


THE GOOD, THE BAD AND THE UGLY CALCIUM

UNLOCKING THE MYSTERIES

alzheimer's • arrhythmia • autism • darier • deafness • diabetes
hailey-hailey • huntington's • infertility • mh • migraine • miyoshi
parkinson's • pkd • sds



ABSTRACTS OF PAPERS AT THE SIXTY-SECOND
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and

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Session 1: Pumps

1. The Plasma Membrane Calcium Pump in Health and Disease: Focus on Hereditary Deafness. **ERNESTO CARAFOLI**, *Venetian Institute of Molecular Medicine, University of Padova, 35129 Padova, Italy*

One of the distinctive properties of the calcium signal is ambivalence. When the control of the cellular homeostasis of calcium becomes defective, calcium becomes a conveyor of doom. Dysfunctions of the calcium signal may involve proteins that process it and/or proteins that transport calcium across membranes to buffer its concentration in cells (e.g., pumps). Genetic defects of the plasma membrane calcium pump (PMCA) produce hereditary deafness. Calcium plays an essential role in the hearing process. It enters the stereocilia of hair cells of the Corti organ through mechanoelectrical transduction (MET) channels opened by the deflection of the hair bundles and is exported back to the endolymph that bathes them by an unusual splicing isoform (*w/a*) of the PMCA2 pump. Precise control of the homeostasis of endolymph calcium is essential to operation of the MET channels. Because the PMCA2 pump is the only system that ejects calcium to the endolymph, it has a vital role in the control of its homeostasis. The *w/a* isoform carries inserts at site A in the N-terminal half of the pump structure and at site C in its C-terminal tail. The C insert induces premature truncation of the pump. Ablation or missense mutations of the pump cause deafness, as described for the first time in G283S mutation of the deafwaddler (*dfw*) mouse. In organotypic cultures, Ca^{2+} imaging of vestibular hair cells has shown that the dissipation of stereociliary Ca^{2+} transients induced by Ca^{2+} uncaging was compromised in the *dfw* (and PMCA2 KO) mice. Novel deafness-inducing mutations have been identified in two human families. That identified in our laboratory (G293S) was close to the site of the *dfw* mutation. The wild-type PMCA2 *w/a* isoform and its G283S and G293S mutants were overexpressed in CHO cells. The other splice variants of PMCA2 (*w/b*, *z/a*, and *z/b*) were also expressed as controls. Recombinant aequorin was used to monitor Ca^{2+} . At variance with the other PMCA2 isoforms, the *w/a* variant reacted poorly to the arrival of a Ca^{2+} pulse induced in CHO cells by InsP_3 . The G293S and G283S mutations did not further reduce the poor ability of the *w/a* variant to become rapidly activated but delayed the longer term dissipation of the Ca^{2+} transients, compromising the long-term nonactivated export of calcium from the stereocilia and thus its homeostasis in the endolymph.

A digenic mechanism was operational in the G293S human family case. The family was screened for mutations in cadherin 23, which had been shown to accentu-

ate hearing loss in the other human family with a PMCA2 mutation. A T1999S substitution was detected in the cadherin 23 gene of the healthy father and affected son but not in that of the unaffected mother, who instead presented the PMCA2 mutation.

2. Unique Gating Properties of CLC Anion Channel Splice Variants are Determined by Altered CBS Domain Secondary Structure. **SONYA DAVE**,^{1,2} **JONATHAN SHEEHAN**,³ and **KEVIN STRANGE**,^{1,2} ¹*Department of Anesthesiology*, ²*Department of Molecular Physiology and Biophysics*, and ³*Center for Structural Biology, Vanderbilt University Medical Center, Nashville, TN 37232*

Eukaryotic CLCs are homodimeric proteins with large cytoplasmic C termini containing two cystathionine- β -synthase (CBS) domains. The role played by CBS domains in CLC channel structure and gating is unclear. *clh-3* encodes two splice variants, CLH-3a and CLH-3b, of a *Caenorhabditis elegans* CLC channel that exhibit striking differences in gating kinetics and sensitivity to depolarizing voltages, pH and Cl^- . Mutagenesis studies have shown that the C terminus of CLH-3b gives rise to its unique gating properties. Splice variation of the C terminus includes the last six amino acids in the second CBS domain (CBS2). Mutating these amino acids in CLH-3b to those present in CLH-3a gives rise to CLH-3a gating properties. Similarly, CLH-3b gating properties are induced in CLH-3a by mutating the last six amino acids of CBS2 to those present in CLH-3b. To define how these amino acids alter CBS2 structure, we developed homology models based on crystal structures of C-terminal cytoplasmic domains in vertebrate CLCs using Modeller and Rosetta software. CBS domains have a highly conserved secondary structure consisting of an N-terminal β sheet ($\beta 1$) followed by an α helix ($\alpha 1$), two β sheets ($\beta 2$ and $\beta 3$), and an α helix ($\alpha 2$). Our models show that the six-amino acid splice variation is part of $\alpha 2$. Measuring the length of this helix in 10,000 candidate structures generated by Rosetta demonstrated that the splice variation has a higher probability of forming a helical turn in CLH-3b. Thus, $\alpha 2$ of CBS2 in CLH-3a is predicted to be shorter compared with that of CLH-3b. The model also suggests that the length of $\alpha 2$ could alter an interface with either a transmembrane α helix that comprises part of the channel pore and/or the interface between cytoplasmic C-terminal monomers. We are currently carrying out modeling and mutagenesis studies to determine how CBS2 splice variation alters these interfaces and channel gating.

3. The Dopamine Transporter as a Possible Mediator of Extrasynaptic Transmitter Release. **MARIA DEL PILAR GOMEZ**,^{1,2} **JUAN MANUEL ANGUEYRA**,^{1,2}

ISABELLE MINTZ,³ and ENRICO NASI,^{1,2} ¹*Universidad Nacional de Colombia, Bogota, Colombia;* ²*Marine Biological Laboratory, Woods Hole, MA 02543;* ³*Northwestern University, Evanston, IL 60208*

The canonical mode of chemical communication among nerve cells is vesicular release of the neurotransmitter (NT); however, scattered reports suggest alternative mechanisms operating in a Ca-independent manner in regions that are devoid of NT-filled vesicles. This study intended to develop an experimental model to test the proposition that the release of dopamine reported in the pars compacta of the Substantia nigra (SN) of rat brain, which is comprised only of somata and proximal dendrites, may be mediated by reverse-mode operation of the dopamine transporter (DAT). The task requires the use of isolated cells in which localization of the required machinery can be ascertained, and the release of NT can be monitored under conditions that exclude conventional exocytosis. We established by Western blot analysis that DAT expresses massively in the SN; this is essential if DAT functions effectively as a release mechanism, compensating for the sluggishness of its transport cycle. Second, we confirmed by immunocytochemistry that the DAT is present in the membrane of the soma and proximal dendrites of SN neurons. Next, we turned to the conditions that would promote reversal of the transport cycle: localized increase in $[Na^+]_i$ and sustained depolarization. We used confocal microscopy on dual-labeled cells with anti-DAT Abs as well as monoclonal Abs against voltage-dependent sodium channels. We observed that DAT and Nav 1.2 colocalize. Moreover, current-clamp recordings indicate the presence of plateau potentials providing the sustained depolarization that may be needed, together with $\Delta[Na^+]_i$, to drive a reversal of DAT transport. Finally, amperometric measurements on voltage-clamped SN cells show oxidation currents that are graded with depolarization and attenuated by GBR-12395, a specific antagonist of DAT. These preliminary observations lend credence to the conjecture that this transporter may indeed contribute to the release of dopamine in the pars compacta of the Substantia nigra. (Supported by National Institutes of Health [National Institute on Drug Abuse] grant RO1 DA016298.)

4. Examining the Ancient Phototransduction Mechanisms in a Primitive Chordate. MARÍA DEL PILAR GOMEZ,^{1,2} JUAN MANUEL ANGUEYRA,^{1,2} and ENRICO NASI,^{1,2} ¹*Universidad Nacional de Colombia, Bogota, Colombia;* ²*Marine Biological Laboratory, Woods Hole, MA 02543*

Two lineages of photoreceptors underlie spatial vision in different organisms: microvillar, which is typical of invertebrates, and ciliary, which is like rods and cones. Additional photosensitive cells have recently been identified among the ganglion cells of mammalian retina; these mediate nonvisual light sensitivity, which is crucial

for the regulation of circadian rhythms, the pupillary reflex, and the modulation of melatonin secretion. A novel photopigment, melanopsin, underlies these photoresponses, and current evidence points to the possibility that its transduction mechanisms may be akin to the lipid signaling scheme of microvillar receptors rather than the cyclic-nucleotide cascade of rods and cones. However, rapid progress toward rigorously testing this conjecture has been hindered by the difficulty of subjecting melanopsin-expressing cells to physiological analysis because of their extreme scarcity (<1% of retinal ganglion cells). Recently, it has been discovered that melanopsin has an ancient origin in the vertebrates and expresses in two morphologically distinct classes of cells (pigmented ocelli and Joseph cells) scattered along the neural tube of a primitive prechordate, the *Amphioxus*. Should these be bona fide photoreceptors, this organism could constitute an appealing model to investigate melanopsin-mediated light transduction. By microdissection and enzymatic dissociation of the neural tube of *Amphioxus*, we obtained morphologically intact isolated cells representative of both cell types and used patch-electrode recording to determine that they are indeed primary photoreceptors. The spectral sensitivity is reminiscent of that of melanopsin-expressing mammalian ganglion cells. Both cell types produce a transient depolarizing receptor potential; under voltage clamp, the photocurrent strongly rectifies in the inward direction and does not revert at potentials up to 100 mV. Manipulations of extracellular calcium and of cytosolic Ca-buffering capacity alter the photocurrent in a manner that parallels the effect of calcium on the light response of invertebrate microvillar receptors, providing preliminary supporting evidence for a kinship between the two phototransduction cascades.

5. Smelling Light: a Novel Olfactory-like Phototransduction Cascade. MARÍA DEL PILAR GOMEZ,^{1,2,3} FRANCISCA SILVA,² JUAN FELIPE DIAZ,^{1,3} and ENRICO NASI,^{1,3} ¹*Universidad Nacional de Colombia, Bogota, Colombia;* ²*Boston University School of Medicine, Boston, MA 02118;* ³*Marine Biological Laboratory, Woods Hole, MA 02543*

The distal photoreceptors of mollusk *Pecten irradians* functionally resemble those of vertebrate rods and cones in that (1) the light-sensing structures are modified cilia, (2) the receptor potential is hyperpolarizing, and (3) cGMP operates as an internal messenger. However, key properties of these cells diverge from those of their vertebrate counterparts: (1) they do not express transducin, (2) the light-induced changes in cGMP reflect regulation of its synthesis rather than hydrolysis, and (3) the receptor potential is caused by an increase in a K-selective conductance. We obtained a full-length clone of the α subunit of a G_o from *Pecten* retina cDNA and localized it to the distal retina by in situ hybridization;

functional data support its involvement in the photoresponse. A β subunit was also identified. A novel guanylate cyclase (GC) mediates downstream effects of light: the photoresponse is insensitive to manipulations of NO (ruling out a soluble GC) and of $[Ca^{2+}]_i$ (unlike rod/cone GCs); moreover, regulation by a G-protein is not contemplated by either canonical GC class. Western blots with antibodies raised against the nonconventional 12-TMD GC of *Paramecium* detected a band of the appropriate molecular mass (242 kD); such GCs have been reported to be regulated by G_o in *Dictyostelium*. PCR amplifications yielded the sequence of two catalytic domains. The last element of the phototransduction cascade of *Pecten*, the light-dependent ion channel, represents the first documented functional link between cyclic-nucleotide-dependent channels and K-selective channels, two ancestrally related protein families. Antibodies raised against the olfactory cyclic nucleotide-gated channel CNG2 but not anti-CNG1 or anti-CNG3 recognize a protein of ~ 73 kD in Western blots and selectively stain the distal retina, localizing primarily in the ciliary appendages of the hyperpolarizing photoreceptors, as revealed by confocal fluorescence microscopy. A novel cascade is proposed to underlie light transduction, which parallels that of olfactory receptor cells.

6. Identification of Ste20 Kinase Regulatory Phosphorylation Sites in a Cell Cycle- and Cell Volume-sensitive Clc Anion Channel. REBECCA A. FALIN,^{1,2,3} REBECCA MORRISON,^{1,2,3} AMY-JOAN L. HAM,⁴ and KEVIN STRANGE,^{1,2,3} ¹Department of Anesthesiology, ²Department of Pharmacology, ³Department of Molecular Physiology and Biophysics, and ⁴Mass Spectrometry Research Center, Department of Biochemistry, Vanderbilt University Medical Center, Nashville, TN 37232

Members of the ClC superfamily of transport proteins function as anion channels and Cl^-/H^+ exchangers in plasma and intracellular organelle membranes and play key roles in diverse and fundamental physiological processes. Despite intensive study, little is known about how ClCs are regulated. *clh-3* encodes two splice variants, CLH-3a and CLH-3b, of a *Caenorhabditis elegans* voltage-gated ClC Cl^- channel. CLH-3b is expressed in the worm oocyte and is activated during meiotic cell cycle progression or in response to cell swelling. Channel activation is brought about by serine/threonine dephosphorylation mediated by the type 1 phosphatases GLC-7 α and GLC-7 β . GCK-3 is a Ste20 kinase and homologue of mammalian PASK/SPAK, which regulates cell volume-sensitive cation-coupled Cl^- cotransporters. GCK-3 binds to a 101-amino acid C-terminal splice insert unique to CLH-3b. Knockdown of GCK-3 in worm oocytes constitutively activates CLH-3b. When coexpressed in HEK293 cells with CLH-3b, GCK-3 dramatically inhibits channel activity and alters gating kinetics and voltage sensitivity. Cell swelling reverses the effects

of kinase inhibition. Using mass spectrometry, we identified two serine residues, S742 and S747, which are phosphorylated when CLH-3b is coexpressed with GCK-3. These residues conform to a recently identified Ste20 kinase phosphorylation motif. GCK-3-mediated channel inhibition is prevented when either residue is mutated to alanine. When S742 and S747 are mutated individually to the phosphomimetic amino acids aspartate or glutamate, channel activity is normal and fully inhibited by GCK-3. Unlike the wild-type channel, cell swelling did not reverse this inhibition. Mutation of both S742 and S747 to aspartate or glutamate constitutively inhibits CLH-3b and renders it insensitive to GCK-3 activity. We conclude that both S742 and S747 must be phosphorylated for inhibition of CLH-3b and that both residues must be dephosphorylated for channel activation in response to cell swelling.

7. Rostafuroxin Antagonizes Adducin-mediated Activation of Na-KATPase-Src Signaling Pathway in Congenic Hypertensive Rats. M. FERRANDI,¹ I. MOLINARI,¹ M.P. RASTALDI,² P. FERRARI,¹ and G. BIANCHI,³ ¹Praxis Research Institute, Sigma-Tau, 20019 Settimo Milanese, Milan, Italy; ²Fondazione D'Amico per la Ricerca sulle Malattie Renali, San Carlo Hospital, 20122 Milan, Italy; ³San Raffaele Hospital, Vita-Salute San Raffaele University, 20132 Milan, Italy

Adducin mutations are associated with genetic hypertension in humans and Milan hypertensive rats (MHS). Mutated Adducin affects actin cytoskeleton organization and increases renal Na-K pump activity in MHS rats and in transfected cells. In congenic rats (NA) having the mutated α -adducin locus introgressed into the normotensive MNS background, hypertension was associated with activation of the Na-KATPase-Src-EGFr- and α v-integrin-dependent signaling pathways within renal caveolae. Treatment of NA rats with the antihypertensive compound Rostafuroxin (100 μ g/kg os) normalized blood pressure and the enhanced expression of Na-KATPase subunits, Src-Tyr⁴¹⁸, EGFr, and integrins, as demonstrated by immunofluorescence analysis on renal tissues and Western blotting on renal caveolae. In a cell-free system, human recombinant adducin dose-dependently activated Src kinase Tyr⁴¹⁸ phosphorylation, with the mutated variant displaying a higher apparent affinity than the wild type (46 ± 3.8 vs. 86 ± 5.7 nM; $P < 0.01$). In turn, Src-dependent Tyr phosphorylation of the mutated adducin in the absence or presence of Na-KATPase was higher than the wild-type adducin (35%; $P < 0.05$). Src-SH₂ and SH₃ domain-GST pull-down and competition experiments with Src-SH₂ peptides showed that mutated adducin preferentially associated to Src-SH₂ rather than Src-SH₃ domain (85 vs. 35%), whereas the wild type equally bound to Src domains (50%). Both adducins did not interact with the Src-kinase domain. 10^{-11} M Rostafuroxin selectively reduced the Src-Tyr⁴¹⁸,

adducin-, and Na-KATPase-Tyr phosphorylation (-30% ; $P < 0.05$) induced by the mutated, but not wild-type, adducin. This effect occurred at a site within the phospho-Tyr-binding pocket of Src-SH₂ domain, where the mutated, but not the wild-type, adducin binds.

These findings provide a molecular explanation for the adducin-mediated activation of signaling pathways associated with hypertension and related organ complications and shed light on the molecular mechanism of Rostafuroxin antihypertensive effects.

8. Disruption of Plasma Membrane Calcium ATPase Isoform 2 (PMCA2) Alters Glutamate-evoked Calcium Transients in Enriched Cultures of Purkinje Cells. AMANDA K. LEE,¹ LARRY D. GASPER,² ANDREW P. THOMAS,² and STELLA ELKABES,¹ ¹*Department of Neurology and Neuroscience and* ²*Department of Pharmacology and Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07101*

The cerebellum plays critical roles in motor coordination, learning, and certain cognitive functions. Cerebellar deficits are observed in several neurological disorders, including ataxia, multiple sclerosis, and autism spectrum disorders. Purkinje cells (PC), the output neurons of the cerebellar cortex, receive glutamatergic inputs that signal through metabotropic glutamate receptor 1 (mGluR1) and AMPA receptors (AMPA-R). Both receptors are important in cerebellar development, function, and plasticity. PCs also highly express a neuronal Ca²⁺ pump, PMCA2. Morphological, cellular, and molecular alterations in the cerebellum of PMCA2^{-/-} mice have been reported, emphasizing the importance of PMCA2 in the development of this region.

