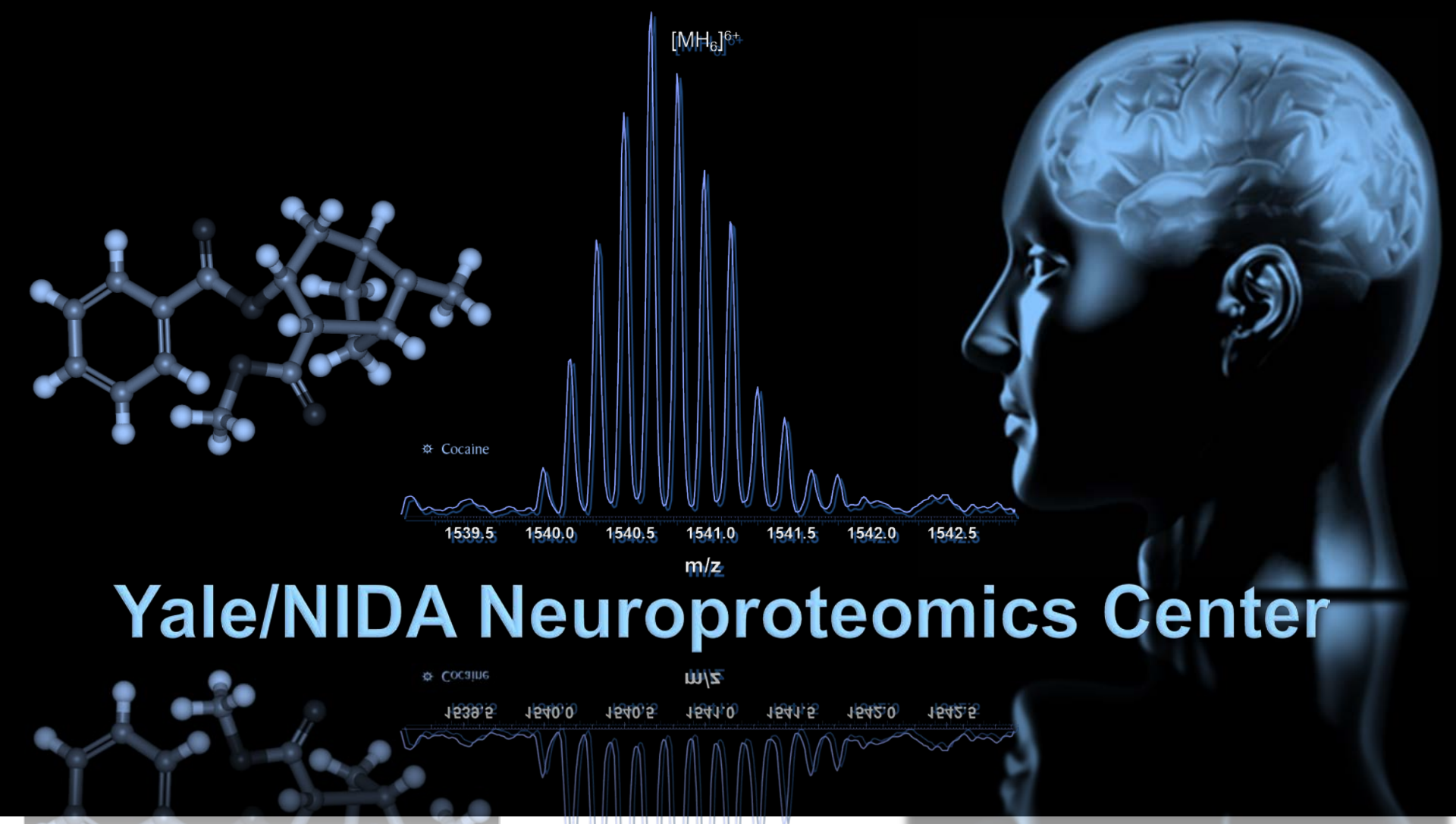
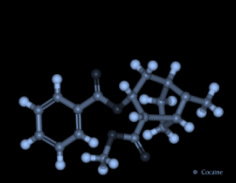


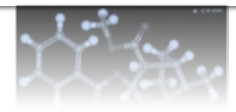
Yale/NIDA Neuroproteomics Center

Angus C. Nairn
Charles B.G. Murphy Professor of Psychiatry

Current and Future Challenges in Quantitative Neuroproteomics

Current and Future Challenges in Quantitative Neuroproteomics





<u>General challenges with particular nuances in studies of the CNS</u>

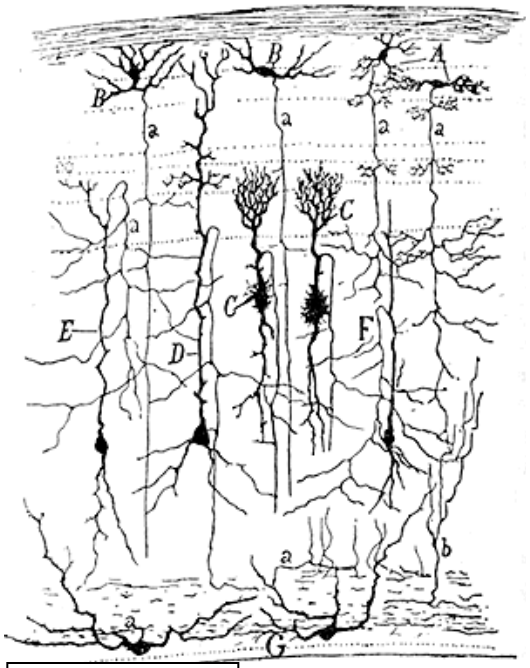
- Application of robust and sensitive mass spectrometric methods
- Accurate reproducible and quantitative procedures
- Appropriate experimental design and assessment of factors that influence technical and biological variance

<u>Specific challenges for proteomic studies of the CNS</u>

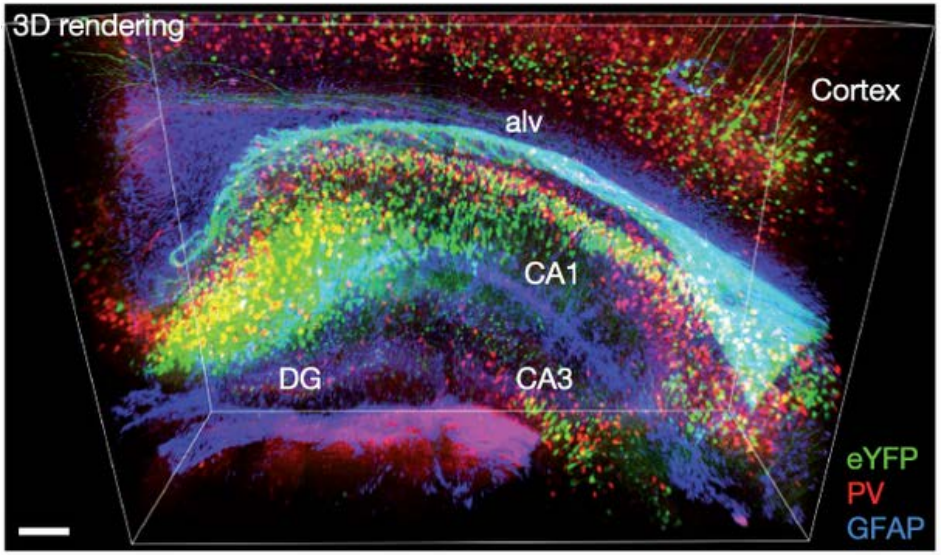
- Huge amount of cell type variability with specific and distinct patterns of gene/protein expression and regulation
- Complex intermingling of neuronal sub-types
- Complex cell shapes and sub-compartments
- Low amounts of proteins to analyze

Current and Future Challenges in Quantitative Neuroproteomics – Cell type Heterogeneity

