C-terminal region regulates the functional expression of human noradrenaline transporter splice variants

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The NET [noradrenaline (norepinephrine) transporter], an Na⁺/Cl[−]-dependent neurotransmitter transporter, has several isoforms produced by alternative splicing in the C-terminal region, each differing in expression and function. We characterized the two major isoforms of human NET, hNET1, which has seven C-terminal amino acids encoded by exon 15, and hNET2, which has 18 amino acids encoded by exon 16, by site-directed mutagenesis in combination with NE (noradrenaline) uptake assays and cell surface biotinylation. Mutants lacking one third or more of the 24 amino acids encoded by exon 14 exhibited neither cell surface expression nor NE uptake activity, with the exception of the mutant lacking the last eight amino acids of hNET2, whose expression and uptake resembled that of the WT (wild-type). A triple alanine replacement of a candidate motif (ENE) in this region mimicked the influences of the truncation. Deletion of either the last three or another four amino acids of the

C-terminus encoded by exon 15 in hNET1 reduced the cell surface expression and NE uptake, whereas deletion of all seven residues reduced the transport activity but did not affect the cell surface expression. Replacement of RRR, an endoplasmic reticulum retention motif, by alanine residues in the C-terminus of hNET2 resulted in a similar expression and function compared with the WT, while partly recovering the effects of the mutation of ENE. These findings suggest that in addition to the function of the C-terminus, the common proximal region encoded by exon 14 regulates the functional expression of splice variants, such as hNET1 and hNET2.

Key words: alternative splicing, membrane trafficking, neurotransmitter, noradrenaline (norepinephrine) transporter (NET), noradrenaline uptake.

INTRODUCTION

The NET [noradrenaline (norepinephrine) transporter] plays an important role in terminating noradrenergic synaptic transmission through the reuptake of released NE (noradrenaline). Psychostimulants (e.g. cocaine and amphetamine) and tricyclic antidepressants (e.g. desipramine) are known to act on monoamine transporters including NET to achieve their pharmacological effects [1–3]. NET is a member of the family of Na⁺/Cl[−]-dependent neurotransmitter transporters that includes the DAT (dopamine transporter), SERT (serotonin transporter), γ -aminobutyric acid and glycine transporters [4]. The hNET (human NET) is a 617-amino-acid protein with 12 hydrophobic transmembrane domains and intracellular N- and C-termini [5].

It is believed that the transport activity and cell surface expression of neurotransmitter transporters are regulated by various cellular mechanisms. Modulation by protein kinase C is well characterized for several of these transporters including NET, which participates in both the down-regulation of cell surface expression and the transporter function through the phosphorylation either of the transporter itself or of specific transporter-associated proteins [6].

Recent studies on the expression and function of neurotransmitter transporters in model cell expression systems have demonstrated that the C-terminal region plays an important role in the cellular regulation of trafficking to the plasma membrane

[7–11]. The C-termini of hDAT (human DAT) and hNET but not SERT contain a type II PDZ (postsynaptic density 95/Disks large/zonula occludens 1)-binding motif, $\Phi X\Phi$ (Φ is any hydrophobic amino acid and X is any amino acid), as the last three amino acids (L⁶¹⁸KV⁶²⁰ for hDAT and L⁶¹⁵AI⁶¹⁷ for hNET), which has been shown to interact with the PDZ-domain-containing protein PICK1 (protein interacting with C-kinase 1) [7]. Subsequently, Bjerggaard et al. [8] suggested that the C-terminus of hDAT actually has dual roles, one independent of PDZ-related interactions for ER (endoplasmic reticulum) export and cell surface targeting, and one involving interactions with proteins such as PICK1, which has yet to be fully clarified. Furthermore, Miranda et al. [9] demonstrated that several amino acid residues at the C-terminus proximal to the last transmembrane domain are essential for the ER export of hDAT. Deletion mutants of NET [10] and SERT [11] also demonstrated that the C-terminus is critical for expression and function. Therefore, the evidence suggests that there are multiple molecular determinants in the C-terminus that regulate the trafficking.

Alternative splicing of the neurotransmitter transporters has been reported to regulate their expression and function. Our own and other laboratories have reported various NET splice variants in different species including hNET (see [12] for a review). These variants, produced by alternative splicing in the 3'-region, possess different C-termini (see Figure 1 for hNET variants), and differ in cell surface expression and transport activity [13–18].

Abbreviations used: bNET1b, bovine NET splice variants lacking exon 14 to exon 15; bNET2b, bovine NET splice variant lacking exon 16; DAT, dopamine transporter; ER, endoplasmic reticulum; hDAT, human DAT; HEK-293, human embryonic kidney 293; hNET, human NET; KRH, Krebs–Ringer Hepes-buffered solution; MDCK cells, Madin–Darby canine kidney cells; NE, noradrenaline; NET, noradrenaline transporter; PDZ, postsynaptic density 95/Disks large/zonula occludens 1; PICK1, protein interacting with C-kinase 1; SERT, serotonin transporter; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; WT, wild-type.

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Figure 1 Summary of the NET isoforms produced by alternative splicing in the C-terminal region and truncated and amino acid-substituted mutants of these isoforms

(A) The amino acid sequences at the C-termini of hNET splice variants are shown with the sites of mutation under investigation in the present study indicated by arrows. The region divided in the deletion mutant is underlined, and named cassettes C1 to C5. Important motifs examined are indicated in the shaded column. (B) A list of mutants used in the present and previous [16] studies with amino acid sequences at exons 13–16.

These results emphasize the involvement of alternative splicing in the role of the C-terminus as mentioned above. However, the characteristics of NET splice variants have not been detailed in full.

Previously, we identified novel isoforms of bovine NET produced by alternative splicing [17], with different splicing patterns from human [14,16] and rat [15] NET. A functional characterization of these variants based on assays of the uptake of [³H]NE and binding of [³H]nisoxetine, a specific radioligand for NET [19], suggested that the C-terminal region encoded by exon 14 is indispensable for the functional expression of bovine NET isoforms [17]. However, the mechanism by which this region regulates the expression and/or function of NET remains unclear. Questions to answer include whether there is a molecular determinant within the region encoded by exon 14 that regulates NET trafficking, and how this region relates to the different C-termini of NET variants encoded by exons 15 and 16.

