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Gamma-protocadherins are enriched and transported in specialized vesicles associated with the secretory pathway in neurons

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Abstract

Gamma protocadherins (Pcdh- γ s) resemble classical cadherins and have the potential to engage in cell-cell interactions with homophilic properties. Emerging evidence suggests non-conventional roles for some protocadherins in neural development. We sought to determine if Pcdh- γ trafficking in neurons is consistent with an intracellular role for these molecules. Here we show that, in contrast to the largely surface localization of classical cadherins, endogenous Pcdh- γ s are primarily intracellular in rat neurons *in vivo* and equally distributed within organelles of subsynaptic dendritic and axonal compartments. A strikingly higher proportion of Pcdh- γ -containing organelles in synaptic compartments was observed at post-natal day 16. To determine the origin of Pcdh- γ trafficking organelles, we isolated organelles with Pcdh- γ antibody coupled magnetic beads from brain organelle suspensions. Vesicles with high levels of COPII and endoplasmic reticulum-Golgi intermediate compartment (ERGIC) components were isolated with the Pcdh- γ antibody but not with the classical cadherin antibody. In cultured hippocampal neurons, Pcdh- γ immunolabeling partially overlapped with calnexin and COPII- positive puncta in dendrites. Mobile Pcdh- γ -GFP profiles dynamically codistributed with a DsRed construct coupled to ER retention signals by live imaging. Pcdh- γ expression correlated with accumulations of tubulovesicular and ER-like organelles in dendrites. Our results are consistent with the possibility that Pcdh- γ s could have a unique function with the secretory pathway in addition to their documented surface roles.

Keywords

adhesion; organelle; live-cell imaging; dendrite; protocadherin

Introduction

The clustered protocadherins (Pcdhs) are a family of transmembrane proteins clustered in the genome (Kohmura *et al.*, 1998; Wu & Maniatis, 1999). The gene clusters (α , β , and γ) have variable exons coding for amino-terminal, transmembrane and juxtamembrane cytoplasmic domains of adhesive-like molecules (Wu & Maniatis, 1999). Three exons at the

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3' end of Pcdh- α and Pcdh- γ clusters encode a constant cytoplasmic domain which is fused by RNA splicing (Tasic *et al.*, 2002) to the variable exons. The amino-terminal domains of Pcdhs contain cadherin repeats which are calcium binding units that mediate homophilic (Nose *et al.*, 1988) adhesive interactions through dimerization (Shapiro *et al.*, 1995; Pertz *et al.*, 1999) and transcellular interactions. For Pcdh- γ s, these domains also mediate transcellular interactions (Frank *et al.*, 2005) with homophilic properties (Fernandez-Monreal *et al.*, 2009). The Pcdh- α s appear not to engage in homophilic recognition but do bind integrins (Mutoh *et al.*, 2004; Morishita *et al.*, 2006). Despite the low similarity between the constant cytoplasmic domains of the Pcdh- γ and Pcdh- α clusters, both were shown to interact with PYK2 and FAK kinases in the cytoplasm (Chen *et al.*, 2009).

There appear to be major differences as to the respective roles of classical cadherins and Pcdhs in neural development and synaptogenesis. Synaptic localization of Pcdhs have been confirmed (Kohmura *et al.*, 1998; Wang *et al.*, 2002; Phillips *et al.*, 2003) but the molecules also exhibit a substantial non-synaptic distribution (Wang *et al.*, 2002; Phillips *et al.*, 2003) representing an intracellular pool (Phillips *et al.*, 2003; Fernandez-Monreal *et al.*, 2009). Knockout studies have pointed to possible non-conventional roles for Pcdh- γ s in neural development (Wang *et al.*, 2002; Weiner *et al.*, 2005; Lefebvre *et al.*, 2008; Prasad *et al.*, 2008; Su *et al.*, 2009). Total genetic deletion caused neonatal death due to two possible factors: neural apoptosis and/or synaptic defects (Wang *et al.*, 2002) with apoptosis being the most prominent among the regions examined so far (Lefebvre *et al.*, 2008). The effect of Pcdh- γ deletion on neuronal survival could reflect a non-conventional role.

Pcdh- γ s trafficking in organelles during neural development is likely to be an important aspect of the function of these molecules (Fernandez-Monreal *et al.*, 2009) and Pcdh- γ s can generate tubular structures emanating from late endosome/lysosome substrates when expressed in HEK293 cells (Hanson *et al.*, 2010). It remained to be determined the types of compartments that Pcdh- γ s are trafficked in neurons and how they might be affected by Pcdh- γ expression. We found by quantitative immunogold electron microscopy that endogenous Pcdh- γ s are primarily intracellular in neurons. This was compared to classical cadherins which are located at the synaptic cleft. Organelles isolated with anti-Pcdh- γ antibodies contained high levels of the post-ER organelle markers COPII and ERGIC-53. In neuron cultures, GFP tagged Pcdh- γ s colocalized with and moved along dendrites in concert with ER-like vesicle markers and correlated with the accumulation of specific tubular and other organelles. Our results suggest that Pcdh- γ s could function in membrane trafficking in neurons in addition to their transcellular binding activity at synapses.

