Identification of Drugs for the Treatment of Spinocerebellar Ataxia Type 2

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THE UNIVERSITY OF UTAH

Disclosures: none

What's ahead…

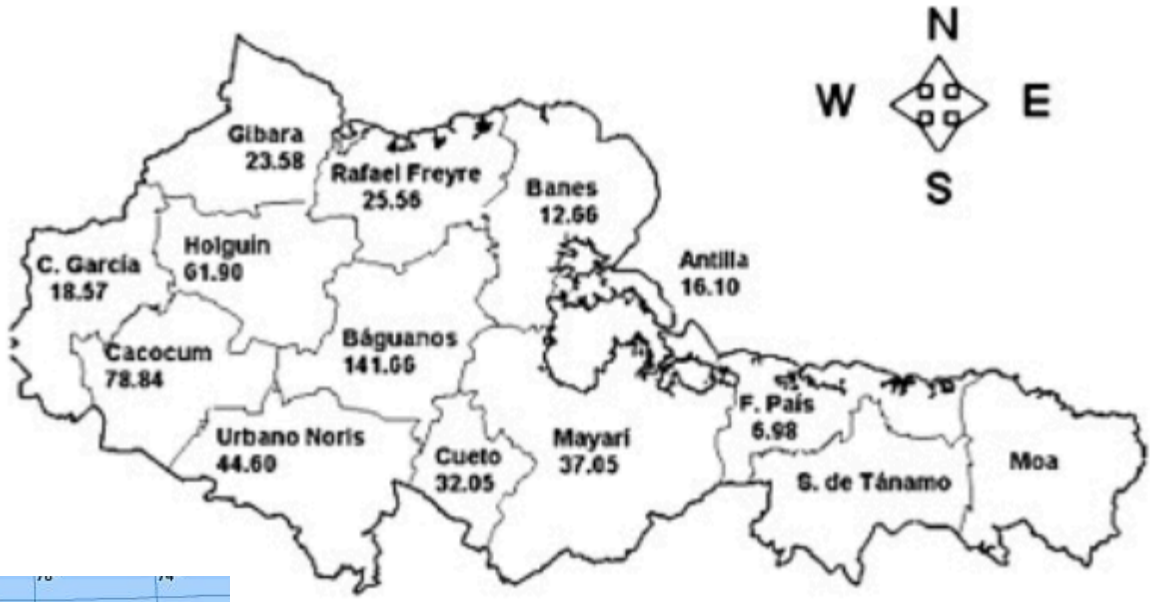
- Introduction to SCA2
  - Clinical Features, Epidemology & Molecular Biology
- Compound screening
  - Secondary screens
  - Biological relevance tests
    - *In vitro* tests
      - Endogenous ataxin-2 expression
      - *In vitro* functional model
    - *Promoter & 3'-UTR characterization*
      - Transcription and translation control
    - *Mouse models*
      - Reporter mouse
        - » Bioavailability, expression & localization
      - Transgenic mouse
        - » Phenotype

SCA2: A brief introduction to its clinical features, epidemiology and molecular biology.

Spinocerebellar Ataxia Type 2 (SCA2)

- Patients are characterized by gait ataxia, frontal executive dysfunction, slow saccades, and DOPA-responsive Parkinsonism.
- SCA2 is a polyglutamine disorder.
- Age of onset varies with CAG length (anticipation).
- Rare (~$1/10^6$) but common in Cuba (3/2000).
- Disease of the cerebellum (SCA2 accounts for 13% of ADCA patients).
- Characterized by Purkinje cell death.

Prevalence rate in Eastern Cuba (per 100,000 people)

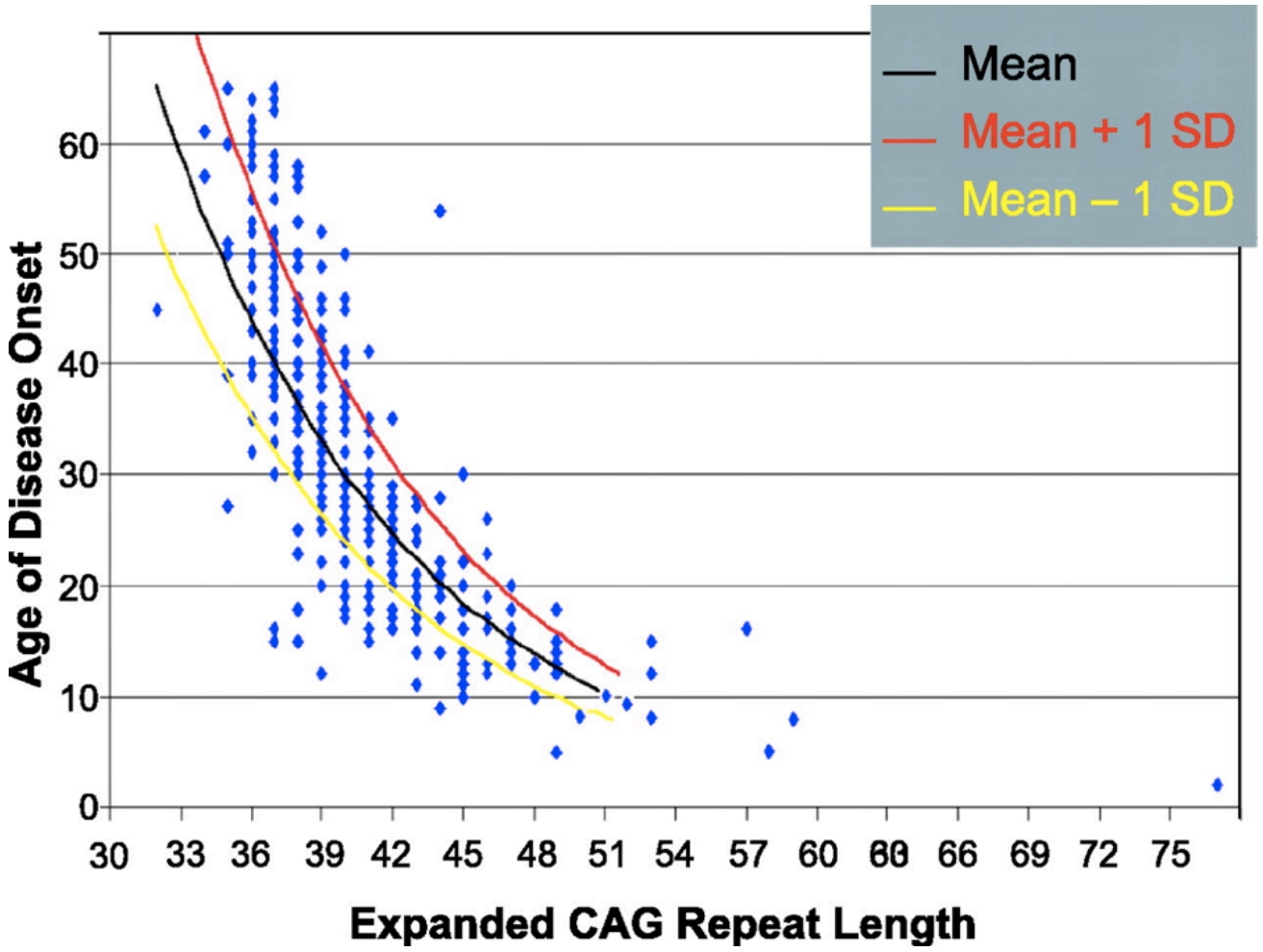


Velazquez Perez et al. Neurosci. Lett. 454:157-60; 2009.

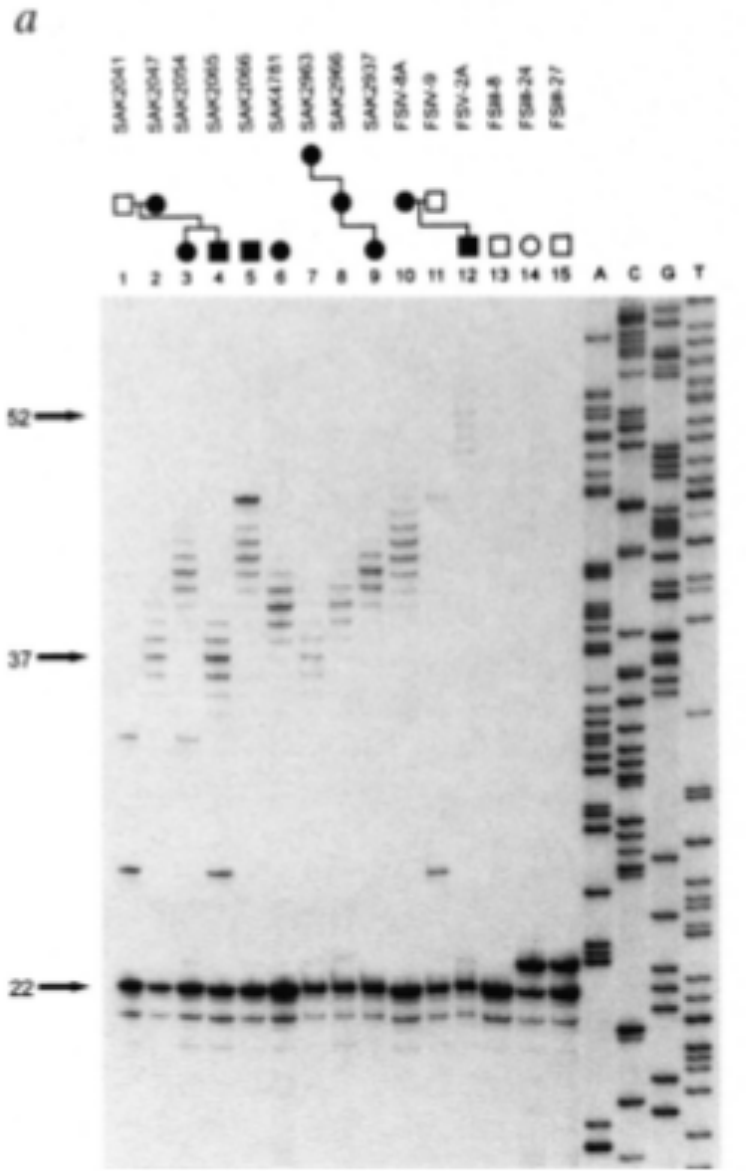
Molecular Genetics of SCA2

- Caused by CAG expansion in the *ATXN2* gene coding region encoding a polyglutamine.
- CAG <32 is normal, >32 is disease-causing.
- In patients the CAG lengthens with each generation.
- Longer CAG tracts are associated with earlier age of onset and greater severity of disease.

Anticipation



Pulst et al. Brain 2005



PAGE

Most common normal allele

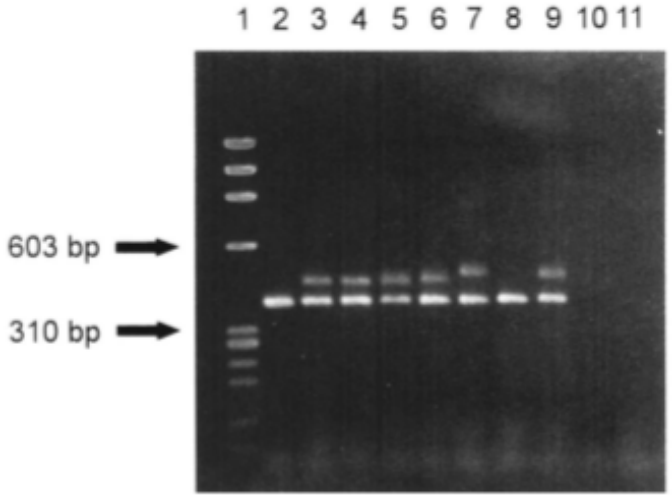
$(CAG)_8CAA(CAG)_4CAA(CAG)_8$

Also observed frequently in the normal population

$(CAG)_{14}CAA(CAG)_7$

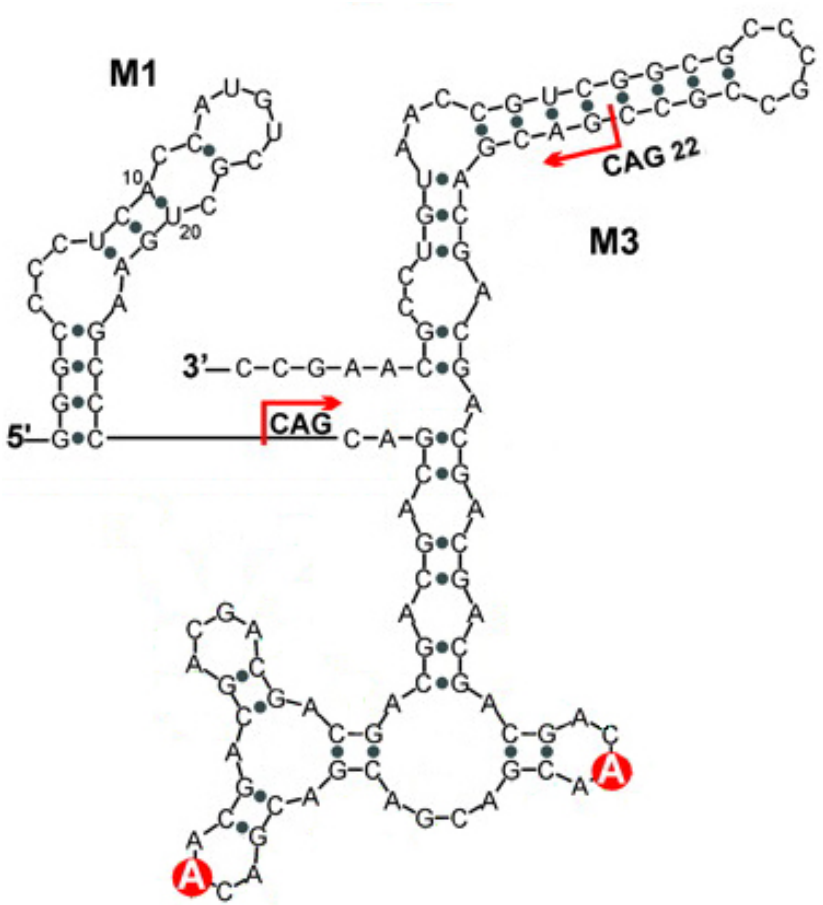
Disease allele

$(CAG)_{>32}$



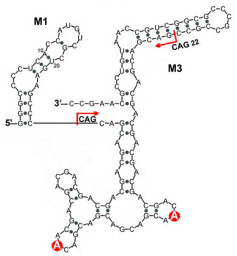
AGE

Secondary RNA structure of the most common normal allele $(CAG)_8CAA(CAG)_4CAA(CAG)_8$

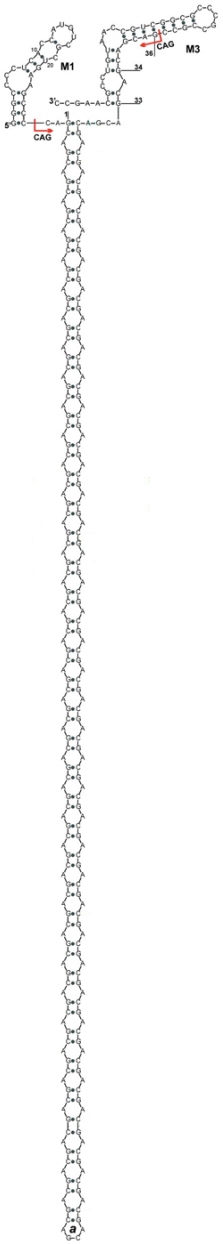


Sobczak, K. et al. J. Biol. Chem. 2005;280:3898-3910

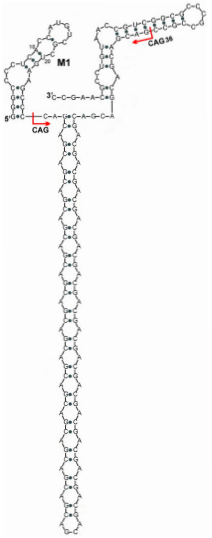
CAG22



CAG110



CAG36

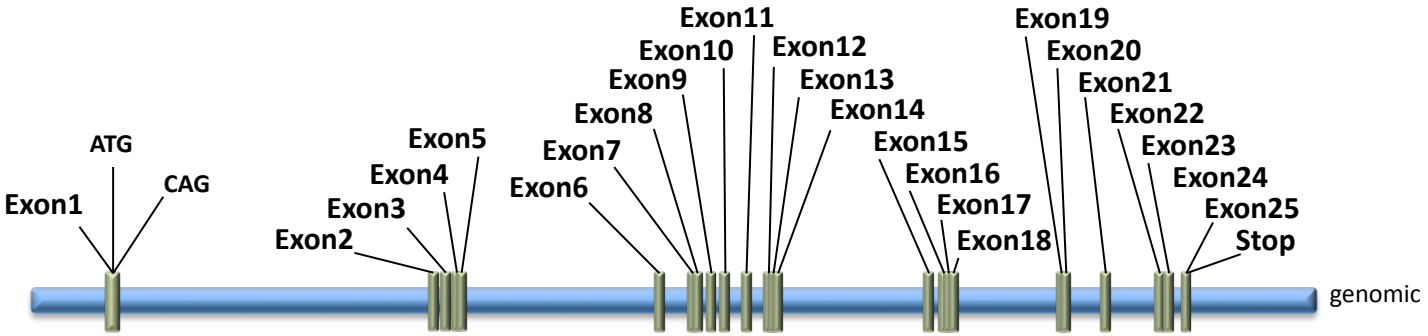


What would this feature do to…

Expression?