We have previously found that PMCA2 forms a complex with mGluR1, its downstream effectors, and AMPA-R subunits, GluR2/3 (Kurnellas, M.P., A.K. Lee, H. Li, L. Deng, D.J. Ehrlich, and S. Elkabes. 2007. *Mol. Cell Neurosci.* 34:178–188). The current studies investigated the role of PMCA2 and especially PMCA2 in the clearance of AMPA-R and mGluR1-mediated Ca²⁺ transients. Using enriched PC cultures, we found that carboxy eosin (CE), a PMCA inhibitor, increases the amplitude of AMPA-R-mediated Ca²⁺ responses and slows the decay of Ca²⁺ transients. In contrast, CE decreases the amplitude of mGluR1-mediated Ca²⁺ transients and accelerates the clearance rate. Interestingly, we observed a reduction in the amplitude of AMPA-R-mediated Ca²⁺ transients in PCs of PMCA2^{-/-} mouse cerebellum. This may be caused by developmental changes, including alterations in PC dendrites (Empson, R.M., M.L. Garside, and T. Knöpfel. 2007. *J. Neurosci.* 27:3753–3758), synapses, expression of AMPA-R, and endoplasmic reticulum Ca²⁺ homeostasis. In contrast, an increase in AMPA-R-mediated Ca²⁺ transients occurred in PCs of PMCA2^{+/-} mouse cerebellum, reminiscent of the aforementioned effects observed after inhibition of PMCA2

by CE. These data indicate that disruption of PMCA2 activity or expression differentially alters glutamate receptor-mediated Ca²⁺ signaling in cerebellar PCs. Future studies will determine how reductions in PMCA2 affect mGluR1 and AMPA-R-mediated responses in PCs and whether PMCA2 plays an essential role in PC pathology.

9. Development of an Intracellular cAMP “Sponge”. KONSTANTINOS LEFKIMMIATIS,^{1,2} JESSICA ROY,^{1,2} MEERA SRIKANTHAN,^{1,2} MARY PAT MOYER,³ SILVANA CURCI,^{1,2} and ALDEBARAN M. HOFER,^{1,2} ¹*VA Boston Healthcare System and* ²*Department of Surgery, Brigham and Women’s Hospital, Harvard Medical School, West Roxbury, MA 02132;* ³*NCELL Corporation LLC, San Antonio, TX 78249*

Cyclic AMP is a fundamental second messenger known for its ability to modulate a wide variety of cellular responses. How one simple signaling molecule is able to simultaneously control so many (sometimes contradictory) cellular functions is an extremely active area of investigation. The existence of spatially distinct cAMP microdomains has been invoked to explain this paradox, but do these signaling microdomains really exist? To determine the importance of these putative cAMP domains, it would be extremely useful to selectively perturb the cAMP concentration in situ, limiting its ability to activate localized effectors. This strategy has been successfully used for other soluble second messengers (“InsP3 sponge” for InsP3 and BAPTA for Ca²⁺). Here, we present the generation and validation of a novel buffer for cAMP based on the cAMP-binding domains of PKA regulatory subunit type Iβ (RIβ). To quantitatively assess the expression of our “cAMP sponge” construct at the single-cell level, we labeled it with the fluorescent protein mCherry. As measured by FRET-based sensors for cAMP, expression of sponge constructs targeted to cytoplasm, plasma membrane, and nucleus in NCM460 cells significantly reduced cAMP responses to 10 nM PGE₂, 10 nM VIP, and 5 μM forskolin. As expected, attenuation of agonist-induced cAMP signals also resulted in reduction in PKA activation as measured by the FRET-based sensor AKAR2. Interestingly, we found that the extra cAMP buffering power provided by the cAMP sponge can be compensated by second messenger derived from connected neighboring cells that do not express the exogenous buffer, revealing new aspects of gap junction-mediated communication. This new tool should also prove valuable for assessing the importance of cAMP microdomains in secretion, migration, and transcriptional regulation.

10. How the Sarcoplasmic Reticulum Ca²⁺-ATPase Pumps Ca²⁺ and is Inhibited by Thapsigargin. JESPER V. MØLLER,¹ CLAUS OLESEN,¹ MARTIN PICARD,²

PREBEN MORTH,² ANNE-MARIE L. WINTER,² POUL NISSEN,² SØREN B. CHRISTENSEN,³ and HELMER SØHOEL,³ ¹*Institute of Physiology and Biophysics and* ²*Department of Molecular Biology, PUMPKIN Research Center, Aarhus University, DK-8000 Aarhus C, Denmark;* ³*Department of Medicinal Chemistry, University of Copenhagen, DK-1165 Copenhagen K, Denmark*

The recent explosion of structures of sarcoplasmic reticulum (SR) Ca^{2+} -ATPase in various functional states, solved at atomic resolution by x-ray diffraction analysis of protein crystals, enables a description of the whole enzymatic transport cycle of this actively $\text{Ca}^{2+}/\text{H}^{+}$ -exchanging protein. We have recently analyzed three new structures (one E1P and two E2P) in detail (Olesen, C., M. Picard, A.M. Winther, C. Gyurup, J.P. Morth, C. Oxvig, J.V. Møller, and P. Nissen. 2007. *Nature*. 450: 1036–1042). They reveal how cytoplasmic Ca^{2+} after phosphorylation of Ca^{2+} -ATPase by ATP is occluded inside the membranous domain and released by opening of the compact 10-transmembrane domain into three lobes to expose the intramembranously bound Ca^{2+} toward the luminal space. This is consistent with a transport mechanism in which the Ca^{2+} -binding sites communicate either with the cytoplasm or with the SR lumen. The new E2P structures were obtained without stabilization by thapsigargin (TG). They show how TG, when bound to the Ca^{2+} -ATPase, is situated at the protein–lipid interphase in a preformed notch between transmembrane segments M3, M5, and M7, with minor adjustments of some of the amino acid side chains. They also provide hints to why TG inhibits the binding of Ca^{2+} . These structural data are useful in the attempt to synthesize new sesquiterpene prodrugs (Søhoel, H., A.M. Jensen, J.V. Møller, P. Nissen, S.R. Denmeade, J.T. Isaacs, C.E. Olsen, and S.B. Christensen. 2006. *Bioorg. Med. Chem.* 14:2810–2815) that can be targeted to produce apoptosis in prostatic cancer cells (Denmeade, S.R., C.M. Jakobsen, S. Janssen, S.R. Khan, E.S. Garrett, H. Lilja, S.B. Christensen, and J.T. Isaacs. 2003. *J. Natl. Cancer Inst.* 95:990–1000).

11. Secretory Pathway Stress as a Common Disease Mechanism in SERCA2 or SPCA1 Ca^{2+} Pump Deficiency. **GARY E. SHULL**, GBOLAHAN OKUNADE, MARIAN L. MILLER, and VIKRAM PRASAD, *Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45231*

Gene-targeting and human genetics studies show that heterozygous-null mutations in the genes encoding the SERCA2 (*Atp2a2*) or SPCA1 (*Atp2c1*) Ca^{2+} pumps, which are expressed in endoplasmic reticulum (ER) or Golgi apparatus, respectively, cause squamous cell tumors in mice and acantholytic skin disease (Darier Disease and Hailey-Hailey Disease) in humans. Despite species differences in disease manifestations, the similarities between the target cell (keratinocytes in both)

and phenotypic effects of the two Ca^{2+} pump deficiencies suggest a common disease mechanism. In the case of SERCA2 deficiency, the involvement of ER stress responses is a likely possibility. Loss of both copies of the SPCA1 gene in mice leads to embryonic death at gestation day 10.5; however, cardiovascular development and hematopoiesis, which are often associated with death at this stage, were normal. Apoptosis was increased in null mutant embryos, suggesting a general loss of cell viability. Membrane structures such as caveolae, junctional complexes, and desmosomes were normal, which is consistent with a functional secretory pathway; however, Golgi membranes were dilated, had fewer stacked leaflets, and were greatly expanded in amount. These studies of SPCA1-null embryos reveal clear evidence of Golgi stress, with both proapoptotic and prosurvival stress responses that are analogous to the well-known ER stress responses observed when SERCA2 activity is deficient. The available data support a model in which prosurvival responses to ER or Golgi stress predominate in mouse keratinocytes and lead to cancer, whereas proapoptotic responses predominate in humans and lead to the acantholytic skin diseases Darier Disease or Hailey-Hailey Disease.

Session 2: Cardiac and Skeletal Muscle

12. The α_{1S} III-IV Loop Influences DHPR Gating but is Not Directly Involved in Excitation–Contraction Coupling Interactions with RyR1. **ROGER A. BANNISTER** and **KURT G. BEAM**, *Department of Physiology and Biophysics, University of Colorado-Denver, Aurora, CO 80045*

In skeletal muscle, coupling between the dihydropyridine receptor (DHPR) and type 1 ryanodine receptor (RyR1) underlies excitation–contraction (EC) coupling. The III-IV loop of the DHPR α_{1S} subunit binds to a segment of RyR1 in vitro (Leong, P., and D.H. MacLennan. 1998. *J. Biol. Chem.* 273:29958–29964), and mutations in the III-IV loop have been linked to malignant hyperthermia (Monnier, N., V. Procaccio, P. Stieglitz, and J. Lunardi. 1997. *Am. J. Hum. Genet.* 60:1316–1325), raising the possibility that this loop is directly involved in signal transmission from the DHPR to RyR1. To clarify the role of the α_{1S} III-IV loop in EC coupling, we examined the functional properties of a chimera (GFP- α_{1S} [III-IVa]) in which the III-IV loop of the divergent α_{1A} isoform replaced that of α_{1S} . Dysgenic myotubes expressing GFP- α_{1S} [III-IVa] yielded myoplasmic Ca^{2+} transients that were ~65% smaller than those of GFP- α_{1S} and that displayed an ~10-mV hyperpolarizing shift in voltage dependence of activation. Relative to GFP- α_{1S} , L-type Ca^{2+} currents mediated by GFP- α_{1S} [III-IVa] were ~40% smaller and activated at ~5 mV less depolarized potentials. The altered gating of GFP- α_{1S} [III-IVa] was accentuated by exposure to \pm Bay K

8644, which caused a much larger hyperpolarizing shift in activation compared with its effect on GFP- α_{1S} . GFP- α_{1S} [III-IVa] channels appeared to traffic less efficiently to the membrane, as indicated by an $\sim 65\%$ reduction in voltage-dependent charge movements, which was similar in magnitude to the reduction of maximal Ca^{2+} transients. Collectively, our observations indicate that the α_{1S} III-IV loop is not directly involved in EC coupling but indirectly influences EC coupling by regulating DHPR gating.

13. Interplay of Calcium and Calmodulin Binding Motif in Regulation of Cardiac Sodium Channel Gating. SUBRATA BISWAS, DEBORAH DISILVESTRE, YANLI TIAN, VICTORIA L. HALPERIN, and GORDON F. TOMASELLI, *Department of Medicine, Division of Cardiology, Johns Hopkins University, Baltimore, MD 21287*

Intracellular Ca^{2+} can mediate bimodal regulation of the voltage-gated cardiac sodium channel ($\text{Na}_V1.5$) through its C terminus (CT) via the calmodulin (CaM)-binding IQ motif and the direct Ca^{2+} -binding EF hand-like (EFL) motif of the channel. Although the EFL and IQ motifs are at opposite ends of the proximal structured portion of the CT- $\text{Na}_V1.5$, mutations in either motif have been associated with arrhythmogenic changes in expressed $\text{Na}_V1.5$ currents. Ca^{2+} binding to $\text{Na}_V1.5$ shifts steady-state inactivation in the depolarizing direction and delays entry into inactivated states. Mutation of the four EFL residues E1788A, D1790A, D1792A, and E1799A ($\text{Na}_V1.5_{4X}$) stabilizes inactivation compared with the wild-type channel and abolishes the Ca^{2+} sensitivity of inactivation gating. Modulation of the steady-state availability of $\text{Na}_V1.5$ by intracellular Ca^{2+} is more pronounced after the truncation of CT proximal to the IQ motif ($\text{Na}_V1.5_{\Delta 1885}$), which retains the EFL. In contrast, CaM has no apparent effect on $\text{Na}_V1.5$ channel function at physiological $[\text{Ca}^{2+}]$, yet FRET between fluorophore-conjugated CaM and CT of intact $\text{Na}_V1.5$ channels demonstrate CaM tethering to channel, indicating a latent CaM regulation of the wild-type channel. Mutating the EFL unmasks CaM-mediated regulation of the kinetics and voltage dependence of inactivation. The latent CaM modulation of inactivation is eliminated by mutation of the IQ motif ($\text{Na}_V1.5_{4X\text{-IQ/AA}}$). Thus, Ca^{2+} binding to the EFL motif plays a critical role in controlling $\text{Na}_V1.5$ availability in the heart through direct binding of Ca^{2+} independent of any CaM-IQ interaction. Unlike other isoforms of the Na channel, the IQ-CaM interaction in the CT- $\text{Na}_V1.5$ is latent under physiological conditions but may become manifest in the presence of disease-causing mutations in the CT of $\text{Na}_V1.5$ (particularly in EFL), contributing to the production of potentially lethal ventricular arrhythmias. However, the concerted action of the two motifs finely tunes normal Na channel inactivation.

14. Calcium Transients Evoked by Electrical Stimulation are In Part Dependent on ATP Release and P2X/P2Y Receptor Activation in Skeletal Muscle Cells. SONJA BUVINIC,¹ JAVIERA LÓPEZ,² JUAN PABLO HUIDOBRO-TORO,² JORDI MOLGÓ,³ and ENRIQUE JAIMOVICH,¹ ¹*Centro de Estudios Moleculares de la Célula, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70005, Santiago, Chile;* ²*Centro de Regulación Celular y Patología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile;* ³*Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif sur Yvette, France*

ATP released to the extracellular medium by all cell types fulfill several physiological roles through activation of plasma membrane P2X (ion channels) or P2Y (metabotropic) receptors. Several subtypes of these receptors are differentially activated by ATP, UTP, or their metabolites (ADP and UDP) generated by ectonucleotidase activities. Skeletal muscle cells express several P2X and P2Y receptor subtypes, and ATP is profusely released during muscle activity. We previously demonstrated that in skeletal myotubes, depolarizing stimuli induces two calcium signals: a fast signal associated with excitation-contraction coupling and a slow signal that has an important component in the nucleus and regulates gene expression. Here, we propose that extracellular nucleotides released by electrical stimulation are in part responsible for intracellular calcium homeostasis. In rat skeletal myotubes, we demonstrated that a tetanic stimulus (45 Hz, 400 pulses, 1 msec each) rapidly increased extracellular levels of ATP, ADP, and AMP from 15 s to 3 min, with different half-life times. Exogenous ATP applications induced a dose-dependent increase in intracellular calcium with an EC_{50} value of $7.8 \pm 3.1 \mu\text{M}$. Exogenous ADP, UTP, and UDP also promote calcium transients. By RT-PCR, we detected mRNA expression for P2X₁₋₇ and P2Y_{1,2,4,6,11} in these cells. Both fast- and slow-calcium signals evoked by tetanic stimulation were partially inhibited by either 10–100 μM suramin (nonselective P2X/P2Y blocker) or 2 U/ml apyrase (nucleotidase that metabolizes ATP and ADP to AMP). In hemidiaphragm preparations, we demonstrated that apyrase reduces both twitch- and tetanus-evoked increase in tension. Our results suggest that nucleotides endogenously released during skeletal muscle activity act through P2X and P2Y receptors to modulate both calcium homeostasis and muscle physiology. (Supported by FONDAP 15010006, FONDAP 13980001, and Fondecyt 3080016.)

15. Maintenance of Muscle Cell Membrane Integrity and the Pathogenesis of Muscular Dystrophy. KEVIN P. CAMPBELL, *Department of Physiology and Biophysics, Roy J. and Lucille A. Carver College of Medicine, Howard Hughes Medical Institute, University of Iowa, Iowa City, IA 52242*

Muscular dystrophy is a group of inherited myogenic disorders characterized by progressive muscle weakness and wasting caused by muscle fiber necrosis. A large number of genes associated with various types of muscular dystrophies have been identified. Interestingly, 12 of these muscular dystrophic genes are related to the dystrophin–glycoprotein complex (DGC). This multi-subunit complex is present at the muscle sarcolemma and connects the basement membrane surrounding the muscle fiber to the intracellular cytoskeleton. The genes affected encode for integral proteins of the DGC, or extracellular matrix proteins that bind the DGC, or enzymes necessary for glycosylation of the central DGC component, dystroglycan. Emerging cell and molecular data on mouse models and patient biopsies strongly support the hypothesis that defects in dystroglycan are central to the pathogenesis of structural and functional brain abnormalities seen in congenital MD. Another class of muscular dystrophies in which repair, not structure, of the plasma membrane is abnormal is linked to mutations in dysferlin. The elucidation of the molecular pathogenesis of various forms of muscular dystrophy is providing new therapeutic strategies for the treatment of these diseases.