In the present study, we have examined the physiological relevance of the regulated expression of NET splice variants by using mutant NETs and conducted kinetic analyses of NE transport and immunological experiments to explore the cell surface expression and function of NET. We found that the region encoded by exons 15 and 16 of the human splice variants hNET1 and hNET2 respectively affected the cell surface expression and transporter function. Furthermore, our results suggest that the common proximal region encoded by exon 14 corresponds to the C-terminal regions of hNET1 and hNET2.

EXPERIMENTAL

Materials

Drugs used in this study were noradrenaline tartrate, pargyline hydrochloride (Nacalai Tesque, Kyoto, Japan) and nisoxetine hydrochloride (Sigma–Aldrich Corporation, St. Louis, MO,

U.S.A.). Reagents for cell culture were from Sigma or Invitrogen (Carlsbad, CA, U.S.A.). [³H]NE was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, U.K.), KOD-plus DNA polymerase from Toyobo (Tokyo, Japan), restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.), and DNA purification kits from Qiagen GmbH (Hilden, Germany). The mouse anti-human NET (NET17-1) antibody was from MAb Technologies (Stone Mountain, GA, U.S.A.). The polyclonal antibody against β-actin was from Sigma–Aldrich. Secondary antibodies conjugated with horseradish peroxidase and FITC were from Amersham Biosciences. Sulfo-NHS-SS-biotin [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] and NeutrAvidin™ beads were from Pierce (Rockford, IL, U.S.A.).

DNA constructs and mutagenesis

The full-length cDNAs encoding hNET1 and hNET2 were subcloned into the mammalian expression vector pcDNA3 (Invitrogen), as described previously [16]. PCR-based mutagenesis was used to delete the various regions encoded by exons 14 and 15, and to selectively replace amino acids by using overlapping primers, in which mutated nucleotides were introduced (Figure 1). C-terminally-truncated mutants of hNET1 were created by introducing stop codons into the coding region of NET. After the mutagenesis, the products were subcloned into the pGEM-T Easy TA cloning vector (Promega), and the results were verified by automated sequencing. Then, restriction fragments containing mutated sequences were digested with appropriate restriction enzymes, and subcloned into pcDNA3.

Cell preparation and transfection

COS-7 cells and MDCK cells (Madin–Darby canine kidney cells; RIKEN Cell Bank, Tsukuba, Japan) were maintained in

Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum at 37 °C in a 5 % CO₂/95 % air atmosphere.

Transient transfections were performed using electroporation (for uptake assays) and liposomes (for immunoblotting and immunocytochemical analyses). COS-7 cells at subconfluence were harvested and transfected with the cDNA (10 µg/10⁷ cells) by electroporation for the [³H]NE uptake assay [16]. A parallel transfection with the pcDNA3 vector alone was performed each time as a negative control. After the electroporation, cells were diluted in the culture medium, plated in 24- or 48-well culture plates and cultured for 2–3 days. For the immunological analysis, cells at subconfluence in 60 mm diameter culture dishes (for immunoblotting) or in 4-well CultureSlides (Falcon Biocoat, Becton Dickinson Labware, Bedford, MA, U.S.A.) were transfected with the cDNA (0.2 µg/cm² of culture plasticware) using FuGENE6^{TM} transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

To establish lines of MDCK cells stably expressing NET isoforms, cells at subconfluence in 100 mm diameter culture dishes were transfected with NET isoform cDNA using FuGENE6^{TM}, as described above. After transfection the cells were incubated for 24 h and then placed in culture medium containing 500 µM G418 for 2–3 weeks to select for stably transfected cells. Cells were initially screened for expression using an uptake assay (see below) and the positive cell lines were confirmed to express the isoforms using indirect immunofluorescence by the anti-NET antibody. For each construct the results were verified with at least two independently derived stable cell lines.

Uptake assay

Cells were washed three times with oxygenated KRH (Krebs–Ringer Hepes-buffered solution; 125 mM NaCl, 5.2 mM KCl, 1.2 mM CaCl₂, 1.4 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM glucose and 20 mM Hepes, pH 7.3 ± 0.1) and incubated for 10 min at 37 °C with 10 nM [³H]NE in the presence of 0.1 mM ascorbate and 50 µM pargyline, as described previously [16]. After the removal of excess radioligands, the cells were washed three times rapidly with ice-cold KRH and any radioactivity remaining in the cells was extracted with NaOH and measured by liquid scintillation spectrometry (RI Research Center, Okayama University Dental School, Okayama, Japan). Non-specific uptake was determined in the mock-transfected cells and also in each plate in the presence of 10 µM nisoxetine. For the kinetic analysis, cells were incubated in KRH containing 10 nM [³H]NE and 0.1–100 µM unlabelled NE. Data were analysed by Eadie–Hofstee plots using Prism3 (GraphPad Softwares, San Diego, CA, U.S.A.). Statistical analyses were performed with Student's *t*-test.

Immunocytochemistry and confocal microscopy

For immunostaining experiments, MDCK cells stably expressing NET variants were grown on Costar Transwell filter supports (Corning, Acton, MA, U.S.A.) at a density of 3.5 × 10⁴ cells/well. The cells were initially rinsed with Ca²⁺- and Mg²⁺-containing PBS, then fixed in 4 % (w/v) paraformaldehyde. After three washes with PBS, they were permeabilized in PBS containing 0.1 % Triton X-100 for 10 min and incubated in blocking solution [5 % (v/v) goat serum] for 1 h. The cells were incubated with the mouse anti-hNET monoclonal antibody NET17-1 (diluted 1:100) for 1 h at room temperature (20–22 °C) followed by incubation with a FITC-conjugated goat anti-mouse secondary antibody. They were then washed three times with PBS, and the filter containing the cells was excised from its support and mounted

on a slide with Perma Fluor aqueous mounting medium (Thermo Shandon, Pittsburgh, PA, U.S.A.). Immunostaining of COS-7 cells grown on Biocoat culture slides was performed as above. After a final wash, the cells were covered with the mounting medium and a coverslip.

