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Norepinephrine transporter splice variants and their interaction with substrates and blockers

Shigeo Kitayama[a,*], Toshihiro Dohi[b]

[a] Department of Dental Pharmacology, Okayama University, Graduate School of Medicine and Dentistry, Shikata 2-5-1, Okayama 700-8525, Japan
[b] Department of Dental Pharmacology, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Minami, Hiroshima 734-8553, Japan

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Abstract

Norepinephrine transporter (NET), a member of the Na^+/Cl^--dependent neurotransmitter transporter family, displays species-specific isoforms produced by alternative RNA splicing. This occurs at 3′-flanking coding and noncoding regions, resulting in different carboxy-terminals. When these NET splice variants were expressed in cultured cell lines, the characteristics of substrate transport and the sensitivity to uptake inhibitors differed between isoforms. The different functional expression suggests the physiological importance of the action and interaction of NET splice variants in synaptic transmission.

Keywords: Norepinephrine transporter; Gene expression; Alternative splicing; Methamphetamine; Uptake inhibitor

1. Introduction

Noradrenergic neurons in the central nervous system (CNS) originate from the brainstem including the *Locus ceruleus* (A6), and project to various regions including the prefrontal cortex, hippocampus, hypothalamus, cerebellum, medulla and spinal cord. In these synaptic regions, norepinephrine transporter (NET) in the plasma membrane acts to terminate noradrenergic transmission by reaccumulating released norepinephrine in a Na^+/Cl^--dependent fashion (Iversen, 1971). Various CNS acting drugs, for example, psychostimulants including cocaine and antidepressants (such as desipramine or imipramine), are known to act on monoamine transporters including NET, thereby facilitating monoaminergic synaptic transmission (Langer and Schoemaker, 1988; Graham and Langer, 1992; Kitayama and Dohi, 1996).

Human NET cDNA has been cloned from human neuroblastoma cell line SK-N-SH cells by expression cloning (Pacholczyk et al., 1991). Hydropathy analysis of the deduced amino acid sequences of human NET suggested there were 12 hydrophobic regions, predicting a putative transmembrane domain and amino- and carboxy-terminal cytosolic structures. There is a large extracellular loop between the putative transmembrane domains 3 and 4, having several consensus sequences for N-linked glycosylation. The amino- and carboxy-terminal regions and some intracellular loops contain sites showing consensus sequences for phosphorylation by cyclic AMP-dependent protein kinase (PKA), Ca^{2+}/diacylglycerol-dependent protein kinase (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II). Human NET is a 69-kDa protein consisting of 617 amino acids (Pacholczyk et al., 1991).

Subsequently, other NETs were identified from various species including monkey (Miller et al., 2001), cow (Lingen et al., 1994; Jursky et al., 1994), rat (Brüss et al., 1997; Kitayama et al., 1999), mouse (Fritz et al., 1998) and medaka fish (Roubert et al., 2001). Comparison of these NETs demonstrated the presence of NET isoforms produced by alternative RNA splicing and their different functional characteristics. In this study, we summarize and discuss recent findings on the functional expression of NET isoforms produced by alternative splicing and its physiological importance in the regulation of NET function.

2. Identification of NET splice variants

In 1994, two laboratories independently reported homologous bovine NET (bNET) cDNAs which encoded different

* Corresponding author. Tel.: +81-86-235-6660; fax: +81-86-235-6664.
E-mail address: shigeok@md.okayama-u.ac.jp (S. Kitayama).