Diversity of neuronal subtypes



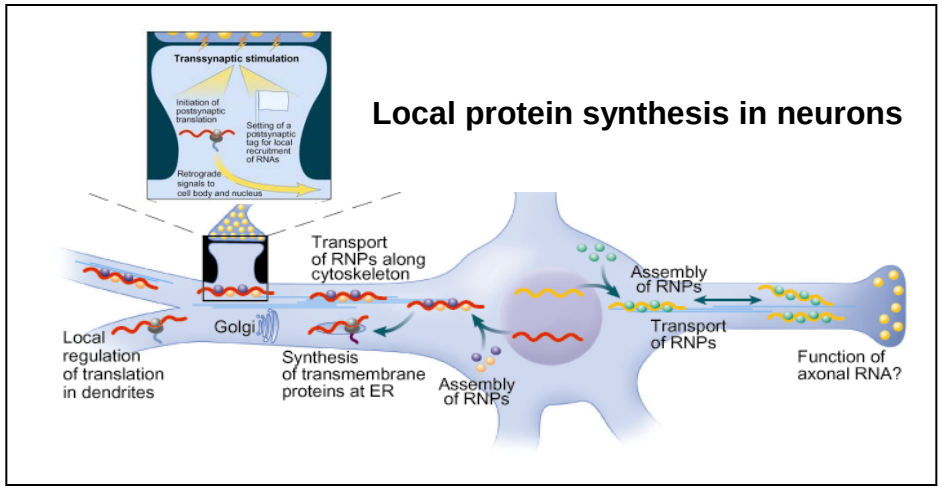
Ramón y Cajal

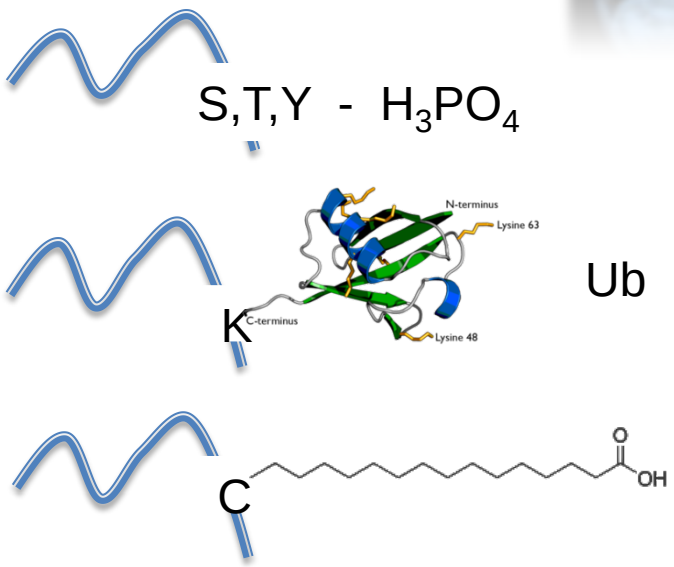


Deisseroth et al Nature 2013 - Three-dimensional view of hippocampus in **c** showing eYFP-expressing neurons (green), parvalbumin-positive neurons (red) and GFAP (blue). Alv, alveus. Scale bar, 200 μm

Current and Future Challenges in Quantitative Neuroproteomics – Sub-cellular analysis

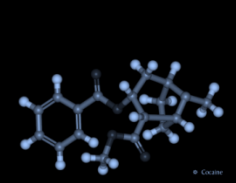
- However, particularly in neurons there is also the added feature of local protein synthesis and possibly local proteosome-mediated degradation





- Finally, there is the desire to identify many different types of post-translational modifications, alternative splicing etc which are key in understanding the control of protein function

Current and Future Challenges in Quantitative Neuroproteomics



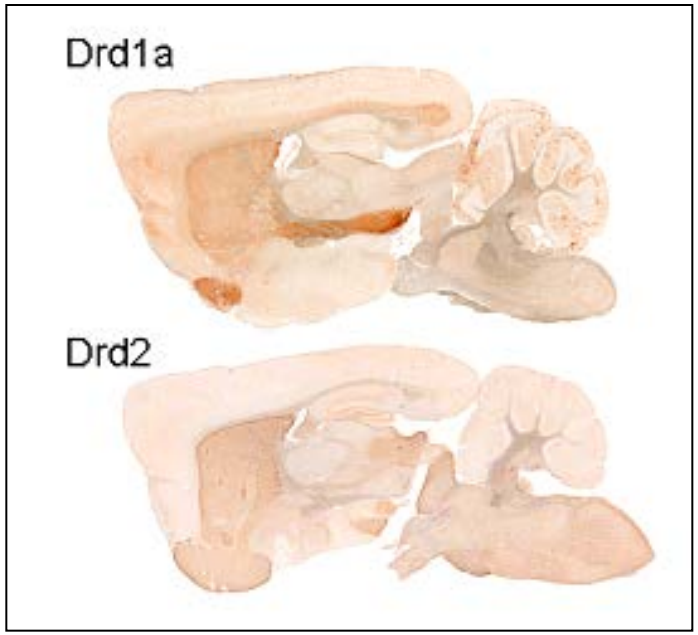
Can proteomics keep up with and complement other genomic approaches?

- The major advances in generation of BAC transgenic mice under the control of gene promoters with specific cell type expression combined with conditional viral expression is driving many aspect of neuroscience, including functional anatomy, definition of novel neuronal circuits and the rapidly progressing field of optogenetics

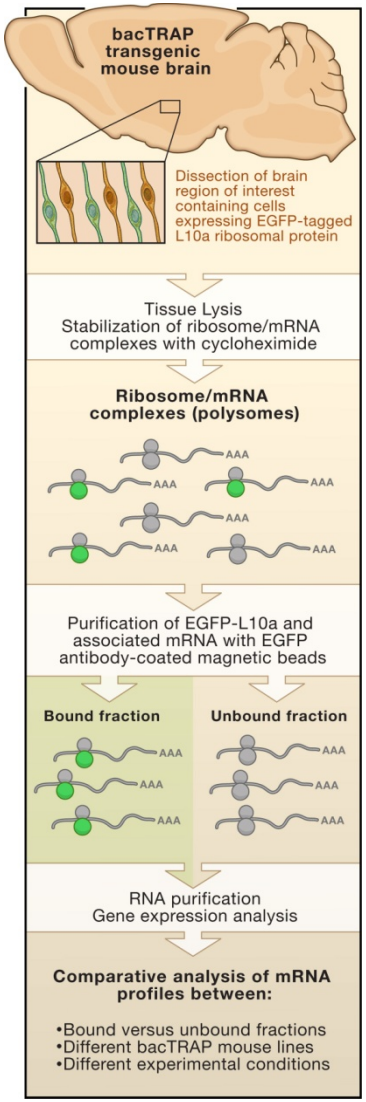
- And is also enabling –omic level analysis of DNA methylation, epigenetic modifications, mRNA expression and ribosomal profiling

BAC transgenic mice expressing tagged proteins can allow expression analysis of ribosomal mRNA in defined populations of neurons

NINDS **GENSAT** BAC TRANSGENIC PROJECT Gene Expression Atlas - http://www.gensat.org/



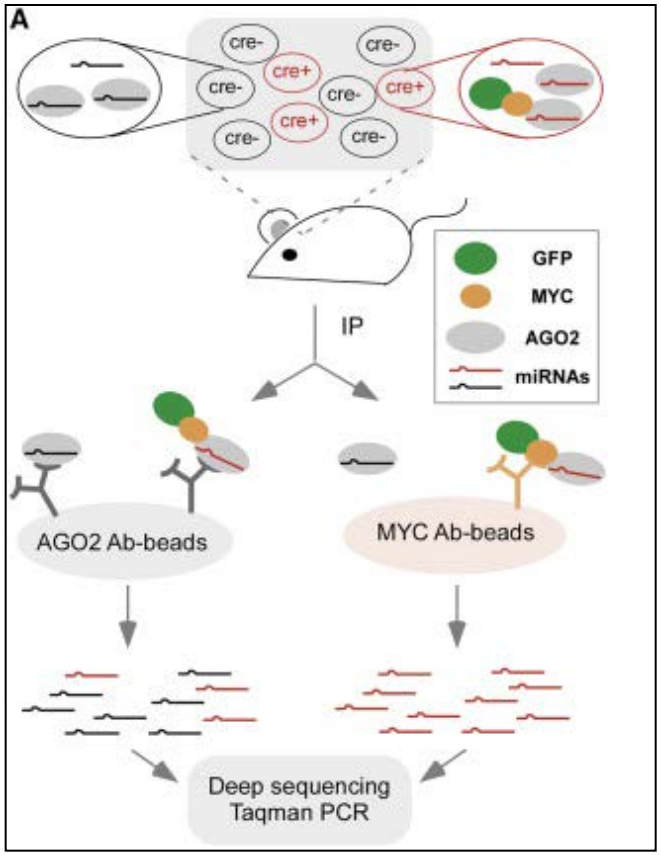
GFP expression in either direct (Drd1a) or indirect (Drd2) pathways of striatum – BAC transgenic mice generated by GENSAT (Rockefeller Univ)



- BacTRAP uses a GFP-tagged ribosomal protein expressed in unique populations of neurons under the control of a specific promoter - method has raised the bar in terms of analyzing mRNA translation

Heiman et al. 2009

Cell type based analysis of microRNA profiles in mouse brain



Huang et al Neuron 2012

- Used Cre-loxP methods to selectively express tagged-Argonaute (AGO2) and identify differential miRNA expression

Targeted Neuroproteomic Approaches (Work in Progress)

Wide array of transgenic mice and different approaches that now allow for GFP labeling of defined cell populations – can we apply to proteomics

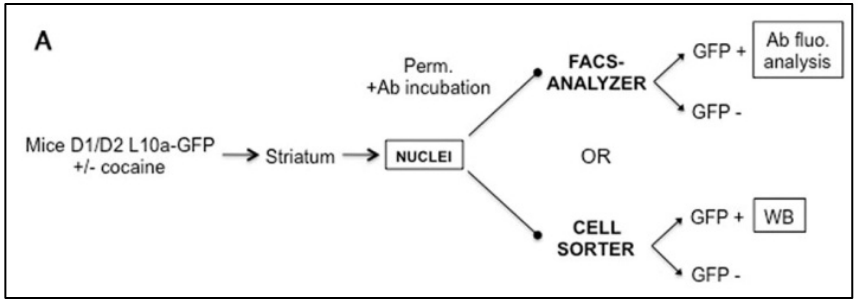
1. FACS sorting?

