Local Traffic

Thursday 02 May 2019 - Thursday 02 May 2019

Book of Abstracts
## Contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Arrestins Rod1 and Rog3 regulate trafficking of glucose transporters in response to starvation and 2-deoxyglucose</td>
<td>1</td>
</tr>
<tr>
<td>Extracellular zinc Contributes to the Slow Polyspermy Block</td>
<td>1</td>
</tr>
<tr>
<td>New optical approaches to investigate GABA type A receptor assembly, trafficking and neuroadaptation to drug treatment and pathology</td>
<td>2</td>
</tr>
<tr>
<td>Revealing a new role for alpha-arrestins in the regulation of autophagy</td>
<td>3</td>
</tr>
<tr>
<td>Uncovering properties of dynamic signal propagation in single cells</td>
<td>3</td>
</tr>
<tr>
<td>Effects of Stretch on the Bladder Umbrella Cell Apical Junctional Ring</td>
<td>4</td>
</tr>
<tr>
<td>Patronin-mediated microtubule minus end growth populates dendrites with minus-end-out microtubules during development and regeneration</td>
<td>4</td>
</tr>
<tr>
<td>Quality control of polycystin 2 missense mutants in the endoplasmic reticulum</td>
<td>5</td>
</tr>
<tr>
<td>SunRISE: a labeling system for long-term imaging of single mRNA molecules in live cells</td>
<td>6</td>
</tr>
<tr>
<td>Fluorogen-Activating Protein (FAP)-tagging vectors and organelle markers for quantitative imaging</td>
<td>7</td>
</tr>
<tr>
<td>Cellular degradation of Nephrogenic Diabetes Insipidus AQP2 Variants in a Yeast Model</td>
<td>7</td>
</tr>
<tr>
<td>Genetic analysis of the Drosophila ESCRT-III Complex protein, VPS24, reveals cell-autonomous and -nonautonomous requirements in proteostasis</td>
<td>7</td>
</tr>
<tr>
<td>Using Mass Spectrometry to Identify GABA Type A Receptor Trafficking Changes In Vivo</td>
<td>8</td>
</tr>
<tr>
<td>A screen for ROMK gain-of-function mutations that may lead to hypertension</td>
<td>9</td>
</tr>
<tr>
<td>Revealing how plasma membrane microdomains influence diffusion of PtdIns (4,5)P2 in living cells</td>
<td>9</td>
</tr>
<tr>
<td>Cross-talk between ligand- and p38-MAP-kinase-induced endocytosis of the EGF receptor</td>
<td>10</td>
</tr>
<tr>
<td>Dual binding affinities for megalin and cubilin receptors accommodate wide variations in filtered albumin load</td>
<td>11</td>
</tr>
<tr>
<td>Protein folding pathway modulation upon Hsp70 inhibition in cancer cells</td>
<td>11</td>
</tr>
<tr>
<td>Altered megalin trafficking in Dent disease</td>
<td>12</td>
</tr>
</tbody>
</table>
Visualization of Annular Gap Junction Vesicle Processing: Formation, Interplay with Cytoplasmic Organelles, and Degradation

PIP2 POTENTIATES THE CA2+-ACTIVATED CL- CHANNEL TMEM16A IN XENOPUS LAEVIS OOCYTES

Ischemic stroke impairs chloride homeostasis and GABAA receptor inhibition

PACS1-Syndrome patient cells contain aberrant microtubule-based structures which can be rescued by inactivation of HDAC6

Acute control of plasma membrane PtdIns(4,5)P2
α-Arrestins Rod1 and Rog3 regulate trafficking of glucose transporters in response to starvation and 2-deoxyglucose

Authors: David Augustine1; Kenny Callahan2; Dakshayini Chandrashekarappa1; Leo Garnar-Wortzel3; Martin Schmidt1; Allyson O’Donnell1

1 University of Pittsburgh
2 Marlboro College

Cells maintain internal homeostasis by reshuffling the complement of transmembrane proteins at the cell surface and intracellular membranes. Processes such as endosomal recycling, clathrin-dependent endocytosis, and clathrin independent endocytosis work in tandem to assist in this reshuffling. A common presence in these processes are the α-arrestins, a family of trafficking adapter proteins conserved from yeast to man. The α-arrestins contain a PPxY motif that allows for binding to the E3 ubiquitin ligase Rsp5 in yeast. This ubiquitination is necessary for targeting of transmembrane proteins for endocytosis, and the α-arrestins mediate this crucial interaction. In yeast, members of the hexose transporter family (Hxts) are differentially expressed depending on nutrient availability. Two conditions that change Hxt expression levels are glucose starvation or treatment with the glucose analog 2-deoxyglucose, which is toxic to cells and thought to block glycolysis. We tagged Hxt1 and Hxt4 with GFP and observed their localization via fluorescent microscopy after either starvation in low glucose media or treatment with 2DG. We found that deletion of nine α-arrestins significantly reduce trafficking of Hxt1 and Hxt4 after starvation or treatment with 2DG. Also, we found that complementing 9ArrD with Rod1, and in some instances Rog3, restored normal trafficking of these transporters, demonstrating that Hxt1 and Hxt4 are trafficked by these α-arrestins. We also show that phosphorylation of Rog3 C-terminal tail is important for Rog3-mediated regulation of these glucose transporters. It is important to note that in mammalian cells, analogous α-arrestin regulation of glucose transporters, known as the GLUTs, has been demonstrated and that α-arrestins are the most highly up-regulated genes upon glucose feeding in many tissue types consistent with an important role in regulating glucose homeostasis. We propose that the studies here define new modes of regulation for α-arrestins and glucose transporters that may be directly applicable to our understanding of metabolic diseases in humans.

Author Affiliation:
Biological Sciences, University of Pittsburgh

Extracellular zinc Contributes to the Slow Polyspermy Block

Authors: Katherine Wozniak1; Wesley Phelps1; Rachel Bainbridge2; Anne Carlson1

1 Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA
2 University of Pittsburgh

Ensuring that an egg is fertilized by a single sperm is essential for progression through embryogenesis. Eggs therefore have multiple mechanisms to prevent sperm entry into an already fertilized egg. In the slow polyspermy block, fertilization induces exocytosis of cortical granules, which are enriched with compounds that transform the extracellular matrix (ECM) surrounding eggs into a barrier impenetrable by sperm. The underlying mechanisms that enable the creation of this protective barrier are largely-unknown. We hypothesize that extracellular zinc may contribute to the slow block. It has recently been shown in mice, primates, and humans that cortical granules are enriched with zinc; furthermore, extracellular zinc impairs sperm motility and binding to the ECM of eggs. Using confocal microscopy with the zinc indicator FluoZin-3, we demonstrate that fertilization and egg activation evoke zinc release from eggs of the African clawed frog, Xenopus laevis, and zebrafish, Danio rerio. Using the appearance of cleavage furrows 90-120 minutes after sperm addition as an indicator of embryonic development, we show that insemination of X. laevis eggs in extracellular
ZnSO4 blocks development in a concentration-dependent manner (IC50 = 20.5 ± 3 mM). To hone-in on when extracellular zinc interferes with development, we inseminated X. laevis eggs in 0 or 1 mM ZnSO4 for 30 minutes, then transferred these eggs to new solutions with or without ZnSO4. None of the eggs inseminated in zinc developed cleavage furrows; whereas, eggs inseminated without zinc, but transferred to a zinc-containing solution, developed normally. The effect of zinc on the ECM of the egg is irreversible, as pretreatment with 1 mM ZnSO4 prior to sperm addition was sufficient to prevent embryonic development. Overall, our data demonstrates that fertilization-induced zinc release is conserved in vertebrates, and this zinc may contribute to the slow polyspermy block.

