133-1147) for $\alpha_2\delta$-2, α_2-3(71-90) and δ-3(1035-1049) for $\alpha_2\delta$-3, monoclonal $\alpha_2\delta$-1 (Sigma), anti-rat α_2-1(1-15), and δ-1 (1030-1044) for $\alpha_2\delta$-1. The numbers refer to the amino acid residues used to generate the anti-peptide Abs. Other primary antibodies used were anti-*Akt* (Cell Signaling Technology), flotillin-1 (BD Biosciences), 5′NT (Santa Cruz Biotechnology), anti-p75 Ab (MC192; Biosensis), and cross-reacting determinant (CRD) Ab (obtained from Nigel Hooper, Leeds University, Leeds, UK).

Preparation of Triton X-100–Insoluble Membrane Fractions. All steps were performed on ice. Confluent cells from four 175-cm^2 flasks or pelleted whole homogenate derived from brain tissue were taken up in 1.5-mL Mes-buffered saline [MBS; contains 25 mM Mes (pH 6.5) 150 mM NaCl, and complete protease inhibitor mixture (Roche)] containing 1% (vol/vol) Triton X-100 (Perbio). It was resuspended by five passages through a 23-gauge needle and then left on ice for 1 h. An equal volume of 90% (wt/vol) sucrose in MBS was then added. The 3-mL sample was transferred to a 13-mL ultracentrifuge tube and overlaid with 10 mL of discontinuous sucrose gradient, which consisted of 35% (wt/vol) sucrose in MBS (5 mL) and 5% (wt/vol) sucrose in MBS (5 mL). The sucrose gradients were centrifuged at 140,000 × *g* for 18 h at 4°C in a Beckman SW40 rotor; 1 mL fractions were subsequently harvested from the top to the bottom of the tube. When necessary, protein fractions from the gradient were washed free of sucrose by dilution into 25 volumes of MBS and centrifuged (100,000 × *g* for 1h at 4°C) to pellet the cholesterol-enriched microdomain material. Triton X-100–insoluble fractions were prepared from rat heart and rabbit skeletal muscle using the same protocol with the following modification: ∼1 g of frozen tissue was cut into small pieces, resuspended in total volume of 1 mL of MBS containing complete protease inhibitor mixture, and thoroughly homogenized with an Ultra Turrax mixer (T25 basic; IKA Labortechnik). An equal volume of MBS containing 2% Triton X-100 was added and mixed with the homogenate (final concentration of Triton X-100 1%) and incubated for 1 h on ice. The rest of the procedure was performed as described above.

Cell-Surface Biotinylation Assay. At 48 h after transfection, cells were rinsed three times with PBS and then incubated with PBS containing 0.5 mg/mL Sulfo-NHS-SS-Biotin (Perbio) for 30 min at room temperature. The biotin solution was removed and replaced with PBS containing 100 mM glycine for 2 min at room temperature to quench the reaction. The cells were gently rinsed three times with PBS, then harvested in PBS containing 1 mM EDTA and protease inhibitors, and transferred to centrifuge tubes. The cells were pelleted by centrifugation and then lysed in PBS, 1% Igepal, and protease inhibitors for 30 min on ice. The detergent lysates were then clarified by centrifugation (14,000 × *g* for 30 min at 4°C). One-tenth of the cell lysate was loaded onto a 3–8% Tris-Acetate gel to determine total protein expression. Biotinylated proteins were precipitated by adding 50 μl of streptavidin-agarose beads (Perbio) and incubated overnight at 4°C. The streptavidin-agarose beads were washed three times and incubated with 100 mM DTT for 30 min at room temperature followed by 2× Laemmli sample buffer. Eluted proteins were then resolved by SDS/PAGE.

Deglycosylation. Triton X-100–insoluble protein (5–10 μg) or affinity-purified HA-tagged $\alpha_2\delta$-2 protein (1–2 μg) was resuspended in a buffer containing 15 mM Tris (pH 8), 75 mM β-mercaptoethanol, 0.5% Triton X-100, 0.05% SDS, and protease inhibitors, and it was incubated for 5 min at 37° C. Two units of endoglycosidase F (Roche) were added, and the reaction proceeded for 5 h at 37°C.

Treatment of Triton X-100–Insoluble Protein Fractions with PI–Phosphatidylinositol-Phospholipase C or GPI–Phosphatidylinositol-Phospholipase C . Triton X-100–insoluble fractions from transfected tsA 201 cells or brain tissue were collected, washed free of sucrose, and centrifuged as described above. The resultant pellet of Triton X-100–insoluble material was resuspended in an appropriate volume of PI-PLC reaction buffer [10 mM Tris (pH 7.4) and 150 mM NaCl containing complete protease inhibitor mixture (Roche)] or GPI-PLC reaction buffer [50 mM hepes (pH 7.5), 2.5 mM EDTA, 1% Triton X-114 containing complete protease inhibitor mixture (Roche) modified from a previous study (8) to a final protein concentration of ∼2 mg/mL]. The samples were sonicated and treated with 3 μg/μl PI-PLC enzyme (Prozyme Glyko) for 1–3 h or for 4 h with 10 μL of *Trypanosoma. brucei* GPI-PLC (per 300 μL sample as described previously) (8). GPI-PLC was a gift from M. Carrington, Cambridge, UK. Alternatively, several hippocampi or cerebella were pooled together and homogenized in a small volume (∼100 μl) of PI-PLC or GPI-PLC– reaction buffer and subjected to PI-PLC treatment (3 μg/μl PI-PLC) for 1–3 h at 37°C or GPI-PLC treatment as noted above. Afterward, the volume was adjusted to 2 mL with MBS (final concentration of Triton X-100 was 1%). Detergent-insoluble fractions were prepared as described above.