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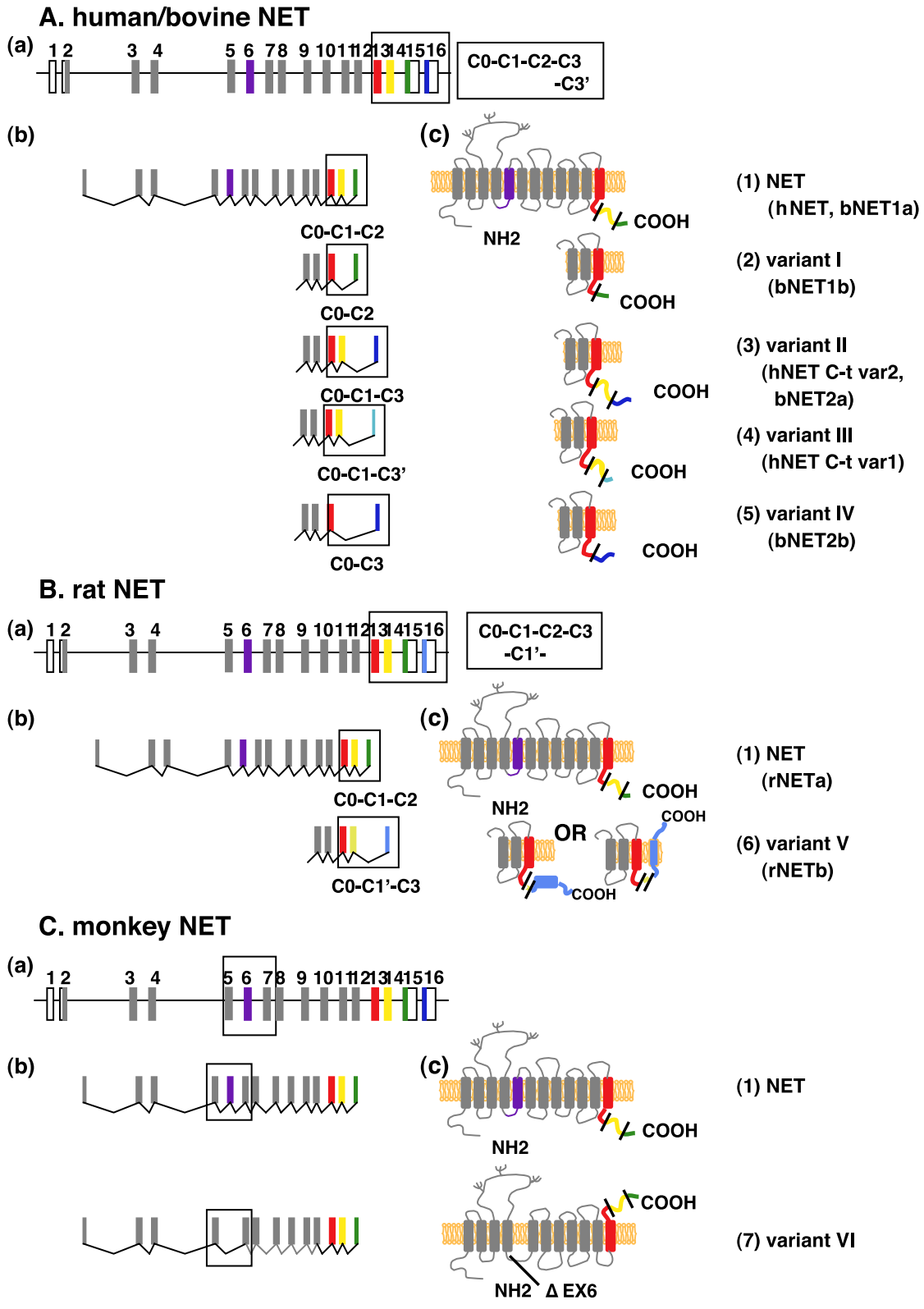

Fig. 1. Schematic representation of (a) NET gene organization, (b) NET mRNA splicing, and (c) membrane topology of NET proteins in various species. In (a), boxes and lines represent exons and introns, and closed boxes represent open reading frames. Since the monkey NET gene has not been identified, the organization in C-(a) is tentative.

carboxy-terminal tails (Lingen et al., 1994; Jursky et al., 1994). bNET1, reported by Lingen et al. (1994), is homologous to human NET (hNET) (Pacholczyk et al., 1991), whereas bNET2, reported by Jursky et al. (1994), has a different carboxy-terminal tail from hNET and bNET1. Therefore, it has been suspected that these isoforms are produced by alternative pre-mRNA splicing (Fig. 1).

