Published in final edited form as:

J Neurochem. 2010 August ; 114(4): 1019–1029. doi:10.1111/j.1471-4159.2010.06828.x.

Real-time, Spatially Resolved Analysis of Serotonin Transporter Activity And Regulation Using the Fluorescent Substrate, ASP⁺

M. Oz¹, T. Libby^{1,*}, B. Kivell^{1,*}, V. Jaligam¹, S. Ramamoorthy², and T.S. Shippenberg¹

¹ Integrative Neuroscience Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, U.S. Department of Health and Human Services, Baltimore MD 21224, USA

² Dept of Neurosciences, Medical College of South Carolina Charleston, SC, 29425 USA

Abstract

The serotonin transporter (SERT) mediates clearance of serotonin from the synapse, thereby, regulating extracellular serotonin concentrations. Radioligand uptake techniques are typically used to assess SERT function in tissue and heterologous expression systems. The need for sufficient protein in samples, however, requires use of homogenate preparations, potentially masking effects limited to specific cell populations. 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP⁺) is a fluorescent monoamine transporter substrate that has been used for real-time monitoring of dopamine and norepinephrine transporter function in single cells. The present live cell imaging studies examine the utility of ASP⁺ for quantifying hSERT function in HEK-293 and neuroblastoma cells. We show rapid membrane binding and intracellular ASP⁺ accumulation in hSERT expressing cells. Accumulation is saturable; dependent on temperature and the presence of sodium and chloride in the media, and attenuated by serotonin. Acute or prolonged exposure of cells to serotonin re-uptake inhibitors produces a concentration-dependent decrease in accumulation. Similar effects are produced by PKC activation whereas p38MAPK activation increases ASP⁺ accumulation. These data demonstrate the validity of ASP⁺ as a probe for monitoring SERT function in living cells. Alterations in SERT binding and uptake can be quantified in the same cell and use of a within cell design permits analysis of time-related alterations in SERT function.

Keywords

live-cell imaging; 4-(4-(dimethylamino)-styryl)-N-methylpyridinium; p38 MAPK; monoamine transporter; PKC

INTRODUCTION

The serotonin transporter (SERT), a member of the SLC6A4 family of sodium-chloride symporter proteins (Ramamoorthy et al. 1993), mediates reuptake of serotonin (5-HT) from the synaptic cleft. By clearing released 5-HT, it plays an essential role in the regulation and maintenance of 5-HT neurotransmission. Dysregulation of SERT function has been implicated in the pathogenesis of a number of psychiatric disorders, including depression (Neumeister 2004; Vergne and Nemeroff, 2006), bipolar disorder (Rotondo et al., 2002;

Correspondences To: TS Shippenberg, Integrative Neuroscience Section, NIDA IRP, 333 Cassell Dr., Baltimore, MD 21224, tshippen@intra.nida.nih.gov, Phone: 410 550 1451, Fax: 410 550 1692.

*Present Address: Victoria University of Wellington, PO Box 600, Wellington 6140, New Zealand

@These authors contributed equally to this work

Cannon et al., 2006), anxiety (Lee et al., 2005; Hu et al., 2006), Tourette's syndrome (Muller-Vahl et al., 2005), autism (Tordjman et al., 2001; Brune et al., 2006) and addiction (Ramamoorthy et al., 1993).

Regulation of SERT activity by several serine/threonine kinases has recently been described. Activation of protein kinase C (PKC) decreases SERT activity and cell-surface expression in heterologous expression systems (Ramamoorthy and Blakely 1999), human platelets (Jayanthi et al., 2005; Carneiro and Blakely 2006) and nerve terminals (Samuvel et al., 2005). In contrast, protein kinase G (PKG) and p38 mitogen-activated protein kinase-linked pathways increase SERT surface expression and function (Miller and Hoffman, 1994; Zhu et al., 2004; 2005). These data indicate the existence of post-translational mechanisms that allow hSERT to respond to changes in the extracellular and intracellular milieu. Importantly, however, in the majority of studies, SERT function was assessed after prolonged kinase activation (e.g., 30 min) and at only a single time point. Therefore, understanding of the dynamic regulation of hSERT function remains limited.

Increasing evidence indicates that regulation of monoamine transporters by GPCRs, kinases and their substrates may differ as a function of the duration of ligand exposure (Jayanthi et al., 2005; Johnson et al., 2005; Zapata et al., 2007). Five-min stimulation of PKC by phorbol 12-myristate 13-acetate (BPMA) increased the K_m and decreased the V_{max} of [3H] serotonin (5-HT) uptake whereas after 30 min stimulation, only a decrease in V_{max} was seen (Jayanthi et al., 2005). Studies of the dopamine transporter (DAT) have revealed that the function and cell surface expression of DAT increases within minutes after D3 receptor activation (Zapata et al., 2007). However, as the duration of agonist exposure increases, DAT function and cell surface expression decrease. Similarly, the DAT substrate, amphetamine, induces a rapid increase in DAT cell surface expression, whereas after prolonged drug exposure transporter internalization is seen. (Johnson et al., 2005). Together these data demonstrate that monoamine transporters are rapidly regulated by substrates, GPCRs and GPCR-linked signaling cascades. Furthermore, they highlight the need to assess rapid as well as more prolonged effects of a manipulation on transporter activity.

SERT function is typically quantified using radioligand uptake assays. Although this method enables quantification of transporter kinetics, examination of rapid changes (≤ 2.5 min) in function is precluded due to the time required for ligand addition and washout. Time course studies cannot be conducted in the same sample but require parallel assays. As such, a within-cell design is precluded. Furthermore, since large populations of cells are sampled, differences in protein (e.g. transporter, GPCR) expression and localization may mask effects limited to a specific cell population.

