

Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor

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A human neurotensin receptor (hNTR) cDNA was cloned from the colonic adenocarcinoma cell line HT29. The cloned cDNA encodes a putative peptide of 418 amino acids with 7 transmembrane domains. The amino acid sequence of the hNTR is 84% identical to the rat NTR [Neuron, 4 (1990) 847–854]. Transfection of this cDNA into COS cells results in the expression of receptors with pharmacological properties similar to those found with HT29 cells. Northern blot analysis using the hNTR cDNA probe indicated a single transcript of 4 kb in the brain, the small intestine and blood mononuclear cells.

Neurotensin; Receptor; Cloning; Binding; HT 29 cell

1. INTRODUCTION

Neurotensin (NT) is a tridecapeptide originally isolated from calf hypothalamus [1]. Thereafter, the presence of high concentrations of neurotensin was found in numerous areas of the central nervous system (CNS) and gastro-intestinal tissues [2]. In these tissues, neurotensin produces a diversity of pharmacological effects [3]. Evidence of a close connection between the NT and dopaminergic systems has recently increased the interest for NT-containing circuits considering their potential implications in abnormal behavior and neuropsychiatric disorders [4].

The pharmacological effects displayed by neurotensin are mediated by specific membrane receptors. The biochemical and pharmacological properties of these binding sites have been extensively studied using mammalian brain homogenates as well as membrane preparations from neuronal and certain non-neuronal cell lines [5,6]. It has been shown that the interaction with these receptors modulates intracellular levels of cGMP, cAMP and inositol phosphates [7,8].

Different groups were able to solubilize and purify to homogeneity NT receptors (NTRs) from bovine [9], rat [10] and mouse brains [11,12], and more recently, Tanaka et al. [13] reported the molecular cloning of the rat neurotensin receptor.

In the present study, a cDNA encoding the human

neurotensin receptor was isolated by screening a human colon adenocarcinoma cell line (HT-29) cDNA expression library using a radioligand-binding strategy. This cDNA encodes a 418 amino-acid protein with a transmembrane topology similar to that of other G-protein coupled receptors. The properties of the cloned binding site expressed in COS cells are identical to those found in HT29 cells. In addition, besides the small intestine and brain, the expression of neurotensin receptor mRNAs was also detected in peripheral blood mononuclear cells (PBMC).

2. MATERIALS AND METHODS

2.1. Drugs and peptides

Labeled (¹²⁵I-labeled [monoiodo-Tyr³]neurotensin, 2,000 Ci/mmol) and unlabeled neurotensin were purchased from Amersham (Buckinghamshire, UK) and Bachem (Bubendorf, Switzerland), respectively. Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS) and phosphate-buffered saline (PBS) were from Gibco (Paisley, Scotland). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF) and 1,10-orthophenanthroline were from Sigma (St. Louis, MO).

A selective, non-peptide antagonist SR 48692 was synthesized at Sanofi Recherche, Montpellier, France [14]. The anti-histamine agent, levocabastine, was obtained from Janssens Pharmaceutica (Geel, Belgium).

2.2. Cell culture

The human colonic adenocarcinoma HT29 cell line (kindly provided by Dr. P. Kitabgi) and the COS-7 cell line were cultured as previously described [8].

2.3. RNA extraction and analysis

Total RNA was extracted from HT29 cells by using the acid-guanidinium isothiocyanate-phenol-chloroform method [15]. Poly(A)

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RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described by Aviv and Leder [16]. RNA analysis was performed by electrophoresis on 1% agarose/formaldehyde gels then transferred to nitrocellulose and probed with a 32 P-labeled hNTR cDNA as described [17].

2.4. cDNA library construction and DNA sequencing

The cDNA library was constructed using the primer-adaptor procedure [18] and the pSVL vector (Pharmacia). Approximately 5×10^5 primary recombinants were produced and organized in pools of 1,000 clones. DNA sequences were determined by the method of Sanger [19].

