Fluorescence-based Neurotransmitter Transporter Uptake Assay in Primary Neuronal Cultures. Parsa Safa, MDS Analytical Technologies, Sunnyvale, CA 94089 & Lesley Radov, AstraZeneca Pharmaceuticals, CNSP Discovery, Wilmington, DE 19803

Abstract

The use of a fluorescence-based assay kit for the measurement of neurotransmitter transporter (NT) uptake activity in cell lines stably expressing the human DAT, NET and SERT gene has been previously reported. We describe here the application of this fluorescence-based method to measure NT uptake activity in primary E-18 rat neuronal cultures. Real-time changes in NET, DAT & SERT transport activity were monitored in these neurons. Since these 3 biogenic amine transporters show overlapping localization and expression in the brain, NT uptake in sister cultures derived from the hippocampus, cortex, midbrain, striatum and nucleus accumbens were compared.. Hippocampal cultures were used to assess NET uptake activity; nucleus accumbens cultures were used to assess DAT uptake activity; and midbrain cultures were used to assess SERT uptake activity. Reference NET, DAT & SERT inhibitors from various pharmacological classes were evaluated for their ability to inhibit transport in these neuronal cultures and the IC_{50} values generated compared to literature values.

Introduction

Depression is a common metal disorder. It is estimated that the lifetime prevalence of major depressive disorder (MDD) is ~7%.[1] Approximately two dozen marketed antidepressants are currently available worldwide. Many of these drugs increase the concentration of the 3 biogenic amine neurotransmitters most closely linked to depression (serotonin, dopamine, norephinephrine/noradrenaline) in the synaptic cleft. This is accomplished by inhibiting their reuptake back into the presynaptic neuron. Drugs targeting a single neurotransmitter transporter, such as the selective serotonin reuptake inhibitors (SSRIs) or selective noradrenalin reuptake inhibitors (SRNIs), were developed to limit the untoward side effects of the tricyclic antidepressants while mimicking their efficacy. The next step forward in depression treatment was the development of dual uptake inhibitors (SSRI/SSNI). These agents have gained acceptance in the clinic, but are not totally satisfactory due to delayed onset, low response rates and their side effect profile. The latest advance in depression therapy has been the development of the triple uptake inhibitors (SSRI/SSNI/selective dopamine uptake inhibitor).

The most common technique used to estimate biogenic amine transport has been monitoring the accumulation of radiolabeled substrate or inhibitor. One major drawback to the use of these radioactive methods is the difficulty in monitoring real-time changes. A second consideration is the increased pressure to minimize the use of radiolabeled materials due to public safety concerns and increasing disposal costs. An alternative, non-radioactive approach for determining biogenic amine transport is available. The use of a fluorescence-based assay kit for the measurement of neurotransmitter transporter uptake activity in cell lines stably expressing the human DAT, NET and SERT gene has been previously reported. A question that constantly arises is the relevance of the use of cell lines over-expressing the recombinant SERT, DAT or NET vs. the use of native tissue protein to determine uptake. We describe here the application of this fluorescence-based method to measure NT uptake activity in a native tissue system. The native tissue system we have chosen to employ is the primary E-18 rat neuronal culture model.

Materials & Methods

Materials: The reference agents Paroxetine, GBR12909, McN5652, Citalopram, Fluvoxamine, Fluoxetine, Nomifensine, Sertraline, Reboxetine, Nisoxetine and Bupropion were were obtained from Tocris. Duloxetine, Desipramine, and Imipramine were obtained from Sigma. All the tissue culture media and supplements were purchased from Invitrogen. The cultureware was purchased from Corning. Other biochemicals and buffers were purchased from Mediatech/Thermo Fisher.

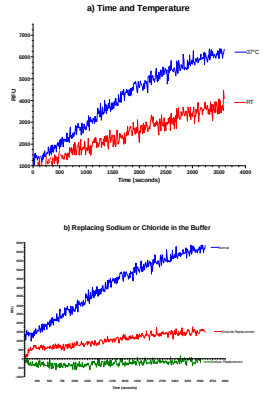
Cell Culture – Primary Neurons: Brains from 18-day-old rat (Holtzman) fetuses (E-18) were aseptically removed from pregnant females and placed into a petri dish containing chilled Hank's Buffered Saline solution (HBSS). The tissue was digested in a solution of 0.25% tissue culture grade trypsin with DNAse (1 ug/ml) for ~ 10' at 37°C. Following the incubation, the tissue was then gently agitated and the material centrifuged at 4°C at 200g for 10 minutes. The supernatant containing cellular debris was aspirated and the cells were washed a second time.. At the end of the second centrifugation, the cells were resuspended in neurobasal media containing B-27 and 0.5mM glutamine, and passed through a sterile cell strainer to remove large cellular clumps. The cell suspension was then resuspended with a large bore 5 ml pipette, followed by a small bore transfer pipette. The cells were counted with a hemocytometer and plated at a concentration of 125,000 cells/well in poly-L-lysine coated 96 well-plate. The plates were maintained in a humidified 37°C CO_2 incubator and were used after 5-9 days in culture.

