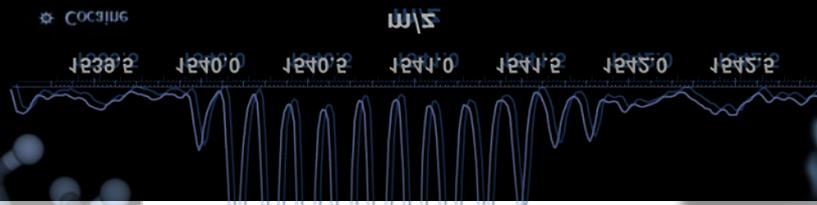


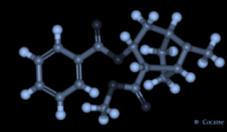
# Yale/NIDA Neuroproteomics Center



Protein Post Translational Modification (PTM)  
& Profiling Core

Erol E. Gulcicek





# Outline



## Determine sites of Post Translational Modifications and changes in their Levels

- Phosphorylation\*\*\*
  - Ubiquitination
  - Palmitoylation
  - Glycosylation
- } Poster Presentation (TuKiet Lam)

## New Methods Recently Used at Yale/NIDA Neuroproteomics Center

- **Pulsed SILAC (Nairn Lab: Shari Wiseman)**
  - Look at differential protein expression rates with or without treatment
- **SILAM - Stable Isotope Labeling of Amino acids in Mammals (or Mice)**  
**Alexandre Stipanovich, Angus Nairn and Paul Greengard**
  - A novel tool by which to quantitatively compare proteomes from tissue
  - Look at both the protein expression and phosphorylation changes
  - WT and CK1 $\Delta$  overexpressed mice to determine **CK1 $\Delta$**  function in striatal brain under *in vivo* conditions:

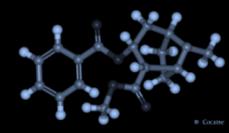


# NIDA Overall PTM Project Base



NIDA Neuroproteomics Protein Post-Translational Modification Identification & Profiling Core Participation						
Investigator	Protein Posttranslational Modifications					Project Description
	Phosphorylation	Ubiquitinylation	Palmitoylation	Glycosylation	Others	
Eipper, Betty	X			X		- Extensive Kalirin7 phosphorylation specific to drugs of abuse phosphorylated by recombinant CaMKII,
Green, William	X		X			- "familial amyotrophic lateral sclerosis(ALS)-linked SOD1 mutants are abnormally palmitoylated".
Greengard, Paul	X					- SILAM - CK1Δ over-expressing mice vs WT mice
Kaczmarek, Leonard	X					- Regulation of Slack channel proteins by phosphorylation sites.
Lombroso, Paul	X	X				- Phosphorylation sites of STEP treated by DHPG of SK-SY5Y cells.
Morabito, Maria	X	X				- Ubiquitination of the PSD-95 protein
Nairn, Angus	X	X			Methylation	- Global Ubiquitination in brain - 18 novel phosphorylation sites in the EF2 Kinase (EF2K) - Ser499, a site regulated by PKA, plays a causal role in turnover of EF2K. - Ser885 of LFC in striatal tissue is regulated by acute exposure to cocaine.
Nestler, Eric	X					- in vitro phosphorylation of ΔFosB by CaMKII in the Nucleus Accumbens Shell"
Picciotto, Marina	X					- nAChR-associated phosphoproteome in smokers vs. non-smoker subjects by IP of several proteins
Taylor, Jane	X					- phosphorylation changes induced by chronic corticosterone exposure in adolescence, indicative of increased risk for developing addictive behaviors.
Tomita, Susumu	X					- identify and quantitate phosphorylation levels of purified TARP g-8 from rodent brain.





