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Substantial loss of substrate by diffusion during uptake in HEK-293 cells expressing neurotransmitter transporters

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Abstract

Human embryonic kidney 293 (HEK-293) cells stably transfected with the human serotonin (5-HT) or dopamine transporter (hSERT, hDAT), or the rat GABA transporter GAT-1 were incubated with saturating concentrations of transporter substrates (hSERT: [^{3}H]5-HT, [^{3}H]N-methyl-phenyl-pyridinium (MPP+); hDAT: [^{3}H]dopamine, [^{3}H]MPP$^+$; rGAT: [^{3}H]GABA). Uptake velocities decreased significantly over time for [^{3}H]5-HT and [^{3}H]dopamine (already visible at 1 min), but not for [^{3}H]MPP$^+$ or [^{3}H]GABA. In efflux experiments cells were preloaded and substrate diffusion into the medium was studied following the addition of appropriate uptake inhibitors. Fractional effluxes were (% min^{-1}) 1.27, 0.72, 0.27 and 0.08 for [^{3}H]5-HT, [^{3}H]dopamine, [^{3}H]MPP$^+$ and [^{3}H]GABA, respectively. The results suggest that in uptake experiments the more lipophilic substrates [^{3}H]5-HT and [^{3}H]dopamine leave the cells by diffusion already after a short time (1 min) of accumulation.

Keywords: HEK-293 cells; hSERT; hDAT; rGAT; Transporter, Uptake; Superfusion; Diffusion

Na^+/Cl^- -dependent transporters such as the dopamine, serotonin, norepinephrine or GABA transporter are well-known proteins, terminating chemical transmission by removing the neurotransmitter from the neuronal vicinity. The proteins have been of intense research interest and are recognized targets of drugs with widespread therapeutic use.

In addition to study transporter function in classical models such as synaptosomes or brain slices, it has become increasingly popular to use heterologous expression systems. It is regarded as particular advantage of this approach that only the transporter of interest is expressed by the cells and can thus be studied without the interference of other components of the presynaptic machinery (e.g. vesicular storage;[3]). While this is true, it should be recognized that one is dealing with an artificial system that may create its own problems hitherto not encountered in routine experimentation. We report such an occurrence in cells expressing the human serotonin (hSERT) or dopamine (hDAT) transporter.

The cDNA for the human plasmalemmal serotonin transporter [11] was a gift of Dr R.D. Blakely (Department of Pharmacology and Center for Molecular Neuroscience, School of Medicine, Vanderbilt University, Nashville, TN). The coding region was subcloned into pEGFP-C1 (Clontech, Palo Alto, CA) removing the GFP coding region as described previously[14]. The cDNA for the human plasmalemmal dopamine transporter inserted in the vector pRC/CMV (Invitrogen, Carlsbad, CA) was a gift of Dr Ch. Pifl (Brain Research Institute, Vienna, A;[8]). The cDNA for the rat GABA transporter (GAT-1) was a gift of Dr P. Schloss (ZI für seelische Gesundheit, Mannheim, D;[13]). The coding region was subcloned into pRC/CMV (Invitrogen, Carlsbad, CA).

For stable expression into HEK-293 cells the same method was used as described in[9]. The stable transfectants (hSERT cells; hDAT cells and rGAT cells) were grown in Dulbecco's minimal essential medium with L-analyl-L-glutamine (L-glutamax I™; Gibco Life Technologies, Grand Island NY), 10% heat-inactivated fetal bovine serum, 50 mg/l gentamicin and 500 μg/ml geneticin (G418) on 100 mm-diameter cell culture dishes at 37°C in an atmosphere of 5% CO_2, 95% air.

Uptake experiments were performed as described previously[14]. In brief, 1×10^5 cells were seeded onto poly-D-lysine-coated 24-well plates, and influx was measured 2 days after plating. Each well was washed once with 1 ml of KRH buffer (Krebs–Ringer Hepes buffer; Hepes 10 mM, NaCl 120 mM, KCl 3 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2

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mM, glucose 20 mM, final pH 7.3). hSERT cells were incubated at 10 μM [^{3}H]5-HT (0.11 Ci/mmol; 0.22 μCi/well) or 200 μM [^{3}H]MPP$^+$ (0.019 Ci/mmol; 0.78 μCi/well). hDAT cells were incubated at 10 μM [^{3}H]DA (0.34 Ci/mmol; 0.68 μCi/well) or 200 μM [^{3}H]MPP$^+$ (0.019 Ci/mmol; 0.78 μCi/well). The medium used for hDAT cells contained 10 μM pargyline and 1 mM tropolone to inhibit metabolism[5]. rGAT cells were incubated at 50 μM [^{3}H]GABA (0.01 Ci/mmol; 0.09 μCi/well). After the given incubation periods (room temperature), the uptake buffer was aspirated, the cells washed twice with ice-cold buffer and lysed with 0.5 ml of 1% sodium dodecyl sulfate (SDS). Radioactivity in the lysates was measured by liquid scintillation counting. Non-specific uptake was defined as uptake in the presence of 100 μM clomipramine, 100 μM cocaine or 10 μM tiagabine for hSERT, hDAT and rGAT cells, respectively, after 5 min preincubation with the appropriate drug.

Superfusion experiments were performed as described previously[14]. In brief, cells were grown overnight onto poly-D-lysine coated round glass cover slips (diameter 5 mm) at 4×10^4 cells/well. Cells were loaded with radiolabelled substrate for 20 min at 37°C in a final volume of 100 μl KRH buffer: SERT cells were incubated with 10 μM [^{3}H]5-HT (0.44 Ci/mmol, 0.44 μCi/well) or 200 μM [^{3}H]MPP$^+$ (0.078 Ci/mmol; 1.6 μCi/well); hDAT cells were loaded in the presence of 10 μM pargyline and 1 mM tropolone with 10 μM [^{3}H]DA (0.34 Ci/mmol, 0.34 μCi/well) or 200 μM [^{3}H]MPP$^+$ (0.078 Ci/mmol; 1.6 μCi/well). GAT cells were incubated with 50 μM [^{3}H]GABA (0.24 Ci/mmol; 1.2 μCi/well). The cover slips were then transferred to superfusion chambers (200 μl) and superfused with KRH buffer (0.7 ml/min) at 25°C. All experiments with hDAT cells were performed in the presence of 10 μM pargyline and 1 mM tropolone. After a washout period of 45 min a stable efflux was obtained and the experiment was started with the collection of 2 min fractions. After three fractions ($t = 0$) the cells were exposed to the drug of interest and another five fractions were collected. At the end of the experiment cells were lysed in 1% SDS. Tritium in the superfusate fractions and in the SDS lysates was determined by liquid scintillation counting. The release of ^{3}H was expressed as fractional rate, i.e. the radioactivity released during a fraction was expressed as a percentage of the total radioactivity present in the cells at the beginning of that fraction.

All curve fitting was done using Prism (GraphPad, San Diego, CA) non-linear fitting and plotting software. All results were expressed as mean ± SEM.

Tissue culture reagents were from Gibco Life Technologies. [1,2-^{3}H[N]] 5-hydroxytryptamine creatinine sulfate (5-HT) and [N-methyl-^{3}H]-4-phenylpyridinium acetate (MPP$^+$) were obtained from New England Nuclear Life Sciences Products (Boston, MA); [2,3,4-^{3}H]γ-Amino-*n*-butyric acid (GABA) from ICN (Irvine, CA) and [2,5,6-^{3}H]DA from Amersham Pharmacia Biotech (Buckinghamshire, UK). 5-HT, GABA, DA, clomipramine, pargyline and tropolone were from Sigma-Aldrich Handels GmbH (Vienna, Austria); MPP$^+$ from RBI (Research Biochemicals International, Natick, MA). tiagabine hydrochloride was generously provided by Sanofi–Synthelabo (Montpellier, F). All other chemicals were from commercial sources.

On incubation with saturating concentrations of labelled substrates (10 μM [^{3}H]5-HT, 200 μM [^{3}H]MPP$^+$, 10 μM [^{3}H]DA, 50 μM [^{3}H]GABA) all transporter expressing cells accumulated their substrates in a time-dependent fashion. The results for different incubation periods between 0.5 and 10 min, expressed as pmol/10^6 cells, are given in Fig. 1A. The accumulation of radioactivity appeared to be linear in all cases for at least 3 min. (A linear regression analysis of the first four datapoints revealed a $r^2 = 0.998$; 0.963, 0.987, 0.981 and 0.998 for the accumulation of [^{3}H]5-HT in SERT cells, [^{3}H]DA in DAT-cells, [^{3}H]GABA in GAT-cells, [^{3}H]MPP$^+$ in SERT-cells and [^{3}H]MPP$^+$ in DAT-cells, respectively).

The data of Fig. 1A are replotted in Fig. 1B. Uptake velocity (pmol/min/10^6 cells) at each measured time point

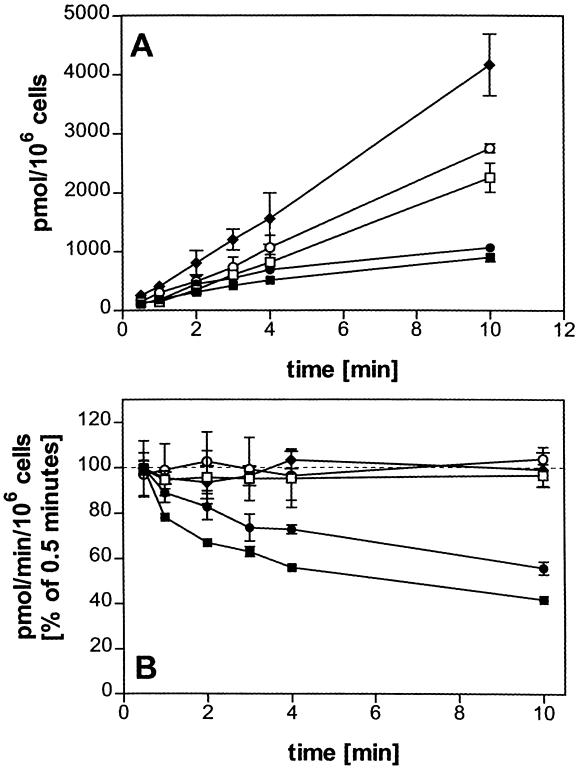

Fig. 1. Uptake of labelled substrates in HEK-293 cells expressing neurotransmitter transporters. hSERT cells were incubated with 10 μM [^{3}H]5-HT (■) or 200 μM [^{3}H]MPP$^+$ (□), hDAT cells with 10 μM [^{3}H]DA in the presence of 10 μM pargyline and 1 mM tropolone (●) or with 200 μM [^{3}H]MPP$^+$(○), rGAT cells with 50 μM [^{3}H]GABA (◇) at room temperature for the times indicated. Symbols represent mean ± SEM of three independent experiments, performed in triplicates each. Panel A: results are shown as total amount of substrate accumulated in the cells (pmol/10^6 cells); panel B: same data as in panel A, calculated as velocities (pmol/10^6 cells/min) and expressed as percent of the value at 0.5 min.

was expressed as percentage of the velocity at 0.5 min. While uptake velocity for the substrates [^{3}H]MPP$^+$ and [^{3}H]GABA did not change over time, the velocity for [^{3}H]5-HT and [^{3}H]DA decreased progressively over the entire observation period, already measurable between 0.5 and 1 min of incubation.

As it was suspected that diffusion of substrate out of the cells was responsible for the observed progressive decrease in uptake velocity a set of superfusion experiments was performed to test this possibility. Cells were preloaded with their respective radiolabelled substrates and superfused (Fig. 2). Fractional efflux of radioactivity under baseline conditions was highest with [^{3}H]5-HT, followed by [^{3}H]DA, [^{3}H]MPP$^+$ and [^{3}H]GABA. On addition of an appropriate uptake inhibitor, there was little or no change in efflux of [^{3}H]MPP$^+$ and [^{3}H]GABA and a clear-cut increase in the efflux of [^{3}H]5-HT and [^{3}H]DA. Effluxes in the presence of reuptake inhibitors amounted to $1.27 \pm 0.06\%$ min^{-1} ($n = 6$) for [^{3}H]5-HT; $0.72 \pm 0.02\%$ min^{-1} ($n = 9$) for [^{3}H]DA; 0.082 (0.008% min^{-1} ($n = 8$) for [^{3}H]GABA and $0.27 \pm 0.01\%$ min^{-1} ($n = 9$) for [^{3}H]MPP$^+$ in both SERT and DAT cells.

As efflux in the presence of an uptake inhibitor can be regarded as exclusively due to diffusion it was of interest to estimate the intracellular substrate concentrations at the time of drug addition. Using an intracellular volume of 1.3 pl [15] the values were (mM) 2.34 ± 0.83, 1.94 ± 0.21, 10.0 ± 0.31, and 14.5 ± 0.24 for [^{3}H]5-HT, [^{3}H]DA, [^{3}H]MPP$^+$ and [^{3}H]GABA, respectively.

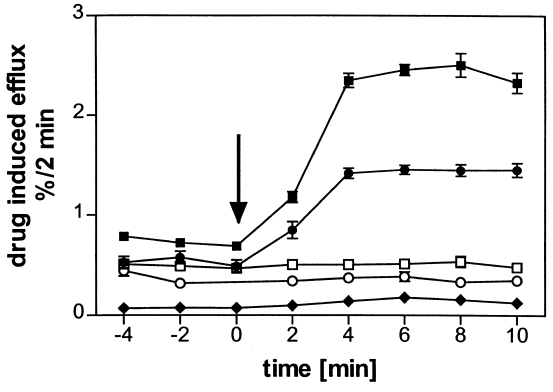

Fig. 2. Efflux of labelled substrates from HEK-293 cells expressing neurotransmitter transporters. Influence of reuptake inhibitors HEK 293 cells stably transfected with the hSERT, hDAT and rGAT were loaded with saturating concentrations of substrate (see Methods), superfused and 2 min fractions were collected. After three fractions (6 min) of basal efflux, the buffer was switched to a buffer containing a high concentration of an appropriate uptake inhibitor: for hSERT cells 100 μM clomipramine (■), preincubated with [^{3}H]5-HT; (□), preincubated with [^{3}H]MPP$^+$); for hDAT cells 100 μM cocaine (●), preincubated with [^{3}H]DA; (○), preincubated with [^{3}H]MPP$^+$); for rGAT cells 10 μM tiagabine (◆). Data are presented as fractional release (i.e. each fraction is expressed as a percentage of radioactivity present in the cells at the beginning of that fraction). Symbols represent mean ± SEM of six observations (one observation = one superfusion chamber, three independent experiments performed in duplicate).

Cells expressing plasmalemmal neurotransmitter transporters enjoy widespread use in studies on transporter function and have in many instances replaced more classical paradigms such as brain slices, synaptosomes or isolated organ preparations. The present report describes a possibly confounding phenomenon occurring in such cells when (1) the expression level of the transporter protein is high and (2) the substrate used is readily diffusible.

On incubation of hSERT and hDAT cells with [^{3}H]5-HT and [^{3}H]DA, respectively, stably transfected HEK-293 cells accumulated label in a time-dependent fashion. When plotted as amount of label accumulated inside the cells, the process appeared to be time-linear over at least 3 min. This was not only the case on visual inspection of the data but also when data points were subjected to linear regression analysis (no significant deviation from linearity with correlation coefficients close to unity). However, on closer scrutiny, viz. by calculating uptake velocities for each determined time point, it became obvious that accumulation was not time-linear for both substrates, and uptake velocity apparently decreased over the entire observation period (Fig. 1B). This was clearly not the case for the substrates [^{3}H]MPP$^+$ and [^{3}H]GABA.

A possible explanation for the observed results would be rapid intracellular metabolism of [^{3}H]5-HT and [^{3}H]DA to tritium water or metabolites which are not retained in the cell. This was most likely not the case in our experiments. The intracellular stability of [^{3}H]5-HT in transporter expressing cells over prolonged incubation periods has been shown in several reports, including our own[15,17]. In the case of [^{3}H]DA inhibitors of monoamine oxidase as well as catechol-O-methyl-transferase were included in the medium, thus preventing metabolism of this substrate[5]. A more likely explanation for the observed non-linearity would be diffusion of substrate out of the cell. Since the cells do not possess a vesicular storage mechanism, substrates reach high concentrations at the inside of the plasma membrane and leave the cell following their concentration gradient. With the two more lipophilic substrates [^{3}H]5-HT and [^{3}H]DA diffusion may be expected to be substantial, whereas the more hydrophilic compounds [^{3}H]MPP$^+$ and [^{3}H]GABA should not display this effect to a noticeable degree.

The hypothesis was tested using superfusion of prelabelled cells. Under such conditions release of radioactivity into the medium represents diffusion if care is taken to prevent reuptake of effluxed substrate back into the cells. In fact, following the addition of appropriate reuptake inhibitors efflux of [^{3}H]5-HT and [^{3}H]DA, but not of [^{3}H]MPP$^+$ or [^{3}H]GABA, were substantially increased, which indicates ongoing reuptake of the former two substrates (see also[14,15]). In the presence of uptake inhibition, the rank order of fractional efflux rates was [^{3}H]5-HT > [^{3}H]DA > [^{3}H]MPP$^+$ > [^{3}H]GABA with the rate for [^{3}H]5-

HT being about 15 times higher than the rate of [^{3}H]GABA. It is also noteworthy that the estimated intracellular concentrations of [^{3}H]GABA and [^{3}H]MPP$^+$ were about 5 times higher than those of [^{3}H]5-HT or [^{3}H]DA, indicating better retention of the former two substrates. Overall, the results of the superfusion experiments support the idea that the observed time dependent decrease in uptake velocity for [^{3}H]5-HT and [^{3}H]DA is a consequence of substrate loss by diffusion which becomes quantitatively substantial already within the first 60 s.

The described phenomenon may be of importance in experiments in which quantitative analyses of transporter function are made, V_{max} being the most prominently affected parameter. For instance, performing a saturation analysis of [^{3}H]5-HT uptake in our cells using an incubation time of 5 min as opposed to 30 s would lead to an underestimation of V_{max} by a factor of 3 (112 pmol/min/10^6 cells vs. 367 pmol/min/10^6 cells; not shown). Thus, even using a relatively short incubation time of 3 min [16] might render a somewhat underestimated V_{max}. However, incubation times of 10–20 min are common[1,2,7,10,12]. The deviation may be particularly high when an incubation temperature of 37°C is chosen[1,2,6,10,12], or misleading conclusions may be drawn when V_{max} values of different substrates are compared (e.g. DA vs MPP$^+$,[4]). In conclusion, the use of heterologous expression systems has made it possible to study various aspects of transporter function in greater detail than has been possible previously. However, one might have to consider other system-inherent problems that must be adequately addressed. Loss of substrate by diffusion after a relatively short incubation time may be one of them.

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