Phase Separation of PI-PLC– and GPI-PLC–Treated Proteins in Triton X-114. Membrane-associated proteins were separated from soluble proteins in two phases of Triton X-114 as described previously (9). Briefly, the pellet of detergent-insoluble material was resuspended in an appropriate volume of reaction buffer (final concentration of ∼2 mg/mL protein) and incubated with PI-PLC or GPI-PLC as described above. Control experiments omitting enzymes were also performed. At the end of this in-

cubation, PI-PLC–treated samples were supplemented with Triton X-114 to a final concentration of 1%. For the phase separation, a cushion of 6% (wt/vol) sucrose, 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.06% Triton X-114 was placed at the bottom of a 1.5-mL Eppendorf tube. GPI-PLC treated samples (already containing 1% Triton X-114 during the incubation with the enzyme) were supplemented with fresh Triton X-114 to 2%. The sucrose cushion used for the phase separation of GPI-PLC treated samples was composed of 6% (wt/vol) sucrose, 50 mM hepes (pH 7.5), 2.5 mM EDTA, 0.06% Triton X-114, and complete protease-inhibitor mixture (Roche). The rest of the procedure for the phase separation of GPI-PLC– or PI-PLC–treated samples was the essentially the same.

The protein sample was then overlaid on this sucrose cushion, and the tube incubated for 3 min at 30°C. The tube was centrifuged at 300 × *g* for 4 min at room temperature in a swinging bucket rotor. After centrifugation, the detergent phase was found as an oily droplet at the bottom of the tube. Fresh Triton X-114 was then added to the upper aqueous phase to 0.5%, and the procedure was repeated using the same sucrose cushion. In the last step, the aqueous phase was removed from the cushion, supplemented with fresh Triton X-114 to 2%, and subjected to another centrifugation. The detergent phase of this last condensation was discarded. The aqueous and detergent phases from this procedure were adjusted to the equal volume with 10 mM Tris (pH 7.4) and 150 mM NaCl containing protease inhibitors. The proteins in the detergent and aqueous phases were precipitated with four volumes of cold acetone for 1 h at −20°C, centrifuged at 16,000 × *g* for 10 min, washed one time with 4:1 acetone:water, and resuspended in an appropriate volume of gel-loading buffer.

Affinity Purification of HA-Tagged $\alpha_2\delta$-2 Proteins and Immunoblotting. Pooled Triton X-100–insoluble protein fractions (1-mL samples containing 5 mg/mL protein) were treated with or without 5U/mL PI-PLC (Sigma) for 1 h at 37°C in the PI-PLC reaction buffer noted above. Proteins were solubilized in 2% (wt/vol) n-octyl glucoside (OG) for 30 min on ice, and the detergent lysates were clarified by centrifugation (40,000 × *g* for 1 h at 4°C). Solubilized proteins were applied to and recirculated through 1-mL columns of HA antibody immobilized on Sepharose CL4B (Sigma) that was preequilibrated in 1% OG. The columns were washed extensively in wash buffer (50 mM Tris, 300 mM NaCl, 1% OG (pH 7.4), and protease inhibitors), and then, bound HA-tagged proteins were eluted with 1 mg/mL HA peptide (Roche) in elution buffer (same as wash buffer except with the addition of 150 mM NaCl) prewarmed to 37°C; 0.5-mL fractions were collected. Peak protein-containing fractions (A_{280nm}) were dialyzed against 50 mM Tris, 150 mM NaCl, and 0.5% OG (pH 7.4) and then analyzed by SDS/PAGE and silver staining. The major HA-tagged α2δ-2 protein-containing fractions (>90% purity) were pooled, and protein was concentrated by acetone precipitation as described above. Samples of purified proteins resolved by SDS/PAGE were transferred to PVDF membranes and probed with antibodies to the HA tag and CRD. Then, secondary antibodies were conjugated to horseradish peroxidize followed by enhanced chemiluminescence detection.

^{3}H Myo-Inositol Labeling of tsA 201 Cells. Two T75 flasks of tsA 201 cells stably expressing HA-tagged $\alpha_2\delta$-2 [α_2-2(HA)-δ-2] or control untransfected cells (1 in 14 dilutions from confluent cell stocks) were grown for 12 h at 37°C in a low-inositol medium (Gibco medium 199 containing Earle's salts, L-glutamine, and 25 mM hepes) supplemented with 10% (vol/vol) dialyzed FBS (Gibco) and the relevant antibiotics. To each flask was added 250 μL 1mCi/mL *myo*-[2-^{3}H] inositol (Amersham; GE Healthcare) in fresh medium, and the final concentration of label was 31 μCi/mL. Cells were grown for an additional 60 h at 37°C, harvested, and washed in PBS (pH 7.4) containing 1 mM EDTA, and the resultant cell pellets were stored at −80°C.

For immunoprecipitation of HA-tagged proteins, the cell pellets were resuspended in 300 μL 50 mM Tris (pH 8.0) to which was added an equal volume of 2× solubilization buffer (final detergent concentrations of 1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS) containing complete protease inhibitors (Roche).