ASP⁺, 4-(4-(dimethylamino)-styryl)-N-methylpyridinium, an analog of the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺), is a monoamine transporter substrate that fluoresces upon contact with lipids (Schwartz et al., 2003). Several laboratories have demonstrated the utility of ASP⁺ in conjunction with live cell imaging techniques for the quantification of dopamine and norepinephrine transporter function in real time (Schwartz et al., 2003; Bolan et al., 2007; Zapata et al., 2007). Binding and transport of ASP⁺ can be continuously monitored, enabling analysis of the time course of a specific effect.

ASP⁺ binds to SERT with micromolar affinity and accumulates in SERT-expressing cells (Schwartz et al., 2003). The use of ASP⁺ for the high-throughput measurement of SERT function was recently described (Fowler et al., 2006). However whether ASP⁺ can be used with live-cell imaging techniques to monitor changes in SERT function in real time is unknown. The present studies used EM4 cells, a variant of HEK-293 cells, expressing the human SERT (hSERT) to address this issue.

MATERIALS AND METHODS

Cell Culture and Transfections

Experiments were conducted in EM4 cells, a variant of HEK293 cells stably expressing a macrophage scavenger to increase adherence to cell culture plastic (R. Horlick, Pharmacopeia, Cranberry, NJ; (Robbins and Horlick 1998). EM4 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Cellgro, Mediatech Inc., Herndon, VA)/10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) in a humidified atmosphere at 37°C and 5% CO₂. Additional experiments were conducted in Neuro2A (N2A) cells, and HEK 293 cells maintained under identical conditions. Cells were seeded in 35mm glass-bottomed Delta T culture dishes (Biotechs, Butler, PA) at 1.25×10^5 cells/ml and 1 ml/dish. Transient transfections were performed 20–24hr after plating. Cells were transfected with pEGFP-hSERT (a generous gift of Dr. H. Sitte) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (for each Delta T dish transfected, 50µl OptiMEM, 2 µl Lipofectamine 2000 reagent, 0.25µg pEGFP-hSERT). Previous studies have shown that addition of fluorescent tags does not modify SERT function, sub-cellular localization or kinase modulation (Sakai et al., 2000; Schmid et al., 2001). Imaging experiments were performed 32–48 hrs after transfection when cells were 70–90% confluent.

[³H]5-HT Uptake in hSERT and pEGFP-hSERT Transfected Cells

[³H]5-HT uptake studies were conducted to confirm that addition of the pEGFP tag to the hSERT N-terminus does not affect kinase regulation of SERT. HEK 293 cells were seeded on poly-D-lysine coated 24-well (150,000 cells/well) plates and maintained for 24 hr prior to transfections. Cells were transfected with hSERT or pEGFP-hSERT plasmids using Lipofectamine 2000 transfection reagent as per the manufacturer instructions. After 24 hr, transfected cells were washed with KRH assay buffer, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 10 mM HEPES, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM Tris, and 10 mM D-glucose) containing 0.1 mM ascorbic acid, 0.1 mM pargyline and preincubated with the PMA (0.5µM) or vehicle (ethanol) for 30 min at 37°C followed by addition of 1 µM 5-HT (30 nM [³H]5-HT plus 970 nM unlabeled 5-HT) to initiate SERT activity. 5-HT uptake was terminated after 10 min incubation at 37°C by rapid washings with KRH buffer containing 10µM imipramine. Cells were lysed with 1% SDS, and the accumulated radioactivity was measured by liquid scintillation. Nonspecific 5-HT uptake was defined as the accumulation in the presence of 0.1 µM fluoxetine and was subtracted from total 5-HT uptake. Data were analyzed using ANOVA followed by the Dunnett's test or the Student's t-test.

Live-Cell Confocal Imaging

Time-resolved live cell images were obtained using a PerkinElmer Ultraview LCI confocal system on a Nikon Eclipse 2000-S inverted microscope equipped with PlanApo objectives (PerkinElmer, Waltham, MA). Images were collected at room temperature (22°C) using a 60X/1.2NA water immersion objective. Images were captured using a Hamamatsu CCD (Hamamatsu, Bridgewater, NJ) and Ultraview software. For imaging, cells were rinsed twice with Krebs-Ringer-HEPES (KRH; 130mM NaCl, 1.3mM KCl, 2.2mM CaCl₂, 1.2mM MgSO₄, 1.2 mM KH₂PO₄, 1.8g/l glucose, and 10mM HEPES, pH 7.4; all chemicals from Sigma-Aldrich, St. Louis, MO). KRH was added to the dish, which was then mounted on a Delta T microscope stage insert (Biotechs). A field of cells was selected, avoiding heavy EGFP over expression. KRH was removed from the dish, an initial image was taken, and 1ml of 10µM ASP⁺ (Sigma-Aldrich) in KRH was added to the dish. EGFP and ASP⁺ were excited by an argon laser (Melles-Griot, Carlsbad, CA) with excitation peak at 488nm. EGFP fluorescence was captured at 525–575nm and ASP⁺ fluorescence at 607–652nm.

ASP⁺ Binding

Utilizing ASP⁺ to probe NET function, Schwartz et al. (2003) distinguished a rapid (msec) increase in ASP⁺ fluorescence intensity limited to the cell surface, representing binding of substrate to the transporter, followed by a second, linear phase of intracellular ASP⁺ accumulation. To visualize binding of ASP⁺ to pEGFP-hSERT, images were collected using a continuous image capture protocol. To accommodate laser settle time, the system collected images approximately every 200msec. For all binding experiments, data were background-subtracted and ASP⁺ fluorescence intensity was expressed as a function of initial fluorescence intensity in order to enable correction for differences between cells in hSERT expression levels. A within cell design was used to characterize binding in control cells. The effects of various manipulations were assessed using a between cell design.