# Phosphopeptide Enrichment - Necessary Step for Differentially Determining Phosphorylation Sites

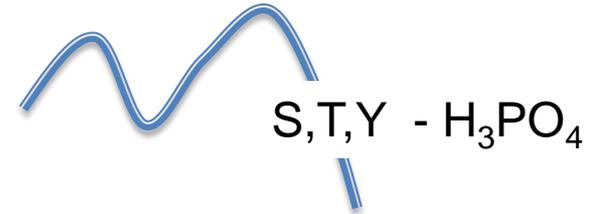


## -Phosphorylation

MS Analysis Requires Phosphopeptide Enrichment

Ser/Thr phosphorylation –  $\text{TiO}_2$  enrichment

Tyr phosphorylation – Phospho-Tyr Antibody



NIDA Investigators utilizing  
PTM Core for phosphorylation:

Eipper, Betty  
DeCamilli, Pietro  
Green, William  
Greengard, Paul  
Kaczmarek, Leonard  
Lombroso, Paul  
Morabito, Maria  
Nairn, Angus  
Nestler, Eric  
Picciotto, Marina  
Sathyanesan, Samuel  
Taylor, Jane  
Tomita Susumu



# Locating sites of phosphorylation in Kalirin-7



- Kalirin-7 participates in the formation and maintenance of dendritic spines
- Kal7 has long been implicated in long term potentiation, fear memories, and addiction like behaviors

## Identification of Kalirin-7 as a Potential Post-Synaptic Density Signaling Hub

Drew D. Kiraly,<sup>1</sup> Kathy L. Stone,<sup>1</sup> Chris M. Colangelo,<sup>2</sup> Tom Abbott,<sup>1</sup> Yanping Wang,<sup>1</sup> Richard E. Mains,<sup>1</sup> and Betty A. Eipper<sup>1,2,3</sup>

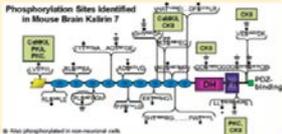
<sup>1</sup>Department of Neuroscience and <sup>2</sup>Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06030, United States

<sup>3</sup>W.M. Keck Facility, Yale University, New Haven, Connecticut 06510, United States

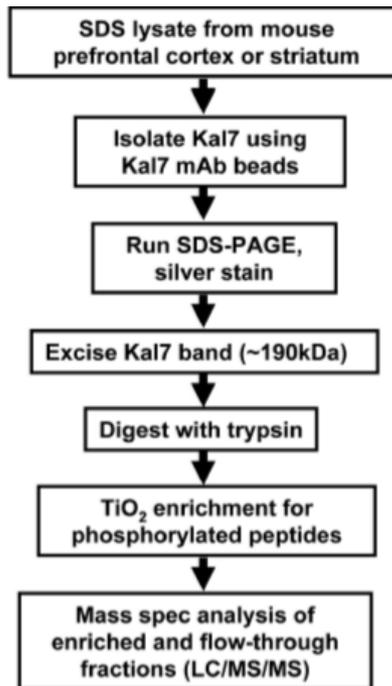
Supporting Information

**ABSTRACT:** Kalirin 7 (Kal7), a multifunctional Rho-GDP/GTP exchange factor (GEF) for Rac1 and RhoG, is embedded in the postsynaptic density at excitatory synapses, where it participates in the formation and maintenance of dendritic spines. Kal7 has been implicated in long-term potentiation, fear memories, and addiction-like behaviors. Using liquid chromatography and tandem mass spectrometry, we identified sites phosphorylated by six PSD-localized kinases implicated in synaptic plasticity and behavior, sites phosphorylated when myc-Kal7 was expressed in non-neuronal cells and sites phosphorylated in mouse brain Kal7. A site in the Sec1/p domain phosphorylated by calcium/calmodulin-dependent protein kinase II, protein kinase A and protein kinase C, was phosphorylated in mouse brain but not in non-neuronal cells. Phosphorylation in the spectrin-like repeat region was more extensive in mouse brain than in non-neuronal cells, with a total of 20 sites identified. Sites in the pleckstrin homology domain and in the linker region connecting the GEF domain to the PDZ binding motif were heavily phosphorylated in both non-neuronal cells and in mouse brain and a second GEF activity. We postulate that the kinase convergence and divergence observed in Kal7 identifies it as a key player in integration of the multiple inputs that regulate synaptic structure and function.