Initial study of the organization of the human NET gene by Pörzgen et al. (1995) did not support the above possibility of NET splice variants in humans. The human NET gene (SLC6A2) is localized to chromosome 16q12.2 (Brüss et al., 1993; Gelernter et al., 1993), spans >45 kb and consists of 14 exons separated by 13 introns, with consensus sequences for RNA splicing at each exon–intron junction (Pörzgen et al., 1995). At that time, Pörzgen et al. (1995) identified exon 14, which encodes the carboxy-terminal reported by Pacholczyk et al. (1991). Many individual exons encode a single intra- or extracellular domain and transmembrane domain as a common feature with other members of the Na^+/Cl^--dependent neurotransmitter transporter family. Rapid amplification of cDNA ends (RACE), primer extension and ribonuclease protection experiments revealed a newly identified exon and multiple trancription start sites for the human NET gene at the 5′-flanking region (Kim et al., 1999). The protein-coding region thus begins within exon 2.

Further analyses by Pörzgen et al. (1998) of the human NET gene at the 3′-flanking region led to the first identification of NET isoforms produced by alternative splicing in humans. These authors found two additional NET transcripts, designated hNET C-t var1 and hNET C-t var2, which were produced using a different 3′-acceptor site of exon 16 for splicing (Pörzgen et al., 1998). Regarding these results, the hNET C-t var1 carboxy-terminal encoded by exon 16 has three amino acids while that of hNET C-t var2 has 18 amino acids. The latter amino acid sequence is homologous to that of bNET2, except for the portion encoded by exon 14, which is lacking in bNET2, suggesting that bNET2 skips both exons 14 and 15 to exon 16 for splicing.

Kitayama et al. (1999) cloned several cDNAs of rat NET (rNET) variants from the brain and PC12 cells. In addition to rNETa, a homologous counterpart of hNET and bNET1, they found rNETb, a variant having a different carboxy-terminal tail without homology to human NETs or bNET1/bNET2. By analyzing the rat NET gene 3′-flanking region, these authors found that these variants are produced by alternative splicing, and that their sites are different from those of human NET or bovine NET. rNETb is produced by splicing from the middle of exon 14 to a site near the end of exon 15 for rNETa-L, thus encoding a different carboxy-terminal tail. Hydropathy analysis showed that rNETb has one additional hydrophobic region within a unique carboxy-terminal tail that is capable of spanning the plasma membrane, resulting in a possible 13th transmembrane domain (Kitayama et al., 1999).

We recently examined bovine NET mRNA expression in the brain and adrenal medulla by RT-PCR and found two additional transcripts produced by alternative splicing (Kitayama et al., 2002). These are produced by different use of exon 13 (cassette C0), exon 14 (C1), exon 15 (C2) and exon 16 (C3): a newly identified transcript consists of C0-C1-C3, designated bNET2a, and because of the inclusion of C1 it differs from bNET2 (Jursky et al., 1994), thus we designated bNET2 as bNET2b. bNET2a is a homologous counterpart of hNET C-t var2. Another transcript, designated bNET1b, consists of C0-C2 in contrast to C0-C1-C2 of bNET1 (Lingen et al., 1994), with skipping of C1, thus we designated bNET1 as bNET1a. We failed to find a homologous counterpart of hNET C-t var1 in bovine NET transcripts. Analysis of the nucleotide sequences of the bovine NET gene at the 3′-flanking region including exon 16 and upstream indicated no another splice acceptor site, as observed in the human NET gene which resulted in hNET C-t var1 (Kitayama et al., unpublished observation).

Miller et al. (2001) reported the cloning of NET cDNA from the monkey brain. They found a new NET variant which lacks a portion encoded by exon 6. Exon 6 encodes the 5th transmembrane region and part of the third extracellular loop. The lack of exon 6 does not cause a frame shift, and this predicts a truncated form of NET protein if it is entirely translated.

3. Functional expression of the NET splice variants

3.1. Expression of NET splice variants

The expression of NET mRNA (Lorang et al., 1994; Hoffman et al., 1998) and protein (Schroeter et al., 2000) was thoroughly investigated in the rat brain. The probes used in these studies, however, were unable to distinguish between rat NET subtypes. Moreover, limited information about the expression of NET splice variants is available for other species.