ASP⁺ Uptake

Saturability, Temperature and Ion-Dependence of ASP⁺ Accumulation—To assess saturation of ASP⁺ (1–50 μM), a single concentration of ASP⁺ was added to a plate and fluorescent accumulation was measured over 5 min. After subtracting nonspecific fluorescence (obtained from nontransfected cells in the same dishes) and normalizing for SERT expression, ASP⁺ fluorescence intensity data for each cell per condition were averaged and nonlinear regression was applied. The influence of temperature on ASP⁺ (10 μM) accumulation was assessed by comparing 5-min uptake values in cells maintained at 25°C to those maintained at 6°C using a between cells design. The Na⁺ dependence of ASP⁺ accumulation was assessed in KRH buffer using isotonic replacement of NaCl with *N*-methyl-D-glucamine (NMDG; Sigma). The buffer composition was: NMDG (130 mM), KCl (1.3 mM), CaCl₂ (2.2 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), HEPES (10 mM), glucose (10 mM). Cl[−] dependence was determined in KRH buffer in which NaCl was substituted with an equimolar concentration of sodium isethionic acid (Sigma): sodium isethionic acid (130 mM), CH₃COOK (1.3 mM), Ca(CH₂CO₂).H₂O (2.2mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), HEPES (10 mM), glucose (10mM). The buffers were adjusted to pH 7.4 to control for potential effects of pH on transporter function using KOH. Data assessing temperature-and ion-dependence were background-corrected and normalized for SERT expression. Five min values were then compared using the Student's t-test.

Influence of Substrate and Transport Inhibitors on ASP⁺ Accumulation—5-HT competition experiments were performed by replacing the KRH buffer with that containing a single concentration of 5-HT (30 nM - 3 μM) 15–30 sec before ASP⁺ (10 μM) addition. Data were background-subtracted and normalized for SERT expression as described above. The effects of increasing concentrations of 5-HT were assessed by comparing the normalized fluorescence values in the absence (controls) and presence of a given 5-HT concentration. The concentration-response curve for the effect of 5-HT was calculated using non-linear curve fitting function of the computer software OriginTM (Originlab Corp. Northampton, MA).

The acute effects of the selective serotonin re-uptake inhibitors (SSRIs) 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro[2]benzofuran-5-carbonitrile (citalopram, 0.1–100 μM) and 3-(4-bromophenyl)-*N,N*-dimethyl-3-pyridin-3-yl-prop-2-en-1-amine (zimeidine, 10 μM) on ASP⁺ accumulation were studied using a within-cells procedure. Inhibitor or vehicle was added to the plates 5 min following the addition of ASP⁺ (10 μM). Image capture proceeded for 5 min. To correct for background, initial ASP⁺ fluorescence determined from images taken prior to addition of ASP⁺ was subtracted from all subsequent values. The slopes of ASP⁺ accumulation during 1-min time periods before and after drug/vehicle addition were normalized for SERT expression, then averaged and

compared. The resultant data were analyzed using a one-way ANOVA followed by the Dunnett's post hoc test or the Student's t-test.

A between-cells design was used to examine the influence of prolonged SSRI exposure on ASP⁺ accumulation. Drug or vehicle was added to the plate, which was then returned to the incubator for the desired incubation period. The plate was then placed on the microscope stage and accumulation was assessed in the continued presence of drug(s)/vehicle(s). After background correction and normalization for SERT expression levels, 5 min uptake values were compared by Student's t-test.

ASP⁺ Accumulation Following Acute or Prolonged Kinase Activation—

Activation of p38 MAPK increases SERT activity and cell surface expression (Samuvel et al., 2005; Zhu et al., 2005; Steiner et al., 2009). A between-cells design was used to determine whether exposure of cells to the p38 MAPK activator, anisomycin (1 μ M), increases ASP⁺ accumulation and whether this effect is reversed by the selective p38 mitogen-activated protein kinase inhibitor, PD169316 (1 μ M). The concentrations of drugs employed were those shown to be kinase selective in other assays (Zhu et al., 2005). Vehicles and drugs were pre-mixed with ASP⁺ solution, and were applied immediately following capture of the 0 sec images. Data were background-corrected, normalized, and compared by one-way ANOVA followed, when appropriate, with the Dunnett's post hoc test.

The effects of prolonged exposure to anisomycin or the PKC activator, β PMA (0.1 μ M) were assessed using a between cell design. Drug or vehicle was added to the plate, which was then returned to the incubator for the desired preincubation time. The plate was then placed on the microscope stage and accumulation was assessed in the continued presence of drug(s)/vehicle(s). After background subtraction and correction for SERT expression levels, 5 min uptake values were compared using a one-way ANOVA followed by the Dunnett's post hoc test.

Data Analysis—Quantification of pEGFP and ASP⁺ fluorescence intensity data (average pixel intensity in arbitrary fluorescence units, AFUs) was accomplished using Ultraview software. All ASP⁺ data were background-corrected as described above. Since ASP⁺ fluorescence intensity was shown to be proportional to pEGFP fluorescence intensity (e.g., hSERT expression), all experiments using a between-cells design were normalized relative to expression level using the formula: $[(ASP^+ \text{ fluorescence intensity})_{\text{background-corrected}} / (\text{pEGFP-hSERT fluorescence intensity})] * 100$. For each set of experiments, images were collected from 2–4 dishes from each of three experimental days (three separate transfections). All statistical analyses, which included correlations, t-tests, ANOVAs with post hoc tests, and nonlinear regression, were performed using GraphPad Prism (GraphPad Software, San Diego, CA). A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

[³H]5-HT uptake in cells transfected with hSERT or pEGFP-hSERT was significantly inhibited by fluoxetine (hSERT: $p \leq 0.001$, $t=17.1$; $df=13$; pEGFP-hSERT: $p \leq 0.001$, $t=9.6$; $df=16$) No difference between constructs in the magnitude of this effect was observed (inhibition: hSERT: 96.1%; pEGFP SERT: 97.9%; $p > 0.05$, $t=0.3$) Treatment of cells with the PKC activator, β PMA, for 30 min significantly inhibited 5-HT uptake in cells expressing hSERT ($p \leq 0.001$, $t=4.5$; $df=8$) or pEGFP-hSERT ($p \leq 0.001$; $t=4.7$; $df=8$) and there was no difference between constructs in this effect (inhibition: hSERT: 74.8%; pEGFP-hSERT: 73.7%; $p > 0.05$, $t=0.2$). These data confirm that addition of the fluorescent tag does not alter transporter regulation.