16. Effect of Adrenergic Stimulation on Resting $[Ca^{2+}]$ in the Sarcoplasmic Reticulum (SR) of Frog Skeletal Muscle Assessed with the Indicator Tetramethylmurexide (TMX). GABOR GYURKOVICS and PAUL C. PAPE, *Université de Sherbrooke Faculté de Médecine et des Sciences de la Santé, Département de Physiologie et Biophysique, Sherbrooke, Québec J1H5N4, Canada* (Sponsor: Paul C. Pape)

Several past studies indicated that adrenergic stimulation enhances the directly stimulated twitch response of fast-twitch skeletal muscle. Arreola et al. (Arreola, J., J. Calvo, M.C. García, and J.A. Sánchez. 1987. *J. Physiol.* 393:307–330) reported that the potentiation required a train of closely spaced action potentials (aps) and attributed this requirement to the build up of voltage activation of L-type Ca channels during the train of aps. In contrast to this idea, other researchers report that adrenaline greatly enhances the first twitch response after adrenergic stimulation, which suggests that the enhanced Ca entry occurred in the resting state (Gonzalez-Serratos, H., L. Hill, and R. Valle-Aguilera. 1981. *J. Physiol.* 315:267–282). To resolve this issue, we evaluated the effect of adrenergic stimulation (10 μ M isoproterenol) on the fraction of the Ca indicator tetramethylmurexide (TMX) complexed with Ca (denoted f_{Ca}), an indication of $[Ca^{2+}]$ in the SR and, less directly, the total Ca content in the fiber (Pape, P.C., K. Fénelon, C.R. Lamboley, and D. Stachura. 2007. *J. Physiol.* 581.1:319–367). These experiments were done on cut fibers from frog mounted in a double-Vaseline gap chamber and held at the normal resting potential of -90 mV. For the main set of results, the internal solu-

tion was nominally physiological (high K-glutamate) with 0.1 mM EGTA and no added Ca. The external solution was normal Ringers. Before the addition of isoproterenol to the external solution, f_{Ca} had a maintained, steady value of 0.08 (SEM = 0.02; $n = 8$). With an exponential delay (τ) after the start of isoproterenol exposure ($\tau = 7.07$ min; SEM = 0.55 min), f_{Ca} progressively increased, reaching a sustained rate of increase of 0.0039/min (SEM = 8). Isoproterenol had no effect on f_{Ca} with no Ca in the external solution. These results indicate that adrenergic stimulation causes a significant net entry of Ca in resting muscle.

17. Mitochondrial Calcium Signaling in Skeletal Muscle Disease. GYÖRGY HAJNÓCZKY, CHRISTOPHER J. BUZAS, MUQING YI, PAL PACHER, and HENRY ROSENBERG, *Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107*

Calcium oscillations exert physiological control on mitochondrial energy metabolism but also induce mitochondrial membrane permeabilization and ensuing cell death. The specificity of the mitochondrial calcium signaling is dependent on the coincident presence of stress factors like reactive oxygen species (ROS), ceramide, and on the amount and spatiotemporal pattern of the Ca^{2+} transfer to the mitochondria. The latter factor is coming into focus based on recent evidence revealing that many human cardiac/skeletal muscle and neurodegenerative diseases are associated with mutations of the intracellular Ca^{2+} release channels and their regulatory proteins and present early mitochondrial impairments. Sensitization of IP3R/RyR-mediated Ca^{2+} release is caused by a variety of mutations associated with malignant hyperthermia (MH)/central core disease, idiopathic paroxysmal ventricular tachycardia, and Huntington disease. However, dependence of the mitochondrial injury on the altered Ca^{2+} mobilization remains elusive. To test the hypothesis that mutations promoting Ca^{2+} release through RyR1 may elicit mitochondrial Ca^{2+} overload and make mitochondria vulnerable to membrane permeabilization, we obtained primary skeletal myoblasts from MH-negative (MHN) and malignant hyperthermia susceptible (MHS; e.g., RyR1 A2347V) individuals and investigated the effect of halothane, a toxic anesthetic for MH patients, on mitochondrial morphology and function. Imaging of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) and mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) showed that the halothane-induced $[Ca^{2+}]_c$ signal is propagated to the mitochondria, giving rise to a large $[Ca^{2+}]_m$ elevation. The halothane-induced $[Ca^{2+}]_c$ signal was also associated with a decrease in mitochondrial membrane potential and in NAD(P)H fluorescence. In a side-by-side comparison of MHN and MHS myotubes, accentuated halothane-induced depolarization was observed in the MHS cells. After

permeabilization of the plasma membrane, both halothane and Ca^{2+} pulses evoked a relatively large mitochondrial depolarization in MHS cells. This was associated with release of cytochrome c from mitochondria to the cytosol, activating a cell killing mechanism. Thus, the halothane-induced sarcoplasmic reticulum Ca^{2+} mobilization is effectively propagated to the mitochondria. In MHS cells, the enhanced Ca^{2+} mobilization may provide a mechanism to damage the mitochondria. Furthermore, halothane may also directly target a mitochondrial factor that synergizes with $[\text{Ca}^{2+}]_m$ to cause impairment of the mitochondrial membrane barrier and metabolic function.

18. The Total Ca Contents of Skeletal Muscle from Frogs and Rats – Implications for the Ca-binding Capacity of Calsequestrin. CÉDRIC R.H. LAMBOLEY and PAUL C. PAPE, *Université de Sherbrooke Faculté de Médecine et des Sciences de la Santé, Département de Physiologie et Biophysique, Sherbrooke, Québec J1H5N4, Canada* (Sponsor: Cedric Lamboley)

Results in Table III of Pape et al. (Pape, P.C., K. Fénelon, C.R. Lamboley, and D. Stachura. 2007. *J. Physiol.* 581.1:319–367) indicate that >90% of the total Ca in the sarcoplasmic reticulum (SR) is complexed to the high capacity Ca-binding protein calsequestrin in resting fast-twitch muscle of frog. In stark contrast to this result, Royer et al. (Royer, L., S. Pouvreau, Y. Wang, G. Meissner, J. Zhou, A. Nori, P. Volpe, J.W. Bain, D.A. Riley, R. Fitts, and E. Rios. 2008. *Biophys. J.* 94:2685) recently reported no significant changes in total releasable Ca when calsequestrin (CSQ1) is knocked down in fast-twitch skeletal muscle of mice. To resolve this issue, we developed a quantitative method for measuring the total amount of Ca in a muscle (or any other tissue, for that matter) using the Ca-dependent UV absorbance properties of the high-affinity Ca buffer BAPTA. In brief, whole fast-twitch muscle (*ileo-fibularis* from frog and *EDL* from rat) were placed in a Ringers (frog) or Krebs solution (rat) with 0 Ca and 0.1 mM EGTA for ~1.5 h to remove external Ca. The muscle was then added to a solution containing 0.1 mM BAPTA and homogenized with a blender. After removal and weighing of small pieces of tendon, an additional 0.1-mM BAPTA solution was added to give a total volume of 50 ml. 1% Triton X-100 was added to permeabilize membranes. Total muscle Ca ($[\text{Ca}_T]$; given in units millimoles per 1,000 grams of wet weight of muscle, less the tendon weight) was determined from absorbances at 306 nm of the supernatant and the supernatant with three levels of Ca: 0Ca, ∞ Ca, and with addition of a known Ca standard. In frog, the average value for $[\text{Ca}_T]$ of 1.89 (SEM = 0.1; $n = 5$) is very close (within 1%) to that expected from previous measurements in cut fibers (Pape, P.C., D.-S. Jong, and W.K. Chandler. 1995. *J. Gen. Physiol.* 106:259–336). In rat,

the average value of 3.3 (SEM = 0.2; $n = 3$) indicates that Ca binding to calsequestrin must be even more important in rat than in frog.

19. Heart Failure and Sudden Cardiac Death: Causes and Cures. **ANDREW R. MARKS**, *Department of Physiology and Cellular Biophysics, Clyde and Helen WU Center for Molecular Cardiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032*

Heart failure and sudden cardiac death are leading causes of mortality. Developing effective therapy has been hampered because of a lack of understanding of the mechanisms that cause these disorders. We have shown that “leaky” ryanodine receptor/calcium release channels (RyR2) contribute to heart failure and sudden cardiac death in humans and relevant animal models. RyR2 is the major calcium release channel on the sarcoplasmic reticulum and is required for excitation-contraction coupling. RyR2 becomes leaky in failing hearts as a result of PKA hyperphosphorylation of the channel that results from chronic activation of the “fight or flight” stress response. PKA hyperphosphorylation decreases the binding affinity of calstabin2, a subunit of the channel, rendering the channels leaky. Treatment with a novel 1,4 benzothiazepine derivative known as a rycal that enhances the affinity of calstabin2 for PKA-phosphorylated RyR2 improves cardiac function and prevents sudden cardiac death in animal models.

20. Calcium Dysfunction in Ankyrin-based Human Cardiac Arrhythmias. **PETER J. MOHLER**, *Department of Internal Medicine and Department of Physiology, University of Iowa Carver College of Medicine, Iowa City, IA 52242*

A number of serious human genetic diseases are caused by abnormal targeting and expression of ion channel and transporters at specialized membrane domains. Our research focuses on the molecular mechanisms underlying ion channel and transporter targeting in cardiac and other excitable cells. In particular, we are interested in the role of the membrane-associated ankyrin family of polypeptides in the targeting and function of ion channels and transporters. Our recent work establishes that loss-of-function mutation in ankyrin-B is the basis for a new human cardiac arrhythmia syndrome associated with bradycardia, abnormal heart rate variability, repolarization defects, and polymorphic tachyarrhythmia in response to stress and/or exercise. Additionally, our work revealed that reduction of ankyrin-B in mice results in reduced levels and abnormal localization of Na/Ca exchanger, Na/KATPase, and InsP3 receptor at transverse-tubule/sarcoplasmic reticulum sites in cardiomyocytes and leads to altered Ca^{2+} signaling and extrasystoles that provide a rationale for the arrhythmia. Current work is focused on two ankyrin-based areas of research directly related to human

disease. Specifically, we are interested in identifying the molecular determinants of ankyrin-B-dependent targeting of ion channels and transporters to specialized T tubule/SR membranes required for normal calcium homeostasis and cardiac function. Additionally, we are actively characterizing the role of ankyrin-G in targeting of voltage-gated sodium channel Nav1.5 (*SCN5A*) to excitable membranes of ventricular cardiomyocytes.

21. Cross Talk between Myostatin and IGF-1 Transduction Pathways Mediated by Calcium in Skeletal Muscle Cells. JUAN ANTONIO VALDÉS and ALFREDO MOLINA, *Departamento de Ciencias Biológicas, Facultad de Ciencias de la Salud, Universidad Andrés Bello, Santiago, Chile*

Skeletal muscle growth and development are very complex and regulated processes that begin during the early embryonic muscle myogenesis and continue throughout life during muscle regeneration and repair. The myogenic program involves several coordinated steps regulated by extracellular cues, like myostatin and insulin-like growth factor-1 (IGF-1). IGF-1 is a positive regulator in proliferation and differentiation of skeletal muscle cells, whereas myostatin is a member of the transforming growth factor β superfamily that acts as a negative regulator of skeletal muscle mass. These growth factors exert their antagonist functions, activating different transduction pathways mediated by their receptors. Myostatin has been shown to bind to Activin type II receptors, activating a signal transduction pathway that leads to phosphorylation of the transcription factors Smad2/3, which ultimately leads to suppression of myogenesis. On the other hand, almost all biological actions are mediated by binding to the IGF-1 receptor, activating transduction pathways like phosphoinositide 3-kinase (PI3K)–phospholipase C (PLC) that will induce inositol triphosphate (IP₃) production and subsequently calcium release from intracellular stores. However, experimental data obtained by our group strongly suggest a negative feedback through a cross talk between IGF-1 and myostatin receptors mediated by calcium: (1) myostatin inhibits calcium release induced by IGF-1 in C2C12 myoblast; (2) myostatin inhibits the activation of NFAT, a calcium-dependent transcription factor; (3) myostatin inhibits NFAT-dependent transcription during myoblast differentiation and fusion. Considering the fact that IGF-1 can induce calcium release from IP₃R intracellular stores mediated by the activation of PI3K-PLC, we propose that myostatin diminishes the PI3K or PLC activity, thereby inhibiting the IP₃-induced calcium release and subsequent activation of calcium-dependent transduction pathways. Actually, we are performing experiments to analyze the contribution of IP₃ pathways in the cross talk between myostatin and IGF-1.

Session 3: Neurodegeneration

22. REST-dependent Epigenetic Remodeling of AMPA Receptor Ca²⁺ Permeability is Critical to Ischemia-induced Neuronal Death. M.V.L. BENNETT, K.M. NOH, A. FOLLENZI, T. MIYAWAKI, J.M. GREALLY, and R.S. ZUKIN, *Albert Einstein College of Medicine, Bronx, NY 10461*

AMPA receptors (AMPArs) mediate fast synaptic transmission at excitatory synapses of the CNS and are crucial to neuronal development, synaptic plasticity, and structural remodeling. The subunit composition and Ca²⁺ permeability of AMPA receptors are not static but are dynamically remodeled in a cell- and synapse-specific manner. The precise mechanisms underlying the switch in AMPAR phenotype remain controversial. Here, we show that after a 10-min period of global ischemia, the transcriptional repressor REST acts via epigenetic modifications to repress GluR2 expression, leading to formation of GluR2-lacking Ca²⁺-permeable AMPARs by neurons in the adult hippocampal CA1 (but not CA3). Increased Ca²⁺ influx in response to glutamate released at excitatory synapses or by activated astrocytes leads to neuronal death specific to CA1. Global ischemia promotes assembly of the transcriptional repressor REST, CoREST, mSin3A, and HDACs (the REST corepressor complex) over the GluR2 promoter specifically in CA1 pyramidal neurons. The REST–corepressor complex promotes deacetylation of the core histone protein H3, dimethylation of H3 at lysine 9 (H3K9me₂) but not lysine 4 (H3K4), and association with MeCP2, an epigenetic signature of gene silencing. Inhibition of REST by lentiviral delivery of a recombinant mutant REST (REST-VP16) or of a REST RNA interference (RNAi) sequence into the hippocampus of rats before the ischemic insult rescues GluR2 expression and ameliorates hippocampal injury. These findings document a causal role for REST-dependent epigenetic remodeling of target genes that regulate AMPAR Ca²⁺ permeability in delayed neurodegeneration after ischemia and identify REST as a novel therapeutic target for ischemic stroke.

23. Src Family Kinases Link Death Receptors to the Apoptotic Calcium Machinery. DARREN BOEHNING, ASKAR AKIMZHANOV, and XINMIN WANG, *Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555*

Inositol 1,4,5-trisphosphate receptor (IP₃R) channels mediate apoptotic calcium release in response to both intrinsic and extrinsic cell death signals. We recently demonstrated that apoptotic calcium release mediated by the death receptor Fas is dependent upon phospholipase C (PLC)– γ activity and subsequent IP₃R-mediated calcium release. However, the mechanism by which Fas receptor is coupled to PLC- γ activation is unknown.

Here, we show that Fas receptor ligation in T cells is associated with the recruitment and activation of src family kinases canonically associated with T cell receptor engagement. Genetic inactivation of Lck and Zap70 eliminated PLC- γ phosphorylation, activation, and subsequent calcium release. These effects could be rescued by stably overexpressing the respective kinases. Recruitment and activation of Lck and Zap70 after Fas receptor clustering was dependent on segregation of these signaling components to lipid rafts, where they combined with the canonical components of the death-induced signaling complex (DISC). These results reveal the surprising finding that Fas receptor signaling mimics T cell receptor signaling and uncovers a novel pathway that is absolutely required for apoptotic calcium release and cell death after death receptor activation.

24. Absence of InsP₃R Ca²⁺ Signals Induce AMPK-dependent Prosurvival Autophagy. CESAR CARDENAS, KING-HO CHEUNG, JUN YANG, HORIA VAIS, and KEVIN FOSKETT, *Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104*

Inositol trisphosphate receptor (InsP₃R) Ca²⁺ release channels are ubiquitously expressed as three different isoforms. Knockdown of InsP₃R expression induced autophagy, a conserved cell survival mechanism that may also promote cell death (Criollo, A., M.C. Maiuri, E. Tasdemir, I. Vitale, A.A. Fiebig, D. Andrews, J. Molgó, J. Díaz, S. Lavandero, F. Harper, et al. 2007. *Cell Death Differ.* 14:1029–1039). Alternately, InsP₃R-mediated Ca²⁺ release was shown to induce autophagy (Høyer-Hansen, M., L. Bastholm, P. Szyniarowski, M. Campanella, G. Szabadkai, T. Farkas, K. Bianchi, N. Fehrenbacher, F. Elling, R. Rizzuto, et al. 2007. *Mol. Cell.* 25:193–205). In normal growth medium, a significantly higher percentage of chicken DT40 B cells genetically deficient in all InsP₃R isoforms (KO) were autophagic compared with InsP₃R-expressing WT cells as measured by electron microscopy, LC3-II formation, and LC3-GFP localization. Viability was not different between the two lines in normal growth medium, but KO cells were resistant to nutrient depletion-induced cell death. Pharmacologic inhibition of autophagy was without effect on WT cell survival in normal growth medium, whereas it caused the death of KO cells, and it eliminated enhanced resistance of KO cells to nutrient deprivation, indicating that autophagy becomes activated as a prosurvival mechanism in the absence of InsP₃R expression. Autophagy was induced specifically by loss of InsP₃R because expression of rat InsP₃R-3 in KO cells reduced autophagy to control levels. Ca²⁺ release through InsP₃R was required because blocking InsP₃R with Xestospongin B induced autophagy, and expression of permeation and gating-deficient InsP₃R failed to reduce autophagy in KO cells. Suppression of autophagy was specific for InsP₃R because expression of RyR failed to reduce au-

tophagy in KO cells. The metabolic sensor kinase AMPK was activated in KO cells, although phosphorylation of mTOR was unchanged. Inhibition of AMPK with compound C or addition methyl-pyruvate reduced autophagy. We conclude that InsP₃R-mediated Ca²⁺ signaling is fundamentally required to preserve optimal bioenergetic status in cells. Its absence triggers a compensatory prosurvival autophagy mediated by AMPK and unidentified downstream effectors that do not include mTOR.

