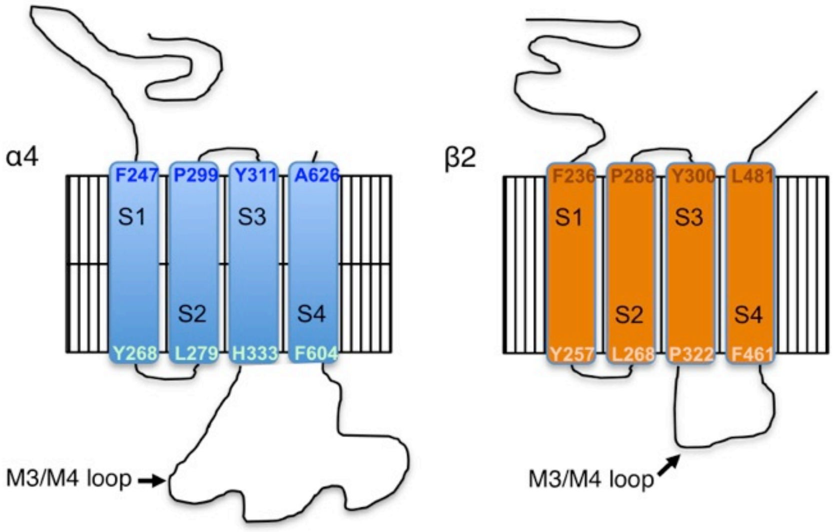
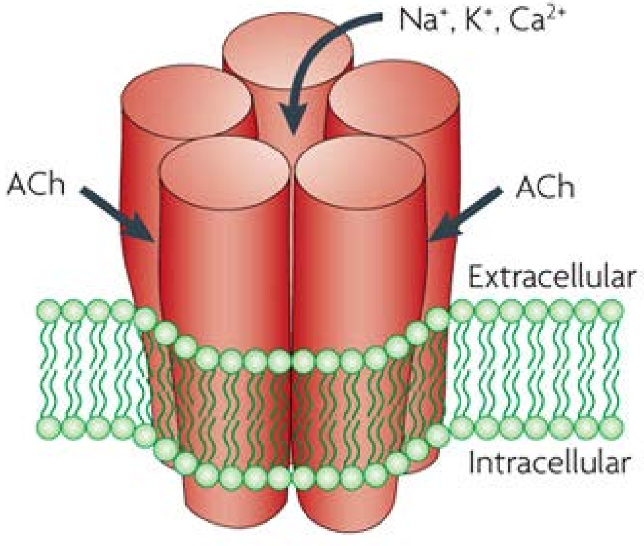


A proteomics approach to understanding nicotine-dependent signaling

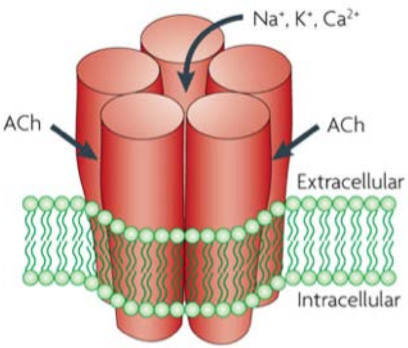
Marina Picciotto and Angela Lee
Depts. of Psychiatry, Neuroscience & Pharmacology
Yale University School of Medicine

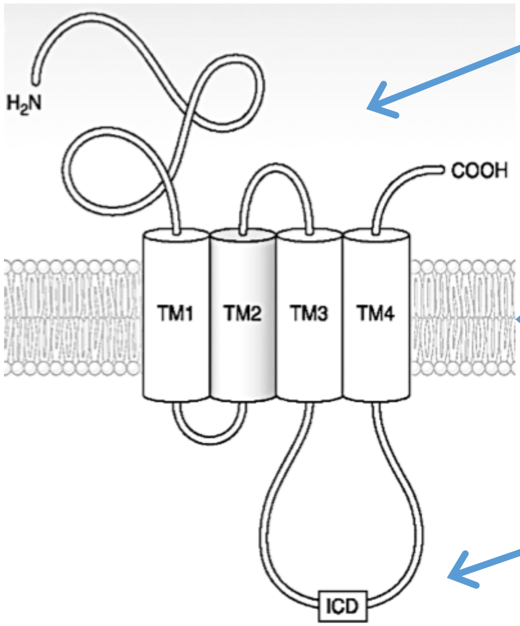
Nicotine activates Nicotinic Acetylcholine Receptors



- Pentameric ligand-gated cation channels; receptors for acetylcholine
- Many different subunits:
  - α2-10, β2-4 in brain
  - Differ in agonist binding specificities, channel properties, Ca-permeability, expression levels, localization, etc.
  - Most common in brain: **α7** and **α4β2**

nAChR Dynamics and Phosphorylation





Ligand binding

4 Transmembrane domains

Large intracellular loop (site of most protein interactions)

- Phosphorylation of muscle-type nAChRs involved in receptor localization and desensitization
- More recent studies of α4:
  - Several putative PO4 sites identified
  - PKA activation → receptor maturation/ upregulation in cell culture
  - PKC phosphorylation promotes recovery from desensitization

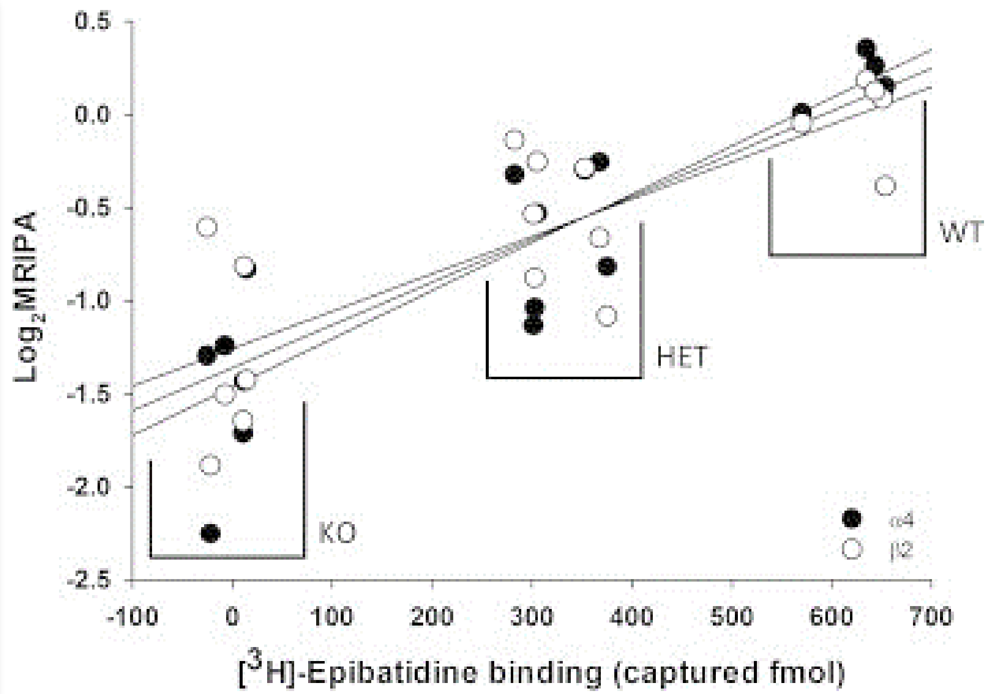
- nAChR phosphorylation has not been demonstrated in vivo
- β2 phosphorylation has not been demonstrated
- Other important brain kinases not evaluated

Exploring the Nicotinic Acetylcholine Receptor-associated Proteome with iTRAQ and Transgenic Mice

Tristan D. McClure-Begley [1,2], Kathy L. Stone [3], Michael J. Marks [2,4], Sharon R. Grady [2], Christopher M. Colangelo [3,5], Jon M. Lindstrom [6], Marina R. Picciotto [1,*]

Quantitative analysis of β2* nAChR-associated proteome

- Brain lysates from α4 and β2 WT, HET and KO mice
- IPed with β2 nAChR antibody
- Precipitated proteins subjected to Mass Spec/ iTRAQ
- Identify proteins significantly correlated with gene dose



Tristan McClure-Begley

McClure-Begley et al. GPB. 2013 Aug;11(4):207-18.

