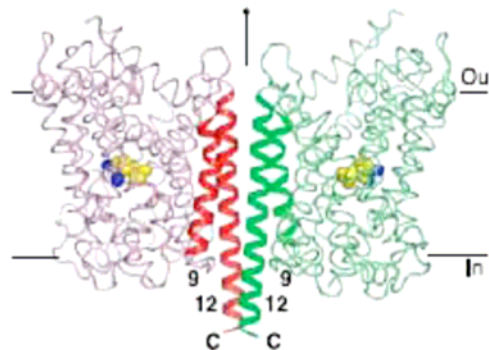


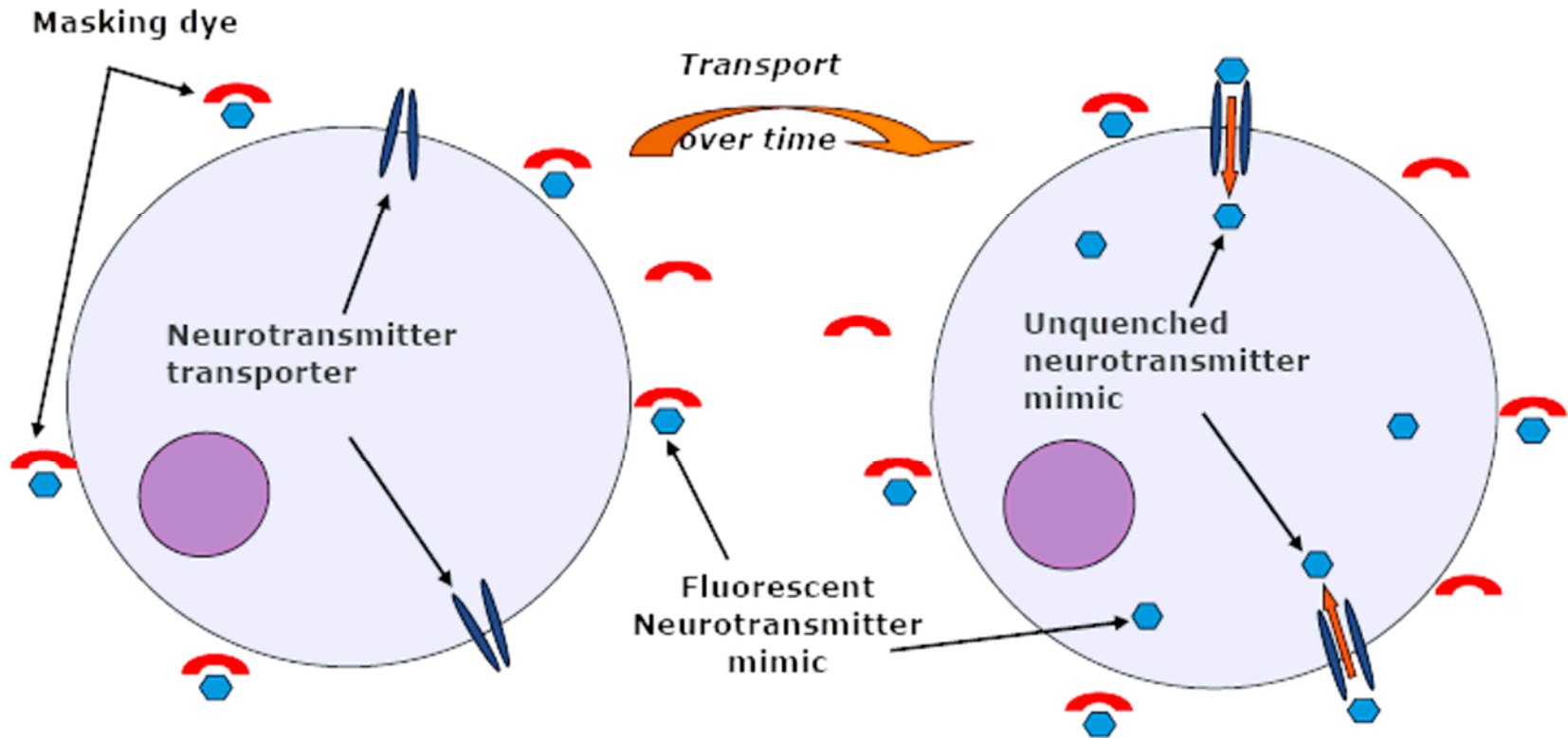
# Evaluation of a Fluorescent Dye-Based Biogenic Amine Transporter Uptake Assay Using Primary Neuronal Cortical Cultures



