Chapter 2

Tracer Flux Measurements to Study Outward Transport by Monoamine Neurotransmitter Transporters

Thomas Steinkellner, Felix P. Mayer, Tina Hofmaier, Marion Holy, Therese Montgomery, Birgit Eisenrauch, Michael Freissmuth, and Harald H. Sitte

Abstract

The physiological role of neurotransmitter transporter (NTT) proteins is the reuptake of released neurotransmitter from the synaptic cleft. NTTs accomplish uptake by undergoing a transport cycle, which relies on a return step in the empty state. In addition, NTTs can also run in the reverse direction and transport substrates out of the cells. This can be observed under conditions, where the transmembrane sodium gradient dissipates, e.g., if sodium accumulates within the cell. This reverse transport mode is also induced by amphetamines and the exact mechanism underlying the amphetamine action is still enigmatic and involves complex regulatory processes. In the current chapter, we describe various methods that can be used to assess the efflux of neurotransmitter from cells heterologously expressing the NTTs of interest or from preparations derived from intact brain tissue.

Key words Carrier-mediated efflux, Transport reversal, Neurotransmitter transporter, Superfusion, Radiolabeled tracer flux, Heterologous cell expression systems, Synaptosomes, Brain slices

1 Introduction

Three different possibilities exist to terminate synaptic transmission: (1) diffusion of neurotransmitter out of the synaptic cleft, (2) enzymatic degradation, or (3) reuptake by neurotransmitter transporters (NTT; [1]). The latter results in reaccumulation of neurotransmitters; the sodium gradient provides the driving force for reuptake from the synaptic cleft. Thus, it is by definition a secondary-active transport [2], which affords an economical and rapid reuse of released neurotransmitter. The monoamine transporters of the solute carrier 6 family (SLC6) comprise the transporters for dopamine, DAT (SLC6A3), norepinephrine, NET (SLC6A2), and serotonin, SERT (SLC6A4) [3], also abbreviated as 5-HTT [4]. The monoamine NTT family is of clinical

importance since they serve as target to alleviate or effectively treat a number of psychiatric disorders including depression and attention deficit hyperactivity disorder [5].

Chemical neurotransmission was established by the pioneering experiments of Otto Loewi: electrical stimulation of a nerve released a diffusible neurotransmitter—in Loewi's case the "Vagusstoff," i.e., acetylcholine, and this principle was shown to be universally true in both the peripheral and the central nervous system. It took some 40 years until Axelrod and Hertting documented that neurotransmission by monoamines (and most other released neurotransmitters) was terminated by a transport process rather than enzymatic degradation. [6]. Several models were proposed to account for the ability of a protein to translocate a solute over the lipid bilayer: Jardetzky condensed these ideas into a concept, which posited an alternating access mechanism [7]. This model was vindicated by the X-ray crystal structures of many transporters, including those of the bacterial transporter LeuTAa, which revealed several conformational states consistent with the sequence of events postulated by the alternating access model [8–10]. The fact that LeuTAa is highly homologous to NTTs allows for educated guesses on the mechanistic details of the transport process [11–14] and provides a reference framework for dynamic studies at the single-molecule level [15, 16].

Importantly, reuptake from the extracellular space to the cytosol is the major but not the only possible transport direction: changes in the intracellular milieu, in particular the sodium concentration, can result in reverse transport and thus lead to NTT-mediated efflux. Reverse transport can also be observed after the administration of sympathomimetic amines such as tyramine or amphetamine-like drugs [6, 17–21]. In vivo, reverse transport can be detected by microdialysis in the awake animals [22–24] and by high-speed chronoamperometry [25]. As an alternative approach, reverse transport by psychoactive amines has also been extensively studied in brain slices or synaptosomes [26–30]. However, the interpretation of mechanistic studies is confounded by two inherent limitations: (1) both slices and synaptosomes often contain several different NTTs (e.g., all monoamine transporters are included in a striatal slice preparation); (2) in addition, slices—and to a lesser extent synaptosomes—also contain the complete machinery for synaptic vesicle exocytosis [31, 32]. Accordingly, isolation of a single transporter requires the others to be blocked by specific NTT-blockers (to detect only effects mediated by the transporter of interest) and/or receptor inhibitors (because autoreceptors may be stimulated by the psychoactive compounds under study and regulate both vesicular and carrier-mediated release).

The cloning of the monoamine NTT cDNA's made it possible to heterologously express a specific monoamine transporter in appropriate cell lines such as human embryonic kidney 293 cells (HEK293). Thereby, the problems are eliminated, which arise from interferences with vesicular storage and the stimulation of monoaminergic

receptors: these paradigms have been used to study reverse transport induced by any substrates of NSS members (e.g., dopamine, tyramine, amphetamine and its derivatives or others [33–37]).

In this chapter, we outline the techniques, which have been successfully used to assess reverse transport mediated by monoamine transporters. Initially, we provide the methodologies to prepare brain slices and synaptosomes and subsequently describe the procedures to work with heterologous expression systems in both static and dynamic systems, i.e., using batch release and a superfusion system, respectively. After a description of the superfusion system, we then elaborate on the experimental procedures of the release experiment. The next part focuses on the evaluation and interpretation of the data. Finally, we will discuss troubleshooting issues and point out limitations and drawbacks of the methods.

2 Materials

2.1 Synaptosome Preparation from Animal Brain Tissue

Krebs-HEPES buffer or Krebs–Henseleit buffer:

Krebs-HEPES buffer (KHB): 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl$_2$, and 1.2 mM MgSO$_4$ supplemented with 5 mM d-glucose, adjusted with NaOH to pH = 7.4.

Krebs–Henseleit buffer (KHensB): 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.25 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 11 mM glucose.

Prepare KHensB freshly every day and oxygenate with 95 % O$_2$/5 % CO$_2$ for 1 h before use to adjust pH to 7.4.

Animal brain removed from either mouse or rat.

Tissue douncer with appropriately sized teflon-coated pestle for the preparation of synaptosomes.

Phosphate-buffered saline (PBS) containing protease inhibitors (Roche Complete™).

Protein determination kit (e.g., BCA kit, Pierce/Thermo Scientific).

24-well plates.

Whatman GF/B filters (1 mm diameter).

2.2 Slice Preparation from Animal Brain Tissue

Krebs-HEPES buffer or KHensB buffer (see above for composition).

Animal brain removed from either mouse or rat.

McIlwain tissue chopper (Fig. 1) for the preparation of brain slices.

2.3 HEK293 Cell Culture and Reagents

DMEM medium (any source is good provided that they adhere to the original recipe, e.g., Gibco, Invitrogen Life Science, Bethesda, MD).
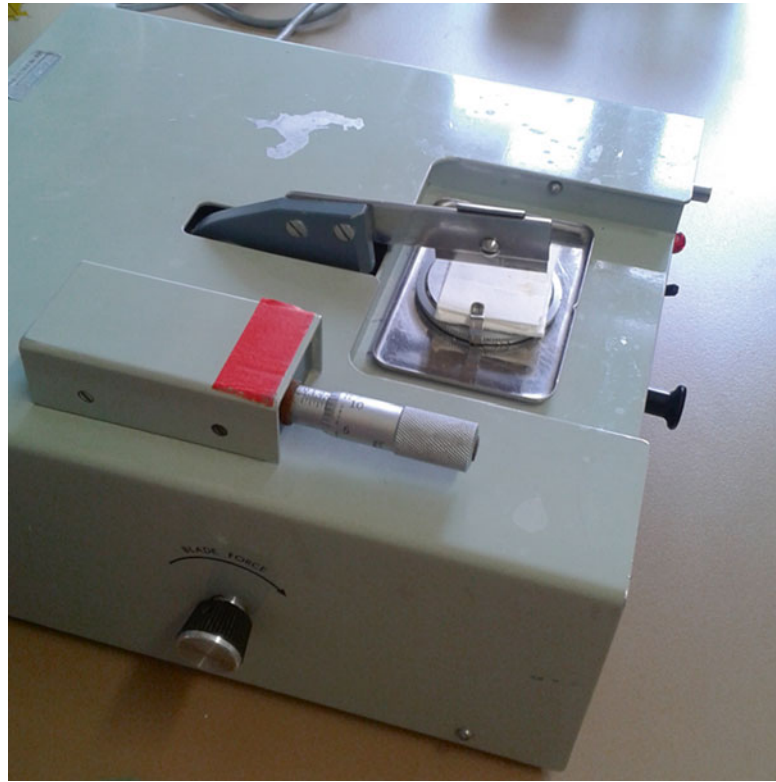
Fetal bovine serum (FBS).

Fig. 1 A McIlwain tissue chopper, ready for use

0.05 % Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO).

l-Glutamine (Gibco, Invitrogen Life Science, Bethesda, MD).

10 cm or 15 cm dishes; 24-well or 96-well culture plates (Greiner, Sarstedt, BD Biosciences, Falcon).

Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solutions are frozen at –20 °C (Gibco, Invitrogen Life Science); 5 mL is added to 0.5 L of DMEM complete culture medium.

Cell line: HEK293 cells transiently or stably expressing NTT of interest.

0.1 mg/mL poly-d-lysine solution in sterile H_2O.

Round coverslip (5 mm diameter).

2.4 Equipment, Software, and Accessories   Cell culture incubator, 37 °C, 5 % CO_2.

Sterile hood for the handling of the cell lines.

Vacuum pump for cell washes.

A personal computer with appropriate data calculation and graphics software.

For the identification of the brain structures, use pertinent brain atlases (e.g., Franklin and Paxinos for mouse brain or the rat brain atlas by König and Klippel). Note: for the preparation of synaptosomes and brain slices, brains need preferentially to be dissected freshly and on a cold plate (not frozen).

3 Methods

In the methods section, we will describe in the first paragraphs how the three preparations covered in this chapter are produced (Sects. 3.1–3.3) before we describe the superfusion assay (Sect. 3.4) and the evaluation of the data (Sect. 3.5).

3.1 Preparation of Rodent Brain Synaptosomes

Kill mouse or rat by cervical dislocation or decapitation.

Remove the brain carefully (Note: make sure that the meninges are removed thoroughly otherwise you risk serious damage to the delicate brain tissue during dissection) and keep on ice.

Dissect both left and right region(s) of interest.

Homogenize in ice-cold 0.32 M sucrose in phosphate-buffered saline (PBS) containing protease inhibitors (Roche Complete™).

Centrifuge the suspension for 10 min at $1000 \times g$. Keep the supernatant, discard the pellet.

Centrifuge the supernatant for 15 min at $12,600 \times g$. Discard the supernatant and resuspend the pellet (referred to as P_2) in KHB.

Measure the wet weight of P_2 or determine the protein concentration of P_2 after resuspension in buffer to estimate the amount of synaptosomes.

Use the resulting synaptosomes directly or freeze at –80 °C. Synaptosomes can be stored at –80 °C for at least 2 years with only minor loss in functional activity.

3.2 Preparation of Mouse Brain Slices

Retrieve a rodent brain as described above and place it into ice-cold buffer (KHB or KHensB).

Dissect the regions of interest (e.g., striatum, hippocampus, or cortical tissue) and store in ice-cold buffer in a watch glass on ice.

Take dissected brain region and place onto a Whatman filter paper saturated with ice-cold buffer; place filter with brain onto the cutting stage of the McIlwain tissue chopper and operate the knife slowly to cut the first tissue slice (usually, 0.3 mm thickness is recommended). Discard the first section and start collecting subsequent sections using a fine brush by gently swiping them and placing them in ice-cold buffer for storage (Fig. 2).

Repeat until all slices of your region of interest are retrieved.

Handle the slices cautiously with the brush avoiding damage to the tissue.

Tissue slices can be kept in ice-cold buffer for a couple of hours but tissue quality and integrity decrease with time.

3.3 Heterologously Expressing Cell Lines

The expression of the NTT of interest in appropriate cell lines can be bothersome from time to time and expression levels may vary significantly. This may cause differences in results for NTT-mediated efflux from lab to lab but even among different cell lines
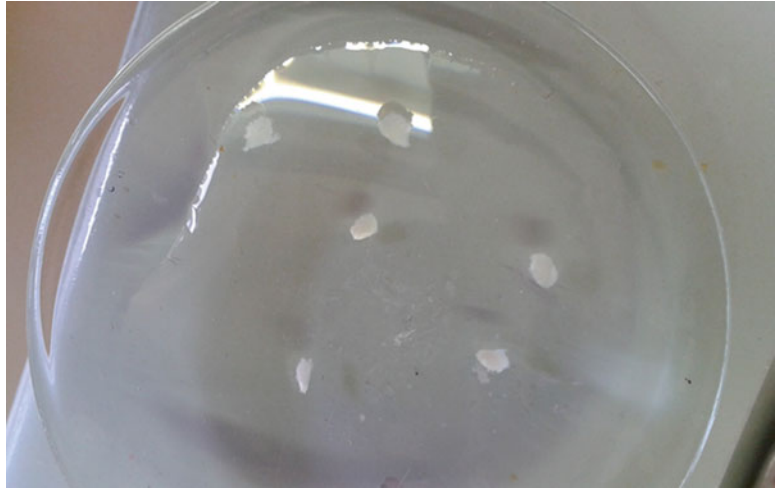
Fig. 2 Watch glass holding ice-cold buffer with floating striatal sections prepared from rat brain tissue

within one lab. The reason for the differences might simply be that the properties of the cell lines differ considerably with regard to the expression of kinases and other important regulatory factors, e.g., protein kinase C, [38], or αCamKII, [39–43]. Hence, it would be good to start the approach by screening the literature and selecting the right cell line based on the experience of other groups. For instance, DAT has been expressed in a number of different cell lines, including HEK293 cells [32], PC-12 cells, [44], SK-NMC cells [45] and LLC-PKC1 cells [46, 47]. All cell lines have their unique advantages: HEK293 cells for instance can be most easily transfected and afford high expression levels. In contrast, LLC-PKC1 cells support much lower expression levels but these cells are endowed with a large complement of protein kinases. Clearly, it depends on the goal, which is being pursued: the high expression in HEK293 cells is best suited to study transporter-ligand interactions while the low expression profile of LLC-PKC1 cells can be utilized to assess the influence of kinases on transporter regulation [47].

For transfection of HEK293 cells we usually resort to the $CaPO_4$ transfection method since it is cheap, easy and reliable; only if this method does not lead to the desired cell expression result, we use a lipofection method. In the following, we outline how we perform $CaPO_4$ transfections, followed by one lipofection method which is given as an example for a large variety of different commercially available products. Still, the number of products is increasing and it provides a simple and efficient way of transfection. However, lipofection interferes with the lipidome of the cells and may therefore interfere with lipid-transporter interactions [48, 49].

At the very least, these aspects must be considered, when selecting the transfection method.

Ca2+ Transfections

HeBS buffer (2×):	
HEPES	50 mM
NaCl	280 mM
$Na_2HPO_4 \cdot 2H_2O$	1.5 mM

Adjust the pH to 7.08 with 10 N NaOH; accurate pH is critical for efficient transfection. Sterilize by filtration through a 0.45-μm nitrocellulose filter. Store at –20 °C. Thaw only once!

Phosphate buffered saline (PBS):	
KCl	2.7 mM
KH_2PO_4	1.5 mM
NaCl	137 mM
$Na_2HPO_4 \cdot 2H_2O$	4.3 mM

Dissolve in sterile water (Milli-Q), adjust to pH 7.3–7.4 (by adding 10 N NaOH).

Seed cells to achieve approximately 40 % confluence in a 10 cm dish on the day of transfection. Mix reagents as follows $(CaCl_2 + H_2O = 1{:}8.6)$:

H_2O	430 μL	
$CaCl_2$	(2.3 M)	50 μL
DNA (1 μg/μL)	20 μL	
HeBS (2×)	500 μL	

Incubate the mixture for 6 min at room temperature to allow the reaction to take place: here, DNA-Ca^{2+} should form a fine precipitate. The precipitate should not be too coarse. Drop the suspension onto the medium covering the cells.

Apply the "glycerol shock" after 4–6 h of incubation time at 37 °C to increase transfection efficiency: remove the transfection media, add 1 mL of glycerol shock solution (thoroughly mix 13.8 mL glycerol and 86.2 mL PBS, sterile filtered afterwards) and remove it immediately after and rapidly by aspiration.

Wash the cells with 10 mL PBS and supply 10 mL of fresh, warm media.

Wait for at least 24 h before performing experiments on transiently expressing cells. To establish a stably expressing cell line, start the selection process with the appropriate antibiotic (e.g., geneticin) 48–72 h after the glycerol shock.

Turbofection

Seed cells into 10 cm dishes and use them for turbofection when they are approximately 80–90 % confluent.

Combine 1 μg of DNA of interest with "empty" vectors (e.g., pcDNA3.1) to reach a total amount of 5 μg of DNA (Note: dilution of transporter DNA is usually recommended as lipofection can induce massive overexpression of the protein of interest; in order to achieve more "physiological" expression, dilution series of transporter DNA should be performed and tested).

Add 500 μL of DMEM to the DNA mix followed by the addition of 5 μL Turbofect (Fermentas; vortex Turbofect well before adding).

Vortex the mixture and incubate for 15–20 min at room temperature.

In the meantime, remove the media from the cells and replace with 4.5 mL of fresh media including FCS and antibiotics.

After the incubation of Turbofect–DNA mixture is ready, add the solution to the cells. Incubate the cells for another 24–48 h at 37 °C.

It is advisable to first verify that the monoamine NTT of interest is expressed in the given cell line at adequate levels prior to carrying out a release assay. Expression can be confirmed by performing an uptake assay or by labeling the NTT of interest with a fluorescent protein (e.g., GFP or mCherry). Note: the addition of a fluorescent protein can compromise NTT function; for instance, only SERT tolerates the addition of a fluorescent protein to the carboxyl terminus [38], whereas in all other monoamine NTT surface expression is substantially reduced because their C-terminal PDZ-binding motif cannot be masked [50, 51].

Intuitively, stable transfection is more appealing, but it may not be necessary to have a cell line with a homogeneous expression level. In addition, it may not be possible to express a transporter of interest in a stable manner. This is for instance true for mutants, which generate a large leak current. A point in case is the mutant DAT-Y335A [52]; this mutation converts the transporter into a ion-channel-like pore [53]. Here, stable expression has not been possible, because the large leak conductance of DAT-Y335A apparently precluded long term cell survival (unpublished results).

3.4 Release Assays   While the preparations (synaptosomes, brain slices or cells on coverslips or in plates) differ, the superfusion is done in a very similar manner; the variations are modest. The static batch release assay will be described in Sect. 3.4.2.

3.4.1   Superfusion Assay   We will shortly describe the superfusion apparatus and how to set it up before each experiment before the actual experiments involving the three different preparations will be explained.

The system is designed to allow for rapid removal of the released neurotransmitter by continuous superfusion of the preparation of interest. The goal is to preclude reuptake of the released neurotransmitter (regardless of whether released spontaneously or after a stimulus) by the cognate NTT or other transporters. An initial superfusion is employed to define the baseline, i.e., to estimate the spontaneous release or leak in the respective preparation.

Our superfusion apparatus was designed to comprise 12 individual channels (Fig. 3). Hence, 12 brain slices, 12 synaptosomal fractions or 12 cell coverslips can be used per assay in parallel. This allows for an adequate number of replicates; we rely on triplicate determinations for each experimental condition. A schematic representation of a superfusion chamber is given in Fig. 4b. Of importance to control the temperature in the chambers, the tubing ought to be immersed in a water bath, which should be kept at a temperature higher than the desired value in the chamber to yield a temperature of 25 °C in the chambers proper.

The preparations are continuously superfused with buffer at a flow rate of 0.7 mL/min. It is essential that no air bubbles are trapped in the superfusion tubings. Air bubbles can be easily removed by rinsing the superfusion system with 30 % isopropanol in ddH$_2$O before the experiment. After the isopropanol, the system is washed extensively with ddH$_2$O (10 min) to remove any residual isopropanol before equilibrating the system buffer (10 min).

The superfusate must obviously have a constant temperature to eliminate a source of variability. The control of the temperature is achieved by immersing the tubing (total diameter = 1 mm, a luminal diameter = 0.35 mm) over a length of 60 cm in a water bath set at the pertinent temperature (usually 25 °C). Note that
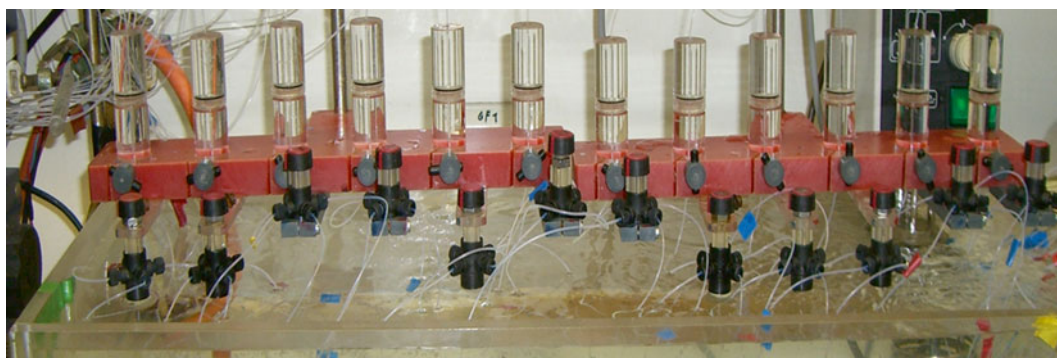

Fig. 3 Twelve-channel superfusion apparatus. Note that the valves in front allow for rapid and simple exchange of solutions, i.e., from baseline buffers to drug containing buffers. The tubing is typically immersed in a water bath, which should be kept at a temperature higher than the desired value in the chamber
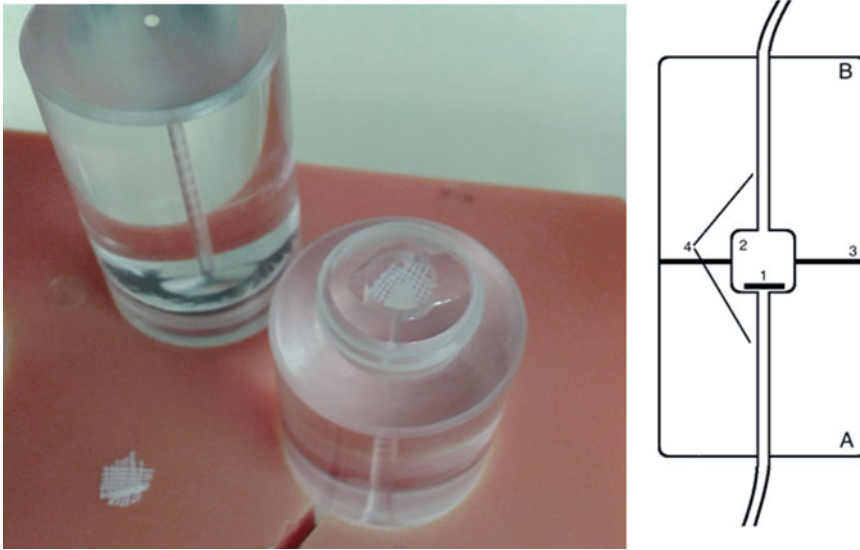
Fig. 4 (**a**) Detailed view into the superfusion chamber. The volume of the central chambers is 200 μL and contains a grid which holds the slices, glass coverslips or synaptosome-loaded filters. (**b**) Schematic representation of the superfusion system. The investigated material (*1*) is placed into the superfusion chamber (*2*; volume = 200 μL). A rubber gasket (*3*) ensures tight closure of the two hemispheres (A and B with a total dimension of 8 cm × 2.5 cm). The flow direction of the superfusion buffer is from A to B via channels (*4*) cut into the hemispheres. Adapted from Singer [68]

the flow rate of the buffer results in mechanical stress for the tested preparations. The flow-related shear forces are limited by the size of the chambers, which harbor the preparations: their volume is 200 μL with a diameter of 8 mm.

For superfusion of synaptosomes, synaptosomal P_2 pellets are resuspended in the respective buffer to achieve a concentration of 1 mg wet weight/15 μL or 100 μg of protein.

For superfusion of brain slices, individual sections are used per channel.

Synaptosomes are incubated with a [³H]-labeled substrate of interest: for instance when DAT is the NTT in focus, [³H]MPP+ or [³H]dopamine can be used to load the tissue. When SERT is the NTT of interest, [³H]5-HT should be used.

CAVEAT: biogenic amines are substrates for monoamine oxidases and prone to spontaneous oxidation. Hence, they can get rapidly metabolized in native tissue such as synaptosomes or tend to spontaneously oxidize. It is therefore advisable to block MAOs throughout the assay by adding for instance 100 nM pargyline (a MAO blocker) and to prevent spontaneous oxidation by including 100 nM ascorbate (as antioxidant) in the buffer.

CAVEAT: radiolabeled substrates may be taken up by multiple transporters, e.g., [³H]dopamine is not only a substrate for DAT but also for NET and SERT. Hence, when DAT is the transporter

of interest, NET and SERT should be blocked. We therefore add 100 nM desipramine (NET blocker) and 100 nM paroxetine to the buffer. In contrast, when SERT is the transporter of interest, we add 100 nM nomifensine to the buffer, which blocks both DAT and NET at similar potencies. If NET is to be investigated, the addition of a 100 nM GBR12909 or GBR12935 (specific DAT inhibitors) and 100 nM paroxetine is recommended.

Preincubation time for synaptosomes and slices:

Preincubate synaptosomes and slices for 30 min at 37 °C with [³H]-labeled substrate (e.g., 100 nM [³H]dopamine or 100 nM [³H]5-HT) in working buffer (+100 nM pargyline + 100 nM ascorbate + respective blockers for other NTT, e.g., 100 nM desipramine (to block NET) and 100 nM paroxetine (to block SERT) or 100 nM nomifensine to block DAT and NET, respectively).

Preincubation time for cells:

Preincubate cells for 20 min at 37 °C with [³H]-labeled substrate in buffer (depending on the cell line using, addition of pargyline to block MAO or ascorbate as an antioxidant is recommended. Also, if neuronal-like cell lines are used, it might be advisable to add the respective inhibitors for NTT that are not the primary focus of investigation, e.g., both SHY5Y cells and PC12 cells endogenously express NET)

Insert preparations into superfusion apparatus and perform washout:

After preincubation, preparations are transferred to the superfusion apparatus.

To insert preparations, stop perfusion flow and open all the channels.

Synaptosomes: prepare a plastic dish with GF/B filters (8 mm diameter) and pipet 15 µL of preincubation solution onto Whatman GF/B filters. Immediately put one loaded filter into one channel, close the channels and perfuse with buffer.

Slices: insert one slice per channel.

Cells: insert one coverslip per channel.

After the preparations have been placed into the chambers, a washout phase is started to equilibrate the system and to establish a stable baseline efflux of radioactivity. Usually a washout time of 45 min is sufficient (keep in mind that MAO blocker, ascorbate and inhibitors of the other NTTs should also be present in the buffer during this washout phase).

During washout, prepare the required amount of scintillation vials in a rack and fill with 2 mL scintillation cocktail (we usually use 50 mL scintillation vials filled with 2 mL of scintillation liquid).

Also during the washout period, prepare the working solutions needed for the efflux experiment, i.e., prepare the releaser solution:

For example "*releaser*" *solution* (for DAT, we usually use 3–10 µM of d-amphetamine, for SERT 3–10 µM of *para*-chloroamphetamine [*p*CA] in working buffer since this allows for

maximal substrate release at monoamine transporters; keep in mind that amphetamines usually have a bell-shaped dose-response curve, i.e., further increases in d-amphetamine or *p*CA concentrations do not increase efflux but rather have inhibitory effects on release; see Seidel et al. [38] for details).

Note: depending on the transporter under investigation, one should choose the respective amphetamine: amphetamines usually are promiscuous molecules but they still show preferences for the one or other transporter. For instance, d-amphetamine has higher affinities for DAT and NET, whereas *p*CA shows an increased affinity for SERT. Hence, it is advisable to select the most potent amphetamine for a given transporter under investigation, because this minimizes off-target effects.

"*Releaser + inhibitor*" (*control*): another way to test, whether release through the NTT of interest is specific it is recommended to include a condition where release is blocked by the pertinent inhibitor for this transporter, e.g., 3–10 µM d-amphetamine + 1 µM GBR12909 for DAT.

After washout, the actual experiment is started by collecting at least 3×2-min fractions where the preparation is superfused with buffer only. After the first three fractions, the tubings can be switched to the releaser solution. When also looking at the effect of an inhibitor, we usually collect three 2-min with inhibitor only (e.g., 1 µM GBR12909) or buffer only before collecting five 2-min fractions with releaser and releaser + inhibitor, respectively.

At the end of the experiment, synaptosomes or slices are collected in tubes filled with 2 mL of 1% SDS to recover the remaining amount of [³H] substrate present in the tissue.

Superfusion experiments with cells grown on coverslips are terminated by collecting three 2-min fractions where the superfusion system is washed with 1% SDS to lyse the cells.

At the end of the experiment, put the lid onto the scintillation vials and shake tubes well before measuring in the liquid scintillation counter.

3.5 Data Analysis   Calculations are based as fractional release: hence the first step is to calculate the radioactivity initially present in the preparation, which is sum of the radioactivity present in all collected 2-min fractions and radioactivity present at the end, i.e., the radioactivity released after solubilization of the tissue with 1% SDS. The released radioactive [³H]substrate in any given 2-min fraction is expressed as percentage of the radioactivity present in the slice at the start of this very collection period, i.e., fractional release = $\text{cpm}_{\text{2-min fraction}}/$ (total radioactivity − sum of previously release) × 100.

Usually the baseline efflux of radioactivity is stable and amounts to about 1–1.5% fractional release per 2-min buffer superfusion. Efflux rises to 5–10% after the addition of amphetamine (Fig. 5).
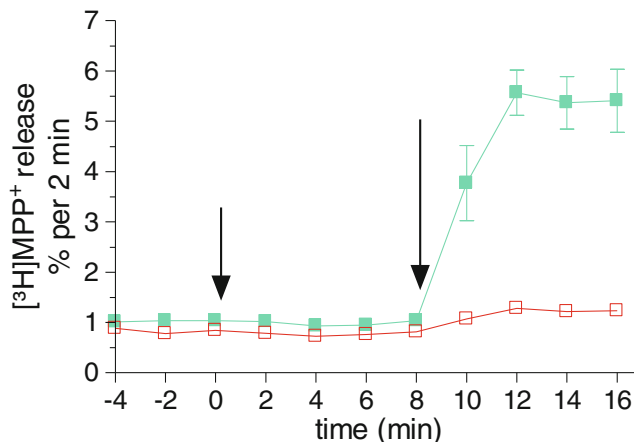
Fig. 5 Superfusion assay, evaluated and displayed: representative experiment. Cells expressing human DAT were preloaded with [³H]MPP⁺ and superfused until a stable baseline was reached. The experiment was started with the collection of 2-min fractions. After three fractions (min-6 until 0 min; *first arrow*) of basal efflux, cells were exposed to cocaine (10 μM), or left at control conditions as indicated. After four fractions (from 8 min onwards; *second arrow*), amphetamine (10 μM) was added to all superfusion channels. After four fractions (i.e., after 16 min), all channels were switched to SDS conditions and the remaining radio-activity lysed from the cells. Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three observations (one observation equals one superfusion chamber)

3.5.1 Static Batch Release Assay

The problem of the batch release assay format is its static nature: Here, the diffusion of substrate and of the compounds under investigation cannot be controlled like in the superfusion apparatus. Superfusion provides a robust assay format to assess transporter-mediated efflux, because confounding effects arising form back diffusion are eliminated [54]. The disadvantage of superfusion, however, is the large volume of superfusate and hence the need of a larger amount of the compound under study. Therefore, static batch release is the preferred method when the amount of compound under scrutiny is small [55], but its limitations must be kept in mind. In the following, we will outline how the static release assay has worked out well in our hands.

Cells are grown in poly-d-lysine-coated 96-well plates (4×10^4 cells per well). The cells are preloaded with 0.05 μM [³H]substrate for 20 min at 37 °C in a final volume of 0.1 mL/well. The residual extracellular radioactivity is removed by three gentle wash steps with Krebs-Ringer-HEPES buffer. The cells are then incubated with the test compounds at room temperature and compared to a reference compound (for instance, d-amphetamine can be used for all three monoamine transporters). All compounds are used at the concentration, which results in a 50% inhibition of substrate

uptake. The specificity of drug-induced release needs to be assessed by the addition of a specific inhibitor at appropriate concentration (for instance, 10 µM of mazindole for DAT and NET or 10 µM paroxetine for SERT) to the test compound. After 10 min, the incubation buffer is removed and transferred into a counting vial; the cells remaining in the well are overlaid with a solution containing 1 % SDS to extract the radioactivity. The resulting solution is transferred into a counting vial. All samples are subjected to standard liquid scintillation counting. It is preferable to perform all determinations in triplicates. The sum of the radioactivity in the incubation buffer and the cell lysate represents the total [³H]substrate included in the assay. This sum is the reference value, to which the released radioactivity is related: the data are expressed as released [³H]substrate as percent of total available radioactivity.

4   Notes

The application of the above mentioned superfusion apparatus provides a robust technique to measure NTT-mediated reverse transport. However, various circumstances must be taken into consideration, which might result in flawed data interpretations.

1. Liquid scintillation counting is our method of choice to determine the amount of tritium within the superfusates. Liquid scintillation cocktails (e.g., Rotiscint®) convert the energy of the beta-decay of tritium into light signals. Some drugs may absorb photons emitted by scintillators and quench the cognate signal. Therefore, one has to ensure that the applied substances do not influence the readout at the tested concentrations. For example, a substance-related quench would seemingly reduce the amount of released tritiated substrate and could be misinterpreted as transporter-related effect.

2. Uptake inhibition assays do not provide the possibility to differentiate between non-transported competitive NTT inhibitors or amphetamine-like releasers [56]. In general, amphetamines are accepted as NTT substrates and compete with the endogenous substrate for the binding site. As a result, amphetamines dose-dependently inhibit uptake of tritiated substrates. The use of the superfusion technique allows bypassing this limitation of inwardly directed radiotracer flux assays. The presence of amphetamines enhances the efflux of tritiated substrates whereas non-transported inhibitors, e.g., cocaine, do not result in NTT-mediated reverse transport [57]. In addition, NTTs utilize the preexisting sodium gradient as driving force for their concentrative transport.

Disrupting the sodium gradient by addition of the Na^+/H^+ ionophore monensin [58] will selectively enhance efflux triggered by true substrates/amphetamines [56, 59]. However, the use of monensin alters the intracellular pH, as monensin serves as a leak for intracellular protons. Serotonin (5-HT) is weak base with a pKa value of 10.4 [60]. A reduction in intracellular H^+ inevitably increases the deprotonated fraction of 5-HT, which can passively diffuse across the plasma membrane. This results in a transporter-independent "pseudo efflux" [59]. It is worth mentioning that inhibition of NTTs by various inhibitors can unmask a basal loss of substrates by diffusion. Biogenic amines do have a limited—albeit measurable—potential to cross the plasma membrane by passive diffusion [61]. Therefore, if results obtained from superfusion experiments with [^{3}H]-5HT or [^{3}H]-DA are ambiguous, [^{3}H]-MPP$^+$ should be used as NTT substrate. MPP$^+$ carries a permanent charge and passive diffusion is thus negligible [54]. The cells or the synaptosomal preparation under investigation may also express additional gradient-driven transporters: Members of the SLC22 family, epitomized by organic cation transporters 1–3 (OCTs, SLC22A1–3) or the plasmalemmal monoamine transporter (PMAT, SLC29A4), translocate DA, NE, 5-HT, and MPP$^+$ across cellular membranes [62, 63]. Their coexistence with SLC6 family members may confound the interpretation of outwardly directed transport of tritiated biogenic amines or MPP$^+$. OCTs and PMAT are very broadly expressed; they can be found in both peripheral tissue and in the central nervous system, where they are expressed in neuronal and non-neuronal cell types [63–67]. As a consequence, gradient-driven transporters may contribute to efflux measured during superfusion experiments. OCTs and PMAT are not blocked by most NTT-inhibitors. Therefore, in the presence of OCTs and/or PMAT, inhibition of NTTs can enhance a basal loss of substrate, because the compensatory re-uptake by NTTs is eliminated by the NTT-inhibitor. This effect is pronounced in systems containing vesicular storage pools and/or upon application of amphetamine-like releasers. Efflux triggered by substances targeting NTTs, in particular their blockers, should be interpreted cautiously, if there is evidence for the presence of other gradient-driven transporters. As a safeguard, we recommend the use of the OCT and PMAT blocker decynium-22 (D22). D22 exhibits rather low affinities for NTTs. Therefore, as control experiment, the experimenter might test, if the efflux in presence of NTT blockers is sensitive to increasing concentrations of D22.

Acknowledgements

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