Identification and expression of a variant isoform of the levocabastine-sensitive neurotensin receptor in the mouse central nervous system

Jean-Marie Botto, Philippe Sarret, Jean-Pierre Vincent, Jean Mazella*

Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, UPR 411, 660 Route des Lecuioles, 06560 Valbonne, France

Received 14 November 1996

Abstract This work describes the molecular cloning of a variant isoform of the low-affinity levocabastine-sensitive neurotensin receptor isolated from mouse brain. Although the corresponding mRNA encodes for a 282 amino acid protein unable to bind neurotensin after transient transfection in COS-7 cells, this non-functional neurotensin receptor is expressed in cerebral neocortex, cerebellum, olfactory bulb, striatum and hypothalamus with a level similar to that of the full-length low-affinity neurotensin receptor. By contrast, this receptor form is very weakly expressed in mesencephalon and absent in the pituitary, but is the major product in the spinal cord.

Key words: Neurotensin; Receptor; Low affinity; Levocabastine; Variant; Cloning

1. Introduction

Among the numerous effects induced after central administration of neurotensin (NT) in murine species, hypothermia, analgesia and increase in the dopamine turnover and release were studied in most detail (for review, see [1]). The recently developed non-peptidic antagonist SR48692 [2] was shown to be an efficient blocker of the NT-induced modulation of the dopaminergic systems via the high-affinity NT receptor (NTRH) cloned from the rat brain [3] and from the human colonic carcinoma HT29 cell line [4]. By contrast, SR48692 failed to antagonize NT-induced hypothermia and analgesia in mouse and rat [5], likely suggesting that other types of NT receptors (cloned and/or not yet identified) could be implicated in these effects. The cloning of cDNAs encoding the low-affinity levocabastine-sensitive NT receptor (NTRL) from mouse and rat brain [6,7] was recently described. This new NT receptor type is pharmacologically distinct from the NTRH and also belongs to the family of G-protein-coupled receptors identified by their seven hydrophobic domains. This receptor family is characterized by the frequent occurrence of several subtypes for each natural ligand. An increasing number of G-protein-coupled receptor subtypes are also expressed under the form of spliced variants with variable parts of the transmembrane domains have been lost. This protein failed to bind NT after transient expression in COS-7 cells, although the corresponding mRNA was expressed in several brain areas.

2. Materials and methods

2.1. Materials

Neurotensin was purchased from Peninsula Laboratories. 125I-Tyr3-NT was prepared and purified as previously described [11]. The expression vector pcDNA3 was from Invitrogen. Dulbecco's modified Eagle's medium and fetal calf serum (FCS) were from Life. Gentamycin, 1-10-phenanthroline were from Sigma. FCS was from Boehringer Mannheim. Oligonucleotides and restriction endonucleases were purchased from Eurogentec. 32P-dATP, 35S-Met were from ICN Pharmaceutical.

2.2. cDNA cloning and expression of the NTRL variant

The mouse brain cDNA library was constructed as initially described [6]. High-stringency screening was performed by hybridization with a randomly primed, 32P-dATP-labeled probe corresponding to the total open reading frame of the cDNA encoding mouse NTRL [6]. Hybridization and filter washing were carried out at 68°C under previously described conditions [12]. One of the isolated clones, bearing 5' and 3' ends identical to the cDNA encoding the functional mNTRL (1.55 kb), was only 1.35 kb long. The total nucleotide sequence of this clone was determined in both strands by using the ABI-prime DNA sequencing kit (Applied Biosystems, Foster City, CA). The 1.35 kb EcoRI–Apal fragment of the variant NTRL cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) containing the cytomegalovirus promotor and the neomycin resistance gene as a selective marker.

Transient transfections were performed with 5 μg of recombinant pcDNA3 plasmid by the DEAE-dextran precipitation method [13] onto semiconfluent COS-7 cells grown in 100 mm cell culture dishes. Binding experiments were performed 60 h after transfection.

Binding experiments were performed on freshly prepared cell membrane homogenates as described previously [14]. Cell membranes (50 μg) were incubated in 250 μl of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin and 0.8 mM 1-10-phenanthroline (binding buffer) with increasing concentrations of 125I-Tyr3-NT of these variant forms have lost their ability to bind the natural ligand either because they have lost typical transmembrane-spanning domains as described for the non-functional dopamine D3 receptor [9] or because several transmembrane-spanning domains have been deleted as observed for variants of the human growth hormone-releasing hormone receptor [10].

In the present work, we demonstrate the existence of a NTRL variant isoform that has been isolated by high-stringency screening of a mouse brain cDNA library. This variant form was characterized by total sequencing and RT-PCR experiments performed on several mouse tissues and brain regions. The deletion of 181 bp, possibly generated by alternative splicing, induced a frame shift which prematurely introduced a stop codon. The translation product of this isoform mRNA is a protein of 282 amino acid residues, instead of 417 for the long mRNA form, in which the last two transmembrane domains have been lost. This protein failed to bind NT after transient expression in COS-7 cells, although the corresponding mRNA was expressed in several brain areas.

