Single Nucleotide Polymorphisms in the Human Norepinephrine Transporter Gene Impact Expression, Trafficking, Antidepressant Interaction and Protein Kinase C Regulation

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Abbreviations: NE, norepinephrine; NET, norepinephrine transporter; hNET, human norepinephrine transporter; SNP, single nucleotide polymorphism; PKC, protein kinase C; KRH, Kreb’s-Ringer’s-HEPES; BIM, bisindolylmaleimide I; β−PMA, phorbol-12-myristate-13acetate; sulfo-NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate; RIPA, radioimmunoprecipitation; PMSF, phenylmethanesulfonyl fluoride; OCT1, organic cation transporter; TM, transmembrane domain; MTS, methanethiosulfonateSERT, serotonin transporter; DAT, dopamine transporter; 5-HT, serotonin; ADHD, Attention Deficit/Hyperactivity Disorder
ABSTRACT

The role of norepinephrine (NE) in attention, memory, affect, stress, heart rate and blood pressure implicate NE in psychiatric and cardiovascular disease. The norepinephrine transporter (NET) mediates reuptake of released catecholamines, thus playing a role in the limitation of signaling strength, in the central and peripheral nervous systems. Nonsynonymous single nucleotide polymorphisms (SNPs) in the human NET (hNET) gene that influence transporter function can contribute to disease, such as the nonfunctional transporter, A457P, identified in Orthostatic Intolerance. Here, we examine additional amino acid variants that have been identified, but not characterized, in populations that include cardiovascular phenotypes. Variant hNETs were expressed in COS-7 cells and assayed for protein expression and trafficking using cell-surface biotinylation and Western blot analysis, transport of radiolabeled substrate, antagonist interaction and regulation through protein kinase C (PKC) linked-pathways by the phorbol ester, β-PMA. We observed functional perturbations in 6 out of the 10 mutants studied. Several variants were defective in trafficking and transport with the most dramatic effect observed for A369P, which was completely devoid of the fully glycosylated form of transporter protein, was retained intracellularly and lacked any transport activity. Furthermore, A369P and another trafficking variant, N292T, impeded surface expression of hNET when coexpressed. F528C, demonstrated increased transport and, remarkably, exhibited both insensitivity to down-regulation by PKC and a decrease in potency for the tricyclic antidepressant desipramine. These findings reveal functional deficits likely to compromise NE signaling in SNP carriers in the population and also identify key regions of NET contributing to transporter biosynthesis, activity and regulation.
Norepinephrine (NE) released at central and peripheral synapses is inactivated through active transport into terminals by the presynaptically-localized norepinephrine transporter (NET) (Iversen, 1961). NET recaptures as much as 90% of released NE in the heart, making it a critical mediator of NE inactivation and presynaptic catecholamine homeostasis (Schomig et al., 1989). Indeed, NET knockout mice, exhibit a diminished rate of extracellular NE clearance in the brain, elevated extracellular NE concentrations in brain and plasma, reduced tissue NE concentrations in brain and heart, and altered dopamine (DA) signaling in brain (Xu et al., 2000; Keller et al., 2004). NET is also a target for tricyclic antidepressants, NET-selective reuptake inhibitors, and psychostimulants, including cocaine, methylphenidate and amphetamine (Fuller and Hemrick-Luecke, 1983; Tatsumi et al., 1997). The human NET (hNET) gene is a single copy gene (SLC6A2) located on chromosome 16 and containing 16 exons (Hahn and Blakely, 2002b). hNET is a member of the SLC6A family of Na+/Cl−-dependent transporters with a predicted protein topology of 12 transmembrane domains with intracellularly localized NH2 and COOH termini (Pacholczyk et al., 1991; Hahn and Blakely, 2002a). A large extracellular loop contains 3 N-glycosylation sites (Melikian et al., 1996). NET transport activity can be regulated by multiple signal transduction mechanisms, such as protein kinase C (PKC) (Apparsundaram et al., 1998).

The importance of NET to NE homeostasis suggests a role for NET in disorders of both the central and autonomic nervous systems. Involvement of noradrenergic systems in mood disorders is suggested by evidence that depression is accompanied by altered indices of noradrenergic function and that effective antidepressants enhance extracellular NE levels (Ressler and Nemeroff, 1999). Furthermore, NET binding sites are decreased in brains of patients with major depression (Klimek et al., 1997). The activation and sensitization of NE
systems in response to stress suggest that NE may play a role in disorders triggered by early life trauma, including depression and post-traumatic stress disorder (Heim and Nemeroff, 2001). NE also plays an important role in attention, vigilance, learning, and memory and is hypothesized to contribute to Attention Deficit/Hyperactivity Disorder (ADHD) (Biederman and Spencer, 1999). Stimulant drugs used to treat ADHD act on both the NET and DA transporter (DAT) and atomoxetine, which selectively targets NET, is also effective in treating ADHD (Biederman and Spencer, 1999). The activity of NET at postganglionic sympathetic nerve terminals, especially in the heart, is impacted in diseases of the cardiovascular system (Blakely, 2001; Hahn and Blakely, 2002a). Diminished NE uptake sites and activity have been observed in hypertension, diabetes, cardiomyopathy and heart failure and ischemia-induced efflux of nonvesicular, cytoplasmic NE via NET may also contribute to fatal arrhythmias (Hahn and Blakely, 2002a).

The importance of NET in the homeostasis of NE in brain and autonomic nervous system, evidence of its dysfunction in disease, and its role as a target for therapeutics raise the question of whether alterations in NE homeostasis or drug response derive from hNET genetic variation. A number of hNET promoter, intron, and coding region polymorphisms have been identified through both discovery-oriented studies of hNET in various clinical populations or during the course of genome sequencing efforts (Hahn and Blakely, 2002a). To date, approximately 20 nonsynonymous single nucleotide polymorphisms (SNPs), which result in amino acid substitutions, have been reported in hNET. Many of these variants were derived from psychiatric and cardiovascular phenotypes, yet only a limited number has been examined for alterations in expression or function (Stöber et al., 1996; Halushka et al., 1999; Runkel et al., 2000; Iwasa et al., 2001). Our laboratory identified, in a familial form of Orthostatic Intolerance, a nonsynonymous hNET SNP that produces the protein variant, A457P, a loss of function,
dominant-negative transporter that contributes to increased heart rate and plasma norepinephrine levels (Shannon et al., 2000; Hahn et al., 2003). Overall, these findings suggest that cardiovascular phenotypes could be enriched for hNET SNPs that produce functional alterations. In the present work, we examine the functional impact of amino acid variants, primarily found in cardiovascular phenotypes. We observe changes in protein expression levels, including aberrant processing by glycosylation and altered surface expression, with variants exhibiting abolished or greatly diminished plasma membrane expression. Furthermore, coexpression of hNET with variants exhibiting greatly altered processing generates a dominant negative impact on hNET expression and cell-surface targeting. We demonstrate changes in both NE and DA transport that reveal effects of variants on substrate selectivity. Finally, in a single variant, F528C, we observe both a shift in the potency of the tricyclic antidepressant, desipramine and a novel regulatory phenotype of resistance to regulation through PKC-mediated pathways.
METHODS