Author Affiliation:
Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA

Student/Fellow Presentations / 22

New optical approaches to investigate GABA type A receptor assembly, trafficking and neuroadaptation to drug treatment and pathology

Authors: Matthew Bambino1; Katarina Vajn2; Josh Lorenz-Guertin3; Tija JacobNone

1 University of Pittsburgh School of Medicine
2 University of Pittsburgh
3 Department of Pharmacology and Chemical Biology, University of Pittsburgh, PA

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain making up about 40% of synapses. Due to its ubiquitous nature, dysfunction of this critical inhibition can play a role in a variety of conditions such as autism, insomnia, epilepsy, schizophrenia, and anxiety and panic disorders. GABA type A receptors (GABAARs) are heteropentameric ligand-gated chloride (Cl-) ion channels typically composed of two α (α1-6), two β (β1-3), and one γ (γ1-3) or δ subunit. The channel kinetics and pharmacological sensitivity of a GABAAR is defined by the subunit composition. The most common GABAARs providing fast synaptic inhibition in the adult mammalian cerebral cortex are composed of α1β2γ2 subunits, which are sensitive to the key clinical benzodiazepine drug class that positively modulates GABAARs. Critical determinants of GABAAR neurotransmission are both the receptor composition and regulated trafficking. Synaptic GABAARs are trafficked to the plasma membrane and then tethered across from GABA release sites, allowing efficient phasic inhibitory neurotransmission. Receptors diffuse away from synapses and are internalized by clathrin-dependent endocytosis at extrasynaptic sites, where they can then be either recycled back to the cell membrane or degraded in lysosomes. In order to study these processes, we have developed and validated novel optical methods including: 1) surface Förster resonance energy transfer (FRET) measurements between subunits in a single GABAAR and 2) a fluorogen tagged γ2 subunit (γ2HFAP) for trafficking studies in vitro and in vivo. FRET measurements using a donor α2 pH-sensitive green fluorescent protein (α2pHGFP) and acceptor fluorescently tagged γ2mCherry (γ2RFP) showed a reduction in surface trafficked receptors in response to 24-hour treatment with the benzodiazepine diazepam in cultured cortical neurons. For γ2 subunit trafficking studies, we genetically inserted a fluorogen-activating peptide (FAP) that binds malachite green dyes into a γ2 subunit tagged with pH-sensitive green fluorescent protein (γ2pHFAP). We have used this γ2pHFAP based optical method to track GABAAR dynamics in response to in vitro seizure paradigms, revealing enhanced synaptic turnover, lysosomal targeting, and the trafficking and surface insertion of new, non-recycled GABAARs in an attempt to mitigate this response. We are currently optimizing our γ2pHFAP with adeno-associated virus (PAAV-hSyn1-γ2pHFAP) delivery in vitro and in vivo to visualize GABAAR dynamics. These optical tools provide novel approaches to study GABAAR assembly, trafficking and the neuroadaptive processes undergone in response to drug treatment and pathology.

Author Affiliation:
Department of Pharmacology and Chemical Biology
University of Pittsburgh
Revealing a new role for alpha-arrestins in the regulation of autophagy

**Authors:** Ray Bowman¹; Karandeep Chera²; Allyson O’Donnell¹

¹ University of Pittsburgh
² Duquesne University

Maintaining homeostasis in an ever-changing environment is a hallmark of life. One fundamental way cells achieve this adaptation is by selective redistribution of their proteome. This reorganization is imperative for cell survival and is regulated, in part, by a highly conserved family of protein trafficking adapters called the alpha-arrestins. With previously established roles in endocytosis and recycling of membrane-bound proteins, we have recently implicated the alpha-arrestins in regulation of autophagy, a conserved self-degradative process by which cells recycle aging components and reclaim key nutrients during starvation. This connection was initially identified while screening for modifiers of a resistance to rapamycin, an inhibitor of TORC1 function that mimics nitrogen starvation, conferred by the over-expression of alpha-arrestins Aly1 and Aly2. Since, we have uncovered a suite of genetic interactions with components of the autophagy-related ATG gene family as well as another pair of alpha-arrestins that confer resistance to rapamycin. Live cell imaging and biochemical analysis have revealed alpha-arrestin-dependent changes in autophagic flux and the abundance of autophagy-specific protein Atg8. These data support an exciting novel role for alpha-arrestins as regulators of autophagy, which expands on their known function in sensing and responding to nutrient stress.

**Author Affiliation:**
Department of Biological Sciences
University of Pittsburgh

Uncovering properties of dynamic signal propagation in single cells

**Author:** J. Agustin Cruz¹

**Co-authors:** Gabriel Kowalczyk ²; Chaitanyakusudhir Mokashi ²; David Schipper ²; Sanjana Gupta ²; Qiuhong Zhang²; Robin Lee

¹ Computational and Systems Biology - Pitt
² Department of Computational and Systems Biology - Pitt

How do single cells use signaling pathways to integrate information from cytokine and dose and transmit information to downstream effectors? Inflammatory signaling is commonly studied from the level of cell population, deriving general principles that are not fully representative of individual cell responses. However, even isogenic cells respond differently to the same stimuli, which is caused by intrinsic and extrinsic noise. Noise and cell-to-cell heterogeneity provide means for individual cells to tune their responses to specific ligands and doses, regulating information transmission downstream of a signaling pathway, and orchestrating single cell responses.

Utilizing live-cell imaging and CRISPR-modified human cancer cells, we investigated single-cell responses to inflammatory molecules such as tumor necrosis factor-alpha (TNFα) and interleukin-1 (IL-1). Both signaling pathways converge on the activation of the nuclear factor kappa-B (NF-KB) signaling, inducing formation and activation of the Inhibitor of kappa-B (IKB) kinase (IKK) complex, which releases NF-KB transcription factor proteins from the cytosol to translocate inside the nucleus.

After stimulation with TNF/IL1, U2OS cells that express EGFP-IKK form spots that we observed under a fluorescence microscope. With this approach, we uncovered a dynamic formation and dissociation of EGFP-IKK complexes. Spots size, the intensity of fluorescence, and the rate of formation
are some of their features that we have analyzed. Using computational tools, we are extracting dynamic features from each individual spot in each cell. We aim to determine the relationship between these dynamic features of IKK complex formation and the information transmitted downstream to induce NF-κB nuclear translocation. Thus far, we have discovered that the dose of cytokine used is related to the number of IKK spots formed. We are now accumulating enough quantitative data to cross-reference these dynamics features within the same cell. These analyses will contribute to understanding how intracellular signaling propagates from surface receptors down to transcription factors within an individual cell.

