Dominant Negative Isoform of Rat Norepinephrine Transporter Produced by Alternative RNA Splicing*

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We have cloned from rat brain a family of alternatively spliced cDNAs from a single gene, which encodes a norepinephrine transporter (NET) having variations at the 3′-region including both coding and noncoding regions. This produces two transporter isoforms, rNETa and rNETb, which differ at their COOH termini. The rNETa isoform reveals a COOH terminus homologous to human NET and transports norepinephrine. In contrast, rNETb revealed no detectable transport function but reduced functional expression of rNETa when both isoforms were expressed in the same cell. Thus, rNETb potentially functions as a dominant negative inhibitor of rNETa activity. Co-expression of rNETb with a γ-aminobutyric acid transporter (rGAT1), a serotonin transporter (rSERT), and a dopamine transporter (rDAT) reduced their transport activity. No reduction was found with the glutamate/aspartate transporter (rGLAST). Alternative RNA splicing of NET suggests a novel mechanism for the regulation of synaptic transmission.

The norepinephrine transporter (NET) at presynaptic nerve terminals mediates the uptake of released norepinephrine, resulting in the rapid termination of synaptic transmission and thereby controlling the fine tuning of neuronal activities. Psychostimulants, including amphetamines, and tricyclic antidepressants, such as desipramine, exert their pharmacological effects by acting on NET (1, 2). Molecular cloning studies have resulted in the isolation of human NET cDNA (3), followed by several other monoamine neurotransmitter transporters from several species (4, 5).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB021970 and AB021971.

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FIG. 1. Analyses of two different isoforms of rat NET. A, schematic representation of rat NET mRNAs and their gene organization. Lines and boxes in rNET genes represent introns and exons, respectively. Boxes and lines in rNET mRNAs represent coding and noncoding regions, respectively. Similar organization of NET genes was observed in all species, except that the intron between exons 4 and 5 of rat NET was approximately 10 kb in contrast to 4.5 kb of the mouse or human NET gene. B, deduced amino acid sequences of rNETa and rNETb at the COOH-terminal tail, hydropathy analysis of predicted protein of rat NETs, and the possible structure of their membrane topology. Boxed amino acid sequences indicate the additional hydrophobic region of rNETb presented as an intracellular or transmembrane domain in the scheme. Arrows indicate the RNA splicing site. C, expression of rNETa and rNETb mRNA in rat brain assessed by reverse transcriptase (RTase)-PCR. D, COS cells transfected with five different clones derived from PCR products were subjected to [$^3$H]dopamine uptake assay (left). [$^3$H]Nisoxetine binding in intact COS cells expressing rNETa (rNET2–1) and rNETb (rNET2–4) is shown on the right. COS cells transfected with rNETa, rNETb, or mock cDNA (10 µg/10^7 cells) were incubated with 0.1–20 nM [$^3$H]nisoxetine with or without excess cold nisoxetine for 2 h on ice, and specific binding of [$^3$H]nisoxetine was determined by scintillation counting. *, p < 0.05 versus mock-transfected COS cells.
Detection of rNET mRNA Splice Variants by Reverse Transcriptase-PCR—Total RNA was isolated from rat whole brain (except cerebellum) by acid-phenol extraction (16) and used to synthesize cDNA with avian myeloblastosis virus reverse transcriptase and oligo(dT)15 primers (Boehringer Mannheim). To amplify alternative exons, we used the following PCR primers for rNETa-L (a and b), rNETa-S (a and c), and rNETb (a and d) as indicated in Fig. 1, A and C: a, 5′-TACGTCATCATGAAATTGTCCTGG; b, 5′-CGAAAAGAGGTCCTTGTCCTGG; c, 5′-CAATCTATTTACATACAGATGTAG; and d, 5′-CTGTGGGGCAGAGCTGTGGGGT. PCRs were performed by initial denaturation at 92 °C for 2 min, and then 40 cycles of 92 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 5 min.