Binding and Uptake of ASP⁺

Upon exposure of pEGFP-hSERT transfected EM4 cells (referred to subsequently as hSERT) to ASP⁺, an immediate and persistent increase in ASP⁺ fluorescence intensity was seen. As previously reported for NET (Schwartz et al., 2003) and DAT (Bolan et al., 2007), two phases of accumulation were seen. The initial phase was characterized by a rapid increase in ASP⁺ fluorescence intensity that was restricted to the plasma membrane (Fig. 1A–D). This phase was of short duration (e.g., ≤ 2.5 s) (Fig. 1E; data from three dishes on one experimental day, $n=14$ cells) and consistent with ASP⁺ binding to cell-surface SERT. Half-maximal binding was obtained in less than 1 sec ($\tau_{1/2} = 0.58$ sec, Figure 1E).

During the second phase, ASP⁺ fluorescence intensity continued to increase, but at a slower rate. This was accompanied by gradual intracellular accumulation of ASP⁺ (Fig. 2A–D), which increased linearly over time. Fig. 2E displays total, specific and non-specific accumulation of ASP⁺ fluorescence over five min from one experimental day ($n=38$ –41 cells/condition). Due to low levels of ASP⁺ fluorescence in nonexpressing cells, accumulation was assessed in hSERT-expressing and non-expressing cells in each dish. Statistical analysis of the slope values revealed a significantly greater rate of ASP⁺ accumulation in hSERT-transfected as compared to non-transfected cells. ($p<0.001$, t-test, $t=16.3$, $df=77$, Fig. 2F). Comparison of hSERT-transfected cells to native EM4 cells yielded similar results ($p<0.001$, t-test, $t=7.4$, $df=98$; data not shown).

N2A cells expressing hSERT also showed significantly greater intracellular accumulation of ASP⁺ in transfected cells as compared to nontransfected cells from the same dishes ($p<0.001$, t-test; $t=3.3$, $df=67$; data not shown).

Both binding and uptake of ASP⁺ via hSERT varied as a function of hSERT expression level. Cell-surface ASP⁺ fluorescence intensity measured during the binding phase correlated with the level of hSERT expression as assessed by EGFP fluorescence intensity measured immediately prior to ASP⁺ addition ($r^2=0.587$, Spearman coefficient; Fig. 3a). Likewise, ASP⁺ fluorescence during the second phase of ASP⁺ accumulation correlated with hSERT expression level ($r^2=0.748$, Spearman coefficient). Saturation experiments revealed that ASP⁺ uptake in hSERT-expressing cells is a saturable process, reaching a maximum at 30 μ M (Fig. 3B). Non-linear curve-fitting yielded a one-phase exponential association, with a V_{max} of 0.385 ± 0.02 AFU/sec and K_m of 159 ± 27 nM (nonlinear regression, $R^2=0.97$).

Temperature- and Ion-Dependence of ASP⁺ Uptake

Radioligand uptake studies have shown that SERT function is temperature dependent. Consistent with these findings, a significant, 31% reduction in ASP⁺ accumulation was observed in hSERT expressing cells maintained at 6°C relative to those maintained at 25°C ($P<0.001$, t-test; $t=3.42$, $df=73$; data not shown). hSERT is a Na⁺/Cl[−] symporter (Ramamoorthy et al. 1993). Fig 4 shows that when KRH buffer was replaced with Na⁺- or Cl[−]- free buffer, ASP⁺ binding ($p \leq 0.001$; ANOVA, $F_{(2, 220)} = 124.8$; $p \leq 0.001$, Dunnett's test: Fig 4A) and accumulation significantly decreased ($p \leq 0.001$; ANOVA, $F_{(2, 220)} = 258.6$; $p \leq 0.001$, Dunnett's test: Fig 4B). These findings suggest that ASP⁺ accumulation reflects a transporter-mediated process.

Influence of 5HT on ASP⁺ Uptake

To determine whether ASP⁺ uptake in hSERT expressing cells is inhibited by substrate, the rate of ASP⁺ accumulation was assessed in the presence of varying concentrations of 5-HT (30–3000 nM; Fig 5). Analysis of the slope of ASP⁺ accumulation revealed an IC₅₀ value of 353 nM.

Influence of Acute and Chronic SSRI Exposure on ASP⁺ Uptake

Citalopram is a potent and specific SERT inhibitor that decreases [³H]5HT uptake (Hyttel, 1982). Comparison of 1-min accumulation rates before and after acute citalopram addition revealed a significant, concentration-dependent reduction of ASP⁺ (Fig. 6A, B; $p < 0.001$, ANOVA, $F_{(4,488)} = 61.5$). Even the lowest concentration of citalopram, 100 nM, produced a significant effect ($p < 0.01$, Dunnett's test). In contrast, no difference in ASP⁺ accumulation was observed between untreated and vehicle treated cells (Fig 6A). A decrease in the rate of ASP⁺ accumulation was also observed in response to prolonged citalopram (10 μ M) exposure. In cells preincubated with citalopram for 10 min prior to addition of ASP⁺, accumulation was significantly lower than in vehicle-pretreated cells ($p < 0.0001$, t-test; $t = 6.3$, $df = 158$; Fig. 5C). Acute addition of the SSRI, zimelidine (Ogren et al., 1981), also significantly decreased the rate of ASP⁺ accumulation (vehicle: 5.04 ± 3.4 AFU; zimelidine: -40.9 ± 3.1 AFU; $p < 0.0001$, t-test; $t = 10.1$, $df = 116$; data not shown). A significant decrease in ASP⁺ accumulation was also observed in cells preincubated with zimelidine for 30 min prior to ASP⁺ addition (vehicle: 27.3 ± 3.3 ; zimelidine: 3.6 ± 0.3 AFU; $p < 0.0001$, t-test; $t = 9.5$ $df = 77$). No significant effect of zimelidine was observed in non-transfected cells of the same dishes (data not shown).

