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In vitro assays for the functional characterization of the dopamine transporter (DAT)

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Abstract

This Unit describes procedures for *in vitro* uptake assays for the functional characterization of DAT. All assays are performed using commonly available cell lines that transiently or stably express a particular transporter under investigation. The three main assays described here are: kinetic functional assay to calculate apparent affinity (K_M) and maximal velocity (V_{max}) of radiolabeled DA uptake into cells; dose-response assays to measure potencies (IC_{50}/K_i values) of test compounds for inhibition of transporter function; and efflux assay to determine the ability and potency (EC_{50}) of a ligand to elicit reverse transport of accumulated labeled DA in the cells. These procedures can be reproduced in any pharmacological research laboratory with minimal expertise and resources. Although the methods are described using DAT and its ligands, the same protocol can be directly applied to SERT and NET using their respective ligands.

Keywords

Dopamine transporter; monoamine; neurotransmitter; uptake

Introduction

Monoamine transporters (MATs) are part of the solute carrier 6 (SLC6) family of transporters (Reviewed in Kristensen *et al.*, 2011). They are expressed in the central and peripheral nervous systems where they play a critical physiological role in regulating neurotransmitter homeostasis. There are three types of MAT, the dopamine transporter (DAT; *SLC6A3*), the norepinephrine transporter (NET; *SLC6A2*), and the serotonin transporter (SERT; *SLC6A4*) which mediate the uptake of DA, NE and 5-HT, respectively, from the extracellular space into the intracellular compartment. All three transporters are membrane-embedded proteins exclusively expressed in the presynaptic neuronal terminals of their respective pathways. Their structure consist of 12-transmembrane (TM) spanning domains connected with various extra- and intracellular loops with both N- and C-terminals present in the intracellular region. The highly conserved substrate binding site is located centrally in the transmembrane region between TM1 and TM6 (Figure 1).

The molecular determinants of MAT substrate versus inhibitor binding sites have been investigated through various site-directed mutagenesis, computer-aided molecular modelling

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and crystallographic approaches. Apart from the substrate binding site, these studies have revealed the presence of additional ligand binding sites within MATs of which the location and functional relevance is still under investigation (Coleman *et al.*, 2016, Koldso *et al.*, 2015, Mortensen & Kortagere, 2015, Rothman *et al.*, 2015). MATs mediate a relatively rapid uptake of neurotransmitters (turnover rate ~1 molecule/second) from the synaptic cleft, back into the pre-synaptic neuronal terminals (Kristensen *et al.*, 2011), where the neurotransmitters are sequestered into synaptic vesicles through vesicular monoamine transporters (VMAT, Golovko *et al.*, 2016) for recycling or are degraded by monoamine oxidase enzymes. Along with the transport of one molecule of monoamine neurotransmitter, MATs also co-transport two Na⁺ ions and one or more Cl⁻ ions into the intracellular space. Thus, they are sometimes also referred as Na⁺/Cl⁻-symporters.

MATs play a pivotal role in controlling the signal amplitude and duration of action of monoaminergic neurotransmitters by altering their concentration in the extracellular synaptic space in the CNS (Lin *et al.*, 2011) as well as in the PNS (Ramamoorthy *et al.*, 2011). Therefore, direct or indirect modulation of MATs can markedly affect neuronal activity. Hence, there are a variety of MAT ligands approved as therapeutics or used as tool compounds to investigate the underlying mechanism of CNS-related diseases in which MATs are implicated. MATs are also the primary targets of action of a number of psychostimulant and recreational drugs of abuse such as cocaine, methamphetamine, 3,4-methylenedioxy-methamphetamine (“ecstasy” or MDMA), cathinones (or “bath salts”) and others that block or reverse neurotransmitter transport and increase synaptic neurotransmission leading to and euphoria (Howell & Negus, 2014; Rives *et al.*, 2017). MATs are key targets for the development of CNS-based drugs to restore the homeostasis of neurotransmission. Therefore *in vitro* approaches to the study of MATs is critical in drug discovery. Described in this unit are *in vitro* methods for basic pharmacological/functional characterization of the interactions of ligands with DAT. Protocols for three types of cell-based functional uptake assays for the transporters, i.e. kinetic-uptake assays, dose-response assays and efflux assays are described in detail (Steinkellner *et al.*, 2016; Sucic & Bönisch, 2016). Useful tips for troubleshooting experimental problems and optimization of critical factors that can affect the outcome of the results are also provided. These basic assays are used to determine the main functional parameters (i.e., K_M, V_{max}, IC₅₀, and K_i values) of ligands that interact with DAT.

Basic Protocol 1. Kinetic uptake assay (96 well format) to determine K_M and V_{max} of DAT-mediated DA transport

Introduction

The purpose of this protocol is to measure the maximum velocity of DA uptake (V_{max}) and the affinity of DA (K_M, Michaelis-Menten constant) for DAT. Various concentrations of radioactive DA are incubated for 10 min with cells expressing the hDAT protein. The amount of radioactivity retained inside the cells is measured using a scintillation counter machine. For analysis, a graph of the measured radioactivity counts is plotted against the increasing concentration of the radioactive DA. The V_{max} and K_M values are calculated using this graph. In case if the hDAT-expressing cells are incubated with radioactive DA

concentrations simultaneously in the absence or presence of a particular ligand concentration, then the resulting graph can give information about the type of interaction between DA and the ligand (i.e. competitive or non-competitive/allosteric)

