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SNX27 and SORLA Interact to Reduce Amyloidogenic Subcellular Distribution and Processing of Amyloid Precursor Protein

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Proteolytic generation of amyloidogenic amyloid β (Aβ) fragments from the amyloid precursor protein (APP) significantly contributes to Alzheimer’s disease (AD). Although amyloidogenic APP proteolysis can be affected by trafficking through genetically associated AD components such as SORLA, how SORLA functionally interacts with other trafficking components is yet unclear. Here, we report that SNX27, an endosomal trafficking/recycling factor and a negative regulator of the γ-secretase complex, binds to the SORLA cytosolic tail to form a ternary complex with APP. SNX27 enhances cell surface SORLA and APP levels in human cell lines and mouse primary neurons, and depletion of SNX27 or SORLA reduces APP endosome-to-cell surface recycling kinetics. SNX27 overexpression enhances the generation of cell surface APP cleavage products such as soluble alpha-APP C-terminal fragment (CTFα) in a SORLA-dependent manner. SORLA-mediated Aβ reduction is attenuated by downregulation of SNX27. This indicates that an SNX27/SORLA complex functionally interacts to limit APP distribution to amyloidogenic compartments, forming a non-amyloidogenic shunt to promote APP recycling to the cell surface.

Key words: Alzheimer’s disease; APP; endosome recycling; protein trafficking; SNX27; SORLA

Significance Statement

Many genes have been identified as risk factors for Alzheimer’s disease (AD), and a large proportion of these genes function to limit production or toxicity of the AD-associated amyloid β (Aβ) peptide. Whether and how these genes precisely operate to limit AD onset remains an important question. We identify binding and trafficking interactions between two of these factors, SORLA and SNX27, and demonstrate that SNX27 can direct trafficking of SORLA and the Aβ precursor APP to the cell surface to limit the production of Aβ. Diversion APP to the cell surface through modulation of this molecular complex may represent a complimentary strategy for future development in AD treatment.
APP is proteolytically processed into neurotoxic Aβ peptides through the amyloidogenic pathway or nontoxic APP products such as soluble APPs (sAPPs) and CTs (alpha-APP C-terminal fragment) through the non-amyloidogenic pathway. Amyloidogenic and non-amyloidogenic processing is mutually exclusive; APP cleavage by non-amyloidogenic α-secretases precludes the availability of the rate-limiting β-secretase (BACE1) amyloidogenic cleavage site. Interestingly, non-amyloidogenic cleavage of APP by α-secretases occurs primarily at the cell surface, whereas amyloidogenic cleavage by BACE1 and the γ-secretase complex requires acidified trans-Golgi and endosomal environments (Xu et al., 1997; Jiang et al., 2014). This suggests that intracellular APP trafficking components also likely influence amyloidogenic APP processing.

In support of this notion, APP trafficking components such as SORLA (sortilin-related receptor with A-type repeats) have been found to be linked to AD (Pottier et al., 2012). Originally identified as a component reduced in AD brain (Scherzer et al., 2004), SORLA deletion has been shown to aggravate Aβ plaque formation in AD mouse models (Andersen et al., 2005; Rohe et al., 2008), whereas murine SORLA overexpression was shown to reverse Aβ generation (Caglayan et al., 2014). As an APP trafficking component, SORLA may act as a retention factor to sequester APP at the Golgi away from amyloidogenic processing at the endosome (Andersen et al., 2005; Rogaeve et al., 2007; Fjorback et al., 2012). However, the presence of SORLA at the cell surface and endosomal compartments (Andersen et al., 2005; Offe et al., 2006; Herskovitz et al., 2012) suggests that SORLA may likely have additional roles in mediating endosome-to-cell surface trafficking. Indeed, if mechanisms for SORLA-mediated APP endosome-to-cell surface recycling could be enhanced and restored in SORLA-attenuated AD patients, this may attenuate Aβ neurotoxicity associated with age onset.

SNX27 is a trafficking component required in driving endosome-to-cell surface transport of various endosomal targets through cargo-selective interactions with its N-terminal PDZ domain. Proteomic analysis of cell surface components with siRNA-mediated SNX27 depletion suggests that SNX27 may be required for APP trafficking, possibly through an intermediary trafficking component, because no binding interactions were detected between APP and SNX27 in vivo (Steinberg et al., 2013). Although a role for SNX27 in reducing amyloidogenic Aβ generation through interactions with PS1/γ-secretase has also been implicated (Wang et al., 2014b), whether and how SNX27 can exert cytoprotective effects through its ability to influence APP trafficking remains elusive.

Here, we describe a mechanism for SORLA endosome-to-plasma membrane recycling, which concurrently results in increased surface APP distribution and concomitant non-amyloidogenic α-secretase cleavage. Through an interaction screen to detect binding interactions between the cytosolic SORLA tail region and retromer complex components, we observe strong interactions between the SNX27 PDZ domain and the SORLA tail. We find that overexpression of SNX27 can enhance surface distribution of both SORLA and APP in cultured cells and neurons, whereas SNX27 depletion in cell lines and haploinsufficiency in primary neurons reduces cell surface SORLA and APP levels. SNX27 overexpression was also found to elevate sAPPα generation in cultured cells. Likewise, SORLA overexpression in cultured cells was found to attenuate Aβ levels in a SNX27-dependent manner. Together, these results indicate that SNX27 and SORLA interact and provide an endosomal shunt mechanism to shift the endosomal APP trafficking milieu in favor of non-amyloidogenic processing at the cell surface.

**Materials and Methods**

*Cell culture and transfection.* HEK293T and HEK293 cells stably expressing the Swedish APP KM670/671NL variant (HEKswAPP) were cultured in DMEM supplemented with 10% FBS. Turbofect transfection reagent (Life Technologies) was used for transient transfection of all cell lines described according to specifications from the manufacture supplier. RNaiMAX (Life Technologies) was used for transfection of siRNA oligonucleotides. siRNA targeting sequences to cognate human targets for cell line transfection were 5'-taccagatggacacgta for SNX27 and 5'-caggatttcggagagga for SORLA, all transfected at a final concentration of 10 nM and purchased from Qiagen. AllStars siRNA oligos were transfected as a negative control (Qiagen).

