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Molecular characterisation, expression and localisation of human neurokinin-3 receptor

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The complete amino acid sequence of the human neurokinin-3 receptor was deduced by DNA sequence analysis of human genomic fragments. Comparison of the predicted primary structure with those for the human neurokinin receptors 1 and 2 shows a highly conserved pattern of seven hydrophobic regions with maximum divergence occuring at the amino- and carboxy-termini. The position of intron–exon junctions are identical to those in other reported neurokinin genes. Using a chimeric genomic-cDNA gene, the human NK-3 receptor was expressed in *Xenopus laevis* oocytes where it mediates membrane conductance changes in response to its agonist, neurokinin B. More significantly, expression of the gene in mammalian cells resulted in detection of receptor binding as well as neurokinin-stimulated calcium mobilization and arachidonic acid release, all displaying the pharmacological characteristics expected of a neurokinin-3 receptor. By using the polymerase chain reaction we have shown that mRNA for the human neurokinin-3 receptor is expressed predominantly in the central nervous system.

Neurokinin; Neurokinin-3 receptor (human); Brain localization; Calcium mobilization; Arachidonic acid release

1. INTRODUCTION

Neurokinins are a family of neuropeptides displaying wide-spread distribution and diverse biological activities including regulation of neurotransmission, inflammation and perception of pain [1–3]. Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are mammalian neurokinins showing limited selectivity towards, respectively, NK-1, NK-2 and NK-3 receptors. Pharmacological and biochemical studies, as well as molecular cloning, have shown that all three neurokinin receptors are members of the G-protein-coupled receptor super-family possessing seven putative transmembrane regions [4].

NK-3 receptors have been identified in membranes from guinea pig myenteric plexus and cerebral cortex [5] as well as brain slices of rat, mouse and guinea pig [2,3,6,7]. NK-3 receptors also mediate increased phosphoinositide hydrolysis in guinea pig ileum and neonatal rat spinal cord [2,8]. In addition, NK-3 receptors depolarize guinea pig myenteric neurons to augment release of acetylcholine [9–11], stimulate contraction of the rat portal vein [12] and modulate both serotinergic- and cholinergic-dependent behavioural responses in mouse and rat [13,14]. Despite these observations indicating an important physiological role controlling transmitter release and vascular tone in some species, NK-3 receptors have not been identified in human tissues. Moreover, a recent study employing radioligand binding to brain slices failed to detect NK-3 receptors in either monkey or human [6]. To answer definitively the question of whether the NK-3 receptor is expressed in human tissues, we set out to detect and identify molecularly the NK-3 gene as well as to localise and characterise pharmacologically, the receptor itself.

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2. MATERIALS AND METHODS

2.1. *Materials*

NKB, NKA and SP were purchased from Bachem. The human genomic lambda bank was purchased from Clontech as was the fetal human brain cDNA bank used for amplification of NK-3 cDNA. The plasmids pCR 1000, used to clone amplified DNA and pcDNA-1 NEO were purchased from Invitrogen.

2.2. *Tissues*

Human brain from a deceased 76-year-old patient was obtained from Dr. C. Bouras (University of Geneva). Between 0.3 and 1.0 g of brain tissue was dissected from various neuroanatomical areas as described in the text. Pieces of peripheral tissues (0.2–1.0 g) removed during surgery and frozen quickly at −70°C were obtained from Dr. A.-P. Sapino (Hospital Cantonal, University of Geneva).

2.3. *Gene isolation*

Detection and analysis of recombinant phage was essentially as described previously [15]. Most techniques, including random primer labeling, Southern blotting, dideoxy sequencing, ligation, plasmid DNA isolation and polymerase chain reaction (PCR) were done by standard protocols [16]. Sequence alignments, shown in Fig. 3 were made using 'Pileup' [17].

2.4. *Oocyte electrophysiology*

Oocytes of *Xenopus laevis* at stage VI were prepared by incubation with 0.2% collagenase in OR-2 medium without calcium. Oocyte

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nuclei were injected with 10 nl of injection buffer (88 mM NaCl/1 mM KCl/15 mM HEPES, pH 7.0) containing 1 ng of plasmid DNA. After injection, oocytes were kept at 19°C in OR-2 medium supplemented with 100 units of penicillin, 100 μg of streptomycin, and 20 μg of kanamycin per ml for 1–3 days before being tested electrophysiologically [18].