Proteins significantly correlated with gene dose

- <u>17 proteins</u> identified as interactors of β2 nAChR in mouse brain
- CaMKII isoforms also found in human cortical tissue from smokers and non-smokers (McClure-Begley, 2016)

Table 3 **Proteins with their abundances significantly positively correlated with that of β2 nAChR subunit across genotypes**

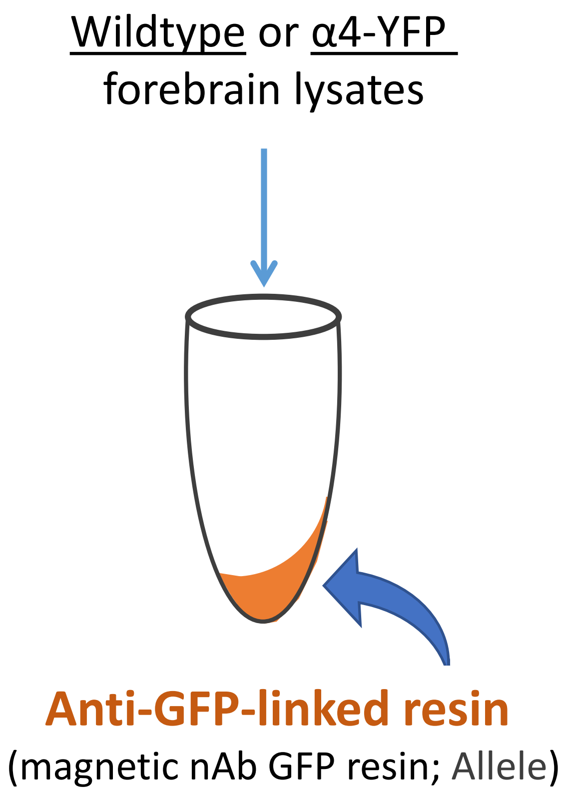
Correlation coefficient	*N*	*F* score	*P* value	Protein	UniProtKB accession No.	Cellular compartment	Previously identified?	Molecular function
0.748	12	20.268	0	Glial fibrillary acidic protein	P03995	Cytoplasm	No	Protein binding, structural molecule
0.858	18	44.542	0	nAChR subunit α4	O70174	Cell junction	No	Neuro-transmitter receptor
1	18	–	0	nAChR subunit β2	Q9ERK7	Cell junction	No	Neuro-transmitter receptor
0.652	18	11.844	0.003	Neurofilament light poly peptide	P08551	Growth cone	No	Protein binding, structural molecule
0.645	18	11.404	0.004	Actin-related protein 3	Q99JY9	Cytoplasm	No	Nucleotide binding, protein binding
0.637	18	10.904	0.004	Calcium/calmodulin-dependent protein kinase type II subunit α	P11798	Cytoplasm	No	Transferase, nucleotide binding, protein binding
0.917	18	21.235	0.01	Calcium/calmodulin-dependent protein kinase type II subunit γ	Q923T9	Sarcoplasmic reticulum membrane	No	Transferase, nucleotide binding, protein binding
0.57	18	7.681	0.014	F-actin				
0.562	18	7.386	0.015	Thyro recept				nding
0.665	12	7.933	0.018	Transc				on
0.539	18	6.563	0.021	Ecton pyropho family member 6				tivity
0.519	18	5.884	0.027	Spectrin β chain, brain 1	Q62261	Cytoplasm	No	Protein binding, lipid binding, structural molecule activity
0.856	6	11.009	0.029	Ras-related protein Rap-1A	P62835	Cell membrane	No	Hydrolase activity, protein binding, nucleotide binding
0.512	18	5.695	0.03	Myosin-10	Q61879	Cytoplasm	No	Protein binding, nucleotide binding, hydrolase
0.506	18	5.496	0.032	Myelin proteolipid protein	P60202	Cell membrane	No	Structural molecular, protein binding
0.502	18	5.378	0.034	Spectrin α chain, brain	P16546	Cytoplasm	Yes	Hydrolase, protein binding, nucleotide binding
0.493	18	5.149	0.037	Tubulin β-3 chain	Q9ERD7	Cytoplasm	No	Hydrolase, nucleotide binding, structural molecular, protein binding, peptide

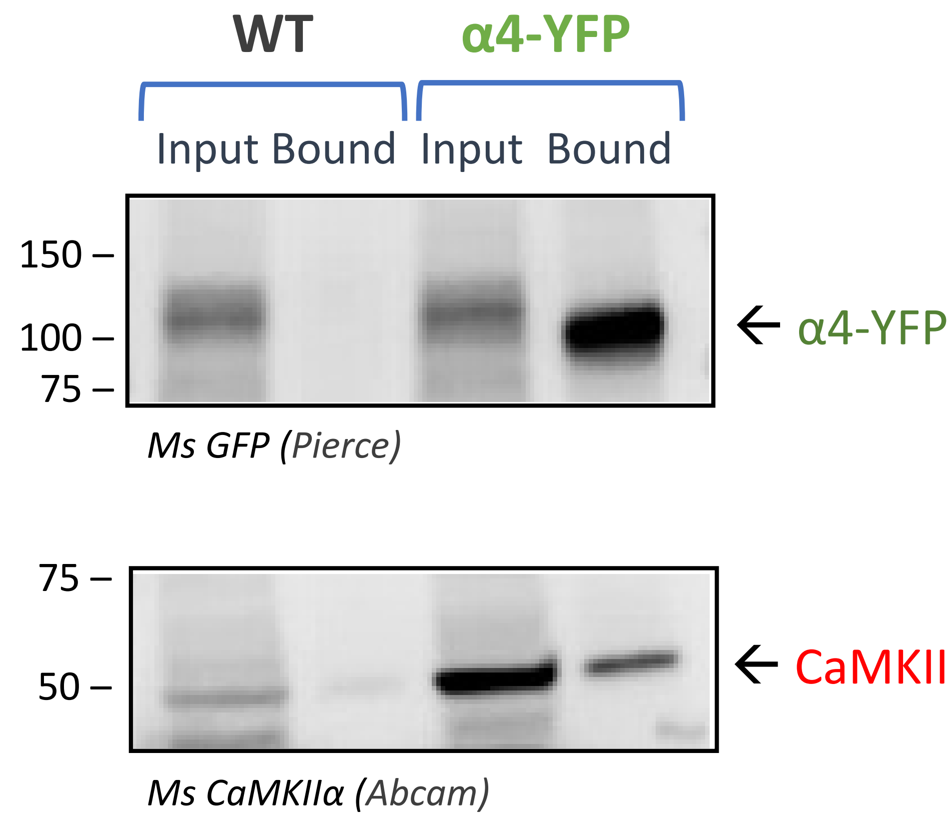
Calcium/calmodulin-dependent protein kinase type II subunit α

Tristan McClure-Begley

McClure-Begley et al. GPB. 2013 Aug;11(4):207-18.