Materials and Methods

Animals

Sprague-Dawley rats were purchased from Charles River Laboratories and housed in the Animal Facilities at the Mount Sinai School of Medicine which holds an Animal Welfare Assurance Number (A3111-01) signifying that it adheres to all National Institutes of Health guidelines for the care and treatment of laboratory animals. All procedures were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee (IACUC).

Post-embed immunogold electron microscopy

Rats were deeply anesthetized and perfused transcardially with 2% dextran (MW 70,000) in 0.1 M phosphate buffer (PB, pH 7.4), followed with a mixture of 4% paraformaldehyde and 0.125% glutaraldehyde in PB. Tissue was removed and postfixed in the same perfusate overnight. Approximately 1 mm thick coronal slices were dissected from the regions of

interest. Freeze substitution and low-temperature embedding of the specimens were performed essentially as described previously with some modifications (Janssen *et al.*, 2005). Slices were cryoprotected by immersion in increasing concentrations of glycerol in PB (10, 20, and 30%) and plunged rapidly in liquid propane cooled by liquid nitrogen at -190°C in a Universal Cryofixation System KF80 (Reichert-Jung, Depew, NY). The samples were immersed in 1.5% uranyl acetate in anhydrous methanol at -90°C for 24 hours in a cryosubstitution Automatic Freeze-Substitution System unit (Leica, Wetzlar, Germany). The temperature was increased in steps of 4°C per hour from -90 to -45°C . The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Hatfield, PA) at 45°C with a progressive increase in the ratio of resin to methanol for 1 hour each, followed by pure Lowicryl overnight. Polymerization was performed with UV light at 45°C for 48 hours followed by 24 hours at room temperature.

The CA1 stratum radiatum region of hippocampus was sectioned. Pairs of ultrathin sections at 75 nm were cut on a Reichert-Jung ultramicrotome and mounted on a nickel mesh grid for immunogold analysis. The grids were treated with a saturated solution of sodium hydroxide in absolute ethanol, rinsed, and incubated in the following solutions at room temperature: 0.1% sodium borohydride and 50 mM glycine, and then in Tris buffered saline (TBS) containing 2% human serum albumin (HSA).

For immunolabeling, grids were incubated with affinity purified rabbit polyclonal antibodies generated against the mouse Pcdh- γ constant cytoplasmic domain (Phillips *et al.*, 2003) or the N-cad cytoplasmic domain (Tanaka *et al.*, 2000) overnight. Grids were then washed, and incubated in secondary 10 nm gold-tagged goat-anti-rabbit IgG F(ab')₂ (Electron Microscopy Sciences, Hatfield, PA) diluted 1:30 in TBS, 2% HSA and 5 mg/ml polyethyleneglycol 20,000. Sections were washed, dried and contrasted with 1% uranyl acetate and Reynolds lead citrate. The sections were analyzed on a JEOL 1200EX electron microscope (JEOL, Peabody, MA). Images were captured by using the Advantage charge-coupled device camera (Advanced Microscopy Techniques, Danvers, MA).

Analysis of percentage of labeled synapses

Images of random fields were taken at a magnification of 7500x. Synapses containing at least two gold particles per synaptic profile (Adams *et al.*, 2001) including the synaptic cleft, spine head and presynaptic compartment, were considered positive for Pcdh- γ or N-cad. At least 150 synapses were counted for each animal in blind and results were expressed as the average of 2–3 different animals per antibody and age.

Subsynaptic distribution

The immunogold particle density and distribution in various subsynaptic compartments were analyzed using the SynBin program (Adams *et al.*, 2001), which is based on principles regarding gold particle proximity to membranes (Ruud & Blackstad, 1999). SynBin assigns a position for each gold particle in relationship to postsynaptic and presynaptic membrane structures (Adams *et al.*, 2001). SynBin has been used to detect and quantify changes in the subsynaptic distribution of various immunogold labeled synaptic molecules in response to estrogen, synaptic activity, or deletion of synaptic molecules (Adams *et al.*, 2001; Adams *et al.*, 2002; Adams *et al.*, 2004; Moga *et al.*, 2006; Park *et al.*, 2008).