Author Affiliation:
Department of Computational and Systems Biology - University of Pittsburgh School of Medicine

Lunch/Poster Session/Vendor Show / 8

Effects of Stretch on the Bladder Umbrella Cell Apical Junctional Ring

Authors: Amity Eaton\None; Dennis Clayton\None; Wily Ruiz\None; Shawn Griffiths\None; Gerard Apodaca\None

The urothelium, which lines the luminal surface of the bladder, must maintain its integrity during cycles of filling and voiding. How this is accomplished is not well understood. A critical component of the epithelial barrier is the apical junctional ring (AJR), which circumscribes polarized epithelial cells at their apical pole. The AJR includes the apical-most tight junction (TJ), subjacent adherens junction (AJ), and associated actomyosin cytoskeleton, which modulates cell tension at regions of cell-cell contact. We previously reported that the TJ ring encircling the outermost umbrella cell layer of the urothelium expands during bladder filling and contracts during voiding. However, whether the AJ is similarly affected, and the identity of the underlying machinery that drives these changes in the AJR remains unknown.

When we examined bladders that were filled and voided in situ we observed the following: (1) Like the TJ ring, the AJ ring also expanded during filling and contracted within minutes of voiding. (2) In addition to actin, which was concentrated in a continuous ring interposed between the TJ and AJ, the actin-binding and bundling protein α-actinin-4, and non-muscle myosin IIA, which is known to regulate AJ dynamics, were also associated with the AJR. (3) The actin disrupting agent cytochalasin D, as well as inhibitors of formins (SMIFH2) or ARP2/3 (CK869), decreased expansion of the AJR indicating a role for active actin polymerization in this process. Surprisingly, treatment with blebbistatin, an inhibitor of myosin II contraction, had no effect on the expansion of the AJR. (4) An inhibitor of exocytosis, brefeldin A, showed a strong inhibitory effect on AJR expansion, indicating that vesicular traffic may regulate AJR dynamics. (5) To target the exocytic machinery more specifically we expressed dominant-negative (DN) mutants of RAB8A, RAB11A, or RAB13 using adenoviral transduction. Whereas DN-RAB13, previously implicated in the regulation of TJ dynamics, significantly decreased AJR expansion during bladder filling, DN-RAB8A and DN-RAB11A, which are involved in stretch-stimulated exocytosis during bladder filling, did not. (6) Unlike expansion of the AJR, its voiding-induced contraction was strongly inhibited by blebbistatin and was also significantly inhibited by cytochalasin D. (7) Intriguingly, AJR contraction was also inhibited by the general endocytosis inhibitor Pitstop2 or expression of a DN mutant of the endocytic regulatory protein Dynamin2. Moreover, DN-RHOA, which we previously implicated in umbrella cell apical endocytosis, similarly impaired junctional ring contraction.

Conclusions: Our studies indicate that a likely mechanism for retention of urothelial continuity in the face of cyclical changes in volume is expansion and contraction of the umbrella cell apical junctional ring (AJR). Not only does AJR expansion depend on actin polymerization, it also requires exocytosis, presumably of vesicles containing AJR-associated membrane proteins. In contrast, constriction of the AJR is likely mediated by actomyosin contraction and endocytosis of excess AJR proteins.

Author Affiliation:
University of Pittsburgh School of Medicine, Renal-Electrolyte Division

Student/Fellow Presentations / 15
Patronin-mediated microtubule minus end growth populates dendrites with minus-end-out microtubules during development and regeneration

Authors: Chengye Feng\textsuperscript{None}; Pankajam Thyagarajan\textsuperscript{None}; Matthew Shorey\textsuperscript{None}; Dylan Seebold\textsuperscript{None}; Alexis Weiner\textsuperscript{None}; Richard Albertson\textsuperscript{None}; Kavitha Rao\textsuperscript{None}; Alvaro Sagasti\textsuperscript{None}; Daniel Goetschius\textsuperscript{None}; Melissa Rolls\textsuperscript{None}

Microtubules are organized independently from the centrosome in mature neurons. Axonal microtubules are plus-end-out. Dendritic microtubule polarity is minus-end-out in C. elegans and Drosophila, and mixed in mammalian neurons. Early in Drosophila neuronal development, g-tubulin, the core microtubule nucleation protein, redistributes from the cell body to dendrite branch points and regulates the microtubule network locally. However, whether microtubules remain anchored on g-tubulin, and how they are organized beyond branch points that contain g-tubulin is unknown. Microtubule minus ends are thought to be stable in cells. Surprisingly, in Drosophila and zebrafish neurons we observed persistent minus end growth with fluorophore labeled EB proteins, with runs lasting over ten minutes. Drosophila Patronin has previously been shown to inhibit minus end growth in vitro. However, we found in vivo it localizes to growing minus ends and is required for persistent minus end growth. When Patronin levels were reduced in neurons, microtubule minus ends had shorter polymerization length and duration. In addition, microtubule polarity in dendrites became mixed. We hypothesized that mixed polarity in the absence of Patronin could be due to failure to grow minus-end-out microtubules into distal dendrites. Indeed, Patronin RNAi lowered the number of minus-end-out microtubules. More strikingly, extra Patronin made terminal dendrites almost completely minus-end-out, indicating low Patronin normally limits minus-end-out microtubules. We also examined the role of minus end growth in dendrite development. During dendrite outgrowth, minus-end-out microtubules are added into dendrites after plus-end-out microtubules, and microtubule polarity gradually shifts to minus-end-out orientation. With reduced Patronin levels, minus-end-out microtubules failed to completely convert the polarity so that it remained mixed. Similar polarity conversion failure happened during dendrite regeneration. In this case very few minus-end-out microtubules were added into regenerating dendrites of neurons with reduced Patronin. In sum, our data suggests that Patronin facilitates sustained microtubule minus end growth, which is critical for populating dendrites with minus-end-out microtubules.

Author Affiliation:
Biochemistry and Molecular Biology and the Huck Institutes of the Life Sciences
The Pennsylvania State University

Lunch/Poster Session/Vendor Show / 11

Quality control of polycystin 2 missense mutants in the endoplasmic reticulum

Author: Christopher Guerriero\textsuperscript{1}

Co-author: Jeffrey Brodsky \textsuperscript{1}

\textsuperscript{1} University of Pittsburgh

BACKGROUND
Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder and is a leading cause of end-stage renal disease. ADPKD arises from mutations in the PKD1 and PKD2 genes, encoding the primary ciliary proteins polycystin 1 (PC1) and polycystin 2 (PC2), respectively. Myriad mutations have been documented throughout the PKD loci, ultimately resulting in aberrant signaling, cell proliferation, and fluid secretion. In addition to the primary cilium, PC2 also localizes to the endoplasmic reticulum (ER). Given PC2’s large size, topological complexity, and localization, we hypothesize that PC2 missense mutants may misfold and be turned over by the endoplasmic reticulum-associated degradation (ERAD) pathway. ERAD triages newly synthesized aberrant proteins and directs misfolded or misassembled conformers for degradation via the ubiquitin proteasome system. Due to an incomplete understanding of the factors that influence early events
during PC2 maturation, we investigated PC2 biogenesis in the genetically-tractable model system, S. cerevisiae.