Confirmation of CaMKII – α4β2* nAChR interaction by co-IP

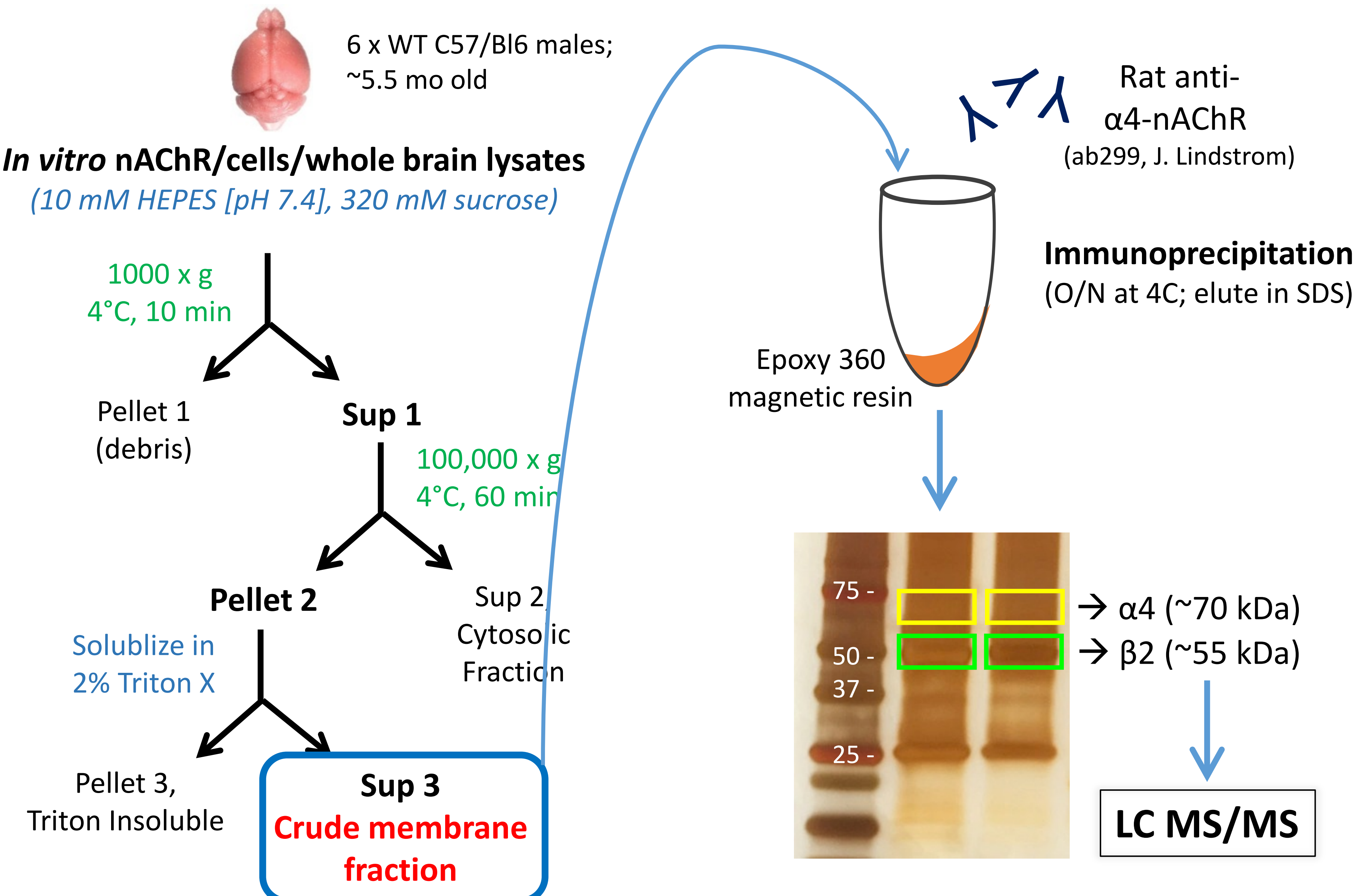




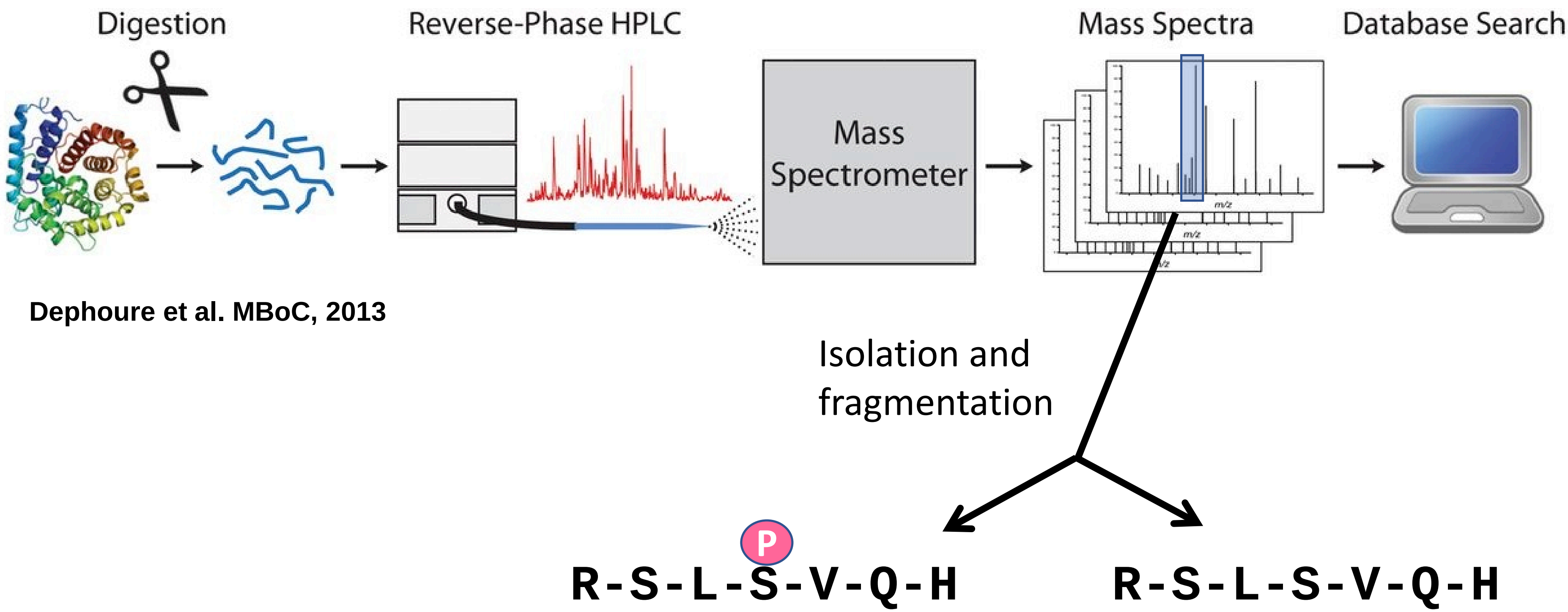
→ Incubate lysate with resin overnight at 4C
→ Separate resin on magnet and remove supernatant (unbound fraction)
→ Thoroughly wash resin (3X with high salt and 0.1% triton X)
→ Elute “bound” fraction by boiling resin in SDS
→ Run “input” and “bound” fractions on gel; WB for CaMKII