Immunofluorescent images were generated using a Zeiss LSM510 laser scanning confocal microscope (Central Research Laboratory, Okayama University Medical School, Okayama, Japan). Images were the product of 2-fold (for MDCK cells) or 4-fold (for COS-7 cells) line averaging. The *z*-series (for MDCK cells) was collected by focusing initially on the middle section of the cells (position 0) and scanning 8 µm above to 4 µm below this plane at 0.5 µm intervals.

Cell surface biotinylation

Cells grown in 60 mm diameter culture dishes were washed with ice-cold PBS, and then incubated for 120 min at 4 °C with 1 mg/ml sulfo-NHS-SS-biotin under gentle agitation. Cells were then washed three times with PBS, collected with and solubilized in RIPA buffer [PBS containing 1 % (v/v) Nonidet P40, 0.5 % sodium deoxycholate and 0.1 % SDS], and incubated at 4 °C for 1 h. Non-lysates were removed by centrifugation for 20 min at 20000 *g*, and the biotinylated proteins were precipitated with NeutrAvidin^{TM} beads by incubating overnight at 4 °C with gentle shaking. The precipitated proteins were then washed five times with RIPA buffer, and denatured by heating the beads in sample buffer at 95 °C for 5 min. The precipitates obtained with the NeutrAvidin^{TM} beads were analysed by SDS/PAGE (20 % gel), and then proteins were transferred on to a PVDF membrane (Amersham Biosciences). Western blotting was performed with a monoclonal mouse antibody to hNET (NET17-1) followed by a secondary antibody conjugated to horseradish peroxidase, and bands were detected by ECL® (enhanced chemiluminescence; Amersham Biosciences) on autoradiographic film (Kodak). Several X-ray films were analysed to determine the linear range of the chemiluminescence signals, and the quantification was performed using densitometry and NIH (National Institutes of Health) Image software. Statistical analyses were performed using Student's *t*-test.

RESULTS

Differential expression and function of hNET splice variants in MDCK cells

Human NET has three isoforms produced by alternative splicing, each with different characteristics of expression and function [16,18]. To clarify the nature of the isoforms and their physiological relevance, we first compared the expression and function of the two major isoforms of hNET1 and hNET2, which have different C-termini encoded by exon 15 and exon 16 respectively, by stably expressing them in polarized MDCK cells.

Figure 2(A) shows the subcellular localization of hNET1 and hNET2 in MDCK cells visualized by immunocytochemistry with confocal microscopy. hNET1 was located mostly in the plasma membrane, whereas hNET2 was present mainly in the intracellular compartment and, in part, in the plasma membrane (Figure 2A); these observations were consistent with those made previously by ourselves [16] and Distelmaier et al. [18]. Confocal images in a vertical section illustrated that both hNET1 and hNET2 were expressed at the basolateral surface. The expression of hNET1 at the basolateral surface in polarized cells has been reported elsewhere [20,21]. The present results demonstrated that

Figure 2 Differential subcellular distribution and transport kinetics of hNET1 and hNET2 in MDCK cells

(A) Immunofluorescence imaging of human NET splice variants. MDCK cells stably expressing hNET1 and hNET2 were stained with the anti-NET antibody as described in the Experimental section. The upper and right-hand panels show z-scanning images of the site on x-y-stacks indicated by white lines respectively. Scale bar, 10 μm. (B) Cell surface biotinylation of hNET1 and hNET2. Cells were incubated with sulfo-NHS-SS-biotin for 120 min at 4°C, and the biotinylated proteins were recovered from lysates with NeutrAvidin^{TM}-conjugated beads as described in the Experimental section. T, total fraction before NeutrAvidin^{TM} precipitation; C, NeutrAvidin^{TM} precipitates. Mature proteins of 80–85 kDa (indicated by arrow) were observed for hNET1 and hNET2 in both fractions. (C) A saturated [3H]NE uptake was observed in MDCK cells stably expressing hNET1 (□) or hNET2 (●).

hNET2, which has a different C-terminus, was also located to the basolateral surface when expressed at the plasma membrane.

Western blot analyses and cell surface biotinylation assays demonstrated that both hNET1 and hNET2 (80–85 kDa) were present in the NeutrAvidin^{TM}-precipitated fraction from the biotinylated extracts of the MDCK cells stably expressing hNET1 and hNET2 (Figure 2B), suggesting that both isoforms were expressed in the plasma membrane. Immunoblots of total extract from the cells expressing hNET2 revealed the presence of a narrow band of immunoreactivity at 60 kDa, which was not observed in the cell surface fraction. The mobility of these bands on SDS/PAGE suggested the presence of both mature N-glycosylated NET proteins (80–85 kDa species for both hNET1 and hNET2) and immature unglycosylated or core glycosylated NET (60 kDa species for hNET2), as demonstrated by Bauman and Blakely [10]. Taken together with the immunocytochemical data, the results of the Western blot analyses of cell surface biotinylation assays demonstrated that hNET1 and hNET2 differ in their biosynthesis, cellular localization, and/or turnover, suggesting an essential contribution of the C-terminal end to these processes.

Figure 2(C) shows the saturated uptake of [3H]NE in MDCK cells stably expressing hNET1 and hNET2 respectively. The kinetic analysis revealed the K_m and V_{max} values for hNET1 and hNET2 to be 5.35 ± 1.94 and 5.95 ± 3.93 μM ($n=3$; no significant difference), and 164.53 ± 9.91 and 69.28 ± 5.08 fmol/min per μg of protein ($n=3$; $P<0.05$) respectively. No significant difference in K_m with the relatively high concentrations observed here compared with our previous observations in COS cells [16], and also in the present study (Table 1), although the

Table 1 Kinetic analyses of the transport of [3H]NE transport by the WT and mutant NET isoforms

COS cells transiently expressing the WT and mutant NETs were incubated with 10 nM [3H]NE and various concentrations of unlabelled NE. The saturated uptake of [3H]NE was kinetically analysed by Eadie–Hofstee plots using Prism3. Results are means $\pm$ S.E.M. for three to six experiments performed in triplicate. * $P<0.05$, ** $P<0.01$ compared with WT. # $P<0.05$, ## $P<0.01$ compared with hNET1 WT. ND, not determined, since the amount taken up was too small to analyse kinetically.