Plasmids constructs

The expression vector pcDNA3 (Invitrogen Life Technologies, Carlsbad, CA) containing the coding sequence for hNET, pcDNA3-hNET, bearing an introduced AflII site was utilized in the construction of the hNET nonsynonymous SNPs, R121Q, V244I, N292T, V356L, A369P, N375S, K463R, F528C, Y548H and I549T. Single-point mutations were generated using the QuikChange site-directed mutagenesis kit (Strategene Cloning Systems, La Jolla, CA) according to the manufacturer’s instructions. A Bgl II site was created at position 436 (Accession no. NM_001043) to facilitate subcloning. Sequences were confirmed using dideoxynucleotide terminators (Center for Molecular Neuroscience Neurogenomics Core, Vanderbilt University). In some experiments, an HA epitope-tagged hNET construct was utilized to distinguish hNET from cotransfected hNET variants.

Cell culture and transfection

All experiments were performed in transiently transfected COS-7 cells (American Type Tissue Collection, Manassas, VA). COS-7 cells were maintained in Dulbecco’s modified eagle medium (Gibco/Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Gibco/Invitrogen) and 100 U/ml penicillin/100 µg/ml streptomycin (Gibco/Invitrogen) in a humidified incubator at 37°C and 5% CO2. One day prior to transfection, cells were plated in individual wells of 24-well plates at a density of 5 x 10^4 cells/well. Transfection was performed using Fugene 6 reagent as described by the manufacturer (Roche Applied Science, Indianapolis, IN). All experimental manipulations
were begun ~24 hours after transfection. Observations in all experiments were obtained using multiple DNA stocks to control for any variability in DNA plasmid preparation.


Transport was assayed essentially as described (Hahn et al., 2003). Briefly, cells were washed twice with Kreb’s-Ringer’s-HEPES (KRH; 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 2.2 mM CaCl₂, 10 mM HEPES, pH 7.4) buffer and preincubated with assay buffer (KRH, 10 mM D-glucose, 100 µM ascorbic acid, 100 µM pargyline and 1 mM tropolone) for 10 min at 37°C, with 1 µM desipramine added to a subset of wells to assess nonspecific accumulation, followed by the addition of radiolabeled substrate for a 10 min transport assay. In experiments using a single concentration of substrate, 50 nM of [[^3]H]NE or [[^3]H]DA (~36 Ci/mmol or ~49 Ci/mmol, respectively; Amersham Biosciences AB, Uppsala, Sweden) was used. Saturation kinetics to determine Kₑₘ and Vₘₐₓ values were carried out using serial dilutions of NE or DA (100 nM-10 µM) with [[^3]H]NE or [[^3]H]DA of constant specific activity, respectively. In experiments assessing the effects of drugs on transport, drugs were added prior to addition of [[^3]H]NE and this preincubation time varied. In experiments of phorbol ester regulation of NET, cells were incubated in 1 µM bisindolylmaleimide I (BIM; Calbiochem, San Diego, CA) or 1 µM staurosporine (Sigma-Aldrich Corporation, St. Louis, MO) for 20 min, followed by the addition of 100 nM phorbol-12-myristate-13-acetate (β−PMA; Sigma-Aldrich), for 5, 10 or 30 min prior to the addition of [[^3]H]NE. In some experiments, cells were treated with 100 nM or 1µM β−PMA for 30 min prior to the addition of [[^3]H]NE. In experiments to determine the inhibition of [[^3]H]NE uptake by antagonists, cells were incubated for 10 min at 37°C with cocaine (Sigma) or desipramine (Sigma) followed by the addition of [[^3]H]NE to a final concentration of 50 nM for 10 min of uptake at 37°C. Following uptake, cells were washed three times in KRH,
incubated for 2 hr in Microscint 20 scintillation fluid (Packard) and accumulated radiolabeled substrate was quantified in a Topcount plate scintillation counter (Packard).

$K_M$ and $V_{MAX}$ values were calculated by nonlinear regression analysis according to a single site hyperbolic model (Prism 4, Graphpad Software, San Diego, CA). $K_I$ values for inhibition of $[^3H]NE$ uptake by antagonists were calculated for data expressed as percent inhibition of total uptake versus the log of drug concentration by non-linear regression analysis according to a single site competition model (Prism).

**Cell surface biotinylation and immunoblots**

To investigate the effect of hNET SNPs on protein expression and trafficking, cell-surface biotinylation was performed on intact cells (Hahn et al., 2003). Cells were washed 4X in 1X PBS and incubated with 1.0 mg/ml sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce Biotechnology, Inc., Rockford, IL) in PBS containing 0.1 mM CaCl$_2$ and 1.0 mM MgCl$_2$ (PBS/Ca/Mg) for 20 min at 4°C, washed, quenched with 3 washes of 100 mM glycine in PBS/Ca/Mg and washed 2 times in PBS/Ca/Mg. Cells were lysed in radioimmunoprecipitation (RIPA) buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 250 µM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µM pepstatin) for 30 min at 4°C, centrifuged at 20,000 x g for 30 min and supernatants were assayed for protein concentration using a BCA kit (Pierce). Equal amounts of protein were incubated with Immunopure Immobilized Streptavidin beads (Pierce) for 45 min at RT. Beads were washed four times in RIPA buffer and proteins bound to beads were eluted in 1X sample buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue). Samples were then separated by 10% SDS-
Proteins were transferred electrophoretically to Immobilon-P membrane (Millipore, Bedford, MA). Membranes were incubated with a monoclonal antibody directed against hNET at a dilution of 1:500 (NET17-1; Mab Technologies, Inc., Stone Mountain, GA), followed by incubation with a goat anti-mouse peroxidase-conjugated secondary antibody at a dilution of 1:5000 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). In cotransfection experiments that utilized an HA-tagged hNET an HRP-conjugated anti-HA antibody was used at a dilution of 1:500 (Roche). Visualization of immunoreactivity was achieved using Western Lightning enhanced chemiluminescent reagent (Perkin Elmer Life Sciences, Inc., Boston, MA).