For the present analysis, 30 nm was chosen for the bin width to assure high resolution and accommodate the theoretical limit of resolution. The bins were as follows: Presynaptic, 0–30 nm intracellular from the presynaptic membrane throughout the width of the synaptic junction; Postsynaptic, 0–30 nm intracellular from the postsynaptic membrane throughout

the width of the synaptic junction; Cleft, between the pre- and postsynaptic membrane throughout the width of the synaptic junction; Presynaptic lateral, 0–30 nm intracellular from the presynaptic membrane not including the width of the synaptic junction; Postsynaptic lateral, 0–30 nm from the postsynaptic membrane not including the width of the synaptic junction; Presynaptic intracellular, greater than 30 nm from the axonal membrane; Postsynaptic intracellular, greater than 30 nm from the dendritic membrane. Gold particle analysis was done on an average of 35 randomly chosen axo-spinous synapses per animal at a magnification of 40,000x. Analysis was done blindly. Any synapse that contained at least two gold particles in any bin was included in the analysis. Synapses that lacked clear visualization and delineation of presynaptic and postsynaptic membranes, cleft or postsynaptic density were excluded. Results are compiled from 3 animals for each antibody.

Subcellular fractionation

Rats were euthanized with CO₂, rapidly decapitated, and brains dissected out. Total rat brains were homogenized in ice cold buffer containing 0.32 M sucrose, 10 mM Hepes, pH 7.4 and protease inhibitors. Cellular and nuclear debris were pelleted by centrifugation at $800 \times g$ at 4°C for 10 min. The post-nuclear supernatant (PNS) was centrifuged at $9,200 \times g$ at 4°C for 15 min to obtain the organelle-enriched fraction S2 in the supernatant. The supernatant was loaded in a discontinuous sucrose gradient (from 0.5 M to 1.3 M sucrose) and centrifuged overnight at 28,000 r.p.m in a SW-40Ti rotor. Fractions were collected from the top of the gradient and analyzed by western blot.

Immunoisolation of organelles

Magnetic Dynabeads M-280 coupled to sheep anti-rabbit IgG (Invitrogen, Carlsbad, CA) were incubated with affinity purified anti-Pcdh- γ constant cytoplasmic domain (Phillips *et al.*, 2003) or anti-N-cad cytoplasmic domain (Tanaka *et al.*, 2000) antibodies or rabbit IgG as control. Antibody-coated Dynabeads were then incubated with S2 fractions, washed, and bound organelles were fixed with 4% glutaraldehyde for electron microscopy, or eluted for gel electrophoresis and mass spectrometry or western blot analyses.

Mass spectrometry

Samples were extracted from gels by tryptic digestion as described (Brasaemle *et al.*, 2004). The resultant peptides were dissolved in 2–25 μ l of HPLC sample solvent (water:methanol:acetic acid:trifluoroacetic acid, 70:30:0.5:0.01, v/v/v/v). Micro-HPLC-MS/MS analysis was conducted on an LCQ electrospray ionization ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) coupled with an online MicroPro-HPLC system (Eldex Laboratories, Napa, CA).

Hippocampal neuronal cultures, immunostaining, confocal and time-lapse microscopy

Neuronal cultures and time-lapse microscopy were performed as described (Fernandez-Monreal *et al.*, 2009). Hippocampi from E18 rat embryos were dissociated and plated on 35 mm glass-bottomed dishes (MatTek, Ashland, MA). Neurons were double-transfected with Pcdh- γ A3-GFP (Phillips *et al.*, 2003; Fernandez-Monreal *et al.*, 2009) and pDsRedER2 (Clontech, Mountain View, CA) at 17 days in vitro by calcium phosphate precipitation. The next day, live images were acquired on a Zeiss LSM510 META laser scanning confocal microscope equipped with a live imaging chamber that maintains cultures at 37 °C and 5% CO₂. Images were acquired by multi-track with a 63X oil objective in 512×512 pixel mode with 1X averaging and a pixel time of 1.61 μ s. GFP was excited using the 488 nm laser at 9% intensity and captured through a 505–550 bandpass filter. DsRed2 was excited with the 543 nm laser at 80% intensity and captured through a 585–615 bandpass filter. Time series

images were imported into Image J (available at <http://rsb.info.nih.gov/ij> and developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) as a stack for image analysis.

For immunostaining with rabbit polyclonal anti-ERGIC53 (obtained from Sigma, St. Louis, MO) after live imaging, neurons were immediately fixed with 4% paraformaldehyde, blocked and immunolabeled in phosphate buffered saline (PBS), 3% goat serum, 3% bovine serum albumin and 0.1% Triton X-100. Secondary antibody was Alexa Fluor 647 conjugated goat anti-rabbit IgGs (Invitrogen/Molecular Probes, Carlsbad, CA). For immunolabeling with anti-Pcdh- γ and monoclonal anti-calnexin or anti-Sec31A (both from BD Transduction, San Jose, CA), neurons were permeabilized with methanol at -20°C after paraformaldehyde fixation. They were washed in PBS and blocked and labeled as above. Secondary antibodies were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (Invitrogen/Molecular Probes, Carlsbad, CA). Calnexin or Sec31A/Pcdh- γ double labeled images were acquired on a Zeiss LSM410 laser scanning confocal microscope using a 63X oil objective. The polyclonal anti-Pcdh- γ constant cytoplasmic domain antibody, generated as described (Phillips *et al.*, 2003), and monoclonal anti-Pcdh- γ constant cytoplasmic domain (clone 159/5, UC Davis/NIH NeuroMab Facility) detected the same puncta in cultured hippocampal neurons (Supplemental Fig. 1).

