in response to a Ca+2 flux. Synaptotagmin 1 is the vesicle-localized, Ca+2-dependent phospholipid binding protein that mediates this Ca+2 sensitivity. Synaptotagmin is composed of a single transmembrane span with an ~130 amino acid unstructured tether to a Ca+2 binding domain (C2A). A second Ca2+ binding domain (C2B) is attached to C2A by a 9 amino acid connector. We have suggested that the high local concentration of vesicle membrane sensed by C2A due to this tethering leads to robust binding to lipid (Kertz. 2007. BJ. *92*:1408). This same concept of tethering may also be applied to C2A with regard to C2B. We hypothesized that C2A is connected to C2B to enhance interactions between the domains. Such an interaction is predicted to alter the ability of C2A and C2B to interact with ligands and transduce binding information. To be reversible, the interaction is predicted to be weak due to the high local concentration of one C2 domain to another. We compared the protein stabilities and affinities in the presence and absence of Ca2+ and/or phospholipid of the isolated C2 domains with the full-length soluble construct. If there is not an interaction between the domains, the stability and binding data of C2AB is predicted to be the average of the stabilities and affinities of the individual domains. Deviation from the mean is predicted if interactions occur between the domains. We do not find the mean to describe the stability and binding properties of C2AB. The data will be discussed in context of a high-resolution crystal structure of human synaptotagmin I C2AB indicating an interaction between the domains.

1288-Pos Identification Of SNARE And SNARE-associated Proteins In Cardiac Myocytes

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Board B264

In addition to its role as a pump, the heart also has endocrine functions. Cardiac myocytes contribute to blood volume control by secreting natriuretic peptide hormones, ANP and BNP by both constitutive and regulated exocytotic fusion. Detailed information, however, regarding the identity and function of specific membrane fusion proteins (SNARE proteins) involved in exocytosis in the endocrine heart is deficient. We identified SNARE and SNARE-associated proteins and determined their association with natriuretic-containing secretory granules using primary cultures of neonatal and adult rat cardiac myocytes. Cardiac myocytes were screened for mRNA transcripts by RT-PCR to determine which SNARE proteins were present and SNARE proteins known to be involved in regulated exocytosis in other cell types were further characterized in cardiac myocytes by western blot and co-immunoprecipitation methods. Localization of SNARE and SNARE-associated proteins was determined using cell fractionation and immunofluorescence methods. This study demonstrated that multiple SNARE proteins are present in neonatal and adult cardiac myocytes suggested the importance of SNARE proteins in exocytosis of natriuretic peptides from the endocrine heart.

Exocytosis & Endocytosis - II

1289-Pos Efficiency of Amperometric Catecholamine Detection with Transparent Microelectrodes: Comparison of ITO with Ultrathin Gold

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Board B265

Chromaffin cells of the adrenal gland constitute a model system for neuronal exocytosis where quantal release of catecholamines can be measured amperometrically, usually employing a carbon fiber electrode. Release from single vesicles is indicated by an amperometric current spike from oxidation of the released molecules (Wightman, et al 1991 PNAS *88*: 10754), often preceded by a foot signal (Chow, et al 1992 Nature *356*: 60) indicating opening and expansion of the fusion pore (Albillos, et al 1997 Nature *389*: 509). The charge obtained from the integrated amperometric spike is proportional to the number of molecules released from this vesicle with two electronic charges transferred per molecule.

To gain information about the mechanisms of transmitter release it is desirable to perform simultaneous fluorescence imaging and electrochemical detection of individual events. Additionally, to minimize diffusional broadening, the electrode needs to be in close contact with the cell membrane. For simultaneous fluorescence imaging through the electrode we fabricated transparent electrochemical detector arrays on glass using photolithography. Transparent planar microelectrode arrays were fabricated from either indium tin oxide (ITO), or ultrathin (~14 nm) gold. Amperometric spikes from bovine chromaffin cells could be detected with both materials at low noise, and fluorescence changes monitored through the electrodes with TIRF microscopy. Foot signals were detected with both types of electrodes. For arrays with similar geometry, the mean amperometric charge was 1.11±0.24pC (SEM, n=9 cells) for ultrathin gold, and 0.48±0.10pC (SEM, n=13 cells) for ITO electrodes, indicating that the gold electrodes are more sensitive to catecholamines then ITO. This result suggests that about half of the electrons from catecholamine oxidation are lost at ITO electrodes, presumably in reduction of some components of the ITO surface.