Kinase Regulation of ASP⁺ Uptake

Acute addition of anisomycin, an activator of p38 MAPK (Nahas et al. 1996), to cells, produced a transient increase in the rate of ASP⁺ accumulation. This effect was apparent within 2 min after drug addition (1 μ M; $p < 0.001$ vs veh; Fig 7A) and reversed by the p38 MAPK inhibitor PD169316 ($p < 0.001$, ANOVA: $F_{(3, 114)} = 12.3$; $p < 0.001$ Dunnett's test). Preincubation with PD169316 alone did not affect ASP⁺ accumulation. The effects of anisomycin diminished following more prolonged kinase activation. Although a significant increase was still apparent after 5 ($p < 0.001$, ANOVA: $F_{(3, 255)} = 5.9$; $p < 0.001$, Dunnett's test; Fig 7B) and 10 min ($p < 0.05$, ANOVA: $F_{(3, 252)} = 3.9$, $p < 0.001$ Dunnett's test, Fig 7C) exposure to drug, no increase was apparent after 30 min ($p \geq 0.5$, ANOVA: $F_{(3, 166)} = 0.8$; Fig 7D).

Prolonged activation of PKC decreases [³H] 5-HT uptake in heterologous expressions systems and synaptosomal preparations (Qian et al., 1997; Jayanthi et al., 2005; Samuvel et al., 2005). To determine whether ASP⁺ could be used to monitor decreases in SERT function, hSERT-expressing cells were incubated with the PKC activator phorbol 12-myristate 13-acetate (β PMA) for 30 min prior to ASP⁺ addition. Fig 8 shows that β PMA significantly decreased ASP⁺ accumulation relative to vehicle exposure ($p < 0.0001$, t-test; $t = 6.9$, $df = 161$).

DISCUSSION

The present imaging studies demonstrate that use of the fluorescent monoamine transporter substrate ASP⁺, in conjunction with confocal microscopy, enables real-time quantification of SERT function in heterologous cells. In EM4 or N2A cells expressing hSERT, accumulation of ASP⁺ was rapid and saturable. Accumulation varied as a function of temperature, the presence of sodium and chloride in the incubation media, and SERT expression levels. Acute or prolonged exposure of cells to the SSRIs citalopram or zimelidine reduced ASP⁺ accumulation. A similar effect was observed in response to 5HT. Activation of p38MAPK rapidly increased ASP⁺ accumulation. In contrast, accumulation was decreased in response to prolonged PKC activation.

ASP⁺ binds with micromolar affinity to monoamine transporters (Schwartz et al., 2003; Mason et al., 2005; Bolan et al., 2007). Although the utility of this fluorescent probe for

monitoring function of DAT and NET has recently been demonstrated, only two reports have assessed its utility for the quantification of SERT function. Using confocal microscopy, Schwartz et al. (2003) reported ASP⁺ accumulation in hSERT-transfected HEK293 cells, but they did not further characterize this phenomenon. More recently, Fowler et al. (2006) used high throughput molecular fluorimetry to assess hSERT-mediated uptake ASP⁺. Like radioligand uptake assays, however, this method yields information on populations of cells at single time points (10 to 60 min), and thus does not permit real time observation or a within-cells design.

The present study shows that ASP⁺ rapidly accumulates in the cytoplasm of EM4 or N2A cells transiently transfected with hSERT. The use of rapid image acquisition rates allowed identification of two phases of ASP⁺ accumulation: an initial, rapid (msec) process associated with substrate binding and a second, slower (sec-min) phase of cytosolic ASP⁺ accumulation.

Intracellular ASP⁺ accumulation in hSERT-transfected cells is specific and varies as a function of hSERT expression level. ASP⁺ accumulation was significantly greater in transfected as compared to non-transfected cells. Importantly, the magnitude of ASP⁺ accumulation in individual hSERT-transfected EM4 cells is proportional to hSERT expression level, suggesting a transporter-mediated effect.

[³H]5HT uptake is saturable and temperature dependent. In the present study, ASP⁺ accumulation was saturable over a concentration range of 1–50 μM, reaching maximum uptake at 30 μM. Accumulation at 25°C was significantly greater than at 6°C, demonstrating temperature dependence of this effect. hSERT is a co-transporter, transferring one sodium ion and one chloride ion with one molecule of 5HT (Rudnick 2006). Consistent with NaCl dependent transport, a reduction in ASP⁺ accumulation was seen when KRH was replaced with that containing low Na⁺ or Cl[−]. These findings suggest that ASP⁺ accumulation reflects an active uptake process.

The acute addition of citalopram to hSERT expressing-cells resulted in a concentration-dependent decrease in ASP⁺ uptake. This effect was rapid; it was apparent within 20 sec after drug addition. A similar increase in uptake was observed following acute addition of another SSRI, zimelidine. These data highlight the utility of live cell imaging procedures in delineating the time course of a drug effect since rapid changes in transporter activity cannot be resolved in radioligand uptake assays. Longer exposure to either citalopram (10 min) or zimelidine (30 min) significantly attenuates ASP⁺ accumulation. This finding is in accord with radioligand uptake studies and indicates that acute tolerance does not rapidly develop to SSRI-evoked increases in SERT function.