Correlative light and electron microscopy

Hippocampal neurons prepared as above were plated on gridded glass bottom dishes (Mattek, Ashland, MA) and transfected at 13–16 days in vitro with Pcdh- γ A3-GFP or N-cad-venus using calcium phosphate precipitation as described. A live image of transfected neurons was acquired on the LSM510 META as outlined above and the neurons were subsequently fixed and processed for correlative light and electron microscopy (CLEM) exactly as described (Hanson *et al.*, 2010).

Results

Pcdh- γ s are primarily intracellular in neurons and synaptic compartments

To determine the relative levels of synaptic and intracellular Pcdh- γ s we used post-embedding immuno-electron microscopy followed by analysis of subsynaptic distribution using SynBin (Adams *et al.*, 2001) which determines the position of gold particles in relationship to postsynaptic and presynaptic membranes and assigns each to a bin. The distribution of Pcdh- γ s was compared to that of N-cadherin (N-cad) in the adult rat hippocampal CA1 stratum radiatum. Seven different bins were established for synaptic compartments (Fig. 1A) and the proportion of gold particles in each bin were counted and averaged over at least 98 synapses from 2 animals each for Pcdh- γ and N-cad. We found that Pcdh- γ s were distributed primarily in the intracellular pre- and postsynaptic compartment bins (34% and 39%, respectively) with only $\sim 2\%$ of the gold particles found in the synaptic cleft, $\sim 8\%$ at the postsynaptic density (PSD) and $\sim 2\%$ at the presynaptic specialization (Fig. 1B). The remainder of Pcdh- γ gold particles were found in the lateral non-cleft pre- and postsynaptic compartments. In contrast, the majority of N-cad labeling was distributed at the presynaptic specialization, the synaptic cleft and the PSD (24%, 19%, and 17%, respectively) with 13% and 12% in the presynaptic and postsynaptic intracellular compartments, and the remainder in the lateral bins. These results are consistent with the previous light level immunolocalizations (Wang *et al.*, 2002; Phillips *et al.*, 2003) in cultured neurons that found only a partial synaptic distribution for Pcdh- γ s. Given the limits of light microscopy, some of the Pcdh- γ labeling these earlier studies that was considered “synaptic” could have been labeling of subsynaptic organelles.

When the lateral and intracellular pre- and postsynaptic compartments were combined and compared with the combined presynaptic, PSD and cleft bins, only approximately 12% of Pcdh- γ gold particles could be considered “synaptic” as compared to approximately 60% of N-cad particles (Fig. 1C). Typical examples of Pcdh- γ and N-cad labeled synapses are shown (Fig. 1D). Intracellular Pcdh- γ labeling in synaptic compartments was generally confined to distinct profiles resembling trafficking organelles (Fig. 1D, bottom panel) with some Pcdh- γ gold particles approaching the synaptic cleft. In contrast, N-cad labeling was overwhelmingly associated with the synaptic specializations or the cleft itself.

We compared the percentage of all synaptic compartments labeled with anti-Pcdh- γ or N-cad antibodies in CA1 at various postnatal developmental time points. The results were averaged over 3 animals per developmental stage. Approximately 10–20% of the synapses and associated pre- and postsynaptic compartments were labeled with the N-cad antibody and this number remained relatively stable throughout development (Fig. 2A). In contrast, while anti-Pcdh- γ s labeled approximately 35% of the synaptic compartments in both postnatal day (PND) 9–13 and adult (PND>20), there was a dramatic peak of labeling at PND 16 with approximately 70% of synaptic compartments labeled (Fig. 2A). The total expression levels of both Pcdh- γ and N-cad in cortex were relatively stable throughout development with a slight decrease in Pcdh- γ in adult, while the expression of a synaptic protein, SV2, increased during development (Fig. 2B). This suggests there may be a translocation of Pcdh- γ organelles at PND 16 to synaptic compartments. This could also account for increased synaptic labeling at the light level observed in cultures of higher density (Phillips *et al.*, 2003).

We used SynBin to compare the distribution of Pcdh- γ in synaptic compartments at PND16 and adult. A significant increase in relative percentage of Pcdh- γ distribution was observed at the synaptic cleft at PND16 when compared to adult ($21.71 \pm 1.7\%$, $N=3$ versus $12.23 \pm 0.56\%$, $N=2$, unpaired two-tailed t-test, $F=13.93$, $P=0.024$, Fig. 2C). Altogether, these data suggest that Pcdh- γ s are mostly intracellular and organelles containing them could be involved in synapse development.