METHODS
We have established a yeast expression system for 3XHA-PC2 to study the early biosynthetic decisions that mediate the folding, maturation, and degradation of PC2 missense mutants. Disease-causing PC2 variants were generated by site-directed mutagenesis, expressed in S. cerevisiae, and their metabolism was examined using cycloheximide chase analysis. Results from the yeast model were further confirmed via transient transfection of GFP-PC2 into HEK293 cells.

RESULTS
Our preliminary data indicate that select PC2 missense mutants are more rapidly degraded in both yeast and HEK293 cells relative to wild type PC2. Moreover, unstable PC2 mutants are more highly poly-ubiquitinated than wild type PC2. In addition, following treatment with the proteasome inhibitor MG132, the level of polyubiquitinated protein increases to a greater extent for PC2 missense variants, suggesting selective turnover by the proteasome.

CONCLUSION
The findings support our hypothesis that PC2 missense mutants are ERAD substrates. Given recent interest in the development of protein folding modulators for other ERAD-related diseases, our data provide a promising hint that the maturation of some PC2 missense mutants may be amenable to pharmacological correction.

FUNDING
NIDDK101584
NIDDK079307

Author Affiliation:
Department of Biological Sciences
University of Pittsburgh

Student/Fellow Presentations / 28

SunRISE: a labeling system for long-term imaging of single mRNA molecules in live cells

Authors: Yue GuoNone; Robin LeeNone

Cells rely on tight control of gene expression to respond accurately to stimuli. However, mRNA as the key regulator in genetic information flow shows a high degree of variability in single cells, including copy numbers, spatiotemporal distribution, and dynamics. To understand how variability and dynamics in gene expression influence cellular behaviors, we have developed SunRISE, a fluorescence labeling system to visualize and track the dynamics of single mRNA molecules over long periods of time in living cells. SunRISE harnesses the principle of recruiting multiple copies (up to ~1000) of fluorescent proteins to single mRNA molecules in two stages, thereby enabling long-term imaging of single mRNAs with minimal photobleaching. Because each fluorescent mRNA molecule has a high signal-to-noise ratio, we can observe the whole lifespan of mRNA continuously in living cells. We will describe a synthetic biology approach, using models to predict how system components interact with each other and then construct the optimal labeling conditions based on these predictions. Together, SunRISE provides a versatile toolbox to image single mRNA molecules in living cells and is a promising approach to reveal the complete dynamics of mRNAs from birth to death.

Author Affiliation:
Department of Physics and Astronomy, University of Pittsburgh
Fluorogen-Activating Protein (FAP)-tagging vectors and organelle markers for quantitative imaging

Authors: Natalie Hager\(^1\); Ceara McAtee\(^1\); Justina Warnick\(^1\); Marcel Bruchez\(^2\); Jeffrey Brodsky\(^1\); Allyson O'Donnell\(^1\)

\(^1\) University of Pittsburgh  
\(^2\) Carnegie Mellon University

Recent advantages of genetically encoded fluorescent probes have led to the development of fluorogen activating proteins (FAPs). This technology has two components: a non-fluorescent single chain antibody (SCA) that can be fused to a protein of interest and fluorogens, which are non-fluorescent dye molecules when free in solution. When the SCA and fluorogen bind, there is a 20,000-fold fluorescent increase relative to unbound dye. This level of fluorescence is comparable to typical fluorescent proteins. However, the FAP-technology has two major advantages; (1) using either a membrane-permeant or impermeant fluorogen dye we are able to selectively label intracellular proteins from proteins at the plasma membrane and (2) since the fluorogen does not fluoresce when it is not bound by SCA, we are able to completely eliminate background fluorescence when imaging in other fluorescent channels. Although developed in yeast, this technology has surprisingly not been used to detect localization in this model system until our recent work looking at the residence of an ectopically expressed mammalian membrane protein, Kir2.1, at the cell surface. In order to make this technology more readily available to the cell biology community, we have first optimized the SCA sequence for expression in yeast, and then created a series of SCA-tagging constructs and organelle markers to be used as tools for the cell biology research community. These tools will allow scientists to quantitatively analyze protein dynamics, minimize the effects of background, observe multiple fluorescent tags simultaneously in a single cell, and selectively illuminate the surface or intracellular population of a tagged protein.

Author Affiliation:
Department of Biological Sciences, University of Pittsburgh

Cellular degradation of Nephrogenic Diabetes Insipidus AQP2 Variants in a Yeast Model

Authors: Nancy Kaufmann\(^\text{None}\); Dawn Bisi\(^\text{None}\); Ling Xu\(^\text{None}\); Sam Estabrooks\(^\text{None}\)

Nephrogenic Diabetes Insipidus causes dehydration, excessive thirst, and excessive urination. Mutations in the human aquaporin 2 water channel gene can cause the congenital form of the disease. We are working to characterize three single amino acid changes in different regions of the aquaporin 2 protein to understand how these cause disease. Using a Saccharomyces cerevisae model system we hope to genetically identify targets to rescue the mutant proteins. We found that all three mutations destabilize the protein in a proteasome-dependent manner. Here we show that loss of the E3 ubiquitin ligase HRD1 significantly stabilizes the mutant aquaporin degradation, while loss of the chaperone HSP90 seems to have little effect. In the future, we hope to identify the chaperone pathway involved in the folding of each of these three aquaporin variants before looking for patterns in location of mutation and chaperones involved in recognizing proteins as misfolded. This could have impact on diabetes insipidus as well as treatment of other membrane protein folding diseases.

Author Affiliation:
Department of Biological Sciences University of Pittsburgh
Genetic analysis of the Drosophila ESCRT-III Complex protein, VPS24, reveals cell-autonomous and -nonautonomous requirements in proteostasis

Authors: Jonathan Florian¹; Samuel DeMattei; Kendall Godwin¹; Devon Sweeder¹; Richard Ordway¹; Fumiko Kawasaki

¹ PSU

The ESCRT (Endosomal Sorting Complexes Required for Transport) pathway plays key roles in a variety of membrane remodeling processes in eukaryotic cells. In vitro studies and genetic analysis have defined the ESCRT pathway, which is composed of four hetero-multimeric complexes (ESCRT-0 to III) as well as several accessory proteins, and characterized its functions. Among these, the ESCRT pathway participates in proteostasis through trafficking of ubiquitinated proteins and organelles for lysosomal degradation. Despite this progress, additional work is required to fully establish the in vivo properties and functions of this pathway. Here we report a new mutation in a Drosophila ESCRT III gene, vps24. This mutant was recovered in our forward genetic screen for mutations that disrupt neural function and has led to broader genetic analysis of the ESCRT pathway at the cellular and organismal levels. Our initial findings indicate that VPS24 functions in proteostasis through both intracellular (cell-autonomous) and, interestingly, intercellular (cell-nonautonomous) mechanisms. These studies suggest that VPS24 is critical for effective proteostasis in specific neuronal and muscle cell types. Moreover, VPS24 can support organismal proteostasis through intercellular signaling mechanisms which are specific with regard to both cell type and direction. These studies may provide new insight into the in vivo roles of the ESCRT pathway in proteostasis and intercellular signaling, as well as our understanding of ESCRT function in neurological disease.