Taken from Yamashita et al (2005) Nature 437 215-233

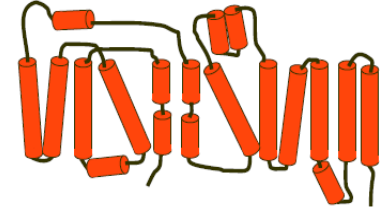
*Lesley A. Radov, Ph.D., AstraZeneca Pharmaceuticals  
Discovery Research - CNS/PAIN; Wilmington, DE*

# Neurotransmitter Transporter Uptake Assay Principle

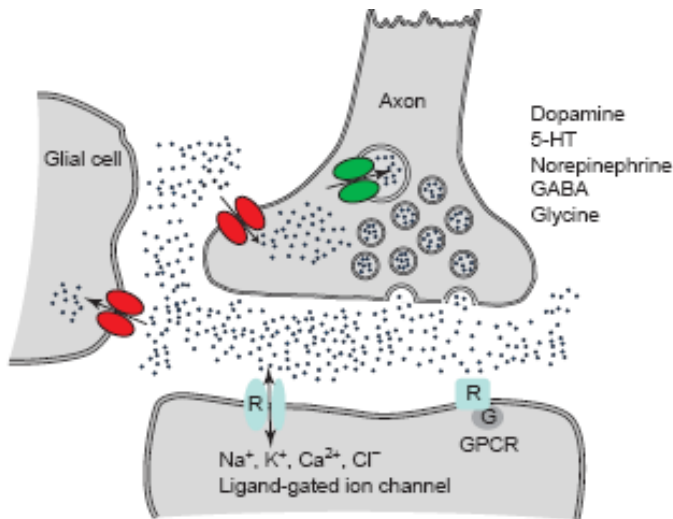


**Principle Identical in Primary Neuronal Cortical Cell as in Recombinant Cell**

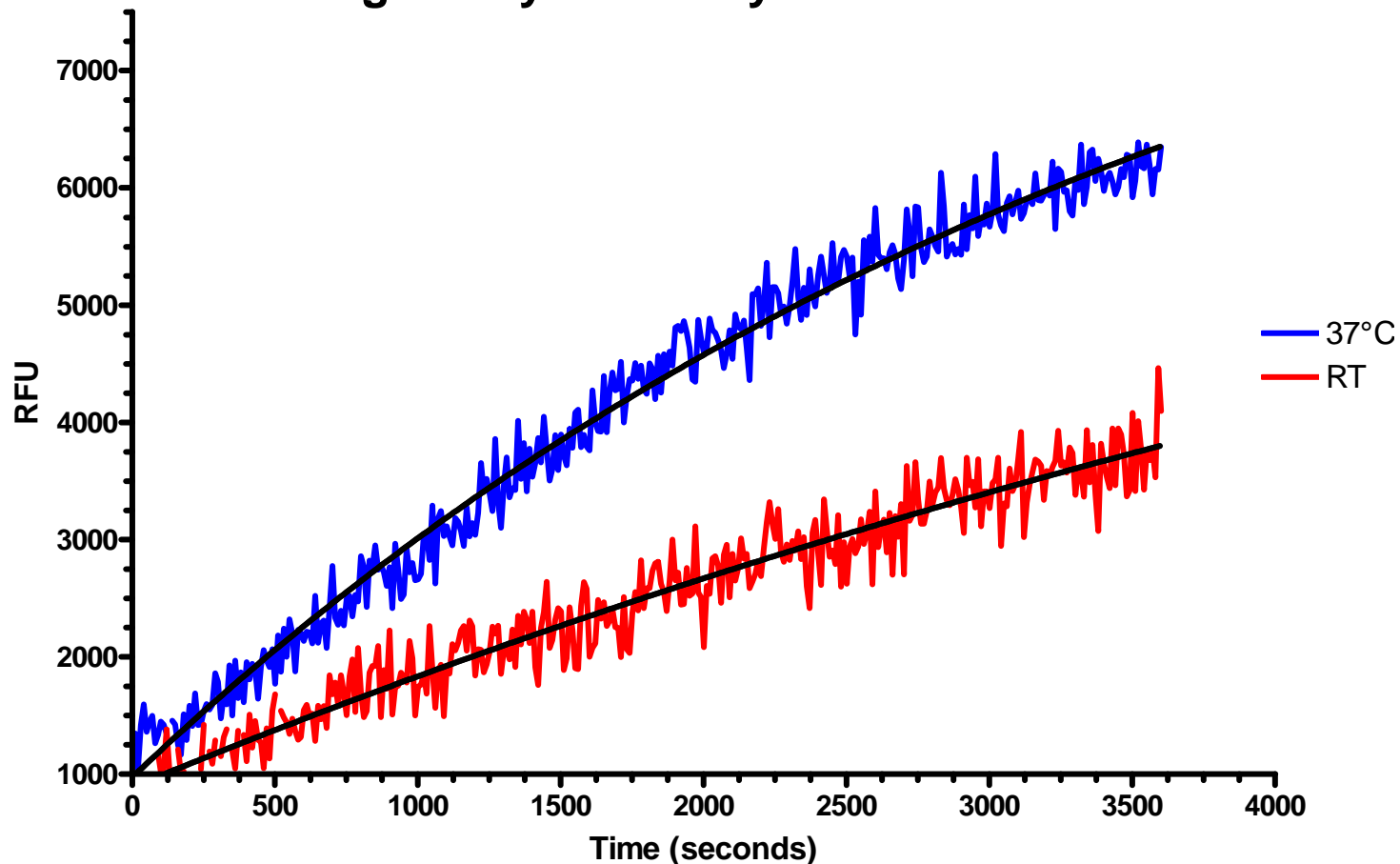
# NET, DAT & SERT belong to the Na<sup>+</sup>Cl<sup>-</sup> Coupled SLC6 Gene Family