Pcdh- γ and N-cad are transported in different compartments

To characterize Pcdh- γ -containing organelles we separated an organelle suspension by sucrose gradient centrifugation and probed the resultant fractions with antibodies to various organelle markers. Pcdh- γ s and N-cad cosedimented in the same membrane fractions, together with some endosomal and ER-associated vesicle markers (Fig. 3A, fractions 11–19) including fractions that contained some EEA1, Rab8, Rab11, Sec23 and ERGIC-53. The marker proteins associated with ER such as protein disulfide isomerase (PDI) and the post-ER markers Sec23 and ERGIC-53 were most abundant in the fraction that pelleted through the gradient (Fig. 3A, fraction 25). Smaller yet significant relative amounts of Pcdh- γ organelles were also observed to pellet as well. There were no differences in relative distribution between PND16 and adult.

To determine whether Pcdh- γ s and N-cad reside in distinct compartments we isolated organelles from the organelle suspension using magnetic beads coupled to Pcdh- γ or N-cad cytoplasmic domain antibodies. The resultant organelles and proteins were analyzed by electron microscopy (Fig. 4A), electrophoresis and mass spectrometry (Fig. 4B) and immunoblotting (Fig. 4C). While control IgG and N-cad beads eluates lacked distinct protein species, eluates from Pcdh- γ beads contained two prominent bands at approximately ~80–90 and ~120–140 kDa on the silver stained gel (Fig. 4B). These bands were excised and subjected to mass spectrometry for protein identification. We detected two Pcdh- γ -containing organelle proteins, Sec23 and Sec24, which are members of the COPII complex involved in transport of cargo proteins from the ER to the Golgi (Fig 4B). Immunoblots of

these samples with anti-Sec23 antibodies confirmed this result (Fig. 4C). We also identified ERGIC-53, another marker for trafficking organelles intermediate between ER and Golgi, in the Pcdh- γ , but not N-cad, bead eluates (Fig. 4C). Of note, we did not identify PDI or other markers of ER in either bead eluate (not shown) which might be expected given that ER consists of large easily pelletable organelles that may not present in the postnuclear supernatant at high levels.

Pcdh- γ s are transported in vesicles associated with the secretory pathway

The biochemical evidence suggests Pcdh- γ s may be enriched in vesicles associated with the secretory pathway in dendrites, in particular, ER and post-ER. We therefore sought to determine the relationship of Pcdh- γ s with markers of these organelles in neuronal cultures and whether they might move coordinately with Pcdh- γ -GFP fusions. Cultured hippocampal neurons at 21 days in vitro were immunolabeled with anti-Pcdh- γ s and either anti-calnexin (Fig. 5A, C) or anti-Sec31A (Fig. 5B, D) for ER and COPII colocalization, respectively. Calnexin and Sec 31A had different overall labeling patterns in neurons (Fig. 5A and B). Calnexin tended to label proximal dendrites (arrowheads, red channel Fig. 5A) while Sec31A labeled apparent vesicles, mostly in the cell body but also in dendrites (arrowheads, red channel, Fig. 5B). In both cases, where there were accumulations of these two markers, there could also be found accumulations of Pcdh- γ labeled structures (arrowheads, green channel, Fig. 5A and B) although many Pcdh- γ puncta did not colocalize with either marker. At sites of co-accumulation of Pcdh- γ s and calnexin, anti-Pcdh- γ labeled organelles often overlapped with these (arrow, Fig. 5C) while other Pcdh- γ puncta were juxtaposed with calnexin labeling (arrowheads, Fig. 5C). At sites where both Pcdh- γ s and Sec31A accumulated, anti-Sec31A detected smaller puncta in dendrites, which overlapped (arrow, Fig. 5D) or juxtaposed with (arrowheads, Fig. 5D) Pcdh- γ organelles in dendrites. Thus, neuronal sites which accumulate Pcdh- γ s also show the clustering of other organelles of the secretory pathway although not in a precisely overlapping arrangement. This suggests that Pcdh- γ labeled organelles associate with secretory organelles.

Pcdh- γ -GFP fusions, which are completely functional in vivo (Wang *et al.*, 2002; Weiner *et al.*, 2005), delineate mobile organelles in dendrites (Fernandez-Monreal *et al.*, 2009). To determine if mobile Pcdh- γ organelles and ER-related vesicles might interact, we cotransfected neurons with Pcdh- γ A3-GFP and pDsRed2-ER, which is a red fluorescent protein coupled to ER targeting sequences. We then recorded the trafficking of the organelles by two-channel time-lapse microscopy at ~10 second intervals over a period of ~6 minutes. Like immunolabeled Pcdh- γ s and calnexin, dendrites tended to accumulate DsRed-ER and Pcdh- γ puncta in the same regions (brackets, Fig. 6A). When examined by time-lapse, mobile DsRed-ER and Pcdh- γ puncta could be observed to move together (see supplemental movie) and dynamically bud and fuse. Mobile DsRed-ER/Pcdh- γ puncta (Fig. 6B–C, arrowheads) were emitted from spots containing both markers, which then joined other DsRed-ER/Pcdh- γ positive sites (Fig. 6B–C, arrows). After live imaging, neurons were fixed and immunolabeled for ERGIC-53, which was associated with most of the Pcdh- γ puncta observed in the live image (Fig. 6D, arrows). This suggests that Pcdh- γ containing organelles dynamically associate with ER and post-ER trafficking organelles in dendrites.