0014-5793/97$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved. PII S0014-5793(96)01391-9
(100 C/mmol) from 1 to 10 nM. Non-specific binding was measured in parallel incubations containing 1 μM unlabeled NT. After 20 min at 25°C, incubation media were filtered through cellulose acetate filters (Sartorius, Bohemia, NY). Filters were rinsed twice with 2 ml of ice-cold binding buffer and counted in a Packard γ-counter, with a 80% counting efficiency.

2.3. RT-PCR and Southern blot analysis

Total RNAs were extracted from different mouse central nervous system (CNS) regions using the Chomczynski and Sacchi method [15]. The reverse transcription reactions were performed with 2 μg of total RNAs by using the 'Reverse Transcription System' kit (Promega) and a 15-mer oligo-dT primer. The resulting single-stranded DNA was used as template for PCR amplifications. The oligonucleotides used (5'-AACCGTCTGCTATCTTCC-3' and 5'-TGGGTTTTCTTGGGATCCC-3') are flanking the region where the deletion occurred and allow the amplification of a fragment of the receptor cDNA from bases 654-1257 in the sequence previously reported [6]. The predicted size of amplified fragments was 604 bp for nNTRL and 423 bp for the variant form. PCR products were analyzed on a 2% agarose gel. In some cases, the gel was denatured in 0.5 M NaOH, 1 M NaCl twice for 15 min and neutralized in 20XSSC and DNA fragments were transferred onto a Hybond-N nylon membrane (Amersham) under 20XSSC overnight. Transferred DNAs were finally fixed by exposure to UV.

The prehybridization was performed at 65°C for 2 h in 5XDenhardt's solution, 6XSSC, 0.2% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed in the same buffer using 32P-labeled specific probes. Probe 1 which is theoretically able to label the two variant products was prepared by PCR using oligonucleotides described above and the recombinant plasmid containing the full-length NTRL as template. Probe 2 which is specific for the greater fragment corresponding to the native NTR was prepared from the first probe by enzymatic restriction with BseXI and BstYI, which led to a 110 bp fragment corresponding to a part of the region missing in the variant form. This fragment was purified from a 5% low gel temperature agarose gel and centrifugation through glass fiber. The probes were labeled with [32P]-dCTP by using the 'Nonprimer labeling kit' from Appiogene. The labeled probes were purified by G50 gel filtration and used for hybridization after denaturation for 5 min at 95°C. The hybridization was carried out overnight at 65°C with 1X106 cpm/ml of labeled probes. Washing steps were carried out as follows: 2X15 min with 0.2 SSC, 0.1% SDS at 65°C; 2X15 min with 0.1 SCC; 0.1% SDS at 65°C.

3. Results

3.1. Molecular cloning, sequencing and identification of the variant form of NTRL

A mouse brain cDNA library consisting of 5X105 clones was screened by hybridization with a cDNA probe corresponding to the open reading frame of the mouse NTRL cDNA clone under high-stringency conditions. From seven positive clones corresponding to either partial or total NTRL cDNA, one clone, whose restriction analysis revealed 5' and 3' ends identical to those of the full-length NTRL cDNA, clearly appeared shorter (1.37 kb). Nucleic acid sequence analysis demonstrated that this cDNA clone was identical to that of NTRL except that a 181 bp fragment corresponding to nucleotides 747-927 of the previously reported cDNA sequence was missing (Fig. 1A). This deletion causes a shift of the reading frame in the corresponding C-terminal amino acid sequence, resulting in a truncated NTR which was 135 amino acid shorter than the full length form and which contained 42 different amino acids in its C-terminus (Fig. 1A). Note that the C-terminal sequence of the short NTRL form, which could be intracellular, contains nine Ser and six Thr residues. The previously described mouse NTRL is a protein of 417 amino acids, whereas the shorter form is constituted of 282 amino acid residues. As shown in Fig. 1B, the nucleotide
sequence of the 5' end of the truncated portion was found to share homology with the consensus sequence of vertebrate splice acceptor sites [16]. Hydrophobicity analysis of the predicted amino acid sequences clearly indicated that the C-terminus of the truncated NTRL missed the two C-terminal transmembrane domains VI and VII (Fig. 2) [17]. As predicted from this observation, COS-7 cells transfection of the recombinant pcDNA3 plasmid encoding the shorter NTRL led to membrane preparations totally devoid of $^{125}$I-Tyr$^3$NT binding (results not shown).