Transporter	K_m (μM)	V_{max} (fmol/min per μg of protein)
hNET1 WT	1.53 ± 0.14	83.96 ± 12.47
1C1	ND	ND
1C2	1.26 ± 0.33	$2.93 \pm 0.10^{**}$
1C3	$0.80 \pm 0.09^{**}$	$1.92 \pm 0.53^{**}$
1C1–2	ND	ND
1C2–3	ND	ND
1C1–3	ND	ND
hNET2 WT	$1.08 \pm 0.08^{\#}$	$32.21 \pm 5.04^{\#}$
2C1	ND	ND
2C2	0.95 ± 0.22	$7.29 \pm 2.61^*$
2C3	1.17 ± 0.10	34.82 ± 7.95
2C1–2	ND	ND
2C2–3	ND	ND
2C1–3	0.83 ± 0.61	$1.80 \pm 1.04^{**}$

V_{max} values revealed similar results. To resolve this discrepancy, we examined the kinetics of [3H]NE uptake in MDCK cells transiently transfected with hNET variant cDNAs. K_m and V_{max} values for [3H]NE uptake by hNET1 and hNET2 transiently expressed in MDCK cells were 1.39 ± 0.20 and 0.75 ± 0.17 μM ($n=7$; $P<0.01$), and 1.80 ± 0.20 and 0.61 ± 0.16 fmol/min per μg of protein ($n=7$; $P<0.05$) respectively. Thus the difference in affinity for NE between hNET1 and hNET2 depends on transfection (stable compared with transient) rather than cell type, when the two isoforms are heterologously expressed, at least, in COS and MDCK cells.

Transfection efficiency of transiently expressed MDCK cells in our preparations was low, and not enough to evaluate further the immunological detection in Western blot analyses and immunocytochemistry.

The region encoded by exon 14 modulates cell surface expression and function

There might be multiple molecular determinants in the C-terminus, which regulate the expression and function of NET, as demonstrated for DAT by Miranda et al. [9]. One candidate exists within the region encoded by exon 14, since we identified bovine NET splice variants lacking exon 14 to exon 15 (bNET1b) or to exon 16 (bNET2b), neither of which displayed [3H]NE transport activity and [3H]nisoxetine binding when expressed in COS-7 cells [17]. We initially explored the occurrence of human counterparts to these variants in human tissues by reverse transcriptase-PCR using total RNA from human placenta and the neuroblastoma cell line SK-N-SH, in which NET expression has been examined [14,16,22]. The mRNA of the exon 14-truncated isoform of hNET1 but not hNET2 was expressed in the placenta and SK-N-SH cells (results not shown). Therefore we constructed the human NET homologues of bNET1b and bNET2b, hNET1ΔEX14 (1C1–3) (the region divided in the deletion mutant has been named cassette C1 to C5; as shown in Figure 1) and hNET2ΔEX14 (2C1–3) respectively, through truncation of the region encoded by exon 14, and examined

Figure 3 The C-terminal region of NET encoded by exon 14 plays an important role in functional expression

(A) Immunofluorescence imaging of the WT and mutant NET variants. COS-7 cells were transiently transfected with WT hNET1, hNET1ΔEX14 (1C1–3), WT hNET2 or hNET2ΔEX14 (2C1–3). After 1 day of transfection, the subcellular localization of the NET variants was assessed by confocal microscopy with immunological detection using a hNET-specific antibody as described in the Experimental section. Scale bar, 10 μm. (B) Cell surface biotinylation of the WT and mutant NET variants expressed in COS-7 cells. After 2 days of transfection, cells were incubated with sulfo-NHS-SS-biotin for 120 min at 4°C, and the biotinylated proteins were recovered from lysates with NeutrAvidin™-conjugated beads as described in the Experimental section. The total fraction before NeutrAvidin™ precipitation (T) and the NeutrAvidin™ precipitate (C) were resolved by SDS/PAGE, and hNET was detected by Western blot analysis using mouse anti-hNET antibody. The mature (80–85 kDa) and immature (55 kDa) NET proteins

are indicated by arrows. (C) Uptake activity of the WT and mutant NETs. Cells were transfected and after 2 days they were subjected to a 10 min transport assay with 10 nM [³H]NE, as described in the Experimental section. Non-specific uptake was determined in parallel assays with 10 μM nisoxetine. The values represent specific uptake from which non-specific uptake has been subtracted. Results are means ± S.E.M. for three or four experiments performed in triplicate. *P < 0.01 compared with WT.

the impact on NET function and expression by comparing these mutants in COS-7 cells. Immunocytochemical observation demonstrated that the transient expression of WT (wild-type) hNET1 in COS-7 cells occurred at the cell surface with an intracellular distribution, whereas WT hNET2 localized mostly in the cytosolic compartment with less expression at the cell surface in COS-7 cells (Figure 3A), in accordance with the observation in MDCK cells (Figure 2). The truncated NET variants, hNET1ΔEX14 (1C1–3) and hNET2ΔEX14 (2C1–3), localized to the cytosol and accumulated near the perinuclear area (Figure 3A).

Immunoblots of extracts from COS-7 cells transiently expressing the WT hNET1 and hNET2 revealed that both the 55 kDa and 80–85 kDa bands for hNET1, and the 60 kDa and 80–85 kDa bands for hNET2 were observed in the total extract from each of the transfected COS-7 cells (Figure 3B). The immunoreactivity of NeutrAvidin™-precipitates from biotinylated extracts to anti-hNET was markedly enriched for both species with the 80–85 kDa band greater than 55 or 60 kDa band, suggesting the cell surface expression of mature and, to a lesser extent, immature forms of the two isoforms. Truncation of the region encoded by exon 14 caused a marked reduction in levels of mature protein (80–85 kDa band for both hNET1 and hNET2) without a change in levels of immature protein (55 kDa band for hNET1 and 60 kDa band for hNET2) in total fractions, in association with a striking loss of mature but not immature protein in cell surface fractions (Figure 3B).