2.5. *CHO cell expression, radioligand binding and intracellular calcium measurements*

The chimeric NK-3 genomic-cDNA gene was ligated to the vector pBGL312 for transfection in Chinese Hamster Ovary (CHO) cells. pBGL312 is a derivative of pBG312 [19] containing resistance genes for both G418 and methotrexate. One transfected CHO cell clone resistant to 400 μg/ml G418 was grown in 6-well plates (1–2 × 10^6 cells), washed twice with phosphate-buffered saline and incubated at 37°C in 0.5 ml of Dulbecco's Modified Eagles medium/Ham's F-12 (v/v) medium (Gibco, Paisley, Scotland, UK) supplemented with 0.6 mM $MnCl_2$, 0.004% (w/v) BSA and containing [^{3}H]Senktide (NEN; specific activity 83.1 Ci/mmol) and other neuropeptides as indicated. After 60 min, cells were washed twice with ice-cold medium (ligand-free), solubilized using 5% (w/v) SDS and bound [^{3}H]Senktide determined by scintillation spectrometry. For saturation analysis non-specific binding was measured in the presence of 1 μM NKB. Intracellular Ca^{2+} concentrations were determined using the fluorescent Ca^{2+} chelator *fura2* (Calbiochem) exactly as described [20].

2.6. *RNA isolation and PCR analysis*

Total RNA was extracted from each tissue by the method of Chomczynski and Sacchi [21]. A 10 μg sample of total RNA was used for the synthesis of cDNA with random primers and AMV reverse transcriptase in a total volume of 20 μl. PCR amplification was performed in 100 μl reactions containing 4 μl of cDNA and primers at 1 mM. The following primers were used (see Fig. 1A):

primer 1: (5′)-GGTGGAGGCGTGGGTGCAGACG
primer 2: (5′)-CACTTACCATATTATGCTCATTATACTGG
primer 3: (5′)-GGGTATATAGGACAGGACTGATAAA

The PCR was carried out for 30 cycles by heating at 94°C for 1 min, followed by annealing at 60°C for 2 min and elongation at 72°C for 3 min. 5 μl of each reaction was loaded on a 0.8% agarose gel after electrophoresis and transferred to GeneScreen Plus (NEN) filters. All cDNA samples were also compared for equivalency by PCR with actin-specific primers (not shown).

3. RESULTS AND DISCUSSION

Because prior pharmacological analysis or radioligand binding studies had not identified any human tissues as positive for NK-3 receptor expression, we chose to screen a human genomic bank and to clone all of the exons encoding the translated portions of NK-3. The obtained sequences permitted the design of human NK-3 specific PCR primers and subsequently the amplification of NK-3 cDNA.

Screening of 5 × 10^5 recombinant phage, bearing inserts of human genomic DNA generated three candidates that strongly cross-hybridized with rat NK-3 cDNA [22]. DNA prepared from these phage was analyzed by Southern blotting and fragments were chosen for subcloning into pUC19 (Fig. 1A). Based on sequence comparison with other neurokinin genes [22–26], we concluded that the fragments contained all of the human NK-3 exons corresponding to translated portions of the gene with the exception of the first 17 bp of exon 2. The intron–exon boundaries in human NK-3 conform in position to other NK genes. Sequence information for the combined exons is shown in Fig. 1B. This sequence begins with a naturally occurring *Pst*I site, 16 bp upstream from the codon for the initiating methionine and ends with an *Xba*I site which is the original terminus of the 3500 bp *Xba*I fragment that contains exons 4 and 5.

PCR primers were designed to several parts of the human NK-3 sequence and tested on a variety of cDNA templates. In no case were we able to generate a full-length cDNA copy using primers 1 and 3 (Fig. 1A), corresponding to the all of the exon sequences. The longest PCR product, generated for human NK-3, came from a fetal brain cDNA template and primers whose positions are shown in Fig. 1A. This 941 bp fragment contains the last 24 bp of exon 1 and all of the exons 2, 3, 4 and 5. The fragment was cloned and its sequence confirmed that shown for the exon sequences as well as the exon–intron junctions.