To demonstrate that this shorter form is actually expressed in the mouse brain, and particularly to eliminate the possibility of an artifactual recombination during the construction of the library, the presence of both long and short receptor mRNAs in the mouse brain was examined by RT-PCR experiments. Amplification products obtained by PCR were visualized in Fig. 3A (lane 2) where two bands with predicted sizes of 604 and 423 bp were actually amplified in the whole brain. The identity of these PCR products was further confirmed by Southern blot analysis, using either a common radiolabeled cDNA probe (probe 1, 604 bp), or a radiolabeled probe specific of the longer form (probe 2, 110 bp). Labeling of the two bands in Fig. 3B, and of only the 604 bp band in Fig. 3C, corresponded to the expected situation in which both PCR products bore common nucleotide sequence, the shorter form lacking a 181 bp fragment as compared to the long form.

To eliminate the possible obtention of an artifactual product generated in mouse brain either by reverse transcription or by PCR reactions, RNAs were extracted from COS-7 cells transiently transfected with the full-length NTRL cDNA, then, the single strand obtained by reverse transcription was used with the same primers for PCR amplification. Only the largest product (604 bp) was detected (Fig. 5, lane 9).

The expression of mRNA encoding two NTRL isoforms in different mouse CNS structures and in the pituitary was examined (Fig. 4). The two forms were clearly expressed in the neocortex, the cerebellum, the striatum, the olfactory bulb and the hypothalamus (lanes 1, 2, 4, 6, and 8). A higher expression of the full-length form was observed in the mesencephalon (lane 3) whereas the spinal cord almost exclusively expressed the truncated form (lane 5). By contrast, the pituitary appeared to be devoid of NTRL isoforms (lane 7).

4. Discussion

This work describes the existence of a variant form of the mouse NTRL. The corresponding receptor cDNA is identical to the full-length NTRL, except for a 181 nucleotide deletion. This deletion leads to a shift of the reading frame which changes the C-terminal amino acid sequence leading to a protein of 282 residues. The resulting protein is devoid of the last two hydrophobic domains VI and VII and fails to specifically bind $^{125}$I-Tyr$^3$-NT after transient expression in COS-7 cells. We know from coupled transcription-translation experiments that the cDNA encoding this truncated receptor is able to produce a protein of 31 kDa as predicted from the amino acid sequence (result not shown). However, the loss of 135 residues, representing almost one-third of the NTRL, could alter the correct addressing to the cell membrane during expression experiments.

Two possibilities could account for the production of this non-functional NTRL (nfNTRL) mRNA. (1) It could result from alternative splicing of the NTRL primary transcript. (2) The variant NTRL cDNA could represent the transcript from an unidentified duplicated gene carrying a deletion. Indeed, if the 181 nucleotides sequence (missing in nfNTRL) is defined as an intron, expression of the corresponding mRNA must be regulated by alternative splicing of the NTRL primary transcript. The consensus sequence for 5' splice sites correctly matches with this putative intron at its 5' end (Fig. 1B), but the 3' cleavage site is CA:N whereas the highly conserved sequence of 3' splice sites is AG:N [16]. Thus, in the case of an alternative splicing, the 3' end of the intron would be unusual. Careful sequencing of several clones isolated from mouse brain by RT-PCR confirms the sequence of the truncated isoform with a 181-nucleotide-long deletion. Therefore, the possibility of a gene duplication with a deletion represents the most probable explanation of the existence of such a truncated receptor form. Further chromosomal location using specific probes will definitively answer the question.

A growing series of truncated G-protein-coupled receptors has been described. Some of these truncations did not modify

![Fig. 3. Hydrophobicity plot of the two NTRL isoforms. Hydrophobicity of predicted amino acid sequences of longer (A) and shorter (B) NTRL was determined by the method of Kyte and Doolittle [17]. Stretches of hydrophobic regions are numbered. Inserts: Transmembrane domains and extra- and intracytoplasmic loops of the respective proteins are schematically represented.](image-url)
NTRL mRNA was predominantly expressed. The discrepancy and is expressed as a protein VII, mone [10]. More recently, a short variant of the 5-hydroxytryptamine 2c receptor produced by alternative splicing has been identified in rat, mouse and human brain [19]. The truncated form of potential intracellular plasma membrane, could play a role in the regulation of intracellular events induced by the full-length NTRL. Indeed, the C-terminal sequence of the truncated NTRL which bears 42 different amino acid residues strongly resembles classical C-terminus of G-protein-coupled receptors rich in Ser and Thr residues [20]. For example, this protein, if correctly inserted into the cell plasma membrane, could play a role in the regulation of internalization of either the NTRL or a structurally related receptor. The functional or pathological importance of the truncated NTRL remains to be elucidated. However, it is important to be aware of the existence of this new family of truncated receptors whose physiological activity is unknown.

Acknowledgements: This work was supported by the Centre National de la Recherche Scientifique. J.M.B. is a fellowship recipient of the Association pour la Recherche sur le Cancer. We thank Franck Aguilera for excellent artwork.

References