*Primary neuronal culture.* Pregnant female mice were collected from timed matings, and embryos were harvested from SNX27+/−/het/wild-type (WT) matings at E15–E17. Primary cortical neurons were obtained by microdissection of the cerebral cortex from embryos using a stereomicroscope and dispersed by digestion in trypsin and DNAseI for 30 min at 37°C, followed by trituration in DMEM. Embryonic tissue was also collected at harvesting and processed for genotype analysis using the MyTaq DNA extraction and PCR genotyping system (Bioline), in which WT and SNX27+/−/−/− neurons from individual embryos were maintained separately. Neurons were plated and maintained on poly-D-lysine-coated coverslips or culture dishes in Neurobasal medium supplemented with B27, glutamine, and penicillin/streptomycin, whereby media was changed every 3 d, when half of the media was replaced.

*Antibodies and plasmid constructs.* GST-fusion constructs were expressed in HEK293T cells using the pRK5mGST vector, in which full-length SNX27 and various domain deletions were cloned downstream in-frame of the N-terminal GST tag. The SORLA cytosolic tail (amino acids 2159–2214) region and deletions (tailΔ2159–2177 comprising amino acids 2160–2124, tail–2182, amino acids 2159–2182, tail–2198, and amino acids 2159–2198), VS26, VS29, and VS35 were also cloned into pRK5mGST for expression in HEK293T cells for interaction studies. Full-length pRK5mGST SNX27 H114A was generated by PCR-mediated site-directed mutagenesis, using a pRK5mGST SNX27 template. Myc-tagged retromer expression vectors were generous gifts from Dr. Wanjin Hong (Institute of Molecular and Cell Biology, Singapore); pDmyc–VS26 and VS35, and pClneo myc–SNX27, SNX1, SNX2, SNX5, and SNX6. pCDNA3 SORLA was obtained as a kind gift from Dr. James Lah (Emory University, Atlanta, GA). Vectors to express recombinant GST and His6 proteins included pGEX4T–SORLA tail in which SORLA cDNA sequences corresponding to amino acids 2159–2214 were PCR amplified and cloned using BamHI/EcoRI sites; likewise, pTRChis6A SNX27, pTRChis6A SNX27 PDZ, and pTRChis6A VS26 were PCR amplified and cloned in-frame downstream of the his6 tag. Coexpression of SNX27 and GFP was mediated by transfection of pIRE52 GFP–SNX27, in which SNX27 was cloned using BamHI/Xhol sites into BglII/Sall vector sites. Lentiviruses for SNX27 transduction were generated by the SBP-MDJ viral vector core facility using a pCDH513 SNX27 vector, in which SNX27 was cloned into pCDH513 using Nhel/BamHI sites. pRK5mGST–SNX27 PDZ constructs were generated by sequence alignment of SNX27, NHERF2, and SHANK2 PDZ domains, in which conserved amino acids within the secondary β-strand and H114A α-helix residues were mutated by site-directed mutagenesis. Because all residues conferred charged or bulky structural elements except for the terminal L130 residue within the terminal β-strand, we switched the hydrophobic L130 residue to Q.

pCDNA3 SORLA–mCherry was cloned by generating an XbaI at the 3′ end to replace the SORLA stop codon, in which mCherry was cloned downstream of the SORLA ORF using XbaI sites. A pCDNA3 GFP–SNX27 expression construct used to detect overlapping SNX27/SORLA localizing interactions in HEK293T cells was generated by cloning the SNX27 ORF downstream of an N-terminal GFP tag. GFP-tagged GLUR1 and SORLA cytosolic tail constructs were cloned into pCDNA3 GFP with a six glycine linker, in which cDNA sequences corresponding to the GLUR1 (amino acids 831–906) and SORLA (amino acids 2159–2214) tail
sequences were cloned in-frame downstream. A pCDNA3–FLAG SNX27 vector construct was generated by cloning SNX27 in-frame downstream of pCDNA3–FLAG.

Antibodies detecting human and mouse SNX27 were generous gifts from Drs. Wanjin Hong and Paul Slesinger (Mount Sinai Hospital, New York, NY). SORLA (LR11) and EEA1 (BD Biosciences), BIP, γ-adaptin, and GST polyconal (Santa Cruz Biotechnology), FLAG M2 (Stratagene), GF-P monoclonal (Genetex), TGN38 and Y188 C-terminal APP rabbit monoclonal (Abcam), actin (Sigma), Xpress (Life Technologies), Lamp1 (LyIC6; Enzo Diagnostics), and Tnflk (Life Technologies) antibodies were all purchased from commercial sources. The 9E10 monoclonal antibody to detect the myc epitope was generated in-house. The B436 monoclonal antibody targeting the human extracellular Aβ transmembrane proximal region has been described previously (Egger et al., 2009) and was used to detect Aβ, sAPPα, and surface APP by immunofluorescence. The 22C11 monoclonal antibody for APP detection was purified in-house.

Recombinant protein purification. GST, GST–SORLA tail, and his6–SNX27, SNX27 PDZ, and VPS26 were expressed from pGEX4T3 and pTRChis6A vectors described above and purified by glutathione Sepharose/Ni-NTA agarose affinity purification methods described previously (Huang et al., 2013). BL21 pGEX4T3 or pTRChis6 transformants were grown to exponential phase and induced with 1 mM isopropyl-β-D-thiogalactopyranoside and cultured for an additional 3–5 h. Bacteria were grown to exponential phase and induced with 1 mM isopropyl-β-D-thiogalactopyranoside and cultured for an additional 3–5 h. Bacteria were pelleted in lysis buffer and lysed by sonication in 10 mg/ml lysozyme in the presence of protease inhibitors (complete; Roche). After clearing lysates by spinning at 10,000 g for 10 min, GST proteins were precipitated using glutathione Sepharose, whereas his6-tagged constructs were precipitated with Ni-NTA agarose in the presence of 10 mM imidazole.