25. Deviant Ryanodine Receptor-mediated Calcium Release Alters Synaptic Activity in Presymptomatic Alzheimer's Disease Mice. SHREAYA CHAKROBORTY, IVAN GOUSSAKOV, MEGAN MILLER, and BETH STUTZMANN, *Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064*

ER calcium signaling is critical for many neuronal signaling events, including modulation of synaptically evoked responses and long-term plasticity. In neurons expressing Alzheimer's disease (AD)-linked presenilin (PS) mutations, ER calcium dysregulation occurs early in the disease process, before the onset of histopathology and memory deficits. Neuronal compartments associated with dense synaptic contacts, such as dendritic spine heads and distal processes, show particularly high relative increases in RyR-sensitive calcium release compared with nontransgenic mice. However, it is largely unknown how synaptic transmission and plasticity mechanisms are affected by exaggerated ER calcium release.

In this study, ryanodine (RyR)-mediated alterations in calcium signaling and synaptic activity were examined in cortical and hippocampal neurons from young (6–8 wk old) triple transgenic (3xTg-AD) and nontransgenic (NonTg) control mice. Using two-photon calcium imaging and electrophysiological techniques, we demonstrate greatly exaggerated (>10-fold) calcium signals in dendritic spine heads and dendritic processes of pyramidal neurons from the AD mice relative to NonTgs. Under control conditions, membrane electrical properties, basal synaptic transmission, and plasticity mechanisms appear similar between the mouse strains. However, manipulating the RyR-calcium stores reveals remarkably different patterns of calcium signaling underlying these synaptic and membrane properties. Blocking the RyR with dantrolene has little effect on synaptic transmission and plasticity in the NonTg animals, yet it increases paired-pulse facilitation and synaptic strength and converts LTP expression to LTD in the 3xTg-AD mice. Activation of RyR by caffeine also alters synaptic transmission differently in AD mice compared with NonTgs. Synaptic stimulation concurrent with RyR activation reveals significantly increased calcium release in spine heads and dendrites in 3xTg-AD neurons. These findings demonstrate that 3xTg-AD

pyramidal neurons operate under an aberrant yet seemingly functional calcium signaling and synaptic transmission system long before AD histopathology onset. These early signaling alterations may underlie the later synaptic breakdown and cognitive deficits characteristic of AD.

26. Mechanism of Calcium Disruption in Alzheimer's Disease by Presenilin Regulation of InsP₃ Receptor Channel Gating. KING-HO CHEUNG,¹ DIANA SHINEMAN,^{2,4} MARIOLY MULLER,¹ CESAR CARDENAS,³ LIJUAN MEI,¹ JUN YANG,¹ TAISUKE TOMITA,⁵ TAKESHI IWASUBO,⁵ VIRGINIA M.-Y. LEE,^{2,4} and J. KEVIN FOSKETT,^{1,3} ¹*Department of Physiology,* ²*Department of Pathology and Laboratory Medicine,* ³*Department of Cell and Developmental Biology,* and ⁴*Center for Neurodegenerative Disease Research, University of Pennsylvania, Philadelphia, PA 19104;* ⁵*Department of Neuropathology and Neuroscience, University of Tokyo, Tokyo 113-0033, Japan*

Mutations in presenilins (PS) are the major cause of familial Alzheimer's disease (FAD) and have been associated with calcium (Ca²⁺) signaling abnormalities. Recently, it was proposed that PS may contain an intrinsic Ca²⁺ and monovalent ion permeability that is impaired in FAD-associated mutant PS (Tu, H., O. Nelson, A. Bezprozvanny, Z. Wang, S.F. Lee, Y.H. Hao, L. Serneels, B. De Strooper, G. Yu, and I. Bezprozvanny. 2006. *Cell*. 126:981–993). Here, using a different electrophysiological technique, nuclear patch clamping, we have been unable to verify that either wild-type (WT) or mutant PS form ion channels in native ER membranes. However, we have identified a molecular interaction of WT and FAD mutant PS with the inositol 1,4,5-trisphosphate receptor (InsP₃R) Ca²⁺ release channel. Both WT and FAD mutant PS1 (M146L) and PS2 (N141I) interact biochemically with the InsP₃R, as evidenced by coimmunoprecipitation using cell culture and brain lysates. In two model cell systems, nuclear patch clamp electrophysiology revealed that FAD mutant PS1 (M146L) and PS2 (N141I) exert profound stimulatory effects on InsP₃R channel gating activity in response to both saturating and suboptimal levels of InsP₃. The stimulatory activity results primarily from destabilization of the channel closed state. These functional interactions result in InsP₃R-dependent exaggerated cellular Ca²⁺ signaling in FAD PS-expressing cells in response to agonist stimulation as well as enhanced low level Ca²⁺ signaling in unstimulated cells. Parallel studies in InsP₃R-expressing and -deficient cells revealed that enhanced Ca²⁺ release from the endoplasmic reticulum as a result of the specific interaction of PS1-M146L with the InsP₃R stimulates amyloid β processing, an important feature of AD pathology. These observations provide molecular insights into the Ca²⁺ dysregulation hypothesis of AD pathogenesis and suggest novel targets for therapeutic intervention.

27. Bcl-2 Interacts with the InsP₃R to Increase Ca²⁺ Oscillations and Antioxidant Capacity. EMILY ECKENRODE,³ LIJUAN MEI,¹ J. KEVIN FOSKETT,^{1,2} and CARL WHITE,³ ¹*Department of Physiology and* ²*Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104;* ³*Department of Physiology and Biophysics, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064*

Expression of Bcl-2 inhibits apoptosis by a variety of mechanisms. Some of the physiological effects of Bcl-2 are mediated by modulation of the endoplasmic reticulum (ER)-localized inositol trisphosphate receptor Ca²⁺ release channel (InsP₃R). In addition, Bcl-2 expression repeatedly correlates with elevated levels of reduced glutathione (GSH), providing increased cellular antioxidant capacity and apoptotic resistance. This study examined the role of the Bcl-2–InsP₃R interaction in regulating Ca²⁺ homeostasis, apoptosis, and total cellular glutathione.

Using the DT40 cell line genetically deficient in all InsP₃Rs (DT40-TKO), stable lines were generated that overexpressed Bcl-2 in both the WT and TKO background. In permeabilized cells, experiments measuring ER Ca²⁺ flux revealed that Bcl-2 expression increased the apparent sensitivity of InsP₃R-dependent Ca²⁺ release to low levels of InsP₃. In intact cells, Ca²⁺ imaging experiments demonstrated that Bcl-2 expression in the WT increased both the frequency of spontaneous Ca²⁺ oscillations and the number of oscillating cells. Importantly, in WT and TKO expressing cells, Bcl-2 protected against apoptotic stimuli, although it provided significantly more protection in the InsP₃R-expressing WT cells. This was correlated with a significant increase in steady-state levels of GSH in WT but not TKO cells expressing Bcl-2.

Together, these data suggest that modulation of InsP₃R-dependent Ca²⁺ signaling by Bcl-2 is required to increase the cellular pool of reduced GSH. This supports a novel model in which Bcl-2 expression leads to InsP₃R-dependent redox adaptations that enable cells to better withstand an apoptotic stimulus.

28. Calcium Involvement in Toxic β Amyloid Peptide's Rapid Suppression of Kv1.1 Channel Activity. JOSEPH FARLEY, ALEC SEXTON, BRENT HALLAHAN, and JORDAN RAYNOR, *Indiana University, Neuroscience Program, Bloomington, IN 47405*

Substantial evidence implicates the amyloid β (Aβ1-42) peptide in the pathogenesis of Alzheimer's Disease (AD). Exactly how Aβ1-42 kills neurons remains unclear; most accounts view disruption of Ca²⁺ homeostasis as critical. Previous studies indicate that Aβ1-42 affects diverse neuronal K⁺ currents, but the molecular composition of the contributing channel subunits has generally been undefined. Kv1.1 is a voltage-dependent K⁺ channel responsible for repolarization of action

potentials in many mammalian neurons, including those affected by AD. Suppression of Kv1.1 activity would be expected to increase Ca^{2+} influx. We report that A β 1-42 produces a profound and rapid suppression of Kv1.1 currents, and this is partially dependent on $[\text{Ca}^{2+}]_i$. Homomeric α -subunit Kv1.1 channels were expressed in *Xenopus* oocytes, and macroscopic Kv1.1 currents were measured using standard voltage-clamp methods. Bath application of 1 μM A β 1-42 produced an $\sim 50\%$ decrease in Kv1.1 current (at 30 min), with no change in voltage dependency nor any indication of use-dependent pore block. Solvent and A β 40-1 control peptide experiments produced little change ($\leq 7\%$). Dose-response studies indicate a clear suppression of Kv1.1 current by A β 1-42 concentrations as low as 1 nM. We have previously studied Ca^{2+} -mediated suppression of Kv1.1 currents in oocytes by a pathway involving tyrosine phosphorylation, PKC, and calcineurin (PP2B) activation. We find that suppression of Kv1.1 by A β 1-42 is partially mediated by this pathway. Although suppression was not dependent on Ca^{2+} influx, because A β 1-42 addition to a Ca^{2+} -free bath still produced strong suppression of K^+ currents ($\sim 50\%$), partial abrogation ($\sim 50\%$) was produced by incubating oocytes in the Ca^{2+} -chelator BAPTA-AM, indicating that $[\text{Ca}^{2+}]_i$ plays a role. Cyclosporine A (a PP2B inhibitor) also partially ($\sim 50\%$) blocked Kv1.1 suppression. Our results suggest that A β 1-42 suppression of Kv1.1 (and related K^+) channels may represent one of the earliest steps in AD neurotoxicity and that $[\text{Ca}^{2+}]_i$ is involved.

29. Heterogeneous Ligand Sensitivities of Single InsP_3 Receptor Ca^{2+} -release Channels within Endoplasmic Reticulum Membrane Patches. MARISABEL FERNÁNDEZ-MONGIL, SUMAN DATTA, J. KEVIN FOSKETT, and DON-ON DANIEL MAK, *Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104*

Modulation of cytoplasmic-free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by the ubiquitous inositol 1,4,5-trisphosphate (InsP_3) receptor (InsP_3R), an intracellular Ca^{2+} -release channel localized to the endoplasmic reticulum (ER) membrane, regulates numerous physiological processes. A fundamental yet controversial aspect of the InsP_3 -mediated Ca^{2+} signaling is the widely observed phenomenon of quantal Ca^{2+} release: the ability of cells to have graded release of Ca^{2+} from intracellular stores in response to incremental levels of extracellular agonist or cytoplasmic $[\text{InsP}_3]$. Many schemes have been proposed to account for this phenomenon, including Ca^{2+} stores containing InsP_3R with different ligand sensitivities, Ca^{2+} stores with different InsP_3R densities, and regulation of InsP_3R channel activities by $[\text{Ca}^{2+}]$ in the ER lumen. Here, we applied rapid solution exchange techniques in nuclear patch-clamp experiments with membrane patches obtained in the cytoplasmic-side out configuration to expose the cyto-

plasmic side of InsP_3R channels in the same membrane patches to alternating buffers containing different ligand (InsP_3 and Ca^{2+}) concentrations under constant luminal $[\text{Ca}^{2+}]$. We observed that even in the same membrane patch, a larger number of InsP_3R channels was activated by more favorable ligand conditions in a graded manner up to the optimal ligand conditions (optimal $[\text{Ca}^{2+}]_i$ and saturating $[\text{InsP}_3]$). Such graded activation of InsP_3R channels in the same nuclear membrane patches was not only observed in endogenous InsP_3R channels in insect Sf9 cells with only one InsP_3R gene but also in recombinant rat type 3 InsP_3R channels stably transfected into DT40 cells with all three genes for the endogenous InsP_3R isoforms knocked out. Thus, a population of InsP_3R channels homogeneous in their primary sequences in the same Ca^{2+} store can nevertheless exhibit graded heterogeneous sensitivities to activation by $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$. (Supported by National Institutes of Health grant 5R01GM074999.)

30. Secretases, Oxidative Stress, and Perturbed Calcium Homeostasis in AD and Stroke. MARK P. MATTON, *Laboratory of Neurosciences, National Institute on Aging Intramural Research Program, Baltimore, MD 21224*

In this presentation, I will describe how cleavage of the β -amyloid precursor protein (APP) by α , β and γ secretases influences neuronal calcium homeostasis in the contexts of synaptic plasticity and Alzheimer's disease. In brief, sequential cleavages of APP by β and γ secretases generate amyloid β peptide (A β). A β induces membrane-associated oxidative stress that impairs the function of ion-motive ATPases and glutamate and glucose transporters, thereby promoting elevations of intracellular calcium levels, which impairs synaptic function and renders neurons vulnerable to excitotoxicity. Cleavage of APP by α secretase generates sAPP- α , which activates a signaling pathway involving cGMP production and activation of potassium channels that hyperpolarizes neurons. Cleavage of Notch by γ secretase generates the Notch NICD, which translocates to the nucleus and modifies the expression of genes encoding proteins that may enhance responses of neurons to glutamate, thereby playing a role in synaptic plasticity. However, under conditions of energetic compromise, as in stroke, Notch activation renders neurons vulnerable to calcium overload and cell death. By modifying APP and Notch processing, secretase inhibitors are valuable research tools and also hold potential as neurotherapeutic agents.

31. Is the Basis of Some Neurodegenerative Diseases Aberrant Control of an Ancient Self-replicating Protein Template? JULIE E.M. MCGEOCH, *Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138*

Water entrapped by a self-replicating membrane of polymer hydrophobic protein has been suggested as the first material-based topology of the universe (McGeoch, J. 2008. *Harvard da Vinci Group, May 12*). That a similar system existed when cells first arose on earth 3.8 million yr ago is supported by evidence that the nucleotide code of one hydrophobic polymer, proteolipid, is the most conserved throughout Archaea, Eubacteria, and Eukaria. Water-tight hydrogen-bonded β sheets of proteolipid can stack as 6-Å-deep layers around centrally entrapped ordered water, providing a topology that separates charge, and intermittent α -helical configurations confer ion channel function for the transport of cations and water between the layers (McGeoch, J.E., and M.W. McGeoch. 2008. *J.R. Soc. Interface*. 5:311–318). Here, we hypothesize that this ancient system of separating charge between insulating layers of hydrophobic protein was incorporated into the first cells as the fundamental component for information storage, later becoming a nervous system. The advent of nucleotide code-based cell chemistry evolved proteins to interact with this system, some of which suppressed its inclination to self-replicate from a template. Today, certain diseases of the nervous system in *Homo sapiens* involving aggregated β -sheet protein might have their basis in age-related imperfect transcription/translation or code mutations, rendering the code-based system of control aberrant. We suggest that Batten's (NCL's) and Alzheimer's disease might be in this category. The ratio of cell proteolipid mass to its P1 and P2 code translation should be higher than that for another control polymer like a skin keratin if there is ancient template-based self-replication for proteolipid, and, in Batten's disease, it should be even higher as a result of aberrant control of the template system.

32. Mechanisms of Altered *N*-Methyl-*D*-Aspartate Receptor-mediated Calcium Signaling in the YAC Mouse Model of Huntington's Disease. A. MILNERWOOD,¹ C. GLADDING,¹ M.R. HAYDEN,^{2,3} T.H. MURPHY,^{1,2} and L.A. RAYMOND,^{1,2} ¹Department of Psychiatry, ²Brain Research Centre, and ³Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC V6T 1Z4

Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative disease predominantly involving the striatal medium-sized spiny neurons (MSNs). HD is caused by expansion of a CAG repeat in the *HD* gene, resulting in a polyglutamine (polyQ) expansion >35 near the N terminus of the protein, huntingtin, and age of onset is inversely correlated with polyQ repeat length. Previous work in human HD brain tissue suggested that overactivation of *N*-methyl-*D*-aspartate (NMDA)-type glutamate receptors (NMDARs) contributes to the selective loss of striatal MSNs. We have tested this hypothesis using the YAC transgenic

HD mouse model, expressing full-length human huntingtin with 18 polyQ (normal; YAC18) or 46, 72, or 120 polyQ (pathologically expanded; YAC46, YAC72, and YAC128, respectively). Striatal neurons from YAC mice expressing expanded polyQ huntingtin show enhanced sensitivity to NMDA-induced apoptotic cell death compared with MSNs from wild-type or YAC18 mice both in vivo and in cultures from postnatal mice. In MSN cultures, sensitization to NMDAR-mediated apoptosis results from increased activation of the mitochondrial permeability transition (mPT) pore and the intrinsic pathway, in part because of enhanced NMDAR activity and calcium mishandling. However, less is known about the mechanisms underlying increased striatal sensitivity to excitotoxicity in these HD mice in vivo. Previous studies demonstrate that apoptotic or survival pathways can be preferentially triggered by activation of extrasynaptic or synaptic NMDARs, respectively. In whole cell patch clamp recordings from cortico-striatal brain slices, we found evidence for increased extrasynaptic NMDAR activity in YAC128 MSNs compared with YAC18 MSNs. NMDAR synaptic current amplitude is increased in YAC128 mice under conditions of augmented glutamate release but not with low levels of synaptic activity. Moreover, treatment with a glutamate uptake inhibitor results in a significant increase in NMDAR-mediated holding current and action potential-evoked charge transfer in YAC128 but not YAC18 MSNs, suggesting activation of a population of extrasynaptic NMDARs in the YAC128 MSNs. Consistent with these electrophysiological data, subcellular fractionation shows enhanced expression of NR1 in the striatal nonpostsynaptic plasma membrane fraction from YAC128 compared with YAC18 mice, whereas no differences are detected in the striatal postsynaptic fractions or either fraction from cortical tissue. These data demonstrate a link between an inherited neurodegenerative disease and extrasynaptic NMDAR neurotransmission. (Supported by the Canadian Institutes of Health Research, the Michael Smith Foundation for Health Research, the Huntington Disease Society of American, the Huntington Society of Canada, and the HighQ Foundation.)