Materials

- Dulbecco's Phosphate Buffered Salt Solution, 10X (DPBS). *Used to prepare buffers.*
- 0.1M CaCl₂ (store at room temperature (RT))
- 1M MgCl (store at RT)
- Ascorbic acid (100 mM stock in deionized water, store aliquots at –20°C)
- Catechol O-methyltransferase (COMT) inhibitor Ro-41-0960 (Source: Sigma Aldrich; Make a 10 mM stock in ethanol, store at –20°C for up to one year). *Used in order to block the enzyme that degrades catecholamines such as dopamine and norepinephrine.*
- Radioactive dopamine [(DA), 3,4-[Ring-2,5,6-3H]-Dihydroxyphenylethylamine from PerkinElmer]
- 96-well plates (e.g., Falcon™ Tissue Culture-Treated 96-well Microplates from Fisher Scientific)
- COS-7 or MDCK cell lines, (ATCC)
- pcDNA3.1-hDAT plasmid #32810 (www.addgene.org)
- DMEM Culture media (eg. Dulbecco's Modified Eagle Medium with L-Glutamine, 4.5g/L Glucose and Sodium Pyruvate, Corning™ 10013CV) with additional 10% Fetal Bovine Serum (VWR #97068-085) and 5% Penicillin/Streptomycin solution (Corning™ cellgro™ 30002CI)
- OptiMem (serum free media, store at –4°C)
- Hemacytometer (eg. Hausser Scientific Bright-Line™ and Hy-Lite™ Counting Chambers, Fisher Scientific #02-671-10)
- TransIT®-LT1 transfection reagent (Mirus Bio LLC, store at –4°C)
- Trypsin 0.05%/0.53mM EDTA (for COS-7, Corning™ 25052CI) or trypsin 0.25%/EDTA 0.1% (for MDCK, Corning™ 25053CI)
- Plate washer (eg. Biotek ELx50 for 96-well plates)
- Scintillation fluid (eg. Scintillation fluid Ecolite (+) (MP BIOMEDICALS INC)
- Microplate Scintillation and Luminescence Counter (Perkin Elmer, Shelton, CT, USA).
- Sealing tape for 96-well plates (eg., adhesive tape from VWR #60941-070)

Protocol

1. Plate COS-7 cells transiently transfected with pcDNA3.1-hDAT at 20,000 cell per well density or stably transfected *hDAT*-MDCK cells at 50,000 cell per well density on a 96-well plate in 150 μ L per well volume of DMEM in a sterile laminar flow biosafety cabinet. For non-specific uptake, plate non-transfected COS-7 or naïve MDCK cells in parallel. Transfected cells are plated in rows A – F and non-transfected cells are plated in row G – H (Figure 2).

Treatment of cells with trypsin in a 75 cm² tissue culture flask with 80% confluency: Aspirate media from the flasks and add 1 mL PBS (autoclaved) at RT to wash the cells of any residual culture media. Add 1 mL trypsin (0.05%/0.53mM EDTA for COS-7 or 0.25%/EDTA 0.1% for MDCK) (for enzymatic detachment of cells) and incubate the flask for 5 min in a 37°C, 5% CO₂ humidified incubator. Add 5 mL DMEM to mix the dissociated cells. Use 10 μ L of this mix to count the number of cells present per volume using a hemacytometer. Finally, calculate the total volume of cells needed for plating the desired cell count per well.

To transfect COS-7 cells for one 96-well plate, add 8.6 μ g of DAT DNA to 864 μ L of OptiMem (serum-free media), followed by the addition of 28.8 μ L of TransIT®-LT1 transfection reagent. Incubate this mix for a maximum 30–40 minutes at RT. Add appropriate quantity of ‘trypsinized’ COS-7 cells to the mix to obtain a density of 20,000 cells per well. Adjust the volume of the mix with DMEM to 20 mL, and then distribute this mix to the 96-well plate with 150 μ L volume per well. For plating stably transfected MDCK cells, the appropriate volume of ‘trypsinized’ cells containing the desired number of cells per plate (50,000 cells per well) are mixed with DMEM up to a total volume of 20 mL. This mix is distributed on a 96-well by adding 150 μ L in each well.

2. Incubate plate in a 37°C, 5% CO₂ humidified incubator for 48 hours (COS-7) or 24 hours (MDCK) to reach up to 80% confluency (observed under a microscope).
3. On the day of the experiment, aspirate the media from the cells and add PBS-CM (at RT) using a plate washer e.g., Biotek ELx50.
4. Aspirate PBS-CM, and add 40 μ L of vehicle (assay buffer) or a specific concentration of a ‘test’ compound (usually its IC₅₀) to each well (from column 1 to 12) at RT. Non-transfected cells only receive 40 μ L of the vehicle (see Figure 2).
5. Incubate the plates for 5–10 minutes.
6. Initiate DA uptake by adding 40 μ L of 6 different radioactive DA substrate dilutions to the wells – two wells per dilution (from rows A to H) (Figure 3). *The six cold + hot DA dilutions are prepared in the range 10 μ M to 0.01 μ M (see the section Reagents and Solutions). However, since 40 μ L of solution is already present in each well, the resulting range of DA concentration received by the wells becomes 5 μ M to 0.005 μ M.*

7. Incubate each well for exactly 10 minutes – timing is critical as the data will be analyzed as velocities.
8. To terminate the assay, wash the plate twice with PBS-CM (at RT) and then add 50 μ L of scintillation cocktail to each well. Seal the plate with sealing tape and quantify the radioactivity in a Microplate Scintillation and Luminescence Counter (Perkin Elmer, USA).
9. For the analysis of the kinetic uptake data, subtract the counts for the non-specific uptake from the corresponding total counts. To convert the specific counts (raw data is obtained in counts per minute (cpm)) into the units of fmol/min/well, divide cpm by the counter efficiency to get the values in disintegrations per minute (dpm). Transform the dpm values to μ Ci (micro Curies) by dividing with 2.2×10^6 ($1\mu\text{Ci} = 2.2 \times 10^6$ dpm). Further divide this by the 'new' specific activity of the DA solution prepared by mixing hot and cold DA substrate to convert Ci into moles (For example if the radiolabeled DA was diluted 100-fold with cold compound the specific activity of the original radiolabeled DA also should be divided by 100). Then calculate the values per minute by dividing by 10. Plot the resulting values in fmol/min/well against increasing concentrations of DA. Perform nonlinear regression analysis using GraphPad Prism software fitting the data to a Michaelis-Menten equation to obtain K_m and $V_{\max}$ values.

Step-wise analysis of a kinetic uptake sample data

Step 1: Obtain the raw data file from the scintillation counter machine and open it in Microsoft Excel. The raw data will typically look like the one given in Table 1.

Step 2: Calculate the averages for the non-specific counts under each DA-concentration as shown in Table 2.

Step 3: Subtract the average 'non-specific' counts from the total count values in each column to get 'specific' counts as shown in Table 3.

Step 4: To convert the values obtained in cpm into the units of fmol/min/well, first convert cpm data into the units of dpm by dividing the counts by the scintillation counter's efficiency. To demonstrate this, let us take the value in well A1 as an example, i.e. A1: $22 \text{ cpm} = 22 \div 0.3 = 73.3 \text{ dpm}$. *The counter efficiency for the machine used in this example is 30%.*

Step 5: Transform all the dpm values to μ Ci (microCuries) by dividing with 2.2×10^6 ($1\mu\text{Ci} = 2.2 \times 10^6$ dpm), i.e. A1: $73.3 \text{ dpm} = 73.3 \div (2.2 \times 10^6) = 33.3 \times 10^{-6} \mu\text{Ci}$ or $33.3 \times 10^{-12} \text{ Ci}$.