Glutathione beads were washed in 10 mM Tris-HCl, pH 8, and 0.5 M NaCl, whereas Ni-NTA beads were washed in the same buffer containing 20 mM imidazole. GST proteins were eluted with 30 mM reduced glutathione in 0.3 M Tris-HCl, pH 8, and his6 proteins were eluted in 0.3 M imidazole in 10 mM Tris-HCl, pH 8, and 0.5 M NaCl. Eluted proteins were then dialyzed in 1X PBS with 5% glycerol and 0.3 mM DTT overnight and frozen at −80°C. The Xpress antibody was used to detect the Xpress epitope downstream of the his6 tag in the pTRChis6A vector by immunoblotting.

Recombinant in vitro, semi-in vitro, and in vivo GST pull-down assays. To assay binding interactions between recombinant GST–SORLA tail and his6 purified SNX27/VPS26 constructs, recombinant purified GST or GST–SORLA tail were incubated with recombinant his6 proteins for 2 h at 4°C rocking in the presence of glutathione Sepharose, precipitated, and washed three times at room temperature 15 min each in lysis buffer containing 0.5 M NaCl. GST and his6 components were then immunoblotted for GST or Xpress bound/coprecipitated by immunoblotting.

Semi-in vitro binding interactions required reimmobilizing recombinant purified GST or GST–SORLA tail constructs on glutathione, in which beads were washed and individually incubated with HEK293T lysates expressing myc-tagged constructs comprising the core retromer complex (Vps26, Vps35, Snx27, Snx1, Snx2, Snx5, and Snx6). After incubation with the immobilized GST constructs, myc-tagged constructs were coprecipitated, washed in lysis buffer, and visualized by immunoblotting.

In vivo GST pull-down interactions involved transfection of HEK293T cells with pRK5mGST vectors expressing retromer components or GST-tagged SNX27 truncation fragments as described above. For detection of semi-endogenous interactions, pRK5mGST vectors expressing SNX27, SNX27 truncations, or other tagged retromer components were transfected in HEK293T and cultured overnight, and lysates were generated, in which GST constructs were precipitated using glutathione agarose. Beads were consequently washed, and endogenous SORLA or APP interactions coprecipitated with the GST precipitates were detected by immunoblotting. In our PDZ mutagenesis screen for mutations attenuating SNX27 PDZ/SORLA interactions, pRK5mGST PDZ constructs were transfected in HEKswAPP cells and precipitated for endogenous SORLA or APP with glutathione Sepharose as above. We note in our pull-down assays that L130Q and L130A mutations have equivalent effects on attenuating SORLA/APP interaction (data not shown).

Coimmunoprecipitation assays. SORLA complexes were immunoprecipitated from mouse brain lysates in lysis buffer using 2 μg of SORLA monoclonal antibody (BD Biosciences), and immune complexes were precipitated with Protein G and washed five times in lysis buffer before immunoblotting.

Surface biotinylation. Cell surface biotinylation using EZ-link Sulfo-NHS-LC biotin in HEKswAPP and primary neurons was performed as described previously (Wang et al., 2013). Briefly, cells or neurons were washed in cold 1X PBS supplemented with 1 mM MgCl2 and 1.3 mM CaCl2 (1X PBS/CaM) and incubated with 500 μg/ml biotin labeling reagent in 1X PBS/CaM at 4°C with agitation. Labeling was repeated and quenched with 7.5 μg/ml glycine in 1X PBS/CaM. Cells were washed, lysed in lysis buffer, and incubated overnight with streptavidin agarose at 4°C with agitation. Streptavidin agarose beads were then washed and boiled in Laemmli buffer, and biotin-labeled precipitates were visualized and quantified by immunoblotting.

Kinetic endocytosis and endosome to surface recycling assays. Measurement of cell surface internalization and endosome-to-cell surface recycling of SORLA and APP was determined in transfected HEKswAPP cells as described previously (Wang et al., 2013).

Sucrose density fractionation. For sucrose density subcellular fractionation, cells were lysed using a steel ball-bearing chamber and subjected to ultracentrifugation as described previously (Zhang et al., 2003). Briefly, cells were washed in 1X PBS and resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM MgAc2 and passed 15–20 times in a steel ball-bearing chamber. Lysates were cleared at 800 × g for 5 min and loaded onto a discontinuous sucrose gradient comprising 1.5 ml of 2 M, 4 ml of 1.3 M, 3 ml of 1.16 M, and 2 ml of 0.8 M sucrose in 10 mM Tris-HCl, pH 7.4, and 1 mM MgAc2. Gradients were spun for 2.5 h at 100,000 × g, and 1 ml fractions were collected and subjected to TCA precipitation overnight at 4°C, washed in acetone, and subjected to immunoblot analysis.

Mouse lines and hippocampal fractionation analysis. TG2576 mouse lines expressing the Swedish APP695 KM760/671N allele were purchased from Taconic and maintained in-house. Mouse lines overexpressing SORLA at the Rosa26 locus were described previously (Caglayan et al., 2014); SORLA–Rosa26 and SNX27+/− lines were crossed with TG2576 transgenic (TGs) to generate SORLA–Rosa26/TG2576 and SNX27+/−/TG2576 lines. Triple TG2576/SORLA–Rosa26/SNX27+/− TG mouse lines were generated by crossing TG2576/SORLA–Rosa26+positive animals with SNX27−/− or SNX27+/− animals with SORLA–Rosa26+ Tggs. Genotypic TG2576 Tggs from these triple crosses were selected for analysis, in which SNX27 WT and SNX27−/− animals were compared in SORLA–Rosa26 WT and TG (overexpression) backgrounds.

Microscope image sample preparation and acquisition. All images were acquired using an inverted Zeiss Axio Observer Z1 fluorescence microscopy system, in which acquisition and processing of images used Slide-
Results

**SNX27 interacts with the SORLA cytosolic tail**

The retromer complex has been previously described to selectively bind and direct cargo from endosomal compartments. Because trafficking mechanisms for SORLA selection and subsequent distribution from the endosome has yet to be described, we determined whether individual components of the retromer complex could interact with the SORLA cytosolic tail region. Using a semi-\textit{in vitro} interaction assay with a recombinant purified GST–SORLA tail fusion peptide immobilized on glutathione beads, we find that SNX27 selectively precipitated with the SORLA cytosolic tail with little interaction observed from other components of the retromer complex (Fig. 1A).