33. Enhanced CREB Phosphorylation by FAD Mutant Presenilin-1 (M146L)-associated Exaggerated Ca^{2+} Signaling. MARIOLY MÜLLER, KING-HO CHEUNG, CÉSAR CÁRDENAS, LIJUAN MEI, and J. KEVIN FOSKETT, Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

Mutations in presenilin 1 (PS1) and presenilin 2 (PS2) genes account for the majority of early onset familial Alzheimer's Disease (FAD). Accumulating evidence suggests that disruption of intracellular Ca^{2+} signaling may play a proximal and perhaps central role in the pathogenesis of AD. Nevertheless, possible links between disrupted intracellular Ca^{2+} homeostasis and

cell signaling are obscure. Here, we demonstrate that PS1-FAD mutant M146L constitutively enhances phosphorylation of the transcription factor cAMP-response element-binding protein (CREB) in a Ca^{2+} -dependent way. Immunoblotting using p-CREB-specific antibody revealed that CREB is constitutively phosphorylated in M146L-expressing PC12 cells. This phosphorylation was completely abolished by depletion of endoplasmic reticulum Ca^{2+} stores with thapsigargin, suggesting an important role of Ca^{2+} release in this phenomenon. By use of specific pharmacological inhibitors, both CAMKIV and Ca^{2+} -dependent protein kinase C but not MAPK were involved in CREB phosphorylation, reinforcing the idea that Ca^{2+} is a primary signal for CREB phosphorylation. The activity of the InsP_3 receptor Ca^{2+} release channel is potentiated by PS1-FAD mutant M146L. Inhibition of PLC, the enzyme responsible for the IP_3 generation, treatment of the cells with Xestospongin B, a specific InsP_3 receptor inhibitor, or RNAi against InsP_3 receptor type 1, the main neuronal isoform, each completely inhibited PS1-FAD mutant M146L-dependent constitutive CREB phosphorylation. Our results demonstrate that exaggerated Ca^{2+} signaling through activation of the InsP_3 receptor in FAD PS1 mutant-expressing PC12 cells affects CREB phosphorylation and may suggest a pathway involved in AD pathogenesis.

34. Calcium Signaling Defects in Familial Alzheimer's Disease Patient: Relevance for Alzheimer's Disease. OMAR NELSON, HUARUI LIU, and IYIA BEZPROZVANNY, *Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390*

Mutations in presenilin 1 (PS1) and presenilin 2 (PS2) are responsible for ~40% of all early onset familial Alzheimer's disease (FAD) cases in which a genetic cause has been identified. In addition, a number of mutations in PS1 have been associated with the occurrence of frontal temporal dementia (FTD), although a formal proof of their causal involvement has not been provided. Presenilins (PSs) are highly conserved transmembrane proteins that support cleavage of the amyloid precursor protein by γ -secretase. Recently, we discovered that PSs also function as passive endoplasmic reticulum Ca^{2+} leak channels (Tu, H., O. Nelson, A. Bezprozvanny, Z. Wang, S.F. Lee, Y.H. Hao, L. Serneels, B. De Strooper, G. Yu, and I. Bezprozvanny. 2006. *Cell*. 126:981–993). We further found that PS1-M146V, ΔE9 , L166P, A246E, E273A, G384A, P436Q FAD, and PS2-N141I FAD mutations in PSs affected their ER Ca^{2+} leak function. In contrast, the ER Ca^{2+} leak function of PS1 appeared unaffected by A79V FAD mutation. Similar to FAD A79V, neither of the FTD-associated mutations in PS1 (L113P, G183V, and Rins352) affected ER Ca^{2+} leak function in our experiments (Tu, H., O. Nelson, A. Bezprozvanny, Z. Wang, S.F. Lee, Y.H. Hao, L. Serneels, B. De Strooper, G. Yu, and I. Bezprozvanny. 2006. *Cell*.

126:981–993; Nelson, O., H. Tu, T. Lei, M. Bentahir, B. de Strooper, and I. Bezprozvanny. 2007. *J. Clin. Invest.* 117:1230–1239). To further validate our findings, we use Ca^{2+} imaging experiments to evaluate ER Ca^{2+} leak in lymphoblast from FAD patients harboring mutations in PS1 (M139V, M146L, H163Y, K239E, V261F, A426P, A431E, P264L, R269G, and C410Y), PS2-N141I, τ -R406W, APP-V717L, and young and old sporadic AD cases. In addition, Ca^{2+} rescue experiments with the PS1 FAD mutations were performed in PS-null mouse embryonic fibroblasts (DKO) to validate ER Ca^{2+} leak function. We found that PS1 FAD mutations M139V, M146L, H163Y, K239E, V261F, A426P, A431E, and PS2-N141I abolished ER Ca^{2+} leak in lymphoblast and Ca^{2+} rescue experiments in DKO, whereas PS1 (P264L, R269G, ΔE9 , and C410Y), τ -R406W, APP-V717L, and young and old sporadic AD had no effect of ER Ca^{2+} leak in lymphoblast. Our observations are consistent with the potential role of disturbed Ca^{2+} homeostasis in AD pathogenesis. (Supported by Alzheimer's Association research grant IIRG-06-24703 and the McKnight Brain Disorders Award to I. Bezprozvanny and by the National Institutes of Health Predoctoral Fellowship Award for Minority Students [F31-AG031692-02] to O. Nelson.)

35. Role of Ca^{2+} Signaling During the Unfolded Protein Response (UPR). R. MADELAINE PAREDES,¹ PATRICIA CAMACHO,^{1,2} and JAMES D. LECHLEITER,^{1,2}
¹*Department of Cellular and Structural Biology and* ²*Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229*

Two of the main functions of the endoplasmic reticulum (ER) are Ca^{2+} storage and protein folding (Berridge, M.J. 2002. *Cell Calcium*. 32:235–249). Although it has been suggested that these two functions are associated (Li, Y., and P. Camacho. 2004. *J. Cell Biol.* 164:35–46), it is not clear how. It is known that disturbing the balance of any function of the ER generates ER stress (Rutkowski, D.T., and R.J. Kaufman. 2004. *Trends Cell Biol.* 14:20–28). Under ER stress, a process known as the unfolded protein response (UPR) is induced. The UPR process is an attempt by the cell to restore balance and maintain normal function in the ER, which otherwise results in cell death.

Previous work in our group has shown a dependent and direct relationship between Ca^{2+} and members of the protein-folding machinery such as Calreticulin (CRT), a luminal chaperone, and ERp57, an oxidoreductase that promotes disulfide bond formation during protein folding. In that work, it is demonstrated that under high Ca^{2+} concentrations in the lumen of the ER, ERp57 forms a complex with CRT, and that complex can bind the SERCA 2b pump, inhibiting its activity. On the other hand, when luminal Ca^{2+} concentrations are low, the CRT-ERp57 complex can no longer bind SERCA 2b, and the pump is able to function properly,

bringing Ca^{2+} back into the lumen of the ER (Li, Y., and P. Camacho. 2004. *J. Cell Biol.* 164:35–46).

Additional data from our group indicate that treatment of *Xenopus* oocytes with an inhibitor of protein glycosylation (Tunicamycin) that mimics ER stress increases cytosolic Ca^{2+} (in conditions of zero extracellular Ca^{2+}), suggesting a partial depletion of luminal Ca^{2+} subsequent to ER stress (unpublished data).

To monitor the effect of luminal Ca^{2+} on protein folding, we have developed an imaging assay to monitor accumulation of protein in the ER. In brief, we label a luminal protein, CPY (carboxypeptidase Y), that is normally secreted outside the cell with strawberry fluorescent protein (SFP). CPY is known to misfold and accumulate in the ER when a single point mutation is introduced into the protein (Mancini, R., M. Aebi, and A. Helenius. 2003. *J. Biol. Chem.* 278:46895–46905). We labeled the single point mutant of CPY with cyan fluorescent protein (CFP) to image its ER accumulation. Our preliminary data indicate that thapsigargin-depleted ER Ca^{2+} stores increases accumulation of SFP-tagged wild-type CPY. Moreover, prolonged partial depletion of luminal Ca^{2+} by low concentrations of thapsigargin also induces protein misfolding accumulation in the ER. Treatment of oocytes with both high and low concentrations of thapsigargin induced ER stress as monitored by phosphorylation of PERK and phosphorylation of eIF2 α .

Collectively, these data suggest that Ca^{2+} depletion from the ER can be both a causative effect as well as a consequence of protein misfolding in the UPR. (Supported by grants PO1 AG19316-06 and RO1 AG29461-01.)

36. Rosiglitazone Treatment Prevents Mitochondrial Dysfunction in Mutant Huntingtin-Expressing Cells. RODRIGO A. QUINTANILLA,¹ YOUNGMAN JIN,² and GAIL V.W. JOHNSON,^{1,2} ¹Department of Anesthesiology and ²Department of Pharmacology and Physiology, University of Rochester, Rochester, NY 14642

Defects in calcium regulation and mitochondrial function have been implicated in the pathogenesis of Huntington's disease (HD). To evaluate the potential role of the calcium deregulation and loss of mitochondrial function in the pathogenesis of HD, we used striatal cells that express wild-type (STHdh^{Q7/Q7}) or mutant (STHdh^{Q111/Q111}) huntingtin protein at physiological levels. In these studies, we examined cytosolic calcium levels, mitochondria calcium changes, and mitochondrial membrane potential using confocal microscopy. Treatment of mutant cells with thapsigargin resulted in a pronounced decrease in mitochondrial calcium uptake, an increase in reactive oxygen species (ROS) production, and a significant decrease in mitochondrial membrane potential. These events were partially prevented by pretreatment with cyclosporine A (CsA), indi-

cating a potential role for permeability transition pore (PTP) opening in this mitochondrial dysfunction. In addition, we evaluated the potential neuroprotective role of PPAR γ activation in preventing the loss of mitochondrial function in HD using striatal cells. PPAR γ activation by rosiglitazone totally prevented the mitochondrial dysfunction and oxidative stress that occurred when striatal cells expressing mutant huntingtin were challenged with pathological increases in calcium. The beneficial effects of rosiglitazone were mediated by activation of PPAR γ , as all protective effects were prevented by the specific PPAR γ receptor antagonist GW9662. Additionally, we observed that mutant huntingtin-expressing cells presented with significant impairment of the PPAR γ signaling pathway, including decreases in PPAR γ expression and reduced PPAR γ transcriptional activity. In addition, we observed that treatment of striatal cells with rosiglitazone increased mitochondrial mass levels, suggesting a role for the PPAR γ pathway in mitochondrial function in striatal cells. All together, this evidence indicates that PPAR γ activation by rosiglitazone attenuates mitochondrial dysfunction in mutant huntingtin-expressing striatal cells, and this could be an important therapeutic avenue to ameliorate the mitochondrial dysfunction that occurs in HD.

37. Functional Studies of a Novel Gene Linked to Late Onset Alzheimer's Disease. ADAM P. SIEBERT,¹ HORA VAIS,¹ LIJUAN MEI,¹ PHILIPPE MARAMBAUD,³ and J. KEVIN FOSKETT,^{1,2} ¹Department of Physiology and ²Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104; ³Litwin-Zucker Research Center for the Study of Alzheimer's Disease and Memory Disorders, Feinstein Institute for Medical Research, Manhasset, NY 11030

Alzheimer's Disease is a common form of dementia involving slowly developing and ultimately fatal neurodegeneration. Mutations in presenilins and amyloid precursor protein cause the majority of early onset autosomal dominant familial cases of the disease. However, complex interactions between different genetic variants and environmental factors are believed to modulate the risk of the vast majority of late onset Alzheimer's disease. A prevailing idea, the calcium hypothesis, proposes that sustained changes in intracellular calcium homeostasis provide the common pathway for age-associated brain changes. Recently, we identified CALHM1, a gene of unknown function, located on chromosome 10 within 1.6 Mb of the late onset Alzheimer's Disease marker D10S1671. The frequency of a nonsynonymous single-nucleotide polymorphism (SNP) in CALHM1, which results in an amino acid substitution, is significantly increased in independent cohorts of AD cases in French and British populations. In preliminary studies, we have determined that CALHM1 expression induces

novel plasma membrane ionic conductances in *Xenopus* oocytes and CHO cells that are significantly permeable to Ca^{2+} . In addition, CALHM1 causes disturbances in intracellular Ca^{2+} homeostasis in both CHO and PC12 cells. The polymorphic AD-associated CALHM1 induces similar plasma membrane conductances, but the Ca^{2+} permeability is reduced. (Supported by National Institutes of Health grant GM/DK56328 and a pilot project from the University of Pennsylvania Alzheimer's Disease Core Center.)

38. Ryanodine Receptor Type 3 Up-regulation is Neuroprotective in Cortical Cultures from Tgcrnd8 Mice. CHARLENE SUPNET, JOHN BRADLEY, CHARMAINE NOONAN, ELISABETH WILLIAMS, KELLY RICHARD, and MICHAEL MAYNE, *Department of Biomedical Sciences, University of Prince Edward Island, Institute for Nutrisciences and Health, National Research Council of Canada, Charlottetown, Prince Edward Island C1A4P3, Canada*

Before the extensive neurodegeneration that occurs in the late stages of Alzheimer's disease (AD), there are marked and sustained changes in intraneuronal calcium (Ca^{2+}) handling. Recent evidence suggests that particular Ca^{2+} -release channels located on the endoplasmic reticulum (ER), the ryanodine receptors (RyRs), could mediate these changes in neurons from AD models via increased protein and Ca^{2+} release from the ER. Interestingly, postmortem tissues from AD patients in the early stages of the disease have elevated ryanodine binding in hippocampal regions compared with nondemented controls. We have reported that up-regulation of RyR type 3 is responsible for the increased Ca^{2+} levels observed in cortical neurons after glutamate and ryanodine treatment from transgenic (Tg)CRND8 mice, which produce high levels of the β -amyloid fragment 1–42. The objective of this study is to investigate the impact of increased RyR3 levels on the neurobiology of TgCRND8 cortical cultures.

Given that increased RyRs and Ca^{2+} levels have been shown to be involved in neurotoxicity in presenilin-1 and -2 mutants, we wanted to investigate the effect of increased RyR3 on the viability of TgCRND8 cortical cultures. After the application of glutamate, H_2O_2 , or staurosporine, Tg cultures did not show increased susceptibility to death compared with non-Tg littermate controls. Furthermore, Tg cultures did not show increased death after long-term culture compared with non-Tg. Considering that Tg mice display no overt neurodegeneration, we investigated the alternate hypothesis that increased RyR3 was involved in maintaining the viability of Tg cultures. To test this hypothesis, we blocked the up-regulation of RyR3 in Tg cultures by treatment with short-interfering RNA (siRNA) directed to RyR3. Interestingly, Tg cultures treated with siRyR3 show decreased viability of $\sim 35\%$ compared with non-Tg treated with siRyR3 as assessed by MTT. After stain-

ing with the neuron marker NeuN, the number of neurons in Tg cultures treated with siRyR3 was decreased by $\sim 50\%$ compared with Tg treated with non-specific siRNA. These data suggest that the increased expression of RyR3 is neuroprotective in TgCRND8 cortical cultures and offer a novel role for RyRs in AD pathology.

39. Calcium and Selective Vulnerability of Dopaminergic Neurons in Parkinson's Disease. **D. JAMES SURMEIER**, *Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611*

Why dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) die in Parkinson's disease (PD) has been an enduring mystery. Our studies suggest that the unusual reliance of adult SNc DA neurons on L-type Cav1.3 Ca^{2+} channels to drive their maintained, rhythmic pacemaking renders them vulnerable to stressors thought to contribute to disease progression. Young SNc DA neurons exhibit Cav1.3 channel-independent pacemaking that is similar in rate and regularity to that seen in adult neurons. The mechanisms underlying this juvenile pacemaking are retained into adulthood but remain latent. Antagonizing Cav1.3 Ca^{2+} channels in adult neurons induces a reversion to these juvenile mechanisms to maintain pacemaking within a normal range. Inducing this reversion renders SNc DA neurons significantly less sensitive to three toxins used to create animal models of PD, pointing to a novel strategy that could slow or stop the progression of PD.

40. Neuronal Calcium Signaling and Striatal Neurodegeneration in Huntington's Disease. TIE-SHAN TANG, HONGYU WANG, and **ILYA BEZPROZVANNY**, *Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390*

Huntington's disease (HD) is a neurodegenerative disorder caused by polyglutamine (polyQ) expansion in Huntingtin protein (Htt). PolyQ expansion in Htt^{exp} causes selective degeneration of striatal medium spiny neurons (MSN) in HD patients. A central question in the study of HD is how this polyQ expansion of Htt leads to the neurodegeneration of MSN. Recently, we discovered that mutant Htt^{exp} selectively associates with and activates type 1 inositol 1,4,5-trisphosphate receptor (InsP₃R1), an intracellular Ca^{2+} release channel (Tang, T.S., H. Tu, E.Y. Chan, A. Maximov, Z. Wang, C. L. Wellington, M.R. Hayden, and I. Bezprozvanny. 2003. *Neuron*. 39:227–239). Based on these results, we proposed that the MSN degeneration in HD results from deranged neuronal Ca^{2+} signaling (Bezprozvanny, I., and M.R. Hayden. 2004. *Biochem. Biophys. Res. Commun.* 322:1310–1317). In support of this hypothesis, we demonstrated that glutamate induces apoptosis of MSN primary cultures from the YAC128 HD mouse model (Tang, T.S., E. Slow, V. Lupu, I.G. Stavrovskaya,

M. Sugimori, R. Llinás, B.S. Kristal, M.R. Hayden, and I. Bezprozvanny. 2005. *Proc. Natl. Acad. Sci. USA*. 102:2602–2607).