Step 5: Next, to convert from Ci to moles, divide the values in Ci by the 'new' specific activity of the prepared radioactive DA solution. For our experiment, the 'new' specific activity is 0.3 Ci/mmol (See Reagents and Solutions section for details).

Therefore, for A1: $(33.3 \times 10^{-12}) \div 0.3 = 111.0 \times 10^{-12}$ mmol or 111.0 femtomole per well (fmol/well).

Step 6: Finally, divide the values in fmol/well by 10 (because the experiment was run for 10 min) to get the units of fmol/min/well, i.e., A1: $111.0 \div 10 = 11.1$ fmol/min/well. Table 4 shows the final data converted into the units of fmol/min/well for all the wells.

Step 7: For analysis, copy the resulting values in fmol/min/well to GraphPad Prism software such that the values for each DA-concentration are aligned horizontally as shown in Table 5.

Step 8: Perform nonlinear regression analysis using GraphPad Prism software fitting the data to a Michaelis-Menten equation to obtain K_M and V_{max} values (see figure 4 as an example).

Basic Protocol 2: Dose-response assay (96 well format) to determine IC_{50} values and apparent K_i of ligands at DAT

Introduction

This protocol provides a straightforward assay to measure the inhibitory potency of new 'test' inhibitors against the transport of radioactive DA into hDAT-transfected cells. This assay measures the accumulated intracellular [3H]-DA in hDAT-expressing cells incubated with various concentrations of the test compound. If the test compound inhibits the uptake of DA in a dose-dependent manner, a plot of measured radioactive against the increasing concentrations of the compound will give a sigmoidal curve which can be used to calculate potency of the compound. The potency is expressed as IC_{50} , which is defined as concentration of a compound required to inhibit the DA uptake by 50%. If the inhibitor competes with DA for the same binding site, then the IC_{50} can also be used to calculate K_i (apparent affinity) using the Cheng-Prusoff equation.

Materials—In addition to the materials listed in Basic protocol 1

1. A test compound for which IC_{50} needs to be determined.
2. Cocaine, a competitive DAT inhibitor, which is used as a reference or standard compound in this experiment (commercially available from Sigma Aldrich).

Protocol

1. Transfect and plate cells as described in Protocol 1.
2. Incubate the plate in a 37°C, 5% CO₂ humidified incubator for 48 hours (COS-7) or 24 hours (MDCK) to reach up to 80% confluency.
3. Aspirate the media from the cells and add PBS-CM at RT using plate washer instrument (For example Biotek ELx50).
4. Aspirate PBS-CM, and add 40 μ L of a vehicle (assay buffer as a control) and 7 test dilutions of test compound(s) or the standard compound (such as cocaine) to

rows A (For example 0 μM) to H (for example 1 mM) of the 96-well plate (see Figure 5).

5. Incubate the plate for 10 minutes.
6. Add 40 μL of the appropriate 50nM [^3H]-DA solution to each well (final effective concentration of [^3H]-DA becomes 25 nM in each well) with gentle shaking and incubate for exactly 10 minutes at room temperature to perform the functional uptake assay (Figure 6).
7. To terminate the assay, wash the plate twice with PBS-CM (at RT) and then add 50 μL of scintillation cocktail to each well. Seal the plate with sealing tape and quantify the radioactivity in a Microplate Scintillation and Luminescence Counter (Perkin Elmer, Shelton, CT, USA).
8. For analysis, normalize the specific counts for zero inhibitor concentration to 100% and plot the relative percentage of [^3H]-DA uptake as a function of the logarithm of the inhibitor concentration (reference or test compound). Perform non-linear regression analysis (Figure 7) with variable slope function using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). The IC_{50} value can be estimated from the plot and used to obtain the K_i by applying the Cheng-Prusoff (1973) equation shown below:

$$K_i = \text{IC}_{50} / (1 + [\text{}^3\text{H-DA}] / K_M)$$

Where K_M is the Michaelis-Menten constant which represents the apparent affinity constant of the monoamine substrate for the transporter and is calculated using the kinetic uptake assays as outlined in Protocol 1.

Step-wise analysis of a dose-response sample data

Step 1: Retrieve the raw data from the counter machine and open it using Microsoft Excel. Table 6 shows an example of a raw data obtained from a dose-response analysis of a test inhibitor and cocaine.

Step 2: Calculate the average of counts for only zero inhibitor concentration (Table 7).

Step 3: Normalize the average counts for zero inhibitor concentration to 100% and then calculate the % of counts obtained for various inhibitor concentrations against the average '0' inhibitor concentration of cocaine or test compound. For example, as shown in Table 8, divide all the counts obtained with cocaine by 2068, and then multiply them by 100 to get the percentages. Similarly, divide all the counts obtained with the test compound by 713, and then multiply them by 100 to get the percentages

Step 4: Plot the relative percentage of [^3H]-DA uptake as a function of the inhibitor concentration (reference or test compound) using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). Perform non-linear regression analysis

with variable slope function to estimate the IC_{50} values for each compound (Figure 7).

Basic Protocol 3: Release/Efflux assays (24 well format) to determine the ability of ligands to modulate DAT-mediated efflux of dopamine

Introduction

DAT substrates (such as dopamine, amphetamine, etc.) also act as releasers of accumulated DA from inside the DAT-transfected cells. Thus, if an unknown 'substrate' is tested in a dose-response assay (i.e. protocol 2), it can be difficult to differentiate if a decrease in accumulated DA inside the cells is a result of inhibition of transport or is rather an effect of increased efflux of DA out of the cells. This protocol describes a simple substrate release assay which measures the amount of efflux of pre-loaded radioactive DA in hDAT-transfected cells induced by exposure to test compounds. Cells expressing DAT protein are incubated for 30 min to 1 hour in radioactive DA. The remaining solution is removed and the cells are then incubated with varying concentrations of control or test compounds for 10 min. The radioactivity of the supernatant and of the cells is measured separately in a liquid scintillation counter. A bar-graph representing the percentage of radioactive DA released by different test compound can be used for the comparison of relative potencies. The test 'substrates' trigger DA release in a concentration dependent manner. Efflux potency is represented as EC_{50} , i.e. the concentration of test compound required to evoke 50% of the preloaded radioactive DA.