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1290-Pos SV2B Regulates Intraterminal Ca^{2+} and Synaptic Vesicle Dynamics in Retinal Bipolar Neurons

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Board B266

Despite its relationship to human disease, the role that synaptic vesicle protein 2 (SV2) plays in the neuronal secretory pathway is

poorly-defined. SV2B is the SV2 isoform found in retinal ribbon synapses of the high-sensitivity rod pathway. Using a combination of capacitance and calcium measurements, we compared the secretory responses of rod bipolar neurons from wild-type and SV2B−/− mice. Bipolar neurons lacking SV2B had a resting intraterminal $[Ca^{2+}]_i$ that was approximately double that of wild-type cells. Relative to wild-type cell, SV2B−/− neurons exhibited a larger increase in cumulative $[Ca^{2+}]_i$ evoked by a short train of depolarizations, a depressed secretory response to the first pulse in the train, and a prolonged rate of membrane recovery at the cessation of the train. When the size of the secretory response evoked by each pulse was evaluated as a function of the change in $[Ca^{2+}]_i$, a rightward shift in the Ca^{2+} sensitivity of release was observed for SV2B−/− neurons. All aspects of the SV2B phenotype were rescued by lowering resting $[Ca^{2+}]_i$ in SV2B−/− neurons to control levels. By contrast, although the SV2B−/− endocytic phenotype could be mimicked in wild-type cells by elevating intraterminal $[Ca^{2+}]_i$, elevated $[Ca^{2+}]_i$ did not mimic the shift in the apparent Ca^{2+}-sensitivity of release. Together, the data suggest that SV2B indirectly regulates synaptic vesicle dynamics via its ability to regulate intraterminal $[Ca^{2+}]_i$. In addition, two distinct sites or pathways of Ca^{2+}-dependent regulation are suggested.

1291-Pos Disassembly of Clathrin Coats Monitored in Bulk and by Single Object Fluorescence Imaging

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Board B267

Endocytic clathrin-coated vesicles go through a life cycle of coated pit assembly, membrane deformation, cargo loading, vesicle budding and coat disassembly. The uncoating process is intiated after vesicle budding by association of the co-chaperone auxilin with the coat, which in turn recruits the uncoating ATPase Hsc70 (Massol et al. PNAS 2006, *103*, 10265).

We have tested the auxilin, Hsc70 and ATP dependent disassembly of coats reconstituted from a series of recombinant clathrin heavy chain mutants with increasing C-terminal truncations in bulk uncoating assays. This uncoating reaction only proceeds normally when the recombinant clathrin contains the QLMLT motif, a putative binding site for Hsc70. To further test the uncoating mechanism we are developing a single object fluorescence microscopy assay to follow the steps of the uncoating reaction on individual objects (instead of an ensemble) in real time. Clathrin coats are assembled in vitro from fluorescently tagged proteins and immobilized on chemically modified glass cover slips to monitor the diffraction limited signals from the co-planar coats using total internal reflection fluorescence microscopy. The goal is to analyze the time dependence of the uncoating reaction determined as disappearance of the fluorescence signals from coats assembled with wild type or mutant clathrin as a function of the concentrations of auxilin, Hsc70 and ATP.

1292-Pos The role of synaptobrevin 2 transmembrane domain in vesicle fusion

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Board B268

The fusion of synaptic vesicles as well as chromaffin granules is driven by the formation of the SNARE complex composed of synaptobrevin 2, the synaptosomal associated protein of 25k (SNAP25) and syntaxin 1a. Previous studies have reported a role of the transmembane (TM) domain in the homodimerization of synaptobrevin 2 in vitro (1). Specific residues were mapped at the TM region of the protein, postulating, a possible biological importance of synaptobrevin 2 TM domain interactions in the fusion machinery. To investigate this hypothesis in cellular exocytosis, we used embryonic chromaffin cells lacking both synaptobrevin2 and cellulebrevin. The double knock-out is necessary because both proteins, synaptobrevin 2 and cellubrevin can support chromaffin granule exocytosis (2). Various combinations of the mutations L99A, C103A and I111A were expressed in the cells in rescue experiments, and the change in membrane area due to exocytosis was quantified by measuring the capacitance increase in the whole-cell patch clamp configuration in response to calcium increase by caged calcium flash photolysis. The initial results suggest that when the TM domaine residues leucine99, cysteine103 and isoleucine111 were simultaneously mutated to alanine, the amplitude of exocytosis was significantly reduced suggesting fusion of fewer vesicles. We are also characterizing the properties of single exocytotic events for these mutants by the analysis of amperometry spikes, patch capacitance measurements in bovine chromaffin cells expressing these constructs.In additional experiments, we modified the intravesicular end of synaptobrevin 2, by adding extra amino acids: two lysine, two histidine or two glutamic acid residues, at the C-terminus. The preliminary results indicate that surprisingly these additions render the protein rather inactive. Expression of these constructs in embryonic mouse synaptobrevin 2/cellubrevin double knockout chromaffin cells show very small capacitance changes indicating that they do not support full excocytosis.