The samples were incubated at 4°C for 60 min with constant gentle mixing and then centrifuged at 16,000 × *g* for 30 min at 4°C. The clarified whole-cell detergent lysates were transferred to fresh tubes and sampled for SDS/PAGE. To the remaining lysate samples (0.5 mL each) was added 3 μg high-affinity anti-HA antibody (Roche), and the samples incubated at 4°C overnight with constant mixing. Immune complexes were precipitated with Protein G-Sepharose beads (25-μL packed beads; Sigma-Aldrich) for 2 h at 4°C with mixing. The pellets were washed six times by centrifugation with 1-mL volumes of 1× solubilization buffer and finally, with PBS (pH 7.4) and protease inhibitors. Bound proteins were eluted from the Protein G-Sepharose beads in 60 μL SDS/PAGE sample buffer containing 50 mM DTT for 60 min at 37°C. Samples of the whole-cell detergent lysates (50 μg total protein) or immunoprecipitated proteins (30 μL) were run on duplicate 4–12% Bis-Tris gels (Invitrogen). One gel was used for Western blotting against anti-HA and δ-2(1080-1094) antibodies. The second gel was prepared for autoradiography. Briefly, after running, the gel was immediately soaked for 30 min in Amplify flurographic reagent (GE Healthcare), rinsed in distilled water, mounted on 3M paper, and then, dried under vacuum. For detection of ^{3}H-labeled proteins, the dried gel was overlaid with Amersham Hyperfilm ^{3}H (GE Healthcare) in a light-tight cassette and exposed for 75 days at −80°C. The film was developed using a standard automated film developer.

Dorsal Root Ganglia Cultures and PI-PLC Treatment. Cell cultures were obtained after enzymatic and mechanical dispersal of rat dorsal root ganglia (DRG) as described previously (3). Briefly, DRGs were removed from adult Sprague-Dawley rats and incubated in Hank's basal salt solution (HBSS) containing 1000 U/mL DNase (Invitrogen), 5mg/mL dispase (Invitrogen), and 2mg/mL collagenase type 1A (Sigma) for 40 min at 37°C. The partially digested ganglia were then washed and triturated in growth medium (DMEM/F12 with 10% FBS), 1% glutamine, 1% penicillin/streptomycin, and 6 g/L glucose). Cells were plated on Matrigel-coated (BD Biosciences) coverslips, and the medium was changed every other day. For PI-PLC treatment of DRGs maintained in culture for 2–6 days, coverslips were washed two times with DMEM + DMEM/F12 (1:1) and incubated with 4 U/mL or 8 U/mL PI-PLC (Sigma; reconstituted in water) or the equivalent volume of water without PI-PLC in DMEM + DMEM/F12 for 1 h at 37°C. The supernatant was collected for Western blotting.

PI-PLC Treatment of Cos-7 and tsA 201 Cells. Cos-7 cells were treated as described for DRG neurons, washed twice with DMEM, and incubated with 4 U/mL or 8 U/mL PI-PLC (Sigma or Glyko) for 60 min or 90 min, as stated, at 37°C. The tsA-201 cells were incubated with 4 U/mL PI-PLC (20 μl/mL of stock solution in 10 mM Tris-HCl (pH 8), 10 mM EDTA, and 60% glycerol; Sigma) as stated. Control cells were treated with an equivalent volume of buffer without PI-PLC.

Immunocytochemistry on DRG Cultures. The immunocytochemical detection of $\alpha_2\delta$-1 was performed as described previously (10). Briefly, cells were washed and fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) for 5 min at room temperature followed by heat-induced antigen retrieval [10 mM citrate buffer (pH 6.0) at 95°C for 10 min] before blocking the samples with

10% goat serum and 4% BSA in TBS for at least 30 min. Samples were then incubated with anti–α_2-1 monoclonal Ab (1:100) at 4°C overnight, washed, and incubated with biotinylated goat anti-mouse IgG (1:500; Invitrogen) for 2 h at 4°C followed by streptavidin-Alexa Fluor 488 (1:500; Invitrogen) for 1 h at room temperature. After washing and DNA staining with DAPI (300 nM; Invitrogen), samples were mounted in Vecta-Shield (Vector Laboratories). Immunofluorescence labeling was detected with a LSM 510Meta (Zeiss) confocal microscope in optical sections of 1-μm thickness. Experiments were performed on four individual cell cultures.

Immunocytochemistry on Cos-7 Cells. The method used is essentially the same as previously described (11). Cells were fixed with 4% paraformaldehyde in TBS for 5 min at room temperature and washed twice with TBS. No permeabilization step was used. The primary anti-α_2-2 (2.9 μg/mL), α_2-3 (0.88 μg/mL), δ-3 (3.5 μg/mL), or anti-5′NT (1:500) Abs were incubated overnight at 4° C. This was followed with incubation by the FITC conjugated anti-rabbit (1:500; Sigma), Texas Red conjugated anti-rabbit (1:500; Molecular Probes), or biotinylated anti-rabbit or anti-mouse Abs (1:500) and streptavidin-Alexa Fluor488 (Invitrogen; 1:500) or streptavidin-AlexaFluor594 (1:500; Invitrogen). DAPI was also used to visualize the nuclei. Cells were mounted in Vectashield (Vector laboratories) to reduce photobleaching. For experiments on transfected α7 nicotinic 5HT3 receptor chimeras, cells were not fixed. They were washed in HBSS buffer [21 mM hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 7.5 mM glucose (pH 7.4)], and the receptors were visualized using Alexa fluor 488-α-bungarotoxin (Invitrogen; 10 μg/mL in HBSS for 20 min at 17°C). Cells were examined on a confocal laser-scanning microscope (Ziess LSM) using a ×40 (1.3 NA) or ×63 (1.4 NA) oil-immersion objective. Confocal optical sections were 1 μm. Photomultiplier settings were kept constant in each experiment, and all images were scanned sequentially. Image processing was performed using ImageJ (http://rsb.info.nih.gov/ij/). Data illustrated are representative of >10 cells from at least three independent experiments, unless otherwise stated. In some experiments, where stated, cells were visualized using an epifluorescence microscope (Zeiss Optiphot).