Toxicity?

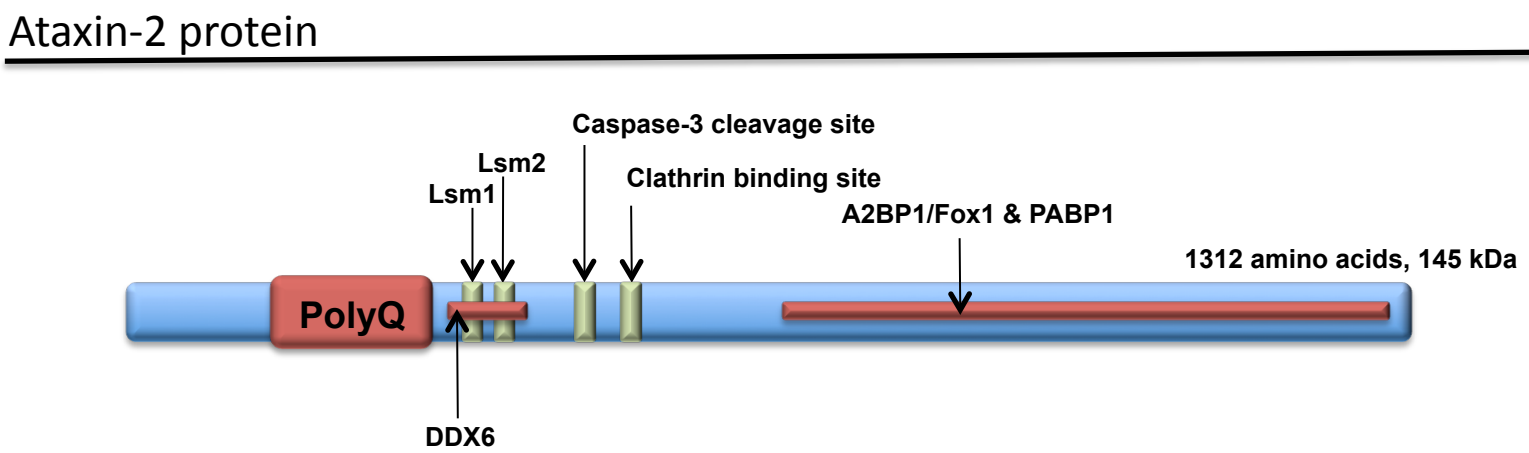
The *ATXN2* gene



150 Kb
25 exons
CHR 12q24.1
3,939 bp cDNA

Ataxin-2 protein & function

- Ataxin-2 appears to control RNA metabolism or RNA expression through its interactions with polyA binding protein (PABP1), RNA splicing factor A2BP1/Fox1, and polyribosomes.
- Ataxin-2 localizes to Golgi, stress granules and p-bodies.
- Overexpression of ataxin-2 induces apoptosis and is toxic.



Towards compound screening: building a valid system.

SCA2 problem

- We have known the *ATXN2* gene causes SCA2 since 1996 but we do not know enough about its function to devise functional therapeutics.

- With the lack of knowledge on its function we decided that the best approach would be to devise therapeutics to knock down its expression.

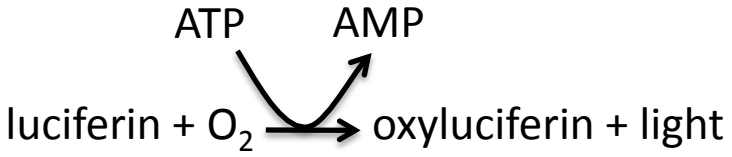
Hypothesis

Compounds that lower ataxin-2 expression will be therapeutic for SCA2

- SCA2 pathology is related to a gain of normal function or gain of toxic *ATXN2* mutant function.
- In humans outcome worsens with increased mutant *ATXN2* dose, and this is supported by studies of transgenic mice (1,2).
- *ATXN2* knockout mice have only mild phenotypes including obesity and reduced fertility (3).
- Reversibility of SCA1&3 transgenic mouse phenotype (4).

1. Ragothaman et al., 2004 & 2008
2. Huynh et al., 2000, 2009
3. Kiehl et al., 2006, Lastres-Becker 2008
4. Zu et al., 2004; Boy et al., 2009.

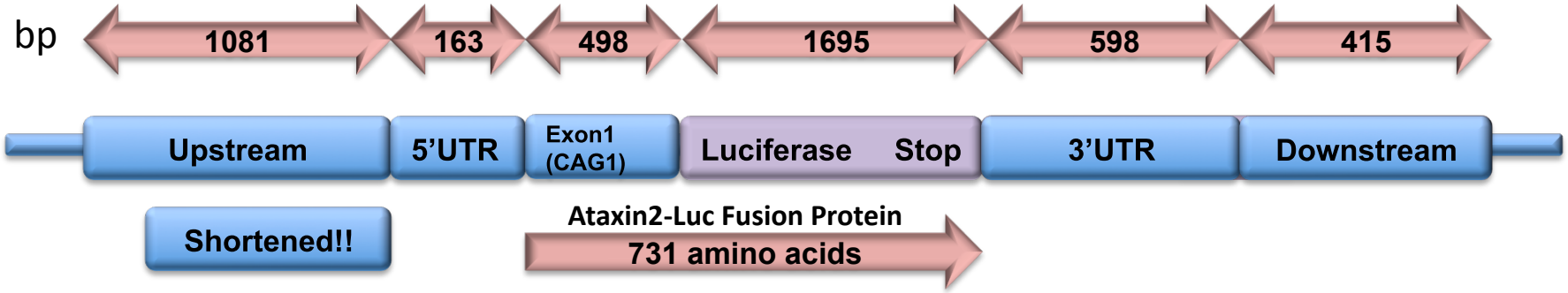
Firefly (*Photinus pyralis*) Luciferase



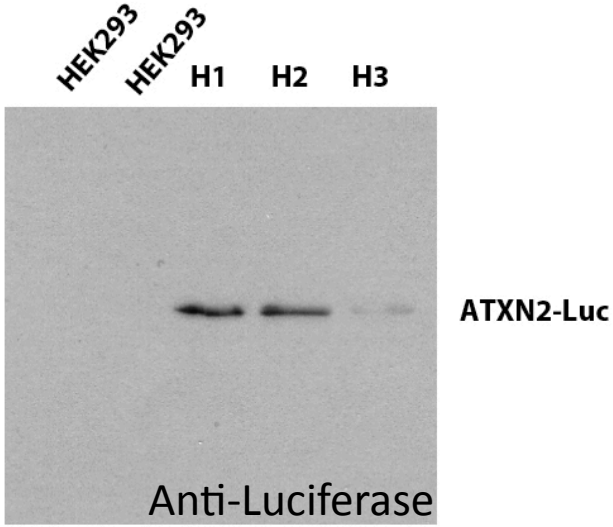
Sea Pansy (*Renilla reniformis*) Luciferase

pGL2-ATXN2-Luc

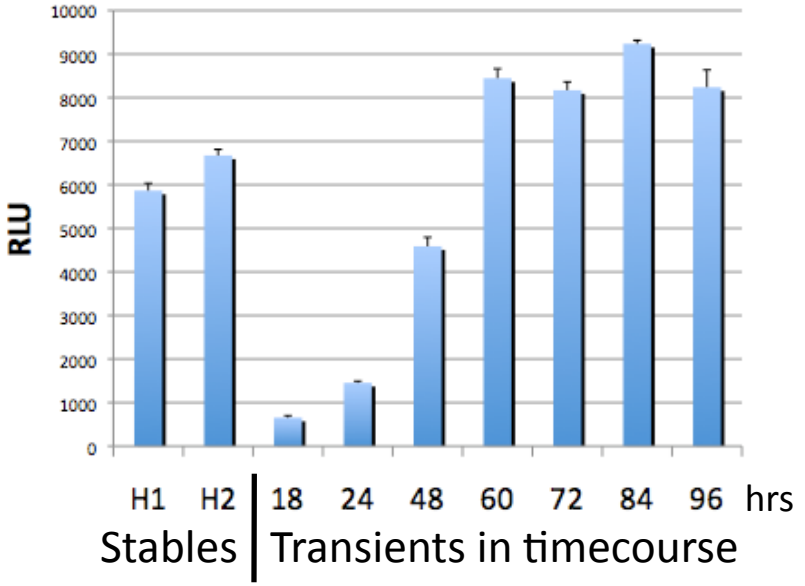
ATXN2-Luciferase Expression Construct



Western Blotting



Luciferase Assays



Optimizations before screening

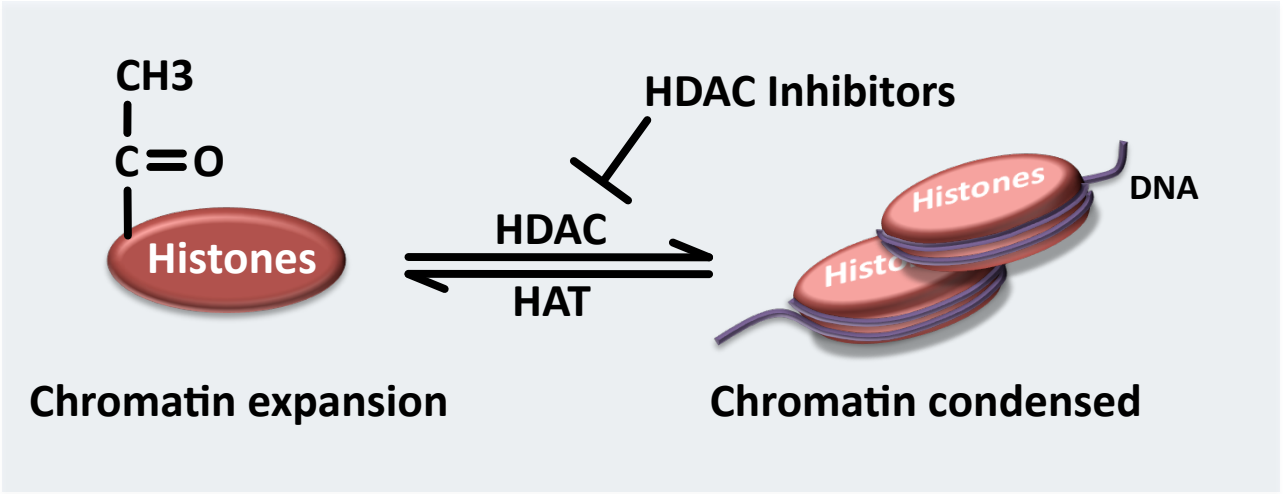
Optimizations

- Effect of DMSO on proliferation.
- Numbers of cells to plate.
- Method of drug addition.
- Length of time to incubate cells on drugs.

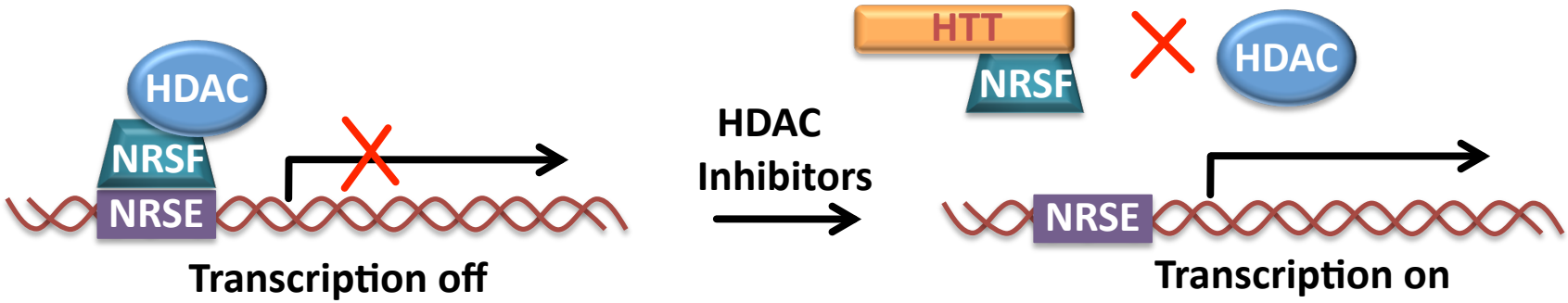
- Positive control compounds.

HDAC inhibitors as therapeutics for polyglutamine disorders

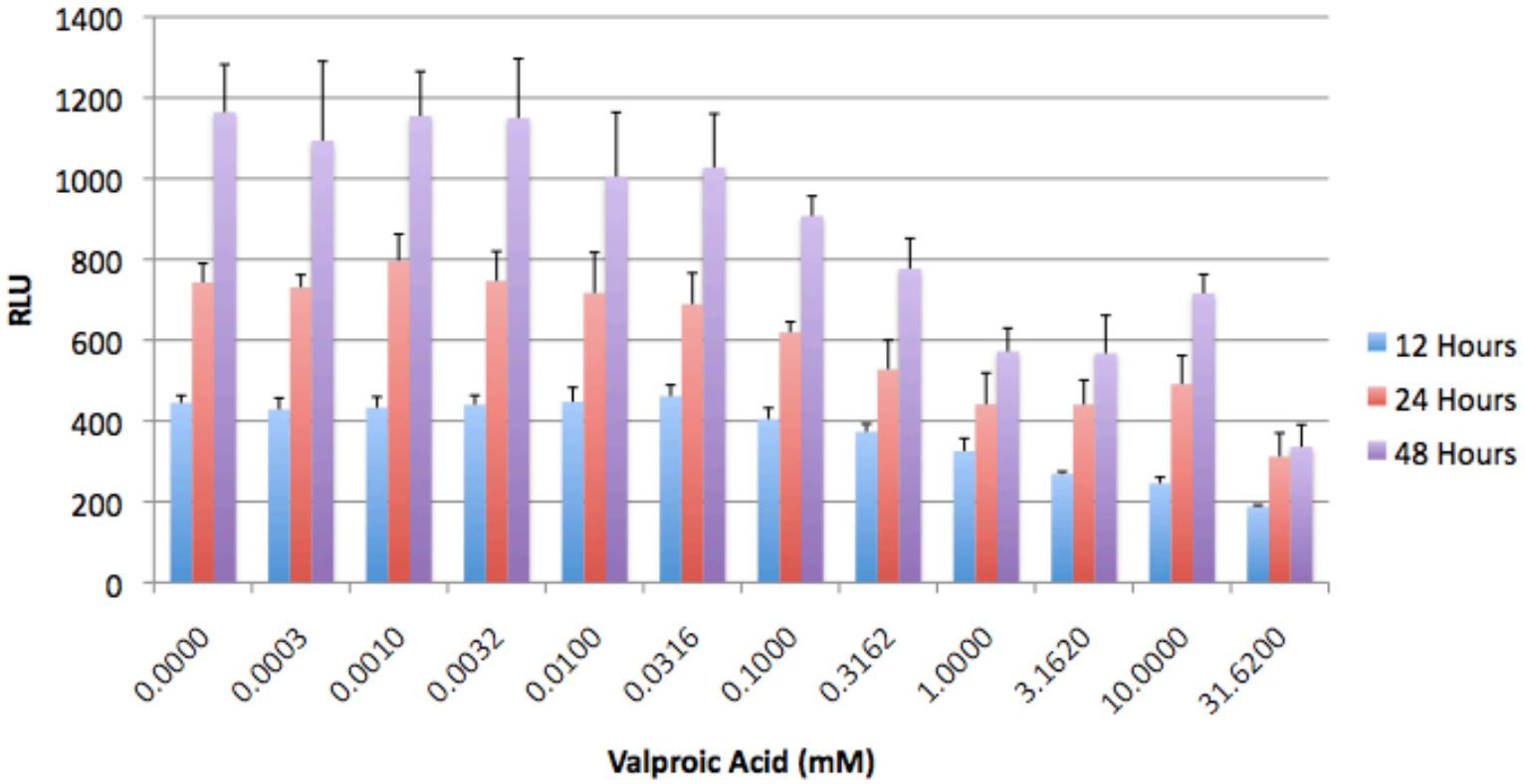
Butler & Bates, Nat Rev Neurosci 7, 2006.