**Quantitation of Immunoblots**

Quantitation of band density was performed on scanned images using ImageJ, a public domain image processing program (W. Rasband, NIMH, Bethesda, MD). The optical density of each lane was plotted and area under the curve measured for both the 110 and 54 kDa NET immunoreactive bands. Analyses were performed on replicate experiments and data are presented both in graph form of the means ± S.E.M. of repeated experiments and with blots of a representative experiment.

**Statistical Analysis**

Data were analyzed using one-way analysis of variance (ANOVA) with levels of significance set at p < 0.05 (SPSS for Windows, release 7.0). The contribution of individual group means to overall significant F values was determined by Fisher’s Least Square Difference post hoc test, with p < 0.05 considered significant.
RESULTS

Multiple naturally-occurring variants have been identified in hNET, though only a few have been functionally assessed (Fig. 1; Halushka et al., 1999; Runkel et al., 2000; Shannon et al., 2000; Iwasa et al., 2001; Hahn et al., 2003). In the present work, we examined a group of these uncharacterized SNPs, most of which were detected in populations with cardiovascular phenotypes. (Fig. 1, white-filled circles; Table 1). Seven of these SNPs, encoding the amino acid changes N292T, V356L, A369P, N375S, K463R, F528C and Y548H, were identified in a study of both Africans and Americans with Northern European ancestry with blood pressure measures in the upper and lower 2.5th percentile using high density microarray chips (Halushka et al., 1999). R121Q was found in a study of Japanese patients with long QT syndrome and was identified in both the control and long QT groups (Iwasa et al., 2001). I549T has been observed by several groups in populations of Asian and European ancestry and is deposited at dbSNP, the SNP database at the National Center for Biotechnology and Informatics (NCBI). These SNPs have been validated by observance of the minor allele in more than one chromosome and/or by multiple independent submissions and Table 1 lists their estimated frequencies (dbSNP; (Halushka et al., 1999; Iwasa et al., 2001). Our laboratory, using denaturing high performance liquid chromatography (DHPLC) followed by dideoxy sequencing confirmation, identified V244I in one individual with no defined phenotype. These SNPs occur at amino acids with extremely high conservation among NETs of various species as well as among NETs, DATs and serotonin transporters (SERTs) (Table 2). The variants occur throughout the transporter, in both transmembrane domains (TMs) and loop regions (Figure 1). Site-directed mutagenesis was used to generate these SNPs in the hNET cDNA in the expression vector, pcDNA3.
All assays were performed 24 hr following transfection of the hNET cDNA (hereafter termed hNET) or variant hNETs at equal DNA concentrations into COS-7 cells. To assess the influence of hNET SNPs on transporter expression, total and cell surface protein was measured using a cell-impermeant biotinylating reagent followed by immunoblotting of Streptavidin-recovered protein. Previous work from our lab demonstrates that steady state profiles of hNET from heterologous expression systems comprise several different species of protein arising from different stages of progression through the biosynthetic pathway (Melikian et al., 1994; Hahn et al., 2003). In transiently transfected COS-7 cells, the major forms observed are 46, 54 and 80-110 (termed 110 hereafter) kDa that represent, respectively, unglycosylated, core glycosylated and highly glycosylated forms of the transporter, with the 110 kDa form preferentially expressed on the plasma membrane (Hahn et al., 2003). Analysis of hNET variants revealed several distinct protein expression profiles (Figs. 2 and 3). Evaluation of the 54 and 110 kDa forms of NET in total and surface pools revealed mutant-dependent changes in protein expression levels suggestive of alterations in both glycosylation and trafficking (total 54 kDa form, $F_{10,41} = 21.25; p < 0.001$; surface 54 kDa form, $F_{10,36} = 10.47; p < 0.001$; total 110 kDa form, $F_{10,40} = 7.64; p < 0.001$; surface 110 kDa form, $F_{10,42} = 10.53; p < 0.001$). A369P expressed the 54 kDa form of hNET at levels comparable to hNET, whereas the 110 kDa form was essentially absent in total lysates and in the biotinylated fraction (Fig. 2A and B; Fig. 3A and B). F528C demonstrated a 22% increase in total lysate levels of the 110 kDa protein and a 35%, increase in 110 kDa surface levels (Fig. 2A and B; Fig. 3A and B). Total and surface levels of the 110 kDa form of R121Q were $83.8\pm4.1$ and $72.1\pm5.9$ of hNET, respectively, with surface levels significantly different from hNET (Fig. 2A and B; Fig. 3A and B). Y548H expressed surface levels of the 110 kDa form that were $71.7\pm10.6$ of hNET, a decrease that did not reach significance (Fig. 2B; Fig. 3B).
N292T shows an aberrant glycosylation pattern, in which a large amount of protein remains in the nonglycosylated 46 kDa form and other forms of varying size are present, including the 54 and 110 kDa forms, creating a smeared appearance on blots (Fig. 2A). The 110 kDa form of N292T in total lysate was 20.0±12.9 of hNET, although this value is difficult to estimate due to the continuous nature of the distribution of N292T protein. Interestingly, the biotinylated fraction reveals that much of the anomalous N292T remains intracellular whereas the 110 and 54 kDa forms are expressed on the surface, as well as the nonglycosylated 46 kDa form (Fig 2B).

The surface levels of the 110 kDa form are decreased to levels 50.7±13.7 of hNET whereas there was a large increase in the surface expression of the 54 kDa form (Fig 2B; Fig 3B). Finally, there was a significant decrease in I549T protein in surface fractions not evident in totals (Fig. 2 and 3).