Our biochemical data indicated that Htt^{exp} specifically binds to the C-terminal region cytosolic of InsP₃R1 (IC10 fragment). To test the importance of Htt^{exp} association with InsP₃R1 for HD pathology, we generated lentiviral constructs expressing GFP-IC10 fusion protein. Control viruses expressing GFP protein were also generated. We found that infection with lentiviruses encoding GFP-IC10 protein stabilized Ca²⁺ signaling in YAC128 MSN and protected YAC128 MSN from glutamate-induced apoptosis. To extend these studies to the whole animal level, we generated adeno-associated viruses (AAV1) encoding GFP-IC10 and control GFP protein. The AAV1-GFP-IC10 and AAV1-GFP viruses were stereotactically injected to the striatal region of YAC128 mice and control wild-type mice. The progression of degeneration in injected YAC128 mice was evaluated by behavioral analysis and by stereological methods as described in our previous whole animal studies of tetra- benazine in YAC128 mice (Tang, T.S., X. Chen, J. Liu, and I. Bezprozvanny. 2007. *J. Neurosci.* 27:7899–7910). Obtained results support an importance of Ca²⁺ signaling and InsP₃R1-Htt^{exp} association for HD pathogenesis. (Supported by National Institutes of Health grants NS38082 and NS056224, the High Q Foundation, Robert A. Welch Foundation, and the McKnight Neuroscience of Brain Disorders Award [to I. Bezprozvanny]).

41. **Inhalational Anesthetics Induces Apoptosis via Activation of IP₃ Receptors.** HUA FENG WEI, GE LIANG, ERIC ZHAO, HUI YANG, QIANRU CHEN, MARYELLEN ECKENHOFF, and RODERIC ECKENHOFF, *Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, PA 19104*

We hypothesize that the inhalational anesthetics induce apoptosis by causing abnormal calcium release from the endoplasmic reticulum (ER) via overactivation of inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃R). We exposed chicken B lymphocytes with total IP₃R knockout (DT40 IP₃R TKO) and the wild-type control to equipotent concentrations of isoflurane, sevoflurane, and desflurane. We then determined the cell damage and the changes of calcium concentrations in the ER ([Ca²⁺]_{ER}), cytosol ([Ca²⁺]_{cyt}), and mitochondria ([Ca²⁺]_m; Liang, G., Q. Wang, Y. Li, B. Kang, M.F. Eckenhoff, R.G. Eckenhoff, and H. Wei. 2008. *Anesth Analg.* 106:492–500; Wei, H., G. Liang, H. Yang, Q. Wang, B. Hawkins, M. Madesh, S. Wang, and R.G. Eckenhoff. 2008. *Anesthesiology*. 108: 251–260). We also determined the effects of the suppression of IP₃R expression using siRNA on isoflurane toxicity in rat primary cortical neurons. Finally, we treated 7-d-old rats with 1 or 1.5% isoflurane for 6 h with or without preexposure to intraventricular injection of the IP₃R antagonist xesto-

spongin C. We measured apoptosis in the hippocampus CA1 region and cortex and evaluated their memory and learning abilities as juveniles (P36–46) using a Morris water maze (MWM).

All three anesthetics caused significant cell damage associated with decreases in the [Ca²⁺]_{ER} and increases in the [Ca²⁺]_{cyt} and [Ca²⁺]_m only in DT40 wild type but not in IP₃R TKO cells, with isoflurane being significantly more potent than sevoflurane or desflurane. Suppression of IP₃R nearly abolished the isoflurane toxicity. 1% isoflurane treatment for 6 h markedly increased the apoptosis in the hippocampus CA1 region and the cortex, which was significantly inhibited by xestospongin C. Isoflurane (1%) for 6 h did not significantly affect memory and learning. 1.5% isoflurane for 6 h impaired the learning and memory transiently, which seemed to be inhibited by xestospongin C.

Inhalational anesthetics induce cell damage by causing abnormal calcium release from ER via excessive activation of IP₃R. Isoflurane has greater potency than sevoflurane or desflurane to induce cell damage, possibly by its greater ability to disturb intracellular calcium homeostasis. (Supported by a National Institute of General Medical Science K08 grant [1-K08-GM-073224-01 to H. Wei], March of Dimes Birth Defects Foundation Research Grant [12-FY05-62 to H. Wei], and the Research Fund at the Department of Anesthesiology and Critical Care, University of Pennsylvania [H. Wei].)

Session 4: Central Nervous System

42. **Calcium Signaling Abnormalities in Familial Hemiplegic Migraine Type 2 (FHM2), a Window into Autism and Polygenic Neuropsychiatric Channelopathies.** J. JAY GARGUS, *Department of Physiology and Biophysics and Department of Pediatrics, Division of Human Genetics, University of California, Irvine, Orange, CA 92868*

Point mutations in the α2 isoform of the Na,K-ATPase cause FHM2, a dominant migraine phenotype identical to that caused by P/Q channel mutations underlying FHM1 and sodium channel mutations underlying FHM3 (Gargus, J.J., and D.W. Dodick. 2008. *Scientific American*. In press). Although FHM2 alleles produce diverse effects on Na pump kinetics (Segall, L., A. Mezzetti, R. Scanzano, J.J. Gargus, E. Purisima, and R. Blostein. 2005. *Proc. Natl. Acad. Sci. USA*. 102:11106–11111), all FHM2 alleles tested, unlike WT, similarly disrupt intracellular Ca²⁺ signaling in stable transformant neuroblastoma (Smith, I.F., C. Shih, R. Blostein, I. Parker, and J.J. Gargus 2008. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* In press). Ca²⁺ signals evoked by carbachol are diminished in mutant transformants, Ca²⁺ oscillations are suppressed, and local Ca²⁺ puffs evoked by photoreleased IP₃ occur with similar frequency yet lower amplitude than in identical WT transformants. These

effects are evident even without inhibiting endogenous Na pumps, simulating the heterozygous disease state. That FHM2 mutants can maintain cation gradients and affect ATPase kinetics in different ways yet similarly cause disease and disrupt Ca^{2+} signaling suggests that regulation of IP_3 -mediated Ca^{2+} signaling is a critical physiological function of the $\alpha 2$ Na pump and that perturbed neuronal calcium signaling is the shared pathogenic mechanism of FHM1/2.

That two of the FHM genes are exclusively neuronal and that different alleles of all three FHM genes cause seizures, the prototypical neuronal channelopathy phenotype (Gargus, J.J. 2006. *Biol. Psychiatry*. 60:177–185), places FHM and likely common polygenic migraine within that disease spectrum. The high familial comorbidity of migraine with seizures as well as a host of the common polygenic neuropsychiatric diseases, including autism, suggests shared heritable factors extending this spectrum. An allele of *CACNA1C*, the FHM1 homologue, causes a monogenic autism/LQT syndrome, and mutations in the FHM3 gene and another FHM1 homologue, *CACNA1H*, have been found in autism; this underscores a vulnerability in calcium homeostasis that is reinforced by findings of functionally altered mitochondrial energetics in autism.

43. Structural Determinants of Phosphoinositide Regulation of M-type Potassium Channels. CIRIA C. HERNANDEZ and MARK S. SHAPIRO, *Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229*

The regulation of M-type (KCNQ, Kv7) K^+ channels by PIP_2 has perhaps the best correspondence to physiological function, but the site of action and structural motif of PIP_2 on these channels have not been established. The C terminus of Kv7 channels contains four highly conserved helical domains, called A–D. Using single-channel recordings of chimeras of Kv7.2–7.4 channels with highly differential PIP_2 sensitivities, we localized the interhelix A-B linker as the primary site of PIP_2 action, a region of 71 residues. Point mutants within this linker in Kv7.2 identified a conserved cluster of basic residues that could interact with the lipid using electrostatic and hydrogen bonds. Substitution of conserved positively charged residues in Kv7.2 identified K425, K452, R459, R461, R463, and R467 as important molecular determinants for the apparent affinity of the channels for PIP_2 . To gain insight into the specific interactions between PIP_2 and the channels, a homology model of this domain of Kv7.2 was constructed using the solved crystal structure of the C terminus of Kir2.1 (Pegan, S., C. Arrabit, W. Zhou, W. Kwiatkowski, A. Collins, P.A. Slesinger, and S. Choe. 2005. *Nat. Neurosci.* 8:279–287) as a template using the program SWISS-MODEL (Schwede, T., J. Kopp, N. Guex, and M.C. Peitsch. 2003. *Nucleic Acids Res.* 31:3381–3385). The

model predicts a structure of the former very similar to the latter with a C α RMS of 0.55 Å and a seven- β -sheet barrel motif common to other PIP_2 -binding domains. Phosphoinositide-docking simulations predict affinities and interaction energies in accord with the experimental data and, furthermore, indicate that the precise identity of residues in the interacting pocket alter channel/ PIP_2 interactions not only by altering electrostatic energies but by allosterically shifting the structure of the lipid-binding surface. These results are likely to shed light on the general structural mechanisms of phosphoinositide regulation of ion channels.

44. The Epilepsy Mutation of BK Channels Alters Ca^{2+} Sensitivity by an Allosteric Mechanism. GAYATHRI KRISHNAMOORTHY,^{1,2} AKANSHA SAXENA,^{3,4} GUOHUI ZHANG,^{2,4} ALEX YANG,^{2,4} JINGYI SHI,^{2,4} DAVID SEPT,^{3,4} and JIANMIN CUI,^{2,4} ¹Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106; ²Cardiac Bioelectricity and Arrhythmia Center, ³Center for Computational Biology, and ⁴Department of Biomedical Engineering, Washington University, St. Louis, MO 63130

Large conductance Ca^{2+} and voltage-activated K^+ (BK) channels regulate neuronal action potential duration and synaptic transmission. It has been shown that an Asp to Gly mutation (the epilepsy mutation) in the BK α subunit increases Ca^{2+} sensitivity of the channel and is linked to generalized epilepsy and paroxysmal dyskinesia (Du, W., J.F. Bautista, H. Yang, A. Diez-Sampedro, S.A. You, L. Wang, P. Kotagal, H.O. Lüders, J. Shi, J. Cui, G.B. Richardson, and Q.K. Wang. 2005. *Nat. Gen.* 37:733–738). Here, we report an allosteric connection important for Ca^{2+} -dependent activation in the AC region, an 80-residue cytosolic domain that links the Ca^{2+} -binding sites to the activation gate. Amino acid residues distributed from the peripheral to the central AC region in discrete areas cooperate in contributing to Ca^{2+} sensitivity. The epilepsy mutation is located in the AC region, and the allosteric connection among these residues also underlies the effect of the epilepsy mutation in enhancing Ca^{2+} sensitivity. Molecular dynamics simulations of the AC region based on the structure of MthK channel reveal that the epilepsy mutation at the peripheral AC region reduces the flexibility of the central AC region. The correlation between experimental results and simulations suggest that the dynamic process of the AC region is an important driving force for the allosteric connection.

45. Palmitoylated $\text{Ca}_v\beta 2a$ Toggles Slow Pathway Modulation of $\text{Ca}_v2.2$ (N-type Ca^{2+}) Current by Tachykinin Receptor Activation. TORA MITRA-GANGULI,¹ IULIA VITKO,² EDWARD PEREZ-REYES,² and ANN R. RITTENHOUSE,¹ ¹Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655; ²Department of Pharmacology, University of Virginia, Charlottesville, VA 22908

The G_q -protein-coupled tachykinin receptor NK-1R modulates $Ca_v2.2$ (N-type Ca^{2+} channel) activity by a voltage-independent slow pathway (unpublished data). This mechanism is not completely understood, yet it has an important role in pain perception. Here, we show that when $Ca_v\beta3$ is coexpressed with $Ca_v2.2$ and $\alpha_2\delta1$, activation of NK-1R by substance P (SP), its preferred natural ligand, inhibits N current. In contrast, coexpression with $Ca_v\beta2a$, which is uniquely palmitoylated on two N-terminal cysteines, enhances N current by acting at a second site. Arachidonic acid (AA) mimics this pattern of modulation. Moreover, antagonists of phospholipase A2 block inhibition, implicating AA as the final effector. Exogenous application of palmitic acid antagonizes current inhibition when $Ca_v\beta3$ is present. This finding suggests that the palmitoyl groups block N-current inhibition by NK-1R-stimulated release of free AA, revealing latent enhancement. Therefore, we tested whether $Ca_v\beta2a$ requires a specific orientation that allows its palmitoyl groups to antagonize current inhibition. $Ca_v\beta$ binds with high affinity to the α -interacting domain (AID) of the I-II linker of $Ca_v2.2$ whereby the rigid segment between domain I S6 (IS6) and AID orients $Ca_v\beta$ with respect to the gating machinery of the channel. We found that rotating $Ca_v\beta2a$ by deleting one or two amino acids in the IS6-AID segment changes the modulation of N current by SP from enhancement to inhibition. These findings suggest that when the palmitoyl groups are sufficiently displaced, endogenously released AA can now bind to the inhibitory site. In support of this interpretation, adding back exogenous palmitic acid rescued the block of inhibition. This study identifies a novel functional role of palmitoylation in affecting modulation of channels. The findings also indicate that a specific orientation of the $Ca_v\beta$ docks its palmitoyl groups close to $Ca_v2.2$, thereby blocking N-current inhibition by SP. (Supported by grants NS38691 and NS34195 and funds from the University of Massachusetts Medical School.)

46. Simulations can Help Demystify Calcium Dynamics under the Presynaptic Membrane. JOHN W. MOORE, *Duke University School of Medicine, Durham, NC 27708*

Several mechanisms regulate the calcium ion concentration, $[Ca]$, within the presynaptic terminal in both time and space. The details of how these processes interact remain mysterious. Computer simulations that include calcium channels, an ATP-driven calcium pump, intracellular calcium buffering and diffusion, and the NaCa exchanger may offer insight by revealing how each component affects the spatial and temporal dynamics of the intracellular $[Ca]$. Furthermore, simulations can show how each mechanism might modulate transmitter release.

Simulations of the intracellular calcium dynamics in response to the invasion of a presynaptic terminal by an action potential were performed using NEURON. They demonstrate the following: (1) calcium enters via the NaCa exchanger during the rising phase of the impulse and only later via the calcium channel, primarily during the impulse's falling phase; (2) this surge in $[Ca]$ is large, but it encounters buffering that restricts the increase to the submembrane region; (3) this restriction provides for the most efficient operation of the calcium pump, which is located in the membrane; (4) the pump can quickly reduce the submembrane $[Ca]$ to a very low level, which is necessary to terminate transmitter release abruptly; and (5) the pump and NaCa exchanger together eject a large fraction of the calcium entering during an impulse, whereas the residual calcium is readily absorbed by the intracellular buffering capacity.

Furthermore, by using a simple transmitter release model (release proportional to $[Ca]^2$), we see that the NaCa exchanger affects release in two important ways: (1) it enhances release in response to a single action potential because the exchanger increases both the speed and amplitude of the submembrane calcium increase; and (2) it minimizes facilitation during a train of action potentials because of the exchanger's powerful extrusion of entering calcium.

47. Coexpression with $\beta2a$ Decreases AA Inhibition of $Ca_v1.3$ L-type Calcium Channels. MANDYL L. ROBERTS-CROWLEY and ANN R. RITTENHOUSE, *Department of Physiology, Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA 01655*

L-type Ca^{2+} (L-) channels are important for gene expression, secretion, and membrane excitability. Specifically, a role for the $Ca_v1.3$ L-channel isoform has been implicated in hearing, sinoatrial node function (Platzer, J., J. Engel, A. Schrott-Fischer, K. Stephan, S. Bova, H. Chen, H. Zheng, and J. Striessnig. 2000. *Cell* 102:89–97), and Parkinson's disease (Day, M., Z. Wang, J. Ding, X. An, C.A. Ingham, A.F. Shering, D. Wokosin, E. Ilijic, Z. Sun, A.R. Sampson, et al. 2006. *Nat. Neurosci.* 9:251–259). L-currents are inhibited by arachidonic acid (AA), but the mechanism of inhibition remains unclear (Liu, L., and A.R. Rittenhouse. 2000. *J. Physiol.* 525:391–404). To test this, L-channels ($Ca_v1.3$, α_{1D}) were transfected in HEK 293 cells with different accessory $Ca_v\beta$ (β_{1b} , β_{2a} , β_3 , or β_4) and $\alpha_2\delta-1$ subunits and GFP. Whole-cell Ba^{2+} currents were measured from recombinant channels expressed in HEK 293 cells at a test potential of -10 mV from a holding potential of -90 mV. A 1-min exposure to $10 \mu M$ AA inhibited currents with β_{1b} , β_3 , or β_4 58%, 51%, or 44%, respectively, but with β_{2a} only 31%. At a more depolarized holding potential of -60 mV, currents were inhibited to a lesser degree. These data are best explained by a simple model in which AA stabilizes $Ca_v1.3$ in a deep, closed channel conformation, resulting

in current inhibition. Consistent with this hypothesis, inhibition by AA occurred in the absence of test pulses, indicating that channels do not need to open to become inhibited. AA had no effect on the voltage dependence of holding potential-dependent inactivation or on recovery from inactivation regardless of $\text{Ca}_v\beta$ subunit. Unexpectedly, kinetic analysis revealed evidence for two populations of L-channels that exhibit willing and reluctant gating previously described for Ca_v2 channels. AA preferentially inhibited reluctant gating channels, revealing the accelerated kinetics of noninhibited willing channels. Our novel findings that the $\text{Ca}_v\beta$ subunit alters the kinetic changes and magnitude of inhibition by AA suggest that $\text{Ca}_v\beta$ expression may regulate how AA modulates Ca^{2+} -dependent processes that rely on L-channels. (Supported by National Institutes of Health grants R01-NS34195 and 5 T32 NS07366-09 and funding from the University of Massachusetts Medical School.)