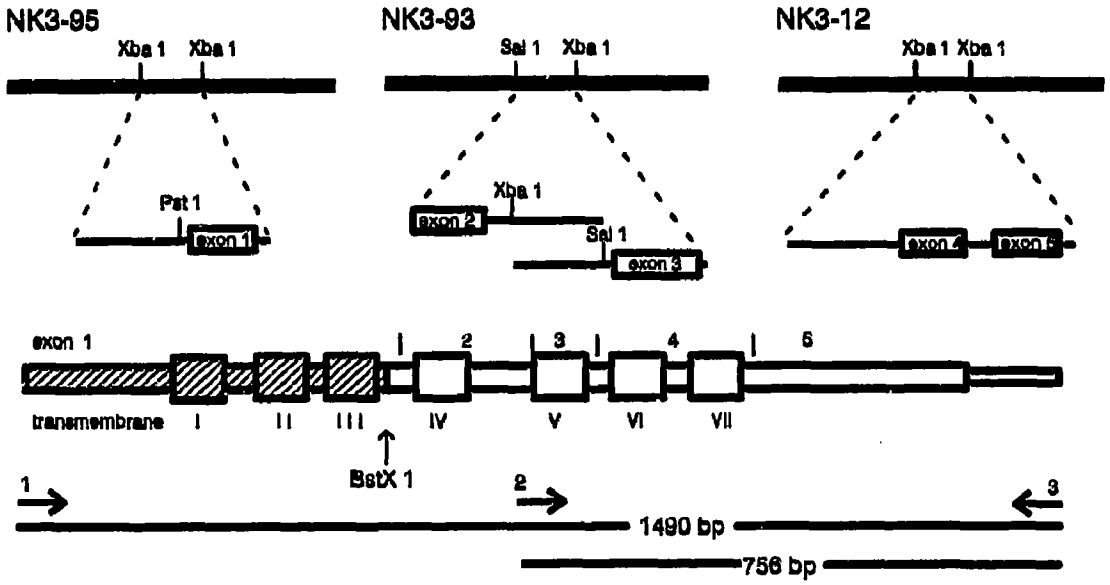
Comparison of the amino acid sequences for the three human neurokinin receptors (Fig. 2) shows a maximum conservation of sequence in the putative transmembrane regions and a minimal identity at the amino- and carboxy-termini. One exception is the region shown between transmembrane region I and II, which is also highly conserved between the three proteins. Closer inspection also permits one to identify some transmembrane regions (TMVII) as more conserved than others. Overall, the amino acid sequence of human NK-3 has 74% and 68% homology with the sequences for human NK-1 and NK-2.

In order to confirm that the genomic sequence encodes a human NK-3 receptor we prepared a chimeric gene by ligating the PCR amplified cDNA to a fragment of exon 1 DNA. This was simplified due to the presence of a unique site for *Bst*XI in both the cDNA and exon 1 DNA. The chimeric gene (shown schematically in Fig. 1A) was subcloned in the plasmid pcDNA1-NEO or pBGL312 and investigated in a series of functional assays.

We began using electrophysiology to test for NK-3 receptor mediated functional activity 2 days after the plasmid pcDNA1-NEO was injected into nuclei of *Xenopus laevis* oocytes. Neurokinins increased current amplitude (data not shown) with the relative rank-order of potency: NKB>NKA>SP which is consistent with the actions of an NK-3 receptor [2,11,12,28]. Half-maximally effective concentrations of NKB and NKA were 4 nM and 40 nM, respectively. A reversal potential of −30 mV, close to the equilibrium potential for chloride, indicates that a Ca^{2+}-sensitive chloride channel is activated [27].

We next expressed the NK-3 receptor gene in CHO cells using plasmid pBGL312 with one G418-resistant subclone employed in all experiments. [^{3}H]Senktide was

A



B

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CTGCAGACCGGTGGCGATGGCCATCCTCCCAGCAGCAGAAACCTGGATAGACGGGGGTGGAGGCGTGGGTGCAGACGCCGTGAACCTGACCGCCTCGCTA
                  MetAlaIleLeuProAlaAlaGluThrTrpIleAspGlyGlyGlyGlyValGlyAlaAspAlaValAsnLeuThrAlaSerLeu    28

GCTGCCGGGGCGGCCACGGGGGCAGTTGAGACTGGGTGGCTGCAACTGCTGGACCAAGCTGGCAACCTCTCCTCCTCCCCTTCCGCGCTGGGACTGCCTG
AlaAlaGlyAlaAlaThrGlyAlaValGluThrGlyTrpLeuGlnLeuLeuAspGlnAlaGlyAsnLeuSerSerProSerAlaLeuGlyLeuProVal    62