- Operate as Na<sup>+</sup> dependent co-transporters
- Use Na<sup>+</sup> gradient to couple the ‘downhill’ transport of Na<sup>+</sup> with ‘uphill’ transport of neurotransmitter from extracellular environment into the cell
- Usually involves the additional cotransport of Cl<sup>-</sup>

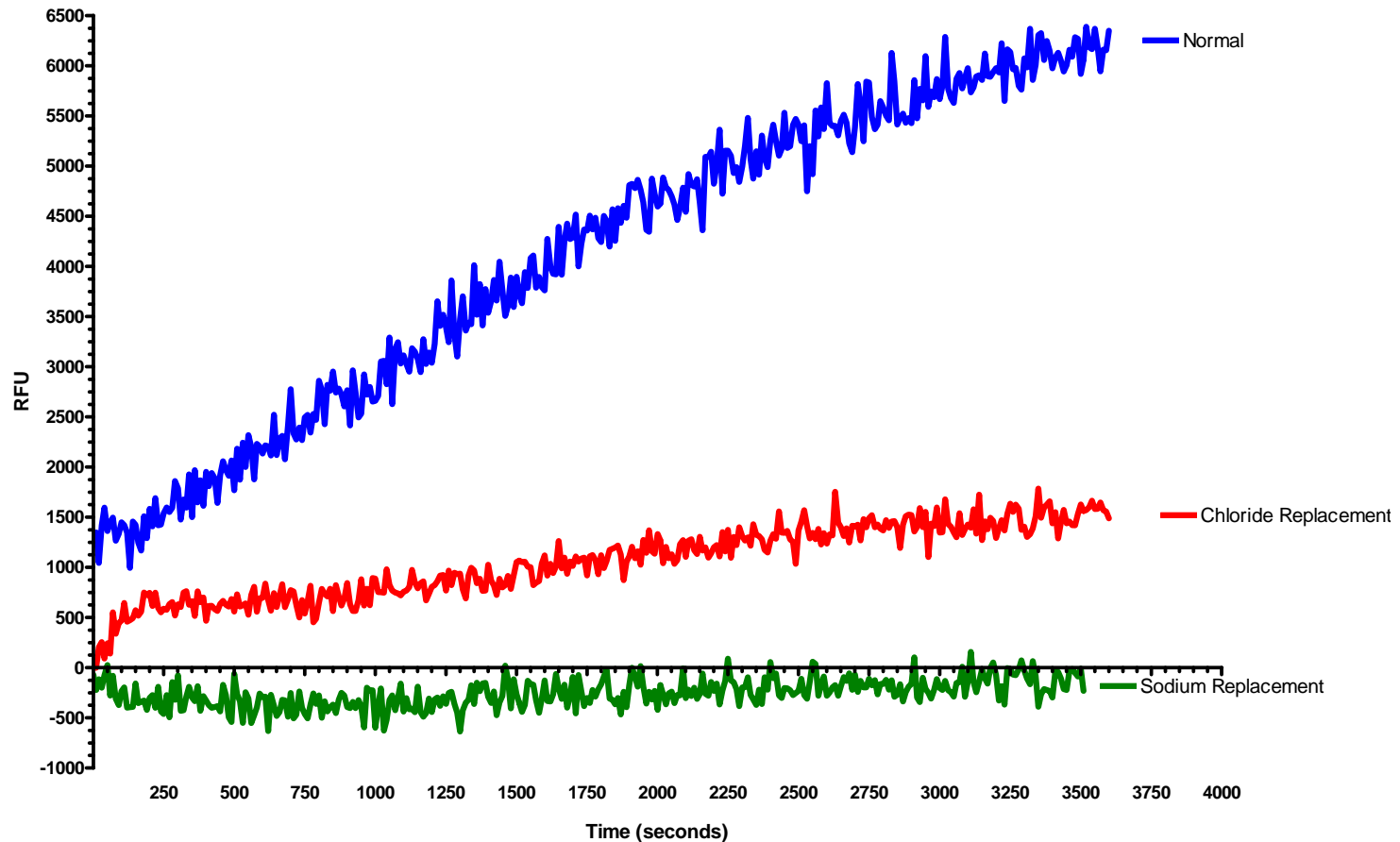


## The Effect of Time and Temperature on Dye Loading in Day 9 Primary Neuronal Cultures



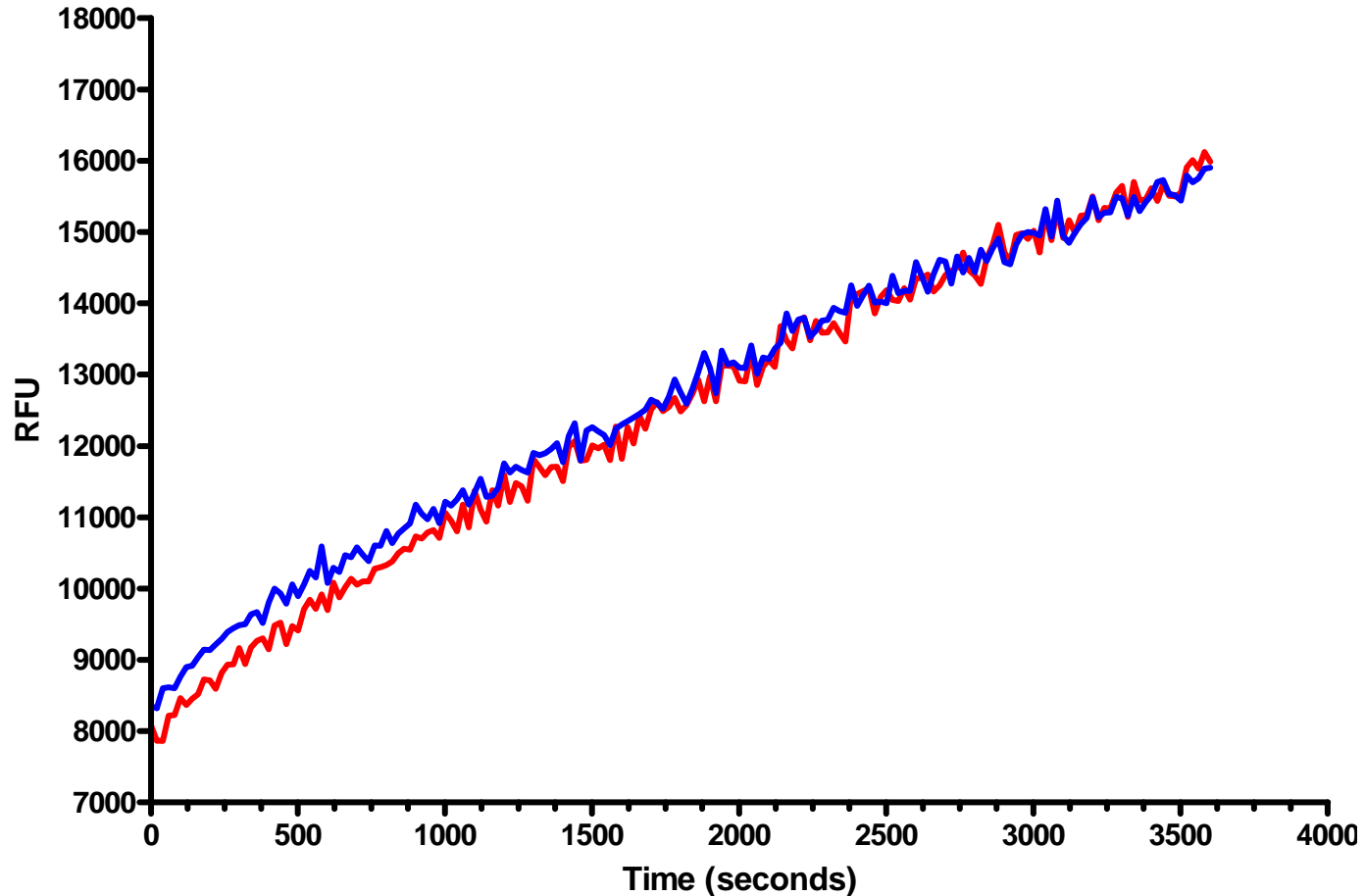
**Uptake proceeds faster at 37°C than at room temperature; adheres to ‘rule of thumb’ that rate of uptake ~doubles for every increase of 10°C**