Transport assays of 50 nM [³H]NE or [³H]DA were next performed to determine the functional capacity of all hNET variants. Several SNPs altered transport of [³H]NE (F₁₀,₃₂ = 23.61; p < 0.001) or [³H]DA (F₁₀,₃₂ = 19.26; p < 0.001; Fig. 4; Table 3). A369P failed to transport either [³H]NE or [³H]DA (Fig. 4). N292T exhibited decreased transport of [³H]NE and [³H]DA (62.2±4.8 and 67.7±6.6% of hNET, respectively), whereas Y548H demonstrated a more modest reduction of transport of [³H]NE and [³H]DA (77.7±6.3 and 79.8±% of hNET, respectively; Fig. 4). Cells expressing F528C yielded an increase in [³H]NE transport to 131.0±11.1% of hNET. Interestingly, [³H]DA transport was unchanged for this variant. R121Q transport rates of [³H]NE and [³H]DA were decreased to 56% and 72% of hNET, respectively. Comparison of the ratios of [³H]NE to [³H]DA transport among transporter variants revealed significant changes in substrate selectivity (F₁₀,₃₂ = 2.64; p< 0.05; Table 3). As noted, F528C demonstrated an increased transport of [³H]NE versus [³H]DA as compared to hNET. Although
the change in the ratio of R121Q \[^3\text{H}\text{NE}\] to \[^3\text{H}\text{DA}\] transport did not reach significance in the single point assay, significant differences were observed in R121Q substrate transport when saturation kinetics were determined (see below).

Saturation kinetics for both \[^3\text{H}\text{NE}\] and \[^3\text{H}\text{DA}\] transport were obtained for mutants that demonstrated altered transport in the single point assays. Due to its complete lack of surface expression and transport, A369P was not included in these assays. Cells expressing R121Q, N292T, F528C or Y548H all demonstrated altered kinetic profiles compared to hNET (NE, F\(_{4,14}\) = 18.50; \(p < 0.001\); DA, F\(_{4,14}\) = 28.15; \(p < 0.001\)). We also examined the \(V_{\text{MAX}}\) to \(K_{\text{M}}\) ratios, as a measure of transporter efficiency and found ratios significantly changed for several mutants (NE, F\(_{4,14}\) = 93.5; \(p < 0.01\); DA, F\(_{4,14}\) = 5.11; \(p < 0.05\)). First, similar to findings in the single point assays, F528C demonstrated an increased the \(V_{\text{MAX}}\) for \[^3\text{H}\text{NE}\] transport (Fig 5A). The F528C \(K_{\text{M}}\) for NE, although consistently somewhat lower, was not significantly different from hNET. DA \(V_{\text{MAX}}\) was also elevated (Fig 5B). The \(V_{\text{MAX}}\) to \(K_{\text{M}}\) ratio of F528C transport of \[^3\text{H}\text{NE}\] was twice that of hNET (6.1 ± 0.3 versus 3.1 ± 0.2, respectively). whereas the \(V_{\text{MAX}}\) to \(K_{\text{M}}\) ratio for \[^3\text{H}\text{DA}\] was not significantly elevated. Saturation kinetics of R121Q revealed a significant decrease in \(V_{\text{MAX}}\) for \[^3\text{H}\text{NE}\] transport to 65.6±1.4% of hNET. In contrast, the \(V_{\text{MAX}}\) for \[^3\text{H}\text{DA}\] transport decreased only to 91.0±8.1% of hNET (Fig. 5A and B). The R121Q \(V_{\text{MAX}}\) to \(K_{\text{M}}\) ratio was decreased to half that of hNET using \[^3\text{H}\text{NE}\] as the substrate (1.4 ± 0.3 versus 3.1 ± 0.2, respectively), but remained unchanged with respect to \[^3\text{H}\text{DA}\]. This was contributed to by a consistent, though not significant, increase in the R121Q \(K_{\text{M}}\) for NE. N292T was diminished in both \[^3\text{H}\text{NE}\] and \[^3\text{H}\text{DA}\] transport, with \(V_{\text{MAX}}\) values 43.5±6.3 and 48.1±5.6% of hNET, respectively (Fig. 5A and B). Y548H NE and DA \(V_{\text{MAX}}\) values were 87.8±5.4 and 82.2±3.2% of
hNET, respectively, changes that reached significance for the decrease in DA transport (Fig. 5A and B).

We also tested the effect of hNET variants on the ability of transporter inhibitors to compete for \[^3H\]NE transport, targeting cocaine and desipramine as a prototypical psychostimulant and tricyclic antidepressant, respectively. In general only modest shifts in $K_i$ values were observed. One exception was F528C, which demonstrated a significant increase in the $K_i$ value for desipramine competition of NE uptake, losing potency by approximately 8-fold (4.7 ± 0.6 vs. 40.9 ± 6.0 nM; $F_{9,30} = 24.50$; p<0.001.

The changes in F528C and R121Q expression levels coupled with substrate-specific transport properties suggested that, in addition to trafficking alterations, there might be compromised regulation by signal transduction mechanisms. A well-studied mechanism of hNET regulation is the PKC-mediated down-regulation that occurs in response to receptor stimulation or direct activation of PKC with phorbol esters (Apparsundaram et al., 1998). We therefore tested the ability of the phorbol ester, $\beta$-PMA, to down-regulate R121Q and F528C compared to hNET. Thirty min of 100 nM or 1 $\mu$M $\beta$-PMA decreased $[^3H]$NE uptake by approximately 40% (Fig 6A). Treatment with either 100 nM or 1 $\mu$M $\beta$-PMA for 30 min resulted in a significantly greater decrease in transport by R121Q compared to hNET, whereas F528C was unaffected by $\beta$-PMA treatment (Fig. 6A). Incubation with 100 nM $\beta$-PMA for 10 min in the presence or absence of PKC inhibitors was performed to examine the specificity of these effects. Ten min $\beta$-PMA treatment resulted in a 25% decrease in hNET $[^3H]$NE transport that was completely blocked by 1 $\mu$M staurosporine or 1 $\mu$M BIM (Fig. 6B; $F_{5,17} = 9.88$; p < 0.001). R121Q again demonstrated a greater decrease in $[^3H]$NE transport compared to hNET in response to 10 min of $\beta$-PMA treatment (p < 0.05; Fig. 6B). The effect on R121Q was also
completely blocked by staurosporine or BIM (F_{5,17} = 6.38; p < 0.01). F528C was completely resistant to the effects of 10 min treatment with 100 nM β-PMA (F_{5,17} = 1.23; Fig. 6B). Interestingly, there was a trend for PKC inhibitors to increase basal levels of transport for hNET and R121Q, an effect that was significant for the staurosporine-induced increase in uptake for hNET (Fig. 6B). This suggests the presence of tonic regulation of hNET in COS-7 cells that can be relieved by staurosporine. Interestingly, F528C showed no such effect of PKC inhibitors on basal transport, again indicating the insensitivity of F528C to regulation by PKC-linked pathways.