Additional materials (see Basic protocol 1)

1. 24-well plates, (eg., Thermo Scientific Biolite #930186)
2. Scintillation vials (eg., Simport™ Scientific HDPE Snaptwist™ Scintillation Vial from FisherBrand #03-342-3)
3. Liquid scintillation analyzer (eg., Tri-carb 3100TR by PerkinElmer)
4. 1% SDS/0.1N NaOH lysis buffer in deionized water, pH = 7.4.

Protocol

1. Plate 24-well plates with COS-7 cells transfected with DAT cDNA at a density of 100,000 cells per well in a laminar biosafety cabinet. A group of non-transfected cells can be plated in parallel to serve as a negative control.

Briefly, for plating one 24-well plate, to 1200 μ L of OptiMem (serum free media), add 12 μ g DAT DNA and 36 μ L of Trans-IT-LT1 transfection reagent. Incubate this mix for maximum 30–40 minutes at RT. Then, add appropriate quantity of 'trypsinized' (see Protocol 1) COS-7 cells to get a density of 100,000 cells per well for a 24-well plate. Then, make up the volume of the mix with DMEM up to 13 mL. Transfer 500 μ L aliquots of this mixture to each of the 24 wells.

2. Incubate the transfected cells at 37°C for 48 hours to reach a confluency of around 80% in all wells (observed under the microscope).
3. Wash the cells once with 500 µL of PBS-CM at RT to completely remove the culture media.
4. Load all wells with 250 µL of a solution of 25 nM [³H]-DA (see Figure 8).
5. Incubate the plate at 37°C for 30 minutes to 1 hour to allow the uptake of [³H]-DA into the cells.
6. Terminate the uptake by aspirating radioactive solution and washing the cells with 500 µL of PBS-CM (at RT) per well.
7. Initiate the efflux by replacing the PBS-CM with 250 µL of a range of conditions and concentrations of DA releasers/substrates such as DA, amphetamine, methamphetamine, etc. and/or blockers for 10 minutes as described below. An unknown compound can also be included in the experiment in order to test its DA-releasing effects as compared to other reference compounds.

Example: For measuring efflux of [³H]-DA in WT-DAT in a 24-well plates, 8 conditions for the efflux can be tested (Figure 9). For example, to each of three wells is added assay buffer (vehicle as a control), 20 µM amphetamine, 20 µM cold DA, 100 µM cocaine, a test compound (20 µM), a mixture of amphetamine (20 µM) and cocaine (100 µM), a mixture of amphetamine (20 µM) and the test compound (20 µM), a mixture of DA (20 µM) and test compound (20 µM) respectively.

8. After exactly 10 minutes, transfer 200 µL of the supernatant from each well to scintillation vials containing 5 mL of scintillation fluid (Ecolite⁺, MP Biomedicals Inc.).
9. Aspirate the remaining liquid from the wells, lyse the cells (in 250 µL of 1% SDS/0.1N NaOH lysis buffer at RT) and transfer into another set of scintillation vials containing scintillation fluid (5 mL).
10. Measure the radioactivity present in all the scintillation vials in a liquid scintillation counter machine.
11. For quantification, add the radioactive counts (raw data in dpm) obtained from the collected supernatant and the lysed cells to get the total radioactivity present. The percentage of efflux induced by test compound is calculated by determining the percent of radioactivity from the supernatant sample relative to the total radioactivity. A histogram can also be plotted to compare the percentage efflux induced or blocked by the compounds (Figure 10).

Step-wise analysis of an efflux assay sample data

Step 1: Open the raw data (counts are obtained in dpm) from the counter machine in Microsoft Excel (Table 9).

Step 2: Since the radioactive counts were obtained from collecting only 200 μL of the supernatant, the counts for the supernatant are corrected for a 250 μL volume (Table 10). *This is because the wells had 250 μL of the solution, and not 200 μL .*

Step 3: Add the radioactive counts obtained from the collected supernatant and the corresponding lysed cells to get the total radioactivity present for each condition (Table 11).

Step 4: Calculate the percent of radioactivity from the supernatant sample relative to the total radioactivity. This gives the percentage of efflux induced by a particular condition (Table 12).

Step 5: A histogram can also be plotted using GraphPad Prism software for a visual comparison of the percentage efflux induced or blocked by the compounds (Figure 10).

Reagent and Solutions

Protocol 1

1. PBS-CM: Dilute 100 mL DPBS (10X, Dulbecco's Phosphate Buffered Salt Solution) in 900mL de-ionized distilled water and adjust the pH to 7.4. Then, add 1mL each of 0.1M CaCl_2 (Final concentration 0.1mM) and 1M MgCl (Final concentration 1mM). *Can be stored at RT for prolonged periods of time.*
2. Assay buffer: To 100 mL of PBS-CM, add 50 μL of 100mM ascorbic acid (final concentration 50 μM) and 50 μL of the COMT inhibitor Ro-41-0960 (10mM solution in ethanol, final concentration 5 μM). *Freshly prepared.*
3. DA dilutions: Prepare six dilutions of a mixture of non-radioactive (cold) and radioactive (hot) DA substrate. The ratio of cold and hot DA should ideally be 10- to 100-folds. *Example: To make 1 mL of 10 μM of hot:cold DA solution in a ratio of 1:100 for one 96-well plate: Add 3 μL of the commercially available [^3H]-DA solution (which has an activity of 1 mCi/mL; specific activity of 30.0 Ci/mmol; molarity of 33.3 μM) to 1 mL of the assay buffer to get a final concentration of 0.10 μM of 1 mL [^3H]-DA solution. To achieve a 100-fold dilution, 0.99 μL of the cold DA (from a 10 mM stock) is added to 1 mL of 0.10 μM [^3H]-DA solution. The final concentration of the cold/hot DA mix thus becomes 10 μM (0.10 μM hot DA + 9.9 μM cold DA). Since the hot DA is diluted 100-folds with cold DA, the original specific activity (30.0 Ci/mmol, as mentioned on the manufacturer's label) of the hot DA now becomes 0.30 Ci/mmol (i.e. 100-fold less) for the final 10 μM cold/hot DA solution.*

Using the 1mL 10 μM cold/hot DA solution, prepare five additional concentrations from 2.50 μM to 10 nM by serial (or logarithmic) dilution. For example, add 250 μL of the 10 μM solution to 750 μL of assay buffer to get a concentration of 1.25 μM . Then, add 250 μL of this 1.25 μM solution to another 750 μL of assay buffer to get 0.625 μM , and so on. Prepare all the dilutions fresh and use within 2 hours.