2.5. Transfection of COS cells and radiolabeled ligand detection

Preparation of recombinant plasmids and the transfection of COS-7 cells by the DEAE dextran procedure were as previously described

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1  TCAAGCTCGCCCGGCGAGCGAGCGGCGCTGCTCTCGGGGCGCTGGGAGC  60
61  GCGCGCTTGGAGATCGGAGCGAGCTGAGACCGCTGGCAGCGCGGAGCGGAGAGC  120
121  CCGAGAGATCGAGGCTTGGAGCTGAGAGCGAGCTGAGAGCGGAGCGGAGAGAGC  180
181  AGCGCGAGCGCGGAGCGGCGGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  240
241  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  300
301  AGCGCGAGCGCGGAGCGGCGGAGCTGAGAGCGGAGCTGAGAGCGGAGCGGAGAGC  360
361  CAGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  420
-3  MRLNSSAPGTPGTAAADPFQRAQAGLEEALLAPGFGNAGSNASERVLA  480
421  GACCGCTTCAGCGGAGCGGAGCGGAGCTGAGAGCGGAGCTGCTGGCGGCGCTGGC  480
17  DPFQRAQAGLEEALLAPGFG  36
481  AAGCGCTTCAGCGGAGCGGAGCGGAGCTGAGAGCGGAGCTGCTGGCGGCGCTGGC  540
37  NASGNASERVLAAPSS  36
541  AACACCGAGATCTACTCCAAAGTCTGAGAGCGGAGCTGCTGGCGGCGCTGGC  600
57  NTDIYSKVLVLA  16
601  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  660
77  GTTGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  720
561  CTCAGAGCGGCTGAGTACCTGAGCGGAGCGGAGCTGCTGGCGGCGCTGGC  720
97  LQSTVHTNHLGLSLALSLDL  116
721  CTCGCGGCTTGGAGATCGGAGCGAGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  780
117  LLANPVEELYNFIWVHPWAF  136
781  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  840
137  GDAGCGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT  840
841  CTCAGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  900
157  LNVASLSVERLYLAICHFPK  176
901  AAGCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  960
177  KTLNRSRSTRKFFISAIWLAS  196
961  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1020
197  ALLTVPMLFTMGLQNRSGD  216
1021  CAGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1080
217  QHAGGLVCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT  236
1081  ATACGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  1140
237  IQVNTFMSTFIPPHMVVISV  256
1141  ACCATCATCGGAGCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1200
257  TIIANRLTVHVRQAAEQGV  276
1201  TCGAGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1260
277  CTYVGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  296
1261  GCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1320
297  ALRHGVLVLA  316
1321  CTCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1380
317  LPHYHRLMFCYISDEQWTF  336
1381  TTCCTGATGCTTACGCTTACCTTACCTTACCTTACCTTACCTTACCTTACCTTAC  1440
337  FLYDFYHYHFIWVHPWAF  356
1441  TCGAGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1500
357  STINPLIYNLVSANFRHIFL  376
1501  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1560
377  ATLAACLPVWRRRRKRPA  396
1561  AGGAGCGCGGAGCGGAGCGGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1620
397  BRADSVSNHTLSNATRE  416
1621  CTCGATAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT  1680
417  L  436
1681  CCGGAGCGGAGCGGAGCGGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  1740
1741  TCGATGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAG  1800
1801  AGTGTCTCCGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  1860
1861  CAGAGAGAGAGAGCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  1920
1921  GTGTGGGAGAGAGAGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  1980
1981  ATGAATGTGCTGGCTGGGCGGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2040
2041  TCTGTGGAGCTGAGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2100
2101  CGTGTGGAGCTGAGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2160
2161  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  2220
2221  CGTGTGGAGCTGAGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2280
2281  CAGCGCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  2340
2341  CTCCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2400
2401  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  2460
2461  GCGCGAGTCCGAGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2520
2521  CGCATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT  2580
2581  TGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2640
2641  CTCGATGAGCTGAGCGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  2700
2701  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  2760
2761  GCGCGCTTGGAGATCGGAGCGAGCGCTGCTGGCTGGCGCTTCCGCTTCCGCTGAGC  2820
2821  ATTCAGGCTTCCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  2880
2881  CTACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  2940
2941  GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3000
3001  GCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3060
3061  TGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  3120
3121  ATGAGAGTCCGATGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  3180
3181  TCTTTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3240
3241  TGGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  3300
3301  GTTCATCGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3360
3361  AGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3420
3421  GAGCTTTCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  3480
3481  GTGGCTCAGAGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCTTCCGCT  3540
3541  GACTTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3600
3601  TAAATTCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3660
3661  CTGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3720
3721  GTCTTTCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3780
3781  GTCCTTTCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3840
3841  CTCCTTTCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  3900
3901  CGACAGCTGATCTGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT  3960
3961  CTTCTGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT  4020
4021  AGCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  4080
4081  ACAAACCGCTGATCTTCAATAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG  4140

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Fig. 1. Nucleotide and predicted amino acid sequences of the hNTR cDNA.