Author Affiliation:
Department of Biology and Centers for Cellular Dynamics and Molecular Investigation of Neurological Disorders, The Pennsylvania State University

Using Mass Spectrometry to Identify GABA Type A Receptor Trafficking Changes In Vivo

Authors: Joshua Lorenz-Guertin¹; Sabyasachi Das¹; Sammy Pardo²; Dana Molleur²; Susan Weintraub²; Tija Jacob¹

¹ University of Pittsburgh Medical School
² UT Health Science Center at San Antonio, San Antonio

Fast neuronal inhibition in the adult brain predominantly occurs through binding of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) to heteropentameric GABA type A receptors (GABAA Rs). Surface and intracellular trafficking of GABAA Rs regulate the strength of GABAergic inhibitory neurotransmission. To identify receptor trafficking changes occurring in vivo after treatment with GABAA R targeted drugs, we immunoprecipitated mouse cortical tissue with a GABAA R γ2 subunit antibody and subjected samples to quantitative label-free HPLC-ESI-MS/MS. Pilot experiments revealed our Co-IP and mass spectrometry conditions were able to successfully identify all expected GABAA R subunits and the GABAergic scaffolding protein gephyrin. To examine drug-induced changes in GABAA R associated proteins, we injected mice I.P. with vehicle or 10 mg/kg diazepam (DZP), a GABAA R positive allosteric modulator, and collected cortical tissue 12 h post-injection. 365 protein hits were identified by Scaffold analysis and peptide spectral counting revealed 46 proteins with elevated levels of interaction with γ2-GABAA Rs and 23 proteins demonstrating diminished association with γ2-GABAA Rs. We examined the biological significance of these altered protein interactions using Ingenuity Pathway Analysis (IPA) and found key pathways including endocytosis and organization of cytoskeleton were activated, while the development of neurons...
pathway was inhibited in response to DZP. Secondary GO analysis of proteins found to be elevated in association with GABAAR post-DZP also suggested accumulation of these receptors into localization and trafficking pathways. Ongoing studies aim to construct a more comprehensive in vivo model of inhibitory synapse plasticity by measuring DZP evoked changes in the scaffolding protein gephyrin through the use of Co-IP and phosphoproteomics. Overall, our work provides an innovative proteomics approach to investigate changes in receptor trafficking in vivo by applying Co-IP and bioinformatics methods to study changes in protein-protein interactions.

Author Affiliation:
Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA

Lunch/Poster Session/Vendor Show / 27

A screen for ROMK gain-of-function mutations that may lead to hypertension

**Authors:** Nga (Katie) Nguyen¹ ; Timothy Mackie¹ ; Jackson Parr¹ ; Xueqi (Shae) Wang² ; Shaohu Sheng² ; Thomas Kleyman² ; Jeffrey Brodsky³

¹ Department of Biological Sciences, University of Pittsburgh  
² Renal-Electrolyte Division, School of Medicine, University of Pittsburgh

Hypertension affects one billion people worldwide and contributes to 13.5 million deaths each year. While genetic causes can be attributed to up to half of all cases, hypertension is highly polygenic, with no known single gene playing a major role. One determining factor of hypertension is the highly efficient process of sodium reabsorption in the kidney, which is achieved through an extensive network of transporters and ion channels. Among these proteins, one critical channel is the renal outer medullary K+ (ROMK) channel. Loss-of-function mutations in ROMK give rise to the salt-wasting Bartter’s Syndrome Type II, and recent work from our lab indicated that these mutated proteins are targeted for premature degradation in the endoplasmic reticulum. Interestingly, heterozygous carriers of these same mutations, estimated to make up 1% of the population, are protected from hypertension. Therefore, our hypothesis is that the opposite may hold true: Specifically, we suggest that a gain-of-function (GOF) mutation in ROMK will predispose people to hypertension. An experimental approach to identify and characterize these mutations will not only identify a trait that determines a predisposition for hypertension, but also strengthen our understanding of the structural dynamics that underlie ROMK function in the kidney and ROMK trafficking.

Toward this goal, we expressed ROMK in a Saccharomyces cerevisiae strain lacking two major K+ transporters so that growth is restricted on low K+. We then screened for clones containing randomly mutagenized ROMK insertions that exhibit augmented growth on low K+. So far, we have identified 11 such mutations, divided into weak or strong rescuers of growth. Preliminary analysis showed that one mutation, K80M, exhibited increased whole-cell currents when introduced into Xenopus oocytes. Other mutations reside near to or even at identical positions in the human genome database. Going forward, we will continue the yeast screen until saturation is reached and then use multiple biochemical, cellular, and electrophysiological approaches to define the mechanisms underlying the altered activity in isolated ROMK variants.

Author Affiliation:
Department of Biological Sciences, University of Pittsburgh

Student/Fellow Presentations / 20

Revealing how plasma membrane microdomains influence diffusion of PtdIns (4,5)P2 in living cells

**Author:** Jonathan Pacheco Romero¹
Co-author: Gerald Hammond

University of Pittsburgh

PtdIns(4,5)P2 is key lipid to control a wide array of cellular processes. These processes include cellular signaling, regulation of endo/exocytosis, modulation of receptors, ion channels and remodeling of cytoskeleton just to name a few. In consequence, alterations of PtdIns(4,5)P2 can lead to several diseases, including diabetes or cancer. Understand how PtdIns(4,5)P2 does to coordinate between different functions is not full understood. Some hypothesis propose a local synthesis of the kinases in charge to produce PtdIns(4,5)P2, otherwise other evidence shown the formation of clusters of PtdIns(4,5)P2. Indeed, PtdIns(4,5)P2 is well recognized on important cellular structures like phagosomes, synaptic terminals or others membrane domains. However, nothing is known about the dynamic of PtdIns(4,5)P2 in these domains. Here, by using genetically encoded sensors of PtdIns(4,5)P2 fused to photoactivatable fluorescent protein pamCherry1, we tracked individual molecules of PtdIns(4,5)P2 at the plasma membrane of living cells. The lateral mobility of PtdIns(4,5)P2 yielded an apparent diffusion coefficient of 0.2 µm²/s. We characterized the diffusion of PtdIns(4,5)P2, showing great portion of molecules with random motion and just a small proportion of molecules presenting directed or confined motion. We detected the presence of two population with different mobility, both of them not getting away from brownian motion. Then we evaluated the effects on PtdIns(4,5)P2 diffusion when individual molecules travel through several plasma membrane microdomains. To label plasma membrane microdomains we used endogenously labeled HELA cells. Results show that some domains reduce PtdIns(4,5)P2 diffusion, but some others do not have any effect on PtdIns(4,5)P2 dynamics. In general, this picture reflects the highly heterogeneity of the plasma membrane and the effects that has on PtdIns(4,5)P2 diffusion.

