SAP97 and CASK mediate sorting of N-Methyl-D-Aspartate Receptors through a novel secretory pathway

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Figure S1. Differential trafficking of GluRs following selective Golgi impairment. P0 hippocampal neurons were transfected at DIV12 with either GFP-GluR1 or NR1-GFP. Neurons expressed exogenous subunit protein for several hours, and were then subjected to either temperature block or BFA treatment, to selectively impair trafficking through early secretory compartments. A) Exit of newly synthesized subunits from the somatic Golgi was assayed by shifting cultures to 20°C for 2h, at 12h post-transfection, and measuring subunit expression in proximal dendrites (identified by MAP2 staining). NR1-GFP expression levels appeared unaltered (top panels), while GFP-GluR1 expression was significantly diminished (bottom panels). B) Quantification of 20°C block experiment, measured as puncta per micron (mean ± S.E.M. from 6-10 neurons per group; p< 0.05). C) Quantification of a 15°C block experiment, which causes an accumulation of membranous cargo at the level of the ER (mean ± S.E.M. from 6-10 neurons per group; p< 0.05). D) Cultures were treated with BFA at 2h post-transfection, for a period of 5h. This resulted in the disruption of Golgi membranes, as indicated by staining for TGN 38 (top panels), with no effect on dendritic expression levels of NR1-GFP (middle panels), but a significant decrease for GFP-GluR1 (bottom panels). E) Quantification of BFA experiment (mean ± S.E.M. from 6-10 neurons per group; p< 0.05). Scale bar, 5 µm.
Figure S2. AMPARs do not accumulate at Golgi outposts following mutant ARF1 expression. E18 hippocampal neurons were transfected at DIV12 with GFP-GluR1 alone, or with ARF-Q71I-HA. Cultures were then fixed at 12-15h post-transfection and labeled with antibodies against GM130 and the HA epitope. A) At this time point, over-expression of GFP-GluR1 resulted in an extensive dendritic distribution (top row). Co-expression with ARF-Q71I-HA significantly increased the Golgi localization of GFP-GluR1 in the soma, resulting in a loss of trafficking beyond proximal dendritic segments (bottom row). B) Higher magnification image of a dendrite illustrating the lack of GFP-GluR1 fluorescence accumulation in the Golgi outpost of neurons expressing ARF1-Q71I-HA. C) Intensity profile for GFP-GluR1 fluorescence over the first 20μm dendritic segment in cells expressing GluR1 alone, or in combination with ARF-Q71I-HA. (individual data points are the mean ± S.E.M. from 10 dendrites per group). Scale bars, 10 μm.
Figure S3. Mutant ARF1 expression does not affect synaptic density. E18 hippocampal neurons were transfected at DIV12, with ARF Q71I-HA. Cultures were fixed at 12-15h post-transfection and labeled with antibodies against the HA epitope, synaptophysin, and PSD95. A) Staining of pre- and post-synaptic markers in control (top row) and ARF-Q71I-HA transfected (bottom row) neurons. Expression of the ARF1 mutant had no qualitative effect on synaptic density (number of co-localized puncta per unit length of dendrite), as illustrated in thresholded, combined channel mask images (third panels). B) Quantitative analysis yielded no significant differences in synaptic density between the two groups (mean ± S.E.M. from an average of 2-3 fields, from 5 neurons, per group). Scale bar, 10 µm.
**Figure S4.** Dendritic ER-NMDAR vesicles contain CASK and KIF17. E18 hippocampal neurons were transfected at DIV12 with NR1-GFP alone, or in combination with DsRed-ER, fixed at 2d post-transfection, and stained with antibodies against CASK or KIF17. NR1-GFP puncta in dendrites co-localized (arrows) with CASK (A) and KIF17 (B) immuno-reactive puncta. Neurons expressing both NR1-GFP and DsRed-ER displayed an overlapping distribution with both CASK (C), and KIF17 (D). Scale bars, 10 µm.
Figure S5. shRNA-mediated knockdown of SAP97 and CASK expression. A) Efficacy of shRNA-mediated, SAP97 knockdown. E18 hippocampal neurons were infected shortly after plating, at DIV0, with a lentivirus coding a hairpin sequence against SAP97, or a lentivirus with a randomized siRNA sequence (scrambled). All shRNA viruses contained a GFP reporter and infection efficiency approached 100% in our cultures. A) Lysates from infected cultures were harvested at DIV 16 and subjected to Western blot analysis using antibodies against SAP97 and tubulin (loading control). We observed a >80% decrease in SAP97 band intensities (2 independent experiments), indicating a significant knockdown of expression. B) Efficacy of shRNA-mediated, CASK knockdown. E18 hippocampal neurons were infected shortly after plating, at DIV 0, with a lentivirus coding a hairpin sequence against CASK, or a scrambled shRNA sequence. Lysates from infected cultures were harvested at DIV16 and subjected to Western blot analysis using antibodies against CASK and tubulin (loading control). We observed a >70% decrease in CASK expression (2 independent experiments), as evidenced by a specific loss in CASK band intensity.
Figure S6. Knockdown of SAP97 or CASK does not affect synaptic density. E18 hippocampal neurons were infected shortly after plating, at DIV0, with lentivirus encoding a randomized shRNA sequence (scrambled, top row), CASK shRNA (middle row), or SAP97 shRNA (bottom row). Cultures were fixed at DIV16 and stained with antibodies against the pre- and post-synaptic markers, synaptophysin and PSD95, respectively. Knockdown of either SAP97 or CASK had no qualitative effect on synaptic density (number of co-localized puncta per unit length of dendrite), as illustrated in thresholded, combined channel mask images (third panels). B) Quantitative analysis yielded no significant differences in synaptic density between the three groups (mean ± S.E.M. from an average of 2-3 fields, from 10 neurons, per group). Scale bar, 10 µm.
Figure S7. Proposed model illustrating key elements of the alternative secretory pathway in neurons, as well as sites of selective sorting perturbations.
Supplementary Results