Electrophysiology. Calcium-channel expression in tsA-201 cells was investigated by a whole-cell patch clamp recording, which was essentially as described previously (12). The internal (pipette) and external solutions and recording techniques were similar to those previously described (13). The patch pipette solution contained (in mM) Cs-aspartate (140), EGTA (5), $MgCl_2$ (2), $CaCl_2$ (0.1), K_2ATP (2), Hepes (10; pH 7.2), and mOsm with sucrose (310). The external solution for recording Ba^{2+} currents contained (in mM) tetraethylammonium (TEA) Br (150), KCl (3), $NaHCO_3$ (1.0), $MgCl_2$ (1.0), Hepes (10), glucose (4), $BaCl_2$ (1 or 5 as indicated; pH 7.4), mosM with sucrose (320). Pipettes of resistance 2–4 MΩ were used. An Axopatch 1D amplifier (Axon Instruments) was used, and data were filtered at 1–2 kHz and digitized at 5–10 kHz. Current records were subjected to leak and residual capacitance-current subtraction (P/8 protocol). Analysis was performed using Pclamp 9 (Molecular Devices) and Origin 7 (Microcal Origin).

Data Analysis. Where data are given as mean ± SEM, statistical comparisons were performed using either Student's *t* test or ANOVA with a post hoc test as appropriate.

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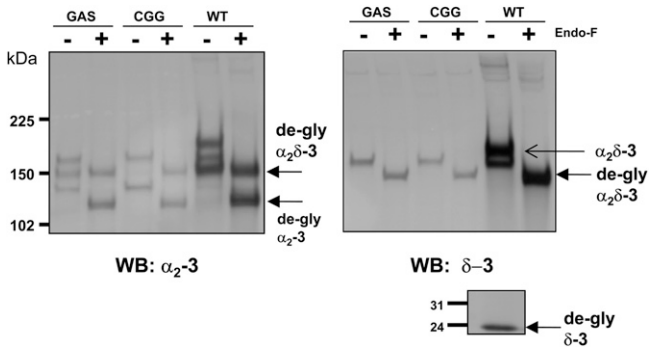

Fig. S1. Immunoblot for wild-type (WT) $\alpha_2\delta$-3 and GPI-mutant $\alpha_2\delta$-3 constructs to show presence of cleaved and uncleaved α_2-3. (*Upper*) Immunoblot showing pattern of protein bands for $\alpha_2\delta$-3 species taken from peak lipid-raft fractions using α_2-3 (71–90) Ab (*Left*) and δ-3 (1035-1049) Ab (*Right*) for $\alpha_2\delta$-3 GAS-WKW (lanes 1 and 2), $\alpha_2\delta$-3 CGG-WKW (lanes 3 and 4), and WT $\alpha_2\delta$-3 (lanes 5 and 6) before and after deglycosylation with endoglycosidase F (EndoF) as indicated. *Lower* shows that the δ-3 Ab also recognized free δ-3 on the same immunoblot. Note that without deglycosylation, multiple glycosylated states of $\alpha_2\delta$-3 species are observed, indicating the presence of both immature and mature glycosylated species. After deglycosylation, only two bands were observed for all $\alpha_2\delta$-3 constructs, the upper band corresponding to full-length $\alpha_2\delta$-3 (~150 kDa) and the lower band corresponding to cleaved α_2-3 (~120 kDa), because it is not recognized by the δ-3 Ab.

