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SHORT COMMUNICATION

Val 70, Phe 72 and the last seven amino acid residues of C-terminal are essential to the function of norepinephrine transporter

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ABSTRACT

The norepinephrine transporter(NET) is a member of the Na^+/Cl- dependent neurotransmitter transporter family and constitutes the target of several clinically important antidepressants. To delineate the critical amino acid residues and the function of C-terminal in regulating transport activity of NET, here we constructed two site mutants (V70F, F72V; V70I, F72V) and one C-terminal truncated mutant (△ 611-617). The wild type and mutants of NET were expressed in Xenopus oocytes by injection of their cRNA. We found that all of these mutants lost their transport activity. These results indicate that the amino acid residues of V70 and F72 , and the last seven amino acids of C-terminal are essential to the transport activity of NET.

Key words: *Norepinephrine, transporter, neurotransmitter uptake, mutagenesis.*

INTRODUCTION

Neurotransmitter transporters control the intensity and duration of signal trans-

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duction by a mechanism of rapidly removing transmitter molecules from the synaptic cleft. The extracellular concentration of norepinephrine (NE) bathing synaptic and extrasynaptic adrenergic receptors is regulated by presynaptic norepinephrine transporter (NET)[1, 2]. NET has received particular attention because it presents the target of clinically important antidepressants as well as drugs of abuse and has been implicated in neurological and psychiatric disorders[3]. However, its precise transport mechanism remains largely undefined. Moore and Blakely[4] constructed several chimeras between human NET (hNET) and rat serotonin transporter (rSERT), and found that the chimera, in which the 76 amino acids in N-terminal of hNET was substituted with parallel part of rSERT, the switch point was at S2 in Fig 1 , existed 30% of the wild-type hNET activity, but for the chimera, in which the switch point was at S1, had 100% of the activity. There are only two amino acids different between these two chimeras (Fig 1), that is V70 and F72. To confirm the critical function of these two amino acids in hNET molecule, here we used site-directed mutagenesis technique to construct two mutants to evaluate the role of these two amino acids.

We previously reported the structure of the hNET gene[5], and found that there was a close relationship between exon splicing of the gene and transmembrane domains of the protein (Fig 1). Similar case also exists in human γ-aminobutyric acid transporter (hGAT)[6]. An interesting feature in the gene structure of hNET is that the last seven amino acid residues in hNET C-terminal is separately encoded by an exon. In this paper, we truncated the seven amino acid residues from the C-terminal of hNET to detect whether they were necessary for transport function of the protein.

MATERIALS AND METHODS

Construction of hNET Mutants

A full length human NET cDNA (gifted from Prof. Susan G. Amara) was subcloned into the pAlter-1 (Promega) to yield clone pAlter-NET for the construction of mutants (V70F, F72V); (V70I, F72V) and △ 611-617. Single-stranded phagemid DNA derived from pAlter-NET was used as template, point mutations and deletion were generated with synthetic oligonucleotides using an *in vitro* Mutagenesis kit (Altered-site; Promega). All mutants were verified by dideoxynucleotide sequencing.

In vitro transcription

Wild type and mutants of hNET were subcloned into expression vector pNWP (constructed by Drs Philip Wood and Fang Huang). DNA were linearized by digestion with restriction enzyme Not I and transcribed into cRNA using SP6 RNA polymerase (Ambion).

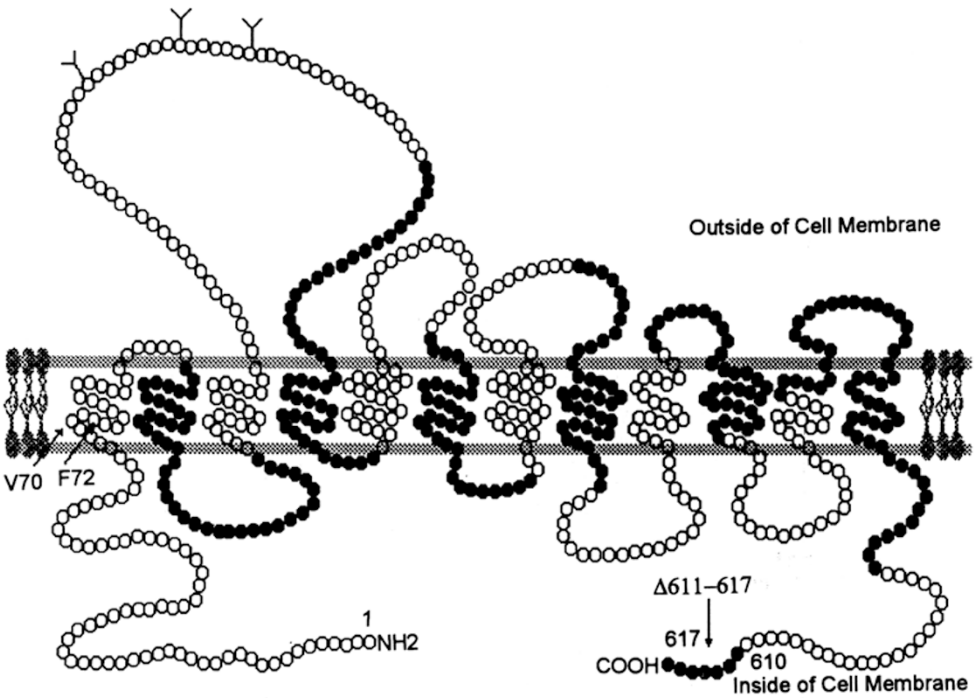
Oocytes injection and transport assays

Oocytes were surgically removed from adult Xenopus laevis females and treated with collagenase to remove the follicular layer. After several hours recovery, each healthy stage V-VI oocyte was injected with 40nl cRNA (0.5 mg/ml) or water using a manual injector. Following incubation at 20 ℃ for three days in incubation solution (Na-Ori solution: 90 m*M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$:, 5 m*M* MOPS, pH7.4), transport activity was measured, eight oocytes from