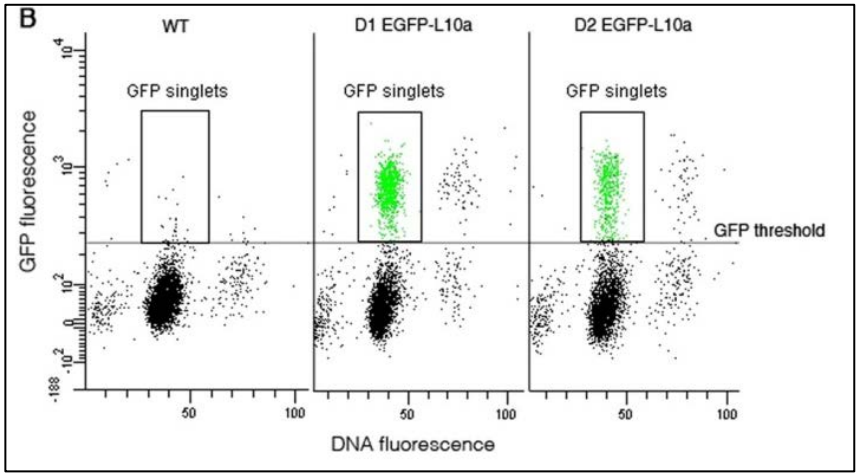
2. Isolation of sub-proteomes from defined neuronal sub-types using rapid immunoprecipitation methods

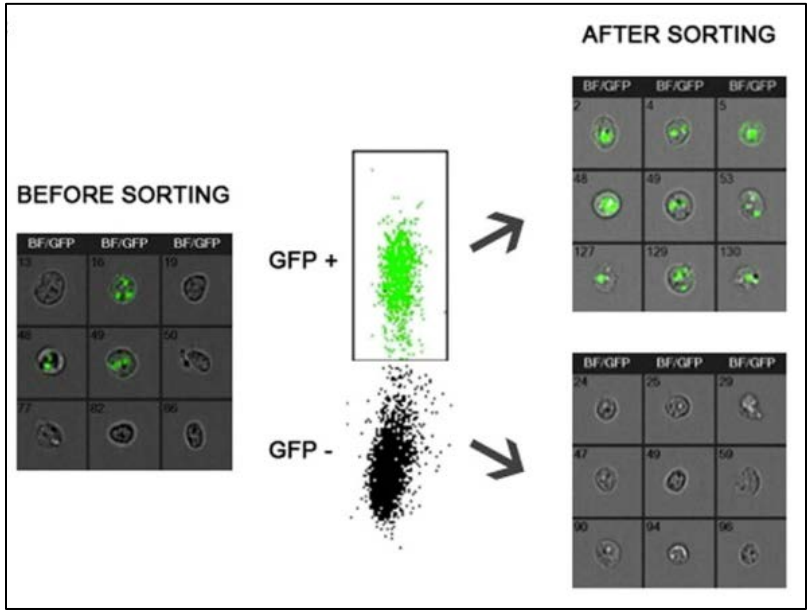
3. Development of large-scale targeted mass spectrometry methods for quantitative analysis of sub-proteomes

FACS sorting D1- and D2-Medium spiny neuron nuclei from mouse striatum

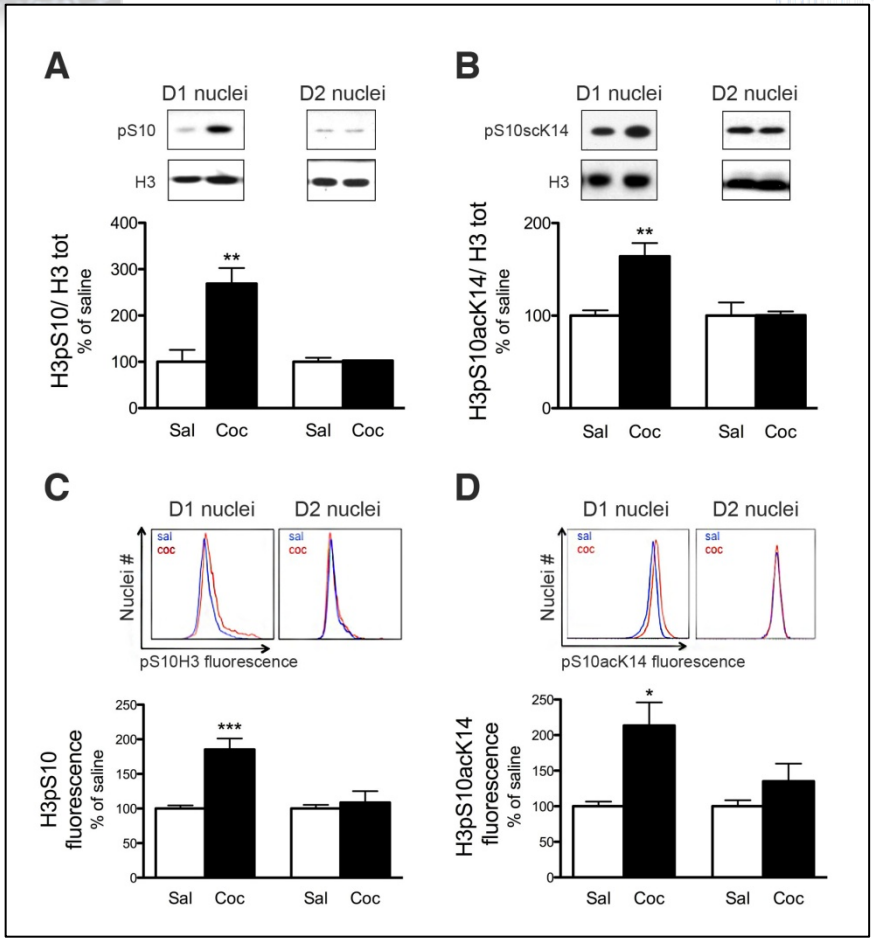
Jordi et al PNAS In Press (2013)



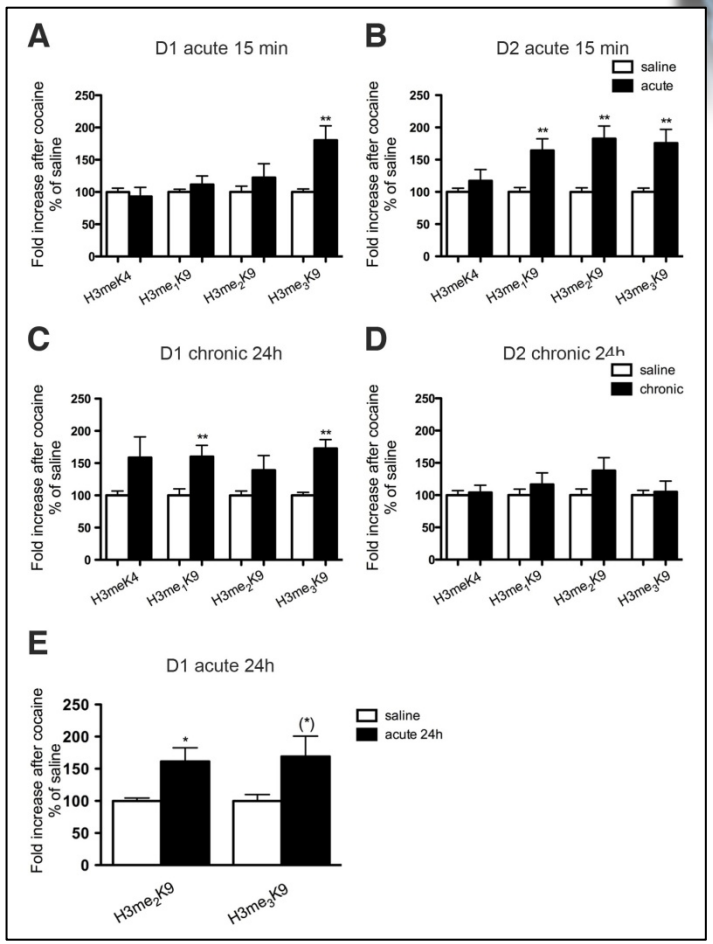




FACS sorting D1- and D2-Medium spiny neuron nuclei from mouse striatum



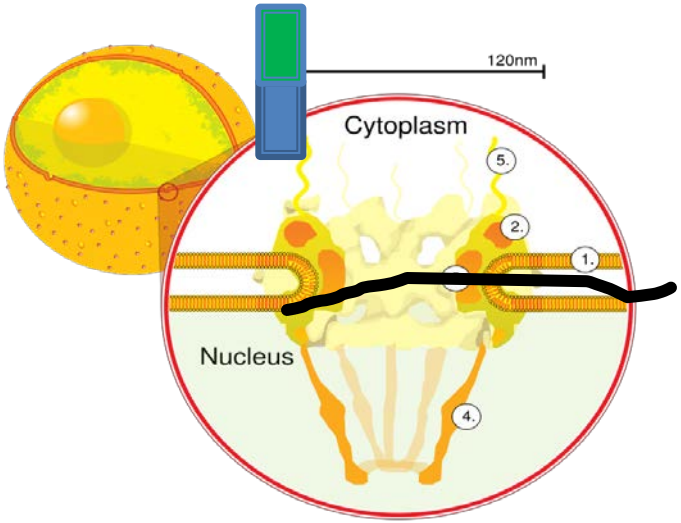
Histone Phosphorylation and Acetylation



Histone methylation

Jordi et al PNAS In Press (2013)