**KEYWORDS:** phosphorylation, CaMKII, CKII, kinase convergence, spectrin repeat, plasticity, dendritic spine, synaptogenesis, Rho-GEF



## Kal7-Work flow

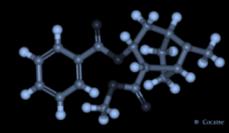


## Phosphopeptides Identified

residue	score	peptide sequence
487	82.08	K.ALLDVLQRPLSPGNSESLTATANYSKA
491	59.87	K.ALLDVLQRPLSPGNpSESLTATANYSKA
493/495	56.12	K.ALLDVLQRPLSPGNSEpSLpTATANYSKA
710	28.31	R.pSAPPSLGPEPTEAR.D
724	47.39	R.SAPPSLGPEPTEARpSAVSNNK.T
963	27.45	K.AEALLQAGHpyDADAIRECAEK.V
1188	22.95	K.LLIQLADpSFVEK.G
1249	22.84	K.DLELDIIPASLpSDRE
1520	53.17	K.LLTpSELGVTEHVEGDPCK.F
1590	61.94	K.GALKEPIQLKpTPAK.L
1598	92.54	R.NNpSKRDGVEDGDSQGDSSQpDTISIAsR
1608	70.43	K.RDGVEDGDSQGDpSSQpDTISIAsR.T
1613	71.06	K.RDGVEDGDSQGDpSSQpDTISIAsR.T
1614	57.92	R.DGVEDGDSQGDGSpSQpDTISIAsR.T
1618	59.31	R.DGVEDGDSQGDGSSQpDTISIAsR.T
1620	65.83	R.DGVEDGDSQGDGSSQpDTIISIAsR.T
1625/1626	63.93	R.pTpSQNTVESDKDGNLVP.RW
1629	54.98	R.TSQNpTVESDKDGNLVP.RW
1632	75.24	R.TSQNTVEpSDKDGNLVP.RW
1652	24.06	R.WHLGPGDPFSpTYV.-

NIDA Investigator: Betty Eipper – More this afternoon



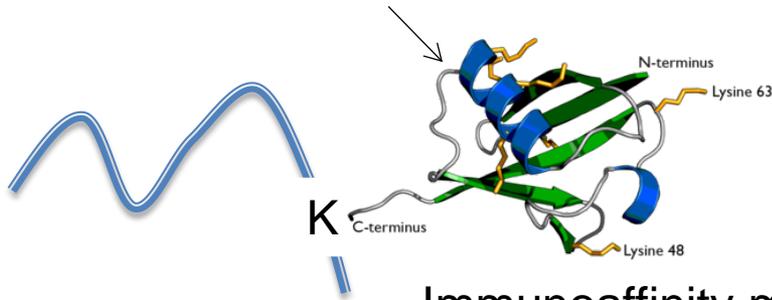


# Phosphopeptide Enrichment - Necessary Step for Differentially Determining Phosphorylation Sites



## -Ubiquitination

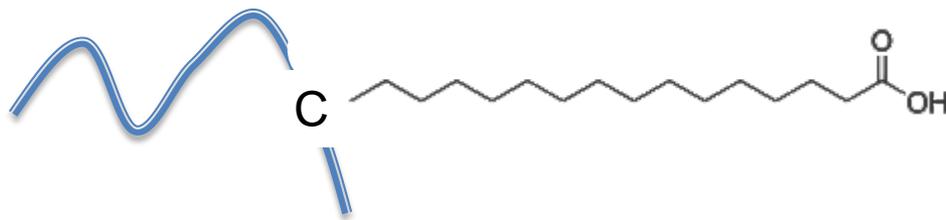
MQIFVKTTLTGKTTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGG---