Pörzgen et al. (1998) first demonstrated the expression of human NET splice variant mRNA in SK-N-SH cells, a human neuroblastoma cell line, from which they cloned NET variant cDNAs. NET is known to exist not only in the CNS but also in peripheral neuronal and non-neuronal tissues. Expression of variant NET mRNA was also found in non-neuronal peripheral tissues, such as the placenta and adrenal medulla (Pörzgen et al., 1998; Kitayama et al., 2001).

Kitayama et al. (2002) demonstrated the expression of bovine NET splice variant mRNA in the bovine brain and adrenal medulla by reverse transcriptase-polymerase chain reaction (RT-PCR). All variants, including bNET1a, bNET1b, bNET2a and bNET2b, were present in these tissues at possibly different levels. bNET1a seems more abundant in these tissues than bNET1b, while bNET2b seems more abundant than bNET2a (Kitayama et al., 2002,

unpublished observation). The precise level of expression needs to be further clarified by quantitative methods.

At present, there is no useful probe (antibody) to specifically identify NET splice variant proteins. Burton et al. (1998) first demonstrated the different intracellular localization of bNET1 (bNET1a) and bNET2 (bNET2b) proteins in stably transfected cells using an antibody that recognizes both isoforms. bNET1a was localized in the plasma membrane, while bNET2b remained mainly within the cytosol.

Kitayama et al. (2001) immunocytochemically demonstrated the expression of hNET, hNET C-t var1 and hNET C-t var2 in transiently transfected COS-7 cells (SV40-transformed kidney cell line from African green monkey). hNET and hNET C-t var2 were localized in the plasma membrane, whereas hNET C-t var2 appeared to be mainly localized in the cytosol, unlike hNET. hNET C-t var1 immunoreactivity was not observed in the plasma membrane.

These results were confirmed by experiments using [^{3}H]nisoxetine, a specific radioligand for NET (Tejani-Butt et al., 1990), in an intact cell assay system (Kitayama et al., 2001, 2002).

3.2. Functional diversity of the NET splice variants

In addition to the different subcellular localization of NET isoforms as mentioned above, functional analysis of these variants showed different transport activity and pharmacology.

Initially, Jursky et al. (1994) reported that bNET2 (bNET2b) lacks norepinephrine transport activity when expressed in COS-7 cells. Later, Burton et al. (1998) examined the expression and function of bNET1 (bNET1a) and bNET2 (bNET2b) in stably transfected cells. In accordance with previous findings (Lingen et al., 1994; Jursky et al., 1994), cells expressing bNET1a showed robust [^{3}H]norepinephrine uptake, whereas cells expressing bNET2b had no [^{3}H]norepinephrine transport activity. Rat NET has a unique variant different from human or bovine NET, rNETb, which shows no [^{3}H]norepinephrine transport activity (Kitayama et al., 1999). These results might suggest that the carboxy-terminal has an important role not only in NET expression, as mentioned above, but also in NET function.

bNET2b shows two differences from bNET1: one is the lack of the region encoded by exon 14, and the other is a different carboxy-terminal end encoded by exon 16. The results of Burton et al. (1998) suggest the importance of these two regions for the functional expression of NET. This was examined further by Kitayama et al. (2001, 2002). hNET C-t var2 displays a carboxy-terminal homologous to that of bNET2 but contains the region encoded by exon 14. Transfection of COS-7 cells with hNET C-t var2 resulted in robust [^{3}H]norepinephrine uptake and the expression of NET in the plasma membrane (Kitayama et al., 2001). These results, along with those of Burton et al. (1998), suggested that the region encoded by exon 14 is important for NET functional expression. This was confirmed by experiments using the novel bovine NET variants mentioned above (Kitayama et al., 2002). Despite there being a choice between either the carboxy-terminal end encoded by exon 15 or that encoded by exon 16, the region encoded by exon 14 is indispensable for NET functional expression (Kitayama et al., 2002).

Since functional expression was observed only with hNET/bNET1a and hNET C-t var2/bNET2a, the characteristics of transporter function were investigated in COS-7 cells transfected with these variants (Kitayama et al., 2001, 2002). Both hNET C-t var2 and bNET2a were associated with a robust uptake of [^{3}H]norepinephrine with higher affinity (lower K_m) and lower initial velocity (V_{max}) than hNET and bNET1a. These results suggested the importance of the carboxy-terminal in the transport activity of NET. The functional importance of the carboxy-terminal region in catecholamine transporters is suggested by the results of investigations performed using site-directed mutagenesis (Bauman and Blakely, 2002) and chimeras between DAT and NET (Buck and Amara, 1994; Giros et al., 1994).