To confirm these interactions, we expressed GST fusions with members of the core retromer components in HEK293T cells and assayed for coprecipitation with endogenous SORLA. We similarly observed selective interactions between SNX27 and endogenous SORLA, with relatively no interaction observed with other retromer components (Fig. 1B). Because SNX27 interactions with endogenous SORLA appeared to be somewhat weak, we also assayed for SNX27/SORLA interactions with SORLA co-overexpression (Fig. 1C). We found that SORLA/SNX27 interactions were indeed markedly improved with SORLA/GST–SNX27 co-overexpression and subsequent precipitation of GST–SNX27 complexes (Fig. 1C). Because SNX27 has been previously characterized to bind various endosomal trafficking components through selective interactions with modular PDZ, PX, RA, and FERM domains (Cullen, 2008), we expressed individual GST-fused SNX27 domain modules in HEK293T cells and assayed for interactions with endogenous SORLA (Fig. 1D). We observed strong interactions with full-length SNX27 and the N-terminal SNX27 PDZ domain with endogenous SORLA (Fig. 1D), in which relatively little interaction was observed in constructs lacking this domain. Using recombinant purified components, we next determined whether interactions between the SORLA tail and SNX27 were direct. We found that full-length SNX27 or the SNX27 PDZ domain coprecipitated with the SORLA cytosolic tail (Fig. 1E). These results suggest that SNX27/SORLA tail interactions may be direct. As interactions between SORLA and VPS26 were previously described (Fjorback et al., 2012), we also could reiterate VPS26/SORLA tail interactions \textit{in vivo} (Fig. 1F) but not \textit{in vitro} (Fig. 1E), suggesting that VPS26 may require complexes formed \textit{in vivo} for SORLA tail interactions.

SORLA interactions with VPS26 were previously reported to require a FANSHY motif within amino acids 2159–2180 within the SORLA tail (Fjorback et al., 2012); we find by deletion and subsequent GST pull-down analysis that deletion of the membrane proximal 2159–2177 region of the SORLA tail attenuated its interaction with both VPS26 and SNX27 in cells coexpressing GST–SNX27 or GST–VPS26 and GFP–SORLA tail constructs (Fig. 1F). Because we were able to reconstitute SORLA tail/SNX27 interactions \textit{in vitro} using recombinant proteins (Fig. 1E), we determined the effects of deletion of GGA (amino acids 2208–2214), acidic (amino acids 2190–2198), and membrane proximal (amino acids 2159–2177) regions in the SORLA tail region for interactions with endogenous SNX27 (Fig. 1G). Likewise, deletion of this 2159–2177 region in GST–SORLA tail construct abrogated its interaction with endogenous SNX27, whereas deletion of 2182–2214 (acidic and GGA) or 2198–2214 (GGA) regions had no effect on their interactions (Fig. 1G). Together, this indicates that SNX27/SORLA interaction likely requires the presence of a membrane proximal region within the SORLA tail.

Extensive evidence suggests that the SNX27 PDZ domain comprises an important cargo selection module required for endosomal sorting. Because SORLA lacks a canonical C-terminal X-S/T-X-PDZ interaction motif, we were interested in determining whether PDZ mutations that abrogate PDZ/PDZ-motif interactions could affect SORLA tail interactions (Lauffer et al., 2010). We found that, although the H114A mutation within an α-helical PDZ-motif-binding interface of SNX27 disrupted its interactions with a PDZ-motif substrate GLUR1 tail (Wang et al., 2013), this mutation had relatively little effect on SORLA tail interactions (Fig. 1H). Quantification and comparison of GLUR1 and SORLA tail interactions with SNX27 suggest that SORLA tail/SNX27 interactions \textit{in vivo} are weak compared with GLUR1 tail/SNX27 binding (Fig. 1H, top graph); however, GLUR1 tail interactions with WT SNX27 are fivefold higher compared with H114A, in which little or no change is observed between WT/H114A SNX27 and SORLA tail (Fig. 1H, middle and bottom graphs). Together, these results indicate a direct interaction between a membrane proximal region of the SORLA cytosolic tail and the SNX27 PDZ domain, which may occur through a different binding mechanism previously described for PDZ/PDZ C-terminal motifs.

SNX27 and SORLA are components that have been previously observed to localize in part to endosomal compartments (Lauffer et al., 2010; Herskowitz et al., 2012). Given their ability to interact physically, we were interested in determining whether SNX27 and SORLA interactions could be reiterated by localization overlap. mCherry-tagged SORLA and GFP-tagged SNX27 appeared to colocalize at punctate subcellular foci as previously described, and, although no specific overlap was observed with GFP alone, we observed an overlap between SORLA and SNX27 puncta in HEK293 cells stably transfected with the Swedish K595N/M596L variant (HEKswAPP) and primary cortical neurons (Fig. 2A, B). Although overexpressed SORLA and SNX27 may not reconstitute true intracellular distribution profiles of these components, colocalizing overlap between SORLA and SNX27 further support binding interactions between these two components.