Immunoaffinity motif: K-  $\epsilon$  -Gly-Gly

NIDA Investigators:  
Paul Lombroso  
Maria Morabito  
Angus Nairn

## -Palmitoylation



NIDA Investigators:  
Bill Green

Please See Tu Lam's Poster for more detail

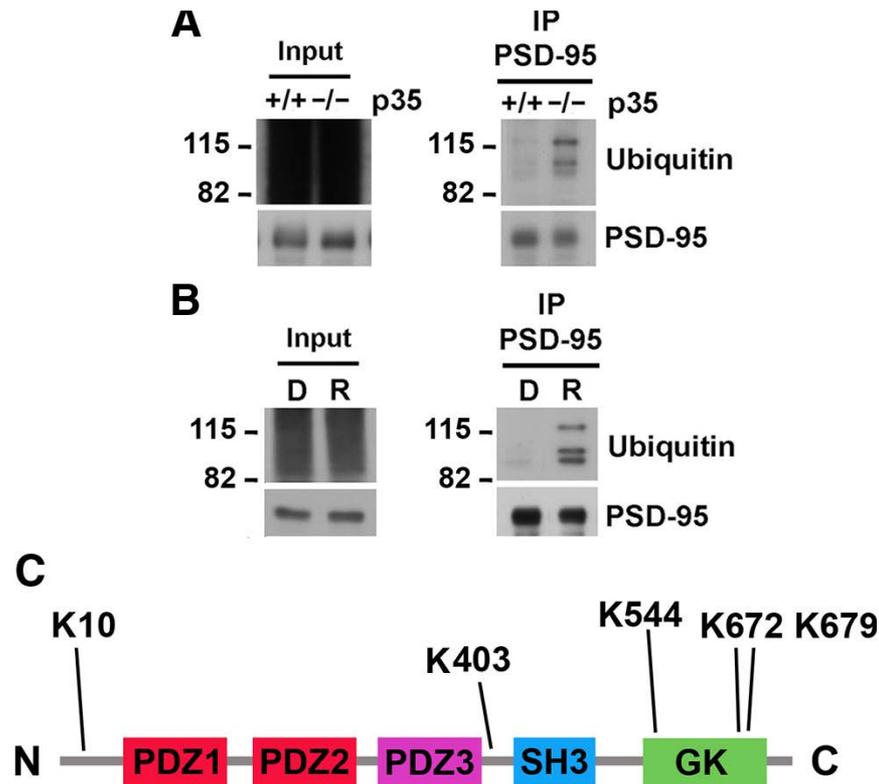


# Reduced Cdk5 activity promotes PSD-95 ubiquitination

## Cyclin-Dependent Kinase 5 Regulates PSD-95 Ubiquitination in Neurons

Michael J. Bianchetta,<sup>1</sup> TuKiet T. Lam,<sup>2</sup> Stephen N. Jones,<sup>1</sup> and Maria A. Morabito<sup>1</sup>

<sup>1</sup>Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655 and <sup>2</sup>W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Connecticut 06511