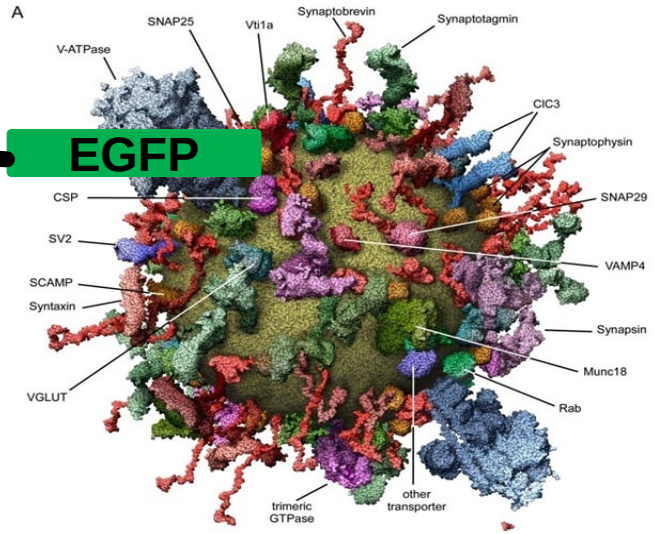
2. Proteomics of Neuronal Subtype-Specific Organelles



EGFP

EGFP

Reinard Jahn, Cell 127, 831-846, 2006



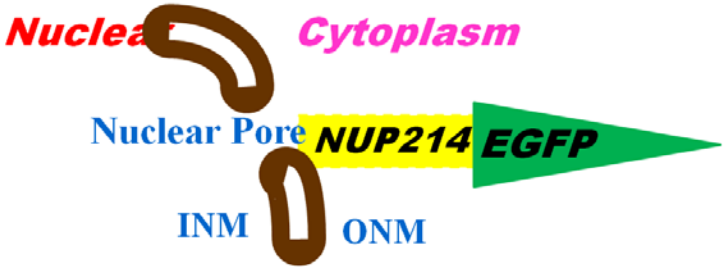
Anshu Shen

2. Isolation of Neuronal Subtype-Specific Organelles

Genetically tag organelles with EGFP and pull down through affinity isolation

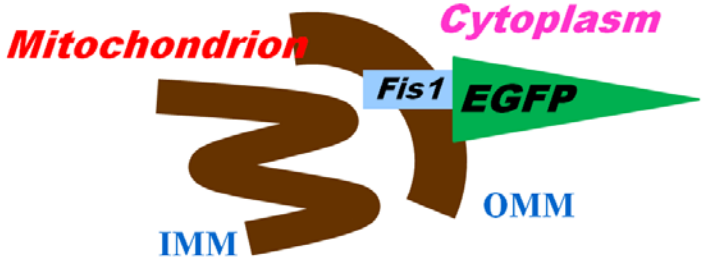
- **Identify candidate proteins that are part of the cytosolic side of organelles.**
- **Label organelles by expression EGFP tagged candidate proteins.**
- **Pull down organelles through immunoprecipitation**

Nuclei were tagged by expression of EGFP-fused Nup214



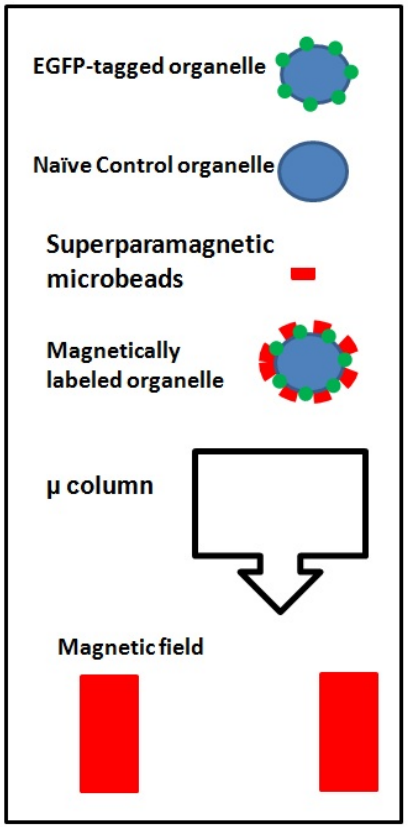
Synaptic vesicles were labeled by expression of EGFP-tagged synaptophysin.

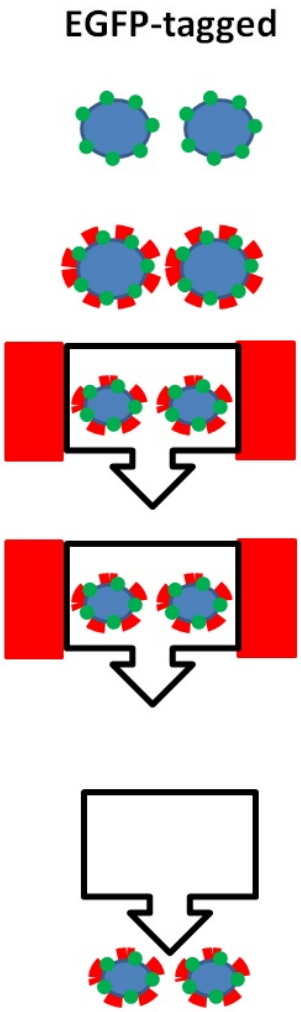
Mitochondria were labeled by expression of EGFP-tagged Fis1