The heterozygous nature of hNET SNPs coupled with evidence of oligomer formation by neurotransmitter transporters compelled us to examine the influence of coexpression of hNET variants on hNET. Variants that exhibited altered processing and trafficking of transporter protein, R121Q, F528C, N292T and A369P, were each cotransfected with an equal amount of HA-tagged hNET followed by cell-surface biotinylation and Western blot analysis. Both N292T and A369P exhibited marked effects on hNET expression when cotransfected (total 54 kDa form, F\(_{(4,14)}\) = 5.61; p < 0.05; surface 54 kDa form, F\(_{(4,14)}\) = 16.58; p < 0.001; surface 110 kDa form, F\(_{(4,14)}\) = 12.05; p < 0.001; total 110 kDa F\(_{(4,13)}\) = 10.39; p < 0.01). N292T produced a dramatic decrease in total hNET expression of both 54 and 110 kDa forms and greatly diminished surface expression (Fig. 7A-D). A369P also diminished total and surface expression of hNET (Fig. 7A-D). [3H]NE transport for all hNET and variant coexpressed conditions was intermediate to transport levels of hNET or the variant expressed alone (data not shown). We also measured the effect of β-PMA in cotransfection experiments, to test the ability of variants to confer functional changes to a presumed transporter complex. Cotransfection of R121Q or F528C with hNET followed by 30 min treatment with 1 µM β-PMA resulted in regulation that
was intermediate between the effects of β-PMA on hNET or either variant expressed singly (data not shown). Similarly, cotransfection of R121Q and F528C, which individually produce the most divergent phenotypes, with respect to β-PMA regulation, also generated a β-PMA response intermediate to that observed in cells transfected with either variant alone (data not shown).

In order to evaluate a potential correlation of functional effects with amino acid substitution of each variant, we examined the BLOSUM62 scores of the hNET SNPs. The BLOSUM62 is a scoring matrix that infers protein function for amino acid substitutions (Henikoff and Henikoff, 1992). In a study that examined gene mutations in the organic cation transporter, OCT1, Leabman and coworkers (2003) predicted that substitutions at residues conserved in all members of mammalian OCT1 orthologs would be more deleterious than those at evolutionarily unconserved residues (Leabman et al., 2003). The majority of OCT1 mutations at conserved residues decreased function whereas none of the unconserved changes affected function. Furthermore, the BLOSUM62 scores were more negative (evolutionarily unfavorable) for the variants that decreased function and most with non-negative scores exhibited normal function. When the hNET SNPs evaluated in the present study were divided into two groups, one with those found to exert effects on protein expression, substrate transport, inhibitor binding or regulation by PKC (R121Q, A369P, N292T, F528C, Y548H and I549T) and a second having no effect on these measures (V244I, V356L, N375S and K463R), the group of variants conferring functional changes had significantly lower BLOSUM62 scores, -1.14 ± 0.28, than those deemed to have no effects on function, 1.75 ± 0.48 (p < 0.05; Student’s t-test, 2-tailed).
DISCUSSION

Although it is likely that complex disorders result from multiple gene and environmental influences, candidate gene approaches remain attractive when a strong argument can be advanced for the role of a gene in disease, particularly when endophenotypes are examined. We previously identified a nonfunctional, dominant-negative hNET mutation that contributes to a phenotype of Orthostatic Intolerance, tachycardia and elevated plasma NE levels (Shannon et al., 2000; Hahn et al., 2003). Most of the SNPs in the present study were also identified in extreme blood pressure or long QT syndrome (Halushka et al., 1999; Iwasa et al., 2001). The present results reveal striking effects of these naturally-occurring SNPs on transporter protein expression, substrate transport, antagonist interaction and regulation by kinase-mediated signaling pathways. The use of selective phenotypes should continue to reveal hNET variants with functional consequences that, though they may be limited to a small number of cases or families, could greatly contribute to phenotypes in those individuals.

Among the hNET variants evaluated in the present study, V244I, V356L, N375S, and K463R, had little effect on hNET expression levels or transport of NE and DA, and could represent variation retained in the population due to a lack of negative selection pressure, as evidenced by the positive BLOSUM62 scores of this group and the presence of alternate amino acids at several of these positions in NET of other species (Table 2). Other amino acids substitutions at these positions, however, have revealed some functional impact. Whereas the extracellular loop 4 variant, N375S, was without effect, N375P increases uptake 2-fold (Roubert et al., 2001). Similarly, the engineered mutant V356S demonstrates an increase in DA uptake, and a decreased affinity for nortriptyline and desipramine, whereas the naturally-occurring polymorphism V356L did not differ in transport or antagonist affinity.
More than half of the SNPs examined resulted in demonstrable changes in expression, activity, binding or regulation, and, moreover, these SNPs correlated with negative BLOSUM62 scores, indicating unfavorable substitutions. A369P generated the most dramatic effect, revealing a complete lack of transport of NE and DA that was accompanied by a total loss of the surface 110 kDa protein. The 54 kDa form of A369P was expressed on the surface, yet this species was nonfunctional. As we detected minimal accumulation of the immature form of A369P as well as a form migrating at a molecular weight higher than the 110 kDa form that we suspect represents intracellular aggregates, A369P is likely a misfolded protein that is targeted for degradation following exit from the ER, a pattern similar to that observed for the hNET A457P mutation (Hahn et al., 2003). Interestingly, an hNET mutant of multiple residues, that incorporates amino acids close to A369 in extracellular loop 4, also does not transport (Roubert et al., 2001) pointing to an important role of extracellular loop 4 in transport biosynthesis and function.