Measurement of Neurotransmitter Uptake Inhibition: The assay was conducted using the MDS Analytical Technologies Neurotransmitter Transporter Uptake Assay Kit®. The media was removed from the neuronal cultures by gently blotting the plate onto absorbent towels. Subsequently, the cultures were washed twice with at least 200µl/well of assay buffer (1X HBSS/20mM HEPES, pH 7.4) followed by the addition of either 100µl of assay buffer alone or assay buffer containing the inhibitor at the desired concentration. The cultures were incubated at 37°C for 15 minutes in the dark and an initial "pre-dye" fluorescence measurement was read using the MDS Analytical Technologies Flexstation II. The cultures were then loaded with 100µl of the neurotransmitter uptake dye (NT) and the plates incubated for an additional 30 minutes at 37°C in the dark. After the final 30-minute incubation period, the plates were read again in the Flexstation II instrument. For both readings, the output was mean relative fluorescence units (RFU).

Data Analysis: Data was collected as mean RFU using the SOFTmax Pro software. In all experiments, the appropriate average buffer control was included. Outliers were detected using the Grubb's Test and removed from the calculations if appropriate. IC_{50} concentration curves for the transporters were determined for the neurotransmitters. Individual IC_{50} values, Hill slopes, 95% confidence intervals, and goodness of fit numbers were computed using the Graphpad Prism software nonlinear regression analysis program. For these analyses, constrained values were used when the fits did not converge using unconstrained parameters. The correlation between the IC_{50} value generated in the primary neuronal cultures and the K_i values from the literature was done using the Spearman method.

Results

Fig. 1. Dye loading occurs in a time and temperature dependent manner in primary neuronal cultures and is sensitive to external sodium and chloride concentrations.



Top: uptake proceeds faster at 37°C than at room temperature. This is consistent with the 'rule of thumb' that rate of uptake approximately doubles for every increase of 10°C.
Bottom: sodium and chloride are essential for transport into primary neurons confirming that transport is via Na^+ / Cl^- coupled transporters

Fig. 2. The Neurotransmitter Transporter Uptake Assay is rapid and robust.

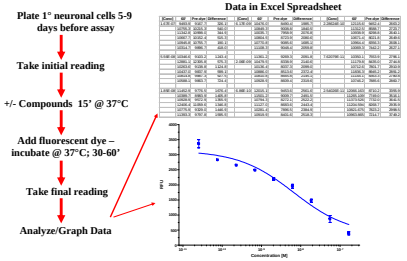
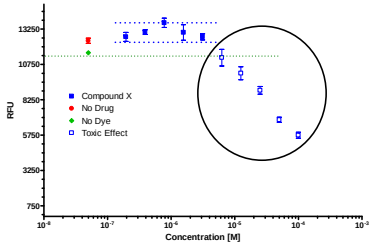
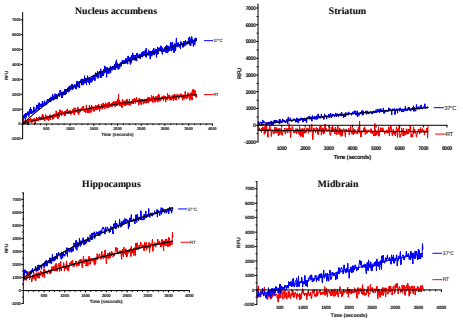


Fig. 3. The Effect of a 15 Minute Pretreatment with Compound X on Uptake in Day 9 Neuronal Cultures



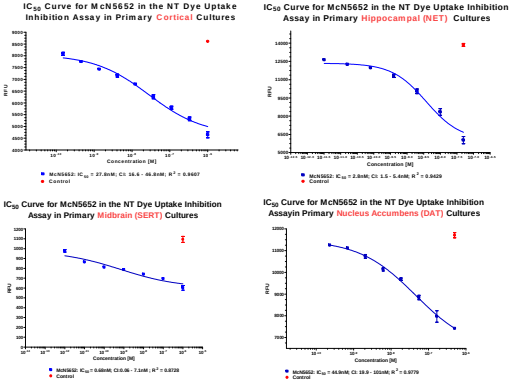
Compound is really not effective: the reduction of fluorescence is actually the result of toxic compound concentrations, causing cell disintegration and thus the appearance of uptake inhibition.