In parallel with the immunocytochemical and immunoblot analysis, we performed a functional assay of these mutants. As shown in Figure 3(C), hNET1ΔEX14 (1C1–3) revealed no significant uptake of [³H]NE over the background level when assayed at a fixed concentration. hNET2ΔEX14 (2C1–3) also revealed a striking reduction in NE transport activity, but the level of activity was sufficient to permit a kinetic evaluation. There was a marked reduction in the *V*_{max} with a modest change in the *K*_m (Table 1).

Assessments of truncated mutation missing exon 14

To explore the importance of the region encoded by exon 14, we constructed mutants in which eight amino acids were deleted sequentially or additionally (Figure 1), and investigated the expression of these mutant NETs in COS-7 cells with assays of the uptake of [³H]NE and immunological examinations of cell expression.

Figure 4(A) shows the cell surface expression of these NET variants assessed using biotinylation assays. In hNET1, the deletion of one and even two parts reduced the total amounts of both mature (80–85 kDa) and immature (50 kDa) NET proteins with a striking reduction in the surface expression of the mature protein and a modest decrease in that of the immature form, similar to the results obtained with a mutant NET lacking all parts encoded by exon 14 (hNET1ΔEX14), as mentioned above. On the other hand, in hNET2, deletion of the N-terminal portions markedly reduced the cell surface expression of the mature NET protein in association with a reduction in the total amount of protein; however, deletion of the last eight amino acids (2C3) only

Figure 4 Effects of truncation in regions encoded by exon 14 on the subcellular distribution and transport activity of NET isoforms

(A) Western blot analyses of the WT and mutant NETs by cell surface biotinylation assays. COS-7 cells were transfected with the WT or deletion mutants of the variants hNET1 and hNET2, and subjected to biotinylation studies, as described in the Experimental section. Blots were probed with mouse monoclonal anti-hNET antibody, followed by goat horseradish-peroxidase-conjugated secondary antibody, and detected by ECL®. The upper and lower panels show the mature (80–85 kDa) and immature (55 kDa) NET proteins respectively. The blot was reprobed with rabbit polyclonal anti-actin antibody as a control for fractionation. Immunoreactivity to actin at 42 kDa was observed only in the total fraction before NeutrAvidin™ precipitation and not in the NeutrAvidin™ precipitates. (B) Quantitative comparison of the cell surface expression of mature (80–85 kDa) NET proteins between WT and mutant NETs. The optical density analyses were performed on the total fraction before NeutrAvidin™ precipitation (closed bar) and on NeutrAvidin™ precipitates (open bar). Results are presented as a ratio with WT band density $\pm$ S.E.M. for three or four separate experiments. * $P < 0.05$. (C) [³H]NE uptake in COS-7 cells expressing the WT and mutant NETs. Cells were transfected and 2 days later they were incubated with 10 nM [³H]NE and increasing concentrations of unlabelled NE. Non-specific uptake was determined in parallel assays with 100 μ M cocaine. The values represent specific uptake from which non-specific uptake has been subtracted. Kinetic data for WT hNET1, WT hNET2, and the mutants 1C2, 1C3, 2C2, 2C3 and 2C1–3 are shown. The specific uptake of [³H]NE by other mutants was not sufficient to analyse kinetically. Results are means $\pm$ S.E.M. of three or four experiments performed in triplicate.

slightly affected the surface expression or total amount of NET protein. Deletion of cassettes 1 plus 2 and cassettes 2 plus 3 repressed the total and cell surface expression of hNET2 variants. Figure 4(B) shows the results of the quantitative analysis of the total and cell surface expression of mature (80–85 kDa) NET protein.

In association with these results, uptake experiments revealed a functional correlation with cell surface expression. Only the mutant of hNET2 (2C3) which lacks the last eight amino acids, revealed a robust uptake of [³H]NE similar to the WT hNET2 (Figure 4C). Other mutants exhibited NE transport activity at levels less than 10% of the WT. Table 1 summarizes the kinetic analysis of the NE transport activity of these mutants. The changes

in $V_{\max}$ observed in 1C2 and 1C3 for hNET1 and 2C2 and 2C1–3 for hNET2 might represent a reduction in the amount of functional transporter present at the cell surface, consistent with the results of the immunoblot analysis mentioned above, while the changes in K_m observed in 1C3 for hNET1 suggest the involvement of functional alterations caused by the mutations.

Assessment of C-terminal region in exon 14 using amino acid substitutions

To further identify the motif important for the expression of NET variants, we focused on a candidate motif for the ER export signal lying in the C2 cassette within exon 14, ENE, corresponding to

Figure 5 Cell surface expression of the mutants of hNET1 and hNET2 with alanine substitutions at exon 14 assessed by biotinylation

(A) Surface biotinylation of COS-7 cells expressing WT hNET1, 1E595A/N596A/E597A (1ENE_AAA), WT hNET2 or 2E595A/N596A/E597A (2ENE_AAA). Surface biotinylated proteins were precipitated with NeutrAvidin beads and analysed by SDS/PAGE, followed by immunoblotting with the hNET (upper panel) and actin (lower panel) antibodies, as described in the Experimental section. (B) Quantitative comparison of the cell surface expression of the mature (80–85 kDa) protein between WT and mutant NETs. The absorbance analyses were performed on the total fraction before NeutrAvidin™ precipitation (filled column) and on NeutrAvidin™ precipitates (open column). Results are displayed as a ratio with WT band density $\pm$ S.E.M. of three separate experiments. * $P < 0.05$.

the known motif FCYENE observed in the K channel [23] or (D/E)X(E/D), a more general motif for trafficking (Figure 1). We performed consecutive triple-alanine replacements of these amino acids within this region of hNET1 and hNET2 and compared the results with those obtained with truncated mutants.

The substitution of alanine residues for E⁵⁹⁵NE⁵⁹⁷ reduced the cell surface expression of both the mature (80–85 kDa) and immature (55 kDa) NET proteins with a modest change in the total amount of NET protein for hNET1 and a marked reduction in that for hNET2 (Figure 5). Parallel uptake experiments showed that, consistent with the cell surface biotinylation immunoassay data, substitution mutants of both hNET1 and hNET2 had a $V_{\max}$ for the uptake of [³H]NE that was 20–30 % of that of WT without showing a change in the K_m value. The $V_{\max}$ values of WT hNET1 and 1(E595A/N596A/E597A) were 58.46 ± 18.17 and 19.46 ± 4.22 fmol/min per μ g of protein ($n = 3$; $P < 0.01$), and those of WT hNET2 and 2(E595A/N596A/E597A) were 25.44 ± 3.41 and 5.52 ± 2.30 fmol/min per μ g of protein ($n = 3$; $P < 0.01$) respectively. On the other hand, the K_m values of WT hNET1 and 1(E595A/N596A/E597A) were 1.17 ± 0.14 and 1.14 ± 0.22 μ M, and those of WT hNET2 and

2(E595A/N596A/E597A) were 0.77 ± 0.09 and 0.74 ± 0.40 μ M respectively.