**SNX27 is important for SORLA and APP cell surface distribution**

As a key trafficking component in mediating the intracellular distribution of APP, SORLA has been shown previously to interact with APP. Therefore, we determined whether endogenous SNX27 could interact with APP and SORLA \textit{in vivo} by coimmunoprecipitation. Using a SORLA antibody, we found that SORLA could coimmunoprecipitate with both APP and SNX27 from
Figure 1. The SNX27 PDZ domain interacts with the cytosolic SORLA tail. A, Probing interactions between the retromer complex and the SORLA cytosolic tail. HEK293T lysates expressing different myc-tagged retromer components were incubated with recombinant purified GST fusions of the SORLA cytosolic tail immobilized on glutathione Sepharose. Unbound components were washed from precipitates, and bound components were detected by myc immunoblots. B, The SNX27 retromer component interacts with endogenous SORLA with a comparatively high affinity. GST-fusion constructs comprising various retromer complex components were expressed in HEK293T cells, and glutathione precipitates were probed for endogenous SORLA by immunoblotting. C, GST, GST–SNX27, and control or SORLA vector constructs were coexpressed by transfection in HEKswAPP cells as indicated, and GST-tagged complexes were precipitated and immunoblotted as in B. D, The SNX27 PDZ domain binds to the SORLA cytosolic tail. GST–SNX27 constructs comprising various SNX27 domains were assayed for coprecipitation with endogenous SORLA in HEK293T cells, in which glutathione precipitates were probed by immunoblotting. E, Reconstitution of SNX27 PDZ domain/SORLA tail interactions in vitro. Recombinant his6–Xpress SNX27, SNX27 PDZ, or VPS26 constructs were incubated with a recombinant purified GST–SORLA tail peptide immobilized to glutathione Sepharose. Bound his6/Xpress-tagged precipitates were detected by Xpress immunoblotting. F, G, SNX27/SORLA tail interactions require residues 2159–2177 in the SORLA tail region. GST-tagged VPS26 or SNX27 was coexpressed with a GFP-tagged construct comprising the SORLA tail or SORLA tail lacking a region containing the FANSHY motif (amino acids 2159–2177) in HEK293T cells, and glutathione precipitates were analyzed by immunoblot. G, GST-tagged SORLA tail deletion constructs were expressed in HEK293T cells, and glutathione precipitates were probed for endogenous SORLA by immunoblotting. H, Vectors expressing GFP-tagged GLUR1 or SORLA cytosolic tail fragments were cotransfected with GST-tagged SNX27 or H114A SNX27 constructs in HEK293T cells, and glutathione Sepharose precipitates from lysates were probed for GFP and GST constructs. Quantification of GFP-tail coprecipitation in the adjacent graphs: bound/input in relation to GLUR1-tail precipitation (GLUR1-tail set to 1.0, top), bound/input of H114A constructs compared with WT for GLUR1 and SORLA tails (WT set to 1.0, middle), and fold increase in GFP-tail binding ratios, WT/H114A for GLUR1 and SORLA tails (bottom).
mouse brain lysates (Fig. 3A). Similar to SORLA, SNX27 coprecipitates with both endogenous SORLA and APP in a manner dependent on the N-terminal PDZ domain as determined by deletion and GST pull-down analysis (Fig. 3B). These results indicate that SNX27, SORLA, and APP can form a ternary complex. SNX27 is an endosomal trafficking component that mediates cell surface distribution of numerous transmembrane cargo. We then determined whether reductions in SNX27 levels could influence cell surface SORLA and APP distribution. We found that SNX27 siRNA treatment in HEKswAPP cells led to a reduction in surface SORLA and APP levels (Fig. 3C, left panel). Similarly, we also found that SNX27 haploinsufficient in dissociated primary neuronal cultures also caused reductions in SORLA and APP cell surface distribution with little influence on surface transferrin

receptor (TfnR) distribution (Fig. 3C, right panels). Using an APP antibody targeting the extracellular surface proximal to the Aβ transmembrane region (B436), we found that APP surface labeling in HEKswAPP cells observed under steady-state conditions were reduced with SNX27 siRNA treatment (Fig. 3D). These results demonstrate that reductions in SNX27 levels can also attenuate cell surface SORLA and APP distribution.

We also determined whether SNX27 overexpression could enhance surface APP and SORLA distribution. We found that overexpression of SNX27 could enhance both surface SORLA (Fig. 4A, left panels) and surface APP (Fig. 4B, left panels) levels in HEKswAPP cells, in which relatively little or no effect was observed with overexpression of other retromer components (Fig. 4A, B). Furthermore, SNX27 overexpression was observed to enhance surface SORLA and APP distribution, with relatively little effect on surface TfnR distribution in cortical neurons transduced with SNX27 lentiviral constructs (Fig. 4C, right panels). In support of our biotin surface labeling results, we also observed an increase in cell surface APP levels by anti-APP antibody surface labeling and immunofluorescence (Fig. 4D).

Together, these results strongly indicate that SNX27 mediates cell surface distribution of SORLA and APP.

**SNX27 and SORLA Mediate Endosome-to-Cell Surface APP Recycling**

Using a cleavable disulfide biotin cell surface labeling method described previously (Wang et al., 2013), we labeled cell surface components in HEKswAPP cells and tracked the internalization and resurfacing kinetics of SORLA and APP. Surface-labeled SORLA and APP both achieved peak internalization at 15 min and consequently sorted back to the cell surface within 30–60 min (Fig. 5A). We note that, although APP almost entirely recycled to the cell surface 60 min after internalization, much of SORLA was retained internally. This might suggest that SORLA may be trafficked to other intracellular sites such as the Golgi after internalization, whereas APP is efficiently recycled to the cell surface. We also tracked APP endosome-to-cell surface trafficking using this method and observed redistribution of internalized APP to the cell surface between 30 and 60 min (Fig. 5B).

We then determined the individual effects of siRNA-mediated SNX27 and SORLA depletion on APP internalization and endosome-to-cell surface recycling kinetics. We found no significant difference in APP internalization in HEKswAPP cells transfected with control, SNX27, and SORLA siRNA (Fig. 5C). However, we observed slower endosome-to-cell surface redistribution of APP in both SNX27 and SORLA siRNA-transfected cells (Fig. 5D). These results suggest that SNX27 and SORLA may both be required for efficient APP endosome-to-cell surface recycling. Because a role for SNX27-mediated endosome-to-cell surface distribution has been established for numerous plasma membrane components, including GLUR1, GIRK, βAR, and GLUT1 (Lunn et al., 2007; Temkin et al., 2011; Steinberg et al., 2013; Wang et al., 2013),
cells (LAMP1/APP overlap in both SNX27 and SORLA siRNA-transfected control siRNA-transfected HEKswAPP cells, we observed increased SORLA depletion could influence APP localization at lysosomes. Although we observed poor overlap between LAMP1 and APP in lysosomal compartments, we also determined whether SNX27 and SORLA have been characterized previously as neuroprotective trafficking components that can affect endosome-to-cell surface clear- ance machinery can induce SORLA and APP accumulation in endosomes, which can enhance subsequent passage of APP to late endosomes/lysosomes.