Megan Miller

Approach: membrane prep and IP



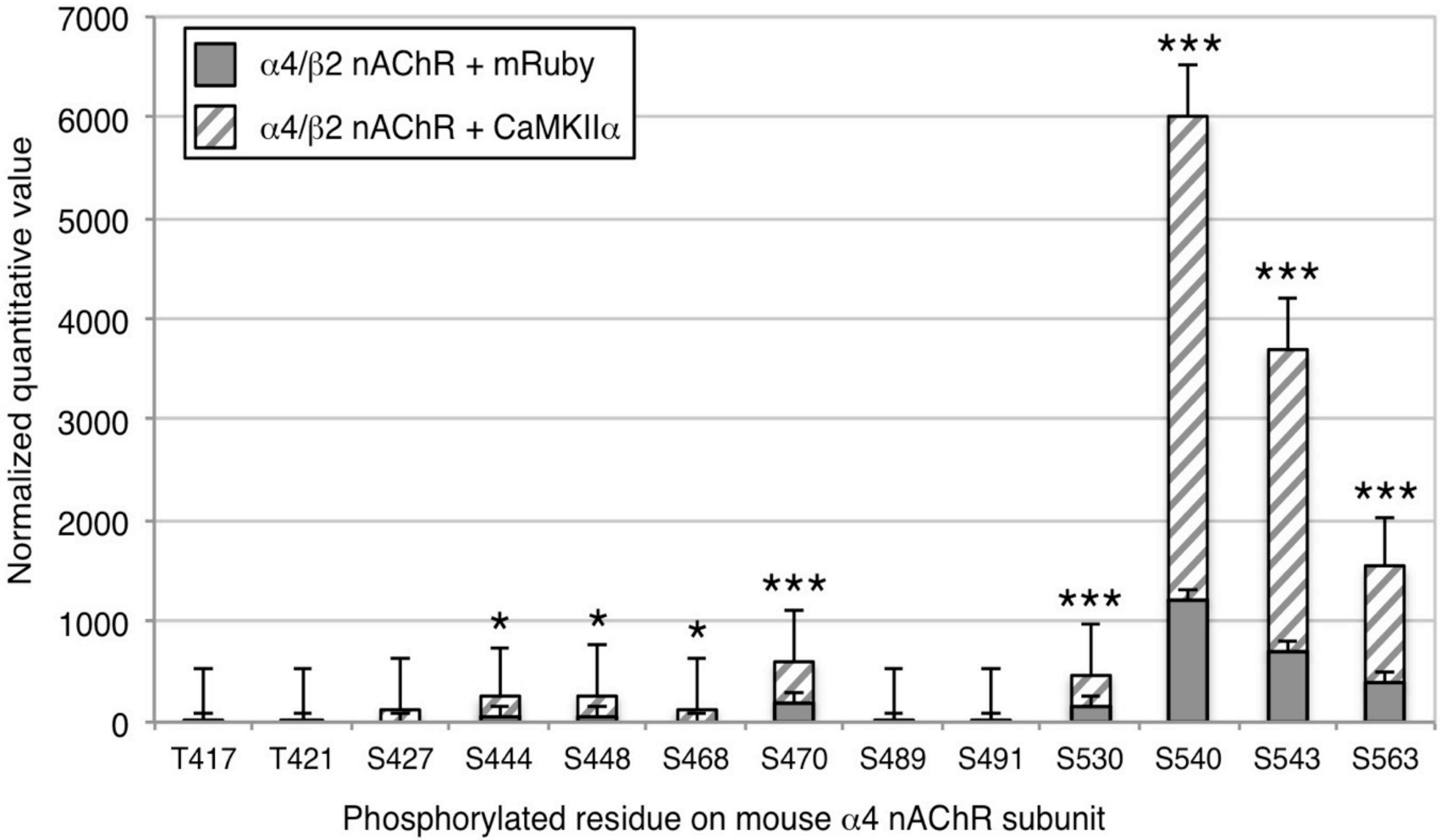
Approach: Tandem MS analysis of phosphorylation



Dephoure et al. MBoC, 2013

→ LC MS/MS → peptide identifications >96% identical
→ Coverage of α4β2 nAChR subunits = 35-50%
→ 80-95% coverage in M3/M4 loop region

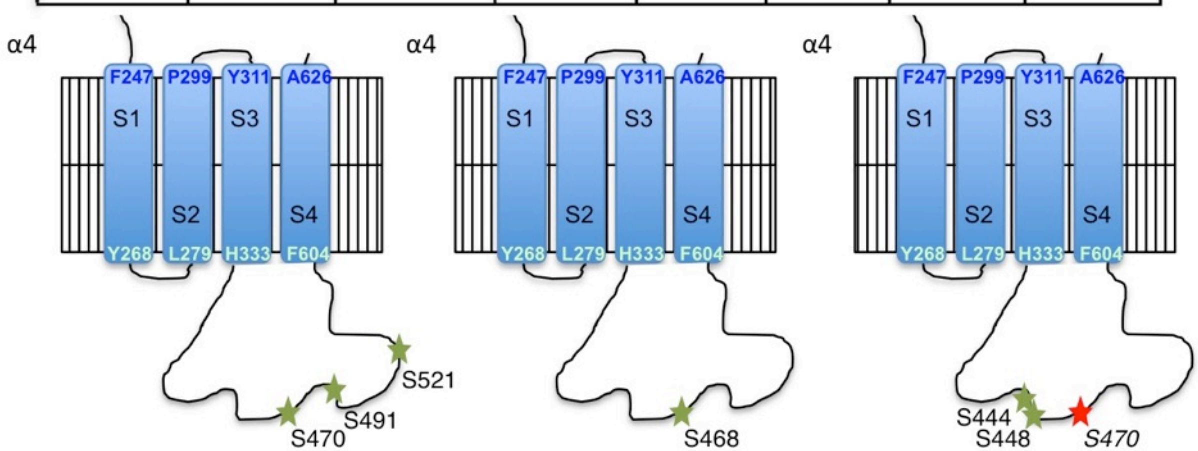
Does CaMKIIα phosphorylate α4β2 nAChRs?



Megan Miller and Rashaun Wilson

What are the phosphorylation sites on α4β2 nAChRs?

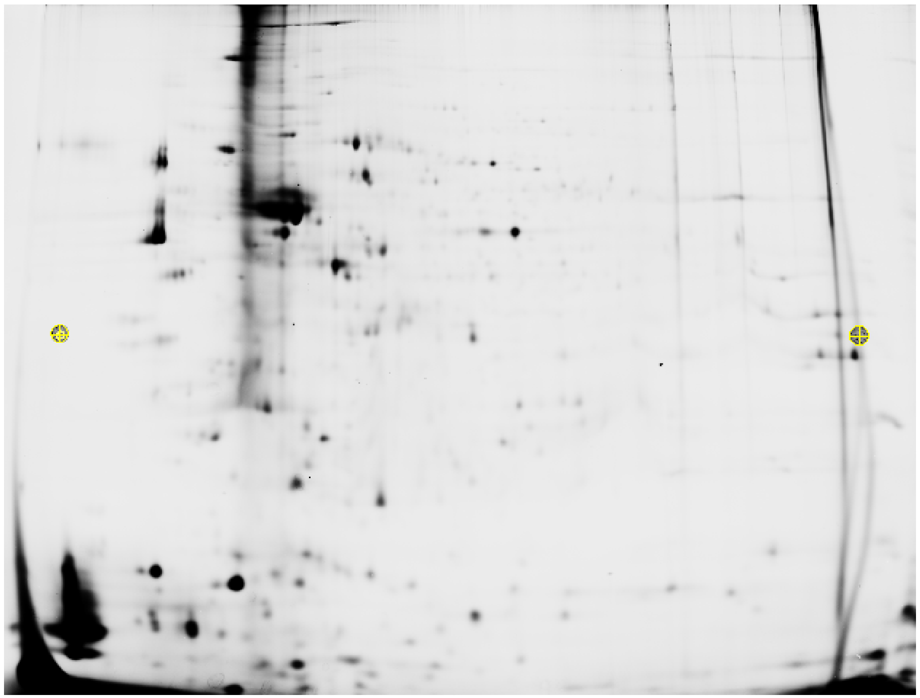
HEK Cells		*In Vitro*			*In Vivo*		
Basal	CaMKII	PPase resistant	CaMKII	PKA	Saline	Acute Nic	Repeat Nic
			T417*				
	S444*					S444*	
	S448*					S448*	
	S468*		S468***				
S470	S470***	S470		S470***		*S470*	*S470*[A]
				S491*	S491		
		S521		S521***			
S530	S530***						
S540	S540***	S540					
S543	S543***				S543		
S563	S563***				S563		



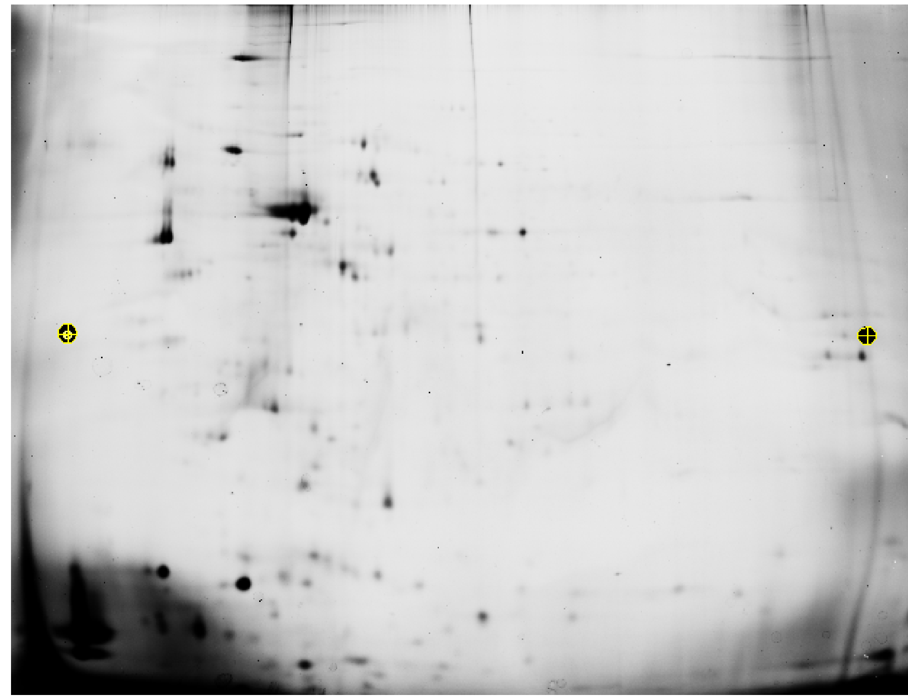
PKA *in vitro* | HEK cells+CaMKIIα / CaMKIIα *in vitro* | Acute Nicotine *in vivo*

Megan Miller and Rashaun Wilson

What is the consequence of nicotine exposure on the proteome in brain areas involved in reinforcement?