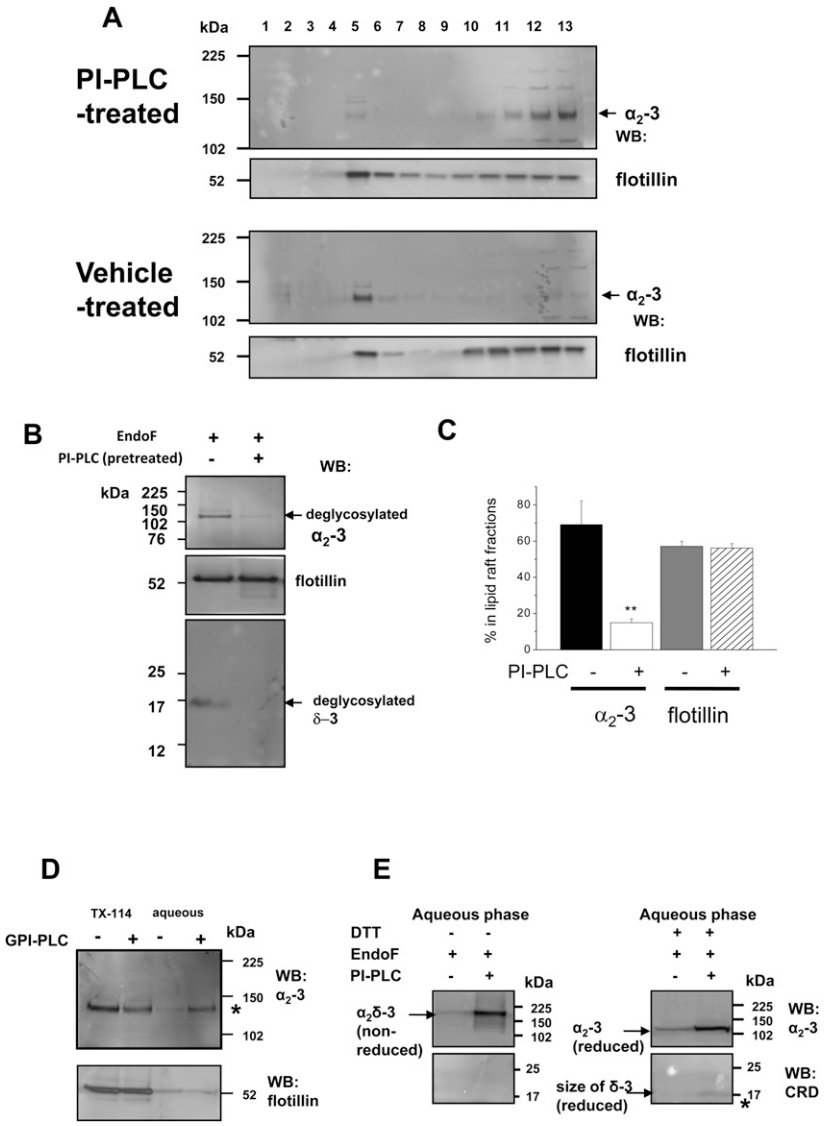

Fig. S2. PI-PLC treatment reduces lipid-raft association of hippocampal $\alpha_2\delta$-3. (*A*) Lysates from the hippocampus were incubated with PI-PLC (*Upper*) or vehicle (*Lower*) for 3 h at 37°C in the presence of protease inhibitors and then subjected to sucrose-gradient centrifugation. The immunoblot profile of $\alpha_2\delta$-3 in sucrose-gradient fractions prepared from hippocampal lysate was then examined using α_2-3 (71–90) Ab (arrowed, rows 1 and 3). Flotillin distribution in same lipid-raft profiles is shown in rows 2 and 4. A similar result was obtained with 90 min incubation with PI-PLC. (*B*) Combined fractions 4–7 from two lipid-raft preparations (±PI-PLC treated before lipid-raft preparation as indicated) were concentrated 10-fold and deglycosylated with EndoF. An aliquot was used to visualize δ-3, which is also decreased in the lipid-raft fractions by PI-PLC pretreatment. This shows that the decrease in α_2-3 in the rafts is not caused by detachment of α_2-3 by disulfide bond reduction. (*C*) Quantification of the amount of α_2-3 (*Left*) and flotillin (*Right*) in lipid-raft fraction five after PI-PLC treatment of hippocampal lysates. Black and gray bars, vehicle treated; white and hatched bars, PI-PLC-treated for 3 h. Data represent the mean ± SEM from three independent experiments. ***P*, 0.0152 from Student's *t* test. (*D*) Material from hippocampal detergent-resistant membranes (DRM) fraction five treated with vehicle or GPI-PLC as indicated in *SI Materials and Methods* and then subjected to phase separation in Triton X-114. (*Upper*) α_2-3 and (*Lower*) flotillin showing protein in the detergent (left two lanes) and aqueous phases (right two lanes) without and with GPI-PLC treatment as indicated. The α_2-3 subunit band in the aqueous phase is indicated by an asterisk. (*E*) PI-PLC–treated material from hippocampal DRM fraction five were subjected to phase separation in Triton X-114 without and with PI-PLC treatment as indicated. (*Upper*) α_2-3 and (*Lower*) CRD showing protein in the aqueous phase without reduction in the absence of DTT (right two lanes) or with reduction by DTT (right two lanes). The band at the level of δ-3 in the CRD blot (indicated with an asterisk) is only seen after PI-PLC treatment and is not observed in the absence of reducing conditions when free δ-3 would not be formed (lower left blot).