Fig. 4. Cultures from several but not all brain regions show significant dye uptake.



The dye uptake signal/noise window in primary cultures of hippocampus (NET), midbrain (SERT), and nucleus accumbens (DAT) is sufficient and reliable enough for these tissues to be used for transporter studies. The dye uptake signal/noise window in primary cultures of striatal cultures is NOT large enough to allow these cultures to be used on a routine basis – especially with the variability inherent in the system.

Fig. 5. The pharmacology of a triple uptake inhibitor: McN5652



Neuronal cultures were pretreated with various concentrations of the triple uptake inhibitor McN5652. The IC_{50} value and 95% confidence intervals for the various tissues were as follows: 1) Cortical IC_{50} = 27.8nM; the confidence interval was 16.6 to 46.8nM; and the R^2 was 0.9607. This is a combined NET/SERT/DAT value since the tissue contained DAT, NET & SERT. To determine individual DAT, SERT & NET numbers, specific tissues and/or procedures must be used to ensure that only a single NT transporter is measured. 2) Hippocampal (NET) IC_{50} = 2.8nM; the confidence interval was 1.5 to 2.4nM; and the R^2 was 0.9429; 3) Midbrain (SERT) IC_{50} = 0.68nM; the confidence interval was 0.06 to 7.1nM; and the R^2 was 0.8728; 4) N. accumbens (DAT) IC_{50} = 44.9nM; the confidence interval was 19.9 to 101nM; and the R^2 was 0.9779.

Table 1. The effect of culture age on compound potency.

Compound IC_{50} (nM)	Confidence Interval (nM)	R^2	Days in Culture
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
Mean±SD			
13.4±7.9			

The experimental data shows that as the culture mature over time, the test compound appears more potent, although the differences (IC_{50} values) are not statistically significant since the confidence intervals are overlapping. The most consistent results are seen when using cultures between days 6-8 of after plating.

Table 2. IC_{50} values are reproducible in primary neuronal cultures of the same age.

		Compound IC_{50} (nM)	Confidence Interval (nM)	R^2	Days in Culture
Week 1	Day 1	10.5	5.8 - 58.9	0.9863	Day 7
	Day 2	8.6	6.1 - 12.1	0.9272	Day 8
Week 2	Day 1	11.3	4.8 - 26.7	0.9130	Day 7
	Day 2	6.8	2.4 - 19.0	0.9914	Day 8
Week 3	Day 1	15.7	11.1 - 22.3	0.9852	Day 7
	Day 2	6.8	3.0 - 15.0	0.9113	Day 8

This table illustrates that the results are reproducible from week to week when cultures of the same age are used. The confidence limits overlap between all 6 studies, showing that there is no significant difference among the 6 the IC_{50} values.

Table 3. Comparison of IC_{50} results from primary neuronal cultures with reported K_i values.

Drug	Syn 1° Cell NET nM		Syn 1° Cell SERT nM		Syn 1° Cell DAT nM	
Duloxetine	15.6	22.8	4.6	4.3	369.2	496
Fluoxetine	281	306	12	11.6	1600	2961
Citalopram	4000	6147	1.3	2.8	28000	33970
Fluvoxamine	620	709	7	3.3	5000	5293
Paroxetine	81	121	0.29	0.47	5100	5800
Sertraline	160	197	0.19	0.11	48	66.1
GBR12909	440	242	170	301	1	1.6
Nisoxetine	1.3	1.7	310	351	510	1000
Nomifensine	5	15.5	1280	1660	51	65.1
Desipramine	0.9	0.24	340	632	5200	6700
Bupropion	2300	2200	15600	11540	630	826
Reboxetine	8.2	15.8	1070	1636		>100000
Imipramine	13	26	42	33.8	5110	8238
McN5652	2.9	2.8	0.68	0.68	36.8	44.9

There was excellent agreement between published literature K*i* values for the 14 drugs evaluated and the IC_{50} values generated in the primary neuronal culture system. The Spearman *r* for each transporter is as follows: NET = 0.9801; SERT = 0.9956; & DAT = 0.9835.

Conclusion

- Dye uptake in primary neuronal cultures is time, temperature, sodium and chloride dependent.
- Assay is reproducible if culture age is kept constant.
- Tissue source is critical to determine valid NET vs DAT vs SERT inhibitor selectivity & potency.
- Excellent correlation exists between the published K_i values for the 14 compounds evaluated and the IC_{50} values generated with the MDS Analytical Technologies Neurotransmitter Transporter Uptake Assay Kit®.

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