## The Effect of Dye Loading in Day 9 Primary Neuronal Cultures of Replacing Sodium or Chloride in the Buffer



**Sodium and chloride are essential for dye transport into primary neurons confirming that transport is via  $\text{Na}^+\text{Cl}^-$  coupled transporters**

## Effect of 2.5mM Probenecid on Dye Uptake in Day 9 Primary Rat Neuronal Cultures



**Unlike in recombinant cells where Probenecid is an absolute requirement, the use of organic anion transporting polypeptides inhibitors has a minimal effect on the dye uptake and retention in the primary neurons**

# NT Uptake Inhibition Assay Procedure

Plate 1° neuronal cells 5-9 days before assay

+/- Compounds 15' @ 37°C

Take initial reading

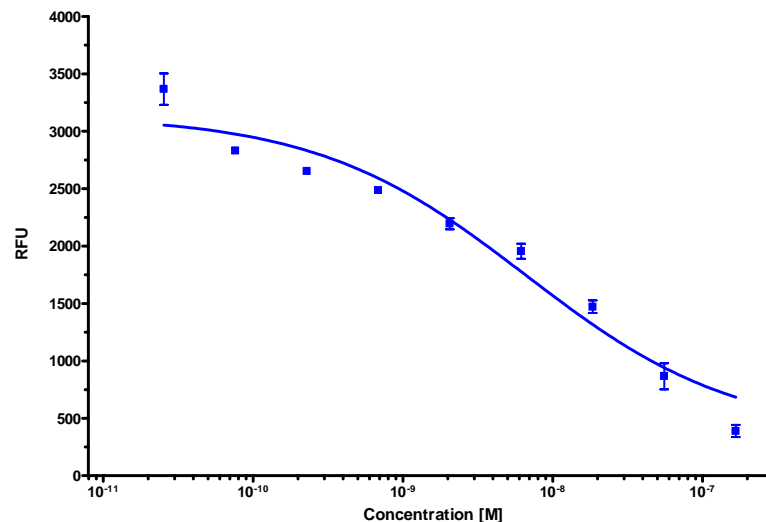
Add fluorescent dye – Incubate @ 37°C; 30-60'