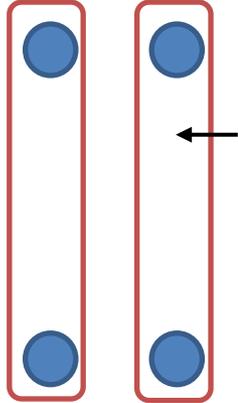




# Use of "Pulsed SILAC" to determine Differential Protein Expression Rates

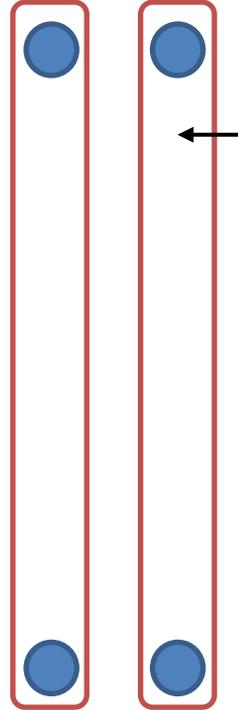


L Control H Treated



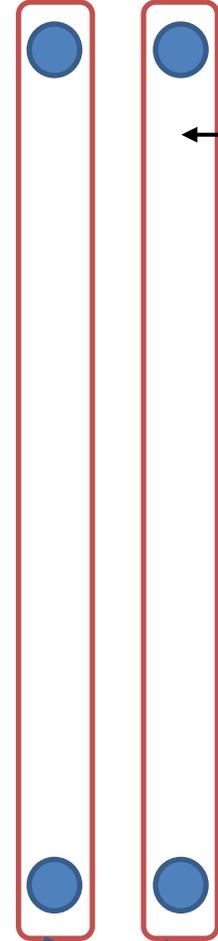
Mix at  $t_1$