Two recent studies have shown that p38 MAPK activation upregulates SERT function (Samuvel et al., 2005; Zhu et al., 2005). Consistent with this finding, acute addition of the p38 MAPK activator, anisomycin, triggered a rapid, PD169316-reversible increase in the rate of intracellular ASP⁺ accumulation. A significant increase in accumulation rate was apparent within 2 min after anisomycin addition. This effect was still apparent after 5 and 10 min of anisomycin exposure. However, this effect dissipated after 30 min anisomycin exposure. As was observed in the present study, the increase in [³H] 5HT uptake observed in platelets was apparent after a 10 min incubation period (Zhu et al., 2005). In both studies, however, uptake returns to basal levels after chronic kinase activation indicating that tolerance develops to this effect. Studies in platelets have shown that anisomycin increases the K_m of 5HT uptake without affecting V_{max} . Although kinetic studies in EM4 cells are lacking, this finding provides suggestive evidence that ASP⁺ accumulation is sensitive to manipulations that affect the affinity of uptake.

Prolonged PKC activation down-regulates SERT function, an effect associated with transporter internalization (Jayanthi et al., 2005)). To determine whether ASP⁺ accumulation is altered in response to PKC activation, transfected cells were incubated with β PMA for 30 min and changes in ASP⁺ accumulation were quantified. β PMA treatment significantly decreased ASP⁺ uptake. These findings and those regarding the effects of p38 MAPK activation suggest that ASP⁺ can be used to monitor alterations in SERT function that result from changes in $V_{\max}$ as well as K_m . In this regard, it should be noted that the kinetics of ASP⁺ and 5HT may differ due to the distinct structure of the two substrates. Therefore, detailed studies are needed to determine the utility of ASP⁺ for kinetic analyses of SERT function.

PKC activation increases SERT phosphorylation in HEK-293 cells (Ramamoorthy et al., 1998) and the time course of this effect parallels decrease in 5-HT transport and SERT surface expression. Thus, SERT phosphorylation by the PKC-dependent pathway may trigger a reduction in 5-HT uptake and SERT internalization. For example, phosphorylation of the intracellular SERT pool may decrease SERT recycling or redirect trafficking events which contribute to changes in surface expression. Alternatively, SERT may be phosphorylated at the cell surface providing a signal for internalization, a change in SERT catalytic properties, or both. In contrast to PKC, p38 MAPK inhibition reduces basal SERT phosphorylation (Samuvel et al., 2005) suggesting that constitutively active p38 MAPK is involved in maintaining SERT basal phosphorylation, expression and activity. The findings that PKC activation increases SERT internalization and phosphorylation whereas inhibition of p38 MAPK reduces basal SERT phosphorylation and plasma membrane insertion suggests that p38 MAPK and PKC must act on different phospho-sites. If direct phosphorylation contributes to both processes, then these kinases may target SERT molecules at different cellular locations and that PKC and p38 MAPK mediated SERT basal phosphorylation may differentially impact SERT trafficking and catalytic activity. Future efforts directed towards the identification of the specific sites of PKC and p38 MAPK mediated phosphorylation will illuminate the cellular and molecular mechanisms of SERT regulation in response to PKC and p38 MAPK activation.

In summary, the present data demonstrate that ASP⁺ is a useful probe for real-time quantification of SERT function in living cells. Our studies demonstrate that changes in SERT function can be detected with < 1 μ m spatial resolution and <100 ms temporal resolution. Accumulation is linear over 10 min, thereby enabling detection of time-related changes in uptake rate relative to basal levels in the same cell. Using a between cells design, the influence of prolonged exposure to various agents can also be determined. These findings and those describing the use of ASP⁺ in high throughput screens of SERT ligands demonstrate that ASP⁺ is a novel tool for probing SERT function in cell expression systems that may allow greater understanding of transporter function and its regulation in normal and disease states.

Acknowledgments

We gratefully acknowledge the gift of pEGFP-hSERT from Dr. Harold Sitte. This research was supported by DHHS NIH/NIDA IRP and NIH grant (MH062612: S.R).

References

- Bereiter-Hahn J. Dimethylaminostyrylmethylpyridiniumiodine (daspmi) as a fluorescent probe for mitochondria in situ. *Biochimica et Biophysica Acta*. 1976; 423:1–14. [PubMed: 764877]
- Bolan EA, Kivell B, Jiligam V, et al. D2 receptors regulate dopamine transporter function via an extracellular signal-regulated kinase 1 and 2-dependent and phosphoinositide 3 kinase-independent mechanism. *Mol Pharmacol*. 2007; 71:1222–32. [PubMed: 17267664]