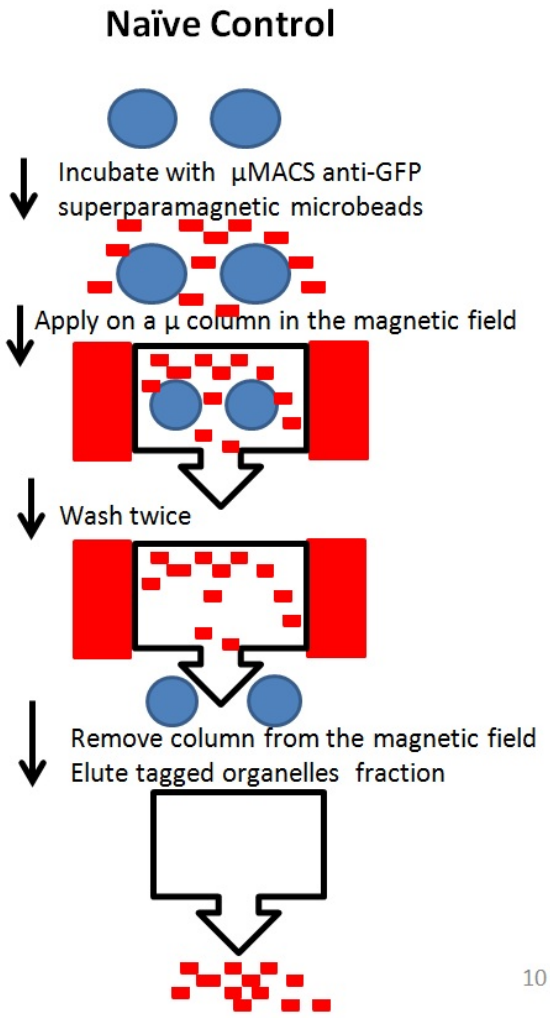


Anshu Shen

2. Isolation of Neuronal Subtype-Specific Organelles





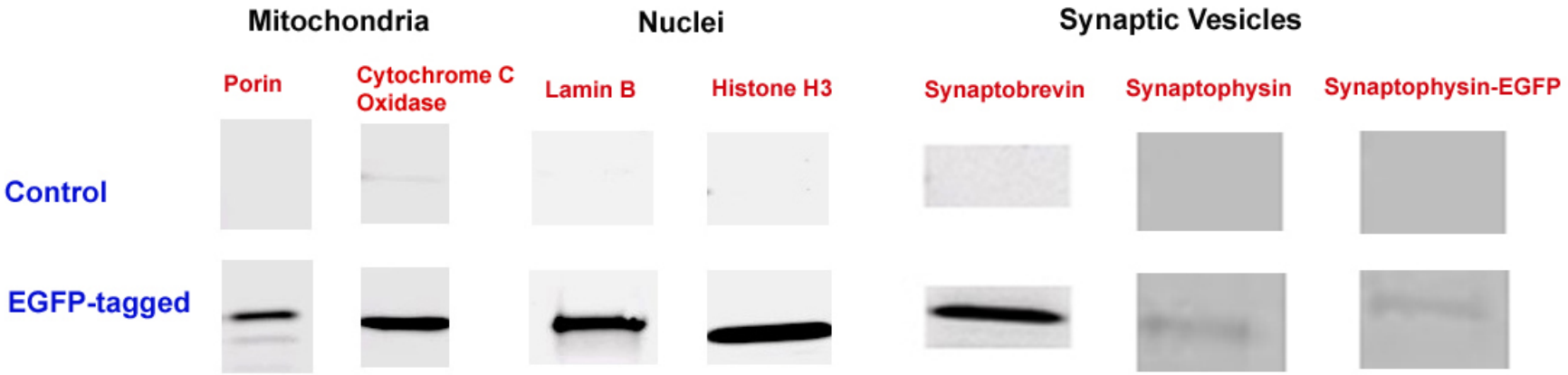


Affinity isolation methods were optimized to allow for specific, efficient and reproducible isolation of pure EGFP-tagged mitochondria, nuclei, and synaptic vesicles respectively.

Anshu Shen

2. Isolation of Neuronal Subtype-Specific Organelles

Immunoblotting analysis



High efficiency of affinity isolation. Immunoprecipitation elution fractions from transfected or naïve control cells samples were analyzed using SDS-PAGE and immunoblotting with antibodies to mitochondrial markers (porin and cytochrome C oxidase), nuclear makers (lamin B and histone H3), and synaptic vesicle marker (synaptobrevin and synaptophysin) respectively.

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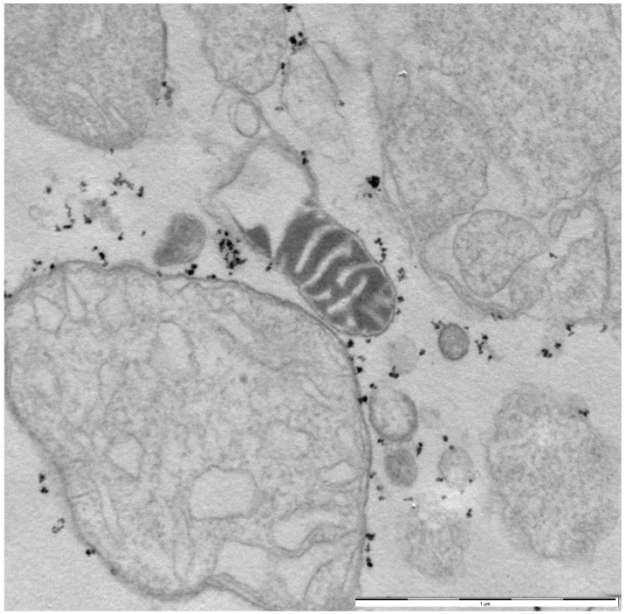
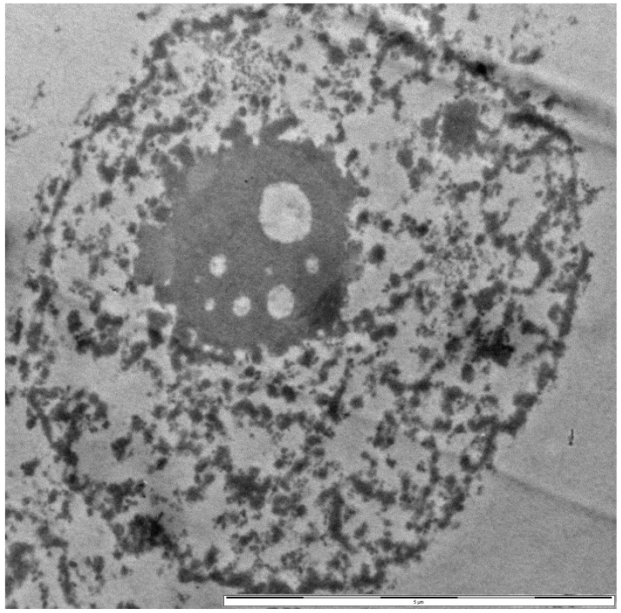
2. Isolation of Neuronal Subtype-Specific Organelles

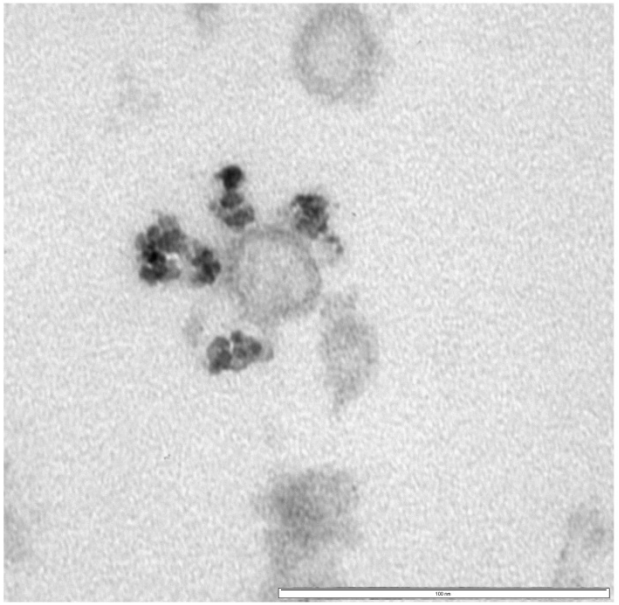
Mitochondria



Nuclei



Synaptic Vesicles



EM analysis reveals that organelles have been enriched and labeled with superparamagnetic microbeads. Image was acquired with an FEI Tenai Biotwin 80-120kV TEM.

Anshu Shen

Top five high- abundance proteins identified by LC-MS/MS analysis

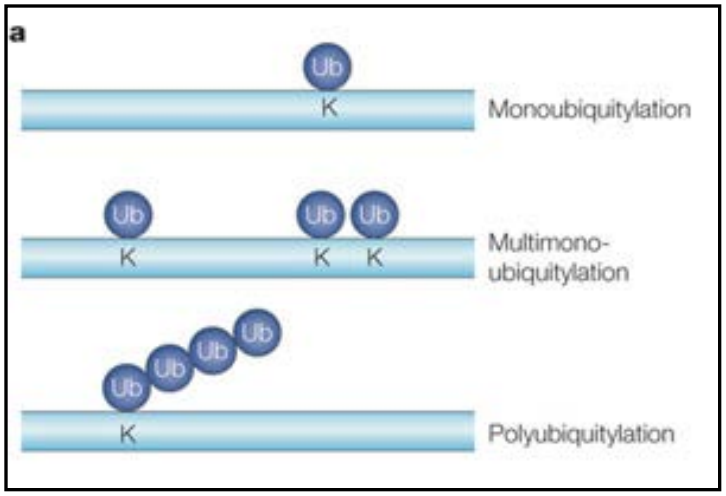
	Protein Name	Protein ID	Expectation	Score	MW	% Coverage
Mitochondria						
1	chaperonin (HSP60)	gi|306890	7.4E-46	505	60986	32.8
2	malate dehydrogenase, mitochondrial precursor	gi|21735621	6.3E-26	306	35481	32.8
3	mitochondrial short-chain enoyl-CoA hydratase	gi|433413	0.0000027	109	31260	12.1
4	chaperonin 10	gi|4008131	0.000033	99	10576	34.3
5	mitochondrial acetoacetyl-CoA thiolase	gi|499158	0.00034	88	45252	4
Nuclei						
1	histone H2B	gi|1568551	1.8E-23	281	13928	40.5
2	histone H4	gi|4504301	1.7E-22	271	11360	57.3
3	histone H2A.Z	gi|4504255	1E-15	203	13545	20.3
4	histone H1.3	gi|4885377	4.8E-14	187	22336	14
5	histone H3	gi|386772	3.1	49	15238	26.7
Synaptic Vesicles						
1	cell cycle exit and neuronal differentiation protein 1	gi|62079059	0.000021	95	15034	19.5
2	syntaxin-binding protein 1 isoform a	gi|4507297	0.000062	90	68692	2.5
3	synaptogyrin 3	gi|149052040	0.00059	81	24497	6.6
4	unnamed protein product	gi|57429	0.00061	80	49931	2.7
5	ADP-ribosylation factor 5	gi|4502209	0.021	65	20517	5.6