Author Affiliation:
Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA

Student/Fellow Presentations / 24

Cross-talk between ligand- and p38-MAP-kinase-induced endocytosis of the EGF receptor

Authors: Mireia Pérez Verdaguer; Mads Breum Larsen; Marcel P. Bruchez; Simon C. Watkins; Alexander Sorkin

Department of Cell Biology, University of Pittsburgh School of Medicine
Department of Biological Sciences, Department of Chemistry, and Molecular Biosensor and Imaging Center, Carnegie Mellon University

Cells exquisitely fine-tune responses to environmental cues while using no more than a handful of major signaling cascades. Endocytosis, initially seen as a signal attenuation mechanism, is nowadays believed to orchestrate signaling networks in space and time. Activation of epidermal growth factor (EGF) Receptor (EGFR) through ligand binding initiates signal transduction through several pathways and also results in rapid internalization of the receptor via clathrin-mediated (CME) and -independent endocytosis (CIE). In addition, activation of stress-induced p38 MAPK leads to non-canonical endocytosis of ligand-free EGFR via CME. However, the mechanisms of ligand- and stress-induced EGFR endocytosis remain elusive, and whether simultaneous ligand binding and p38/MAPK activation, that is a likely scenario under physiological conditions in vivo, results in cross-talk interactions between these two processes is unknown. Hence, we have developed a novel experimental system that allows quantitative analysis of ligand-bound and free EGFR endocytosis by tagging the endogenous EGFR with fluorogen activating protein (FAP) at the amino-terminus using CRISPR/Cas9 gene-editing. When FAP-EGFR was activated by low, physiological ligand concentrations (max. at 1-3ng/ml), internalization of ligand-bound receptors and p38-dependent internalization of ligand-free receptors were observed simultaneously, with both internalization pathways merging to the same endosomes. Likewise, in the presence of both EGF and tumor necrosis factor α (TNFα), that robustly activates p38, most extensive clathrin-mediated endocytosis of FAP-EGFR was observed. Interestingly, such an additive effect of co-stimulation on FAP-EGFR internalization was observed only at low EGF concentrations. With the increase of FAP-EGFR ligand occupancy, the
p38 component of FAP-EGFR CME was proportionally decreased. Ultimately, high concentrations of EGF re-directed the bulk of the receptor to the CIE pathway, virtually abolishing the p38-dependent endocytosis. We hypothesize that ligand-induced post-translational modifications of the EGFR inhibit p38-induced interactions of the receptor with the clathrin associated machinery, thus preventing receptor internalization through the non-canonical mechanism. Analysis of dose-dependent, mechanistic cross-talks between these two physiological endocytic processes is in progress in the laboratory.

Author Affiliation:
Department of Cell Biology, University of Pittsburgh School of Medicine

Lunch/Poster Session/Vendor Show / 10

Dual binding affinities for megalin and cubilin receptors accommodate wide variations in filtered albumin load

Authors: Qidong Ren1; Youssef Rbaibi2; Ossama Kashlan2; Ora A. Weisz2

1 School of Medicine, Tsinghua University; Renal-Electrolyte, University of Pittsburgh
2 Renal-Electrolyte, University of Pittsburgh

Albuminuria is one of the most important early symptoms and pathologic factors of kidney disease. The very small fraction of serum albumin that normally escapes the glomerular filtration barrier under physiologic conditions is efficiently recovered by the kidney proximal tubule (PT). Two multiligand receptors, megalin and cubilin, are expressed at the apical surface of PT cells and are believed to function together to internalize filtered albumin and other proteins via clathrin-mediated endocytosis. PT cells express a single high-affinity surface binding site for albumin with a Km similar to the estimated tubular concentration of 23 µg/mL (~0.4 µM). Although this site should be readily saturable, PT cells in vivo can readily internalize at least 50-fold higher concentrations of albumin. We performed experiments to examine how the PT accommodates the uptake of albumin within this extraordinary range of physiologic to pathologic concentrations. Consistent with previous findings, only a single site was detected by surface binding of albumin on ice, whereas exposure of PT cells to increasing amounts of fluorescently-conjugated albumin at 37ºC revealed a biphasic uptake profile. Deconvolution of the profile revealed two uptake sites for albumin with affinities of ~1.2 and 25 µM. We hypothesized that cubilin and megalin receptors function independently of one another to mediate high- and low-affinity uptake of albumin under normal and pathologic conditions, respectively. Consistent with this, knockdown of cubilin using siRNA selectively reduced the binding capacity of the high-affinity site. Digital PCR to quantify the ratio of megalin:cubilin transcripts in mouse kidney revealed that megalin mRNA is expressed at higher levels than cubilin. Current studies to further test our hypothesis are focused on creating the high- and low-affinity albumin uptake sites by megalin or cubilin transfection of non-expressing cells, and assessing whether megalin: cubilin protein ratios in cells and kidney are consistent with the binding capacities of the low and high affinity uptake sites.

Author Affiliation:
School of Medicine, Tsinghua University, Beijing, China; Renal-Electrolyte, University of Pittsburgh, Pittsburgh, PA

Student/Fellow Presentations / 9

Protein folding pathway modulation upon Hsp70 inhibition in cancer cells

Authors: Sara Sannino1; Christopher J. Guerriero1; Amit J. Sabnis2; Jeffrey L. Brodsky1

1 Department of Biological Sciences, University of Pittsburgh
Cancer cells experience acute stress conditions such as low oxygen and energy, and exposure to toxic agents. To survive proliferate without accumulating toxic misfolded proteins, cancer cells constantly modulate protein homeostasis. Thus, it is not surprising that molecular chaperones, like Hsp70, as well as protein degradation pathways are upregulated in cancer cells compared to their normal counterparts. These data suggest that chaperones are potential targets for cancer therapy. We previously demonstrated the dependence of patient-driven rhabdomyosarcoma cell survival on cytoplasmic Hsp70 activity, thanks to the use of a specific Hsp70 inhibitor, MAL3-101. In particular, we discovered that MAL3-101-mediated Hsp70 inhibition activates the PERK arm of the unfolded protein response (UPR) that results in CHOP-dependent cell death (Sabinis et al., 2016). Moreover, by taking advantage of a MAL3-101-resistant cell line (RMS13-R), we recently determined which compensatory mechanism alters MAL3-101-driven cell death. We found that both endoplasmic reticulum-associated degradation (ERAD) and autophagy are upregulated in RMS13-R cells, underlying increased demand on two protein degradation pathways upon inhibition of Hsp70. However, only autophagy inhibition—but not inhibition of ERAD—re-sensitized RMS13-R cells to Hsp70 inhibition, suggesting that autophagy was the key compensatory mechanism for Hsp70 inhibition. Autophagy was further induced by MAL3-101 treatment in RMS13-R cells, as evidenced by an increase in the messages and proteins corresponding to key autophagy components as well as to the accumulation of autophagic-like structures detected by electron microscopy (Sannino et al., 2018). These data highlight a pro-survival role for autophagy induction upon exposure to an Hsp70 inhibitor in cancer, and provide a link between Hsp70, proteasomal degradation, UPR, and autophagy in rhabdomyosarcoma. We next asked if other cancer types might be sensitive to Hsp70 inhibition, and we investigated the potential benefit of combined treatment with autophagy and/or proteasome inhibitors together with MAL3-101. Specifically, we are investigating the effects of Hsp70 inhibition in breast cancer cells, a cancer type in which higher levels of Hsp70 correlate to increased metastasis and poor prognosis in patients. Our preliminary data suggest that HER2-expressing cells are less sensitive to MAL3-101-mediated Hsp70 inhibition and combinatorial treatments including, MAL3-101 and autophagy inhibitors promoted HER2-breast cancer cell death. Further investigations will reveal the potential carcinogenic role of Hsp70 inhibitors in breast cancer treatment and highlight which pathways reduce proteotoxicity in different breast cancer subtypes.