Neuron restrictive silencer factor & Huntingtin

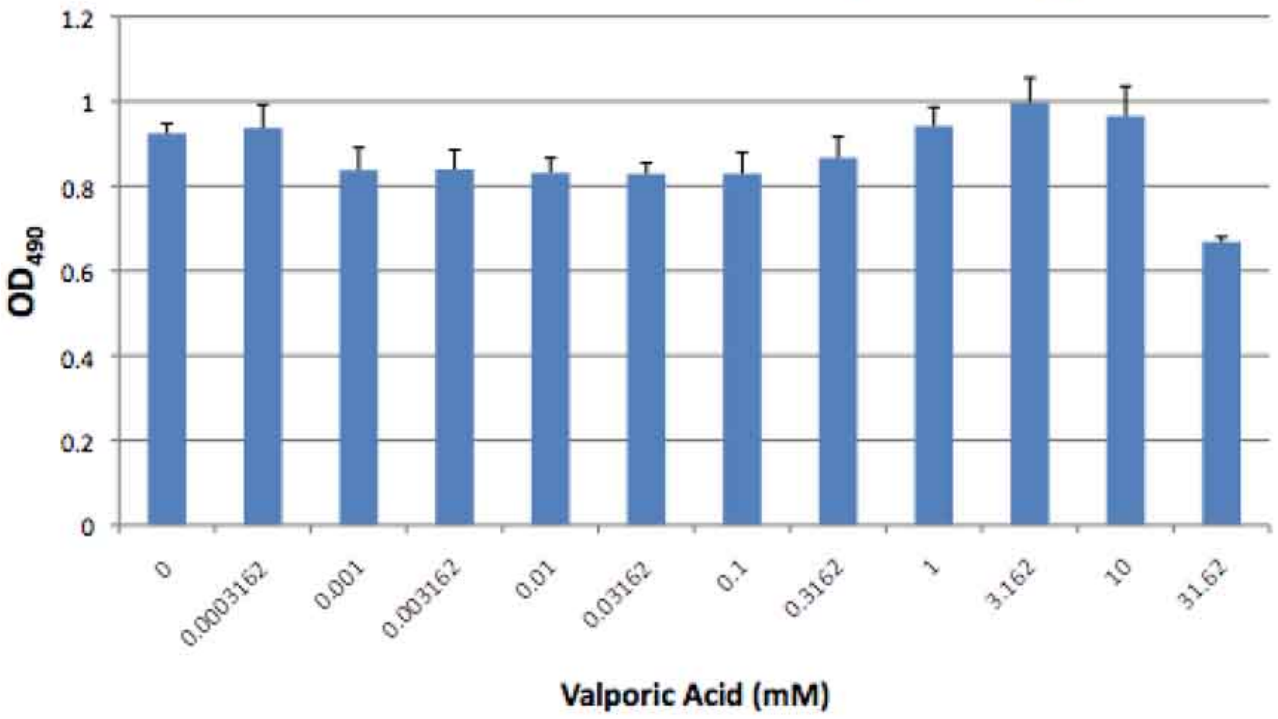


Valproic acid inhibits ATXN2-Luc



The level of VPA that is achievable in patient serum is ~0.3 mM

VPA does not inhibit cell proliferation at the physiological dose



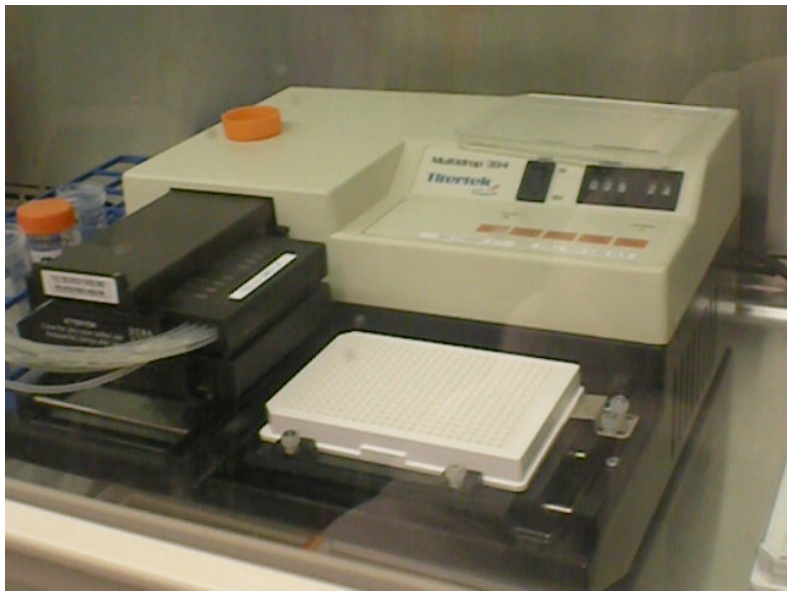
Compound Screening

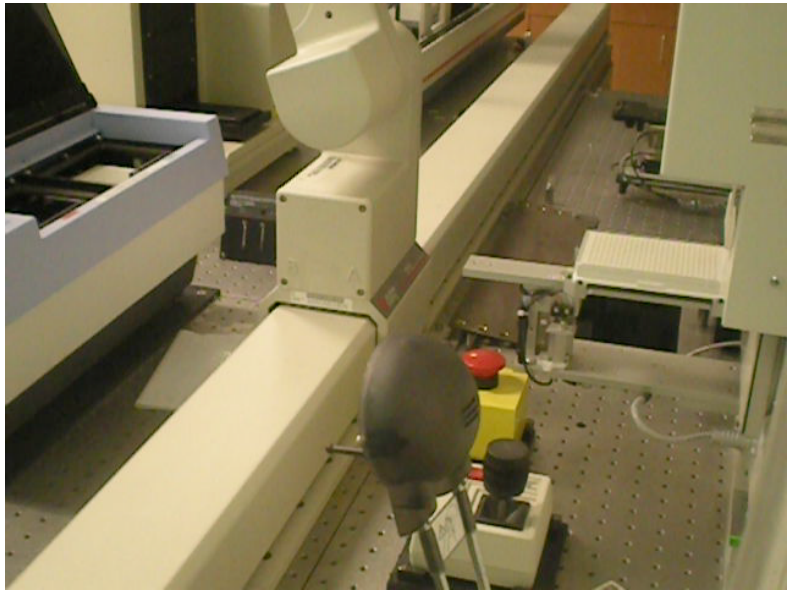
Libraries Screened

Library	Source	Plates	Compounds	Hits (3SD)
Enzymes	Biomol International	1	300	7
Lipids	Biomol International	1	204	0
PW	Prestwick Chemical (FDA approved)	4	1120	27
MS	Microsource Spectrum	7	2000	41
TAR	Asinex (Targeted Library)	27	8505	19
NIH	BioFocus DPI (NIH Clinical Collection)	2	607	15
ES	Unpublished	4	1408	7
S	Unpublished	4	1408	2
DL	Asinex (Platinum Collection)	53	19,570	74
UCLA	ChemBridge Corp.	94	30,000	171

Total number of 384 well plates = 197
Total number of compounds = 65,122
Total hits(3SD down)=363
Hit rate=363/65,122 x 100 = 0.5%

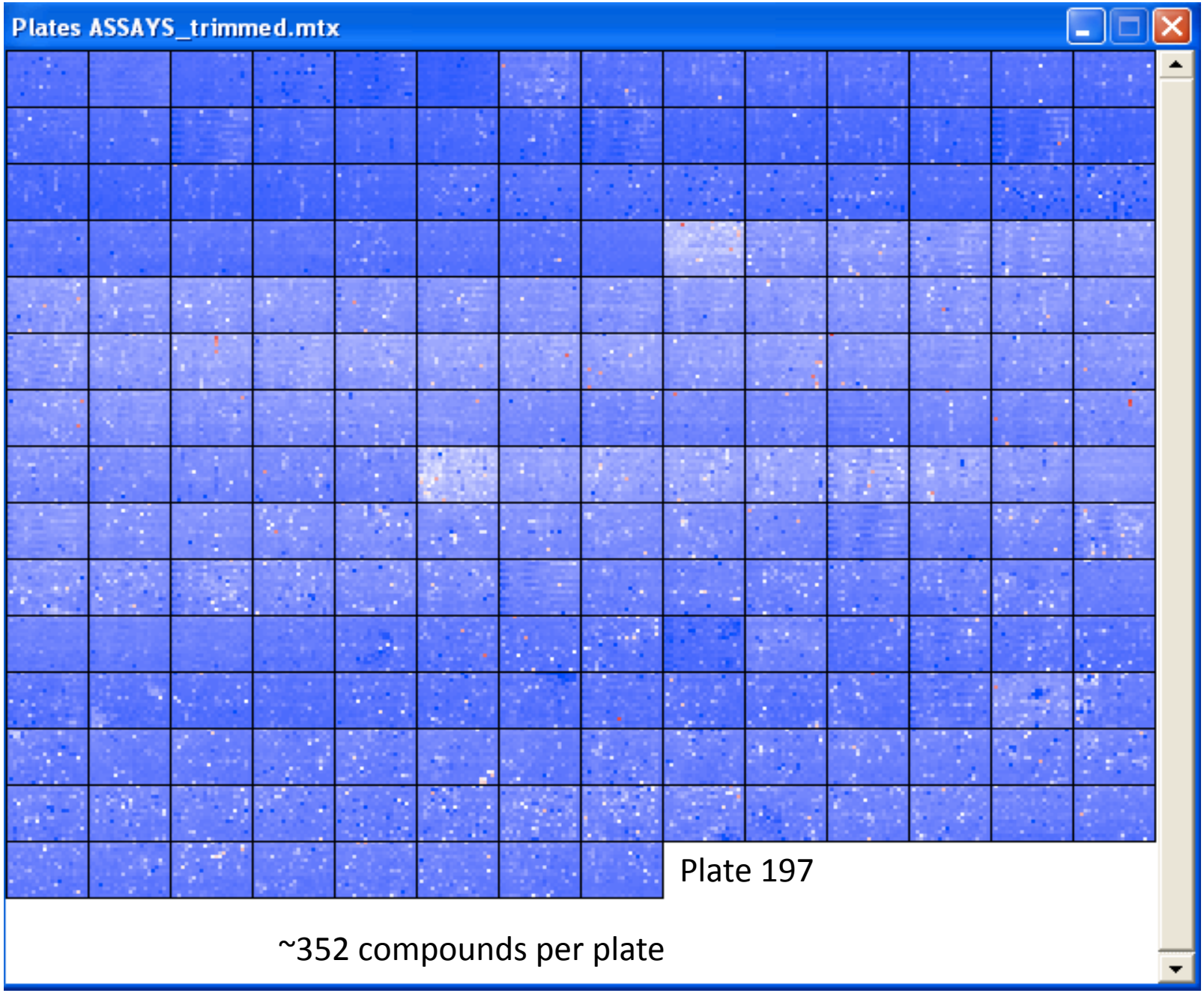
Conducting the screen





Uncorrected RLU Values

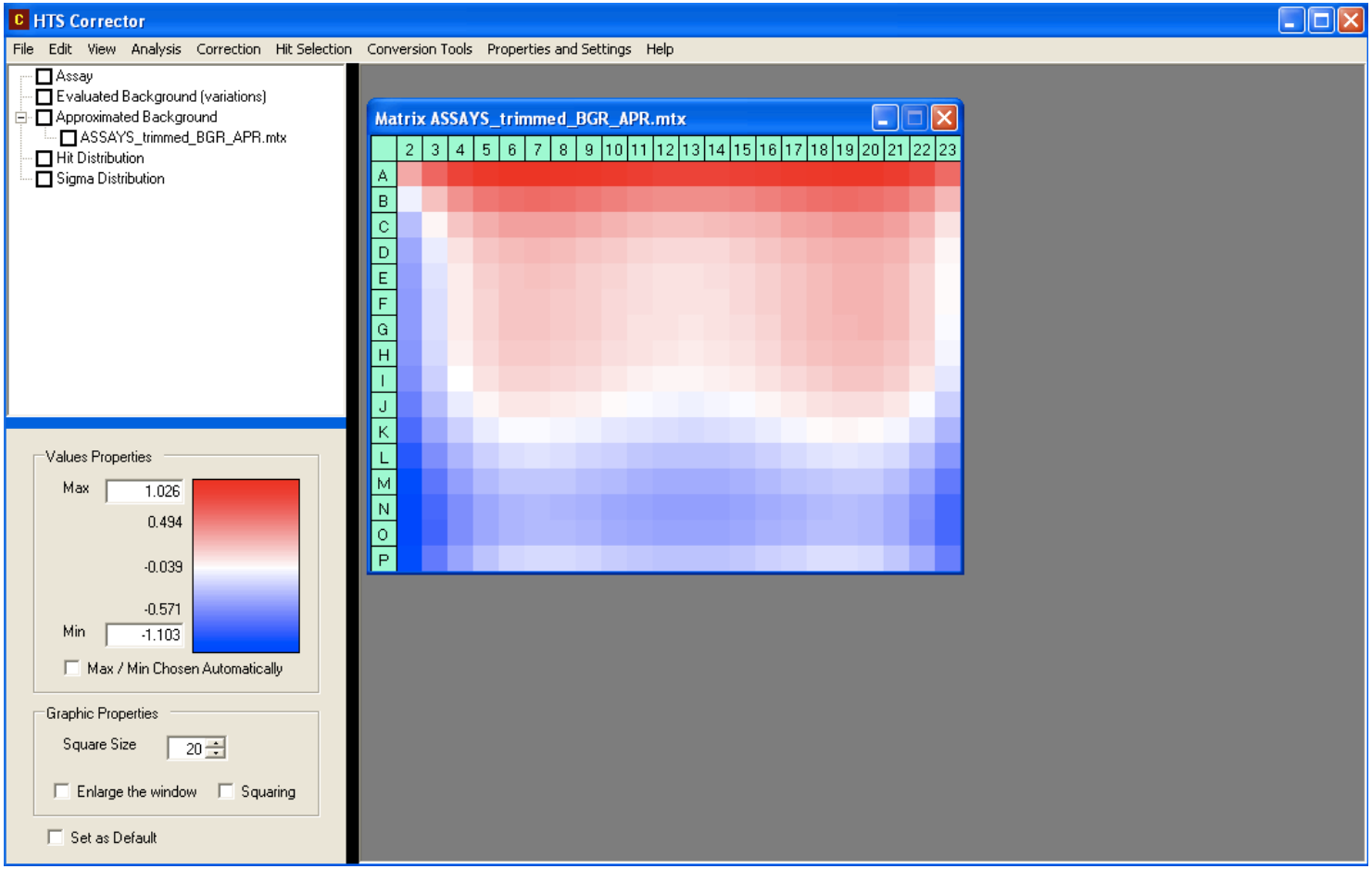
Plate 1



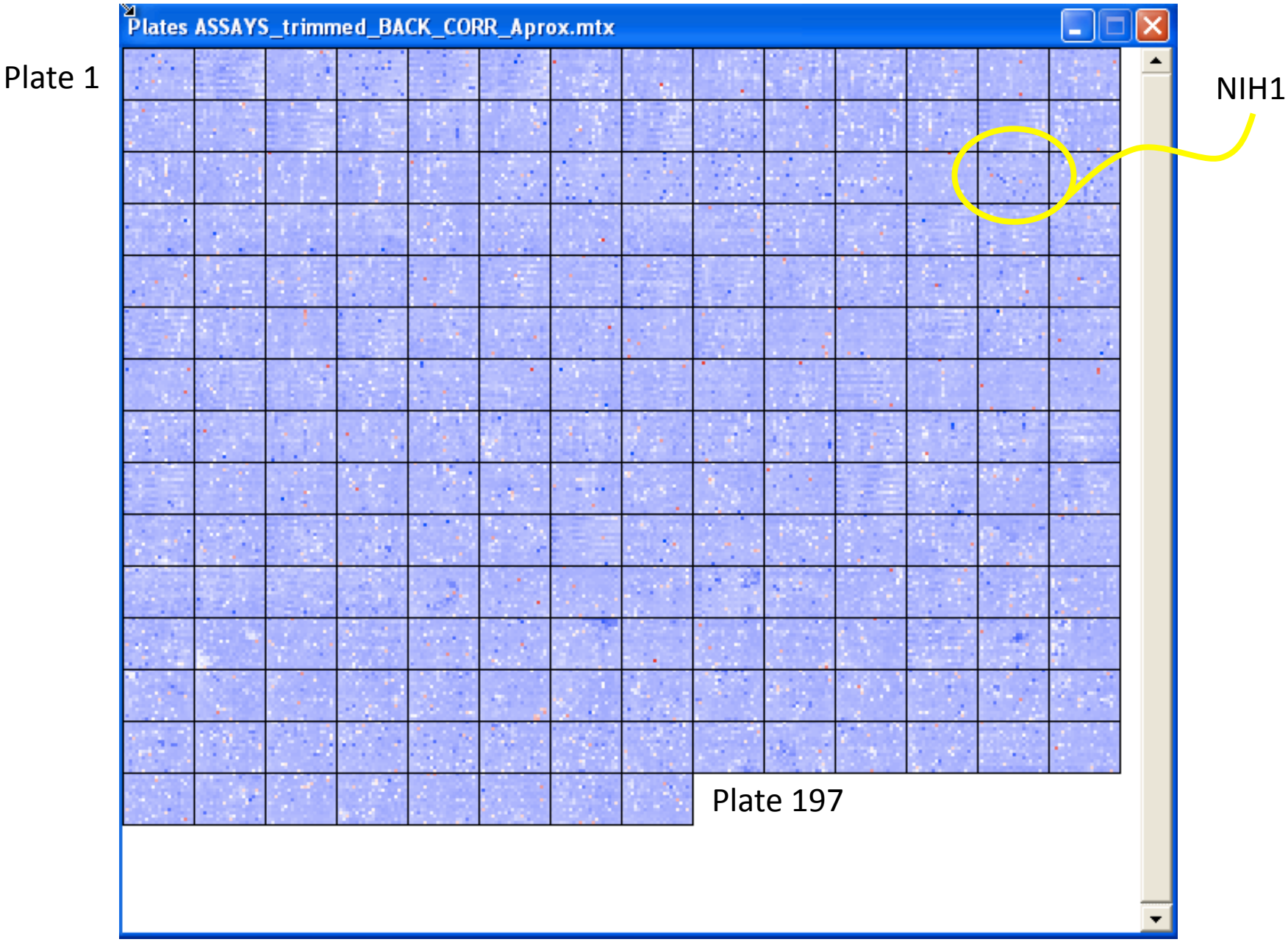
First all values are normalized to a mean of zero and standard deviation of one…