Pcdh- γ A3-GFP induces tubulovesicular organelles

We used correlative light and electron microscopy (CLEM) (Razi & Tooze, 2009) to determine how Pcdh- γ expression might alter the complement of organelles in dendrites. Pcdh- γ A3-GFP or N-cad-Venus transfected neurons were imaged, fixed, processed for electron microscopy, and the imaged neurons were relocated under the electron microscope. Regions of dendrites containing Pcdh- γ A3-GFP puncta (Fig. 7A, arrowhead and arrow) corresponded to accumulations electron-dense tubulovesicular organelles (Fig. 7C–E,

arrowheads). In addition, larger vesicles coated with ribosomes (Fig. 7C–E, arrows) were observed along with darker mitochondrial, lysosomal or multivesicular- like organelles. In contrast, N-cad-Venus (Fig. 7D–F) induced elongated sheets or cisternae (arrowheads, Fig. 7D–E) that resembled expansion of dendritic ER (Takei *et al.*, 1994). Serial sections illustrate the lack of continuity of Pcdh- γ A3-GFP induced tubules (Fig. 7E, arrowheads) and the continuous sheet like morphology of N-cad-Venus induced organelles (Fig. 7F, arrowheads). Thus, Pcdh- γ effect on organelle biogenesis is distinct from N-cad, a surface protein that traverses the conventional secretory pathway.

Discussion

Pcdh- γ knockout phenotypes include defects in synapse number, synaptic transmission and neural survival while neural histogenesis are unaffected (Wang *et al.*, 2002; Weiner *et al.*, 2005; Lefebvre *et al.*, 2008; Prasad *et al.*, 2008). Little is known about Pcdh- γ function at the cellular level. All Pcdhs contain calcium binding cadherin repeats within their amino-terminal domains which suggests a role in cell-cell interactions (Morishita *et al.*, 2006). Alternative splicing within Pcdh clusters and potential epigenetic regulation (Esumi *et al.*, 2005) would therefore create unique combinations of recognition units on the neural cell surface. Thus, the Pcdh cluster is thought to be critical for neural identity possibly dictating how neurons establish synaptic connections via homophilic binding (Shapiro & Colman, 1999; Frank & Kemler, 2002) or by astrocyte-neuron interactions (Garrett & Weiner, 2009). However, cell-cell adhesive activity is relatively weak when tested in cellular aggregation assays (Frank *et al.*, 2005). Transcellular binding activity, at least for the Pcdh- γ s, is enhanced upon deletion of the intracellular domain (Fernandez-Monreal *et al.*, 2009). We now show here by quantitative immunoelectron microscopy that, unlike classical cadherins, Pcdh- γ s are primarily intracellular in neurons. The Pcdh- γ -containing organelles were associated with two proteins of the coat protein complex II (COPII) as well as ERGIC proteins. These target proteins from ER to Golgi and are involved in the modulation of receptor surface expression in dendrites and neuronal function (Aridor *et al.*, 2004) but may also be involved in other functions including autophagy (Ishihara *et al.*, 2001; Fu & Sztul, 2003; Hamasaki *et al.*, 2003). It is possible that Pcdh- γ s are trafficked within these vesicles to surface membranes. It is also possible that Pcdh- γ s, as intracellular proteins, participate in a still uncharacterized vesicle trafficking process in dendrites given that Pcdh- γ bearing organelles are different from those transporting N-cad.

Consistent with its role as a synaptic adhesion molecule, N-cad mediates stable homophilic cell surface adhesion at synaptic junctions (Fannon & Colman, 1996; Uchida *et al.*, 1996; Togashi *et al.*, 2002; Bozdagi *et al.*, 2004). Our study confirms the mostly synaptic distribution of N-cad in contrast to Pcdh- γ s which were associated with trafficking organelles in neurons. Increasing evidence suggests that the function of some protocadherins, including Pcdh- γ s may be more dynamic than classical cadherins, although still featuring transcellular binding. For example, the protocadherin arcadlin/Pcdh8, is induced by neural activity where it translocates to synapses and functions to downregulate the surface expression of N-cadherin (Yasuda *et al.*, 2007). Surface arcadlin/Pcdh8 was found to be rapidly endocytosed along with N-cad upon induction of homophilic interactions. The *Xenopus* ortholog of arcadlin/Pcdh8, paraxial protocadherin, also can downregulate classical cadherin activity (Chen & Gumbiner, 2006). In another example, OL-protocadherin was shown to reduce the binding activity of N-cad in astrocytoma cells by recruiting molecules that disrupt the actin cytoskeleton to cell-cell contact sites (Nakao *et al.*, 2008). New evidence suggests that another family of cadherin repeat containing proteins exhibit striking effects on membrane trafficking. Calsyntenins, intracellular transmembrane cadherin repeat proteins, are involved in trafficking of TGN vesicles (Konecna *et al.*, 2006; Ludwig *et al.*, 2009). More recently, in non-neural cells, intracellular Pcdh- γ s were found to