Together, these results indicate that SNX27 and SORLA are trafficking components that can affect endosome-to-cell surface APP recycling and redistribution.

SNX27 and SORLA interact to mediate non-amyloidogenic APP processing
Our results so far indicate that SNX27 interacts with a SORLA/APP complex and that SNX27 and SORLA can influence endosome-to-cell surface APP trafficking. Both SNX27 and SORLA have been characterized previously as neuroprotective components that are required to limit amyloidogenic processing of APP to Ab. The results here suggest that SNX27 and SORLA may function cooperatively as a protective shunt mechanism to redistribute APP from the endosome to the cell surface. Because amyloidogenic APP processing at acidified endosomes and non-amyloidogenic APP cleavage at the cell surface occurs in a mutually exclusive manner, our results so far suggest that SNX27 may enhance SORLA/APP surface cleavage and SNX27-dependent inhibition of the γ-secretase complex described previously (Fig. 9A; Wang et al., 2014b).
Figure 6. SNX27 and SORLA depletion can influence subcellular APP distribution. **A**, HEKswAPP cells were transfected with control, SNX27, or SORLA siRNAs, harvested and subjected to sucrose density fractionation as described in Materials and Methods, and immunoblotted for the components indicated. Untreated cells were surface biotinylated, and sucrose density fractions were probed with streptavidin–HRP to detect cell surface components as indicated. **B**, Lysates from the input from **A** were probed for SORLA and SNX27 depletion by immunoblotting.
Given that SNX27 affects cargo distribution to numerous targets through its PDZ domain, we questioned whether attenuating SNX27/SORLA interactions through mutation of the PDZ domain could affect SORLA/APP surface distribution and consequent APP processing. To this end, we individually introduced mutations into residues conserved between SNX27, SHANK2, and NHERF2 PDZ regions within each secondary structural element in a GST–SNX27 expression construct and assayed for interactions with APP and SORLA in HEKswAPP cells (Fig. 9B). We found that SORLA/SNX27 interactions were influenced most dramatically by mutation of an L130 residue within the terminal strand within the PDZ domain (Fig. 9B). Integrating this mutation into a FLAG–SNX27 overexpression construct, we found that SNX27 overexpression could enhance SORLA/APP surface distribution and sAPPα generation, whereas overexpression of the SORLA-refractory L130Q mutation had markedly reduced effects on surface SORLA/APP distribution and sAPPα production (Fig. 9C). This provides an additional indication that SNX27-mediated surface SORLA/APP distribution and sAPPα generation is dependent on SNX27/SORLA interactions.

We next determined the effects of modulating SORLA levels on APP processing. We observed a decrease in sAPPα with SORLA overexpression and increased sAPPα accumulation with SORLA depletion (Fig. 9D,E). Similarly, dramatic increases in sAPPα were observed with SORLA deletion in mouse knock-out models (Rohe et al., 2008). Because our results suggest that surface SORLA/APP levels and APP cleavage may be dependent on SNX27, we assayed whether SNX27-mediated elevation of sAPPα levels was sensitive to reductions in SORLA by siRNA transfection. We observed an attenuated SNX27-dependent increase in both CTFα and sAPPα levels with SORLA siRNA transfection, indicating that SNX27-dependent enhancement of non-amyloidogenic APP processing is SORLA dependent (Fig. 9F,G). This suggests that
SNX27 facilitates SORLA and APP distribution to PSD-enriched membranes

Having established that SNX27 mediates SORLA and APP distribution to the cell surface and because SORLA-mediated reductions in Aβ production are dependent on SNX27 in HEKswAPP cell lines (Fig. 9H), we determined whether SNX27 had similar effects on SORLA/APP membrane distribution in TG2576 mouse lines expressing the human Swedish APP variant. To this end, we combined TG2576, SORLA–Rosa26, and SNX27 deletion alleles through consecutive mating of TG2576 in combination with SORLA–Rosa26 and SNX27+/−/− animals and screened for TG2576 SNX27 WT and SNX27+/− progeny in SORLA–Rosa26 WT and TG overexpression backgrounds. TG2576 mice were killed at 8 weeks of age, and hippocampal tissue was processed for membrane/PSD enrichment and assayed for APP/SORLA distribution by immunoblotting.

We observed that SORLA and APP appeared in cytosolic and membrane fractions and were relatively less abundant in PSD-enriched fractions (Fig. 10A). Our fractionation protocol partitioned presynaptic markers such as synaptophysin (SVP38) to Triton X-100-soluble (TX-sol) membrane fractions and PSD markers such as PSD95 to Triton X-100-insoluble PSD-enrichment fractions (Fig. 10A). Although we observe mild elevations in SORLA in total lysates from our mixed SORLA–Rosa26 background (~50%), we find that SORLA enrichment is nearly threefold in PSD fractions (Fig. 10B,C). We find that SNX27 haploinsufficiency in SORLA–Rosa26 TG mouse lines had a significant effect on reducing SORLA and APP levels in PSD-enriched fractions, in which SNX27+/− had less of an effect in reducing SORLA and APP in TX-sol membrane fractions (Fig. 10B,C). Because our results in cells and dissociated neurons indicate that SNX27 can enhance SORLA and APP distribution to the cell surface, these results give an additional indication that SNX27 can mediate distribution of SORLA and APP to PSD-associated membranes in vivo.

Discussion

The distribution of the amyloid precursor component APP to the cell surface or endosomal compartments is critical in determining its eventual processing through non-amyloidogenic or amyloidogenic pathways. Because proteolytic cleavage through these pathways occur in a mutually exclusive manner, enhanced distribution in favor of either pathway may ultimately influence proteotoxic Aβ generation and eventual AD pathophysiology (Fig. 10D). Here, we describe physical and functional interactions between the APP trafficking component SNX27 and SORLA, which has essential functions in diverting internalized transmembrane endosomal components back to the cell surface (Fig. 10D). Although previous evidence has indicated that SORLA has a predominant role in trafficking APP to the Golgi (Andersen et al., 2005; Rogaeva et al., 2007; Fjorback et al., 2012), whether SORLA could also mediate endosome-to-cell surface APP recycling has been essentially unexplored.