- Brune CW, Kim SJ, Salt J, Leventhal BL, Lord C, Cook EH. 5-HTTLPR Genotype-Specific Phenotype in Children and Adolescents With Autism. *Am J Psychiatry*. 2006; 163:2148–56. [PubMed: 17151167]
- Cannon DM, Ichise M, et al. Serotonin transporter binding in bipolar disorder assessed using [11C]DASB and positron emission tomography. *Biol Psychiatry*. 2006; 60:207–17. [PubMed: 16875929]
- Carneiro AM, Blakely RD. Serotonin-, protein kinase C-, and Hic-5-associated redistribution of the platelet serotonin transporter. *J Biol Chem*. 2006; 281:24769–80. [PubMed: 16803896]
- Fowler A, Seifert N, Acker V, Woehrle T, Kilpert C, de Saizieu A. A nonradioactive high-throughput/high-content assay for measurement of the human serotonin reuptake transporter function in vitro. *J Biomol Screen*. 2006; 11:1027–34. [PubMed: 17099247]
- Hu XZ, Lipsky RH, Zhu G, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet*. 2006; 78:815–26. [PubMed: 16642437]
- Hyttel J. Citalopram--pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. *Prog Neuropsychopharmacol Biol Psychiatry*. 1982; 6:277–95. [PubMed: 6128769]
- Jayanthi LD, Samuvel DJ, Blakely RD, Ramamoorthy S. Evidence for biphasic effects of protein kinase C on serotonin transporter function, endocytosis, and phosphorylation. *Mol Pharmacol*. 2005; 67:2077–87. [PubMed: 15774771]
- Johnson LA, Furman CA, Zhang M, Guptaroy B, Gnegy ME. Rapid delivery of the dopamine transporter to the plasmalemmal membrane upon amphetamine stimulation. *Neuropharmacology*. 2005; 49:750–8. [PubMed: 16212991]
- Lee HJ, Lee MS, Kang RH, et al. Influence of the serotonin transporter promoter gene polymorphism on susceptibility to posttraumatic stress disorder. *Depress Anxiety*. 2005; 21:135–9. [PubMed: 15965993]
- Mason JN, Farmer H, Tomlinson ID, Schwartz JW, Savchenko V, DeFelice LJ, Rosenthal SJ, Blakely RD. Novel fluorescence-based approaches for the study of biogenic amine transporter localization, activity, and regulation. *J Neurosci Methods*. 2005; 143:3–25. [PubMed: 15763132]
- Miller KJ, Hoffman BJ. Adenosine A3 receptors regulate serotonin transport via nitric oxide and cGMP. *J Biol Chem*. 1994; 269(44):27351–6. [PubMed: 7525554]
- Muller-Vahl KR, Meyer GJ, Knapp WH, Emrich HM, Gielow P, Brucke T, Berding G. Serotonin transporter binding in Tourette Syndrome. *Neurosci Lett*. 2005; 385:120–5. [PubMed: 15936877]
- Nahas N, Molski TF, Fernandez GA, Sha'afi RI. Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists. *Biochem J*. 1996; 318:247–53. [PubMed: 8761479]
- Neumeister A, Young T, Stastny J. Implications of genetic research on the role of the serotonin in depression: emphasis on the serotonin type 1A receptor and the serotonin transporter. *Psychopharmacology (Berl)*. 2004; 174:512–24. [PubMed: 15249991]
- Ogren SO, Ross SB, Hall H, Holm AC, Renyi AL. The pharmacology of zimelidine: a 5-HT selective reuptake inhibitor. *Acta Psychiatr Scand Suppl*. 1981; 290:127–51. [PubMed: 6452789]
- Qian Y, Galli A, Ramamoorthy S, Risso S, DeFelice LJ, Blakely RD. Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression. *J Neuro*. 1997; 17:45–57.
- Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T, Chang AS, Ganapathy V, Blakely RD. Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc Natl Acad Sci U S A*. 1993; 90:2542–6. [PubMed: 7681602]
- Ramamoorthy S, Blakely RD. Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. *Science*. 1999; 285:763–6. [PubMed: 10427004]
- Robbins AK, Horlick RA. Macrophage scavenger receptor confers an adherent phenotype to cells in culture. *Biotechniques*. 1998; 25:240–4. [PubMed: 9714883]
- Rotondo A, Mazzanti C, Dell'Osso L, Rucci P, Sullivan P, Bouanani S, Gonnelli C, Goldman D, Cassano GB. Catechol o-methyltransferase, serotonin transporter, and tryptophan hydroxylase

- gene polymorphisms in bipolar disorder patients with and without comorbid panic disorder. *Am J Psychiatry*. 2002; 159:23–9. [PubMed: 11772685]
- Rudnick G. Structure/function relationships in serotonin transporter: new insights from the structure of a bacterial transporter. *Handb Exp Pharmacol*. 2006; 175:59–73. [PubMed: 16722230]
- Sakai N, Kodama N, Ohmori S, Sasaki K, Saito N. Involvement of the actin cytoskeleton in the regulation of serotonin transporter (SET) activity: possible mechanism underlying SET regulation by protein kinase C. *Neurochem Int*. 2000; 36:567–79. [PubMed: 10771115]
- Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S. A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. *J Neurosci*. 2005; 25:29–41. [PubMed: 15634764]
- Schmid JA, Scholze P, Kudlacek O, Freissmuth M, Singer EA, Sitte HH. Oligomerization of the human serotonin transporter and of the rat GABA transporter 1 visualized by fluorescence resonance energy transfer microscopy in living cells. *J Biol Chem*. 2001; 276:3805–10. [PubMed: 11071889]
- Schwartz JW, Blakely RD, DeFelice LJ. Binding and transport in norepinephrine transporters. Real-time, spatially resolved analysis in single cells using a fluorescent substrate. *J Biol Chem*. 2003; 278:9768–77. [PubMed: 12499385]
- Steiner JA, Carneiro AM, Wright J, et al. cGMP-dependent protein kinase Ialpha associates with the antidepressant-sensitive serotonin transporter and dictates rapid modulation of serotonin uptake. *Mol Brain*. 2009; 2:26. [Epub ahead of print]. [PubMed: 19656393]
- Tordjman S, Gutknecht L, Carlier M, et al. Role of the serotonin transporter gene in the behavioral expression of autism. *Mol Psychiatry*. 2001; 6:434–9. [PubMed: 11443529]
- Vergne DE, Nemeroff CB. The interaction of serotonin transporter gene polymorphisms and early adverse life events on vulnerability for major depression. *Curr Psychiatry Rep*. 2006; 8:452–7. [PubMed: 17094925]
- Wong DF, Brasic JR, Singer HS, et al. Mechanisms of Dopaminergic and Serotonergic Neurotransmission in Tourette Syndrome: Clues from an In Vivo Neurochemistry Study with PET. *Neuropsychopharmacology*. 2008; 33:1239–1251. [PubMed: 17987065]
- Zapata A, Kivell B, Han Y, et al. Regulation of dopamine transporter function and cell surface expression by D3 dopamine receptors. *J Biol Chem*. 2007; 282:35842–35854. [PubMed: 17923483]
- Zhu CB, Carneiro AM, Dostmann WR, Hewlett WA, Blakely RD. p38 MAPK activation elevates serotonin transport activity via a trafficking-independent, protein phosphatase 2A-dependent process. *J Biol Chem*. 2005; 280:15649–58. [PubMed: 15728187]
- Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD. Adenosine receptor, protein kinase G, and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. *Mol Pharmacol*. 2004; 65:1462–74. [PubMed: 15155839]
- Zhu CB, Hewlett WA, Francis SH, Corbin JD, Blakely RD. Stimulation of serotonin transport by the cyclic GMP phosphodiesterase-5 inhibitor sildenafil. *Eur J Pharmacol*. 2004; 504:1–6. [PubMed: 15507214]