each experiment group were incubated in the same medium containing 2.5 μM NE (with suitable ^{3}H-NE as tracer or ^{14}C-sucrose for the purpose to detect the oocytes in use were leaky or not). Uptake assays were performed at 20 ℃ for 20 min, the oocytes were washed six times with ice-cold incubation solution. Each oocyte was then transferred into a scintillation vial, dissolved in 0.1 ml of 10 % SDS and counted in 2 ml of scintillation fluid.

RESULTS AND DISCUSSIONS

In the experiment of Moore and Blakely[4], if the 76 amino acid residues in N-terminal of hNET was substituted with parallel part of rSERT, the chimera retained 30% of the wild-type hNET activity, however, when the 63 amino acids in N-terminal



S1 ★★ S2

rSERT	(74)	IRQGERETWGKKM DFLLSVIGYAVDL GNIWRFPYI(108)
hNET	(51)	GDAQPRETWGKKI DFLLSVVGFAVDL ANVWRFPYL(85)

Fig 1. (Upper) Schematic representation of the human norepinephrine transporter protein (hNET). A stretch of sequence consisting of the same circles (open or closed) represents an exon encoding region. Potential glycosylation sites present in the largest loop outside marked by "Y". The mutation sites are indicated by arrow. (Lower) sequences of transmembrane domain 1 and their near part of N-terminal of rSERT and hNET, the first transmembrane domain was underlined.

of hNET was replaced by corresponding part of rSERT, the resulting chimera retained 100% of the NE transport activity. There are only two amino acids different between two chimeras (Fig 1), the Val70 in hNET is changed to Ile and Phe72 is changed to Tyr. To study the function of these two amino acid residues in the activity of the hNET, we generated two kinds of mutants of the hNET by using the method of site-directed Mutagenesis. They are (V70I, F72V) and (V70F, F72V). The latter is just to exchange the position of these two amino acid residues in the protein. The mutant (V70F, F72V) is non-functional and mutant (V70I, F72V) represents less than 10% of NE transport activity compared to wide type hNET (Fig 2). Putting together the results of chimeras and our experiment, it suggests that V70 and F72 may be important for appropriate substrate recognition of the hNET, and that one aromatic amino acid residue exists at position 72 and Val at position 70 are critical for the protein activity.

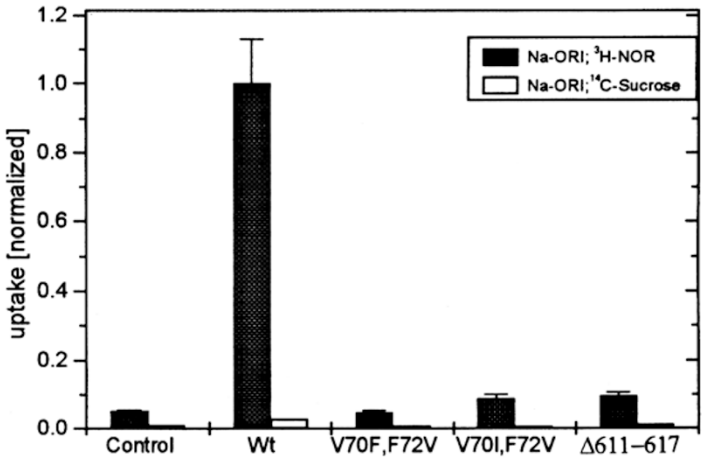


Fig 2. Functional analysis of wild type (WT) and mutants (V70I, F72V) (V70F, F72V) and △ 611-617.

The hNET protein is encoded by 14 exons, splicing of exons has a close relationship with structural domains on the protein, except transmembrane domain 10 and 11, all others are encoded by a separate exon, the sequence encoding for C-terminal of hNET is from three exons (Fig 1), the last 7 amino acid residues (LQHWLAI) in C-terminal is encoded by exon 14. In order to know whether these 7 amino acid residues are important for the function of the hNET, we made a truncated mutant (△ 611-617) of hNET. The mutant presented less than 10% of transport activity of wild-type (Fig 2). This result indicated that the last 7 amino acid residues of the C-terminal may constitute a separate functional domain or motif which is important for the function of hNET. Bendahan and Kanner[7] had made a series of deletions concerning C-terminal of GAT, and found that mutant with last 24 amino

acid residues truncated had 100% transport activity, mutant with last 39 amino acid residues truncated decreased its transport activity dramatically. In GAT1 the C-terminal is encoded by two exons, the last 34 amino acid residues is encoded by exon15[6]. Although the topological structure of hNET is much similar to that of GAT, their amino acid sequence and exon division of gene in C-terminal are not conserved. It is reasonable to deduce that the last seven amino acids in C-terminal of hNET is essential to the function of the protein and play different roles in transporting function comparing with the GAT1.

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