2. Cell-Specific Expression Of tagged Ubiquitin Using Tg-BAC

6xHis-HA

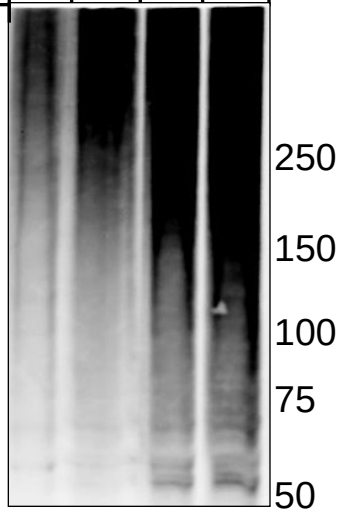
Lys-6 Lys-11 Lys-27 Lys-29 Lys-33 Lys-48 Lys-63

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG



Hicke et al, 2005

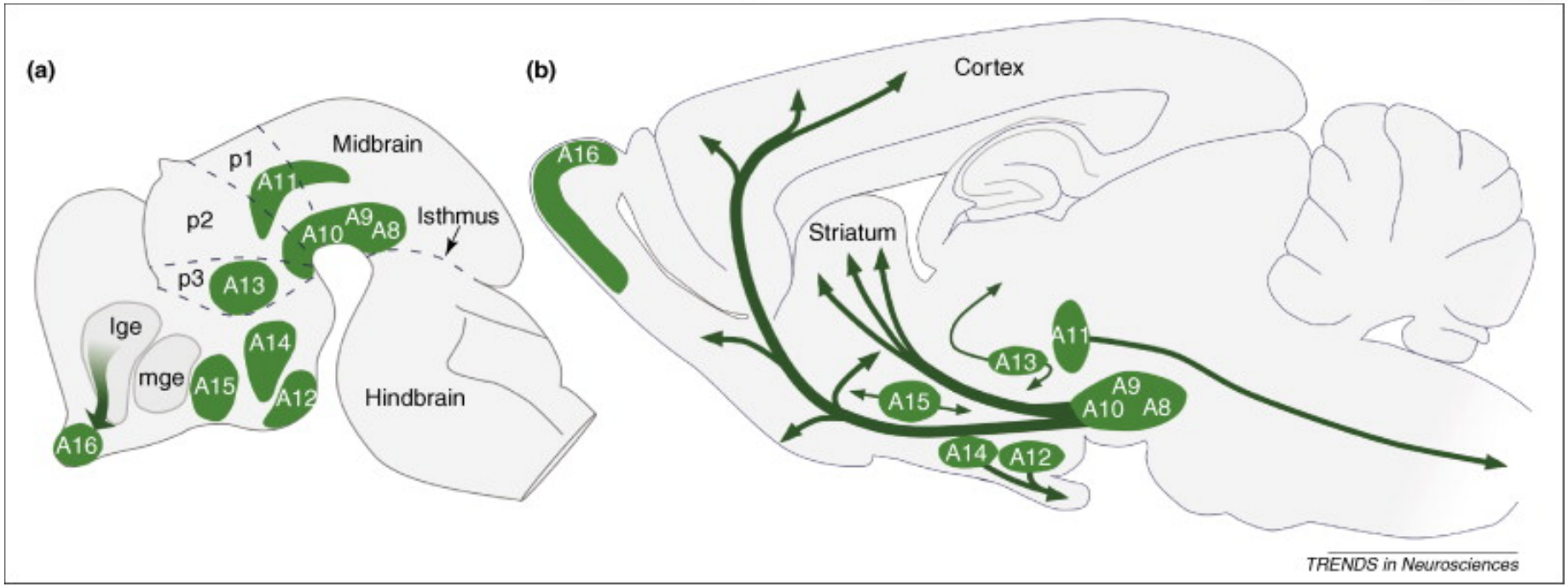
MG132 (μM)	0	1	2	5
6xHis-Ubiquitin	+	+	+	+



George Craft

Dopamine projection neurons

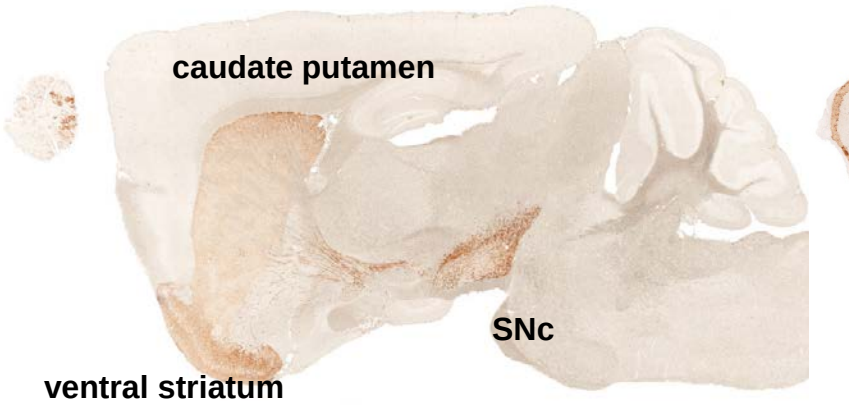
Distribution of DA neuron cell groups in the developing (a) and adult (b) rodent brain

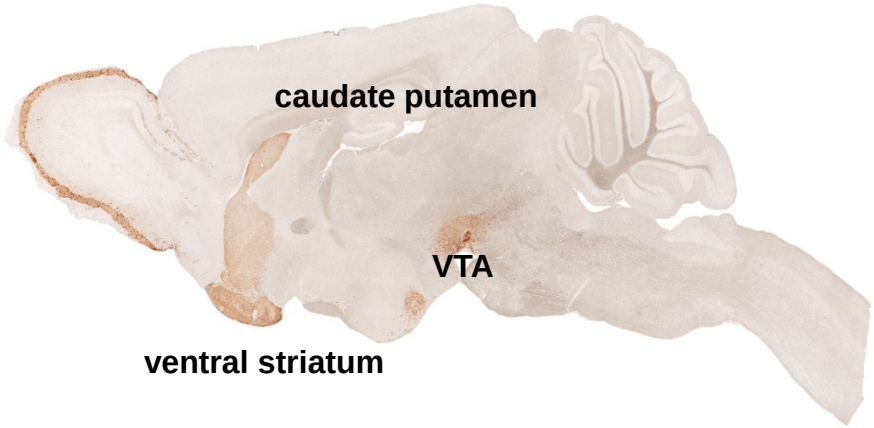


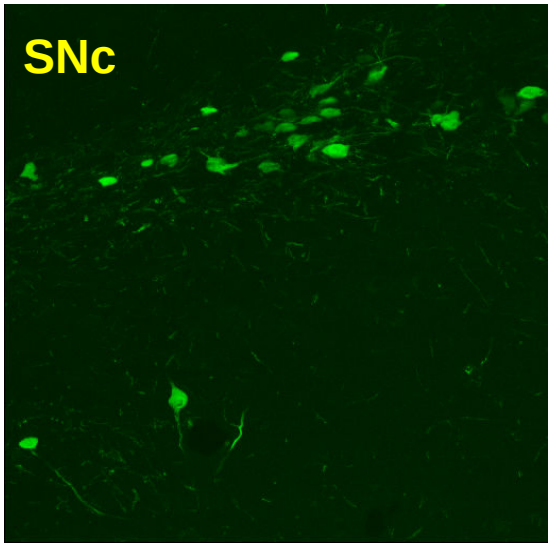
Bjorklund and Dunnett, TINS 2007

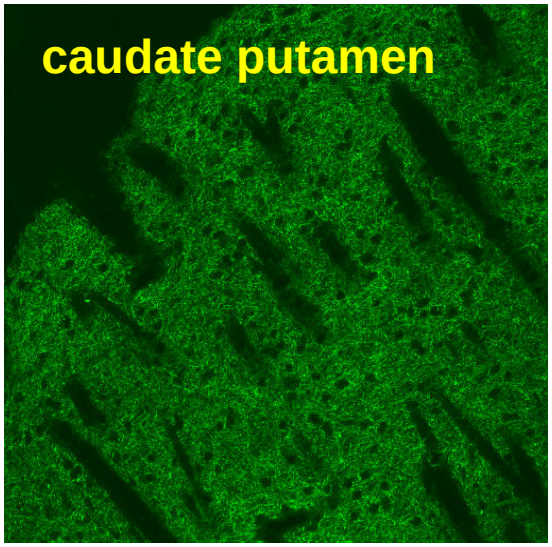
Midbrain DA neurons – 20-30,000 in mice, to 400-600,000 in humans

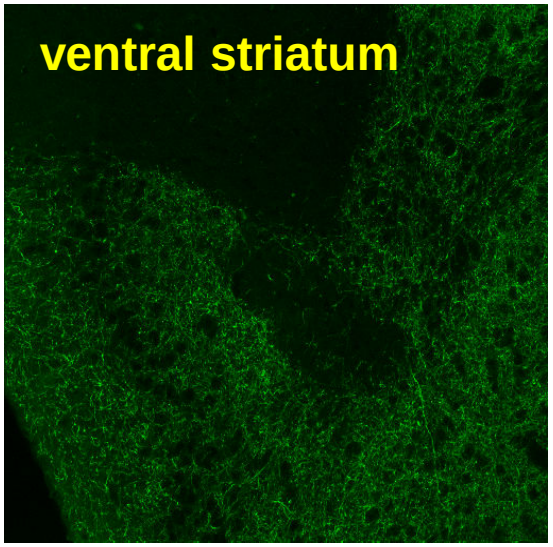
2. Utilize the Transcriptional Unit and Its Associated Regulatory Elements of DAT.