Two of the mutants, Y548H and N292T displayed a graded loss of both DA and NE transport. Transport deficits of N292T were similar in degree to reductions in levels of the 110 kDa form, indicating that the 54 kDa form, which demonstrated increased surface expression, is not functional. Interestingly, a triple mutation in the third extracellular loop that includes an N292R substitution exhibited a 7-fold increased capacity for DA (Roubert et al., 2001). Y548H, located in extracellular loop 6, reduced both NE and DA uptake and surface transporter by approximately 20%. I549T also demonstrated a decrease in protein, but not in transport. The disparity between these measures for I549T remains to be clarified in future studies, but its proximity to Y548 suggests a sensitivity of this region of the transporter to substitutions.
The variants discussed thus far that affected transport, A369P, N292T and Y548H, are located in extracellular loops 3-6, and these loops, as well as proximal TMs, have been implicated in transport function. Studies of DAT/NET chimeras suggest that the region spanning TMs 4-9 are critical for substrate translocation (Buck and Amara, 1994). Chimeras of SERT containing the NET extracellular loops 4, 5, or 6 retain antagonist selectivity but demonstrate greatly reduced transport activity (Smicun et al., 1999). Furthermore, MTS reagent sensitivity studies reveal that residues close to the extracellular face of TM 7 or 8 of human DAT are important for substrate and antagonist interactions (Norregaard et al., 2003).

F528C, located in TM 11, demonstrated a selective change in transport of NE versus DA, evident by an increase in both the ratio of NE to DA transport and ratio of $V_{\text{MAX}}$ to $K_{M}$ for NE compared to hNET. F528C does not differ from hNET in $K_{i}$ for NE competition of either $[^3H]$nisoxetine binding or $[^3H]$DA uptake, suggesting that there is an increase in translocation efficiency of NE at F528C (data not shown). Residues in TM 11 and 12 of SERT influence potencies of both substrates and tricyclic antidepressants (Barker et al., 1994). Similar to F528C, S545A in TM 11 of SERT, corresponding to S525 in hNET, increases the $V_{\text{MAX}}$ for serotonin transport and decreases the binding affinity for the tricyclic antidepressant, imipramine (Sur et al., 1997). Furthermore, a single F in this region directs potency of hSERT for tricyclic antidepressants (Barker and Blakely, 1996). In contrast, F530A in rat DAT, corresponding to F528 in hNET, demonstrates little effect on expression levels, yet transport activity is reduced by greater than 90% (Lin et al., 1999). To the extent that F528C may be enriched in an extreme blood pressure phenotype, it could influence tricyclic treatment response in such individuals. Taken together, our data and the results of mutational analyses support the involvement of
TM11, generally, and F528 in hNET, specifically, in both substrate affinities and translocation and antagonist affinities.

The phenotype of R121Q highlights the important role of intracellular loop 1 in transporter trafficking and regulation. The greater impact on NE transport, compared to either DA transport or surface protein levels, is inconsistent with an impact conferred solely by altered surface density. Sucic et al. noted that R121 is part of a conserved sequence GXXXRXG that is similar to a GXXXXRXG motif present in the bacterial Tn10 tetracycline antiporter, and in which the positively charged R is required for transport (Yamaguchi et al., 1992). These authors also observed reductions in transport and expression with substitution of G at R121 in NET (Sucic and Bryan-Lluka, 2002). Interestingly, conversion of rat DAT intracellular loop 1 to the corresponding hNET residues decreases basal uptake and blocks ethanol-induced activation of rat DAT (Maiya et al., 2002). Our preliminary studies indicate that R121Q also appears more sensitive to the effects of ethanol (data not shown).

The ability of variants R121Q and F528C to selectively confer changes to NE versus DA transport has precedent in other studies of mutations that produce a substrate-selective impact on transport. For example, species differences between drosophila and human SERTs in binding and transport affinities for substituted tryptamines, but not serotonin, can be closely recapitulated by the single mutation, Y95F, in human SERT (Adkins et al., 2001). Studies of rat and human SERT chimeras indicate that TM 11 and 12 of human SERT account for the difference in potency of d-amphetamine relative to rat SERT, whereas serotonin recognition is unaffected (Barker et al., 1994). Differences in substrate utilization by hNET variants suggest a potential impact of variants on the balance of catecholamine neurotransmission in areas of the central nervous system where dual innervation by NE and DA occurs. In the prefrontal cortex, where
both NE and DA fibers are present, released DA is cleared from the extracellular space by NET (Gresch et al., 1995). The therapeutic effect of selective NET blockers in the treatment of ADHD may lie in their ability to elevate both NE and DA.

The observation that F528C was insensitive and R121Q more sensitive to $\beta$–PMA suggests that similar mechanisms may underlie differences observed in both basal and regulated transport. F528C was insensitive to staurosporine inhibition, supporting that PKC activity present in untreated cells may play a part in differences we observe in basal transport. In addition, PKC-induced down-regulation results not only in a decrease in hNET $V_{MAX}$ but also influences NET intrinsic activity (Apparsundaram et al., 1998; Sung et al., 2003) and such a function may play a role in effects observed on hNET variants in the present study.

A369P and N292T both decreased total and surface levels of hNET supporting growing evidence that intracellularly-retained or mistargeted mutants exert dominant-negative effects to decrease surface expression and activity of wild type transporters facilitated by formation of oligomeric complexes (Hahn et al., 2003; Sitte et al., 2004). Elevated levels of unglycosylated and anomalously glycosylated N292T may additionally saturate synthesis and glycosylation processes. That N292T accumulates in lower molecular forms, whereas it greatly diminishes cotransfected hNET, suggests that N292T may be a more stable protein. The normal core glycosylation but defect in late-stage glycosylation exhibited by A369P is similar to that of our previously described variant, A457P, a variant that restricts hNET surface expression and has a dominant-negative impact on hNET transport (Hahn et al., 2003). It was somewhat surprising that neither N292T nor A369P diminished transport to an extent that might be predicted from the effect on hNET protein. A457P exhibits measurable surface expression of a nonfunctional 110 kDa form that may contribute to its dominant-negative influence on transport. Additionally, the
lack of effect on transport may reflect a limitation of heterologous expression where overexpression and unknown stoichiometry of interacting proteins may mask effects. Cotransfection of hNET with F528C or R121Q yielded shifts in the β-PMA response that were intermediate to the effects on either hNET or variant alone. Thus, one regulation phenotype did not dominate function under conditions of coexpression. Taken together, these results, coupled with our previous data and that of other groups, support that dominant-negative associations with variants can occur at early stages of transporter biosynthesis to influence hNET biosynthetic progression, whereas evidence of functional interactions between subunits at the level of the plasma membrane remain to be explored further.