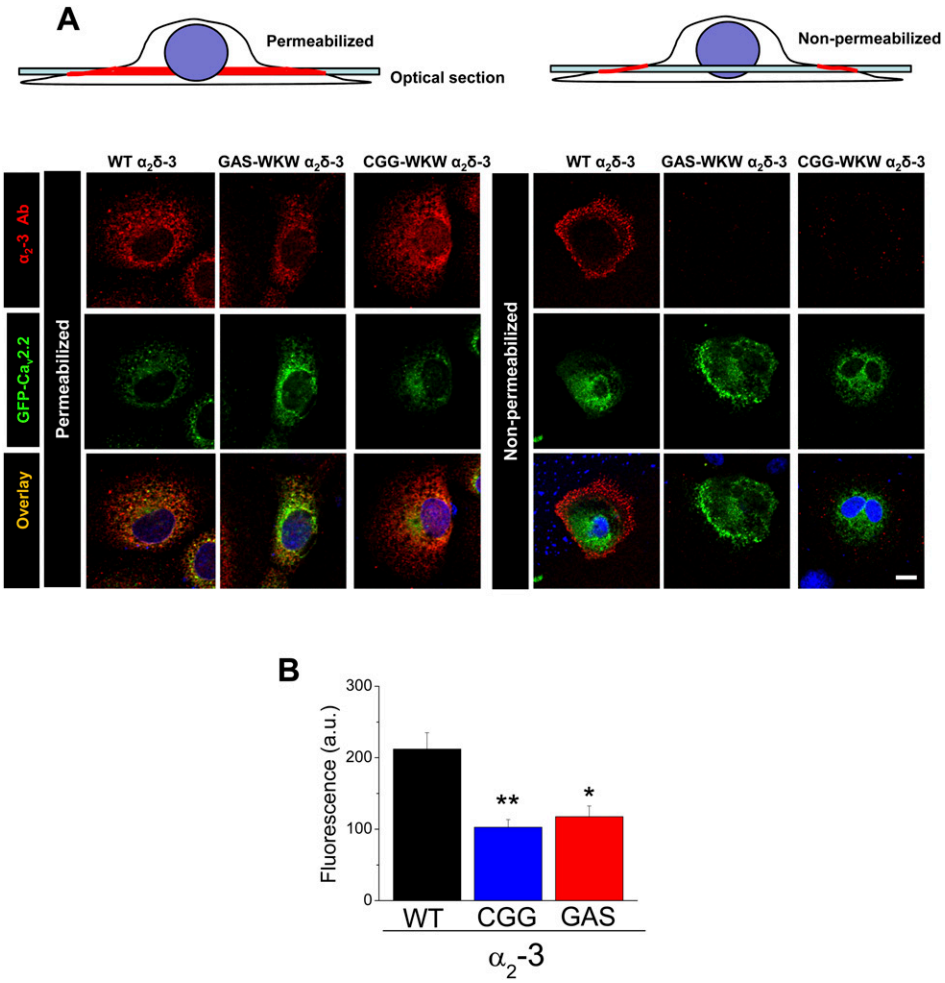

Fig. S3. Cell-surface localization and total expression of WT and GPI-mutant $\alpha_2\delta$-3 revealed using the α_2-3 Ab (*A*). (*Upper*) Diagram of Cos-7 cell to explain the annular cell-surface staining observed in nonpermeabilized cells. (*Lower*) Confocal microscopic images showing membrane localization of $\alpha_2\delta$-3 (top row; using α_2-3 Ab; red) for WT $\alpha_2\delta$-3 (*Left*), GAS-WKW $\alpha_2\delta$-3 (*Center*), and CGG-WKW $\alpha_2\delta$-3 (*Right*) when coexpressed with GFP-$Ca_V2.2$ (*middle row*) and β1b and examined in permeabilized (*Left*) or nonpermeabilized (*Right*) Cos-7 cells. Merged images are shown in the bottom row together with nuclear staining using DAPI (blue). (Scale bar: 20 μm.) *(B)* Quantification of cell-surface immunofluorescence using α_2-3 Ab for WT $\alpha_2\delta$-3 (black bar; $n = 54$), CGG-WKW $\alpha_2\delta$-3 (blue bar; $n = 29$), and GAS-WKW $\alpha_2\delta$-3 (red bar; $n = 31$). *, $P < 0.01$; **, $P < 0.001$ compared with WT $\alpha_2\delta$-3 in an one-way ANOVA and Tukey's post hoc test.

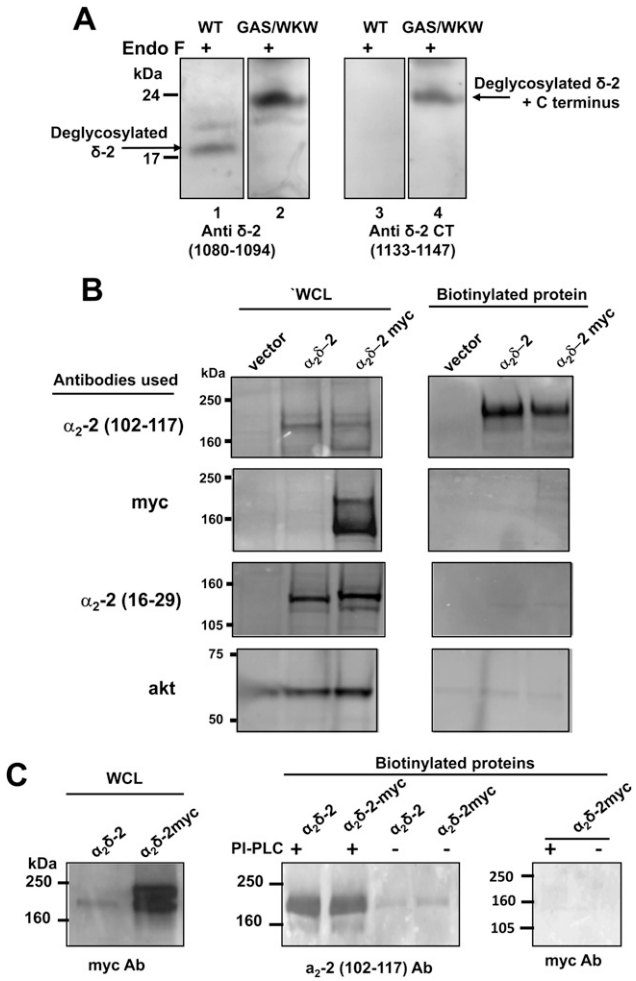

Fig. S4. Additional biochemical evidence that $\alpha_2\delta$-2 is anchored by GPI. WT and C-terminal myc-tagged $\alpha_2\delta$-2 but not the GPI-anchor site mutant of $\alpha_2\delta$-2 are C-terminally truncated. (*A*) This immunoblot shows peak lipid-raft fractions containing δ-2 subunits from WT $\alpha_2\delta$-2 (lanes 1 and 3) and $\alpha_2\delta$-2 with a mutation at the predicted GPI-anchor site (GAS to WKW; lanes 2 and 4) after deglycosylation with EndoF. The blots were probed with anti δ-2 Abs. (*Left*) Anti–δ-2 (1080-1094) Ab; (*Right*) Anti–δ-2 (1133-1147; CT) Ab. For GAS-WKW $\alpha_2\delta$-2, a species of apparent MW ~23 kDa, the correct MW to represent mutant δ-2 with its C terminus still attached, is recognized by both Abs (lanes 2 and 4) and is absent in WT $\alpha_2\delta$-2 (lanes 1 and 3). A band of ~18 kDa representing WT δ-2 was recognized by anti δ-2 (1080-1094; lane 1) but not by the Ab to the C terminus (1133-1147; CT; lane 3). The ~18-kDa species is absent in the mutant GAS-WKW $\alpha_2\delta$-2 lanes. The antibody labeling and the MW of the δ-2 species indicates that unlike WT $\alpha_2\delta$-2, the $\alpha_2\delta$-2 (GAS-WKW) mutant is not C-terminally truncated. Data are representative of three independent experiments. (*B*) Vector-transfected, WT $\alpha_2\delta$-2 and $\alpha_2\delta$-2 with a C-terminal myc tag were expressed in Cos-7 cells. The Cos-7 cells were then subjected to cell-surface biotinylation. The biotinylated proteins were concentrated using streptavidin beads. Blots for whole cell lysate (WCL) (*Left*) and biotinylated proteins (*Right*) are shown for α_2-2 (102-117) Ab (row 1), myc Ab (row 2), α_2-2(16-29) N-terminal signal sequence Ab (row 3), and Akt as a control that cells were not permeabilized by the biotinylation process (row 4). A biotinylated band corresponding to $\alpha_2\delta$-2 is present on the cell surface of Cos-7 cells, expressing both WT $\alpha_2\delta$-2 and $\alpha_2\delta$-2-myc (row 1). This cell-surface $\alpha_2\delta$-2 protein does not have a myc tag associated with it (row 2), and the N-terminal signal sequence has also been cleaved (row 3). (*C*) A similar experiment to that in *B* was performed, except that cells were treated with PI-PLC. WT $\alpha_2\delta$-2 and $\alpha_2\delta$-2 with a C-terminal myc tag were expressed in Cos-7 cells (shown in WCL panel; *Left*). The Cos-7 cells were subjected to cell-surface biotinylation before PI-PLC treatment. The biotinylated proteins released into the supernatant were then concentrated using streptavidin beads. A biotinylated band corresponding to $\alpha_2\delta$-2 was released by PI-PLC from Cos-7 cells, and it expressed both WT $\alpha_2\delta$-2 and $\alpha_2\delta$-2-myc (*Center*). This released $\alpha_2\delta$-2 protein did not have a myc tag associated with it (*Right*).