Figure 1. ASP⁺ binding to hSERT in transfected EM4 cells. *A–D*, Live cell images prior to and during ASP⁺ binding to cell-surface hSERT. *A*, pEGFP-hSERT fluorescence at time 0; *B–D*, ASP⁺ fluorescence at 0, 2 and 10 sec, respectively. *E*, Example of ASP⁺ binding to pEGFP-hSERT in EM4 cells ($n=14$) as a function of time (sec). Total (filled circle), specific (unfilled circle) and non-specific (filled triangle) ASP⁺ accumulation. Non-specific accumulation was defined as ASP⁺ accumulation in non-expressing cells. Error bars represent $\pm$ SEM.

Figure 2. Time-related intracellular ASP⁺ accumulation in EM4 cells transfected with pEGFP-hSERT. *A–D*, Live cell images of ASP⁺ intracellular accumulation. *A*, pEGFP-hSERT fluorescence at time 0; *B–D*, ASP⁺ accumulation at 0, 1 and 5 min, respectively. *E*, Total (filled circle), specific (open circle) and non-specific (filled triangle) ASP⁺ accumulation ($n=14$ cells) as a function of time (sec). The concentrated intracellular areas of ASP⁺ fluorescence likely reflect mitochondrial accumulation of ASP⁺ (Bereiter-Jahn, 1976). Non-specific accumulation was defined as accumulation in non-expressing cells. Error bars represent $\pm$ SEM. *F*, Comparison of ASP⁺ accumulation rate in pEGFP-hSERT transfected vs non-transfected cells. * denotes significant difference between groups ($p \leq 0.001$; Student's *t*-test)

Figure 3.

ASP⁺ binding and uptake of as a function of hSERT expression level. A, ASP⁺ binding is significantly correlated to hSERT cell surface expression (as measured by pEGFP fluorescence); $r^2=0.587$, $p=0.003$; $n=33$. B, ASP⁺ uptake is significantly correlated to hSERT cell surface expression; $r^2=0.748$, $p<0.001$; $n=51$. (Spearman coefficient) C, Saturation curve for ASP⁺ uptake in pEGFP-hSERT transfected cells. Nonlinear regression yielded a one phase exponential association, $V_{\max}=0.385 \pm 0.02$ AFU/sec, $K_m=159 \pm 27$ nM; goodness of fit, $r^2=0.97$. Number of cells: $n=39-56$.

Figure 4.

Ion dependence of ASP⁺ uptake. *A*, ASP⁺ binding is attenuated in the absence of sodium or chloride. Each column represents the mean $\pm$ SEM of $n=70-78$ cells. Asterisks denote significant difference from control (ANOVA: $p \leq 0.0001$; Dunnett's test: $p \leq 0.01$). *B*, ASP⁺ uptake is attenuated in the absence of sodium or chloride. Each column represents the mean $\pm$ SEM of $n=72-78$ cells. Asterisks denote significant difference from control (ANOVA: $p \leq 0.0001$; Dunnett's test: $p \leq 0.01$).

Figure 5. Inhibition of ASP⁺ uptake by increasing concentrations of 5-HT. The rate of ASP⁺ accumulation was assessed in the presence of varying concentrations of 5-HT (30–3000 nM). Ordinate: slope of ASP⁺ accumulation. Abscissa: 5-HT concentration. Each point represents the mean ± SEM of n=31–57 cells.

Figure 6.

Effects of citalopram on ASP⁺ uptake. A, Left: Mean ASP⁺ uptake prior to and after acute addition of vehicle in a single dish of cells (n=20). Right: Mean ASP⁺ uptake prior to and after acute addition of citalopram (10 μ M) in a single dish of cells (n=14). Ordinate: slope of ASP⁺ accumulation (AFU). Abscissa: time (sec) B, Concentration-dependent effects of acute citalopram on ASP⁺ accumulation rate (AFU). Each column represents the mean $\pm$ SEM of n=79–107 cells. Asterisks denote significant difference from vehicle (ANOVA: p<0.001, Dunnet's test: p<0.01). C, Effect of prolonged citalopram exposure on ASP⁺ uptake. ASP⁺ accumulation was quantified in cells incubated for 10 min in the presence of vehicle (n=81) or citalopram (10 μ M; n=81). Asterisk denotes significant difference from vehicle (Student's t-test: p<0.001).

Figure 7.

Effects of p38 MAPK stimulation on ASP⁺ uptake. A, ASP⁺ accumulation is significantly increased within 2 min after acute addition of 1 μ M of the p38 MAPK stimulator, anisomycin (Ani: ANOVA, Dunnett's test: $p < 0.001$). Addition of the p38 MAPK inhibitor PD169316 (PD: 1 μ M) does not alter ASP⁺ accumulation (ANOVA, Dunnett's test: $p > 0.05$) but significantly attenuates the effect of anisomycin (ANOVA, Dunnett's test: $p < 0.001$). B, C, Increased uptakes is still apparent 5 min and 10 min after anisomycin addition and is blocked by PD169316 (PD: 1 μ M). D, After 30 min preincubation, no effects of either anisomycin or PD169316 are observed (ANOVA: $p > 0.05$). Number of cells: vehicle, $n = 25$; anisomycin, $n = 53$; PD169316, $n = 45$; anisomycin + PD169316, $n = 47$.

Figure 8.

Effect of prolonged PKC activation on ASP⁺ accumulation. Cells were incubated with vehicle (n=86 cells) or βPMA (0.1 μM; n=77 cells) for 30 min prior to ASP⁺. Asterisk denotes significant difference from vehicle.