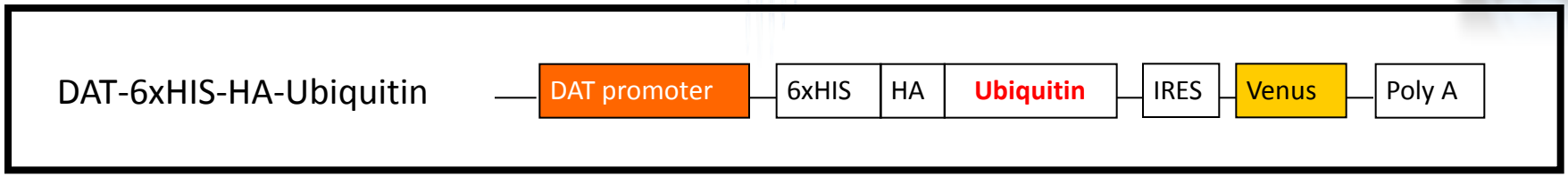




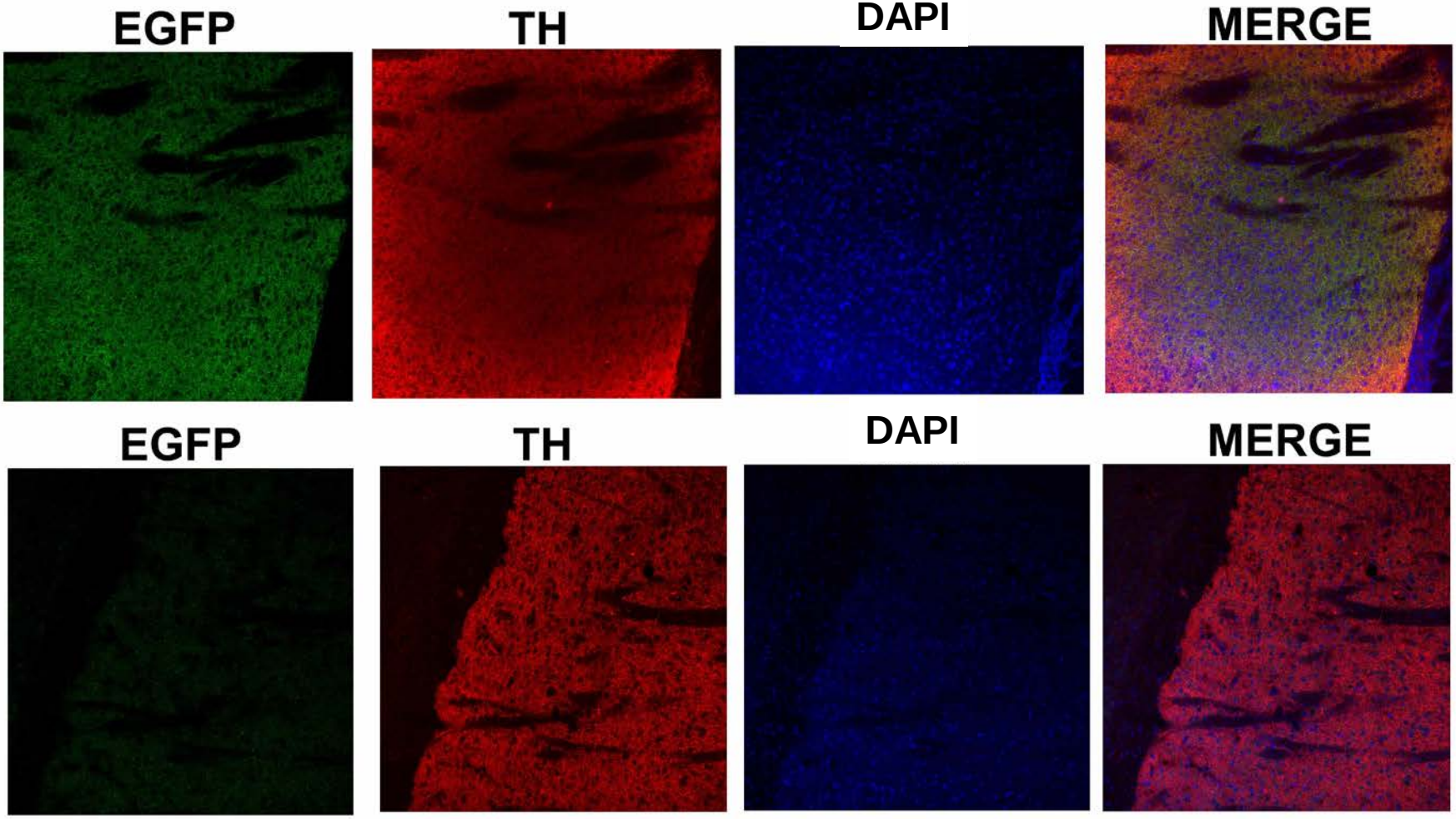


The axons of the DA neurons in the SNc mainly project to the dorsal striatum, with a small percentage innervating specific areas of the cortex and the ventral striatum. *www.gensat.org*

2. Cell-Specific expression of Ubiquitin using Tg-BAC under the control of the dopamine transporter



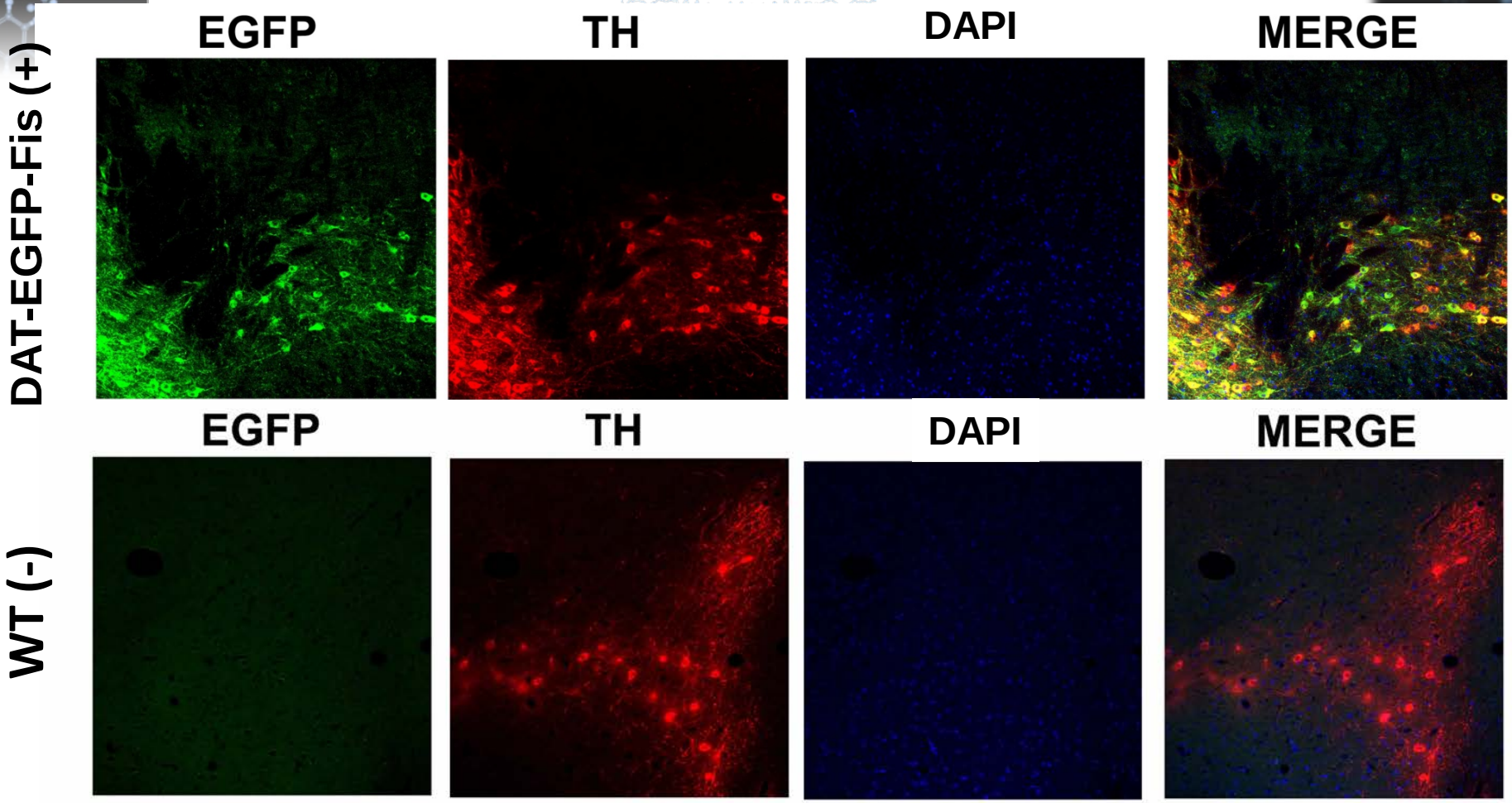
3. Verification EGFP-Fis1 Expression Profile (Genotyping Positive Founder 1, striatum)



- The green-red-blue merged image indicates the co-localization of EGFP and TH in caudate putamen.