Temperature Block and BFA experiments

The sorting of proteins bound for the plasma membrane has traditionally been thought to occur at the level of the TGN. To explore whether the initial sorting of NMDA and AMPA receptors also occurs in this compartment, we conducted classic temperature shift experiments to slow the trafficking of membrane through the ER and/or Golgi. In our initial experiments, neurons were incubated at 20°C for 2hr, a condition shown to inhibit the exit of proteins from the TGN\(^1\). Newly synthesized and assembled receptors were monitored by transfecting hippocampal neurons with EGFP-tagged subunits of either the AMPA or NMDA receptor at 12 days post plating. At 12hr post transfection, cells were either maintained at 37°C, or subjected to a 2hr, 20°C temperature shift before being fixed and stained with antibodies against the dendritic microtubule-associated protein, MAP2, to assess the ability of these de novo synthesized receptors to exit the TGN and traffic into dendrites. As expected, EGFP-tagged GluR1 subunits of the AMPAR (GluR1-GFP) were found to accumulate in the cell soma (data not shown) and exhibited a dramatically reduced dendritic localization following the 20°C temperature shift (Fig. S1A,B). Surprisingly, we failed to detect either an accumulation of EGFP-tagged NR1 subunits of the NMDAR within the cell soma (data not shown), or a significant reduction in the number of NR1-GFP puncta per micron of dendrite (Fig S1A,B) when the neurons were incubated at 20°C for 2hrs. These data suggest that while AMPARs normally traffic through the TGN, NMDARs may utilize an alternative secretory pathway/mechanism that bypasses somatic Golgi. For example, previous studies have shown that mRNAs encoding NMDARs are dendritically localized\(^2,3\) and thus could be synthesized and processed solely by dendritic biosynthetic machinery\(^4\). To rule out this possibility in the current experiments, transfected neurons were grown under more stringent conditions (e.g. 15°C for 2hrs) that block the exit of proteins in the secretory pathway at the level of the ER\(^5\). Here, both NR1-GFP and GluR1-GFP fluorescence was restricted to the cell soma and virtually absence from dendrites (Fig. S1C). These data suggest that, in contrast to AMPARs, NMDARs traffic via somatic ER...
but bypass or exit early from the Golgi after leaving the ER.

To explore further the conclusion that NMDA receptors are not trafficking through somatic Golgi, we employed Brefeldin A treatment (BFA), another manipulation that directly disrupts the trafficking of integral membrane proteins through the Golgi. Similar to neuronal cultures grown at 20°C, neurons treated with BFA exhibited a significant reduction in the trafficking of newly synthesized GluR1-GFP into dendrites (Fig. S1D,E). In contrast, no effect on NMDAR trafficking to dendrites could be detected (Fig. S1D,E). These data together with the ARF1-Q71I data described in the main text (Fig. 1) support the conclusion that NMDARs do not traffic through somatic Golgi.

**AMPARs fail to accumulate at Golgi outposts following mutant ARF1 expression**

Data presented in Fig. 1 illustrate a differential sorting phenotype for AMPA and NMDARs with respect to somatic Golgi membranes. A constitutively active ARF1 mutant, ARF1-Q71I, was used to perturb COPI-dependent vesicle budding within Golgi membranes in the soma and dendrites (outposts). While somatic exit of exogenously expressed NR1-GFP was not attenuated, there was a pronounced and significant buildup at Golgi outposts, indicating that NMDAR secretion within this organelle is COPI-dependent. Conversely, AMPAR trafficking out of the soma was significantly reduced, as evidenced by an increase in GluR1-GFP fluorescence intensity in somatic Golgi (Fig 1A,B). Here we provide further evidence for the impaired somatic exit of GluR1 in neurons expressing mutant ARF1, by measuring the intensity of GFP-GluR1 fluorescence along proximal dendritic processes (Fig. S2). Co-expression of GFP-GluR1 with ARF1-Q71I-HA resulted in an accumulation of the subunit in somatic golgi membranes (Fig. S2A), as evidenced by increased co-localization with GM130, and a loss of GFP-GluR1 fluorescence in dendrites. Note that Golgi outposts in these cells did not exhibit any GluR1 accumulation (Fig. S2B), unlike what we observed with NMDARs. This can be accounted for by the almost total loss of GluR1 signal in proximal dendritic segments of cells with impaired Golgi function (Fig. S2C).
**ARF1-Q71I expression does not affect synapse stability or formation**