Heterologous Expression and Analysis of Transport Activity—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 5% CO2. Cells at subconfluence were harvested and transfected with the cDNA by electroporation (17). Parallel transfection with a pcDNA3 vector alone was performed every time for the negative control. After electroporation, cells were diluted in the culture medium and plated in 24- or 48-well culture plates. After the 2-day culture, cells were washed three times with oxygenated Krebs-Ringer HEPES-buffered solution (KRH: 125 mM NaCl, 5.2 mM KCl, 1.2 mM CaCl2, 1.4 mM MgSO4, 1.2 mM KH2PO4, 5 mM glucose, 20 mM HEPES, pH 7.3) and incubated for 10 min at 37 °C with [3H]noradrenaline (538.72 GBq/mmol, NEN Life Science Products) in KRH containing an inhibitor of its catalyzed enzymes, 50 μM pargyline and 100 μM ascorbate. After 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. Nonspecific uptake was determined in the mock-transfected cells and also in each plate in the presence of 100 μM cocaine. Uptake of [3H]GABA (1409.7 GBq/mmol), [3H]glutamate (658.6 GBq/mmol), [3H]dopamine (888 GBq/mmol), and [3H]serotonin (1028.6 GBq/mmol) was performed in the same way (NEN Life Science Products).

Nisoxetine Binding Assay—Specific binding of nisoxetine, a specific ligand for NET (18), to the plasma membrane NET was evaluated using radiolabeled nisoxetine and intact cells on ice (19). Transfected COS cells were washed with ice-cold KRH and incubated with 2 nM [3H]nisoxetine (3145 GBq/mmol, NEN Life Science Products) in KRH for 2 h on ice. Nonspecific binding was examined in the presence of 10 μM nisoxetine. For cold saturation analysis, cells were incubated in KRH containing 2 nM [3H]nisoxetine and 1–1000 nM cold nisoxetine. For hot saturation analysis, cells were incubated with 0.1–100 nM [3H]nisoxetine in the presence or absence of 10 μM nisoxetine. Data were analyzed by Scatchard plot using kcat (BioMetallics).

RESULTS AND DISCUSSION

Cloning and Expression of Splicing Variants of Rat NET—From 5′- and 3′-RACE and subsequent nested PCR, we obtained from 5′-RACE two positive groups of clones of different sizes (about 0.7 and 0.9 kb) and one positive pool of several clones (about 2.0 kb) from 3′-RACE. Sequence analysis of these amplified fragments indicated that they were the 5′- and 3′-portions of the rat NET coding and noncoding regions. Because we failed to amplify the full-length cDNA from Marathon-Ready cDNA used in RACE, the full-length cDNA of rat NET was cloned by PCR with reverse transcribed mRNA from rat brain and PC12 cells. The resulting amplifications showed two positive bands on agarose gel with lengths of approximately 4.0 and 2.4 kb, respectively. These were cloned into a mammalian expression vector pcDNA3 (Invitrogen) and analyzed by re-
striction enzyme XbaI and by functional expression in COS cells and [3H]dopamine uptake assay. The results showed that the shorter band consisted of two different messages, one of which lacked transport activity. The larger clones displayed [3H]dopamine transport activities (Fig. 1D). Nucleotide sequence analyses of each clone indicated that there were at least three different forms of rat NET (Fig. 1A). Deduced amino acid sequences indicate that there are two NET proteins having different COOH-terminal tails, designated rNETa and rNETb (Fig. 1B). The COOH-terminal tail of rNETa was homologous to human (3), bovine (9), and mouse (12) NET but not to another reported bovine NET (10). On the other hand, the COOH terminus of rNETb showed no similarity to any known protein, including the latter bovine NET (10). Hydropathy analysis (20) demonstrated one additional hydrophobic region in rNETb capable of membrane spanning a structure. The scheme in Fig. 1B displays possible structures of rNETb having a hydrophobic region in the cytosol or in the plasma membrane. Precise membrane topology of rNETb needs to be determined using a specific antibody to the predicted COOH-terminal tail.