Protocol 2

1. Test inhibitor compound dilutions: Make 8 working dilutions for new test compounds (from 20 mM stocks in pure DMSO). Since the IC_{50} of new compounds is unknown, a 'test' range of working dilutions is made. *For example, the experiment can be initially performed by using 8 logarithmic dilutions of the new test compound in the range of 0 to 1 mM. If the resulting IC_{50} does not lie in the middle of the range, then a different range of working dilutions should be tested. The concentration range of the test or reference compound can be different depending on the potency or affinity of the compounds. All working dilutions are freshly prepared from the stocks. The final DMSO concentration in the working dilutions should not exceed 1–2%. The 20 mM drug stocks can be stored in $-20^{\circ}C$ for up to 6 months.*
2. Radioactive DA: Make a [3H]-DA concentration of 50 nM in the assay buffer. *Ideally, the DA concentration should be lower than its anticipated K_M value. Prepare fresh and use within 2 hours.*

Commentary

Background information

Three basic types of functional uptake assays used to evaluate the properties of ligands that interact with DAT are kinetics, dose-response and efflux (See Kenakin 2014). Kinetic uptake assays are performed by incubating the transporter-expressing cells with different concentrations of a suitable radiolabeled substrate. These studies are used for experimental determination of K_M (Michaelis-Menten constant, see Johnson and Goody, 2011) and V_{max} (maximum rate of uptake) values. K_M is defined as substrate concentration at half maximum velocity and is an indicator of apparent binding affinity of a substrate for DAT. The nonlinear regression analysis of the data gives a hyperbola shaped curve with a linear relationship during initial concentration (say, zero concentration) and then non-linear at higher concentrations (ideally, 90% of the maximal transport velocity). For all concentrations it is important to perform the assay within a limited time period (< 10 minutes) such that the accumulation of substrate is linear as the data is analyzed as uptake rates/velocities. While using cell lines, stably or transiently transfected with different forms of DAT, such as chimeras or mutants, the kinetic uptake assay can be used to determine their function.

The dose-response assay measures the ability and the potency of a ligand to inhibit the uptake or transport (a biological response) of DAT. The assay provides an estimate of the IC_{50} value which only indicates the functional potency of an inhibitor and does not directly indicate its binding affinity (i.e. K_i), its interaction with the binding site or the type of inhibition (i.e., competitive, non-competitive or allosteric). IC_{50} values vary between experiments, and is dependent on the experimental conditions such as the concentration of radiolabeled DA. However, since the IC_{50} values of compounds are indicative of their effect on the functional response of a transporter, they are more precise predictors of *in vivo* efficacy and are usually preferred over direct displacement assays (which calculates the K_i , the affinity constant). If the functional inhibition of transport is competitive, i.e. the inhibitor

binds and competes with the radiolabeled substrate for the same site, then the Cheng-Prusoff equation can be applied to calculate affinity (expressed as K_i) from IC_{50} .

$$K_i = IC_{50} / [1 + ([S]/K_M)];$$

where $[S]$ is substrate concentration.

Moreover, the dose-response assays also serve as alternative methods (in contrast to direct displacement assays) for evaluating affinity of ligands against novel or unknown binding sites within DAT for which suitable competing radioactive ligands are not known.

In efflux or release assays, cells expressing the DAT are pre-loaded with a radioactive monoamine substrate or a similar synthetic compound. Then the ability of a ligand to initiate efflux or release of the intracellular radioactive substrate is calculated as a percentage of the total radioactivity loaded into the cells. The ability of a ligand to elicit efflux indicates that the ligand is a DAT substrate. If a ligand can evoke monoamine efflux in a concentration-dependent manner, then the efficacy of a ligand to function as a releaser to promote efflux can be represented by EC_{50} value (the concentration of a releaser required to produce an efflux of 50% of maximal efflux).

For all the uptake assays, it is also essential to consider the specific and non-specific uptake (see Figure 11 for an example). Specific uptake refers to the accumulation of ligand accumulated only by DAT. Non-specific uptake is defined as the amount of ligand accumulated/bound by/to other sites such as the wall of the sample tube, cell membrane, etc. In the case of kinetic assays, non-specific uptake is obtained by performing the assays simultaneously with non-transfected cells or in the presence of a high concentration of a DAT-specific inhibitor for transfected cells. The raw data obtained from the analysis represents the amount of total uptake. Specific uptake to the target is then obtained by subtracting non-specific uptake from the total uptake. The non-specific uptake curve is typically linear and non-saturable with respect to the concentration of the ligand. Specific uptake, on the other hand, is non-linear and saturable.

Another type of assay routinely used for the direct determination of affinities of inhibitors for the transporter binding sites is the binding-displacement assay (not covered in this unit). This experiment is used to quantitate the ability of a test ligand to compete with and inhibit the binding of another radiolabeled inhibitor (traceable ligand should not be the substrate) to the monoamine transporter for the same binding site (therefore not relevant for allosteric ligands). Although this assay provides direct evidence of an interaction of a ligand with the transporter (expressed as the affinity constant, K_i), it is dependent on the availability of selective radioligands, and is less sensitive, and is only indirectly relevant to the transporter function in a physiological system.

Critical parameters and troubleshooting

In order to design and execute an assay protocol and ensure its robustness, quality and consistency, there are several important parameters to be considered. These variables also aid in troubleshooting an experiment in case of problems and irreproducibility.

Understanding and optimization of each of these parameters is important to reduce variability in the results and increase the validity of the assays (Brouwer *et al.*, 2013; Volpe, 2016).

1. Target protein source: Variable results can be obtained based on the factors such as the source of primary cells (human or animals), their condition (frozen or fresh), and the method of transfection and quality of cDNA (transiently or stably). For cell-based assays, most common types of cell lines used are Madin-Darby canine kidney (MDCK), human embryonic kidney (HEK-293), monkey kidney (COS-7), and Lewis-lung cancer porcine kidney 1 (LLC-PK1).