Verification EGFP-Fis1 Expression Profile

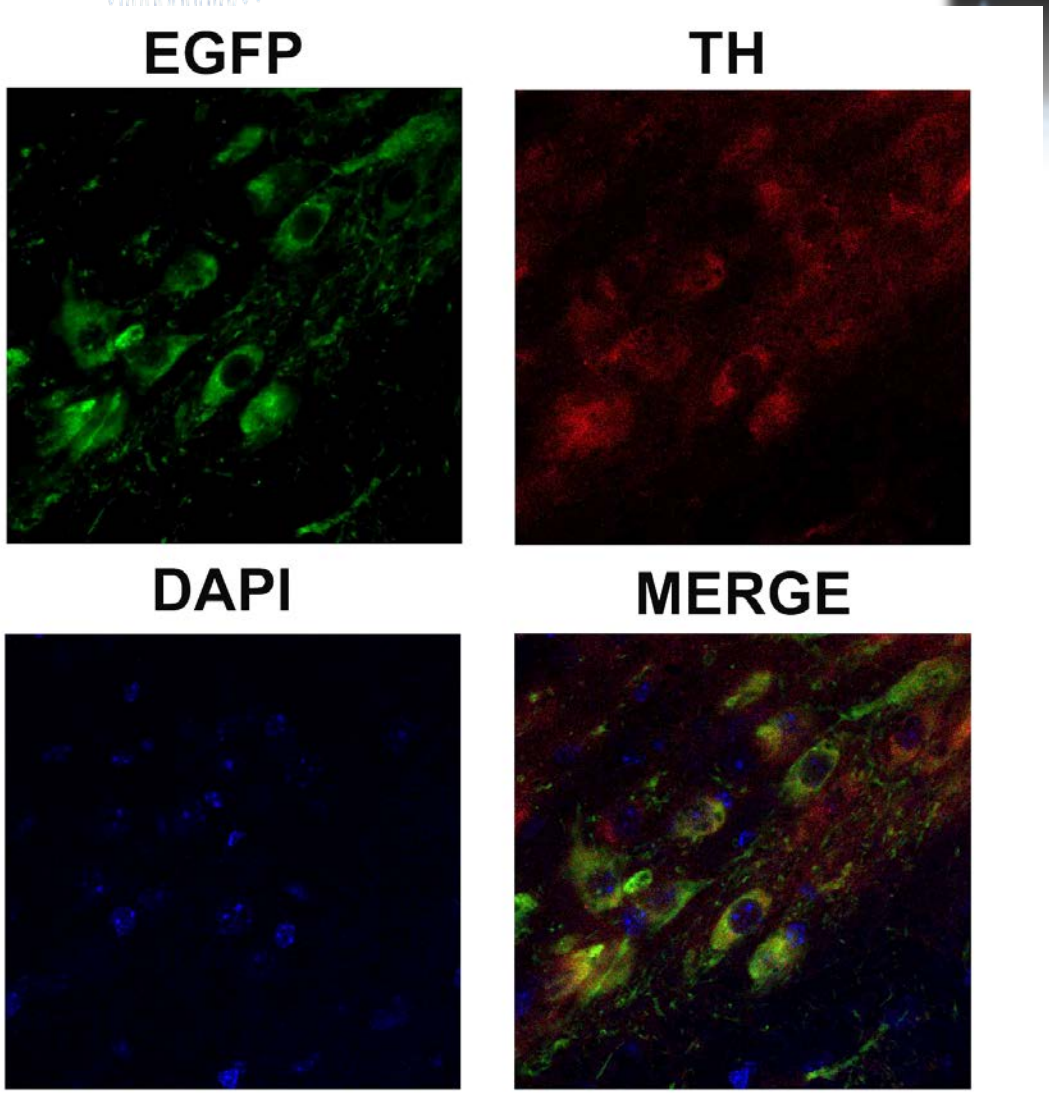
(Genotyping Positive Founder 2, DA neuron cell body)



- The green-red-blue merged image indicates the the co-localization of EGFP and TH.
- Compare with pervious founder, the signal to noise ratio of EGFP channel is relatively lower.

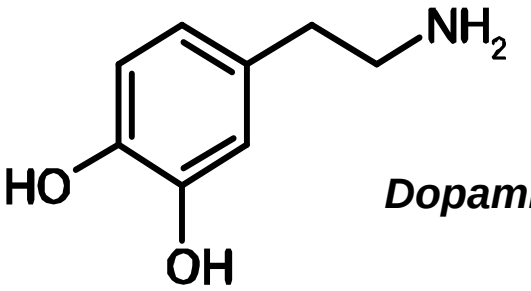
Verification EGFP-Fis1 Expression Profile (Genotyping Positive Founder 1, SNigra)

- **The green-red-blue merged image indicates the the co-localization of EGFP and TH,**
- **The localization of EGFP-Fis1 fluorescence is consistent with mitochondrial localization**

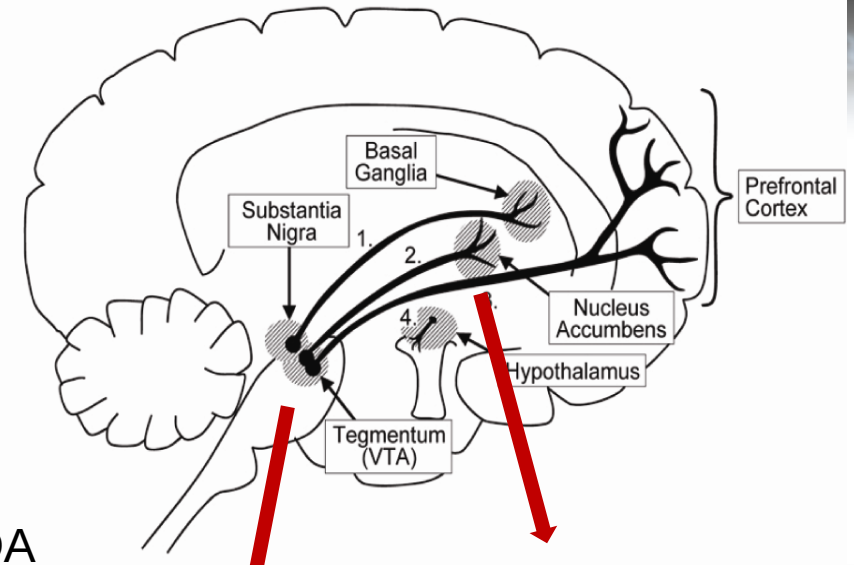


Founder 1,cell body 100X

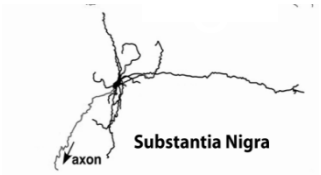
Strategy for ongoing experiments with BAC transgenic mice and Dopamine signaling in mice models

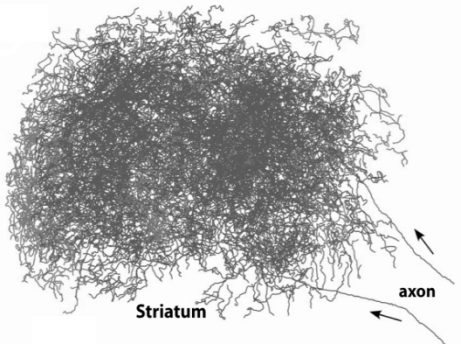


Dopamine



- Examine consequences on organellar proteomes and ubiquitylated proteome in DA neuron sub-compartments – eg axon terminals and projections





Midbrain DA neurons – 20-30,000 in mice, to 400-600,000 in humans

Iversen and Iversen, 2007
Leuner and Muller, 2006
Matsuda 2009

3. Development of large-scale targeted mass spectrometry methods for quantitative analysis of sub-proteomes

- In parallel develop large-scale MRM and SWATH methods to quantitatively interrogate sub-proteomes of interest; eg mitochondria, nuclei, transcription factors, histones, synaptic vesicles
- Optimize MS-based methods to converge at same level of protein isolated from tissue

- Multiple approaches are possible
- eg Target excitatory or inhibitory synapses – proof of principle from studies from Brian Chait and Nat Heintz
- expansion to combined use of viral expression and selective expression of tagged-proteins in BAC-Cre Tg mice
- Potential for combination with FACS sort to isolate specific pre-synaptic/post-synaptic syapses

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