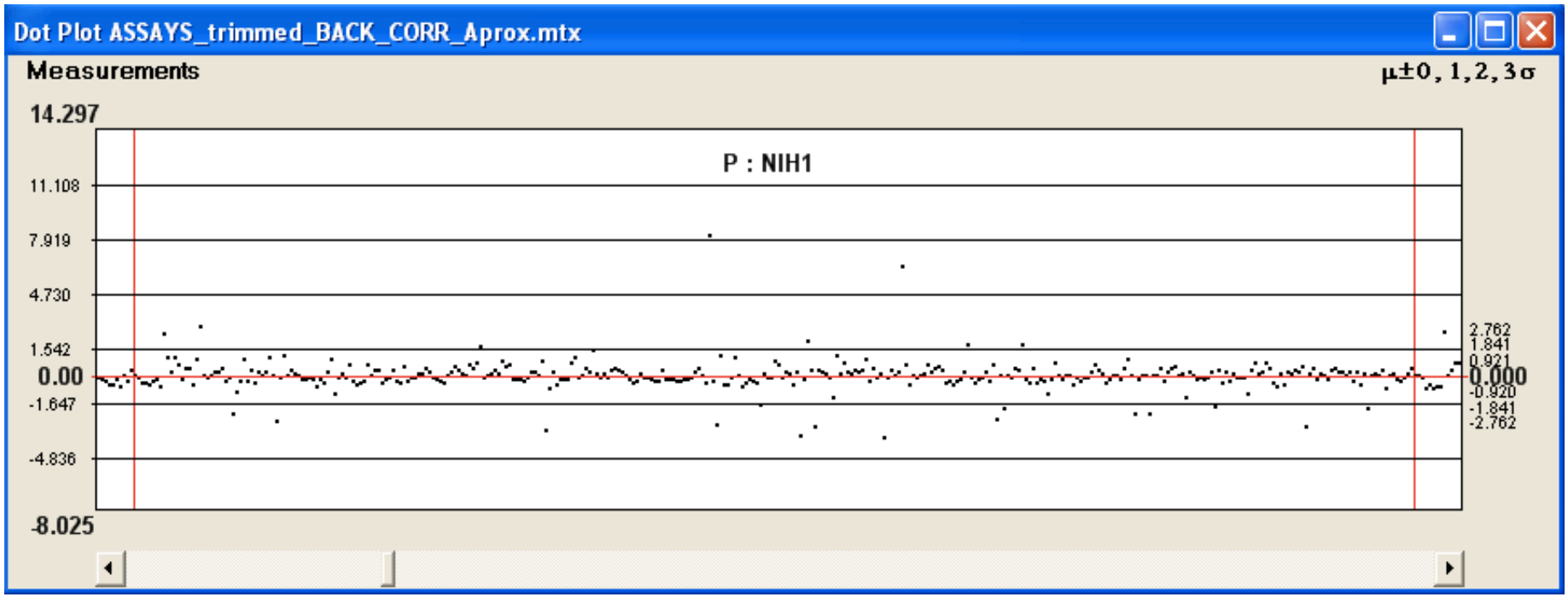
$$x_i' = \frac{x_i - \mu}{\sigma}$$

Then the background is calculated and subtracted

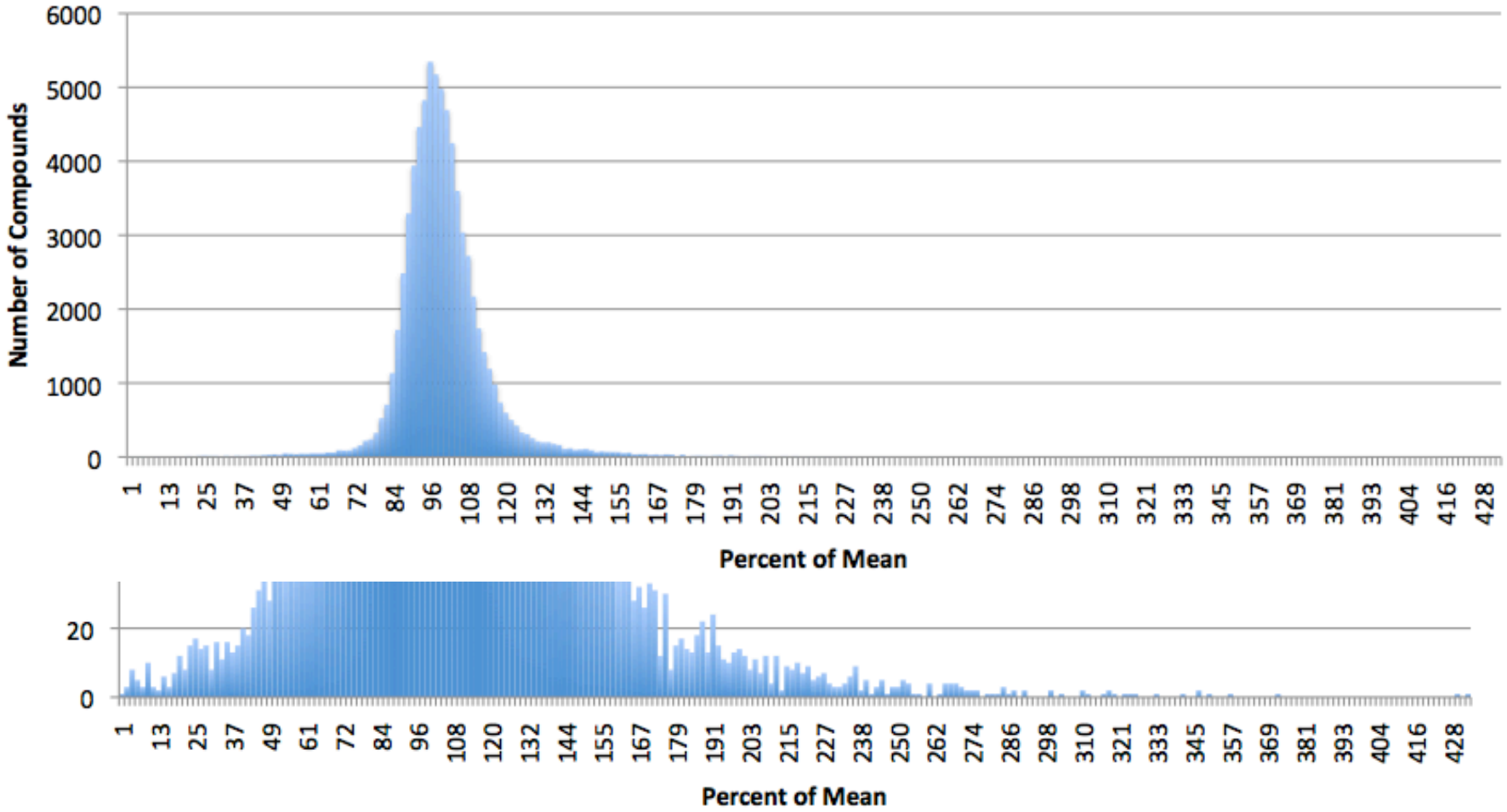


Corrected RLU Scores




Dot Plot ASSAYS_trimmed_BACK_CORR_Aprox.mtx
Measurements
μ±0,1,2,3σ
14.297
P : NIH1
11.108
7.919
4.730
1.542
0.00
-1.647
-4.836
-8.025
2.762
1.841
0.921
0.000
-0.920
-1.841
-2.762

Frequency distribution
(freq of compounds vs effect on expression)



The Hits

Antagonist | Agonist

20 NaK ATPases

Turn up *ATXN2*-Luc ("uppers")

Albendazole
Dantroline
Diphenoxylate
Flufenamic acid
Hydralazine hydrochloride
Ipriflavone
Kaempferol
Nabumetone
Phenelzine sulfate
Piperine
Propafenone
Resveratrol
Riluzole hydrochloride
SDZ-202791 S(+)

Turn Down *ATXN2*-Luc ("downers")

2-amino-9,10-phenanthrenedione
5-Nonyloxytryptamine
Aklavine hydrochloride
Cephaeline dihydrochloride heptahydrate
Cobalamine
Convallatoxin
Cyanocobalamin
Cymarin
Derrubone
Digitoxigenin
Digitoxin
Digoxigenin
Digoxin
Emetine dihydrochloride
Emicymarin
Gitoxigenin diacetate
Gitoxin
Helveticoside
Indatraline
Isopropamide iodide
L-694,247
Lanatoside C
Lomerizine DiHCl
Menadione
Neriifolin
Nicardipine
Ouabain
Periplocymarin
Peruvoside
Pinacidil
Proscillaridin A
Resorcinol monoacetate
Ryanodine
Sanguinarine
Sarmentogenin
SKF-96365
Strophanthidin
Strophanthidinic acid lactone acetate
SU1498
Tegaserod maleate
TMB-8
Tramadol
Trifluoperazine hydrochloride
Trifluridine
U-37883A
U-50488

The Hits

Antagonist | Agonist

10 Calcium Channels

Turn up *ATXN2*-Luc ("uppers")

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The Hits

Antagonist | Agonist

3 Serotonin

Turn up *ATXN2*-Luc ("uppers")

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The Hits

Antagonist | Agonist

3 Dopamine

Turn up *ATXN2*-Luc ("uppers")

Albendazole
Dantroline
Diphenoxylate
Flufenamic acid
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The Hits

Antagonist | Agonist

2 Opioid Receptors

Turn up *ATXN2*-Luc ("uppers")

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20 Others

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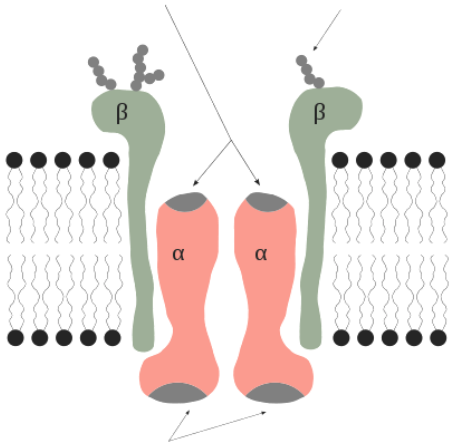
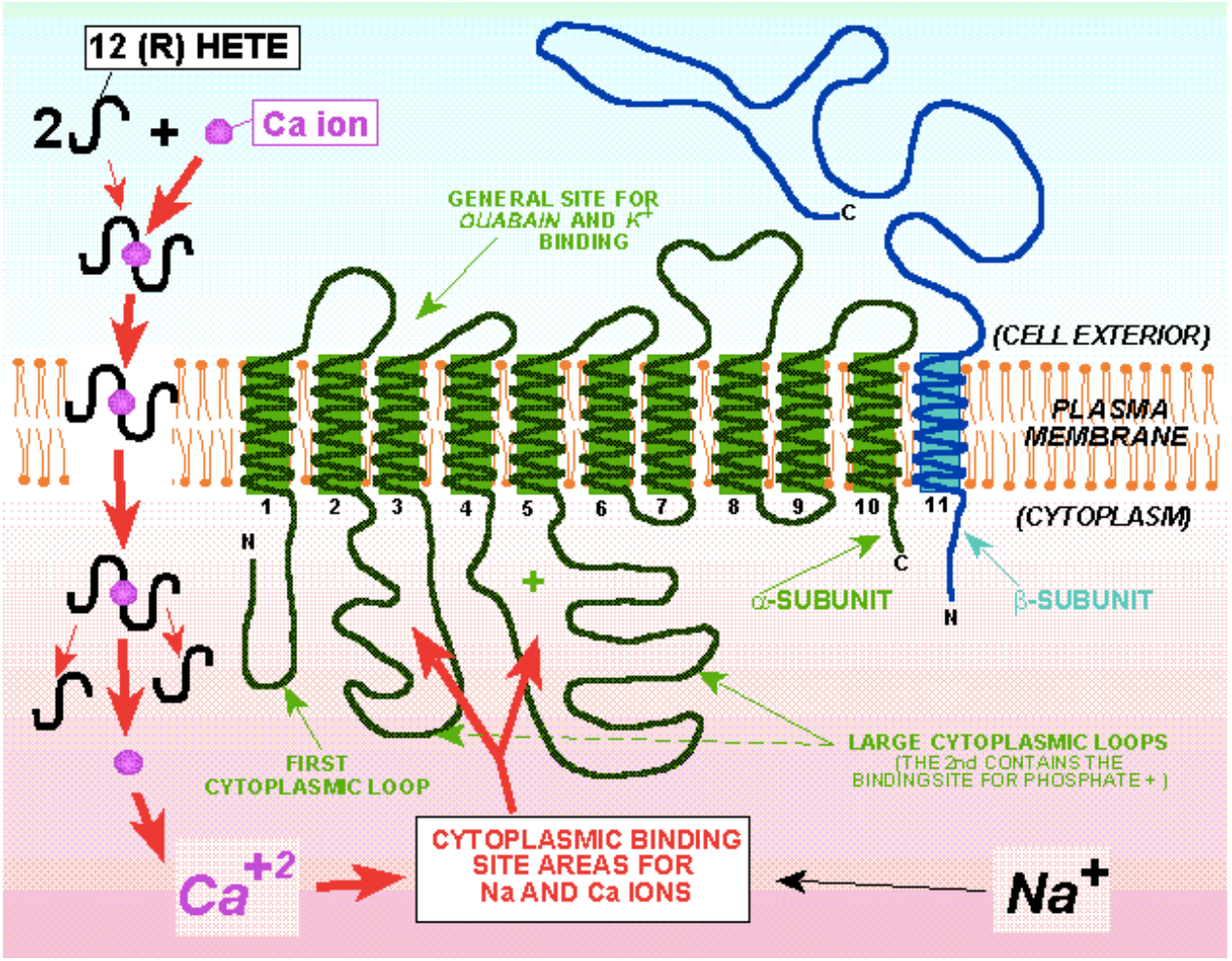
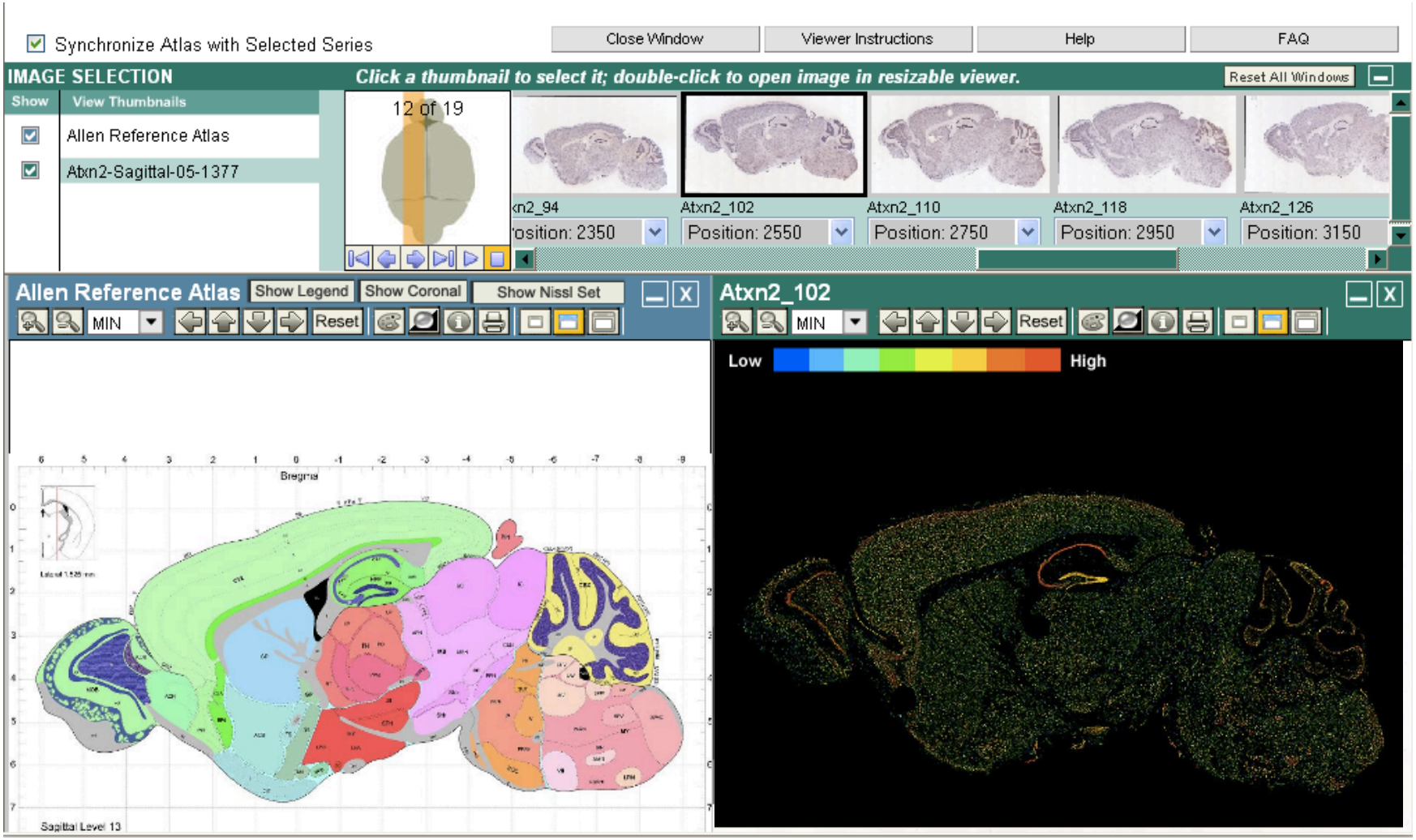
Na K ATPases