Proteomic analysis of cell surface components reduced with SNX27 siRNA downregulation previously implicated APP as a potential SNX27 trafficking target (Steinberg et al., 2013). However, failure to detect APP in the SNX27 interactome might suggest that a bridging component may be required to direct APP...
Figure 9. **SNX27 and SORLA interdependently promote non-amyloidogenic APP cleavage.**

**A**, HEKswAPP cells transfected with control or FLAG–SNX27 vectors were allowed to secrete soluble APP species for the time indicated, and sAPP$_\alpha$ was detected by immunoblotting with the B436 antibody. Lysates were also generated, and APP or CTFs were quantified by immunoblotting as indicated. **B**, GST–SNX27 PDZ constructs were transfected and expressed in HEKswAPP cells and precipitated with glutathione Sepharose. SORLA and APP coprecipitation was visualized by immunoblotting; L130Q PDZ constructs demonstrated marked attenuation in SORLA/APP coprecipitation. **C**, Empty vector, FLAG–SNX27, or L130Q constructs were transfected in HEKswAPP cells, and media were conditioned for sAPP$_\alpha$ secretion as in A or labeled for surface components. Surface SORLA/APP levels were precipitated with streptavidin agarose and visualized by immunoblotting; quantified surface SORLA/APP levels normalized against inputs are shown in the adjacent graphs. **D**, HEKswAPP cells were transfected with control or SORLA-overexpression vectors and immunoblotted for the components indicated. sAPP$_\alpha$ was also detected in conditioned media from transfected cells. **E**, HEKswAPP cells transfected with control or SORLA siRNA were immunoblotted for the components indicated, in which media conditioned for 5 h was collected for sAPP$_\alpha$ immunoblotting. **F**, HEKswAPP cells were transfected with control or SORLA siRNAs as indicated and retransfected with control or FLAG–SNX27 vectors as indicated 24 h later. Cells were incubated for an additional 48 h and immunoblotted for the components indicated. **G**, HEKswAPP cells were transfected with control or SORLA siRNAs as indicated and retransfected with control or FLAG–SNX27 vectors as indicated 24 h later. Cells were incubated for an additional 48 h and immunoblotted for the components indicated. To quantify CTF$_\alpha$ cleavage, band intensity for the four treatments were quantified, and the fold CTF$_\alpha$ increase with SNX27 overexpression was compared between control and SORLA siRNA transfections as indicated. **H**, HEKswAPP cells were transfected with control or SORLA siRNA oligos and control/FLAG–SNX27 vectors as in F, and conditioned media was analyzed for sAPP$_\alpha$ secretion by immunoblot. sAPP$_\alpha$ intensity was normalized relative to control siRNA vector control transfected cells, and relative fold increases with SNX27 overexpression were compared between control and SORLA siRNA samples. **I**, Control or SNX27 siRNA-transfected cells were retransfected with control or SORLA overexpression vectors, and conditioned media was TCA precipitated and immunoblotted for Aβ. All graphs depict mean ± SE from at least three experiments, *p < 0.05, **p < 0.01, ***p < 0.005.
Figure 10. Model for enhanced non-amyloidogenic APP recycling to the cell surface through SNX27/SORLA interactions. A–C, SNX27 haploinsufficiency affects SORLA/APP distribution to hippocampal PSD membranes in vivo in a SORLA–Rosa26 background. A, TG2576 mouse hippocampus was homogenized and subjected to membrane fractionation as described in Materials and Methods. Equal protein quantities were loaded for each fraction, and components were immunoblotted as indicated. B, Hippocampal tissue from TG2576 mice was dissected, homogenized, and subjected to fractionation as in A. Total, cytosolic, TX-sol, and PSD-enriched fractions were generated and immunoblotted for various components as indicated. C, Hippocampal tissue from six mice of each genotype were immunoblotted for SORLA and APP from various fractions, in which SORLA and APP were subjected to densitometric quantification. SORLA/APP from cytosolic fractions were normalized to H9251-tubulin, whereas TX-sol and PSD-enriched fractions were normalized to SVP38 (synaptophysin) and PSD95, respectively. All values were normalized to averages from SNX27/SORLA–Rosa26 WT values set to 1.0. Significance values were determined by nonpaired Student’s t tests (*p < 0.03, **p < 0.01). D, Model for enhanced non-amyloidogenic APP recycling to the cell surface through SNX27/SORLA interactions. Left, Amyloidogenic liberation of the Aβ region (red) is derived from full-length APP through sequential cleavage by BACE1 and the γ-secretase complex; non-amyloidogenic cleavage by α-secretases (blue) at the cell surface precludes Aβ generation. Right, Model for an SNX27/SORLA shunt to limit amyloidogenic Aβ generation. SNX27 interactions with the SORLA cytosolic tail at early endosomes can enhance cell surface APP recycling, thereby enhancing cell surface cleavage products such as sAPPα/CTFα (blue) and attenuating amyloidogenic cleavage of Aβ (red).
trafficking in vivo (Steinberg et al., 2013). Our results implicate SORLA as a likely candidate by which SNX27 diverts APP from the endosome to the cell surface (Fig. 10D). Although both SORLA and SNX27 have been characterized previously as trafficking components that reduce the amyloidogenic production of Aβ, whether these two components worked interdependently to attenuate Aβ levels was unknown. Importantly, our results show that modulation of these two components can coordinately influence non-amyloidogenic sAPPα production and reductions in Aβ generation. However, we note that, under steady-state conditions, APP is primarily absent from cell surface and endosome compartments and may therefore be predominantly affected by Golgi sorting signals. In agreement with this, our results indicate that modulation of SNX27 levels has only modest effects in influencing Aβ generation (Wang et al., 2014b), suggesting that endosomal trafficking pathways normally compete with APP Golgi retention/retromer recycling pathways under steady-state conditions.