induce tubules from late endosomes or lysosomes in a manner dependent on both the amino-terminal cadherin repeat containing domains as well as the cytoplasmic domain (Hanson *et al.*, 2010). It should be noted that cadherin repeats, which bind calcium, project into the lumen of organelles in which they are harbored and could potentially function as luminal calcium sensors.

Pcdh- γ s induce tubules from late endosomal/lysosomal substrates in both HEK293 cells (Hanson *et al.*, 2010) and Neuro-2A cells (HHH and GRP, unpublished data). Deletion of the complete cytoplasmic domain (Δ CCD) of Pcdh- γ A3, including most of the variable intracellular domain and leaving only a 28 amino acid cytoplasmic stub, eliminated the tubules and shifted Pcdh- γ A3 to the conventional secretory pathway (Hanson *et al.*, 2010). In neurons, the tubules at dendritic sites expressing Pcdh- γ A3-GFP were in the vicinity of larger ribosome coated organelles, that could represent a form of ER. These results suggest that Pcdh- γ s may generate different tubulovesicular organelles in neurons as compared to tissue culture cells and this may depend on where they are targeted in the secretory pathway in a particular cell type. The coisolation with COPII and ERGIC components indicates an association with the secretory pathway possibly at a post-ER location. It is also possible that Pcdh- γ s could utilize COPII coats in a non-canonical manner as has been observed in the involvement of COPII in autophagy (Ishihara *et al.*, 2001; Fu & Sztul, 2003; Hamasaki *et al.*, 2003).

The differences between organelles harboring Pcdh- γ A3-GFP and N-cad-Venus in neurons is consistent with a specialized role or position for Pcdh- γ A3-GFP in the secretory pathway. The organelles corresponding to N-cad-Venus positive sites in dendrites resembled those induced when proteins that populate the conventional secretory pathway are overexpressed. For example, the inositol 1,4,5-triphosphate receptor is expressed at high levels in the ER, and when overexpressed, it can induce stacks of ER cisternae (Takei *et al.*, 1994), which have a very strong resemblance to the cisternae induced by N-cad-Venus. Similarly in non-neural cells, expression of ER resident proteins induced membrane whorls that resemble expanded ER (Snapp *et al.*, 2003; Lingwood *et al.*, 2009). These whorls appear to be “non-specific” and inducible by overexpression of generic ER or plasma membrane (Hanson *et al.*, 2010) targeted proteins. In contrast, Pcdh- γ A3-GFP did not induce whorled or expanded ER-like membranes but rather caused the production of an apparently specific type of tubulovesicular organelle, associated with larger vesicles, in dendrites.

If Pcdh- γ s are primarily intracellular, then what is the purpose of their diversity? Although we find that Pcdh- γ s are mostly intracellular, there was a proportion of the molecules at the cell surface as determined by immunogold labeling in this study and in other studies (Murata *et al.*, 2004; Fernandez-Monreal *et al.*, 2009) using extracellular labeling in non-permeabilized cells. We found that Pcdh- γ s are dramatically upregulated in organelles during postnatal neural development at a postnatal time coincident with synapse formation and maturation in rodent brain (Mody *et al.*, 2001), despite no change in total Pcdh- γ levels. It is possible that an event during late neural development causes the production of new classes of organelles containing Pcdh- γ s which may participate in delivering critical synaptic proteins to the plasma membrane. Through intraluminal interactions, a mechanism for the homophilic accumulation and budding of single Pcdh- γ isoforms, and subsequently delivery as “packets”, has been proposed (Hanson *et al.*, 2010). Prior to and/or after this event, the surface activity of Pcdh- γ s may not be involved in neural development. This model should provide a new framework to understand intracellular trafficking during critical periods of postnatal synaptogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Pcdh-γs are primarily intracellular in neurons in vivo

(A) Establishment of bins for SYNBIN analysis. The presynaptic specialization, PSD and lateral domains extend 30 nm in from the plasma membrane. (B) Comparison of bin distribution for Pcdh-γs and N-cad. Pcdh-γs were most prominent in the presynaptic and postsynaptic intracellular compartments while N-cad labeling was found primarily in the synaptic cleft and presynaptic and postsynaptic specializations. (C) Data expressed as comparison of Pcdh-γ and N-cad labeling in synaptic and non-synaptic compartments. Presynaptic and postsynaptic lateral and presynaptic intracellular bins are combined into single presynaptic or postsynaptic compartment bins while cleft, presynaptic and postsynaptic specializations were combined into a single synaptic bin. (D) Representative labeling of Pcdh-γs and N-cad at synapses. High magnification of subsynaptic labeling in organelles (arrows, bottom panel).