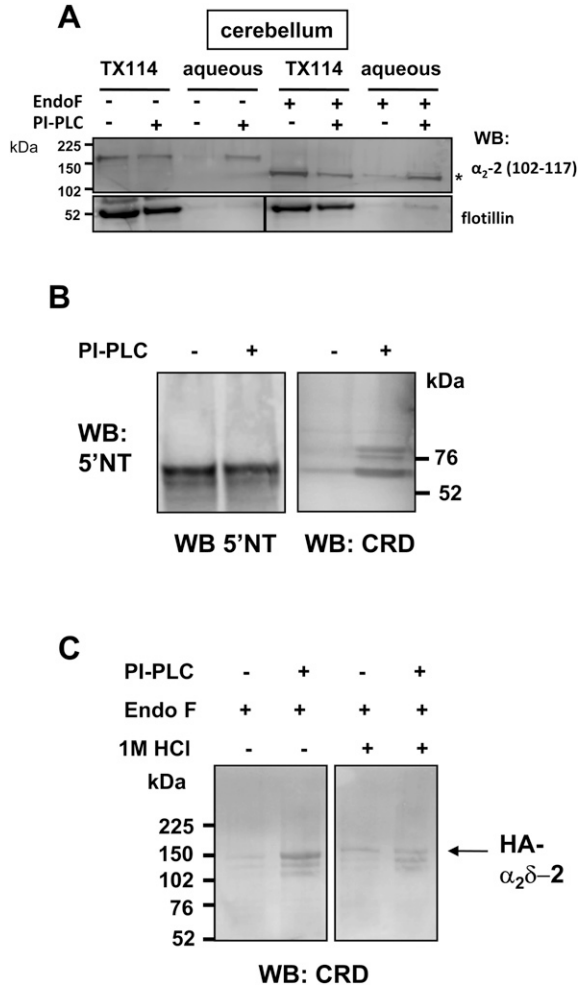

Fig. S5. Effect of PI-PLC treatment of cerebellar $\alpha_2\delta$-2 on its biochemical properties. (*A)* Phase separation in Triton X-114. (*Upper*) α_2-2 and (*Lower*) flotillin show protein in the detergent and aqueous phases with and without PI-PLC treatment both before and after deglycosylation with EndoF as indicated. The α_2-2 partitioning into the aqueous phase after PI-PLC is indicated by an asterisk. (*B)* 5′NT was expressed in tsA 201 cells, and the peak lipid-raft fraction was taken for PI-PLC treatment (right lane of each panel). (*Left*) Western blot with 5′NT Ab; (*Right*) Western blot with CRD Ab. (*C*) Loss of CRD immunoreactivity of PI-PLC–treated HA-tagged $\alpha_2\delta$-2 after 1 M HCl. Material as shown in Fig. 3*E* was treated with 1 M HCl for 1 h at room temperature, and the immunoblot was repeated with anti-CRD Ab. All lanes show deglycosylated HA-$\alpha_2\delta$-2 (arrow). *Left* is without 1 M HCl treatment, and *Right* is after 1 M HCl treatment. The right lane of each panel shows material after PI-PLC treatment, and the left lane is the control that was not treated with PI-PLC.

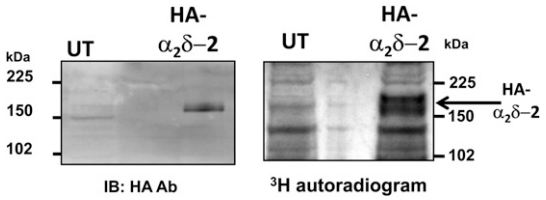

Fig. S6. [^{3}H]*myo*-inositol incorporation into HA-$\alpha_2\delta$-2. Untransfected tsA 201 cells (UT) or cells stably expressing HA-$\alpha_2\delta$-2 were labeled with [^{3}H]*myo*-inositol, as described in *Materials and Methods*, and HA-tagged proteins were subsequently immunoprecipitated with anti-HA Ab as described in *Materials and Methods*. (*Left*) Western blot of immunoprecipitated protein detected with HA Ab. (*Right*) Corresponding ^{3}H autoradiogram of the immunoprecipitated protein. The expected position of HA-$\alpha_2\delta$-2 is arrowed.