Functional response of a transporter can only be measured if the transporter protein is present in an appropriate physiological system. Therefore the source of the transporter can be whole tissues, cells in culture, or membrane preparations. In case of tissues as the protein source, it is important to block other transporters and proteins with selective antagonists/inhibitors to minimize interference and misinterpretation of results. The studies in this unit are performed using naive cells transfected with DAT cDNA to avoid the presence of any other competing protein. When the studies are conducted in isolated tissues expressing DAT, along with other transporter proteins (NET and SERT), a selective antagonist or inhibitor can be added to block the functional responses of the competing transporters. In addition, since VMAT is present in the vesicular membranes of isolated synaptosomal tissues, reserpine is added as a selective VMAT-blocker to prevent any DA uptake into the vesicles. This allows to achieve steady state uptake of DA inside the synapse mediated only by the DAT (Partilla et al., 2016). In contrast, transfected cells provide a more selective system for transporter functional analysis.

2. Culture conditions: Factors such as the number of passages, confluency of cells, type and composition of growth media, incubation temperature and transporter expression can influence the outcome of the transfection procedure or the quality of the cDNA.

A cell density of around 70–80% per well is ideal for appropriate signal amplitude and sensitivity of the functional response. The number of cells plated should be kept consistent throughout the wells of a plate to reduce variability in the data. Most cell lines exhibit optimal function between passages 10 and 25. Appropriate type of cell lines and incubation conditions must be chosen to ensure that the cells remain adhered to the wells throughout the assay procedure. The efficiency of transfection and corresponding transporter expression in the cells can be evaluated using kinetic functional assays (a good uptake signal may reveal transporter presence) as well as surface-biotinylation and subsequent gel-separation/immunoblotting methods. In addition, the quality and integrity of the transporter cDNA must be ensured through sequencing methods and by measuring its concentration and integrity (by using instruments like a spectrophotometer)

3. Experimental conditions: Buffer composition, storage conditions of reagents, pH, drug or substrate concentrations, sampling methods, time points, temperature, physicochemical properties of the drug (such as solubility and stability), and format or layout of the assay plate (e.g., 96-well, 24-well, 12-well, or 6-well) are important variables to be considered while planning an assay protocol. If a transporter variant/mutant for example shows very low activity a larger well size might facilitate more reproducible results.

The symmetrical layout of the assay plates, as demonstrated in all the examples provided in this unit, could be prone to edge artifacts. An edge effect or artifact is a term used to explain an observation where the corner or outmost wells of a plate show different response in an assay as compared to the inner wells. This happens because the wells on the edge are more exposed, and reach the incubation temperature faster than the inner wells. This can cause more evaporation of the media solution from the edges and altered activity of the cells. Edge effects can be reduced by randomization of the layout of the cells or by pre-incubating newly-seeded plates at RT before putting them in the incubator (Lundholt et al., 2003).

Some cell lines are reported to express enzymes such as COMT (Catechol-O-Methyl Transferase) or MAO (monoamine oxidases) that can degrade monoamine substrates. Therefore, the assay buffers should include additives such as Ro-41-0960, which is a specific COMT inhibitor, and ascorbic acid that prevents the oxidation of monoamine substrates.

The time-point for kinetic uptake measurement should be <10-min to ensure the linearity of substrate transport with respect to time.

In dose-response assay, the concentration of [³H]-substrate determines the IC₅₀ of the compound, and should be kept much below the substrate K_M.

Solubility of compounds in the assay buffer is critical as a compound with poor aqueous solubility can give erroneous functional response. Ideally, all drug stock solutions should be prepared freshly in pure DMSO, and the dilutions for pharmacological assays should be made from this solution. If the compound precipitates out from the assay buffer, additives such as DMSO, ethanol or methanol (maximum 1–2%) can be added to increase solubility. Repeated freezing-thawing of stocks and reagents should be avoided, and aliquots should be maintained to minimize degradation.

4. Analytical method to measure the output: Several different kinds of analytical methods (such as LC-MS, radioactivity, or fluorescence) are available depending upon the type of output of an assay.
5. Calculation methods: Various statistical tools and data fit equations are available on software packages for determining the K_M, V_{max}, IC₅₀ and K_i values for an experiment. Use of an appropriate data analysis method is important for accurate interpretation of the results.

6. Control experiments: Establishing suitable negative and positive controls, and the use of reference standards for each experiment is essential to increase the validity and confidence of the analysis.

Understanding results and outcomes of the functional assays

Figure 4 shows a kinetic uptake assay of [^3H]-DA as the substrate of the DAT. The K_M and $V_{\max}$ values were determined as 292 fmol/min/well and 1.34 μM respectively in the absence of any other ligand. When 1 μM of an unknown ‘test’ compound was added, the K_M and $V_{\max}$ values changed to 171 fmol/min/well and 1.5 μM respectively. Since the $V_{\max}$ values for the two experiments changed (i.e., reduced) but the K_M values remained essentially the same, this indicates that the test compound represents an allosteric-type of interaction. In contrast, a competitive ligand would generate the same $V_{\max}$ but a different K_M value with respect to the substrate. Figure 7 illustrates a dose-response curve which measures the potency of dopamine uptake inhibition of cocaine versus another test inhibitor. The IC_{50} values for cocaine and the test inhibitor, i.e. 0.32 μM and 5.9 μM , respectively, represents the concentration of inhibitor required to achieve 50% of the total inhibition of the DAT-mediated DA uptake. These IC_{50} values also indicate that cocaine is a more potent DA-uptake inhibitor than the test compound, as indicated by a “rightward-shift” of the curve in case of the test compound. Figure 10 is a bar graph representation of quantified output from an experiment where percentage efflux of [^3H]-dopamine was measured in the presence of various known DAT substrates and inhibitors. Only substrates (DA and Amphetamine) and not inhibitors (like Cocaine) induce efflux that is greater than the efflux measured in vehicle treated control.

Time considerations

When using COS-7 cells for kinetic and dose-response assays, the non-transfected cells are transfected and plated simultaneously on day 1. The cells are incubated and allowed to grow for 48-hours. In our experience, plating 20,000 cells per well for a 96-well plate is sufficient to reach a confluency of ~80% in 48 hours with cells adhering strongly to the plate. As opposed to assays with COS-7 cells which take a total of 3-days, the stably-transfected MDCK cells can be plated at a density of 50,000 cells per well for a 96-well plate and only need 24 hours incubation time to reach the desired adherence and confluency. On the day of the experiment, the preparation of buffers and drug concentrations takes about 2 hours. Performing the assay itself takes 1–2 hours depending on the number of plates. For example, for a kinetic assay of each 96-well plate, it takes about 20 minutes in total to wash with PBS-CM, add substrate, incubate (10 minutes), wash again and add scintillation fluid. This is followed by additional 15 minutes it takes for the radioactive counter to measure the radioactivity. For dose-response assays, total time required for one 96-well plate to go from washing to radioactive counting is slightly greater as the plate is incubated twice during the experiment.