β
β
α
α


12 (R) HETE
2
+
Ca ion
GENERAL SITE FOR OUABAIN AND K+ BINDING
C
(CELL EXTERIOR)
PLASMA MEMBRANE
(CYTOPLASM)
1
2
3
4
5
6
7
8
9
10
11
N
C
N
+
α-SUBUNIT
β-SUBUNIT
FIRST CYTOPLASMIC LOOP
LARGE CYTOPLASMIC LOOPS
(THE 2nd CONTAINS THE BINDINGSITE FOR PHOSPHATE +)
CYTOPLASMIC BINDING SITE AREAS FOR Na AND Ca IONS
Ca+2
Na+

Allen Brain Atlas

www.brain-map.org

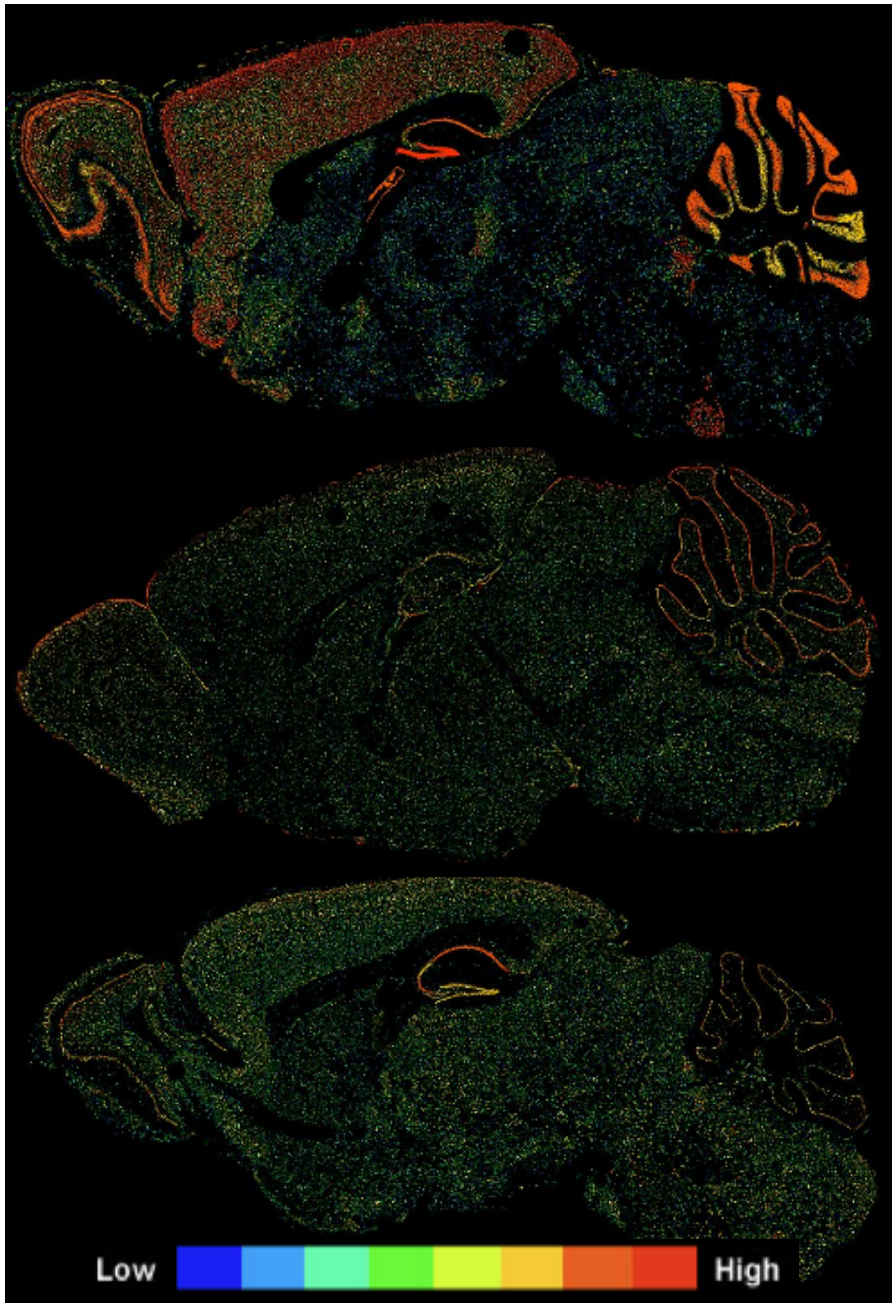


Expression of Na/K ATPases in brain

ATP1α1
ATP1α2
ATP1α3

ATP1α4

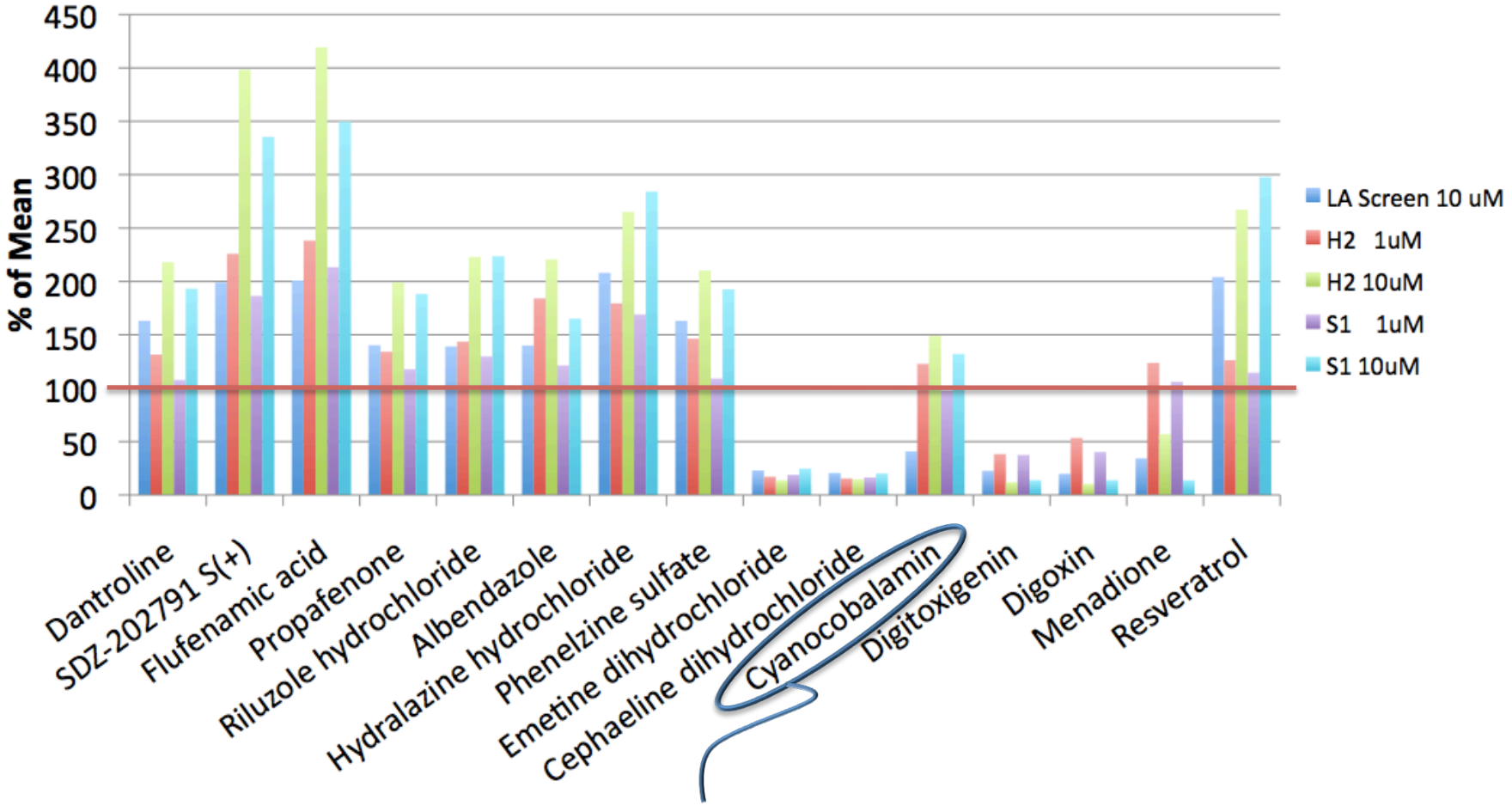
ATXN2



Down-regulation of *ATP1α3* in Purkinje cells by the ATPase inhibitor ouabain (and others) is neuroprotective (Ghoumari et al., FASEB J. 20, 2006)

Validation Assays

Validation assay using HEK293 & SH-SY5Y
(showing only 15 compounds)



No effect in validation assay
Only three out of 225 were like this one

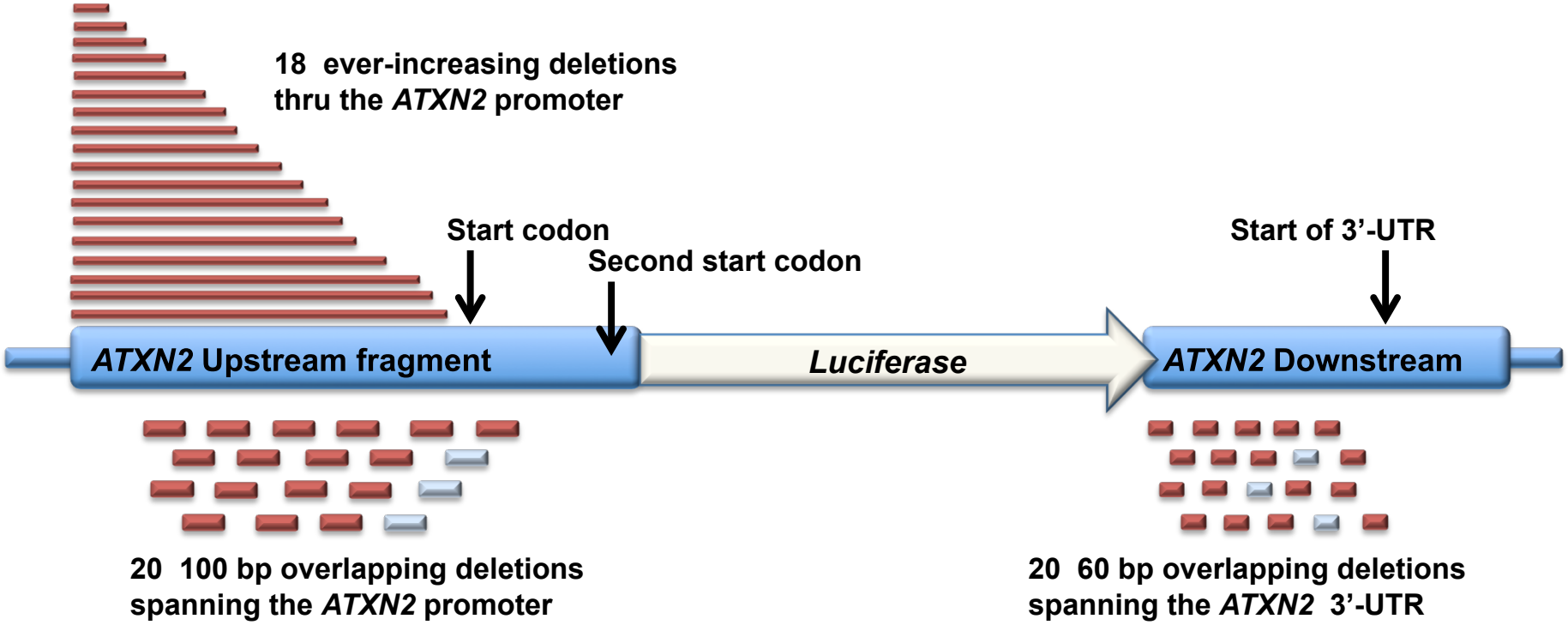
Other controlling constructs

If compounds that reduce *ATXN2*-Luc expression from this construct are specific…

Then they should also effect expression from this construct:

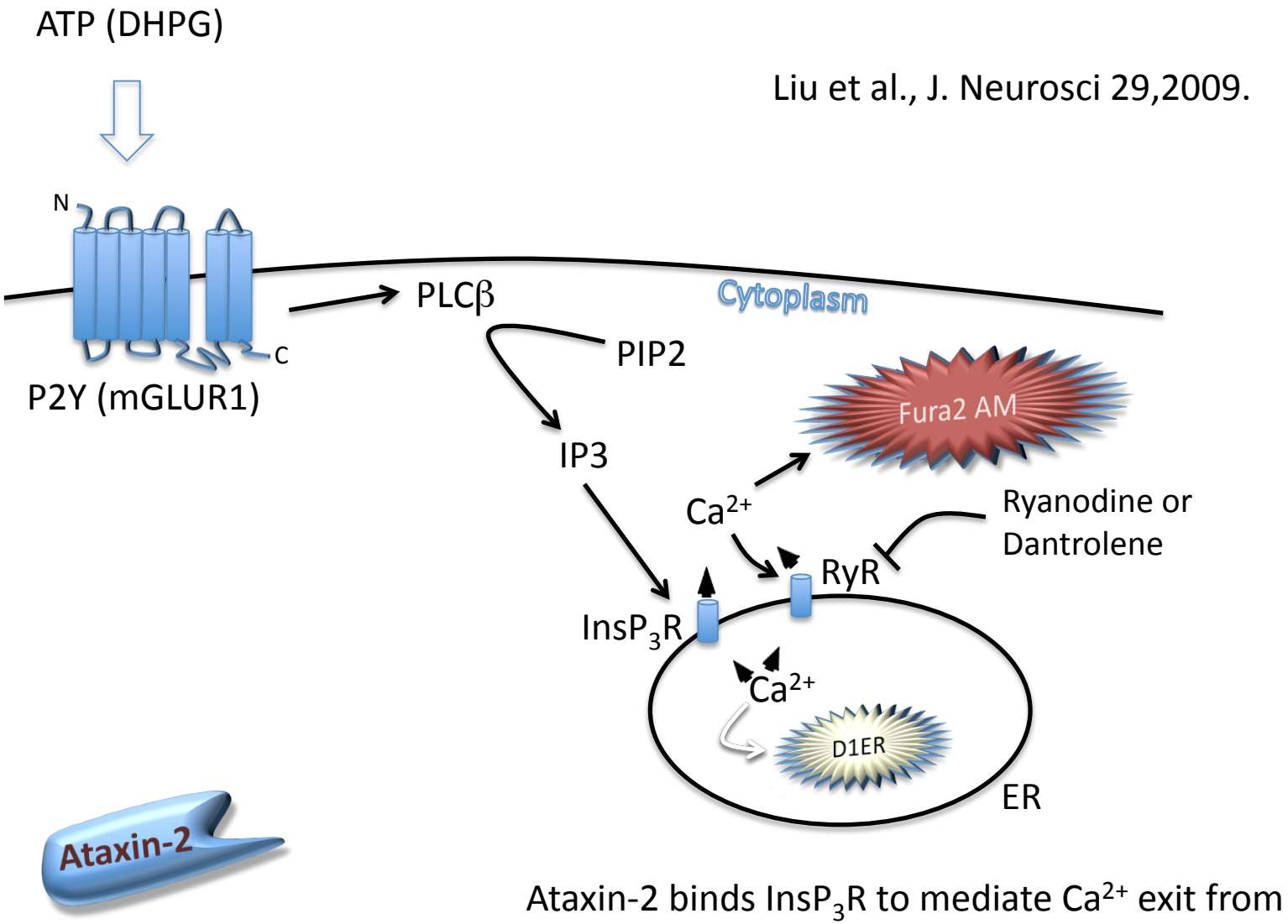
But not this construct:

Deletion constructs

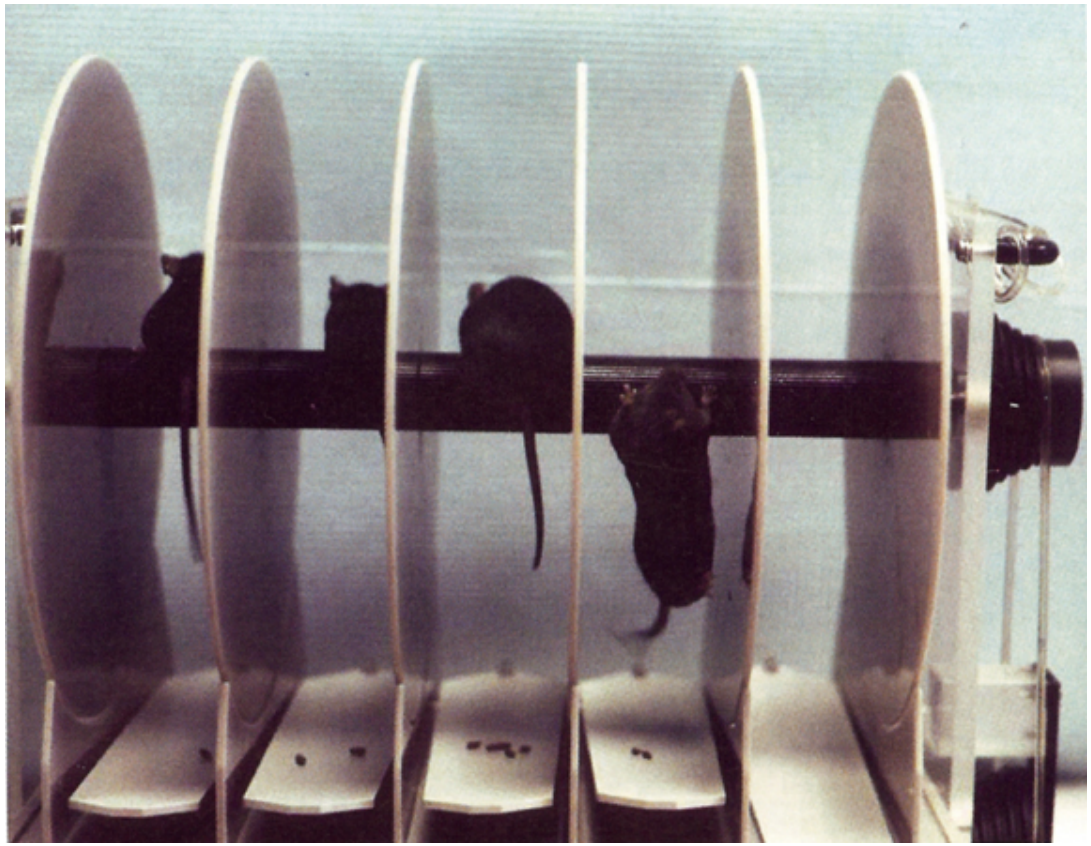


Construction of a new cell line model and its potential use for validating compounds

Cell line model for measuring Ataxin-2 effect on Ca^{2+} movement

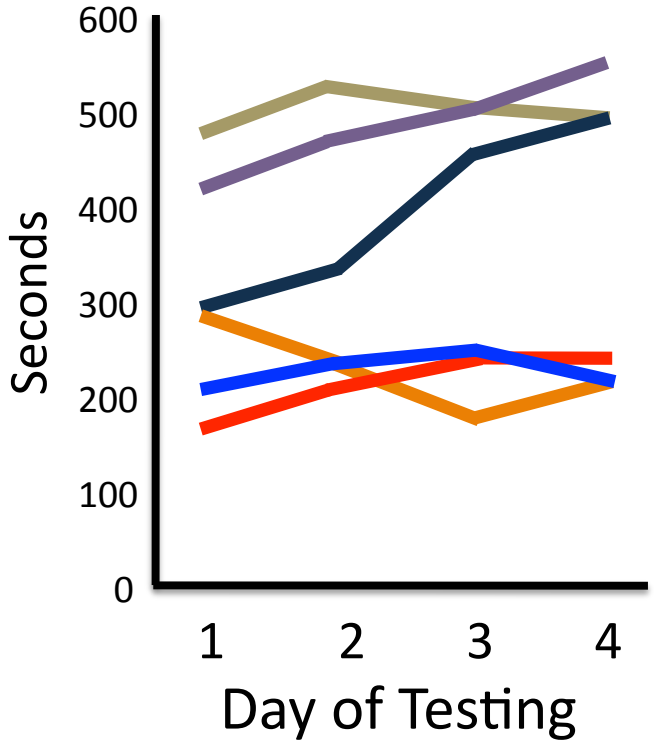


Ataxin-2 binds $InsP_3R$ to mediate Ca^{2+} exit from ER stronger when mutated



Mouse Models

Rotorod performance for SCA2 Q58 transgenic mice

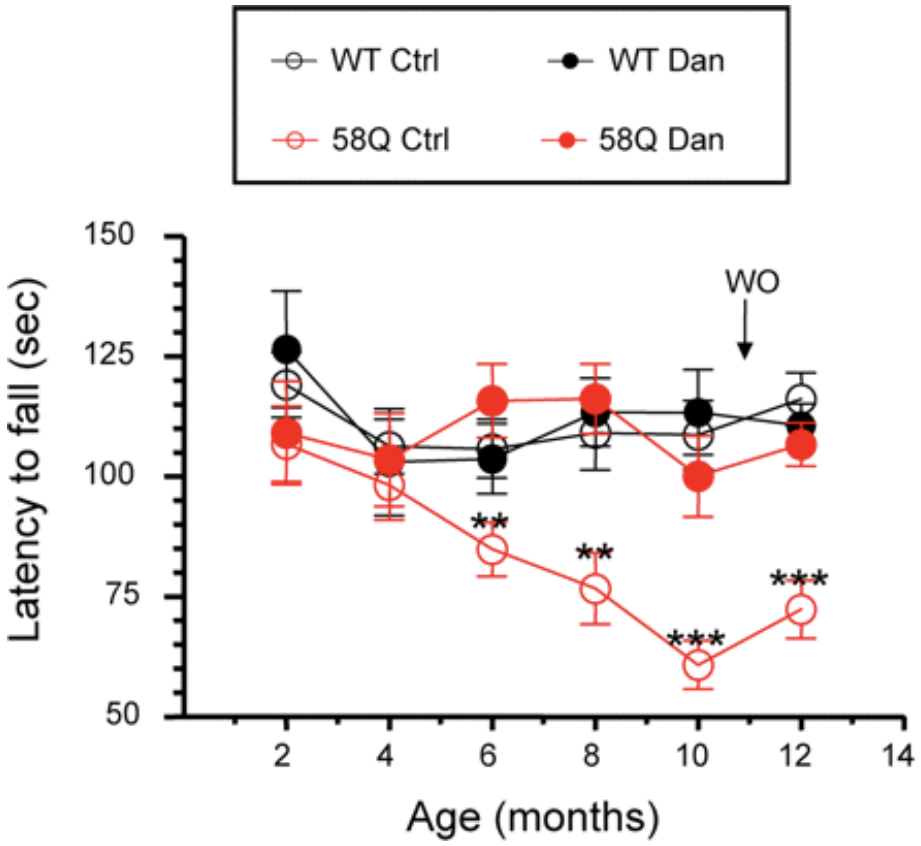


+/+	14 wks (n=10)
+/+	26 wks (n=8)
+/Q58	14 wks (n=8)
+/Q58	16 wks (n=12)
Q58/Q58	26 wks (n=9)
Q58/Q58	26 wks (n=9)

4 to 40 rpm for 10 min

Huynh et al., *Nature Genetics 26, 44 - 50 (2000)*

Rotorod analysis used to measure dantroline effect on SCA2 Q58 transgenic mice

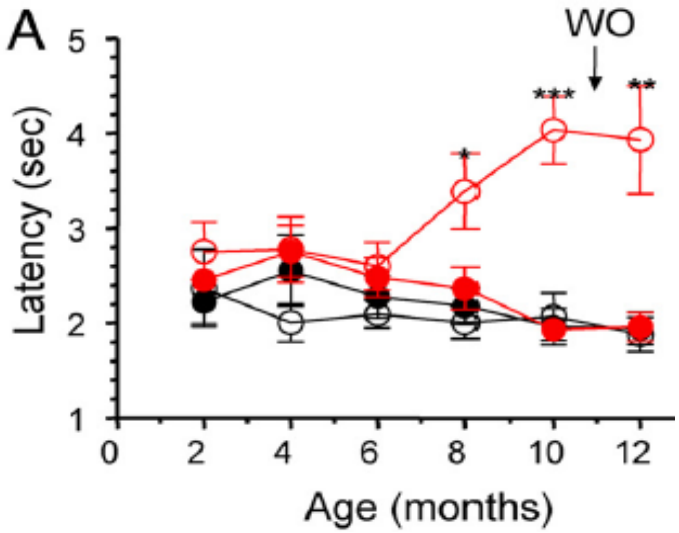


PCP2 promoter

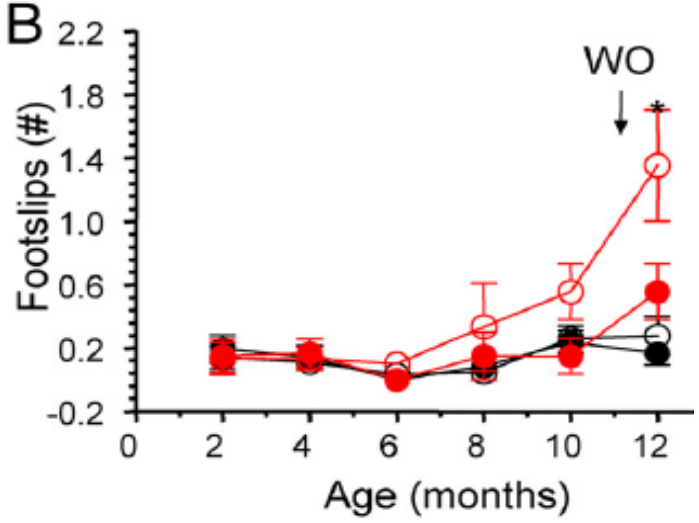
N=9-12 mice per group
0 to 40 rpm over 200 s
Fed 5 mg/kg dantrolene orally twice/wk

Liu et al., J. Neurosci 29,2009.
Ilya Bezprozvanny

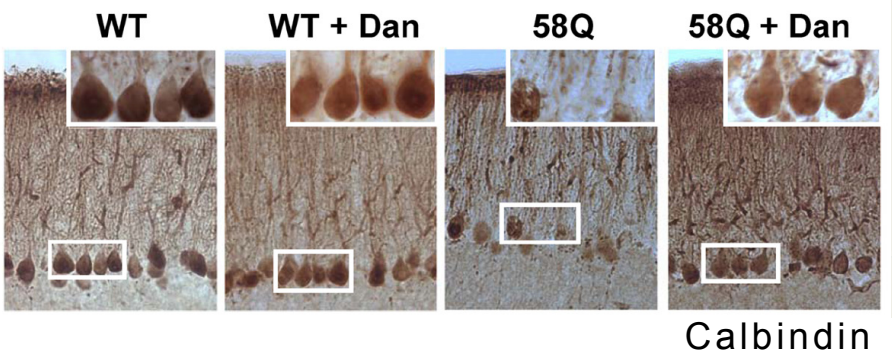
Time to traverse 1cm-wide beam

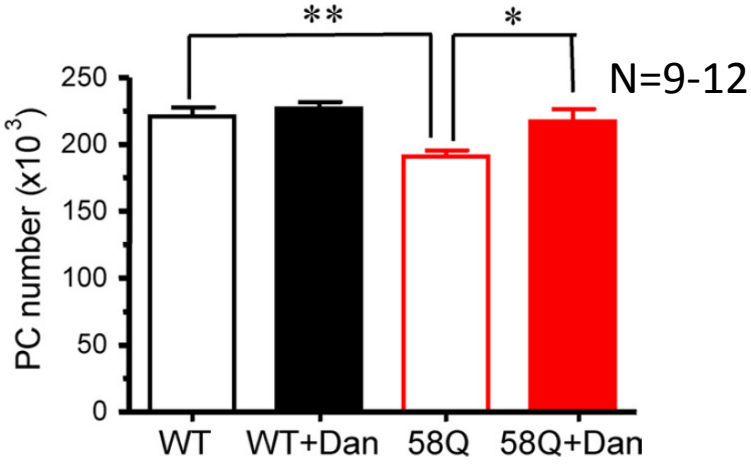


Number of foot slips



Number of Purkinje cells is restored



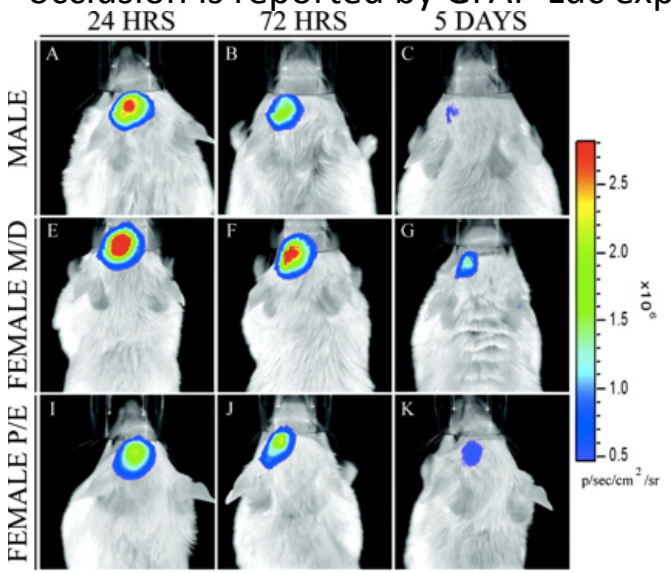


Liu et al., J. Neurosci 29,2009.

Reporter Mouse

- Atxn2-Luc transgenic mice
  - Histology
  - Xenogen IVIS 200 imaging
    - Extracted brains or live animals

Stroke model
Reactive gliosis induced by middle cerebral artery occlusion is reported by GFAP-Luc expression.



Cordeau et al. Stroke 39:935, 2008.

Summary Slide

Several compounds that inhibit *ATXN2*-luciferase expression

Validation tests → Drugs for SCA2

A functional element: We conducted a screen that was not based on *ATXN2* function that resulted in targets of common function.