Changes in K_m for norepinephrine between hNET/bNET1a and hNET C-t var2/bNET2a also suggest different transport properties for methamphetamine, a transportable ligand for monoamine transporters. The finding that hNET C-t var2/bNET2a has a lower K_m for methamphetamine (Kitayama et al., 2001, 2002) suggests that the activity of noradrenergic synapses where hNET C-t var2/bNET2a is present is affected to a greater degree by methamphetamine. Given that the expression of NET isoforms varies in the brain regions, the sensitivity to methamphetamine may differ and be determined by the variant expressed.

There is some evidence indicating different pharmacological profiles of norepinephrine uptake in different brain regions. In accordance with this, it was demonstrated that the potency of uptake inhibitors, including cocaine, desipramine and nisoxetine, in inhibiting norepinephrine transport was similar between hNET/bNET1a and hNET C-t var2/bNET2a (Kitayama et al., 2001, 2002).

4. Regulated expression and functional regulation: physiological relevance of NET splice variants

4.1. Regulated expression via carboxy-terminal

Recent investigations of the role of the carboxy-terminal in regulating the functional expression of neurotransmitter transporters demonstrated that it is involved in various steps including membrane trafficking, stability and recycling processes (see Torres et al., 2003 for review).

The most attractive model for the regulation of NET expression is that implicating an interaction with cytosolic proteins at the carboxy-terminal, one of which is the postsynaptic density 95/Discs large/zona occludens 1 (PDZ) domain-containing protein. hNET has a class II anchor motif at the carboxy-terminal end and the PDZ

domain-containing protein PICK1 (protein that interacts with PKC α1) was suggested as a candidate protein which interacts with NET as well as dopamine transporter (DAT) (Torres et al., 2001). hNET C-t var2/bNET2a does not have this motif in the carboxy-terminal, suggesting that the variant may interact with other protein(s), if any.

4.2. Possible interaction between isoforms

Kitayama et al. (1999) demonstrated the pronounced negative effect of rNETb on the functional expression of norepinephrine transport when co-expressed with rNETa in COS-7 cells. bNET1b and bNET2b also had a dominant negative effect on bNET1a and bNET2a (Kitayama et al., unpublished observation). These results suggest that the interaction between isoforms may play an important role in NET functional expression. A recent observation suggested that neurotransmitter transporters exist as a dimer or tetramer. Assembly of a heterodimer/tetramer may be possible, resulting in a different expression and function from that of a homodimer/tetramer. The finding that co-expression with rNETb decreases the functional expression of not only rNETa but also rat serotonin transporter (rSERT) and rat γ-aminobutyric acid transporter 1 (rGAT1), but not rat glutamate/aspartate transporter (rGLAST) (Kitayama et al., 1999), implies the possible interaction between a subset of the transporter family.

4.3. Possible regulation of NET RNA processing

As mentioned in Section 3, bovine NET variants may have different levels of expression in the brain and adrenal medulla (Kitayama et al., 2002, unpublished observation). Thus, the question arises whether or not NET RNA processing is regulated under physiological conditions to express different isoforms. At present, there is no evidence indicating the regulated expression of NET splice variants or their splice regulation. RNA processing is a highly regulated process occurring in spliceosomes within the nucleus (Manley and Tacke, 1996; Wang and Manley, 1997). Analysis of the 3′-flanking region of NET genes will provide clues to clarify the possible regulation of NET RNA processing.

5. Concluding remarks

NET displays species-specific isoforms produced by alternative RNA splicing, which occurs at the 3′-flanking coding and noncoding regions. NET variants with different carboxy-terminals have different characteristics regarding norepinephrine transport and sensitivity to psychostimulants such as methamphetamine. Furthermore, some isoforms have a dominant negative effect when co-expressed with functional isoforms. The different nature of the functional expression of NET variants suggests the physiological importance of their action and interaction in noradrenergic synaptic transmission.

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