Figure 2. An increase in synaptic compartment labeling by Pcdh-γs at PND 16

(A) The percentage of synaptic compartments labeled plotted as a function of developmental stage. At PND 9, 13, 20 and adult, Pcdh-γs labeled approximately 20–40% of synaptic compartments while at PND 16 approximately 70% of synaptic compartments were labeled. In contrast, N-cad labeling of synaptic compartments was relatively stable at all developmental stages. (B) Increase in Pcdh-γ expression does not account for the increase in synaptic labeling at PND 16. Brain extracts at the indicated developmental stages were immunoblotted with Pcdh-γ, N-cad or SV2 antibodies. (C) SYNBIN analyses comparing PND 16 and adult developmental stages. Data are expressed as a combination of the pre- or postsynaptic bins versus the synaptic bin. The percentage of synaptic labeling at PND 16 was significantly different than adult (* $p < 0.05$).

Figure 3. Co-sedimentation sucrose gradients with different organelle markers
PND16 and adult rat brains were homogenized and an organelle-enriched fraction was isolated from the post-nuclear supernatant. This was loaded on a discontinuous sucrose gradient and fractions were analyzed by immunoblotting for the indicated proteins.

Figure 4. Pcdh- γ -containing organelle isolation

(A) Magnetic beads were coated with the indicated antibodies and incubated with the organelle-enriched fraction from post-nuclear supernatant. Beads were fixed and visualized with electron microscopy Bar=100 nm. (B) Immunoisolated organelles were lysed, electrophoresed and silver stained. Specific bands were then analyzed by mass spectrometry in order to identify proteins from the Pcdh- γ -containing organelles. Two proteins were identified: Sec23 and Sec24. (C) Co-immunoprecipitation with Dynabeads was confirmed by immunoblotting with the post-ER carrier markers Sec23 and ERGIC-53 antibodies. Control or anti-N-cad bead eluates did not contain these proteins. Asterisk indicates the IgG band.

Figure 5. Relationship of Pcdh-γs to ER-related markers

Neurons at 21 DIV were immunolabeled for (A–C) Pcdh-γs and calnexin or (B–D) Pcdh-γs and Sec31A. Arrowheads in A and B indicate coaccumulation of Pcdh-γs and organelle markers. Boxed regions in (C) and (D) are shown magnified. Some Pcdh-γ clusters overlapped with calnexin or Sec31A (C–D, arrows) while others were adjacent (C–D, arrowheads).

Figure 6. Dynamic association of Pcdh- γ and ER-related organelles in dendrites

(A) Dendrite segment from neuron co-transfected with Pcdh- γ A3-GFP and DsRed2-ER plasmids. Brackets indicate areas of coaccumulation. Boxed regions are magnified in the time-lapse images shown in (B) and (C). In (B) a Pcdh- γ A3-GFP/DsRed-ER profile (arrowhead) is stable for 49 sec (top) then emits a mobile Pcdh- γ A3-GFP/DsRed-ER positive organelle (middle) which merges with a similar profile (bottom) and was stable for 20 seconds (see Movie 1). In (C) a Pcdh- γ A3-GFP/DsRed-ER profile (arrowhead) is stable for 78 sec (top), emits an organelle (second from top) that changes direction (third from top) and eventually merges with another Pcdh- γ A3-GFP/DsRed-ER profile which remains stable for at least 108 seconds. (D) Post live imaging immunolabeling for the post-ER trafficking

organelle marker ERGIC-53. Many of the Pcdh- γ puncta distributed with ERGIC-53 (arrowheads).

Figure 7. Pcdh-γA3 and N-cad correspond to different types of organelle accumulations in neuronal dendrites

(A) Pcdh-γA3-GFP or (B) N-cad-Venus expression in the dendrite of a cultured neuron. Arrowhead and arrow indicates regions examined by CLEM. (C) Pcdh-γA3-GFP induced electron-dense tubulovesicular organelles (arrowheads) associating with larger, ribosome-coated, organelles (arrows). This section was taken from area indicated by arrowhead in A. (D) N-cad-Venus induced elongated sheets reminiscent of expanded ER. (E) Serial sections through dendrite area indicated by arrow in A demonstrate that Pcdh-γA3-GFP induced tubules are not sheets. Tubules appear or disappear in continuous sections. (F) Serial

sections through cisternae induced by N-cad-Venus. The same sheets can be viewed in consecutive sections (arrowheads).