Take final reading

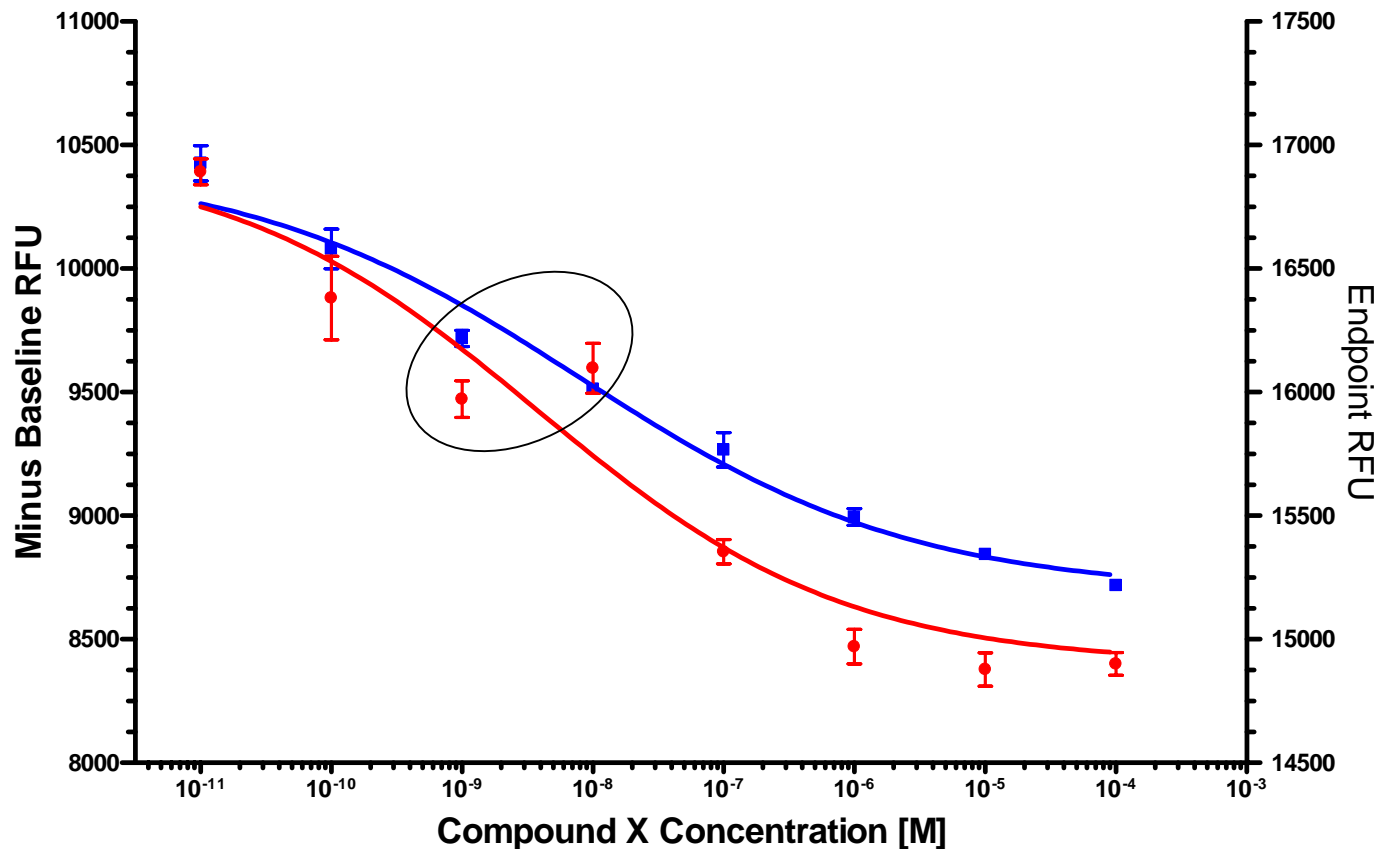
Analyze/Graph Data

Data in Excel Spreadsheet

[Conc]	60'	Pre-dye	Difference	[Conc]	60'	Pre-dye	Difference	[Conc]	60'	Pre-dye	Difference
1.67E-07	9493.9	9167.7	326.1	6.17E-09	10476.0	8490.4	1985.7	2.28624E-10	12115.6	9452.4	2663.2
	10755.3	10215.3	540.0		10849.3	9008.8	1840.5		11312.5	8588.7	2723.7
	11342.8	10998.0	344.9		10035.7	7958.9	2076.8		10938.9	8298.8	2640.1
	10667.7	10152.4	515.3		10804.5	8723.9	2080.6		10671.4	8021.8	2649.6
	10945.8	10755.7	190.1		10770.8	9085.6	1685.1		10964.4	8356.3	2608.1
	10314.7	9896.7	418.0		11108.3	9048.4	2059.8		10069.3	7442.2	2627.1
5.56E-08	10346.6	9103.2	1243.4								
	12881.1	12305.8	575.3	2.06E-09	11361.2	9269.3	2091.8	7.62079E-11	10350.1	7553.9	2796.1
	10263.6	9138.8	1124.8		10479.5	8338.9	2140.6		11179.8	8435.0	2744.8
	10437.0	9837.9	599.1		10136.4	8037.3	2099.0		10712.6	7801.7	2910.9
	10814.8	9887.3	927.5		10886.0	8513.6	2372.4		11836.3	8945.2	2891.2
	10588.1	9863.7	724.4		10810.8	8665.8	2145.1		11144.1	8363.3	2780.8
					10928.9	8609.4	2319.6		10746.2	7885.6	2860.7
1.85E-08	11452.9	9776.5	1676.4	6.86E-10				2.54026E-11			
	10389.7	8983.9	1405.8		12015.1	9453.6	2561.6		12066.163	8710.2	3355.9
	10928.9	9572.9	1355.9		11501.2	9009.7	2491.5		11265.109	7749.0	3516.1
	12406.4	11059.6	1346.8		10794.3	8272.1	2522.2		11373.526	7732.0	3641.5
	10775.9	9329.0	1446.9		11127.0	8683.6	2443.4		11204.594	8268.7	2935.9
	11393.3	9797.8	1595.5		10281.4	7896.5	2384.9		10821.675	7823.2	2998.5
					10919.9	8401.6	2518.3		10963.865	7214.7	3749.2