48. Voltage-gated Ca^{2+} Channels in Trigeminal Nociceptive Processing. JIN TAO and YU-QING CAO, *Department of Anesthesiology, Washington University Pain Center, Washington University School of Medicine, St. Louis, MO 63110*

Migraine is one of the most common neurovascular disorders. Understanding the molecular biology of migraine will help to elucidate the underlying mechanisms as well as lead to more specific treatments. Familial Hemiplegic Migraine (FHM), a rare hereditary form of migraine with aura and hemiparesis, serves as a good model for exploring the mechanisms underlying migraine pathophysiology. Multiple mutations in human *CACNA1A*, the gene encoding the α_{1A} subunit of P/Q-type voltage-gated Ca^{2+} channels (VGCCs), have been identified in patients with FHM type 1 (FHM-1). We set to investigate the effect of FHM-1 mutations on the trigeminal nociceptive processing underlying migraine headache. Wild-type and mutant P/Q-type Ca^{2+} channels are expressed in cultured neurons from trigeminal ganglion and cervical spinal cord dorsal horn, the first and second relay stages in the trigeminal nociceptive pathway. We first test the effects of FHM-1 mutations on Ca^{2+} influx through P/Q-type channels. The possible functional compensation by other VGCCs is also examined. This opens the way for exploring the functional consequences of FHM-1 mutations in neuronal excitability as well as synaptic transmission. Ultimately, this will increase our understanding of the contribution of VGCCs to migraine pathophysiology. (Supported by American Heart Association grant SDG0735081N.)

49. Coupling between Ba^{2+} Extrusion and H^+ Uptake by the Plasma Membrane Calcium ATPase in Snail Neurons. ROGER C. THOMAS, *Department of Physiology,*

Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK

Snail (*Helix aspersa*) neurons extrude Ca^{2+} ions only by an ATP-driven pump, the PMCA, which also takes up H^+ ions (Schwiening, C.J., H.J. Kennedy, and R.C. Thomas. 1993. *Proc. Roy. Soc. Lond. B.* 253:285–289). The coupling ratio between Ca^{2+} ions extruded and H^+ taken up has not hitherto been measured in nerve cells. Using pH- and Ca^{2+} -sensitive microelectrodes in large voltage-clamped neurons (Thomas, R.C. 2002. *J. Gen. Physiol.* 120:567–579), I have found that Ba^{2+} ions are also extruded in exchange for H^+ ions, but more slowly than Ca^{2+} . Both Ba^{2+} and Ca^{2+} extrusion appears to be completely insensitive to increases in the membrane potential from -50 to -90 mV or to removal of external K^+ or Na^+ ions.

I have estimated the Ba^{2+} entry during a 5-s depolarization by measuring the charge carried, assuming that the depolarization generated no other ion fluxes. I have estimated the H^+ uptake during the extrusion by measuring the intracellular pH (pH_i) change, calibrated by iontophoretic injections of HCl into the same cell. I have performed six experiments, and in each at least two Ba^{2+} loads were generated and two HCl injections were made. For each experiment, I calculated the average pH_i change for the Ba^{2+} extrusions and HCl injections. Per μC of charge, the average for the six cells was a pH_i change of 0.29 for Ba^{2+} extrusion and 0.27 for H^+ injection. This suggests that each Ba^{2+} ion was extruded by the PMCA in exchange for two H^+ ions, explaining the pump's insensitivity to changes in membrane potential. It is likely that the Ca/H ratio is the same. (Supported by the Wellcome Trust.)

50. Genetic and Acquired Neural Diseases Involving Voltage-gated Calcium Channels. RICHARD W. TSIEN, CURTIS F. BARRETT, PARSA SAFA, YI-REN CHEN, EDWARD S. BOYDEN, and Y. JOYCE LIAO, *Department of Molecular and Cellular Physiology, Beckman Center, Stanford University, Stanford, CA 94305*

Voltage-gated Ca^{2+} channels link depolarization to Ca^{2+} entry, thereby regulating many important cellular processes. How lesions in Ca^{2+} channels give rise to a wide range of diseases, including genetic and acquired neurological disorders, remains unclear. One approach is to characterize fundamental defects in channel function and to clarify how these might engender pathological behavior, capitalizing when possible on animal models that recapitulate elements of the disorder. Here, we report some early steps toward understanding how specific Ca^{2+} channel defects contribute to a rare type of autism and an acquired form of cerebellar ataxia.

A mutation in the pore-forming subunit of an L-type calcium channel (CaV1.2) causes autism in patients with Timothy syndrome (TS). This rare genetic disorder

is characterized by autism spectrum disorder (ASD) along with cardiac arrhythmias and webbed fingers (Splawski, I., K.W. Timothy, L.M. Sharpe, N. Decher, P. Kumar, R. Bloise, C. Napolitano, P.J. Schwartz, R.M. Joseph, K. Condouris, et al. 2004. *Cell*. 119:19–31). The most common mutation causes a single amino acid change (G406R) near domain IS6. Knock-in mice with the TS point mutation display a surplus of repetitive and stereotyped digging behavior and a paucity of time investigating social stimuli (Wersinger, S.R., R.A. Hesse, M.A. Badura, G.C.L. Bett, and R.L. Rasmuson. 2007. Society for Neuroscience Meeting. Abstr. 62.6/W17). We find that the TS mutation powerfully and selectively slows voltage-dependent inactivation (VDI) while sparing or possibly speeding the kinetics of calcium-dependent inactivation (CDI). The deceleration of VDI was observed when the L-type channels were expressed with $\beta 1$ subunits prominent in the brain as well as $\beta 2$ subunits of importance for the heart. Dissociation of VDI and CDI was further substantiated by measurements of Ca^{2+} channel gating currents and by analysis of another channel mutation (I1624A) that hastens VDI, acting upstream of the step involving Gly406. As highlighted by the TS mutation, CDI does not proceed to completeness but levels off at $\sim 50\%$, consistent with a change in gating modes and not an absorbing inactivation process. Thus, the TS mutation offers a unique perspective on mechanisms of inactivation as well as a promising starting point for exploring the underlying pathophysiology of autism.

Dysfunction of P/Q- and N-type VGCCs may also underlie acquired peripheral and central nervous system diseases (for example, in neurological disorders associated with small-cell lung cancer, including Lambert–Eaton myasthenic syndrome [LEMS] and paraneoplastic cerebellar ataxia [PCA]). Although the pathogenic role of anti-VGCC antibody in LEMS is well established, and anti-VGCC antibody has been found in a significant fraction of PCA patients, its contribution to PCA is still unclear. Using a polyclonal peptide antibody against a major immunogenic region in P/Q-type VGCCs (the extracellular domain III S5–S6 loop), we demonstrated that such antibody was sufficient to inhibit VGCC function in neuronal and recombinant VGCCs. The antibody strongly altered cerebellar synaptic transmission and conferred the phenotype of cerebellar ataxia when administered to the cerebellum of mice. Our data support the hypothesis that anti-VGCC antibody may play a significant role in the pathogenesis of cerebellar dysfunction in PCA.

Session 5: TRP Channels

51. Role of Hydrogen Peroxide in Insulin Release by BRIN BD11 Cells. R. BEAUWENS,^{1,2} R. CRUTZEN,^{1,2}

N. MARKADIEU,^{1,2} A. BOOM,^{1,2} K. LOUCHAMI,^{1,2} W.J. MALAISSE,^{1,2} and A. SENER,^{1,2} ¹Laboratory of Cell and Molecular Physiology and ²Laboratory of Experimental Hormonology, Free University of Brussels, B-1050 Brussels, Belgium

We have previously demonstrated that incubation of β cells and BRIN BD11 cells in a hypotonic medium leads to increased insulin release (Beauwens, R., L. Best, N. Markadieu, R. Crutzen, K. Louchami, P. Brown, A.P. Yates, W.J. Malaisse, and A. Sener. 2006. *Endocrine*. 30:353–363). Hypotonic shock induces opening of volume-sensitive anion channels (VSAC) and, thus, chloride efflux, depolarization, and opening of voltage-sensitive calcium channels leading to insulin release. However, the cascade leading to the gating of VSAC is not characterized. In other cells, a role for increased production of superoxide anion and hydrogen peroxide has been established (Varela, D., F. Simon, A. Riveros, F. Jørgensen, and A. Stutzin. 2004. *J. Biol. Chem* 279:13301–13304). Therefore, we investigated the effect on insulin release by BRIN BD11 cells on exposure to increasing concentration of hydrogen peroxide. An increase in insulin release similar to that induced by a hypotonic shock (by removal of 50 mM NaCl from the medium) was observed upon exposure to 100 μM hydrogen peroxide, and it was also similarly blocked by 100 μM NPPB, suggesting that opening of VSAC also mediates the insulin-releasing effect of hydrogen peroxide.

Hydrogen peroxide is intracellularly produced by different sources, including mitochondria and NAD(P)H oxidases (Nox), among which Nox 1, 2, and 4 have been detected both at the protein and mRNA level of expression. Diphenylene iodonium (DPI), a universal inhibitor of Nox enzyme, was observed to blunt the response to hypotonicity at 10 μM ; other Nox inhibitors tested, phenylarsine oxide (2 μM) and plumbagin (30 μM), also drastically reduced the insulin release induced by hypotonic shock.

Because plumbagin appears to specifically inhibit Nox4, we suggest that cell swelling observed in BRIN BD11 cells exposed to a hypotonic shock leads to increased superoxide production presumably by Nox 4, increased hydrogen peroxide production, and subsequent activation of VSAC. This mechanism may also be operative at a lower level during glucose stimulation, as glucose-stimulated insulin release by isolated islet has also been reported to be decreased by DPI (Uchizono, Y., R. Takeya, M. Iwase, N. Sasaki, M. Oku, H. Imoto, M. Iida, and H. Sumimoto. 2006. *Life Sci*. 80:133–139). We would like to stress that the increase in hydrogen peroxide as suggested here is low and transient as opposed to the high and sustained increase observed in the pathological oxidative stress possibly involved in β cell dysfunction in type 2 diabetes.

52. TRPC6 Expression and Cell Proliferation in Liver Cancer Cell Line. EL BOUSTANY CHARBEL, BIDAUX GABRIEL, ENFISSANTOINE, DELCOURT PHILIPPE, PREVARSKAYA NATALIA, and CAPIOD THIERRY, *Institut National de la Santé et de la Recherche Médicale, Unité 800, IFR143, Villeneuve d'Ascq F-59655, France; University Sciences et Technologies Lille 1, Villeneuve d'Ascq F-59655, France* (Sponsored by the Ligue Nationale contre le Cancer)

We have recently demonstrated that store-operated calcium entry (SOCE) is the main Ca^{2+} influx pathway involved in controlling proliferation of the human hepatoma cell lines HepG2 and Huh-7 (Enfissi, A., S. Prigent, P. Colosetti, and T. Capiod. 2004. *Cell Calcium*. 36:459–467). However, the molecular nature of the calcium channels involved in this process remains unknown. Huh-7 and HepG2 cells express TRPC1 and TRPC6 (transient receptor potential canonical 1 and 6) as well as STIM1 and Orai1, and these four channels are the most likely candidates accounting for SOCE in these cells. Stable TRPC6-overexpressing or TRPC6 knockdown Huh-7 clones were generated to investigate correlations between the presence of the protein, the rate of cell proliferation, and SOCE amplitude. Proliferation rate and SOCE amplitude were increased by 80% and 160%, respectively, in TRPC6-overexpressing Huh-7 cells as compared with control cells. In contrast, proliferation rate and SOCE amplitude were reduced by 50% and 85%, respectively, in TRPC6 knockdown clones compared with untransfected cells. OAG-induced calcium entry was similar in all cells, and siRNA against TRPC1 had no effect on SOCE amplitude, highlighting the relationship between SOCE, TRPC6, and cell proliferation in Huh-7 cells. SOCE amplitude was reduced by STIM1 and Orai1 knockdowns, suggesting possible cooperation between these proteins and TRPC6 in these cells. EGF and HGF increased TRPC6 expression and SOCE amplitude in Huh-7 cells, and cyclin D1 expression was decreased by STIM, Orai1, and TRPC6 knockdowns. TRPC6 was very weakly expressed in isolated hepatocytes from healthy patients and expressed more strongly in samples obtained from tumoral zones from liver of cancer patients. Immunocytochemical staining indicated that TRPC6 was expressed on hepatocytes solely in the tumoral zones of human liver, strongly supporting a role for these calcium channels in liver oncogenesis.

53. Differential Effects of Cholestatic and Choleretic Bile Acids on Store-operated Ca^{2+} Channels in Liver. J. CASTRO,¹ E.C. AROMATARIS,² G.J. BARRITT,¹ and G. Y. RYCHKOV,² ¹Department of Medical Biochemistry, School of Medicine, Flinders University, Adelaide, South Australia 5001, Australia; ²School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

Cholestasis is a pathological condition of reduced bile formation or flow. It arises from hepatocellular dysfunction, in which bile formation and secretion are impaired, or from intrahepatic or extrahepatic biliary obstruction, in which movement of bile along the biliary tree is impeded. Retention of bile salts within the liver in cholestasis plays a critical role in development and progression of the disease. The more hydrophobic, also called cholestatic bile acids, including tauroolithocholate (TLCA) and lithocholate (LCA), amplify the condition by further inhibiting bile flow and causing liver damage. The less hydrophobic bile acids, also called choleretic, including taurodeoxycholate (TDCA) and ursodeoxycholate (UDCA), enhance bile flow and are used to treat some forms of cholestasis. Bile formation, secretion, and flow are largely regulated by Ca^{2+} -mobilizing hormones and intracellular Ca^{2+} . On the other hand, bile salts themselves are known to release Ca^{2+} from intracellular stores and induce Ca^{2+} entry. However, the nature of the Ca^{2+} entry pathways involved is not known. The aim of the present experiments was to identify the Ca^{2+} channels involved in the actions of choleretic and cholestatic bile acids on hepatocyte $[\text{Ca}^{2+}]_{\text{cyt}}$. Using whole cell patch clamping and Ca^{2+} imaging, we show that TDCA and other choleretic bile acids reversibly activate store-operated Ca^{2+} channels in liver cells, whereas LCA and other cholestatic bile acids cause their inhibition. The activation of Ca^{2+} entry by TDCA was observed upon direct addition of the bile acid to the incubation medium, whereas the inhibition by LCA required a 12-h preincubation. TDCA and LCA each caused a redistribution of stromal interaction molecule 1 similar to that induced by thapsigargin, but only TDCA activated Ca^{2+} entry. It is concluded that physiological and pathological effects of bile salts in the liver involve their actions on store-operated Ca^{2+} channels.

54. Multiple Sources of Light-evoked Intracellular Calcium Increases in *Hermisenda* Type B Photoreceptors: Extracellular Calcium Influx and ER Store Release. JOEL CAVALLO, JEFF JOHNSON, NANCY METCALF, EMILY JORDAN, and JOSEPH FARLEY, *Program in Neuroscience, Indiana University, Bloomington, IN 47405*

Previous research suggests that learning-produced changes in excitability and K^{+} currents of *Hermisenda* type B photoreceptors are Ca^{2+} -dependent phenomena. Little information is available concerning the sources and dynamics of Ca^{2+} in these cells. We have used Fura-2 dual-wavelength (340/380 nm) photometry to measure somatic $[\text{Ca}^{2+}]_{\text{i}}$ in B cells. 30-s light steps (LS) produce a large increase in $[\text{Ca}^{2+}]_{\text{i}}$ (~246%). To determine the contribution of Ca^{2+} influx versus Ca^{2+} release, we measured $[\text{Ca}^{2+}]_{\text{i}}$ throughout five consecutive LS in either normal or Ca^{2+} -free ASW (0 mM Ca^{2+} and 30 mM EGTA). For three of seven cells exposed to Ca^{2+} -free ASW, basal $[\text{Ca}^{2+}]_{\text{i}}$ was below detection limits. The other

cells had a basal $[Ca^{2+}]_i$ much lower than when external Ca^{2+} was present. Ca^{2+} -free ASW abolished light-induced $[Ca^{2+}]_i$ increases in all seven cells. We next explored the role of voltage-gated Ca^{2+} channels (VGCCs) to Ca^{2+} influx with the use of 5 mM cobalt. Co^{2+} did not affect either basal $[Ca^{2+}]_i$ or light-induced $[Ca^{2+}]_i$ increases ($n = 5$). To assess the contributions of the ER to light-induced $[Ca^{2+}]_i$ changes, B cells were incubated in 50 μ M of the ryanodine receptor (RyR) blocker dantrolene. Dantrolene reduced the $[Ca^{2+}]_i$ response by $\sim 33\%$ ($n = 5$) and also produced a progressive reduction in basal $[Ca^{2+}]_i$ ($\sim 60\%$). Exposure of Ca^{2+} -free ASW cells ($n = 3$) to 100 μ M–1 mM thapsigargin (TH; a blocker of the ER Ca^{2+} -ATPase pump) increased basal $[Ca^{2+}]_i$, which is consistent with store depletion. Collectively, our results indicate that $[Ca^{2+}]_o$ is necessary for normal basal $[Ca^{2+}]_i$ and critical for light-induced $[Ca^{2+}]_i$ increases, but little Ca^{2+} enters through VGCCs. This suggests that $[Ca^{2+}]_o$ enters the cytosol via other routes (e.g., TRP channels) or that the contribution of Ca^{2+} through VGCCs is slight but serves to trigger Ca^{2+} -induced Ca^{2+} release (CICR) from ER stores.

55. New Ion Channels and Their Potential Physiological Roles. **DAVID CLAPHAM**, *Howard Hughes Medical Institute, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115*

New families of ion channels that increase intracellular or alter organellar calcium levels provide new insights about channel gating and permeation. Unexpectedly, they also alter our understanding of physiological mechanisms and, thus, diseases. These include the TRP family, CatSper, MiCa, Stim/Orai channels (SOC), and HVI1.