An experimental concern regarding the expression of mutant ARF1 in our cultures was the potential for alterations in synaptogenesis or synaptic maintenance due to a reduction in bulk flow processes, which would affect synaptic trafficking of all integral membrane proteins. To ensure that the sorting phenotypes we observed were due to deficits in secretory trafficking and not changes in synaptic density, etc., we quantified the number of synapses in the presence and absence of ARF1-Q71I expression (**Fig. S3**). The density of co-localizing pre- and postsynaptic puncta was unchanged along dendrites of transfected neurons versus untransfected control. This is not surprising considering the short transfection duration and the relative synaptic maturity at this developmental stage.

**Association of CASK and KIF17 with NMDARs on dendritic ER**

Data described in the main text suggest that in bypassing somatic Golgi, NMDARs are transported into dendrites in association with ER-like membranes en route to Golgi outposts. A key to understanding the functional importance of this biosynthetic pathway is defining the molecular composition of these ER-derived, NMDAR-containing vesicles. To accomplish this goal, we drew on previously published data showing that the dendritic transport of at least a sub-population of NMDARs is mediated by the microtubule-dependent motor protein KIF17, and a tripartite complex composed of three multi-domain scaffold proteins CASK (Lin2), Velis/MALS (Lin7), and Mint (Lin10), that can tether NMDARs to KIF17\(^7,8\). To evaluate whether the NMDAR complexes we observed on ER vesicles share features with this previously described complex, we immunostaining hippocampal neurons with antibodies against CASK or KIF17, 48hr after being transfected with DsRed-ER and/or NR1-GFP (**Fig S4**). Here, clusters of both CASK and KIF17 were found to overlap with NR1-GFP (**Fig S4A,B**) and DsRed-ER (**Fig. S4C,D**) puncta. These data suggest that the NMDAR, KIF17, CASK, Velis and Mint transport complex is in fact assembled on ER-like membranes.

**shRNA-mediated knockdown of SAP97 and CASK**

We found that SAP97 and CASK associate with NMDARs, and are subsequently co-transported with the receptor on mobile, non-synaptic vesicles. To specifically test the
requirement for these two MAGUK proteins in NMDAR sorting, we utilized an shRNA approach to selectively knockdown expression in our hippocampal culture system. Target sequences for SAP97 and CASK were generated and inserted into lentiviral vectors for high-efficiency infection of cells. The potency of the different target sequences was first screened in HEK-293 cell-based assays (data not shown), and the most effective of these were then evaluated in our hippocampal cultures (Fig. S5). This was accomplished by probing lysates of lentivirus-infected cells, by Western blotting, with antibodies against tubulin and SAP97 or CASK (Fig. S5A,B). As compared to uninfected neurons or neurons infected with a scrambled shRNA, we observed a 70-90% decrease in expression of SAP97 or CASK, confirming the efficacy of these shRNA in our hippocampal culture system.

**Knockdown of SAP97 or CASK does not affect synaptic density**

To determine whether SAP97 or CASK knockdown yielded any changes in the density of synaptic clusters, the number of co-localizing pre- and postsynaptic puncta were quantified in neurons infected with either the SAP97 or CASK shRNA viruses, or a scrambled control (Fig. S6). Under our experimental conditions, no significant change in synaptic density was measured. This supports the notion that the GluR trafficking phenotypes we observe are not the result of altered synaptic formation or maintenance, but rather processing events in the secretory pathway preceding synaptic delivery.

**Live imaging of ER vesicles carrying NMDARs and SAP97**

Heterologous cell data (Fig. 3) support the notion that SAP97 and CASK regulate the ER exit of NMDARs, thereby promoting delivery of the receptor complex to the plasma membrane. In hippocampal neurons, SAP97, CASK, and NR1 were found to co-distribute synaptically, as well as in small, non-synaptic puncta that may constitute trafficking vesicles (Fig. 4A,B). To specifically ascertain whether or not these puncta might represent a mobile, ER-derived vesicular population, we performed dual wavelength live-imaging experiments of hippocampal neurons expressing either RFP-SAP97 and NR1-GFP, or GFP-SAP97 and DsRed-ER (Fig. 4). We observed many examples of highly mobile puncta containing either combination of proteins/signals, and
static images from representative image sequences are provided in Fig. 4C. Here, we present a movie illustrating one such example of the vesicular movement we observed (Supplementary video 1). A puncta containing RFP-SAP97 and NR1-GFP is observed to move along a dendritic process in a halting manner, consistent with microtubule mediated transport.

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