Taken together, these results were very similar to those obtained with cassette C2-truncated mutants of hNET1 and hNET2, such as 1C2 and 2C2.

Truncation of and substitution at the hNET1 C-terminus encoded by exon 15

A previous investigation demonstrated the role of the PDZ motif located in the C-terminus of DAT in its PICK1-mediated cell surface expression and clustering [6], although recent findings by Bjerggaard et al. [8] demonstrated dual roles for the C-terminus of DAT, in PDZ-dependent and -independent interactions. In addition, the alternative splicing of exon 16 gave two different acceptor sites, resulting in two isoforms: hNET2 (hNET C-t var2 in [14]) and hNET1 Δ C4 Δ C5 + LSF (hNET C-t var1 in [14]; Figure 1). We have demonstrated that the latter hNET2 splice variant, hNET C-t var1 (hNET1 Δ C4 Δ C5 + LSF), exhibited a weaker expression at the cell surface together with no NE transport activity when expressed in COS-7 cells [16]. We therefore examined the effects of the deletion and/or addition of amino acids in this region on the expression and function of NET protein.

The deletion of either three amino acids (LAI) for the PDZ motif at the C-terminus or the four proximal amino acids (LQHW) within the region encoded by exon 15 resulted in a decrease in cell surface expression of mature NET protein as assessed by the biotinylation assay (Figures 6A), but this was not considered significant (Figure 6B). Parallel experiments revealed a marked decrease in the uptake of [³H]NE (Figure 6C). Kinetic analysis revealed a decrease in not only the $V_{\max}$ but also the K_m values (Table 2), suggesting that truncation affects the functional properties of NET proteins. Furthermore, substitution of LSF for the last three amino acids (LAI) resulted in a cell surface expression greater than that of the WT (Figure 6A and 6B) in association with robust transport activity similar to the level of the WT (Figure 6C and Table 2). Since LSF is a type II PDZ-binding motif like LAI, these results taken together with our previous observations [16], suggest an important role for the four proximal amino acids in the expression and function of NET. These results are consistent with the recent findings by Bjerggaard et al. [8] for DAT, as mentioned above.

Interestingly, the deletion of all amino acids in the region encoded by exon 15 caused an increase in surface expression compared with WT hNET1 (Figures 6A and 6B), but caused [³H]NE uptake to decrease markedly (Figure 6C). Kinetic analysis revealed a decrease in both the $V_{\max}$ and the K_m (Table 2). Given that the number of functional transporters at the cell surface does not alter, a decrease in the $V_{\max}$ indicates a reduction in the number of transport cycles. Further deletion at the C-terminus, such as the region encoded by exons 14 and 15 for hNET1 (1C1–5), revealed a reduction in overall expression and cell surface expression (Figures 6A and 6B) in association with a marked decrease in NE transport activity (Figure 6C and Table 2), consistent with the results for the exon 14-deletion mutants, such as hNET1 Δ EX14 (1C1–3) and hNET2 Δ EX14 (2C1–3).

These results suggest that the last part of the C-terminal region of hNET1 not only contains important motifs necessary for cell surface trafficking including the PDZ-binding motif, but also plays an important role in the transport activity of NET.

Assessments of correlation between DxE and RxR motifs in NET2

The C-terminal region of hNET2 encoded by exon 16 contains residues R⁶¹⁷RR⁶¹⁹, an RXR motif for the ER retrieval signal, suggesting that this may cause less cell surface expression

Figure 6 Cell surface expression and transport activity of the NETs mutated at exon 15

(A) Western blot analyses of the WT and mutant NETs by cell surface biotinylation assays. COS-7 cells were transfected with the WT or deletion/substitution mutants of hNET1, and subjected to biotinylation assays, as described in the Experimental section. Blots were probed with mouse monoclonal anti-hNET antibody. The upper and middle panels show the mature (80–85 kDa) and immature (55 kDa) NET proteins respectively. The blot was reprobed with rabbit polyclonal anti-actin antibody (lower panel). (B) Quantitative comparison of the cell surface expression of the mature (80–85 kDa) protein between WT and mutant NETs. The absorbance analyses were performed on the total fraction before NeutrAvidin™ precipitation (closed bar) and on NeutrAvidin™ precipitates (open bar). Results are displayed as a ratio with WT band density $\pm$ S.E.M. for three separate experiments. * $P < 0.05$. (C) [^{3} H]NE uptake in COS-7 cells expressing the WT and mutant NETs. Cells were transfected and after 2 days they were incubated with 10 nM [^{3} H]NE and increasing concentrations of unlabelled NE, as described in the Experimental section. Non-specific uptake was determined in parallel assays with 10 μ M nisoxetine. The values represent specific uptake from which non-specific uptake has been subtracted. Results are means $\pm$ S.E.M. for three experiments performed in triplicate.

of hNET2 than hNET1 ([10,16,18] and the present study). Therefore we decided to substitute alanine for this motif, [2(R617A/R618A/R619A)] and for ENE (E595A/N596A/E597A). Unexpectedly, the 2(R617A/R618A/R619A) mutant of hNET2 displayed a similar cell surface expression and uptake of [^{3} H]NE compared with the WT (Figure 7 and Table 3). On the

Table 2 Effects of C-terminal truncation of hNET1 on the transport of [^{3} H]NE

COS cells transiently expressing the WT and mutant NETs were incubated with 10 nM [^{3} H]NE and various concentrations of unlabelled NE. The saturated uptake of [^{3} H]NE was analysed kinetically by Eadie–Hofstee plots using Prism3. Results are means $\pm$ S.E.M. for three to six experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ compared with WT. ND, not determined, since the amount taken up was too small to analyse kinetically.