56. Calcium Signaling in Polycystic Kidney Disease. **BARBARA E. EHRLICH**, *Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520*

Polycystin-2 (PC2), a member of the transient receptor potential (TRP) family of ion channels (TRPP2), forms a calcium (Ca^{2+})-permeable cation channel. Mutations in PC2 lead to polycystic kidney disease (PKD). The downstream signaling pathway involves activation of intracellular Ca^{2+} release channels, but subsequent steps are still to be identified. Similarly, upstream steps are still unclear but have been hypothesized to depend on changes in intracellular Ca^{2+} levels. Of the two known intracellular Ca^{2+} release channels, the ryanodine receptor (RyR) and the inositol 1,4,5 trisphosphate receptor (InsP₃R), only the RyR has been hypothesized to interact with PC2. We have found a direct physical interaction between PC2 and both intracellular Ca^{2+} release channels and have observed functional consequences of these interactions. Biochemical assays showed that the N terminus of PC2 binds the RyR,

whereas the C terminus only binds to RyR in its open state. *Pkd2*^{-/-} cardiomyocytes had higher frequency of spontaneous Ca^{2+} oscillations, reduced Ca^{2+} release from the sarcoplasmic reticulum stores, and reduced Ca^{2+} content compared with *Pkd2*^{+/+} cardiomyocytes. Epithelial cells primarily contain InsP₃R; the N terminus of PC2 directly interacts with this channel as well. PC2-null ImCD cells exhibited spontaneous Ca^{2+} oscillations. After reintroducing PC2 into the PC2-null cells, no spontaneous oscillations were observed. These interactions among intracellular channel proteins are important for understanding regulation of Ca^{2+} signaling and its role in cyst formation and in PKD.

57. The Role of TRPC3 Protein in Receptor- and Store-operated Calcium Entry in A431 Cells. L. GLUSHANKOVA,¹ E. KAZNACHEYEVA,¹ V. ALEXEENKO,¹ O. ZIMINA,¹ A. SKOPIN,¹ I. BEZPROZVANNY,² and G. MOZHAYEVA,¹ ¹*Institute of Cytology of the Russian Academy of Sciences, 194064 St. Petersburg, Russia;* ²*Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390*

Transient receptor potential (TRP) channels are important players in several human diseases and provide novel targets for pharmacological intervention. TRP channels play a role in human blood pressure regulation and hypertension. It was shown that TRP channel members have a potential role in the growth of human cancer cells and development of drug resistance.

Receptor-activated Ca^{2+} influx is mediated largely by store-operated channels. TRPC channels mediate a significant portion of the receptor-activated Ca^{2+} influx. However, whether any of the TRPC channels function as the store-operated channels remain controversial. In our previous works, we have demonstrated that in human carcinoma, A431 cells are present in the low-conductance moderately selective Ca^{2+} channels as well as in highly selective CRAC channels. These channels can be activated either via PLC-linked receptor stimulation or by passive store depletion. Because it was known that some mammalian proteins related to the TRPC subfamily may mediate both receptor- and store-operated Ca^{2+} influx, we investigated the role of the native TRPC3 in mediating store- and receptor-operated calcium entry in A431 cells. Using the small interfering RNA (siRNA) technique, we have created the cell line with TRPC3 expression knocked down. Using the fluorimetric intracellular calcium concentration measurements, we have found that in these cells, the store-operated calcium influx is significantly decreased, whereas receptor-operated calcium influx is unchanged. Whole-cell current measurements have confirmed this observation. Analysis of the single-channel currents allowed us to conclude that TRPC3 suppression results in the disappearance of the specific subtype of nonselective store-operated Ca^{2+} channels and appearance of the new type

of store-independent channels. Our work suggests that TRPC3 protein is required for the formation of functional store-operated channels in A431 cells. (Supported by the Molecular and Cellular Biology program, Russian Academy of Sciences [to G. Mozhayeva]; Russian Basic Research Foundation grant 07-04-01224 [to E. Kaznacheyeva], grant 07-04-01107 [to G. Mozhayeva], and grant SS-1135.2008.4 [to G. Mozhayeva]; and National Institutes of Health grant NS38082 [to I. Bezprozvanny].)

58. Evidence that TRPM7 is Required for Breast Cancer Cell Proliferation. ARNAUD GUILBERT,¹ MATHIEU GAUTIER,¹ ISABELLE DHENNIN-DUTHILLE,¹ NATHALIE HAREN,¹ HENRI SEVESTRE,^{1,2} and HALIMA OUADID-AHIDOUCH,¹ ¹*Laboratoire de Physiologie Cellulaire et Moléculaire, JE: Canaux ioniques dans le cancer du sein, Faculté des Sciences, 80039 Amiens, France;* ²*Service d'Anatomie Pathologique, CHU Nord, 80054 Amiens, France*

One major clinical problem with breast cancer is the cell's ability to proliferate. Because transient receptor potential (TRP) channels have been implicated in tumor cells, we have investigated the presence of TRPM7 and its role in cell proliferation in the MCF-7 breast cancer cell line, in human breast cancer epithelial primary culture (hBCE), and in breast cancer tissues. Under the patch clamp technique, a spontaneous Mg^{2+} -sensitive cationic current was developed in MCF-7 cells. This current was characterized by an inward current and a strong outward rectification current, both inhibited by intracellular Mg^{2+} and Mg^{2+} -ATP. The inward current was reduced only by La^{3+} , in contrast to the outward current, which was sensitive to 2-aminoethoxydiphenyl borate (2-APB), spermine, La^{3+} , and flufenamic acid. A Mg^{2+} -sensitive current was also recorded in hBCE cells. It was also inhibited by intracellular Mg^{2+} . TRPM7 transcripts were detected in both hBCE and MCF-7 cells. Down-regulation of TRPM7 by siRNA inhibited the currents, particularly the inward one, the basal intracellular Ca^{2+} concentration, and MCF-7 cell proliferation. Moreover, cell proliferation decreased when we reduced the extracellular Ca^{2+} . TRPM7 is expressed at both mRNA and protein levels in 26 normal and carcinoma breast tissues, and its expression in breast cancer depends on the proliferation index (Ki67). TRPM7 is overexpressed in grade III breast cancer tissues when Ki67 is greatest, whereas its expression is unchanged when Ki67 is lowest. Our findings strongly suggest the importance of TRPM7 in generating spontaneous Ca^{2+} influx that is probably associated with the proliferative potential of breast cancer cells.

59. Using High-bandwidth Dynamic Atomic Force Microscopy to Monitor Insulin Secretion in Isolated Islets of Langerhans. G.-H. KIM,¹ P. KOSTERIN,¹ A.L. OBAID,¹ N.M. DOLIBA,² F.M. MATSCHINSKY,² and

B.M. SALZBERG,^{1,3} ¹*Department of Neuroscience,* ²*Department of Biochemistry and Biophysics, and* ³*Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104*

Impairment of insulin secretion is a primary cause of type II diabetes. For this reason, novel technologies capable of monitoring secretion by pancreatic islets in real time may reveal biophysical markers of early molecular events associated with this disease. In this spirit, we implemented a high-bandwidth dynamic atomic force microscope (HBD AFM). A commercially available AFM (ESPM 3D; Novascan) was modified by disabling the X-Y scan and monitoring Z-axis position without feedback at up to 20 kHz using a tipless, uncoated, $350 \times 35 \times 1\text{-}\mu\text{m}$ silicon cantilever (Kim, G.H., P. Kosterin, A.L. Obaid, and B.M. Salzberg. 2007. *Biophys. J.* 92:3122–3129). We then used this device to detect small and rapid mechanical events in real time as β cells in mouse islets of Langerhans responded to the elevated glucose concentrations that stimulate insulin release. Many of the islets that we observed exhibited robust mechanical responses when the bath concentration of glucose was increased from 0 to 10 mM. These responses included an increase in high-frequency mechanical noise and a slow oscillation having a period of ~ 200 s (5 mHz); both of these effects were evident in plots of the power spectral density of the mechanical signal derived from its Fourier transform. The slow component of the mechanical signal is consistent with $[Ca^{2+}]_i$ oscillations recorded in equivalent pancreatic islets under similar conditions (Doliba, N.M., W. Qin, M.Z. Vatamaniuk, C. Li, D. Zelent, H. Najafi, C.W. Buettger, H.W. Collins, R.D. Carr, M.A. Magnuson, and F.M. Matschinsky. 2004. *Am. J. Physiol. Endocrinol. Metab.* 286:E834–E843). The nanometer-scale mechanical phenomena exhibited by pancreatic β cells may help to elucidate the sequence of events that links changes in β cell volume to $[Ca^{2+}]_i$ changes and insulin release. (Supported by U.S. Public Health Service grants NS 40966 to B.M. Salzberg and NIDDK 22122 to F.M. Matschinsky and by Bridge Funding from the University of Pennsylvania School of Medicine to B.M. Salzberg.)

60. Store-operated Cyclic AMP Signaling Mediated by STIM1 and STIM2. KONSTANTINOS LEFKIMMIATIS,^{1,2} MEERA SRIKANTHAN,^{1,2} MARYPAT MOYER,³ SILVANA CURCI,^{1,2} and ALDEBARAN M. HOFER,^{1,2} ¹*VA Boston Healthcare System and* ²*Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, West Roxbury, MA 02132;* ³*INCELL Corporation LLC, San Antonio, TX 78249*

Depletion of Ca^{2+} from the endoplasmic reticulum (ER) results in activation of plasma membrane Ca^{2+} entry channels. This “store-operated” process requires translocation of a transmembrane ER Ca^{2+} sensor protein, stromal interaction molecule 1 (STIM1), to sites

closely apposed to Ca^{2+} channels at the cell surface. It is not known, however, whether a reduction in store Ca^{2+} is coupled to other signaling pathways via this mechanism. We found that lowering free $[\text{Ca}^{2+}]$ within the ER independent of cytosolic $[\text{Ca}^{2+}]$ also led to recruitment and activation of adenylyl cyclase. Reduction of luminal ER Ca^{2+} using ionomycin, agonists, SERCA inhibitors, or the membrane-permeant Ca^{2+} chelator TPEN resulted in enhanced cAMP accumulation, measured in real time using a FRET- and Epac-based cAMP indicator in single NCM460 colonocytes. Similar results were obtained using quantitative competitive immunoassay for cAMP. Depletion of ER Ca^{2+} also resulted in increased PKA activity, as measured using another FRET-based sensor, AKAR3. Translocation of STIM1 and the related protein, STIM2, was required for efficient coupling of ER Ca^{2+} depletion to adenylyl cyclase activity. Knockdown of STIM1 and STIM2 using shRNA (identified using mCherry expression) significantly reduced the cAMP elevation compared with control cells in the same field. Furthermore, the compound ML-9, which was recently shown to inhibit STIM translocation and store-operated Ca^{2+} entry, also significantly inhibited ionomycin-induced elevation of intracellular $[\text{cAMP}]$. We propose the existence of a new pathway (store-operated cAMP signaling [SOcAMPS]) in which the internal store Ca^{2+} content is directly connected to cAMP signaling via STIM proteins. These data may explain how certain cAMP-dependent processes (e.g., fluid and enzyme secretion) become synergized by concurrent activation of GPCRs coupled to cAMP and Ca^{2+} signaling cascades.

61. Role of TRPM Channels Family in β Cell Signaling and Diabetes. **REINHOLD PENNER**, *Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawai'i at Manoa, Honolulu, Hawai'i 96813*

Calcium ions play an important role in regulating numerous cellular functions, controlling muscle contraction, brain activity, or hormone release, including insulin. However, excessive calcium accumulation brought about by calcium influx into cells can lead to cell death. Juvenile or type 1 diabetes is the result of excessive β cell death, whereas relative insulin deficiency and insulin resistance characterizes diabetes type 2. We have identified a number of ion channels of the melastatin-related transient receptor potential (TRPM) subfamily that regulate the transport of calcium into cells and are also expressed in pancreatic β cells: the Ca^{2+} -activated nonselective cation channels TRPM4 and TRPM5, the Mg-nucleotide-regulated TRPM7, as well as the ADP-ribose-activated Ca^{2+} -permeable channel TRPM2. The primary function of TRPM4 and TRPM5 is to depolarize cells, and this function supports electrical activity in β cells. We confirmed that TRPM4 is involved in controlling Ca^{2+} influx in β

cells because cellular knockout of TRPM4 inhibits insulin release. TRPM7 functions as a homeostatic mechanism for Ca^{2+} and Mg^{2+} transport into cells. Like ATP-dependent K^{+} channels, which are crucial for initiating insulin release, TRPM7 is regulated by Mg-ATP, and we found that endogenous TRPM7 channel activity in β cells requires severe Mg^{2+} and Mg-nucleotide depletion in order to be activated. Although the above TRPM channels may participate in insulin release, both in healthy cells and type 2 diabetes, the presence of TRPM2 is particularly interesting in the context of type 1 diabetes because it is a Ca^{2+} -permeable channel that is activated by both immunological stimuli such as $\text{TNF}\alpha$ and environmental factors such as alloxan, a chemical that causes experimental diabetes. This presentation will explore the regulation of selected TRPM channels in β cell signaling and their potential function in diabetes types 1 and 2. (Supported by National Institutes of Health grant R01-GM063954.)

62. Intracellular Ca^{2+} Release Channels as Targets for Immunosuppression. **PRATIMA THAKUR, SEPEHR DADSETAN, LIUDMILA ZAKHAROVA, and ALLA F. FOMINA**, *Department of Physiology and Membrane Biology, University of California, Davis, Davis, CA 95616*

Despite the fact that Ca^{2+} release from IP_3 (IP_3R) and ryanodine (RyR) receptors is an early event after T cell receptor stimulation, their role in prolonged elevation of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is necessary for acquisition of T cell effector functions, is considered to be minor. However, we found the evidence that IP_3R and/or RyR are activated by the store-operated Ca^{2+} entry (SOCE) and control $[\text{Ca}^{2+}]_i$ dynamics in human T cells by regulating Ca^{2+} retention within the store.

Sequential store depletion with cyclopiazonic acid revealed that Ca^{2+} sequestration into the store during SOCE occurred without global $[\text{Ca}^{2+}]_i$ elevation, whereas Ca^{2+} appearance in the cytosol correlated with store depletion. Furthermore, the IP_3R or RyR blockers xestospongins C or ryanodine inhibited $[\text{Ca}^{2+}]_i$ elevation upon SOCE activation in parallel with enhancement of Ca^{2+} retention within the store. These indicate that Ca^{2+} sequestration into the store occurs before the Ca^{2+} appearance in the cytosol and that Ca^{2+} release from IP_3R and/or RyR is the major determinant in shaping $[\text{Ca}^{2+}]_i$ signaling in T cells.

Interestingly, Ca^{2+} sequestration into the store persisted when SOCE was inhibited with 1–10 μM La^{3+} . In addition, perforated-patch and Mn^{2+} -quench experiments revealed the presence of Ca^{2+} influx at unstimulated conditions. We speculate that store-independent Ca^{2+} entry pathway may supply Ca^{2+} for continuous store refilling, whereas the activation of SOCE is necessary for the triggering of Ca^{2+} release from IP_3R and/or RyR .

The importance of intracellular Ca^{2+} -release channels in lymphocyte functioning is underscored by our findings that inhibition of IP₃R or RyR significantly down-regulated T cell proliferation and interleukin 2 production. Thus, our studies revealed a new aspect of $[\text{Ca}^{2+}]_i$ signaling in T cells, i.e., SOCE-dependent Ca^{2+} release via IP₃R and/or RyR and identified the IP₃R and RyR as potential targets for immunosuppression. (Supported by American Heart Association grant-in-aid 0755086Y to A.F. Fomina.)

63. Highly Ca^{2+} -selective TRPM Channels Regulate IP₃-dependent Oscillatory Ca^{2+} Signaling in the *C. elegans* Intestine. JUAN XING,^{1,2,3} XIAOHUI YAN,^{1,2,3} ANA ESTEVEZ,^{1,2,3} and KEVIN STRANGE,^{1,2,3} ¹*Department of Anesthesiology,* ²*Department of Molecular Physiology and Biophysics,* and ³*Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232*

Posterior body wall muscle contraction (pBoc) in the nematode *Caenorhabditis elegans* occurs rhythmically every 45–50 s and mediates defecation. pBoc is controlled by inositol-1,4,5-trisphosphate (IP₃)-dependent Ca^{2+} oscillations in intestinal epithelial cells. The intestinal epithelium can be studied by patch clamp electrophysiology, Ca^{2+} imaging, genome-wide reverse genetic analysis, forward genetics, and molecular biology and thus provides a powerful model to develop an integrated systems level

understanding of a nonexcitable cell oscillatory Ca^{2+} signaling pathway. Intestinal cells express an outwardly rectifying Ca^{2+} (ORCa) current with biophysical properties resembling those of TRPM channels. Two TRPM homologues, GON-2 and GTL-1, are expressed in the intestine. Using deletion and severe loss-of-function alleles of the *glt-1* and *gon-2* genes, we demonstrate that GON-2 and GTL-1 are both required for maintaining rhythmic pBoc and intestinal Ca^{2+} oscillations. Loss of GTL-1 and GON-2 function inhibits I_{ORCa} ~70% and ~90%, respectively. I_{ORCa} is undetectable in *gon-2*; *glt-1* double mutant cells. These results demonstrate that (1) both *gon-2* and *glt-1* are required for ORCa channel function and that (2) GON-2 and GTL-1 can function independently as ion channels, but their functions in mediating I_{ORCa} are interdependent. I_{ORCa} , $I_{\text{GON-2}}$, and $I_{\text{GTL-1}}$ have nearly identical biophysical properties. Importantly, all three channels are at least 60-fold more permeable to Ca^{2+} than either Na^+ or Mg^{2+} . With the exception of TRPV5 and TRPV6, the ORCa, GON-2, and GTL-1 channels thus have the highest Ca^{2+} selectivities of any identified TRP channels. Epistasis analysis suggests that GON-2 and GTL-1 function in the IP₃ signaling pathway to regulate intestinal Ca^{2+} oscillations. We postulate that GON-2 and GTL-1 form heteromeric ORCa channels that mediate selective Ca^{2+} influx and that they function to regulate IP₃ receptor activity and possibly to refill ER Ca^{2+} stores.