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1  MRLNSSAP .GTPGTAAADPFQRAQAGLEEALLAPGFGNAGSNASERVLA  49
1  MHLNSSVPQGTGEPDAPQFSGPQSEMEATFLALSLSNGSGNTSESDTAG  50
50  PSSELDVNTDIYSKVLVTAVYLAFLVVGTVGNTVTAFTLARKKSLQSLQS  99
51  PNSDLVNTDIYSKVLVTAVYLAFLVVGTVGNSVTAFTLARKKSLQSLQS  100
100  TVHYHLGLSLALSDLLTLLAMPVELYNFIWVHPWAFGDAGCRGYFLRD  149
101  TVHYHLGLSLALSDLLTLLAMPVELYNFIWVHPWAFGDAGCRGYFLRD  150
150  ACTYATALNVASLSVERYLAICHFPKAKTLMRSRSTRKFFISAIWLASALL  199
151  ACTYATALNVASLSVERYLAICHFPKAKTLMRSRSTRKFFISAIWLASALL  200
200  TVPMLFTMGEQNRSGDGHAGGLVCTPTIHTATVKVVIQVNTFMSEFFIM  249
201  AIFMLFTMGLQNRSGDGHAGGLVCTPTIHTATVKVVIQVNTFMSEFFIM  250
250  WVISVLNTIANKLTVMVVQAAEQGVCTVGG...EHSTFSMAIEPGRV  295
251  LVISILNTVIANKLTVMVVQAAEQGVCTVGTGTHNGLEHSTFNMTEPGRV  300
296  QALRHGVLVLA  345
301  QALRHGVLVLA  350
346  YMVNTALFYVSSSTINPLIYNLVSANFRHIFLATLACLPVWR .RRRKRA  394
351  YMLTALFYVSSAINPLIYNLVSANFRQVFLSTLACLPGRWRRRRKRPT  400
395  FSRKADSVSSNHTLSSNATRETL  418
401  FSRKPNMSSNHAFSTSATRETL  424

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Fig. 2. Alignment of the hNTR to the rNTR. The complete hNTR and rNTR sequences are compared; gaps for alignment are indicated by dots and identical residues indicated by vertical lines. The solid lines above the sequences indicate the proposed transmembrane domains

[20]. Briefly, 5×10^5 cells were plated in Slides Flasks (Nunc) and grown in DMEM containing 5% FCS in 5% CO_2 at 37°C . After an overnight incubation, the cells were transfected with $3 \mu\text{g}$ of extracted DNA from pools in 2 ml of DMEM containing 400 mg of DEAE dextran (Pharmacia) and 200 μM of chloroquine diphosphate. After 5 h at 37°C , the transfection medium was removed and the cells were treated with 2 ml of PBS containing 10% DMSO for 1 min, rinsed twice with PBS and incubated in DMEM containing 1% FCS for 3 days. Receptor-expressing pools were detected by binding using ^{125}I -labeled [monoiodo-Tyr³]neurotensin (see below) as a probe followed by autoradiography using a PhosphorImager device (Molecular Dynamics, Sunnyvale, CA).

2.6. Binding experiments

The pharmacological and functional characterization of the cloned receptor was performed using COS-transfected cells seeded in 6-well plates (Falcon). Three days after transfection, cell monolayers were washed twice with 50 mM Tris-HCl pH 7.5, 0.2% BSA, 0.1% $\text{Na}_2\text{S}_2\text{O}_8$, 1 mM 1,10-ortho-phenanthroline (binding buffer).

Saturation experiments were done in 1.5 ml of binding buffer containing ^{125}I -labeled [monoiodo-Tyr³]neurotensin over a range from 0.05 to 2 nM in the absence or in the presence of a 500-fold excess of unlabeled neurotensin. After 1 h incubation at room temperature, plates were aspirated and cell monolayers were washed twice with incubation buffer. Finally, 2 ml of 1 N NaOH were added and bound radioactivity was quantified after cell solubilisation. Non-specific binding was defined as binding in the presence of a 500-fold excess of unlabeled ligand and under these experimental conditions was $< 1\%$ of the total counts.