Transporter	K_m (μ M)	V_{max} (fmol/min per μ g of protein)
hNET1 WT	1.43 ± 0.11	81.18 ± 13.72
1C4	$0.45 \pm 0.08^{**}$	$13.80 \pm 3.62^*$
1C5	$0.58 \pm 0.16^*$	$11.73 \pm 1.54^*$
1C4-LSF	1.11 ± 0.06	67.95 ± 12.43
1C4-5	$0.76 \pm 0.12^*$	$22.64 \pm 7.25^*$
1C1-5	ND	ND

other hand, the simultaneous substitution of ENE and RRR led to a partial recovery of cell surface expression in contrast with the complete abolishment observed on the single substitution of ENE (Figures 7A and 7B). However, [^{3} H]NE uptake did not recover on the mutation of both motifs (Figure 7C and Table 3). These results suggest that Arg⁶¹⁷ and Arg⁶¹⁹ by themselves are not important for retention in the ER or trafficking to the plasma membrane but are somehow important for the expression and function of hNET2 in relation to another trafficking motif, ENE.

DISCUSSION

Studies on various members of the Na⁺/Cl[−]-dependent neurotransmitter transporter family regarding the cellular mechanisms of their functional expression have highlighted that the C-terminus is critical for trafficking to the plasma membrane. There is evidence of multiple molecular determinants for the transporter function. NET has splice variants differing at the C-terminus [12], thus it is useful for examining the role of the C-terminus in the trafficking and transport activity.

In the present study, we focused on the role of the C-terminal region encoded by exon 14, proximal to the C-terminal end of human NET splice variants, hNET1 (encoded by exon 15) and hNET2 (encoded by exon 16), in the expression and function of the NET proteins. Previous studies demonstrated differences in the expression and function of NET variants with unique C-termini, suggesting that the C-terminal region plays an important role in the cell surface expression and transporter function of human NET splice variants, hNET1 and hNET2 [12–18]. The present study confirmed this, and found, further, that the region encoded by exon 14 affects hNET1 and hNET2 differently. Interestingly, deletion of the last part of this region did not influence the expression and function of hNET2, although it markedly reduced the cell surface expression and [^{3} H]NE transport activity of hNET1. These results suggest that this proximal region corresponds to the C-terminal regions of hNET1 and hNET2.

Our previous observations [17], and the results presented here, with truncated mutants demonstrated that the region encoded by exon 14 is indispensable for the expression of both human NET isoforms, as it participates in trafficking to, and insertion into, the plasma membrane. However, serial truncations of this part failed to identify an important motif for the trafficking of NET proteins. A candidate for an epitope with a common role in the expression of both isoforms is E⁵⁹⁵NE⁵⁹⁷, which corresponds to the suggested ER export motif, FCYENE, demonstrated for potassium channels [23], and (D/E)X(E/D), a more general motif of the ER export signal. The findings that the substitution of alanine for ENE

Figure 7 Cell surface expression and transport activity of the alanine-substituted mutants of hNET2

(A) Western blot analyses of the WT and mutant NETs by cell surface biotinylation assays. COS-7 cells were transfected with the WT or substitution mutants of hNET2, and subjected to biotinylation assays, as described in the Experimental section. Blots were probed with mouse monoclonal anti-hNET antibody. The upper panel shows the mature (80–85 kDa) and immature (55 kDa) hNET2 proteins respectively. The blot was reprobed with rabbit polyclonal anti-actin antibody (lower panel). (B) Quantitative comparison of the cell surface expression of the mature (80–85 kDa) protein between WT and mutant NETs. The absorbance analyses were performed on the total fraction before NeutrAvidin™ precipitation (closed bar) and on NeutrAvidin™ precipitates (open bar). Results are displayed as a ratio with WT band density $\pm$ S.E.M. for three separate experiments. * $P < 0.05$. (C) $[^3\text{H}]\text{NE}$ uptake in COS-7 cells expressing the WT and mutant NETs. Cells were transfected and after 2 days they were incubated with 10 nM $[^3\text{H}]\text{NE}$ and increasing concentrations of unlabelled NE, as described in the Experimental section. Non-specific uptake was determined in parallel assays with 10 μM nisoxetine. The values represent specific uptake from which non-specific uptake has been subtracted. Results are means $\pm$ S.E.M. for three experiments performed in triplicate.

residues caused a marked reduction in cell surface expression, together with a decrease in NE transport activity for both NET1 and NET2, support this possibility.

We confirmed our previous conclusion concerning the NET splice variants, in that the C-terminus plays an important role not only in cell surface expression and trafficking but also in transport function including substrate recognition. Smaller K_m values for $[^3\text{H}]\text{NE}$ uptake in hNET2 compared with hNET1 were

Table 3 $[^3\text{H}]\text{NE}$ uptake characteristics for WT and amino-acid-substituted mutant hNET2 expressed in COS-7 cells

COS cells transiently expressing the WT and mutant NETs were incubated with 10 nM $[^3\text{H}]\text{NE}$ and various concentrations of unlabelled NE. The saturated uptake of $[^3\text{H}]\text{NE}$ was analysed kinetically by Eadie–Hofstee plots using Prism3. Results are means $\pm$ S.E.M. for three to six experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ compared with WT.