Wildtype NAc
Chronic Nicotine

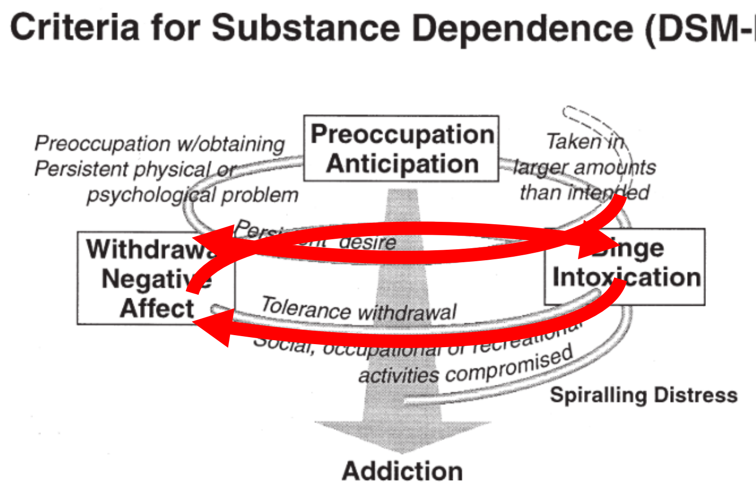


Beta-2 KO NAc
Chronic Nicotine

Rebecca Steiner

Effects of Nicotine on the VTA Proteome in Male and Female Mice

Women are more vulnerable to nicotine addiction

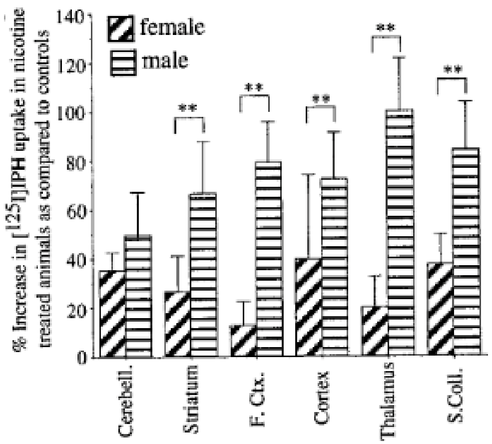


Koob & Le Moal. *Neuropsychopharm* (2001)

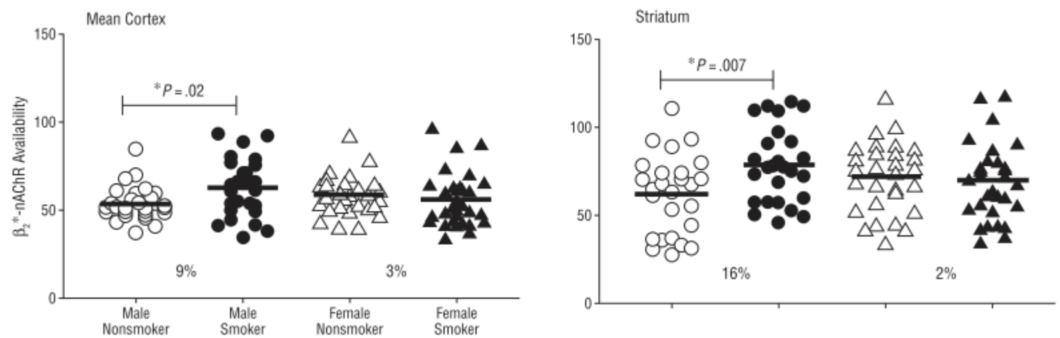
- Teen girls > boys in nicotine dependence milestones (Sylvestre et al. *Am J Epidemiol.* 2018)
- Female rats self-administer more nicotine, more quickly at lower doses (Flores et al. *Physiol Behav.* 2017)
- 31% lower odds of successfully quitting, and 44% greater odds of relapse (Smith et al. *NTR.* 2015; Weinberger et al. *Addiction*. 2014)
- Higher progressive ratio breakpoints in self-administration (Donny et al. *Psychopharm (Berl.).* 2000)

Sex differences in nicotine-induced alterations

nAChR upregulation

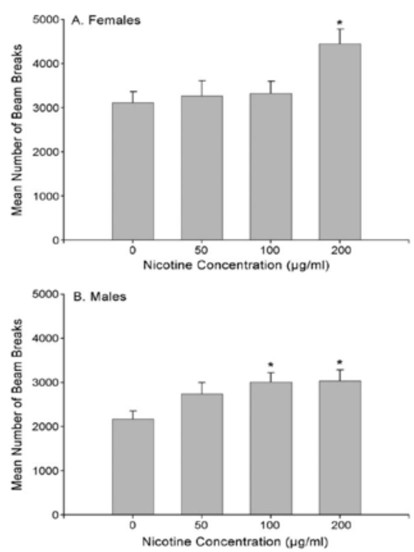


T. MOCHIZUKI ET AL. SYNAPSE 30:116–118 (1998)



Cosgrove et al.
Arch Gen Psychiatry. 2012;69(4):418-427

Mesolimbic function



B.J. Caldarone et al. / Neuroscience Letters 439 (2008) 187–191

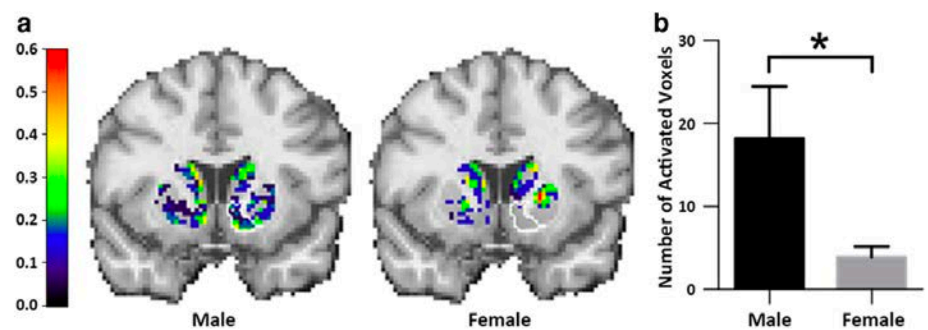


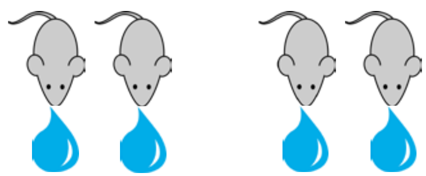
Figure 1. ***a***, Probability of activation maps for male and female smokers. Note the striking difference in the right ventral striatum. ***b***, The mean (and SE) number of voxels activated during smoking in the right ventral striatum for male and female smokers. A permutation test indicated that the mean sex difference in number of activated voxels in the right ventral striatum was highly significant ($p = 0.01$).