L Control H Treated



Mix at  $t_2$

L Control H Treated



Mix at  $t_3$

$t_0=0$

$t_{\text{treatment}}$

$t_1$

$t_2$

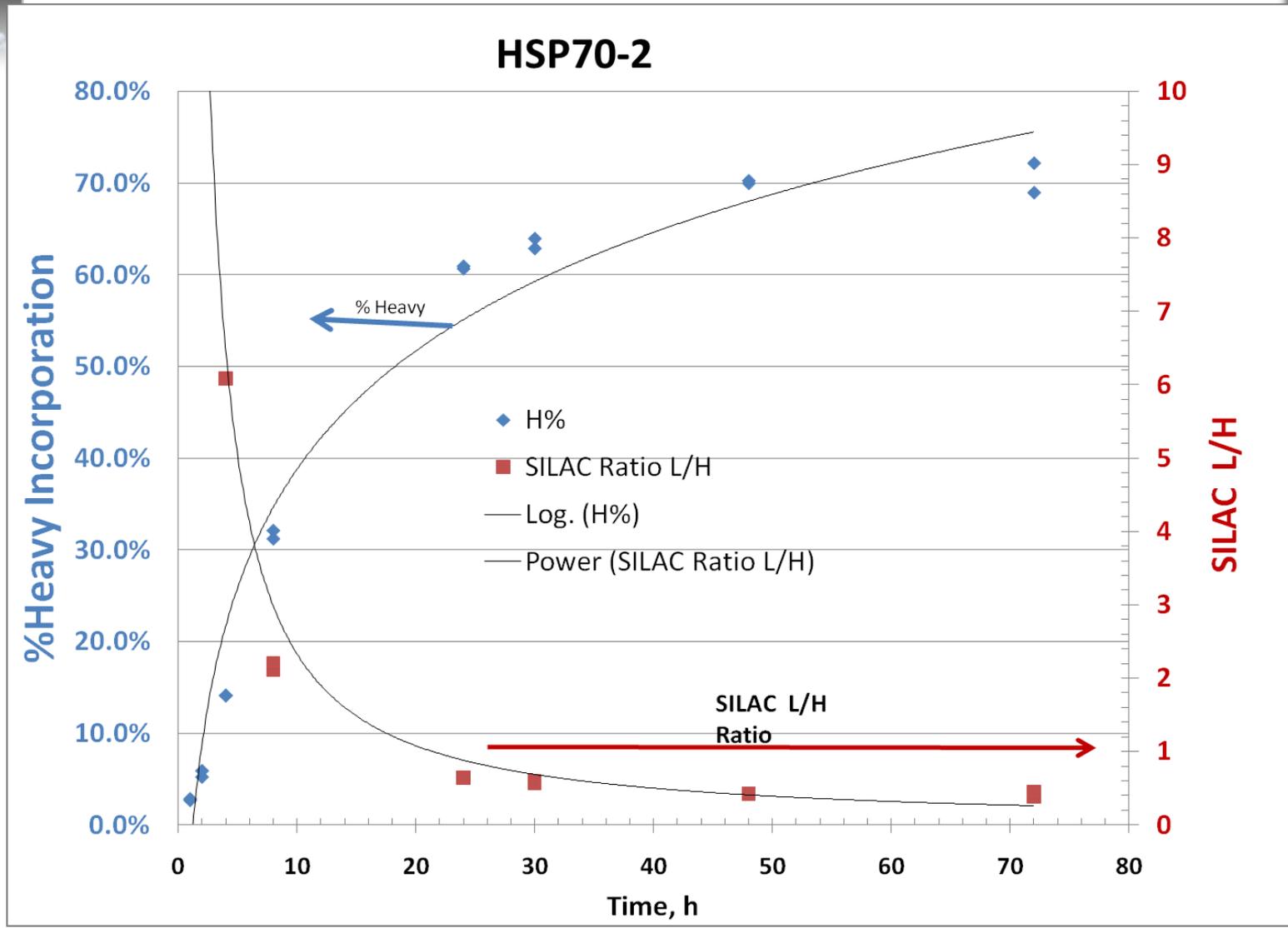
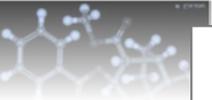
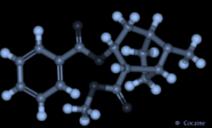
$t_3$