1293-Pos Specific Labeling Of Vesicles Using Targeted Integration Of A Genetically Encoded Fluorescence Marker

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Board B269

Genetically encoded fluorescence proteins have revolutionized microscopic imaging in cell biology. Fluorescent proteins can be used to follow protein trafficking and localization. It further allows labeling organelles of a cell by fusing these markers to organelle specific proteins. For a specific labeling the level of expression plays a critical role. Overexpression of fluorescence fusion protein under exogenous promoters often leads to mistargeting, unspecific label-

ing and artifacts, which may seriously compromise the physiological validity of an experiment. This problem can be avoided by using targeted mutagenesis of the genomic locus to create a labeled fluorescent protein that is under control of its endogenous promoter.

Synaptobrevin2 is located on synaptic vesicles as well as on large dense core vesicles (LDCVs) making it a perfect tool for studying vesicle trafficking and release. We generated a knock-in mouse line expressing synaptobrevin2 fused to monomeric red fluorescent protein (mRFP) from the synaptobrevin2 gene locus. Synaptrobrevin2-mRFP mice show specific and bright fluorescence labeling of synaptic vesicles in neurons and LDCVs in chromaffin cells. The electrophysiological characterization of neurons and chromaffin cells shows no interference of the fluorescence tag with vesicle fusion.

1294-Pos SHP-1/Cdk2 Complexes Associate with beta-Catenin/CEACAM1 and Insulin Receptor/LRP1 to Regulate Insulin-Mediated Endocytosis

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The protein tyrosine phosphatase SHP-1 modulates glucose metabolism and hepatic insulin clearance. We show here that SHP-1 is compartmentalized in endosome (G/E) fractions isolated from rat liver where it associates with the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and the insulin receptor (IR). We also show that the compartmentalized SHP-1 associates with Cdk2. The G/E-compartmentalized Cdk2/SHP-1 complexes are located in a Triton X-100 resistant microenvironment where they associate with multiple phosphorylated proteins. Two of the proteins present in Cdk2 complexes were identified by mass spectrometry as the actin anchor beta-catenin and as the endocytic receptor LRP1. Immunoprecipitations experiments confirmed formation of SHP-1/Cdk2/beta-catenin and SHP-1/Cdk2/LRP1 complexes. We demonstrate that beta-catenin is associated with CEACAM1 in plasma membrane fractions and that LRP1 associates with the internalized IR. Therefore, SHP-1 and Cdk2 are connected with the actin network by beta-catenin and CEACAM1, and also with LRP1. This suggests the presence of signaling strategies whereby both local dynamics of the actin network and the routing of LRP1 participate in a concerted manner to IR sorting.

1295-Pos Improved Nanofabricated Electrochemical Detector Arrays for Monitoring Exocytosis

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Board B271

Micro- and nanofabricated devices emerge as powerful tools to study cellular function on the nanoscale. Our group has previously fabricated electrochemical detector (ECD) arrays of platinum microelectrodes to monitor chromaffin cell exocytosis with simultaneous amperometric recording and fluorescence imaging . This approach allowed correlation of amperometrically detected release with fluorescence signals with precision of a few hundred nm. Here we demonstrate advances in the ECD fabrication and novel software techniques for improved spatiotemporal characterization of exocytosis and stimulus-secretion coupling.

In contrast to the previously photoresist-insulated ECDs, the new devices are insulated with ~300nm of fused silica which allows fast-scan cyclic voltammetric detection of catecholamines released from individual chromaffin granules. The voltammograms exhibit narrow and well-defined redox peaks. We can now functionalize our devices to incorporate surface patterned stimuli using Parylene-C . As proof of principle we surface patterned Poly-D-Lysine molecules at specific locations on the arrays for mast cell stimulation. Mast cells placed on these devices were thus stimulated and serotonin release from their granules was amperometrically detected by the ECD array. We demonstrate excellent signal-to-noise ratio of our devices by resolving the measurement of as few as ~15,000 serotonin molecules at one particular electrode. Novel software techniques performed random walk simulations and fitted the results to the experimental data to reveal the spatial location of secretory events and the time course of release of catecholamines and their diffusion to a particular electrode.

We anticipate ECD arrays to be utilized in various biophysical experiments providing a better understanding of the molecular machinery underlying exocytosis in various cells, including neurons.