The efflux assays in a 24-well plate also take 3-days with transiently transfected COS-7 cells or 2-days for stably transfected MDCK cells. On the day of the experiment, preparation of buffers, stocks and drug concentration can take up to 2 hours. The total length of the actual experiment for one 24-well plate is approximately 1 hour. This is in addition to 1 hour

required for counting 48 samples on a scintillation counter machine generated from one 24-well plate experiment.

At the end of all the assay experiments, adequate time and care must be given in appropriate disposal of radioactive waste, residual liquids, and samples.

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Figure 1. Membrane topology of 12-transmembrane (TM) structure (numbered from I to XII) of MATs with intracellular location of N- and C-termini. The substrate binding site is located between TM1 and 6. Intra- (IL) and extracellular (EL) loops are numbered from 1–6.

Figure 2.

Schematics of a 96-well plate to show the arrangement of transfected and non-transfected cells. Each well receives either 40 µL of vehicle (assay buffer) or a test compound (1 µM) as shown for the kinetic uptakes to be tested in each condition.

Figure 3. Schematic of a 96-well plate showing the addition of various concentrations of cold/hot DA solution for each condition (vehicle or test compound).

Figure 4.

Results from a typical kinetic assay performed in the absence and presence of a 'test' inhibitor. This graph is a result of an average of three independent experiments. The $V_{\max}$ and K_M values were determined to be 292 fmol/min/well and 1.34 μM respectively in the absence of a test inhibitor, whereas in the presence of a test inhibitor, they were 171 fmol/min/well and 1.5 μM , respectively. Since the K_M remains unchanged but the $V_{\max}$ reduces, the test compound therefore acts as a non-competitive inhibitor.

Figure 5.

Schematic of a 96-well plate for dose-response assay (to obtain IC_{50}) when 40 μL of varying concentrations of a test compound and a standard or reference compound (such as cocaine, a DAT inhibitor) are added simultaneously and then incubated for 10 min.

Figure 6.

Schematic of a 96-well plate for dose-response assay when 40 μ L of 50nM [3 H]-DA (final effective concentration becomes 25 nM) is added in all the wells which have already been incubated for 10 min with 40 μ L of varying concentrations of either a test compound or a reference/standard compound such as cocaine (a DAT inhibitor).

Figure 7.

An example of a dose-response curve for cocaine (a DAT competitive inhibitor used as a reference/standard compound) and an unknown 'test' compound (whose IC₅₀ is to be determined) for inhibition of [³H]-DA uptake by the DAT. IC₅₀ was calculated as 0.32 μM for cocaine and 5.9 μM for the test compound.

Figure 8. Schematic of a 24-well plate for an efflux experiment containing hDAT transfected COS-7 cells that has been loaded with 250 μL of 25 nM [³H]-DA solution per well. The plate is incubated for 30 min to 1 hour at 37°C to allow the transport of [³H]-DA into the cells.

Figure 9.

An example of schematic for testing a combination of conditions (in triplicates) for efflux of [^3H]-DA in a 24-well plate. (AMPH = amphetamine, DA = dopamine, Coc = cocaine, Test = an unknown test compound). Each well receives 250 μL of the drug combinations to be tested and incubated at 37°C for exactly 10 min.

Figure 10.

An example of a bar graph comparing the percentage of efflux of [³H]-DA elicited by various known substrates and inhibitors of DAT. The results are a combination of three independent experiments in order to depict the error bars for each condition.

Figure 11.

An example of results from a typical kinetic assay where non-transfected and DAT-transfected cells are simultaneously exposed to the increasing concentrations of radioactive DA for 10 min. Accumulated [^3H]-Dopamine inside the cells is plotted against the concentration of the dopamine to obtain the kinetics of transport. The non-specific “uptake” (linear curve) obtained from the non-transfected cells is subtracted from the total “uptake” to obtain the specific uptake (sigmoidal curve). The values are fit with GraphPad Prism using the “Michaelis-Menten constant” model to obtain the V_{max} and K_M values of DA.

Raw data (in the units of cpm) obtained for a kinetic uptake assay experiment of a 96-well plate containing non-transfected and hDAT-transfected cells incubated with various concentrations of radioactive DA in the absence or presence of 1 μ M concentration of an unknown 'test' compound. The radioactive counts of DA inside the cells is measured using a scintillation counter machine.

Table 1

[DA] μ M		0.005		0.019		0.078		0.312		1.25		5	
		1	2	3	4	5	6	7	8	9	10	11	12
Vehicle	A	32	43	142	206	755	464	2048	1613	4106	3363	6868	5379
	B	44	35	157	130	405	545	1939	1594	3577	3548	7188	6286
	C	35	41	116	127	448	543	1990	1730	3222	3515	6776	6604
	D	13	19	50	62	209	254	1092	795	1854	2023	3580	3845
1 μ M test compound	E	25	13	70	84	236	289	1146	744	1999	1919	3816	4142
	F	21	27	74	68	210	295	1060	849	2489	2359	3934	4261
	G	10	12	9	10	27	27	84	64	162	142	435	559
Non - specific	H	7	10	8	8	27	24	76	50	187	190	509	446

Table 2

Averages are calculated for the non-specific counts obtained from the non-transfected cells incubated with various concentrations of radioactive DA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	32	43	142	206	755	464	2048	1613	4106	3363	6868	5379
B	44	35	157	130	405	545	1939	1594	3577	3548	7188	6286
C	35	41	116	127	448	543	1990	1730	3222	3515	6776	6604
D	13	19	50	62	209	254	1092	795	1854	2023	3580	3845
E	25	13	70	84	236	289	1146	744	1999	1919	3816	4142
F	21	27	74	68	210	295	1060	849	2489	2359	3934	4261
G	10	12	9	10	27	27	84	64	162	142	435	559
H	7	10	8	8	27	24	76	50	187	190	509	446
Avg.	9.75		8.75		26.25		68.5		170.25		487.25	

Table 3

The average values of the ‘non-specific’ counts subtracted from the corresponding total counts to get ‘specific’ counts for each condition. All values have been rounded off for the sake of convenience.