Displacement experiments were done in an analogous manner using 0.2 nM of ^{125}I -labeled [monoiodo-Tyr³]neurotensin as radioligand. The following unlabeled ligands were used as competitors: neurotensin, neuromedin N, SR 48692 and levocabastine.

Binding data derived from saturation and competition experiments were analysed by using the computerized nonlinear curve fitting described by Munson and Rodbard [21].

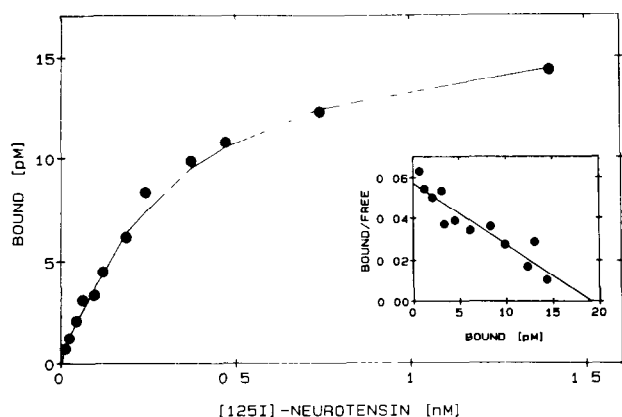


Fig. 3. Saturation isotherm and Scatchard plot (inset) of the specific binding of ^{125}I -labeled [monoiodo-Tyr³]neurotensin to COS-7 cells transfected with the hNTR cDNA. Each point represents the mean of triplicates.

3. RESULTS AND DISCUSSION

3.1. Cloning and sequencing of hNTR

A cDNA expression library containing 5×10^5 recombinant clones was constructed and divided into pools of 10^3 clones. Plasmid DNA from each pool was introduced into COS cells and the binding of neurotensin was detected with a labeled ligand. Only one of the first hundred pools tested showed a significant binding activity. Stepwise fractionations of this neurotensin receptor cDNA-containing pool identified a single recombinant plasmid able to confer a neurotensin binding activity to COS cells.

Fig. 1 shows the 4,140-nucleotide sequence of hNTR

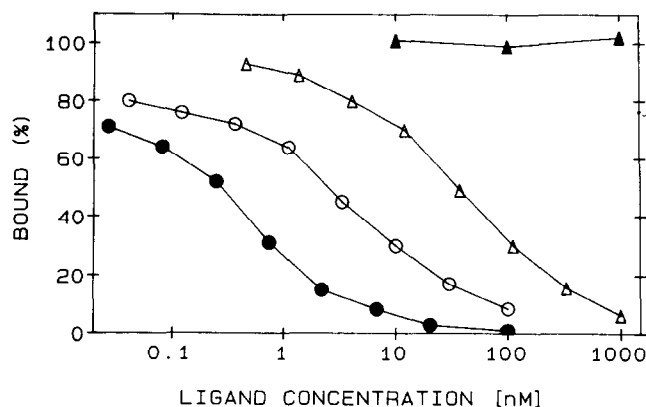


Fig. 4. Inhibition of ^{125}I -labeled [monoiodo-Tyr³]neurotensin binding to COS-7 cells transfected with the hNTR cDNA by unlabeled neurotensin (●), neuromedin N (○), SR 48692 (Δ) and levocabastine (▲). Each point represents the mean of triplicates.

cDNA. Considering the determined size of the NTR mRNA (Fig. 5), hNTR cDNA seems to represent a full length transcript. The longest open reading frame found encodes a protein of 418 residues with a theoretical molecular weight of 46,288 kDa. The protein shows several structural features of G-protein coupled receptors and a high degree of identity (84%) and similarity (92%) with the rat neurotensin receptor [13] (Fig. 2).