Author Affiliation:
Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

Lunch/Poster Session/Vendor Show / 16

Altered megalin trafficking in Dent disease

Authors: Katherine E. Shipman1; Kimberly R. LongNone; Youssef RbaibiNone; Catherine J. BatyNone; Ora A. Weisz2

1 University of Pittsburgh
2 Renal-Electrolyte, University of Pittsburgh

Dent disease is an X-linked disorder that primarily affects the kidney proximal tubule (PT) and is caused by mutations in the Cl-/H+ exchanger CLC-5. Early symptoms include tubular (aka low molecular weight) proteinuria, and patients frequently progress to renal failure. Tubular proteinuria results from inefficient reabsorption of filtered proteins by the PT; however, the role of CLC-5 in this process is unclear. Loss of CLC-5 is known to reduce apical endocytic capacity and to decrease expression of megalin, a multiligand receptor required for the uptake of filtered proteins. We used a previously-developed opossum kidney (OK) cell culture model that recapitulates morphologic and functional features of the PT in vivo to develop a mathematical model of megalin trafficking under normal conditions and in Dent disease models. Data obtained through biochemical approaches were used to estimate endocytic and recycling rates and the half-life of surface megalin in control OK cells and CLC-5 siRNA knockdown (KD) cells. We observed decreases in megalin expression and surface half-life in OK cells depleted of CLC-5. These rates were used to construct a simplified ordinary differential equation model of megalin trafficking in differentiated PT cells describing surface and internalized pools of megalin with estimated kinetic parameters. Gene expression studies
in Clcn5 KO mice suggest there are alterations in cholesterol and lipid metabolic enzymes, and elevated cholesterol levels have been shown to alter recycling endosome organization and impair fast recycling. We hypothesize that loss of CLC-5 disrupts cholesterol homeostasis, resulting in reduced recycling and concomitantly increased degradation of megalin. Consistent with this, we observed a dramatic accumulation and redistribution of cholesterol in CLC-5 KD OK cells. Current and future studies are focused on defining the organization of the apical endocytic pathway using quantitative imaging, quantifying changes in endosomal pH in CLC-5 KO models, and determining the role of altered cholesterol metabolism in megalin traffic. Our comprehensive model of megalin trafficking in PT cells can be used to predict which steps in megalin trafficking are altered in other tubular proteinuric diseases and to identify potential therapeutic targets.

Support: NIH R01 DK101484, R01 DK100357, P30 DK079307, and ASN Pre-Doctoral Fellowship

Author Affiliation:
Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA

Lunch/Poster Session/Vendor Show / 29

Visualization of Annular Gap Junction Vesicle Processing: Formation, Interplay with Cytoplasmic Organelles, and Degradation

Authors: Amber Smith¹; Cheryl Bell¹; Teresa Shakespeare²; Amanda Gamwo³; Sandra Murray⁴

¹ Department of Cell Biology
² Savannah State University
³ Cell Biology Department, University of Pittsburgh
⁴ Dept of Cell Biology, School of Medicine, U of Pittsburgh

Gap junction channels, composed of connexin proteins, allow for molecular communication between adjacent cells. These channels, found in plaques, are removed from the surface by an internalization process that results in annular gap junction vesicles (AGJs) being released into the cytoplasm for degradation. However, we hypothesize that AGJs may have fates other than degradation. In this study, we used confocal, time-lapse, immunofluorescence, transmission, and immuno-electron microscopic techniques to analyze AGJ processing. With time-lapse imaging, 19% of AGJs appeared to re-associate with the plasma membrane, 58% fragmented into smaller vesicles, and 11.5% fused with other organelles. These organelles were positively identified with ultrastructural analysis. Of 549 images of AGJs the percentage in contact with mitochondria (5.2 ± 1.1%) exceeded that in association with lysosomes (2.6 ± 0.6%). While AGJ-lysosome associations are consistent with degradation, the relatively low percent of these associations, the observations of AGJs re-associating with the plasma membrane, and their interactions with mitochondria are inconsistent with degradation being the sole fate of AGJs. Interactions of AGJs with the plasma membrane may represent connexin recycling and AGJ-mitochondria associations may serve to allow ion exchange. We now hope to use the Monte Carlo technique to model and simulate annular gap junction processing. As gap junctions play critical roles in physiological functions, information on AGJ processing will increase our understanding of cell-cell communication in normal and diseased conditions. Supported by NSF grant #MCB-1408986

Author Affiliation:
Department of Cell Biology
Department of Cell Biology
School of Medicine
University of Pittsburgh
Pittsburgh, PA 15206
**PIP2 POTENTIATES THE Ca2+-ACTIVATED Cl- CHANNEL TMEM16A IN XENOPUS LAEVIS OOCYTES**

**Author:** Maiwase Tembo

**Co-authors:** Rachel Bainbridge; Anne Carlson

The Ca2+-activated Cl- channel, TransMEMbrane member 16A (TMEM16A), regulates critical functions including smooth muscle contraction, mucosal secretion, and signal transduction. Despite its importance, we are just beginning to understand TMEM16A's biochemical and biophysical properties. To study TMEM16A regulation, here we recorded endogenous TMEM16A currents from Xenopus laevis oocytes. Using the inside-out configuration of the patch clamp technique, we found that TMEM16A-conducted currents rundown within seconds of patch excision despite the continued presence of Ca2+. Current rundown is common amongst channels regulated by phosphatidylinositol 4,5-bisphosphate (PIP2). Thus, we tested the hypothesis that TMEM16A is potentiated by PIP2 by exposing excised inside-out patches to agents that either increased or sequestered membrane PIP2. We found that following rundown, dioctanoyl-PIP2 (diC8- PIP2) applied with Ca2+ recovered TMEM16A-conducted currents by 3.5-fold, but not when applied without Ca2+. Conversely, application of dioctanoyl-phosphatidyl inositol (diC8-PI), comprised of the backbone of diC8- PIP2 but lacking the two phosphate groups, had only a nominal effect. We also found that PIP2 sequestering agents, neomycin and anti-PIP2, sped TMEM16A current rundown by 2-fold. Current rundown was slowed by at least 2-fold when we enabled rephosphorylation of PI with Mg-ATP application, or by inhibiting PIP2 dephosphorylation by application of the phosphatase inhibitor b-glycerophosphate. In another series of experiments, we also tested our hypothesis that PIP2 potentiates TMEM16A but within intact cells. We used the two-electrode voltage clamp to record the Ca2+-activated Cl- currents from X. laevis oocytes exogenously expressing pseudojanin, a PIP2-depleting enzyme. After the rapamycin-induced dimerization of pseudojanin enabling its translocation to the membrane, we observed that reducing membrane PIP2 also reduced TMEM16A conducted currents in Xenopus laevis oocytes. Taken together, our data demonstrate that PIP2 and Ca2+ are both necessary for TMEM16A to pass current.