Southern blot analysis using the 3′-flanking region of rNETa (rNET2–1) and rNETb (rNET2–4) indicated that three variants of rNET are derived from a single copy of the gene (data not shown). Therefore, it is suggested that rNETa and rNETb are produced by alternative RNA splicing. To confirm this, we isolated this region by PCR from rat genomic DNA using primer sets described in Fig. 1A (P1/R6) and determined the gene organization. Approximately 4.5-kb PCR products contained 4 exons (exon 13–16), which were alternatively spliced as described in Fig. 1A. Notably, rNETb was spliced differently within exon 13, skipping to the middle of exon 16. On the other hand, rNETa-S was spliced at exon 13 like rNETa-L but skipped to the middle of exon 16 at a site different from rNETb. Therefore, these splice variants used different exo-intronic splicing sites within the exons or introns.

The expression of each rat NET mRNA variant was examined by reverse transcriptase-PCR using primers complementary to the regions specific for each variant (Fig. 1A). The majority of the NET mRNA was rNETa-L and, to some extent, rNETb (Fig. 1C). rNETa-S mRNA was not observed in this condition. Pacholezyk et al. (3) demonstrated two different sizes of mRNA encoding human NET having 5.8 and 3.6 kb, the latter being expressed in several brain regions and therefore a possible glial transporter. The present findings suggest that the larger mRNA may encode rNETa-L and the smaller mRNA may encode rNETb and rNETa-S if any, although the difference in sequences may be because of alternative polyadenylation site usage (3). Systematic examination of rNETb mRNA expression in various regions of the brain or multiple tissues should provide clues regarding its physiological significance.

The functional expression of each clone in the COS cells showed that [3H]dopamine uptake was observed in rNETa-
not rNETb-expressing COS cells (Fig. 1D, left). These results suggest that rNETb lacks transport activity and/or that it is not expressed in the plasma membrane. To examine the latter possibility, we performed the binding of [3H]nisoxetine, a specific ligand for NET (18) in COS cells expressing rNETb under these conditions using intact cells (19). A small but significant binding of [3H]nisoxetine was observed in rNETb-expressing COS cells at 10 nM [3H]nisoxetine concentration (Fig. 1D, right). However, further analysis of [3H]nisoxetine binding kinetics for rNETb could not be determined, because considerable binding was observed in mock-transfected control COS cells when the [3H]nisoxetine concentration in the incubation solution increased. In contrast, rNETa-expressing COS cells showed [3H]nisoxetine binding with an affinity of 5.99 ± 0.77 nM $K_D$ and a capacity of 3.50 ± 0.20 fmol/μg of protein $B_{max}$ ($n = 3$). It is therefore probable that expression of rNETb in the plasma membrane is partially restricted and that even expressed rNETb lacks transport activities.

**Dominant Negative NET Isoform**—If rNETa and rNETb are present in the same cell, they might interact as suggested by DAT that makes homomultimeric structures (21). First, we examined their interaction in COS cells by co-expression of rNETa and rNETb. Introduction of both cDNAs resulted in a reduction of [3H]norepinephrine uptake and [3H]nisoxetine binding (Fig. 2A). Expression levels of each mRNA did not change substantially in co-expression, as assessed by reverse transcriptase-PCR (data not shown). Kinetic analyses revealed a decrease in both $K_m$ and $V_{max}$, reflecting an increase in apparent affinity for the substrate and decrease in transport rate for norepinephrine uptake: $2.46 ± 0.28 \mu M$ and $1.45 ± 0.14 \mu M$ of $K_m$ and $68.8 ± 5.8$ fmol/μg of protein/min and $29.6 ± 1.9$ fmol/μg of protein/min of $V_{max}$ in COS cells expressing rNETa alone or rNETa plus rNETb, respectively (Fig. 2B). On the other hand, an increase in $KD$ and decrease in $B_{max}$ of [3H]nisoxetine binding were observed in co-expression: $6.52 ± 1.77 nM$ and $18.28 ± 4.85 nM$ of $K_D$ and $2.00 ± 0.25$ fmol/μg of protein and $1.74 ± 0.27$ fmol/μg of protein of $B_{max}$ in COS cells expressing rNETa alone or rNETa plus rNETb, respectively. These results demonstrated the dominant negative effect of rNETb, suggesting the possible interaction of rNETa and rNETb, which may alter the properties of substrate transport and ligand binding.