3.2. Pharmacological and functional characterization of cloned hNTR

We have examined the binding properties of the human neurotensin receptor expressed in COS cells after transfection of the cloned cDNA. Little or no

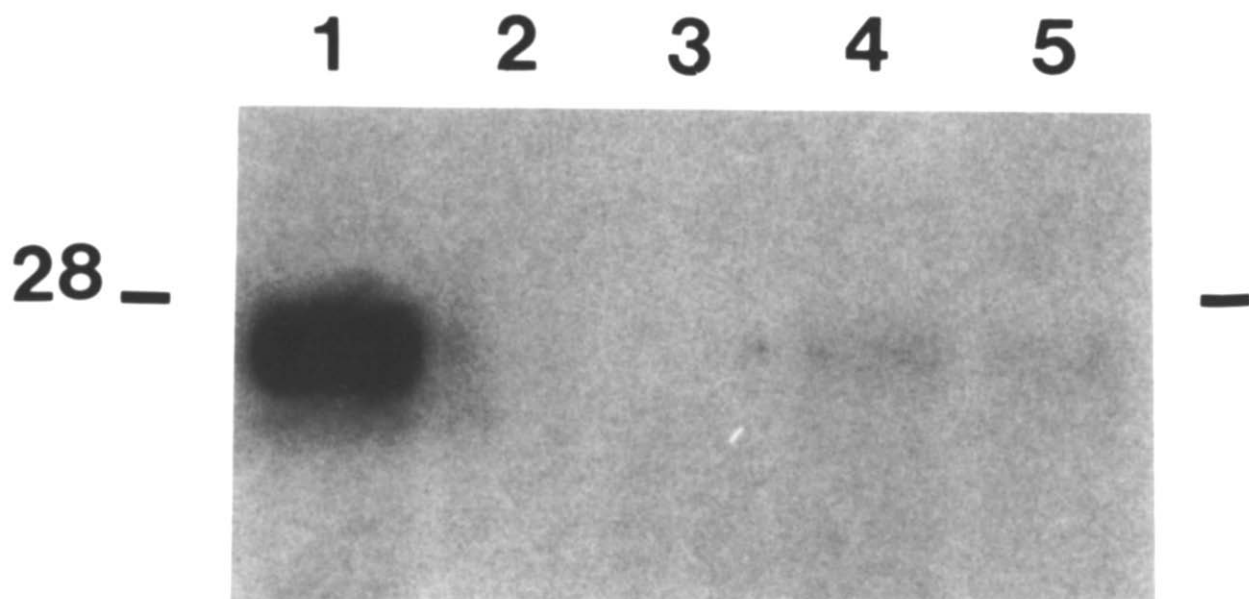


Fig. 5. Northern analysis of poly(A) RNA ($1\mu\text{g}$) from cells and tissues hybridized to ^{32}P -labeled hNTR cDNA. Lane 1, HT29 cells; lane 2, human brain (rear part); lane 3, human brain (front part); lane 4, intestine; lane 5, PBMC.

binding was detected with untransfected cells or cells transfected with the vector DNA alone (not shown). The expressed receptor is able to bind in a specific and saturable manner ^{125}I -labeled [monoiodo-Tyr³]neurotensin (Fig. 3). Scatchard plot analysis of ^{125}I -labeled [monoiodo-Tyr³]neurotensin binding (Fig. 3, inset) showed a single high affinity population of receptors with a dissociation constant (K_d) of 0.56 ± 0.1 nM and a binding capacity (B_{max}) of $30,000 \pm 3,000$ sites/cell.

Fig. 4 shows competition curves of various ligands with ^{125}I -labeled [monoiodo-Tyr³]neurotensin. Agonists and antagonists competed for the binding of [monoiodo-Tyr³]neurotensin with an order of potency identical to the neurotensin receptor expressed in the HT29 cell line [6] and other tissues [14]: (i) neurotensin is the most potent competitor, followed by neuromedin N and SR 48692; (ii) the apparent half maximal concentrations for an inhibition (IC_{50}) derived from the competition curves were 0.3, 2.6 and 38 nM, respectively; and (iii) levocabastine, an anti-histamine agent described as a ligand for the low affinity neurotensin binding site [22] does not compete (using concentrations up to $10 \mu\text{M}$) with ^{125}I -labeled [monoiodo-Tyr³] neurotensin for the binding to COS cells, demonstrating that the transfected cDNA encodes a high affinity NTR.

3.3. Tissue distribution of hNTR mRNA

The tissue distribution of hNTR mRNA was examined by Northern blot analysis. As shown in Fig. 5, ^{32}P -labeled hNTR cDNA hybridized to poly (A) RNAs isolated from HT29 cells, brain, colon and peripheral blood mononuclear cells (PBMC) gave rise to a single band although it is barely detectable in the brain RNAs (lanes 2 and 3). This result was confirmed by polymerase chain reaction (PCR) experiments (not shown).

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