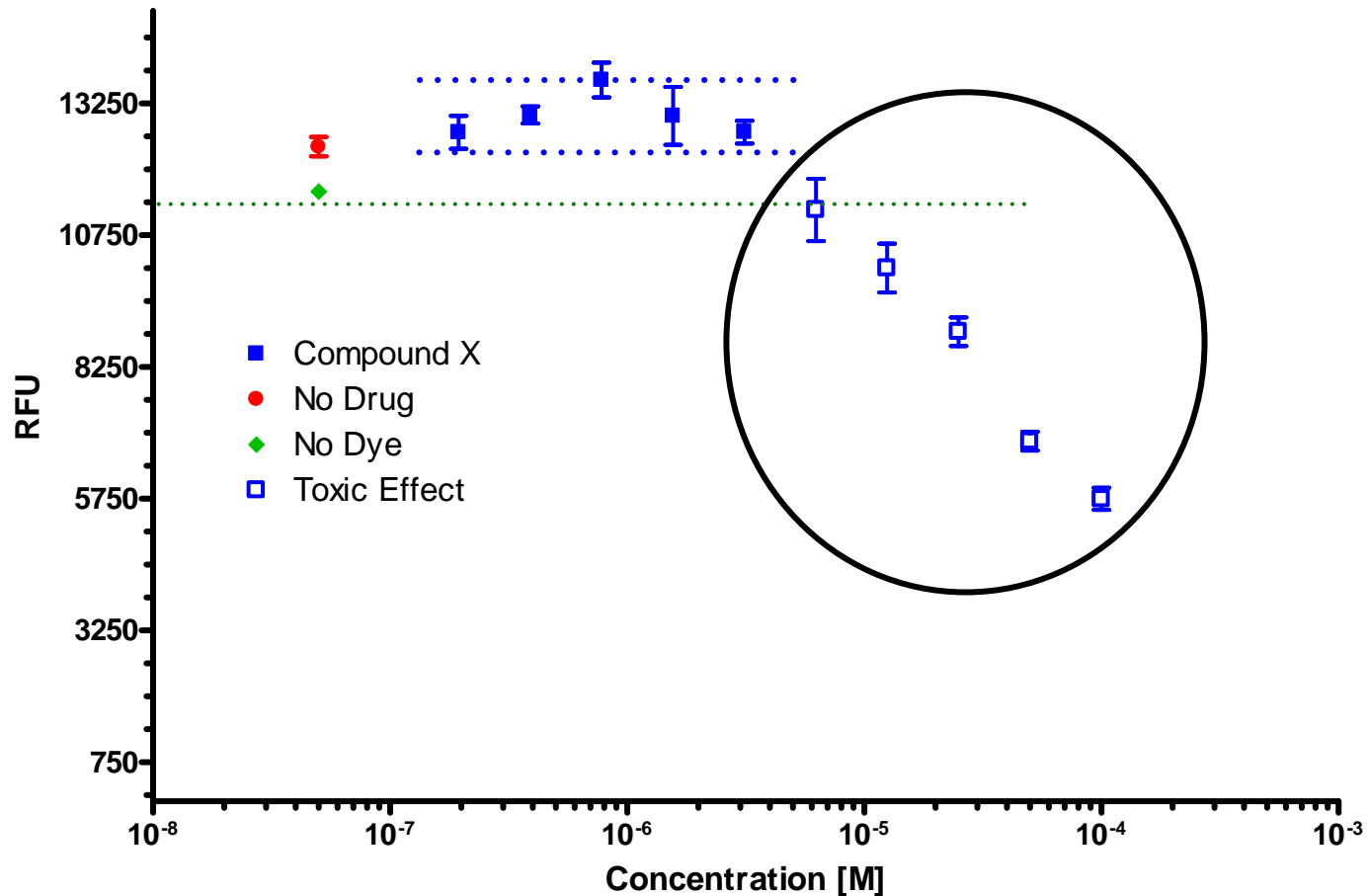
## Comparison of 2 Ways to Calculate Uptake Data