	1	2	3	4	5	6	7	8	9	10	11	12
A	22	33	133	197	729	438	1980	1545	3936	3193	6381	4892
B	34	25	148	121	379	519	1871	1526	3407	3378	6701	5799
C	25	31	107	118	422	517	1922	1662	3052	3345	6289	6117
D	3	9	41	53	183	228	1024	727	1684	1853	3093	3358
E	15	3	61	75	210	263	1078	676	1829	1749	3329	3655
F	11	17	65	59	184	269	992	781	2319	2189	3447	3774

Table 4

The final counts of the specific kinetic uptake of radioactive DA that have been converted to the units of fmol/min/well.

[DA] μ M	0.005		0.019		0.078		0.312		1.25		5	
	1	2	3	4	5	6	7	8	9	10	11	12
Vehicle	A	11	17	67	100	368	221	780	1988	1613	3223	2471
	–											
	B	17	13	75	61	191	262	770	1721	1706	3384	2929
	–											
	C	13	16	54	60	213	261	839	1541	1689	3176	3089
1 μ M test compound	D	2	5	21	27	92	115	367	850	936	1562	1696
	–											
	E	8	2	31	38	106	133	341	924	883	1681	1846
	–											
	F	6	9	33	30	93	136	394	1171	1105	1741	1906

Table 5
Rearranged data of the specific kinetic uptake of radioactive DA to be copied to GraphPad Prism software.

[DA] μ M	Vehicle						1 μ M test compound					
0.005	11	17	13	17	13	16	2	8	6	5	2	9
0.019	67	75	54	100	61	60	21	31	33	27	38	30
0.078	368	191	213	221	262	261	92	106	93	115	133	136
0.312	1000	945	970	780	770	839	517	544	501	367	341	394
1.25	1988	1721	1541	1613	1706	1689	850	924	1171	936	883	1105
5	3223	3384	3176	2471	2929	3089	1562	1681	1741	1696	1846	1906

Table 6

An example of raw data obtained from a dose-response analysis of cocaine versus an unknown 'test' compound. The numbers represent the counts (in the units of cpm) of intracellular radioactive DA in each well measured using a scintillation counter.

[compound]	Cocaine (reference compound)						Test compound					
	1999	2000	2100	1973	1847	2487	728	798	629	791	677	654
0	2182	1798	2173	1801	1742	2083	702	692	630	710	747	689
1 nM	2005	1445	1825	1789	1470	2100	629	682	634	648	642	704
10 nM	1328	1561	1625	1599	1490	1690	583	692	500	607	463	632
1 μ M	464	437	567	628	520	483	482	392	451	458	395	472
10 μ M	118	147	173	164	183	231	340	304	345	352	293	349
100 μ M	65	68	64	71	83	53	182	113	141	159	121	146
1 mM	30	46	55	39	45	49	63	79	66	59	72	80

Table 7

Average counts are calculated of the values obtained for 'zero', inhibitor concentration.

[compound]	Cocaine (reference compound)								Test compound							
	Average = 2068								Average = 713							
0	2182	1798	2173	1801	1742	2083	702	692	630	710	747	689				
1 nM	2005	1445	1825	1789	1470	2100	629	682	634	648	642	704				
10 nM	1328	1561	1625	1599	1490	1690	583	692	500	607	463	632				
100 nM	464	437	567	628	520	483	482	392	451	458	395	472				
1 μ M	118	147	173	164	183	231	340	304	345	352	293	349				
10 μ M	65	68	64	71	83	53	182	113	141	159	121	146				
100 μ M	30	46	55	39	45	49	63	79	66	59	72	80				
1 mM																

Table 8

Percentages of [³H]-DA uptake calculated for corresponding inhibitor concentrations relative to the 'zero' concentration. Calculations are demonstrated for the first well. Values have been rounded up for the sake of simplicity.

[compound]	Cocaine (reference compound)						Test compound					
	$(2068 \div 2068) \times 100 = 100\%$						$(713 \div 713) \times 100 = 100\%$					
0	(2182 $\div$ 2068)	97	70	88	87	84	101	(702 $\div$ 713)	88	96	89	88
1 nM												
10 nM												
100 nM												
1 μ M												
10 μ M												
100 μ M												
1 mM												

Table 9

Example of a raw radioactivity data (counts are in the unit of dpm) obtained for efflux assay conditions depicted in Figure 9 on a 24-well plate containing hDAT-transfected cells.

Supernatant						Lysed cells					
6626	3275	4706	7163	7367	5066	7538	6561	7676	6019	6216	5739
3938	4867	6204	4541	5504	5127	4071	5200	4548	8269	8056	9883
3094	4125	4015	4730	5689	6324	6861	6896	6183	6900	8986	8285
6116	7033	8704	9767	8984	10278	5754	5876	5802	6111	6173	6599

Table 10

The radioactive counts for the supernatant are corrected for the volume of 250. Calculations are demonstrated for the first well. The counts for lysed cells are unchanged.

Supernatant				Lysed cells			
$(6626 \div 200) \times 250 = 8282$	4093	5882	8953	9208	6332	7538	6561
4922	6083	7755	5676	6880	6408	4071	5200
3867	5156	5018	5912	7111	7905	6861	6896
7645	8791	10880	12208	11230	12847	5754	5876
						6111	5802
						6173	8986
						8285	9883
						6599	5739

The data shows the total radioactivity counts for each condition obtained by adding the counts for supernatant with the corresponding data for the lysed cells.

Supernatant counts					Total radioactivity counts = supernatant counts + lysed cell counts						
8282	4093	5882	8953	9208	6332	8282 + 7538 = 15820		13558	14972	15424	12071
4922	6083	7755	5676	6880	6408	8993		11283	12303	13945	16291
3867	5156	5018	5912	7111	7905	10728		12052	11201	12812	16190
7645	8791	10880	12208	11230	12847	13399		14667	16682	18319	19446

Percentage of radioactivity present in the supernatant is calculated relative to the total radioactivity. The corresponding averages of the percentage efflux for each condition is also given.

Table 12

Percentage of radioactivity of the supernatant relative to total radioactivity						
(8282 ÷ 15820)X100 = 52	38	43	59	59	52	
Vehicle = 45%						
54	53	63	40	46	39	Amphetamine = 57%
Dopamine = 57%						
36	42	44	46	44	48	Cocaine = 42%
Test compound = 41%						
57	59	65	66	64	66	Cocaine + Amphetamine = 46%
Test + Amphetamine = 61%						
Test + Dopamine = 66%						