The present data reveal striking influences of naturally-occurring hNET coding variants on hNET function that may indeed contribute to in vivo function, as suggested by their discovery in cardiovascular phenotypes. In this regard, a recently identified SERT variant demonstrates both faster translocation and altered response to second messengers in vitro and can be tracked in carriers within two families presenting with complex psychiatric profiles, including obsessive compulsive disorder and social phobia (Kilic et al., 2003). It will be important to examine the expression of hNET variants in transfected neuronal culture, in which depolarization, second messengers, and voltage-sensitive transporter-associated currents likely all play a role in regulating transporter activity (Galli et al., 1998; Savchenko et al., 2003). Additionally, genetically modified mice bearing functional variants should facilitate the analysis of systems-level questions of transporter response in the face of neuronal excitability and second messenger activation, the influence of substrate selectivity, and the impact of these factors on behavior.
ACKNOWLEDGEMENTS

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REFERENCES


FOOTNOTES

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Figure Legends:

**Figure 1:** Schematic representation of hNET depicting the amino acid variants generated by nonsynonymous SNPs that have been identified (Stöber et al., 1996; Halushka et al., 1999; Shannon et al., 2000; Iwasa et al., 2001), dbSNP. hNET is depicted as a 12 transmembrane domain spanning protein with intracellular NH2- and COOH-termini. For the variants shown, the number refers to the amino acid position in the protein and is preceded by the single-letter code for the amino acid commonly at that position followed by the single letter code for the variant. The approximate positions of the variant residues are indicated by the white- and grey-filled circles. The variants represented by white-filled circles are those evaluated in the present report.

**Figure 2:** A. Western blot analysis of hNET and variant total cellular lysate (A) and surface expression (B). COS-7 cells were transfected with hNET or variant cDNAs as described in Methods. 24 h later cells were incubated in sulfo-NHS-SS-biotin followed by extraction in RIPA buffer containing protease inhibitors. Aliquots containing equal amounts of protein were taken from each sample for total hNET and from the remaining sample aliquots of equal amounts of protein were extracted with streptavidin beads as described in Methods. Blots were probed with a monoclonal antibody to hNET (NET-17-1) followed by a goat anti-mouse HRP-conjugated secondary antibody and chemiluminescent detection. Arrows indicate different molecular weight forms of hNET described in text. Molecular weights indicated are from prestained standards run in parallel and are noted in kDa.
**Fig. 3:** Quantitation of hNET and variant protein expression in total cellular lysate (A) and surface fractions (B) in transiently transfected COS-7 cells described in Fig. 2. Optical density was measured for each band with ImageJ software as described in Methods. Results are expressed as percent of hNET band density for each protein species and are the mean ± S.E.M. of three experiments. Data were analyzed by one-way ANOVA as described in Methods *p < .05.

**Figure 4:** [3H]NE and [3H]DA transport of hNET and variants. COS-7 cells were transiently transfected with hNET or variant cDNAs. 24 h later radiolabeled uptake was performed as described in Methods using 50 nM [3H]NE (light grey bars) or 50 nM [3H]DA (dark grey bars). Nonspecific uptake was defined by 1 µM desipramine. hNET transport of [3H]NE and [3H]DA was 6.3 ± 0.6 x 10^-18 and 1.1 ± 0.04 x 10^-17 moles/cell/min, respectively). Data are expressed as percent [3H]NE or [3H]DA uptake of hNET. Data are the mean ± S.E.M. of three separate experiments and were analyzed by one-way ANOVA as described in Methods. *p < 0.05.

**Figure 5:** Saturation kinetics of [3H]NE (A) and [3H]DA (B) uptake of hNET and variant hNETs. COS-7 cells were transiently transfected with hNET or variant cDNAs. 24 hours later saturation transport assays were performed as described in Methods. Transport was determined by incubating cells for 10 min with 100 nM-10 µM [3H]NE or [3H]DA and nonspecific activity was defined by 1 µM desipramine at each concentration. Data are expressed as the percent of hNET transport at 10 µM substrate and are the mean ± S.E.M of three experiments. KM and VMAX values were determined using Prism as described in Methods. Data were analyzed using one-way analysis of variance followed by Fisher’s L.S.D., *p < 0.05. NE transport VMAX (moles
x 10^{-16}/cell/min) and K_M (µM) values, respectively, were as follows: hNET, 2.1 ± 0.3 and 6.8 ± 1.3; R121Q, 1.4 ± 0.2 and 9.8 ± 2.4; N29T, 0.9 ± 0.02 and 5.2 ± 0.7; F528C, 2.6 ± 0.2 and 4.2 ± 0.7; Y548H, 1.8 ± 0.3 and 7.4 ± 0.8. The DA transport V_MAX (moles x 10^{-16}/cell/min) and K_M (µM) values, respectively, were as follows: hNET, 1.0 ± 0.2 and 1.5 ± 0.7; R121Q, 1.0 ± 0.3 and 1.9 ± 0.9; N29T, 0.5 ± 0.1 and 1.1 ± 0.5; F528C, 1.2 ± 0.2 and 1.4 ± 0.5; Y548H, 0.9 ± 0.2 and 1.4 ± 0.6.

**Figure 6:** Effects of β-PMA treatment on [³H]NE transport by hNET and variants. COS-7 cells were transfected with hNET or variant cDNAs. 24 h later β-PMA and PKC-inhibitor treatments were performed as described in Methods. **A.** COS-7 cells transfected with hNET (black bars), R121Q (striped bars) or F528C (grey bars) cDNAs were incubated in 100 nM or 1 µM β-PMA for 30 min followed by 10 min uptake of 50 nM [³H]NE. [³H]NE transport for vehicle-treated groups were 2.0 ± 0.2, 1.6 ± 0.1 and 4.9 ± 0.4 moles x 10^{-18}/cell/min for hNET, R121Q and F528C, respectively. Data are mean ± S.E.M, n = 3. Data were analyzed by one-way ANOVA, *p < .05 compared to vehicle for that variant; †p < 0.05 compared to PMA-treated hNET. **B.** COS-7 cells transfected with hNET or variant cDNAs were incubated in vehicle (grey bars), 1 µM BIM (striped bars), or 1 µM staurosporine (black bars) for 20 min prior to the addition of 100 nM β-PMA for 10 min followed by 10 min uptake of 50 nM [³H]NE. [³H]NE transport for vehicle/vehicle groups were 5.2 ± 1.4, 3.1 ± 0.9 and 7.0 ± 1.6 moles x 10^{-18}/cell/min for hNET, R121Q and F528C, respectively. Data are expressed as percent vehicle/vehicle treated hNET and are the mean ± S.E.M. of three experiments. Data were analyzed by one-way ANOVA for
each variant, *p < .05 compared to vehicle/vehicle group within hNET or variant; †p < 0.05 compared to hNET vehicle/PMA group.