Modern methods of drug discovery

Acknowledgments

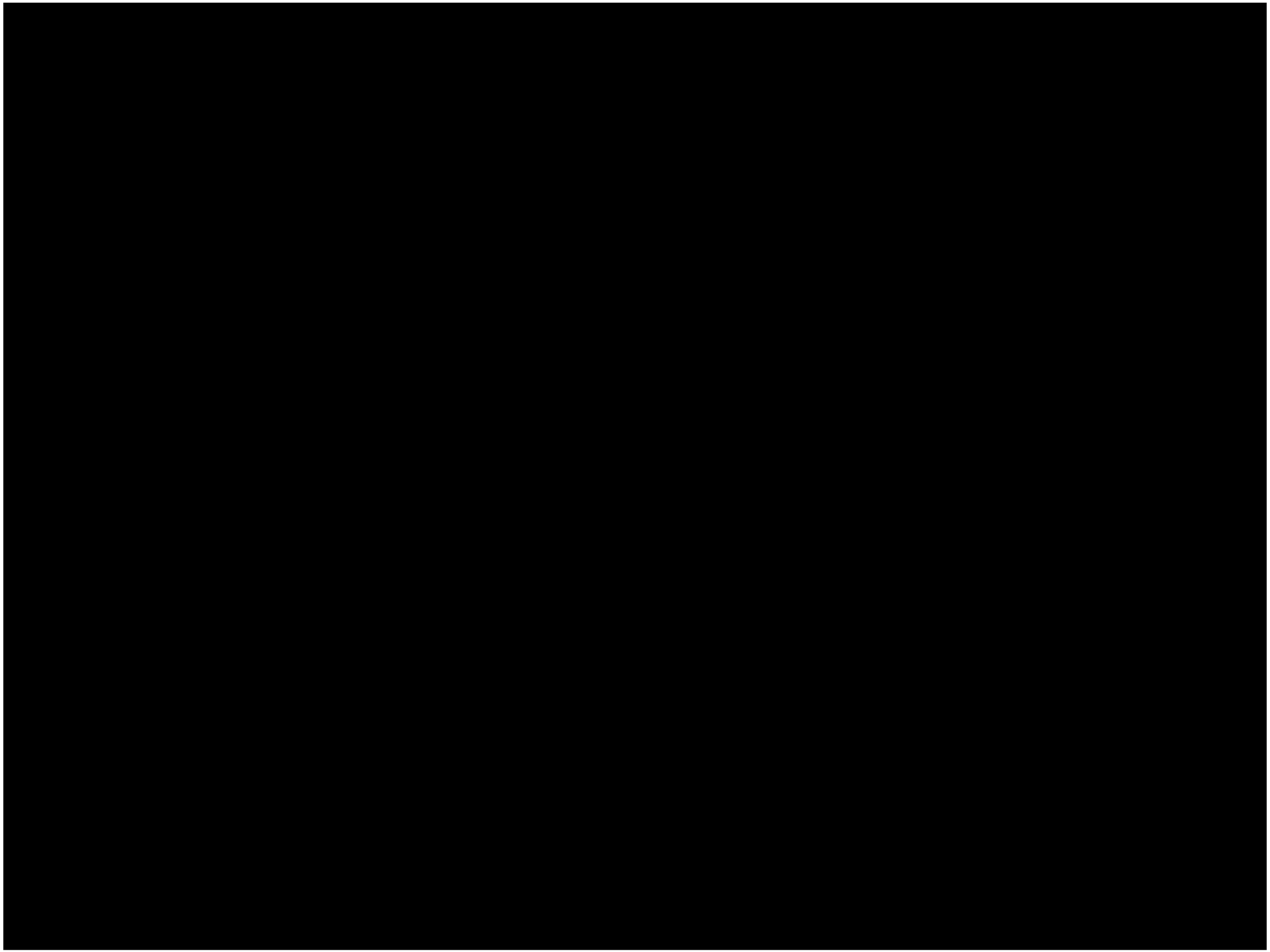
Seth Andrew Christensen

Lance Pflieger

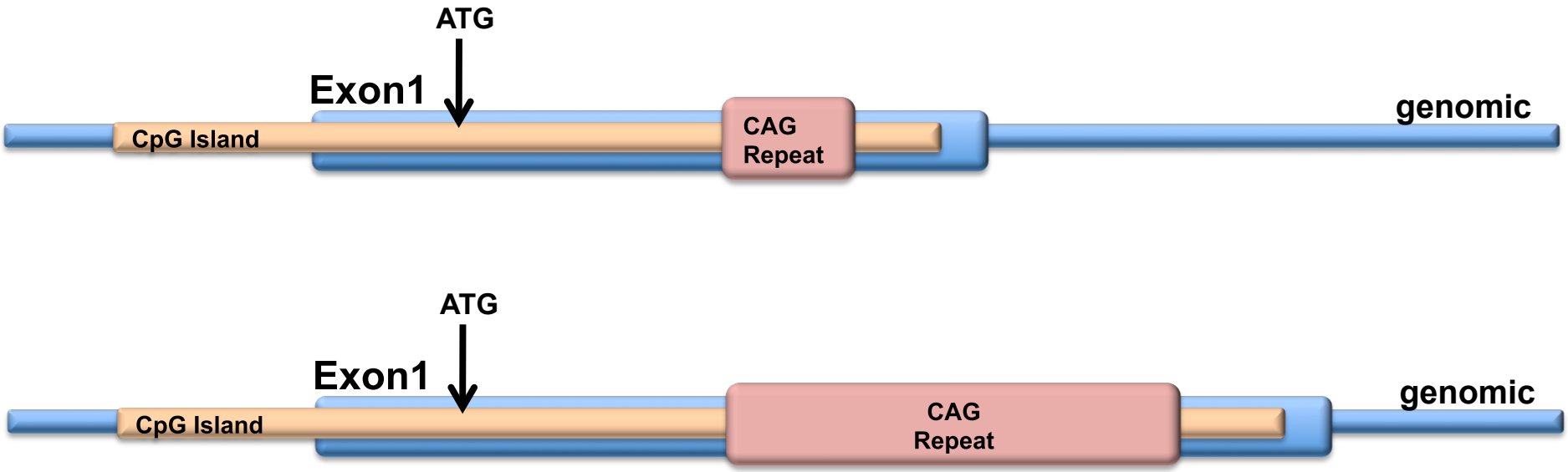
Steven Hansen

Stefan Pulst & the lab

Pilot Grant from the Neurodegenerative Disease Center

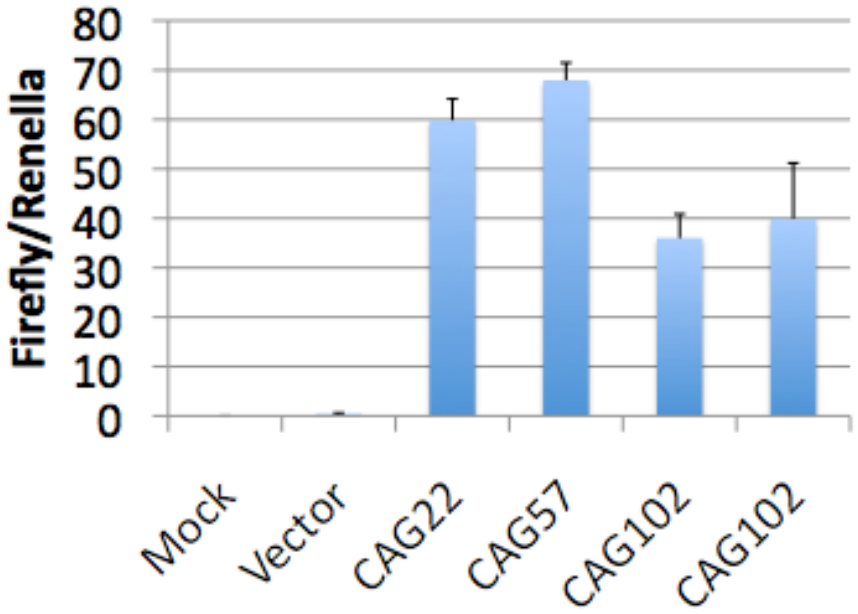


The CAG repeat is flanked by CpG islands suggesting CAG expansion may alter expression



Effect of CAG repeat on expression

ATXN2-Luciferase Expression Construct with expanded CAG repeats



ANOVA: $P<0.01$

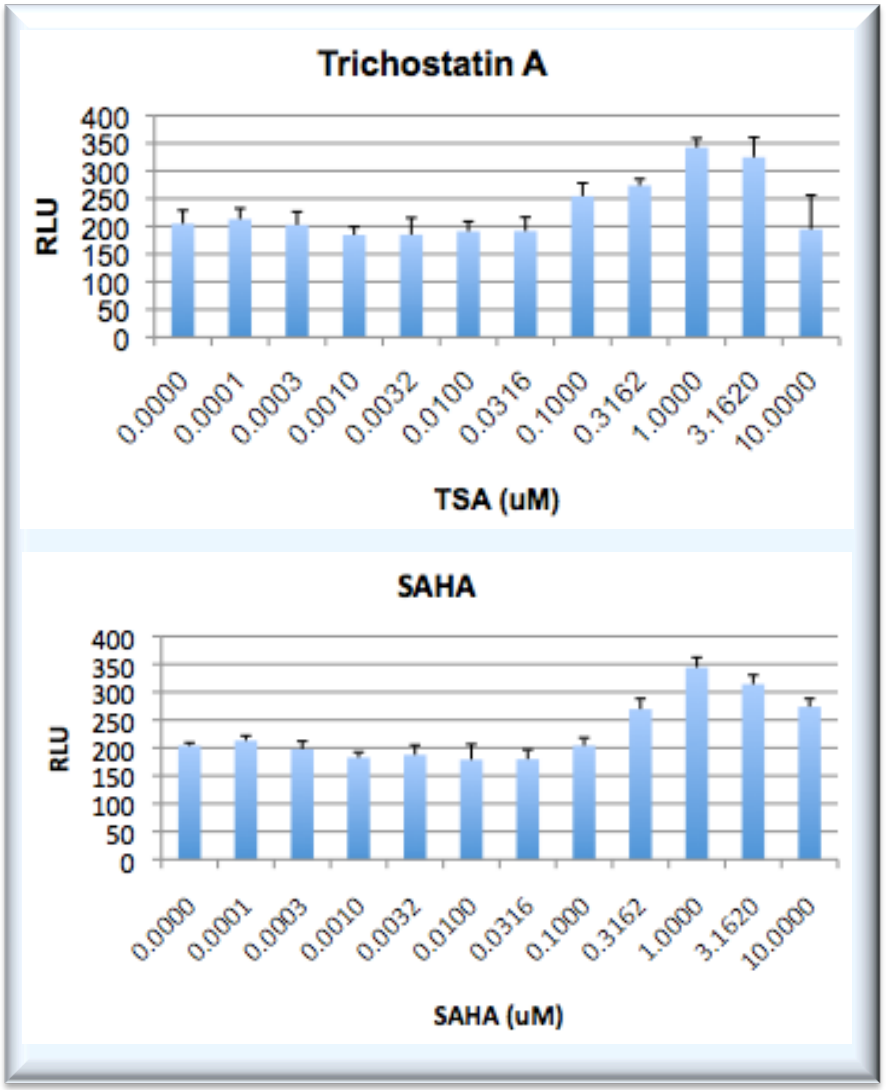
CAG22 vs CAG57 $P<0.05$ but not when the Bonferroni correction is applied

Modest expansion increases expression and perhaps toxicity.

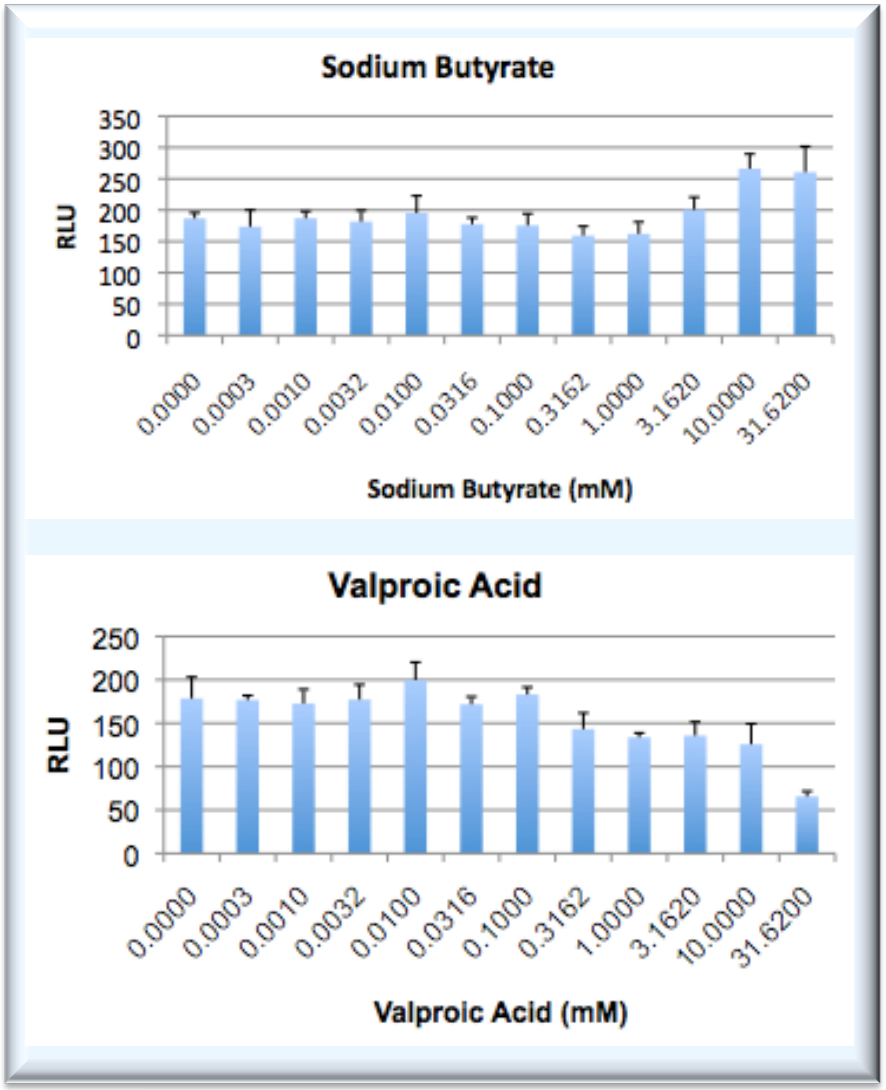
Long expansion reduces expression but is associated with increased toxicity.