1296-Pos A Role For The C-terminus Of The SNARE Protein SNAP-25 In Fusion Pore Opening

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Board B272

The formation of a fusion pore, the connection between vesicular lumen and extracellular space, is a key step of exocytosis. The SNARE hypothesis suggests that the fusion pore opening is driven

by a conformational change in the SNARE complex. Although the structure of the fusion pore remains elusive, current fusion pore models place the C-termini of all three SNAREs in or near the fusion pore. Mutations in the C-terminus of SNAP-25 affects the rate at which fusion pores are formed (1). To study the role of the SNAP-25 C-terminus in determining the structure and dynamics of the fusion pore, SNAP-25 coupled to GFP at its N-terminus was overexpressed in bovine chromaffin cells. Single exocytotic events were characterized by carbon fiber amperometry and cell-attached patch capacitance measurements. Cells overexpressing SNAP-25 delta9 (lacking last nine C-terminal residues) displayed smaller amperometric "foot-currents" indicating reduced flux of transmitter through the fusion pore. Measurements of fusion pore conductance revealed that this is due to reduced fusion pore conductance and lower fusion pore expansion rate. Fusion pores involving SNAP-25 delta9 also showed a markedly prolonged lifetime from formation to rapid expansion. These results indicate that the C-terminus of SNAP-25 not only affects the rate of fusion pore formation, but also determines the structure of the initial fusion pore, the dynamics of fusion pore expansion, and the rate of transmitter release.

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References

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1297-Pos Does the Ca^{2+} Activated Potassium Channel *h*SK3 Play a Functional Role in Endocytosis?

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Board B273

Endocytosis is a mechanism for selective internalization of plasma membrane and membrane proteins. In the nerve terminal, this pathway takes part in the recycling of synaptic vesicles. For clathrin-mediated endocytosis an array of proteins, including dynamin, amphiphysin, synaptojanin, and endophilin, have been implicated as accessory factors in the early steps of clathrin-coated pit formation. Using a *LexA*-based yeast two-hybrid system we identified *h*SK3, a Ca^{2+} activated K^+ channel, as interaction partner of endophilin 3. This interaction was verified by pull-down experiments. The human SK3 channel (*h*SK3) belongs to a family of Ca^{2+} activated K^+ channels that play a role in neuronal function by shaping single action potentials and modifying firing patterns. Sugiura *et al*., 2004 (*J Biol Chem* ***279***:23343) repoted a reduction of endocytosis in COS7 cells expressing endophilin 3. Therefore, in order to determine a physiological consequence of the *h*SK3/endophilin 3 interaction we initially established an endocytosis assay using scanning confocal microscopy to monitor endocytosis in COS7 cells, lacking *h*SK3 channels. The lipophilic styryl dye FM4-64, a general endocytosis marker, was used in order to visualize selectively the plasma membrane and internalized membrane. Cells were incubated with FM4-64 for 30 min either at 4°C (control) or at 37°C and the fluorescence signal of the internal cell lumen was then quantified. Using this assay we did not observe a difference in FM4-64 uptake in COS7 cells expressing EGFP-endophilin 3 and EGFP. This is in contrast to Sugiura et al., (*ibid*) who used labelled transferrin as an endocytotic marker. Future experiments with transferrin will clarify this discrepancy.

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Ligand-gated Channels

1298-Pos Cation and Anion Binding Sites in the Ligand-Binding Domain of Glutamate Receptors of the Kainate Subtype

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Board B274

Ionotropic glutamate receptors (iGluR), activated by the amino acid L-glutamate, form a large family of ligand gated ion channels that mediate the majority of excitatory neurotransmission in the brain. Pharmacologically, the iGluR family is categorized by the selectivity shown by individual proteins towards alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate. Ions modulate the behaviour of many receptors. Recently Plested & Meyer (Neuron 2007, *53*:829) showed that kainate receptors, but not AMPA or NMDA receptors, require both Na+ and Cl– in the extracellular region to function. Whilst only the anion binding site was identified, it was suggested that both anions and cations bind in the ligand binding domain. Using computational approaches, we have identified two cation binding sites located symmetrically opposite the anion binding site and within the same dimer interface cavity. Multiple molecular dynamics simulations and relative binding free energy calculations using thermodynamic integration were performed to study the anion and cation binding sites in detail. Simulations confirm that the identified locations are indeed cation binding sites. The rank order of binding for halide and alkali-metal ions were determined which is indicative of their binding affinities. The computational results agree well with mutagenesis and crystallographic studies.

1299-Pos Investigating the Role of Electrostatic Interactions in Glutamate Receptor Functioning

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Board B275

Elevated levels of glutamate during cerebral ischemia play a major role in "excitotoxicity" of glutamate receptors (GluRs) leading to neuron death and devastating effects on the central nervous system. Clearly understanding the mechanism by which GluRs function is critical for rational drug design while exploring subtle differences in receptor subtypes provide a means of drug specificity, eliminating