**Figure 7:** Effects of cotransfection of hNET variants on hNET expression. COS-7 cells were transfected with HA-tagged hNET or variant hNET cDNAs and experiments were performed 24 h later. Biotinylation and Western blot analyses were performed as described in methods. Total cellular lysate (A) and surface (C) proteins were blotted with an HRP-conjugated anti-HA antibody. Molecular weights standards are indicated in kDa. Quantitation of hNET and variant protein expression in total (B) and surface fractions (D) was performed by determining optical density measurements for each band with ImageJ software as described in Methods. Results are expressed as percent of hNET band density for each protein species and are the mean ± S.E.M. from three experiments. Data were analyzed by one-way ANOVA as described in Methods *p < .05 compared to hNET of corresponding molecular weight form.
Table 1: Nonsynonymous Single Nucleotide Polymorphisms in hNET Gene.

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Subset of identified hNET variants evaluated in the present study. References: 1, Iwasa et al., 2001; 2, Blakely Lab; 3, Halushka et al., 1999; 4, dbSNP. Nucleotide positions are from Genbank Accession numbers X91117-X91127. Exon and intron numbers reflect the revised structure of the hNET gene following identification of a new exon 1 (Kim et al., 1999). IL = intracellular loop, EL = extracellular loop, TM = transmembrane domain (as predicted by Pacholczyk et al., 1991).
Table 2: Sequence alignment among NETs, DATs and SERT for hNET variant amino acid positions.

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<td>K</td>
<td>L</td>
<td>A</td>
<td>S</td>
<td>Y</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>cESERT</td>
<td>R</td>
<td>V</td>
<td>Y</td>
<td>F</td>
<td>S</td>
<td>P</td>
<td>Y</td>
<td>F</td>
<td>Y</td>
<td>T</td>
</tr>
<tr>
<td>manducaSERT</td>
<td>R</td>
<td>V</td>
<td>R</td>
<td>L</td>
<td>A</td>
<td>S</td>
<td>Y</td>
<td>F</td>
<td>Y</td>
<td>T</td>
</tr>
</tbody>
</table>

m = mouse, r = rat, b = bovine, f = frog, h = human, cE = c. elegans, d = drosophila, ET = epinephrine transporter.
Table 3: Transport and Surface Expression of hNET SNPs in COS-7 Cells

<table>
<thead>
<tr>
<th>hNET Variant</th>
<th>Transport (50 nM, % hNET)</th>
<th>Transport Ratio [(^3)H]NE/[(^3)H]DA</th>
<th>Surface Expression (80 kDa form)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R121Q</td>
<td>56.0 ± 5.9*</td>
<td>72.2 ± 5.1*</td>
<td>72.1 ± 5.8*</td>
</tr>
<tr>
<td>V244I</td>
<td>107.4 ± 6.6</td>
<td>99.2 ± 8.8</td>
<td>85.1 ± 4.8</td>
</tr>
<tr>
<td>N292T</td>
<td>62.3 ± 4.8*</td>
<td>67.7 ± 6.6*</td>
<td>50.7 ± 13.7*</td>
</tr>
<tr>
<td>V356L</td>
<td>90.0 ± 7.1</td>
<td>88.9 ± 8.7</td>
<td>116.5 ± 7.9</td>
</tr>
<tr>
<td>A369P</td>
<td>0.5 ± 0.2*</td>
<td>0.7 ± 0.2*</td>
<td>N.D.</td>
</tr>
<tr>
<td>N375S</td>
<td>81.5 ± 5.7</td>
<td>94.6 ± 6.2</td>
<td>105.2 ± 13.8</td>
</tr>
<tr>
<td>K463R</td>
<td>88.8 ± 6.0</td>
<td>95.4 ± 6.9</td>
<td>87.6 ± 16.9</td>
</tr>
<tr>
<td>F528C</td>
<td>131.0 ± 11.1*</td>
<td>95.2 ± 6.1</td>
<td>134.7 ± 20.6*</td>
</tr>
<tr>
<td>Y548H</td>
<td>77.7 ± 6.3*</td>
<td>79.7 ± 6.9*</td>
<td>71.7 ± 10.6</td>
</tr>
<tr>
<td>I549T</td>
<td>94.4 ± 6.1</td>
<td>92.1 ± 7.7</td>
<td>46.1 ± 9.6*</td>
</tr>
</tbody>
</table>

Data are the mean ± S.E.M. of 3 experiments. Transport and surface expression of protein are expressed as percent of wt for each measure. N.D. = not detectable. Data were analyzed using one-way analysis of variance followed by Fisher’s L.S.D., *p < 0.05.
Figure 1
Figure 2

A

Total

B

Surface

hNET  F528C  R121Q  A369P  Y548H  N292T  N375S  K463R  V356L  V244I  I549T

114  88  50.7

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Figure 3

A

TOTAL

110 kDa

54 kDa

Optical Density (Percent hNET)

N.MT  R1212 V1644 N2097 V308 A368P N17299 K403R P3280 R1212 N127 V308

B

SURFACE

110 kDa

54 kDa

Optical Density (Percent hNET)

N.MT  R1212 V1644 N2097 V308 A368P N17299 K403R P3280 R1212 N127 V308
Figure 4

![Graph showing [3H]NE or [3H]DA Transport (Percent hNET) for various proteins.](image-url)
Figure 5

A

\[ ^{3}H \text{NE Uptake (Percent hNET)} \]

\[ ^{3}H \text{DA Uptake (Percent hNET)} \]

NE (µM)

DA (µM)

B

\[ ^{3}H \text{DA Uptake (Percent hNET)} \]

hNET

F528C

R121Q

Y548H

N292T

hNET

F528C

R121Q

Y548H

N292T
Figure 6

A

B

[hNE Transport (Percent Vehicle)]

0 20 40 60 80 100 120

vehicle 100 nM PMA 1 µM PMA

[hNE Transport (Percent Vehicle)]

0 20 40 60 80 100 120 140 160

Vehicle 10 min PMA Vehicle 10 min PMA Vehicle 10 min PMA

hNET R121Q F528C

Vehicle BIM STAUR
Figure 7