**Author Affiliation:**

University of Pittsburgh, Biological Sciences

---

**Ischemic stroke impairs chloride homeostasis and GABAA receptor inhibition**

**Authors:** Katarina Vajn; Sabyasachi Das; Tara Taheri; Tong Jiang; Mohammad Iqbal H Bhuiyan; Victoria Piggot; Gulnaz Begum; Dandan Sun; Tija Jacob

**Affiliation:** Department of Pharmacology and Chemical Biology, University of Pittsburgh

Synaptic and extrasynaptic GABA type A receptors (GABAARs) are heteropentameric ligand-gated chloride (Cl-) ion channels that generate inhibitory neurotransmission in the adult brain. The selective flow of Cl- ions down the concentration gradient into the neuron leads to membrane hyperpolarization and inhibition. Plasticity of GABAergic neurotransmission is essential in healthy brain function but can also be pathological. Ischemic brain injury is associated with reduced GABAAR inhibition, although the underlying mechanisms are not well understood. Intracellular chloride (Cl-)
ion concentration sets the strength and polarity of GABA neurotransmission (inhibitory or excitatory), and is regulated by the Cl- importer Na-K-Cl cotransporter (NKCC1) and neuronal Cl- extruder K-Cl cotransporter (KCC2). Activation of WNK Lysine Deficient Protein Kinase 3 (WNK3) inhibits KCC2 and activates NKCC1 thereby increasing intracellular Cl- concentration and reducing GABAergic inhibition. In this study, we tested whether the regulatory kinase WNK3 plays a role in reducing GABAAR inhibition and development of neurological function deficits in stroke.

Methods: We used 1) Oxygen-Glucose-Deprivation (OGD) model on P18 rat cortical neuron and 2) ischemic stroke by occlusion of cerebral middle artery (MCAO) in wild-type control (WNK3 WT) mice and WNK3 transgenic knockout (WNK3 KO) mice. Western blotting and immunostaining methods measured: KCC2, synaptic α1 GABAAR, extrasynaptic α4δ GABAAR and gephyrin, the major scaffolding protein for synaptic GABAAR. Neurological function was assessed at 1-7 days post-MCAO.

Results: Ischemia triggers KCC2 downregulation, and more notably, a decrease in α1 GABAAR synaptic subunits, extrasynaptic α4δ GABAARs, and gephyrin. In contrast, WNK3 KO mice do not show stroke-induced GABAAR plasticity, and lack KCC2 downregulation. Importantly, we detected less ischemic brain damage and improved neurological function in WNK3 KO mice after stroke.

Conclusion: Together with our prior work showing WNK3 KO mice are resistant to increased NKCC1 levels and phosphorylation in ischemia, these findings suggest that stroke pathology includes reduced GABAAR inhibition via both WNK3 regulation of NKCC1/KCC2 and altered GABAAR subtype expression. Blocking the WNK signaling pathway may present a therapeutic target for stroke.

Author Affiliation:
Department of Pharmacology and Chemical Biology

Lunch/Poster Session/Vendor Show / 18

PACS1-Syndrome patient cells contain aberrant microtubule-based structures which can be rescued by inactivation of HDAC6

Authors: Sabrina Villar-Pazos1; Laurel Thomas1; Troy C. Krzysiak2; Bradley M. Deitch1; Angela M. Gronenborn3; Gary Thomas1

1 Department of Microbiology and Molecular Genetics - University of Pittsburgh School of Medicine
2 Department of Structural Biology - University of Pittsburgh School of Medicine
3 Department of Structural Biology - University of Pittsburgh School of Medicine

PACS1 Syndrome is a recently identified neurodevelopmental disorder that is associated with a recurrent de novo missense mutation in PACS1 (p.Arg203Trp). Patients carrying the PACS1R203W mutation share an overlapping phenotype, including developmental delay, intellectual disability, seizures and characteristic craniofacial dysmorphisms. The mechanism by which PACS1R203W causes PACS1 Syndrome is unknown and there is no curative treatment. Leveraging PACS1 patient-derived fibroblasts and engineered isogenic cell lines, our studies suggest PACS1R203W dysregulates centrosome function, altering Golgi positioning, microtubule organization and integrity of the primary cilium. Biochemical studies suggest the Arg203>Trp disease substitution acts as a gain-of-function mutation that increases the interaction between PACS-1 and the deacetylase HDAC6, which is a key modulator of microtubule and centrosomal/ciliary function and is emerging as a key target to treat neurodevelopmental disorders. Consistent with these findings, HDAC6 inhibitors or siRNA knockdown restore Golgi positioning and stabilize primary cilia in PACS1R203W-expressing cells.

Author Affiliation:
Department of Microbiology and Molecular Genetics - University of Pittsburgh School of Medicine
Acute control of plasma membrane PtdIns(4,5)P2

Authors: Rachel Wills\textsuperscript{None}; James Zewe\textsuperscript{None}; Gerry Hammond\textsuperscript{None}

The inner leaflet of the plasma membrane (PM) is a bustling hub for cellular proteins that facilitate membrane traffic, signal transduction, cytoskeletal assembly and ion flux at the cell surface. Many of these proteins, including ones executing each of the previous functions, engage the PM lipid phosphatidylinositol(4,5)-bisphosphate [PtdIns(4,5)P2]; binding to this lipid recruits and/or activates these proteins. Therefore, harmonious PM function is contingent on tightly regulated control of PtdIns(4,5)P2 levels - and interrogation of these functions is greatly informed by the ability to acutely manipulate PtdIns(4,5)P2 levels. Whereas approaches to acutely deplete PtdIns(4,5)P2 have been available for over a decade, tools that permit acute increases in the lipid have been lacking. We found that expression of constitutive or acutely PM-recruited PI4P 5-kinases (PIP5K) lead to basally elevated PtdIns(4,5)P2 levels as well as depletion of the substrate for this enzyme, PtdIns4P. These alterations were present in the case of the recruitable PIP5K even prior to PM recruitment. We therefore developed a new acutely recruitable PIP5K, based on a mutant kinase domain unable to homodimerize with endogenous PIP5Ks. This enzyme does not effect basal lipid levels, but produces acute (~minutes) elevations in PM PtdIns(4,5)P2 and concomitant depletion of PM PtdIns4P. We are using this tool to evaluate how dynamic PM functions such as clathrin mediated endocytosis, calcium signaling, PI3K-signaling and cortical actin assembly are influenced by acute changes in these lipids. These results provide insights into how homeostasis of one crucial lipid impacts multiple aspects of cellular function.

Author Affiliation:

Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA