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Validation of a fluorescence-based high-throughput assay for the measurement of neurotransmitter transporter uptake activity

Susanne Jørgensen, Elsebet Østergaard Nielsen, Dan Peters, Tino Dyhring *

NeuroSearch A/S, Drug Discovery, 93 Pederstrupvej, Ballerup DK-2750, Denmark

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Abstract

Pre-synaptic dopamine, norepinephrine and serotonin transporters (DAT, NET and SERT) terminate synaptic catecholamine transmission through reuptake of released neurotransmitter. Common approaches for studying these transporters involve radiolabeled substrates or inhibitors which, however, have several limitations. In this study we have used a novel neurotransmitter transporter uptake assay kit. The assay employs a fluorescent substrate that mimics the biogenic amine neurotransmitters and is taken up by the cell through the specific transporters, resulting in increased fluorescence intensity. In order to validate the assay, a variety of reference and proprietary neurotransmitter transporter ligands from a number of chemical and pharmacological classes were tested. The ability of these compounds to inhibit the selective transporter-mediated uptake demonstrated a similar rank order of potency and IC_{50} values close to those obtained in radiolabeled neurotransmitter uptake assays. The described assay enables monitoring of dynamic transport activity of DAT, NET and SERT and is amenable for high-throughput screening and compound characterization.
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Keywords: Neurotransmitter; Uptake; DAT; NET; SERT; High-throughput screening; Fluorescence assay

1. Introduction

1.1. Background

Transporters for dopamine, norepinephrine and serotonin (DAT, NET and SERT) represent established targets for many pharmacological agents that affect brain function, including antidepressants and psychostimulants (Chen and Skolnick, 2007; Rothman and Baumann, 2003; Tamminga et al., 2002). Furthermore, polymorphisms in monoamine transporter genes support a linkage to psychiatric disorders (for review see Hahn and Blakely, 2002). Monoamine transporters are localized at perisynaptic sites, where they are crucial for the termination of monoamine transmission and the maintenance of presynaptic monoamine storage. The sensitivity of monoamine transporters has been examined in brain preparations as well as in heterologous systems with recombinant transporters. The most common method used to estimate the sensitivity of monoamine transport is to monitor cellular accumulation of radiolabeled substrates. The advantages in using a radiolabeled substrate are that the sensitivity is high, the number of radiolabeled molecules taken into the cell can be accurately determined, and detecting radioactivity via scintillation techniques is straightforward. On the other hand, radioactive methods have, in general, an inability to monitor real-time changes in transport function and are not easily amenable for high-throughput screening. In addition, classical methods typically measure accumulation and not intrinsic activity *per se*.

The applicability of fluorescence-based techniques to study transport activity and regulation has previously been investigated in both single cells (Mehrens et al., 2000; Schwartz et al., 2003; Zapata et al., 2007) as well as multiwell platforms (Fowler et al., 2006; Haunsø and Buchanan, 2007; Mason et al., 2005; Wagstaff et al., 2007) using the organic fluorescent compound 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP^+). Unlike radiolabeling-based methods, the use of a fluorescent substrate allows direct visual assessment of preparations, affording significantly enhanced spatial as well as temporal resolution in addition to real-time monitoring. Furthermore, the use of a fluorescent substrate to monitor monoamine transporter function enables implementation of an automated platform compatible with high-throughput screening as well as real-time analysis of transporter function and regulation.

* Corresponding author. Tel.: +45 4460 8277; fax: +45 4460 8080.
E-mail addresses: sjo@neurosearch.dk (S. Jørgensen), eon@neurosearch.dk (E.Ø. Nielsen), dp@neurosearch.dk (D. Peters), tdy@neurosearch.dk (T. Dyhring).

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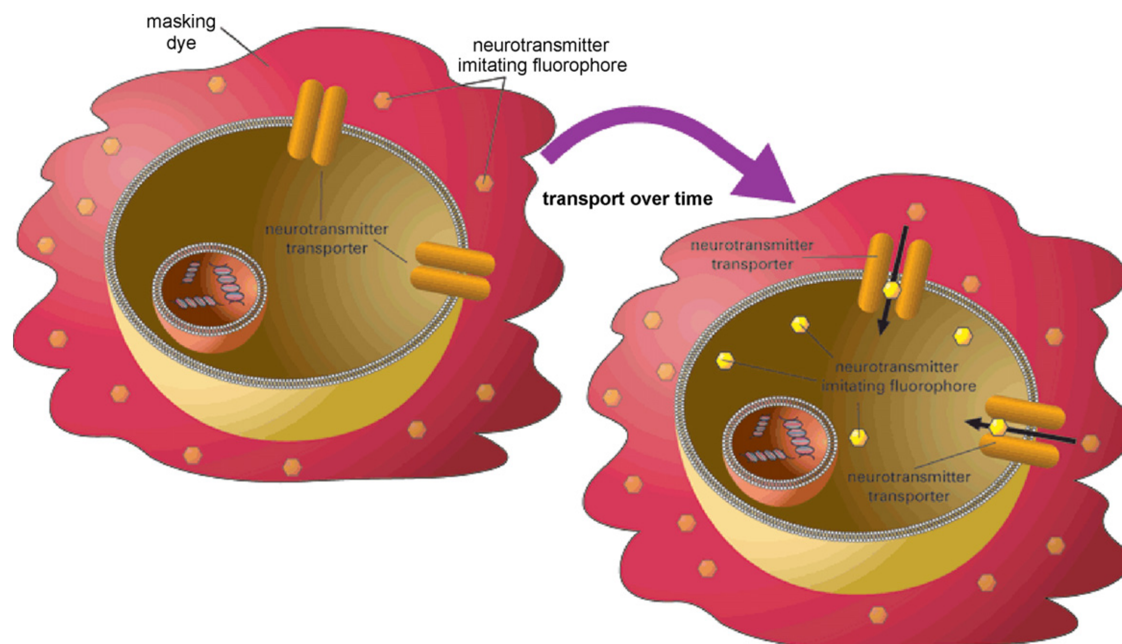

Fig. 1. Principle of the fluorescence-based neurotransmitter transporter uptake assay. Reprinted with permission from MDS Analytical Technologies.

1.2. Neurotransmitter transporter uptake assay principle

Here we describe the evaluation of a novel neurotransmitter transporter uptake assay kit from MDS Analytical Technologies (Sunnyvale, CA) for detection of DAT, NET and SERT activity. The assay utilizes a fluorescent substrate that mimics the biogenic amine neurotransmitters, and is actively transported into the cell by any of the three transporters. The enhanced fluorescence exhibited by intracellular accumulation of the fluorescent substrate offers the possibility to assess transport activity by quantitative fluorescence techniques. In order to ensure low background fluorescence, the neurotransmitter transporter uptake assay kit utilizes a membrane impermeant masking dye that serves to extinguish extracellular fluorescence (Fig. 1). The assay can be performed on practically any bottom-read mode fluorescence microplate reader. In this study we used the FLIPR^{TETRA} (MDS Analytical Technologies), which is a 96/384/1536-well, temperature-controlled fluorescence plate reader that features automated liquid dispensation. This technology platform allows the user to follow the transport of the fluorescent substrate in a kinetic mode involving real-time sampling, rendering the assay suitable for high-throughput screening and compound characterization.

2. Materials and methods

2.1. Materials

^{3}H-dopamine (10.7 Ci/mmol) and ^{3}H-norepinephrine (35 Ci/mmol) were purchased from GE Healthcare UK Limited (Little Chalfont, UK) and ^{3}H-5-hydroxytryptamine (21 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Duloxetine was synthesized at NeuroSearch. Desipramine, bupropion and fluoxetine were purchased from Sigma–Aldrich (Vallensbæk Strand, Denmark). Benztropine and venlafaxine were purchased from Research Biochemicals International (Natick, MA) and Zydus Cadila (Ahmedabad, India), respectively. Reboxetine was extracted from commercially available tablets (Pfizer, Ballerup, Denmark). Citalopram was from Actavis (Gentofte, Denmark). The NeuroSearch proprietary compounds were synthesized according to the procedures described in the following International Patent Publications (WO 97/16451, WO 2004/113334, WO 2005/123679, WO 2005/123715, WO 2006/021564, WO 2006/108789 and WO 2006/131524).

2.2. Cell culture

HEK-293 cells stably expressing human DAT (HEK-hDAT) and CHO-K1 cells stably expressing human DAT (CHO-hDAT) and human NET (CHO-hNET) were a kind gift from Prof. Ulrik Gether (Department of Neuroscience and Pharmacology, University of Copenhagen, Denmark). Non-transfected HEK-293 cells, as well as HEK-293 cells stably expressing human SERT (HEK-hSERT) and HEK-hDAT, were cultured in Dulbecco's modified Eagle's Medium (DMEM, Lonza, Copenhagen, Denmark) supplemented with 10% fetal calf serum (FCS, Invitrogen, Tåstrup, Denmark). The HEK-hDAT cells were cultured in the presence of 150 μg/ml hygromycin (Roche, Hvidovre, Denmark). Non-transfected CHO-K1 cells, CHO-hDAT and CHO-hNET, were cultured in RPMI1640 medium supplemented with L-glutamine (Lonza) and 10% FCS. The CHO-hDAT and CHO-hNET cells were cultured in the presence of 500 μg/ml G418 (Invitrogen). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

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