SHSY5Y cells stably expressing ATXN2-Luc treated 24 hours

Hydroxamates

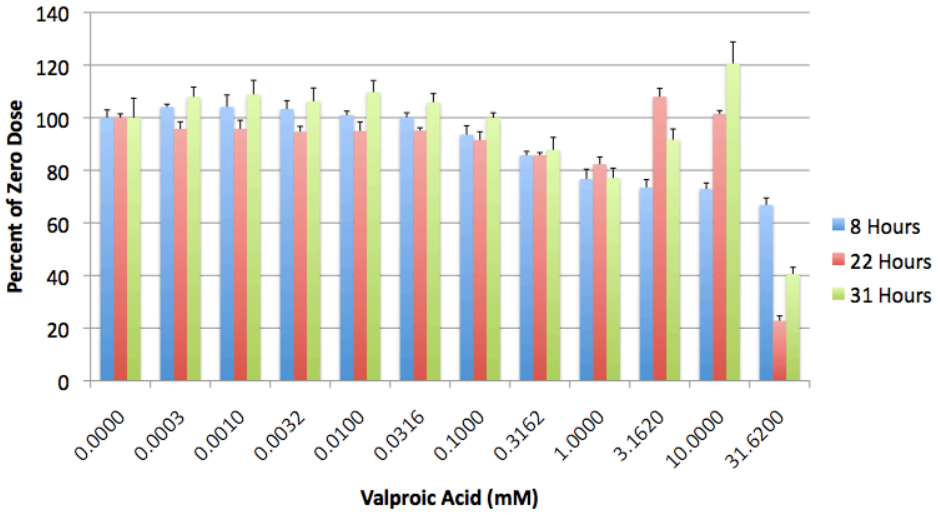


Aliphatic Acids

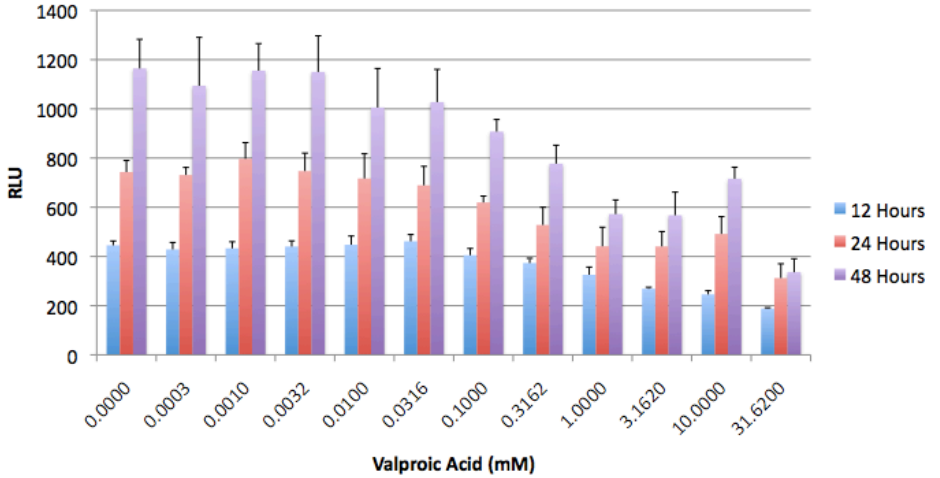


Valproic Acid

HEK293

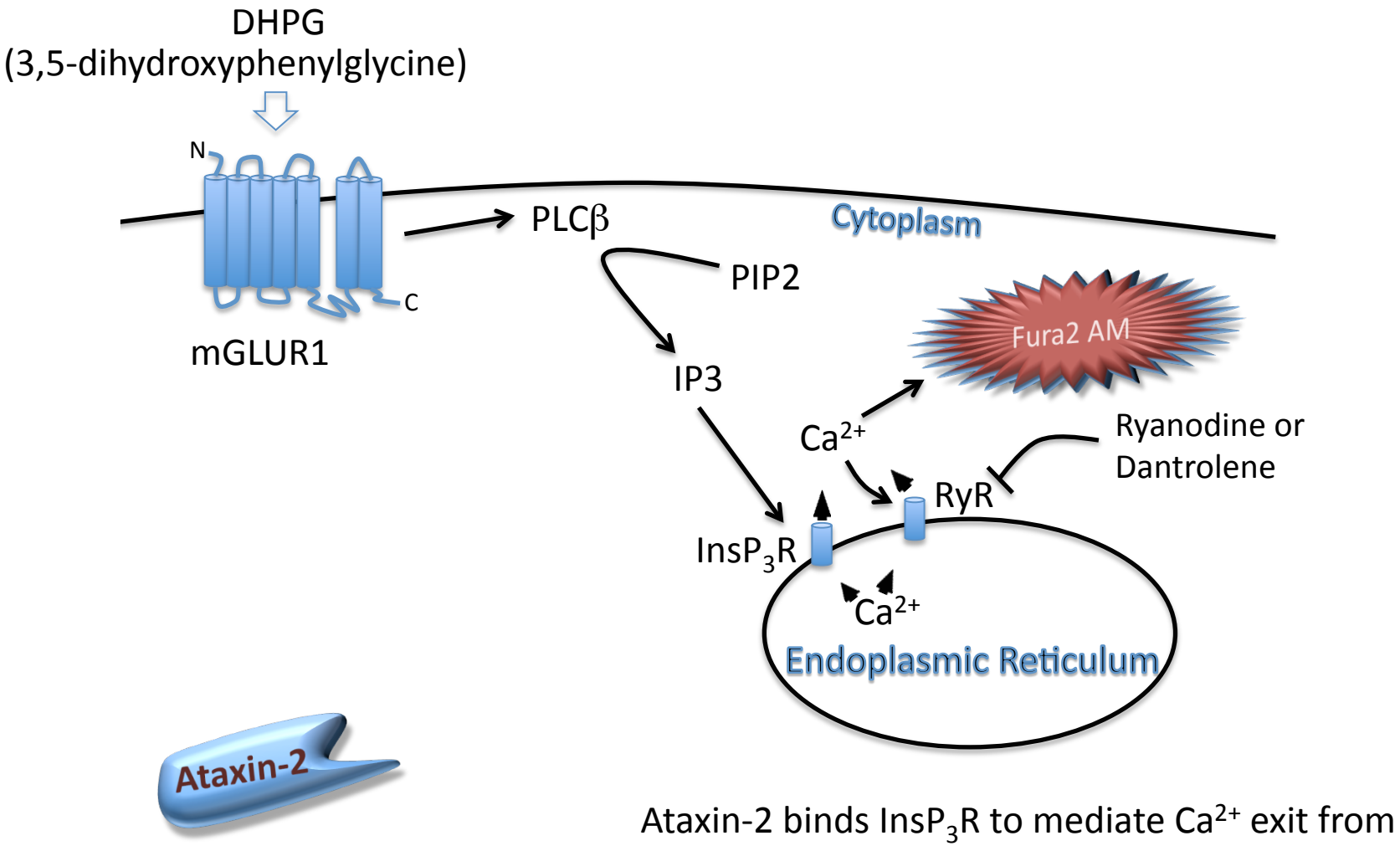


SH-SY5Y



The level of VPA that is achievable in patient serum is ~0.3 mM

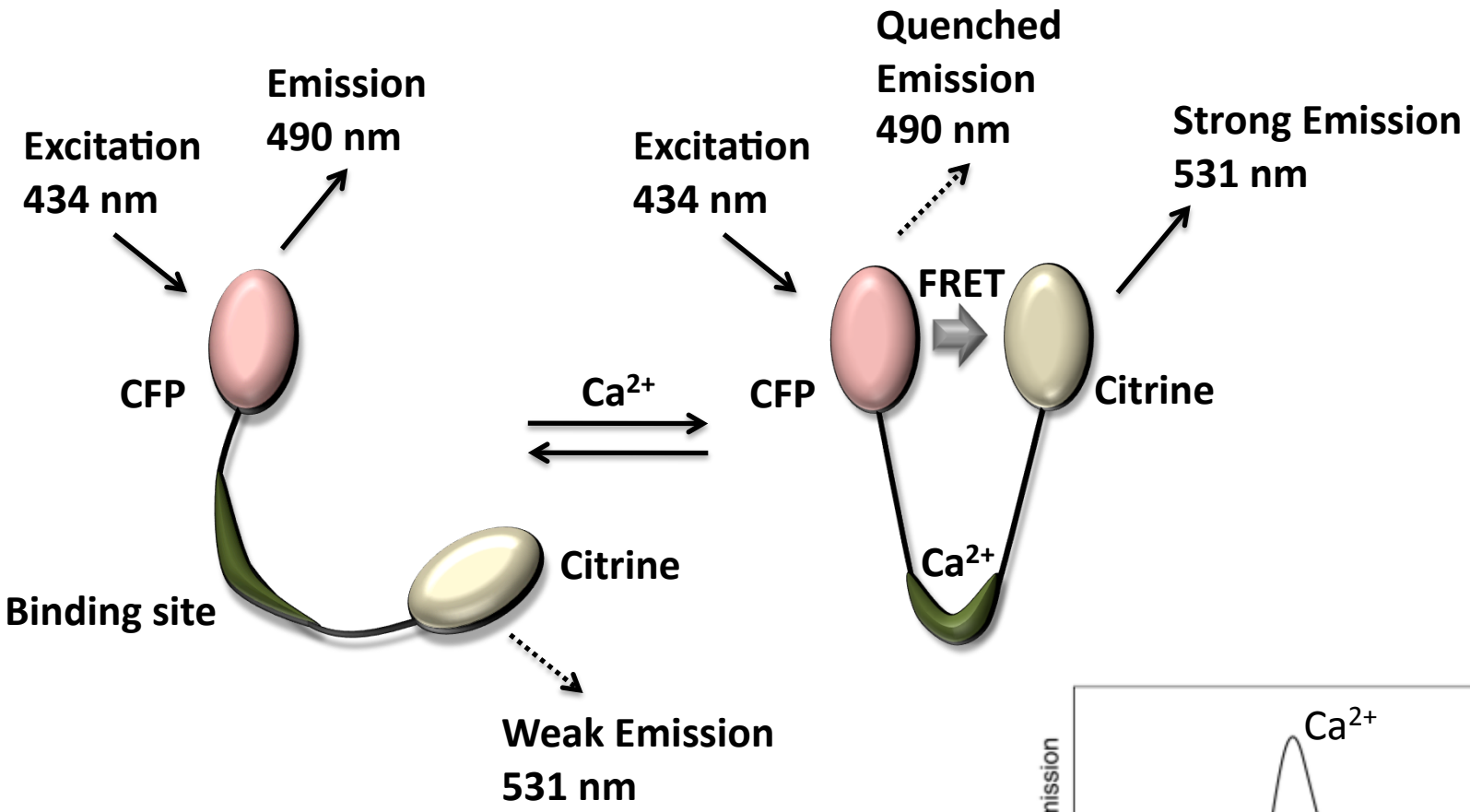
Ataxin-2 action on Ca^{2+} movement in cultured primary Purkinje cells from *ATXN2* transgenic mice



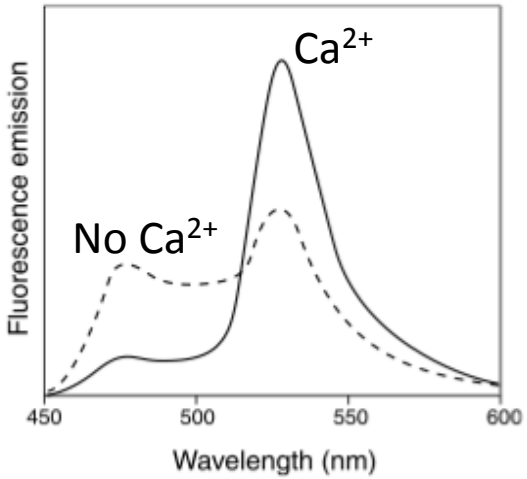
Ataxin-2 binds $InsP_3R$ to mediate Ca^{2+} exit from ER stronger when mutated

Liu et al., J. Neurosci 29,2009.

D1ER Cameleon

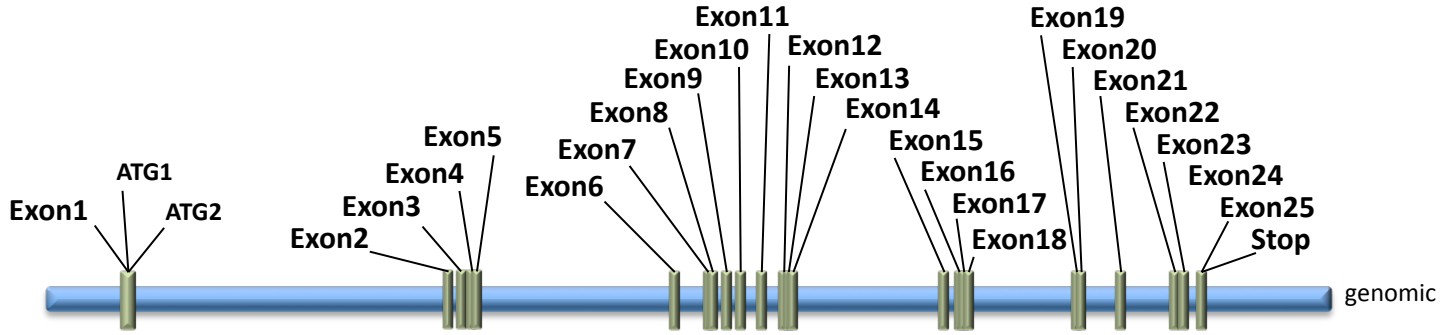


Targeted to the endoplasmic reticulum



The *ATXN2* gene and ataxin-2 protein

ATXN2 gene



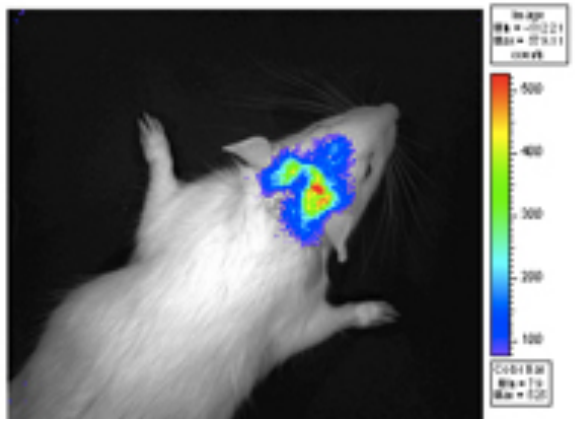
150 Kb, 25 exons, located at CHR 12q24.1, 3,939 bp cDNA

Ataxin-2 protein

Reporter Mouse

- Atxn2-Luc transgenic mice
  - Histology
  - Xenogen IVIS 200 imaging
    - Extracted brains or live animals

Gene delivery model
CMV-Luc delivered by CSF injection
of cationic lipid complex with plasmid



Hauck et al. Mol. Ther. 16:1857, 2008.

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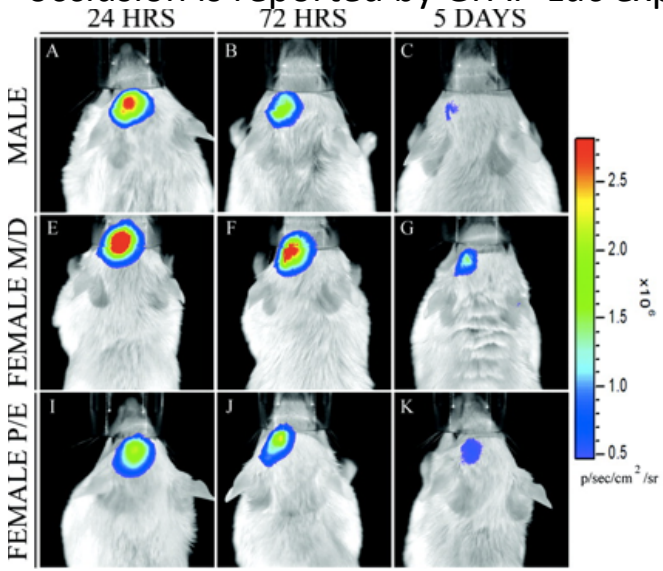
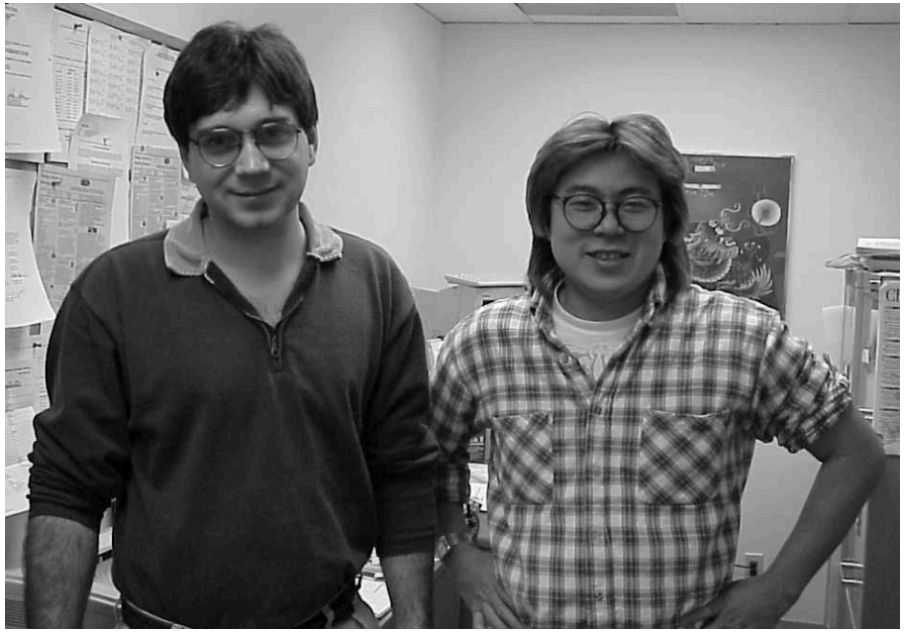
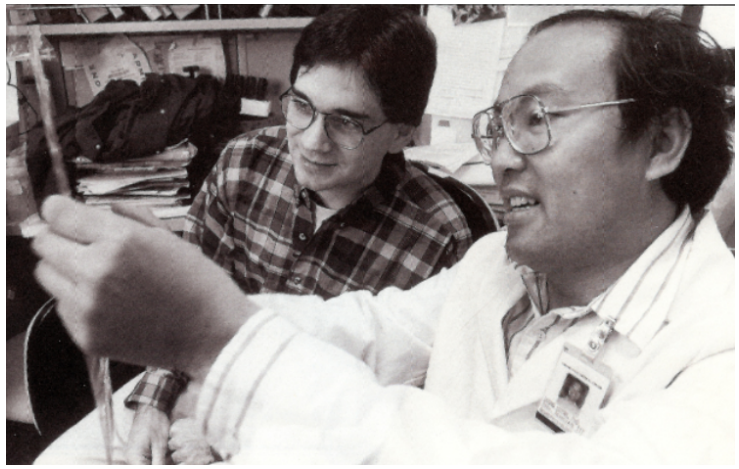


Cordeau et al. Stroke 39:935, 2008.



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