Transporter	K_m (μM)	$V_{\max}$ (fmol/min per μg of protein)
hNET2 WT	0.77 ± 0.05	34.05 ± 8.64
2(R617A/R618A/R619A)	0.76 ± 0.08	29.83 ± 7.12
2(E595A/N596A/E597A)	0.74 ± 0.40	$5.52 \pm 2.30^{**}$
2(E595A/N596A/E597A) + (R617A/R618A/R619A)	0.64 ± 0.11	$8.45 \pm 0.72^*$

observed in both COS-7 and MDCK cells that transiently but not stably expressed hNET1 and hNET2, suggesting the possible involvement of the C-terminus in substrate recognition, although K_m does not necessarily mean the affinity for substrate. Recently, Distelmaier et al. [18] reported that the change in the hNET carboxy terminus induced by alternative splicing had neither an effect on the K_m value for the substrate NE nor on the affinity (K_d) of the NET inhibitor nisoxetine, indicating that the last part of the C-terminus may not be involved in ligand recognition. The reason for this discrepancy remains unclear. hNET1 has a PDZ-binding motif at the C-terminus, whereas hNET2 does not. In the present study, we demonstrated that removal of the last three amino acids (LAI) caused a marked reduction of $V_{\max}$ and K_m in association with a decrease in cell surface expression. Removal or modification of the PDZ-binding motif of hDAT (LKV) also reduced the K_m for the substrate dopamine to half of the WT DAT in HEK-293 (human embryonic kidney 293) cells, suggesting the possible involvement of PICK1 interaction in the transporter function in addition to its importance for expression [8]. Distelmaier et al. [18] observed a reduced K_m for NE and K_d for nisoxetine in the mutant hNETs lacking the last three or seven amino acids transiently expressed in HEK-293 cells, although those were not significant, probably due to large deviation of their results. Therefore one possible explanation is that the transport properties may change in association with the levels of endogenous interacting proteins. Furthermore, hNET2 has a consensus motif for phosphorylation in the C-terminus, which was not observed in hNET1. Phosphorylation may affect the cell surface expression and functional properties of the transporter, as indicated in the neurotransmitter transporters [24]. Further investigations are needed to clarify these possibilities.

The PDZ-binding motif in the C-termini of hDAT and hNET1 have been suggested to play an important role in their trafficking and clustering at the cell surface [7,18]. More recently, Bjerggaard et al. [8] demonstrated that R⁶¹⁵HW⁶¹⁷ preceding L⁶¹⁸KV⁶²⁰, a type II PDZ domain-binding motif of hDAT, acts as an ER export signal independent of PDZ interactions. Miranda et al. [9] also reported evidence of the PDZ-independent involvement of the C-terminus in trafficking and cell surface targeting. In support of this view, we also observed that the deletion of L⁶¹¹QHW⁶¹⁴ of hNET1 decreased cell surface expression and NE transport activity more severely than that of L⁶¹⁵AI⁶¹⁷. These results correlate well with our previous finding [16] that hNET C-t var1, a splice variant from site at exon 16 different from that for hNET2 (hNET C-t var2), which has L⁶¹¹SF⁶¹³ at the carboxy terminal end instead of L⁶¹¹QHWLAI⁶¹⁷, exhibited decreased cell surface expression and transport activity in contrast with the mutant 1LSF (L⁶¹⁵AI⁶¹⁷ of hNET1 replaced by LSF) in the present study, which showed a

similar cell surface expression to the WT. The type II PDZ-binding motif of LSF might preserve the role of LAI, only if the proximal LQHW is present.

However, the present results further demonstrated that deletion of the last seven amino acids of hNET1 did not affect cell surface expression. However, it severely affected the uptake of [³H]NE. These results suggest that the region at least participates in the transport activity, despite not necessarily being involved in trafficking and targeting to the plasma membrane. This is also the case for hNET2, since the mutant 1C4–5 is a mutant hNET2 in which the last 18 amino acids of the C-terminus were deleted. Therefore it is more likely that the interaction of the C-terminus of both isoforms with the proximal region determines their role in the trafficking and targeting. Kinetic analysis demonstrated that deletion of the last seven amino acids of hNET1 (1C4–5) caused a decrease in the $V_{\max}$ without changing the K_m . This together with the finding that the cell surface expression was similar to that of the WT indicates that transport was reduced for this mutant. The results suggest that this region may be involved in the translocation of the transporter, and the deletion caused the transporter to adapt an inward-facing conformation.

Our results from the mutant hNET1, 1C4–5, in which seven amino acids at C-terminal end of hNET1 was deleted, differ from those reported by Distelmaier et al. [18] who showed that deletion of these amino acids decreased both transport activity and cell surface expression. On the other hand, Torres et al. [25] reported that the sequential deletion of amino acids in the C-terminus of hDAT produced a progressive decrease in transport activity, while all those mutants expressed well in HEK-293 cells except the mutant lacking 38 amino acids (S582), which revealed the lack of cell surface expression. These results correlate well with our observations in the hNET mutants, 1C4–5 (lacking seven amino acids) and 1C1–5 (lacking 31 amino acids), in which we observed cell surface expression of 1C4–5 similar to that of WT, and not find any detectable [³H]NE transport activity and cell surface expression of 1C1–5. The discrepancy may be resolved by the recent finding by Miranda et al. [9], who demonstrated that the truncation of the last two, five or ten amino acids did not completely abrogate transport of DAT to the cell surface in either HeLa or HEK-293 cells, rather these truncations substantially delayed expression at the cell surface.

The C-terminal region encoded by exon 16 contains R⁶¹⁷RR⁶¹⁹, an ER retrieval signal, which may contribute to the reduced expression of hNET2 compared with hNET1. However, as mentioned above, deletion of this region (1C4–5) did not affect the cell surface expression of the transporter, suggesting less of a contribution of R⁶¹⁷RR⁶¹⁹ to the decreased expression of hNET2 than to that of hNET1. This is supported by the unexpected observation that the triple substitution, R617A/R618A/R619A, failed to change the cell surface expression or [³H]NE uptake. However, the results do not rule out the participation of the C-terminus of hNET2 in the cell surface expression, since it is assumed that part of the region encoded by exon 14 participates in overcoming the ER retention signal present at the C-terminus of hNET2. Substituting alanine residues for E⁵⁹⁵NE⁵⁹⁷, an ER export motif, markedly diminished the cell surface expression and [³H]NE uptake activity of hNET2. However, a double motif substitution of E595A/N596A/E597A and R617A/R618A/R619A partly recovered the cell surface expression and transport activity, suggesting interaction between these two parts. A more detailed study is needed to clarify this possibility.

In conclusion, in the present study, we have demonstrated that hNET1 and hNET2 differ in subcellular distribution and transport properties, depending on cell type, and that their expression is

regulated differently through the intrinsic C-terminal end and proximal common region encoded by exon 14, and through the interaction of these distinct region for trafficking and recruitment to the plasma membrane. The results also suggest that the C-terminal region affects the functional state of NET, which switches between outward- and inward-facing conformations.

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