Cosgrove, Wang et al. • Dopamine Signature of Smoking J. Neurosci., December 10, 2014 • 34(50):16851–16855

No sex comparisons in proteomics studies

Sex differences in nicotine-induced alterations in mesocorticolimbic system

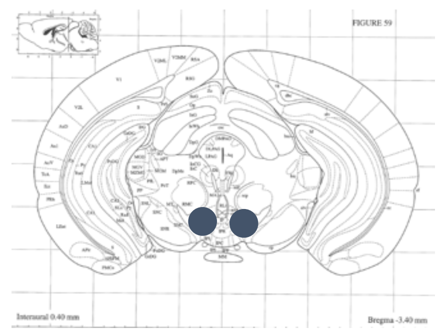
Nicotine administration (chronic, sub-chronic) → Tissue collection (VTA, NAc, mPFC) → Quantitative proteomic analysis



Male
Sacc Nic
(**MS**) (**MN**)

Female
Sacc Nic
(**FS**) (**FN**)

21 days →



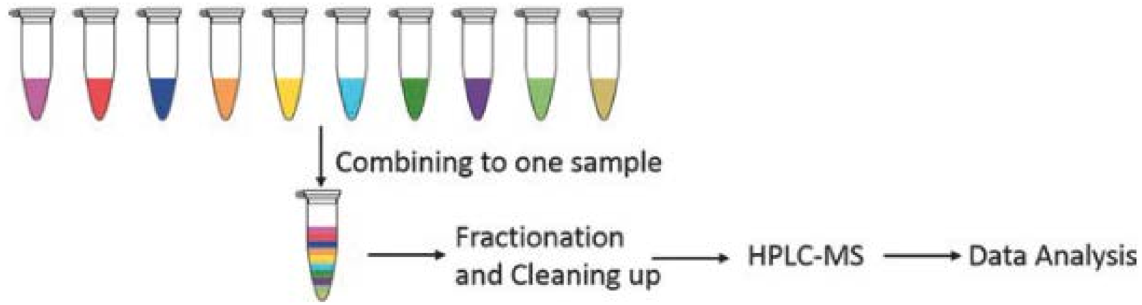
Nic: 200 μg/ml in 2% saccharin
Sacc: 2% saccharin + 0.2% tartaric acid

Experimental Design

Shahid Mansuri

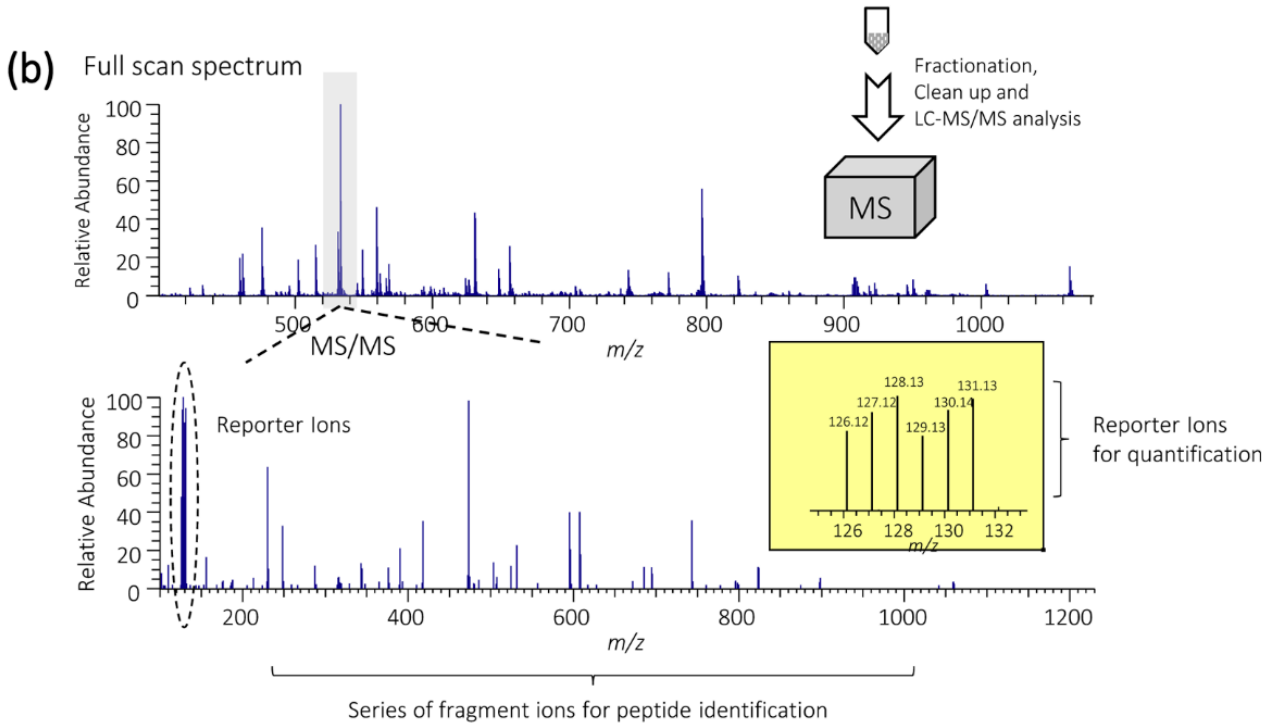
Protein extraction, digestion
TMT 10-plex isobaric labeling

TMT label:	126	127N	127C	128N	128C	129N	129C	130N	130C	131
Run 1:	MixA	FS-1	FS-2	FN-1	FN-2	MS-1	MS-2	MN-1	MN-2	MixB
Run 2:	MixC	FS-3	FS-4	FN-3	FN-4	MS-3	MS-4	MN-3	MN-4	MixD



Adapted from Zhang & Elias in *Proteomics: Methods and Protocols*. 2017

Experimental Design



Adapted from Rauniyar & Yates, J Proteome Res. 2014

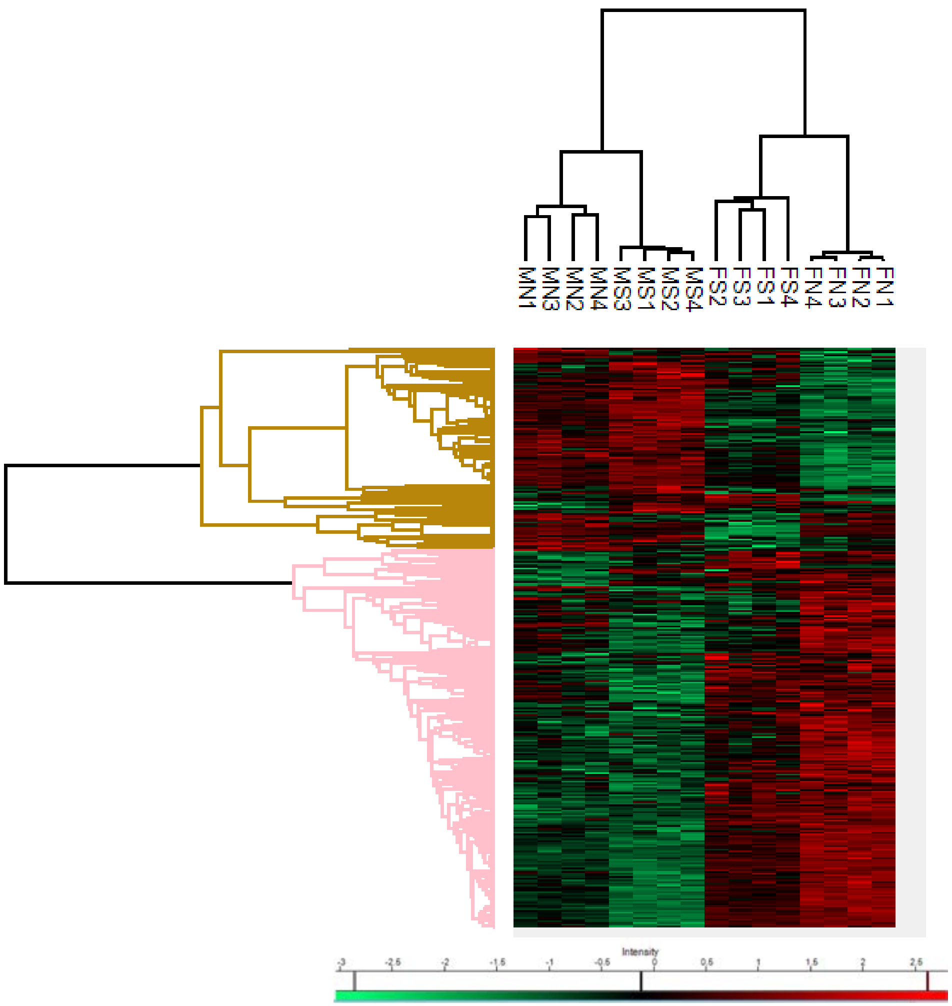
Shahid Mansuri

Results

Identified Proteins (**3431**)