SORLA has been reported to reduce Aβ accumulation through several mechanisms, such as diverting APP traffic from the endosome to the Golgi (Fjorback et al., 2012), inhibiting BACE1/APP interactions (Spoelgen et al., 2006), inhibiting APP oligomerization (Schmidt et al., 2012), and diverting Aβ traffic to lysosomes (Caglayan et al., 2014). Interestingly, SNX27 may similarly have a pluralistic role in limiting Aβ generation in part through inhibiting the γ-secretase complex as reported previously (Wang et al., 2014b) and in part through SORLA/APP trafficking mechanisms described here. Aβ reductions with SNX27 overexpression is typically modest (~10–20%; Wang et al., 2014b), and we also observed SNX27-mediated reduction in Aβ levels with SORLA siRNA transfection (~15%; our unpublished results). Together, this suggests that SNX27-dependent Aβ reduction can occur through the inhibition of γ-secretase in the absence of SORLA and that its ability to limit Aβ generation is not confined to its SORLA/APP trafficking function. Furthermore, we also observe that perturbations in SNX27 or SORLA in our experiments fail to mediate any dramatic changes in APP levels in HEKswAPP cells and in primary neurons. This indicates that, although we describe changes in α, β, and γ-secretase-dependent APP cleavage products with SNX27 and SORLA perturbations, both cells and primary neurons maintain a relatively stable APP pool.

Synaptic loss has been linked to cognitive decline in AD, and it has been postulated that oligomeric Aβ species is a key underly-
ing component in driving pathophysiological synaptotoxicity (Tu et al., 2014). This may suggest that site-specific Aβ generation at synaptic sites may have exaggerated effects on synaptic structure and function in AD brain. SNX27 has been observed previously to be enriched at postsynaptic densities (Hussain et al., 2014), which may affect synaptic AMPAR recycling to the cell surface at synaptic sites (Wang et al., 2013; Hussain et al., 2014). We find that a portion of SORLA and APP partition to PSD-enriched fractions, and SNX27 haploinsufficiency can attenuate SORLA/APP distribution to PSD-enriched preparations in SORLA–Rosa26 TG mice. Because oligomeric Aβ has been correlated previously with synaptotoxicity in mouse models (Koffie et al., 2009), the possibility that SNX27 may regulate APP trafficking and processing at postsynaptic densities indicates that SNX27/SORLA-mediated APP surface redistribution may have site-specific effects at synaptic junctions.

SNX27 has been described previously to bind and function coordinately with members of the core retromer complex, particularly VPS26 (Steinberg et al., 2013). Interestingly, dysfunc-
tion in members of the core retromer complex has also been described in AD. Depleted levels of VPS26 and VPS35 have been observed in AD patient brain (Small et al., 2005), and VPS26, VPS35, and SNX27 haploinsufficiency mouse models have been observed to exacerbate AD phenotypes (Muhammad et al., 2008; Wen et al., 2011; Wang et al., 2014b). Previous studies have also implicated VPS26 to bind the SORLA tail FANSHY region, in which mutation of the interaction site redistributes SORLA to non-Golgi sites, suggesting that VPS26 may also have a role in endosome to Golgi retrieval (Fjorback et al., 2012). Moreover, chemical chaperones that stabilize the retromer complex are found to redistribute APP from endosomes to attenuate amyloidogenic APP processing (Mecozzi et al., 2014). Unlike other members of the retromer complex, SNX27 is primarily involved in promoting endosome-to-cell surface recycling rather than endosome-to-Golgi shuttling mediated by the classical retromer complex (Cullen and Korswagen, 2012). At this point, it is unclear why APP shuttling has diverged two differing sorting pathways from the endosome. However, because both cell surface and Golgi sorting can divert APP from accumulation and amyloidogenic cleavage at lysosomes, both sorting pathways will be of future interest in developing anti-amyloidogenic drug targeting strategies.

Among genetic AD risk factors identified so far, a large number of risk components such as SORLA are found to vary frequently in the general population but demonstrate low penetrance in triggering AD onset. In addition to SORLA, a significant proportion of these frequently-varied/low-risk components comprise endocytic and intracellular trafficking components, including PICALM, BIN1, and CD2AP (Harold et al., 2009; Hollingworth et al., 2011). Together with the observation that retromer trafficking component deficiency can also aggravate AD phenotypes, this suggests that, although intracellular trafficking may not be a primary driver in AD onset, dysfunction of cellular trafficking mechanisms may alter the intracellular distribution of APP or its associated processing components in favor of amyloidogenesis. Because directly altering or inhibiting amyloidogenic processing components such as γ-secretase have failed to be clinically effective in treating AD, targeting AD-associated trafficking components such as SORLA/SNX27 may prove to be a good alternative in ameliorating AD outcome.

The involvement of SNX27 in AD pathogenesis is particularly interesting; although its abundance was not altered in AD, SNX27 was previously observed to disrupt and attenuate γ-secretase activity (Wang et al., 2013, 2014b). Furthermore, SNX27 levels were observed previously to be reduced in Down’s syndrome through a C/EBP-dependent mechanism to induce AMPA receptor trafficking dysfunction and synaptic impairment (Wang et al., 2013). Because the repertoire of SNX27 targets is immense with a diverse subset of membrane proteins now to include SORLA, it is not surprising that SNX27 function is essential to proper physiological neuronal function.

SNX27 belongs to a large family of Sorting Nexin family of PX domain components, of which there are at least 33 members (Cullen, 2008). Because these components are defined by PX domains that primarily binds phosphatidylinositol-3,4,5-trisphosphate (PIP3), a large number of these components may intuitively have some functional role within endosomal compartments. Given that endosomes are aberrantly enlarged in AD and endosome-enriched PIP3 levels are reduced in human AD and AD mouse models (Cataldo et al., 2000; Nixon, 2005; Morel et al., 2013), it will also be interesting whether any of the other Sorting Nexin family members are involved in trafficking amyloidogenic components from the endosome and whether this activity involves SNX27 and SORLA.
In summary, we present evidence for an endosomal sorting system for APP redistribution from endosomes to the cell surface involving SNX27 and SORLA trafficking components.

References
Huang et al. • SNX27 and SORLA Mediate Cell Surface APP Recycling