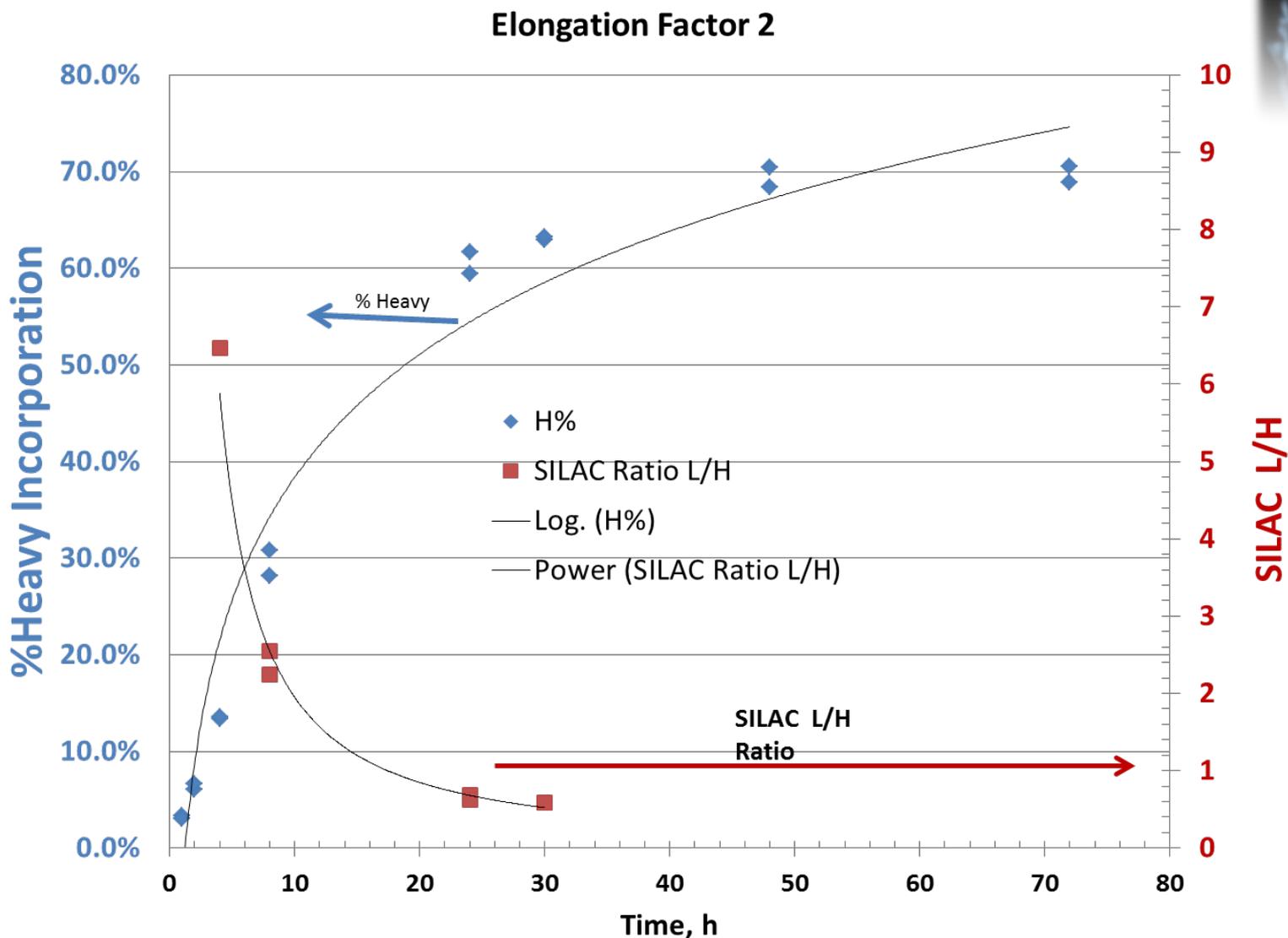
Angus Nairn Lab:  
Shari Wiesman



# HSP 70



# Elongation Factor 2



# SILAC Mouse – Studies of CK1delta overexpression in forebrain



## Project with Greengard and Nairn Labs

- Study the role of CK1 $\delta$ :
- a kinase found to influence dopamine signaling and sensitivity to amphetamine when overexpressed in the mouse forebrain

(Zhou et al. Proc Natl Acad Sci U S A. 2010 107:4401-6. “Forebrain overexpression of CK1delta leads to down-regulation of dopamine receptors and altered locomotor activity reminiscent of ADHD”)



# SILAM - Stable Isotope Labeling of Mammals (or SILAC Mouse)

SILAM is an *in vivo* proteomics approach permitting quantitative determination of protein patterns of mouse organs.

1/4

## Overexpressed Group

Normal Mouse Diet

↓  
Isolate organ

↓  
Prepare organ lysates

## Control Group

Lys(6)-"heavy"-Mouse Diet

↓  
Isolate organ

↓  
Prepare organ lysates

↓  
Proteome Mix (1:1)

↓  
Proceed with 1D SDS Page – 10 bands

↓  
Mass Spectrometric Analysis

3/4

# SILAM - Stable Isotope Labeling of Mammals (or SILAC Mouse)



Mouse Chow with  
**Lys [U-<sup>13</sup>C<sub>6</sub>]**



Breed 3 generations for  
>99% incorporation  
(Brain requires longer  
incorporation time than  
most other organs)