To clarify further the interaction mechanism and specificity, rNETb was co-expressed in COS cells with other rat neurotransmitter transporters including DAT, serotonin transporter (SERT), neuronal GABA transporter type 1 (GAT1) and glial glutamate/aspartate transporter (GLAST). Surprisingly, rNETb reduced the transport function of DAT (Fig. 3A), SERT (Fig. 3B), and GAT (Fig. 3C) but not of GLAST (Fig. 3D). Co-transfection of neurotransmitter transporter cDNAs, e.g. NET and GAT1 or DAT and GAT1, had no effect on any function (data not shown). Furthermore, the functionless mutant of
DAT, e.g. D1A in which Asp79 in the putative 1st transmembrane region was replaced by alanine (19), did not change wild-type DAT activity by co-transfection (data not shown). These results suggest the possibility that NETa inhibits functional expression of the transporter via interaction at some region that is specific for the subfamily of neurotransmitter transporters. Alternatively, if each neurotransmitter transporter has its own alternative spliced form like NET, each transporter may have its own regulatory mechanism via RNA splicing. It is suggested that membrane trafficking and sorting of neurotransmitter transporters are dependent on sequences encoded within the COOH tail as demonstrated in GABA and betaine transporters heterologously expressed in polarized epithelial cells (22) and in the wild-type and COOH-terminal mutated DAT transiently expressed in COS cells (23). Many natural and artificial mutations have been observed to result in endoplasmic reticulum retention and degradation, apparently because they affect protein folding or oligomerization (24). Because oligomeric structure has been inferred for several neurotransmitter transporters including DAT (21), these results suggest the involvement of several amino acids at the COOH terminus in the folding and/or oligomerization process of these neurotransmitter transporters. To date, there are several reports indicating that nuclear and plasma membrane hormone receptors, which were known to form a homo- or heterodimer to act, possessed dominant negative isoforms produced by alternative RNA splicing (see Refs. 25–27 but Ref. 28 for negative evidence). Our results add a new example of this phenomenon specific to the nervous system.

**Physiology and Functional Significance of NET Isoforms—**

The regulation of NET expression in vivo has been investigated in accordance with the physiological and pathological relevance to adrenergic neural transmission, but the results are not consistent. Long term treatment with the tricyclic antidepressant desipramine, for example, has been reported to result in a decrease in norepinephrine uptake sites (29, 30). However, a recent in situ hybridization study on NET mRNA expression following long term desipramine treatment showed a significant increase in hybridization signals in the locus ceruleus of rat brain (31). Taken together, these suggest that the increase in mRNA expression for the NET may not lead to an increased transporter protein expressed on the plasma membrane of norepinephrine neurons. The probe used in the latter investigation was three endelabeled oligonucleotides complementary to the nucleotide sequences encoding the amino acids within 600 of the NET mRNA (3). Therefore, these probes labeled both rNETa and rNETb mRNAs. Based on the present observations, it is reasonable to assume that the increase in rNETb mRNA expression causes a reduction of NET protein on the plasma membrane even though expression of total RNA for NET increases. We propose that measurement of the mRNA expression ratio of rNETa versus rNETb may offer a physiological indicator of NET function.

The regulation of alternative splicing of mRNA is brought about by complex processes in a spatial and/or temporal fashion. Mutation within a gene can lead to inappropriate pairing of 5' and 3'-splice sites resulting in exon skipping or the recognition of cryptic 5'- and 3'-splice sites producing aberrantly spliced RNAs. A recent investigation of the glutamate transporter GLT-1 in relation to the neurodegenerative disease amyotrophic lateral sclerosis suggested a defect in the splicing machinery that leads to the use of inappropriate 5'- and 3'-splice sites (32). These aberrant RNAs function as dominant negative inhibitors of glutamate transport resulting in glutaeminergic excitoneurotoxicity. However, alternative truncated mRNA of GLT-1 similar to this was observed in normal controls (33). This latter observation thus suggested that the alternative splicing form of GLT-1 mRNA plays a physiological role in the central nervous system rather than a pathological one. The finding that alternative splicing of NET mRNA displays a dominant negative effect provides a novel mechanism for the regulation of synaptic transmission by neurotransmitter transporters and highlights the need for further investigation to learn whether this RNA processing is involved in neuronal disorders such as depression or vulnerability to drugs of abuse.

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