Quantified Proteins (**2928**)
3 biological samples out of 4 (75%) contains intensity of identified proteins

Differentially significant (ANOVA) Proteins (**2013**)

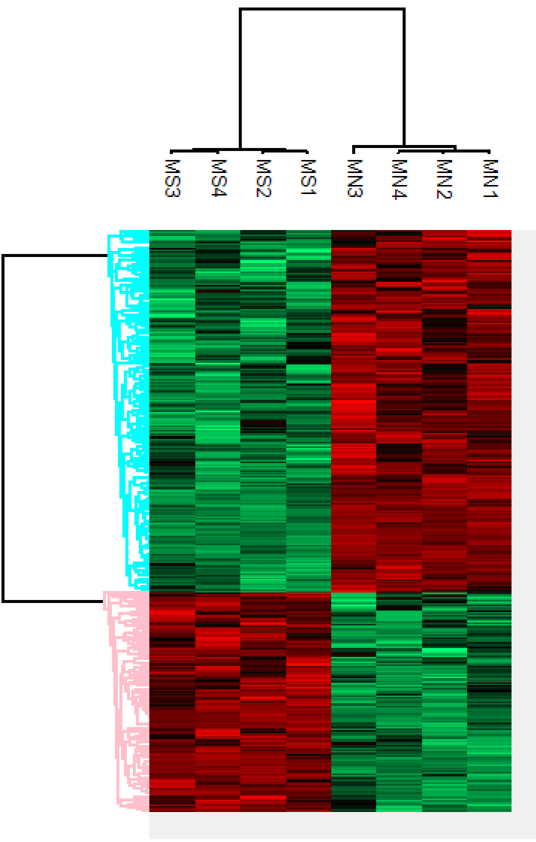


<u>Pairwise Comparisons</u>

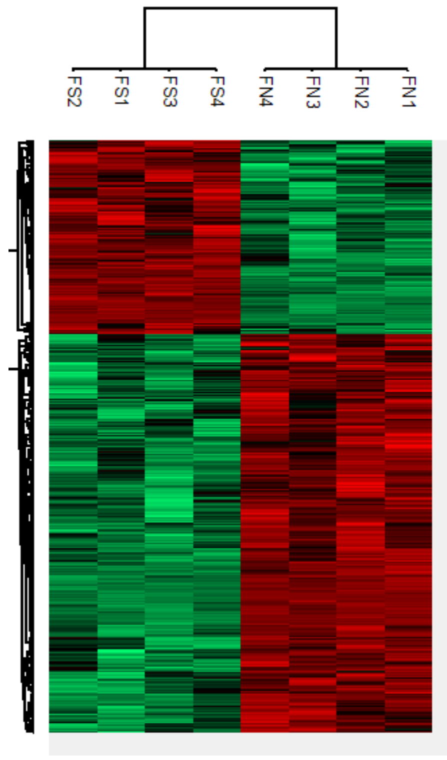
1. Male Nic (MN) vs. Male Sacc (MS)
2. Female Nic (FN) vs. Female Sacc (FS)
3. Male Sacc (MS) vs. Female Sacc (FS)

Shahid Mansuri

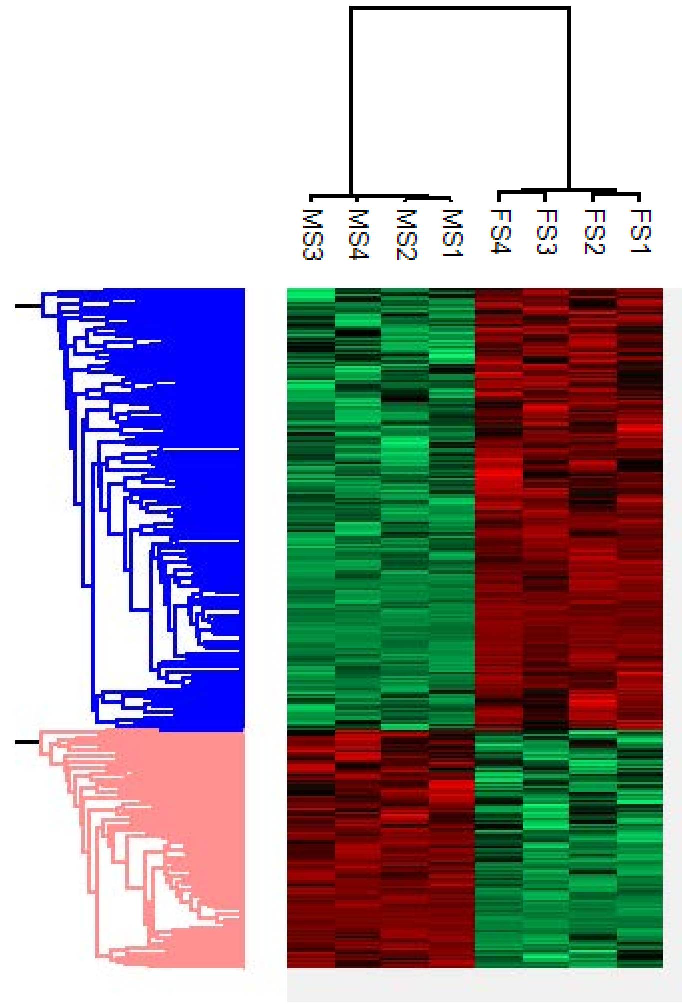
Results of Pairwise Comparisons: Hierarchical clustering



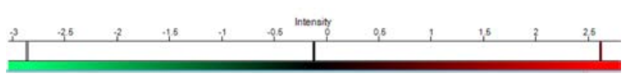
N = 633



N = 868



N = 1299



Future directions

- Validate findings
  - Western blot
  - Planned comparisons of control group male vs female, chronic vs sub-chronic

- Other brain regions collected (NAc, mPFC)

- Sub-chronic nicotine administration

Acknowledgements

Picciotto Lab

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Tukiet Lam
Rashaun Wilson
Jean Kanyo

Thesis Committee

Christopher Pittenger
Ralph DiLeone
Jane Taylor
Michael Nitabach

INP

Charlie Greer
Carol Russo

MSTP

Barbara Kazmierczak
Cheryl DeFilippo
Sue Sansone

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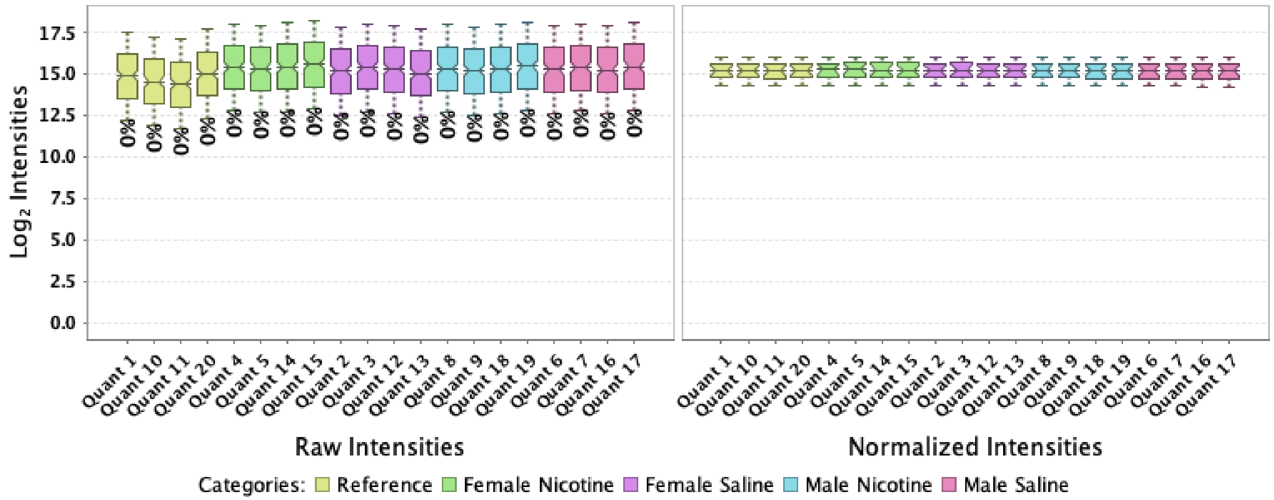
Median normalization