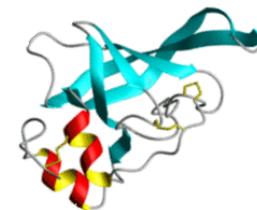
Mice 8-10 weeks



**Tissue Extraction**  
Isolate mouse striatum



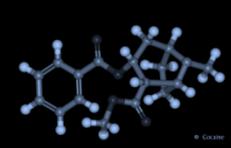
**Normalize protein in lysate**



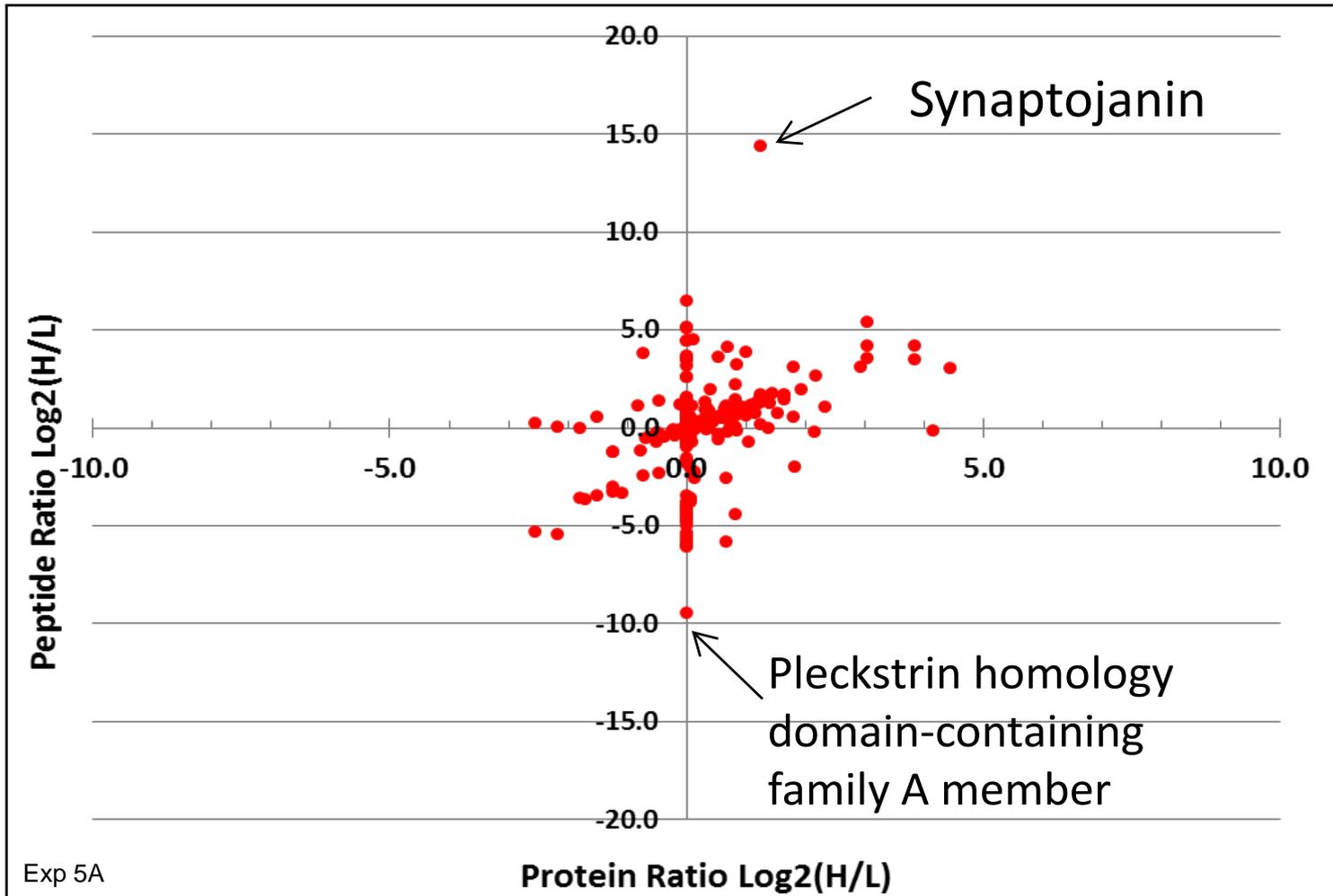
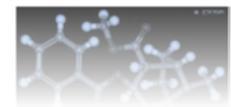
Mouse generation based on:

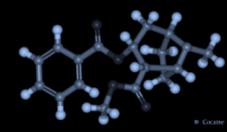
Kruger et. al., "SILAC Mouse for Quantitative Proteomics  
Uncovers Kindlin-3 as an Essential Factor for Red Blood  
Cell Function, Cell, (134), 353-364





# Phosphopeptide vs Protein Ratios





# Conclusion



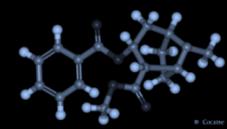
- Phosphorylation of Kal 7 as Potential PSD Signaling Hub
- Reduced Cdk5 activity promotes PSD-95 ubiquitination
- New Methods Recently Used at Yale/NIDA Neuroproteomics Center

## Pulsed SILAC

A new quantitative technique for looking at PTM and expression level differences

**SILAM - Stable Isotope Labeling of Amino acids in Mammals (Mice):**  
quantitatively compare proteomes from forebrain of WT (light) and CK1 $\Delta$  (heavy) overexpressed mice to determine **CK1 $\Delta$**  function under *in vivo* conditions:





# Acknowledgements



## NIH-NIDA Neuroproteomics Funding

DA 018343

### Keck Laboratory

Ken Williams

Kathy Stone

Tukiet Lam

Chris Colangelo

Can Bruce

Jean Kanyo

Mary Lopresti

Kathrin Wilczak

## NIDA Neuroproteomics Leadership

Ken Williams

Angus Nairn

### Rockefeller University

Alexandre Stipanovich