- Minus baseline:  $IC_{50} = 7.9\text{nM}$ ; CI: 3.5 - 18.1nM;  $R^2 = 0.9624$ ; HS = -0.3391
- Endpoint:  $IC_{50} = 3.7\text{nM}$ ; CI: 0.5 - 27.8nM;  $R^2 = 0.8978$ ; HS = -0.3685

The method of data calculation can have an impact on the  $IC_{50}$  calculations; using the endpoint method the CI is greater, there is more variability, and the compound appears twice as potent (which may create a problem for SAR calculations &/or rank order analysis; also potential toxicity problem may be overlooked

# The Effect of a 15 Minute Pretreatment with Compound X on Uptake in Day 9 Neuronal Cultures



Compound is really not effective; the data used in the lower portion of the  $IC_{50}$  curve is actually the result of toxic compound concentrations, where the dye leaks from the cells thus appearing to inhibit uptake.

**To prove that dye uptake is via neurotransmitter transporters the rate of dye uptake should be:**

- temperature dependent**
- time dependent**
- Na<sup>+</sup> dependent**
- Cl<sup>-</sup> dependent**

***And it is!***

## Effect of Culture Age on NT Dye Uptake in Primary Neuronal Cultures

Compound	Confidence	R <sup>2</sup>	Days in Culture
IC <sub>50</sub> (nM)	Interval (nM)		
35.1	15.4 - 79.9	0.9537	Day 4
25.6	17.2 - 38.1	0.9363	Day 4
15.9	5.7 - 44.2	0.9696	Day 5
15.7	1.8 - 13.5	0.9398	Day 5
14.4	8.9 - 23.1	0.9731	Day 6
13.6	1.5 - 126	0.9539	Day 6
12.9	3.3 - 50.2	0.9577	Day 6
11.4	3.6 - 36.6	0.9691	Day 7
11.3	4.8 - 26.7	0.9130	Day 7
10.6	5.1 - 21.7	0.8489	Day 7
8.9	5.2 - 15.4	0.9743	Day 8
8.6	6.1 - 12.1	0.9272	Day 8
7.1	3.0 - 17.2	0.9298	Day 8
5.7	4.4 - 7.3	0.9605	Day 9
4.3	3.2 - 57.0	0.9615	Day 9
<b>Mean±SD</b>			
<b>13.4±7.9</b>			

As the neuronal cultures age, the IC<sub>50</sub> appears to become more potent; however the confidence intervals overlap due to culture variability. It is important to limit experiments to a defined culture age and always run positive standard for comparison & normalization

## **IC<sub>50</sub> Data over 3 Consecutive Weeks using Cultures of the Same Age for Evaluating the Effect of Inhibitor X**

		<b>Compound</b>	<b>Confidence</b>	<b>R<sup>2</sup></b>	<b>Days in</b>
		<b>IC<sub>50</sub> (nM)</b>	<b>Interval (nM)</b>		<b>Culture</b>
Week 1	Day 1	10.5	5.8 - 58.9	0.9863	<b>Day 7</b>
	Day 2	8.6	6.1 - 12.1	0.9272	<b>Day 8</b>
Week 2	Day 1	11.3	4.8 - 26.7	0.9130	<b>Day 7</b>
	Day 2	6.8	2.4 - 19.0	0.9914	<b>Day 8</b>
Week 3	Day 1	15.7	11.1 - 22.3	0.9852	<b>Day 7</b>
	Day 2	6.8	3.0 - 15.0	0.9113	<b>Day 8</b>

**When the culture age is kept constant, the IC<sub>50</sub> value for any specific compound is reproducible from week to week**

# Summary of Salient Findings

- **Principle Identical in Primary Neuronal Cortical Cell as in Recombinant Cell**
- **Uptake is temperature dependent**
- **Uptake is time dependent**
- **Uptake is both sodium & chloride dependent**
- **Organic anion transporting polypeptides inhibitors have a minimal effect on dye and retention in the primary neurons**
- **Time in culture is important to take into account to reduce variability**
- **Must account for baseline reading to avoid erroneous data (or have sufficient wells with no dye as control)**
- **Assay is rapid**
- **Assay reproducible**