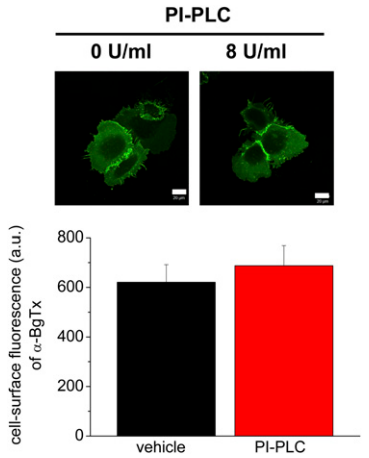

Fig. S7. Lack of effect of acute incubation with PI-PLC on cell-surface localization of a transmembrane receptor expressed in Cos-7 cells. There was a lack of effect of acute incubation of Cos-7 cells for 1 h with PI-PLC (8 U/mL) on cell-surface localization of transfected α7 nicotinic-5HT3 receptor chimeras (α7-V201-5HT3A) (5). This chimeric receptor was used, because it expresses well at the plasma membrane in these cells. The cell-surface expression in transfected nonpermeabilized Cos-7 cells was visualized with AF488 α-bungarotoxin. (*Upper*) Representative examples of cells treated with either vehicle (con; left) or PI-PLC (8 U/mL; right). (Scale bar: 20 μm.) (*Lower*) Bar chart showing cell-surface fluorescence for cells treated with vehicle (black bar; $n = 33$) or PI-PLC (8 U/mL; red bar; $n = 31$) from two independent experiments. NS, Student's t test.

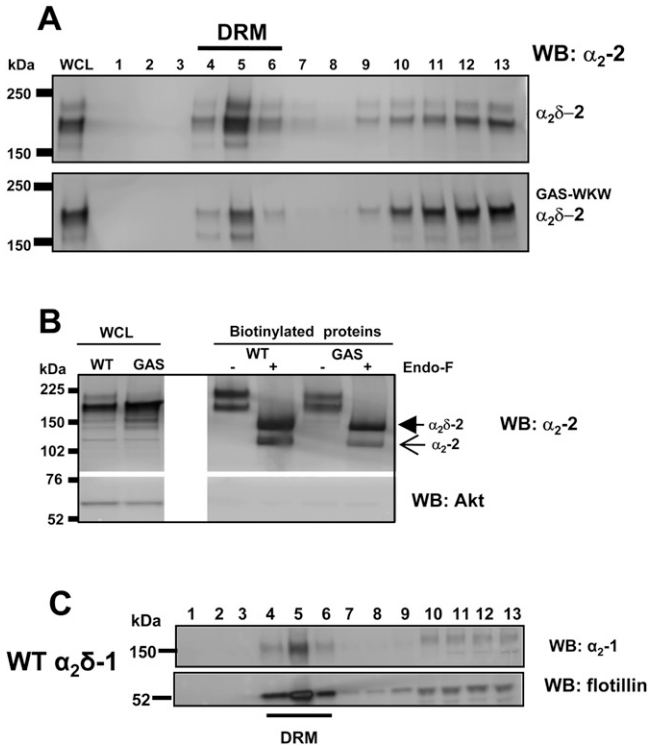

Fig. S8. Evidence that GPI anchoring of $\alpha_2\delta$-2 is important for lipid raft and cell-surface localization. (*A*) WCL (*Left*) and sucrose-gradient fractions (*Right*) 1–13 for $\alpha_2\delta$-2 (*Upper*) and GAS-WKW $\alpha_2\delta$-2 (*Lower*) show the DRM fractions 4–6 and detergent-soluble fractions 9–13. The proportion in the DRM fractions was 55.4% of the total $\alpha_2\delta$-2 protein for WT $\alpha_2\delta$-2 and 22.0% for GAS-WKW $\alpha_2\delta$-2. (*B*) Cell-surface biotinylation of $\alpha_2\delta$-2 and $\alpha_2\delta$-2 GAS-WKW. (*Upper*) The left two lanes show WCL for WT and GAS-WKW $\alpha_2\delta$-2, and the right four lanes show WT and GAS-WKW $\alpha_2\delta$-2 after cell-surface biotinylation before and after de-glycosylation with EndoF. Closed arrow, full-length $\alpha_2\delta$-2; open arrow, cleaved α_2-2. *Lower* shows lack of biotinylation of cytoplasmic Akt. (*C*) Localization of heterologously expressed $\alpha_2\delta$-1 in DRMs. (*Upper*) Immunoblot profile of WT $\alpha_2\delta$-1 in DRMs from transfected tsA-201 cells using α_2-1 monoclonal Ab. (*Lower*) Flotillin distribution was the same as in the DRM profile.

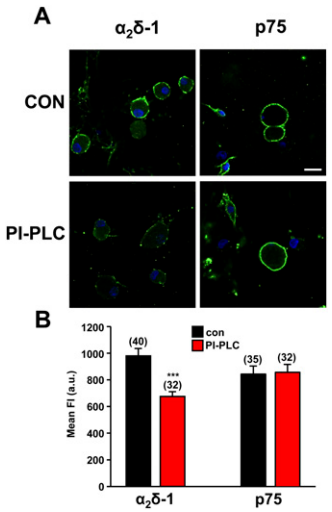

Fig. S9. Lack of effect of acute incubation with PI-PLC on cell-surface localization of endogenous p75 NGF receptor in DRGs. Lack of effect of acute incubation of DRGs for 1 h with PI-PLC (8 U/mL) on cell-surface localization of p75 NGF receptor was visualized with anti-p75 Ab. (*A*) Representative examples of cells treated with either (*Upper*) vehicle (con) or (*Lower*) PI-PLC (8 U/mL) for $\alpha_2\delta$-1 (*Left*) or p75 (*Right*). (Scale bar: 20 μm.) (*B*) Bar chart showing cell-surface fluorescence for cells treated with vehicle (black bars) or PI-PLC (8 U/mL; red bars) for $\alpha_2\delta$-1 (*Left*) and p75 (*Right*) for the number of cells given above each bar from two independent experiments. *** $P < 0.0001$ for Student's *t* test for the effect of PI-PLC on $\alpha_2\delta$-1.