Scaffold parameter

Protein threshold. 99%

Min # peptides. 2

Peptide threshold. 95%

Identified Proteins (**3431**)

→

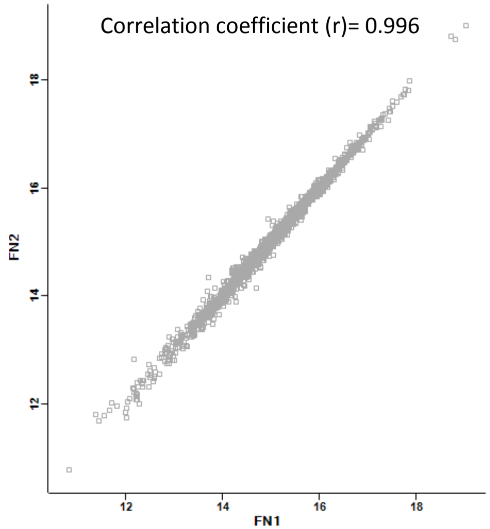
Quantified Proteins (**2928**)
3 biological samples out of 4 (75%) contains intensity of identified proteins

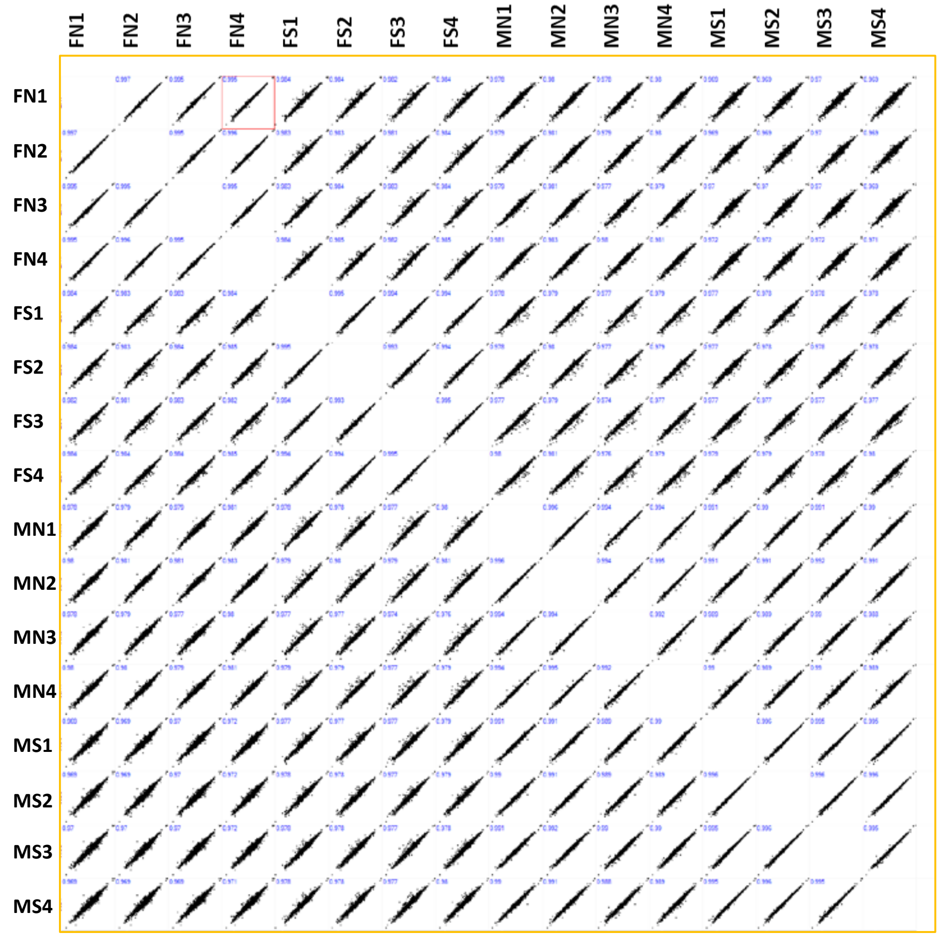
→

Differentially significant (ANOVA) Proteins (**2013**)

Shahid Mansuri

Reproducibility of the samples:
Multi scatter plot: Expression values of each sample are compared to all others.
Correlation calculated as Pearson coefficient

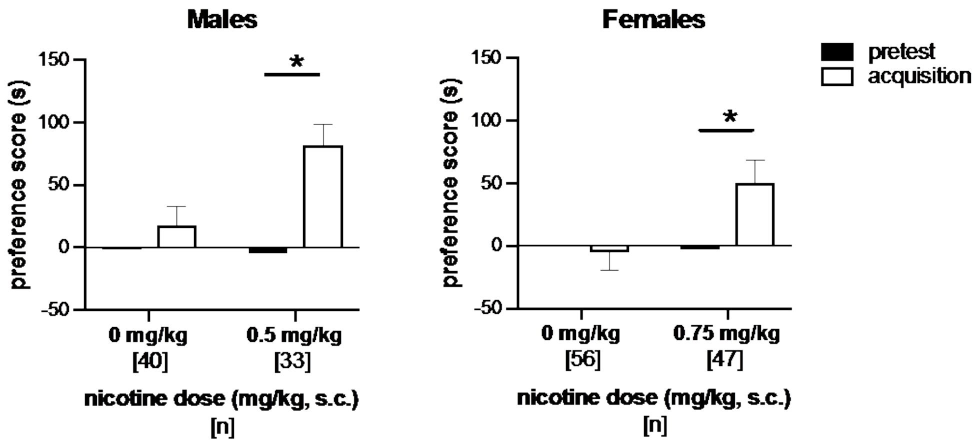




Sub-chronic administration

- CPP-inducing paradigm (3/6 days, alternating)
  - Male: 0.5 mg/kg nicotine, s.c.
  - Female: 0.75 mg/kg nicotine, s.c.

- VTA, NAc, mPFC



Proteomic studies of nicotine-induced alterations

RESEARCH ARTICLE
Nicotine

Proteomics
www.proteomics-journal.com

Multiplexed Isobaric Tag-Based Profiling of Seven Murine Tissues Following In Vivo Nicotine Treatment Using a Minimalistic Proteomics Strategy

Joao A. Paulo, Mark P. Jedrychowski, Edward T. Chouchani, Lawrence Kazak, and Steven P. Gygi**

The influence of chronic nicotine treatment on proteins expressed in the mouse hippocampus and cortex

Kenji Matsuura [a], Mieko Otani [b], Masaoki Takano [b], Keiichi Kadoyama [a], Shogo Matsuyama [a,*]

[a] *Department of Pharmaceutical Health Care, Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, 7-2-1 Kamiohno, Himeji 670-8524, Japan*
[b] *Department of Life Sciences Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University, Kobe 650-8586, Japan*

Techniques and Methods

Biological Psychiatry

Molecular Histochemistry Identifies Peptidomic Organization and Reorganization Along Striatal Projection Units

Akitoyo Hishimoto, Hiroko Nomaru, Kenny Ye, Akira Nishi, Jihyeon Lim, Jennifer T. Aguilan, Edward Nieves, Gina Kang, Ruth Hogue Angeletti, and Noboru Hiroi

6/8 male only
1/8 female only
1/8 sex not reported