TGCGTTCCCCCGCGCCCTCCCAGCCCTGGGCCAACCTCACCAACCAGTTCGTGCAGCCGTCCTGGCGTATCGCGCTCTGGTCCCTGGCGTATGGTGTGGT
  ArgSerProAlaProSerGlnProTrpAlaAsnLeuThrAsnGlnPheValGlnProSerTrpArgIleAlaLeuTrpSerLeuAlaTyrGlyValVal    95

GGTGGCAGTGGCAGTTTTGGGAAATCTCATCGTCATCTGGATCATCCTGGCCCACAAGCGCATGAGGACTGTCACCAACTACTTCCTTGTGAACCTGGCT
 ValAlaValAlaValLeuGlyAsnLeuIleValIleTrpIleIleLeuAlaHisLysArgMetArgThrValThrAsnTyrPheLeuValAsnLeuAla   128

TTCTCCGACGCCTCCATGGCCGCCTTCAACACGTTGGTCAATTTCATCTACGCGCTTCATAGCGAGTGGTACTTTGGCGCCAACTACTGCCGCTTCCAGA
PheSerAspAlaSerMetAlaAlaPheAsnThrLeuValAsnPheIleTyrAlaLeuHisSerGluTrpTyrPheGlyAlaAsnTyrCysArgPheGlnAsn  162

                                        >------ PRIMER ------>
ACTTCTTTCCTATCACAGCTGTGTTCGCCAGCATCTACTCCATGACGGCCATTGCGGTGGACAggtgaggagaggacagacagagaggaaagagggagaa
  PhePheProIleThrAlaValPheAlaSerIleTyrSerMetThrAlaIleAlaValAspArg                    INTRON A                 183

                        GGTATATGGCTATTATTGATCCCTTGAAACCCAGACTGTCTGCTACAGCAACCAAGATTGTCATTGGAAGTATTTGGAT
                          TyrMetAlaIleIleAspProLeuLysProArgLeuSerAlaThrAlaThrLysIleValIleGlySerIleTrpIle  209

TCTAGCATTTCTACTTGCCTTCCCTCAGTGTCTTTATTCCAAAACCAAAGTCATGCCAGGCCGTACTCTCTGCTTTGTGCAATGGCCAGAAGGTCCCAAA
 LeuAlaPheLeuLeuAlaPheProGlnCysLeuTyrSerLysThrLysValMetProGlyArgThrLeuCysPheValGlnTrpProGluGlyProLys   242

CAACATTTCACgtaagttaattctctattatggttttcaattcagttt      INTRON B      catgtgtttttcttatttttcatagTTACCA
GlnHisPheThr                                                                                 TyrHis  248

TATTATCGTCATTATACTGGTGTACTGTTTCCCATTGCTCATCATGGGTATTACATACACCATTGTTGGAATTACTCTCTGGGGAGGAGAAATCCCAGGA
 IleIleValIleIleLeuValTyrCysPheProLeuLeuIleMetGlyIleThrTyrThrIleValGlyIleThrLeuTrpGlyGlyGluIleProGly   281

GATACCTGTGACAAGTATCATGAGCAGCTAAAGGCCAAAAGAAAGgtactggtccatgttgtttaacctag      INTRON C      caaatgact
AspThrCysAspLysTyrHisGluGlnLeuLysAlaLysArgLys                                                  296

ttttctttatagGTTGTCAAAATGATGATTATTGTTGTCATGACATTTGCTATCTGCTGGCTGCCCTATCATATTTACTTCATTCTCACTGCAATCTATC
          ValValLysMetMetIleIleValValMetThrPheAlaIleCysTrpLeuProTyrHisIleTyrPheIleLeuThrAlaIleTyrGln  326

AACAACTAAATAGATGGAAATACATCCAGCAGGTCTACCTGGCTAGCTTTTGGCTGGCAATGAGCTCAACCATGTACAATCCCATCATCTACTGCTGTCT
  GlnLeuAsnArgTrpLysTyrIleGlnGlnValTyrLeuAlaSerPheTrpLeuAlaMetSerSerThrMetTyrAsnProIleIleTyrCysCysLeu  359

GAATAAAAGgtaaaaacaaaactaacgaaatgcaagttgcttgtcac      INTRON D      aaaataacttttcttttctgtggcctgcttttcc
 AsnLysArg                                                                                    362

tcagATTTCGAGCTGGCTTCAAGAGAGCATTTCGCTGGTGTCCTTTCATCAAAGTTTCCAGCTATGATGAGCTAGAGCTCAAGACCACCAGGTTTCATCC
     PheArgAlaGlyPheLysArgAlaPheArgTrpCysProPheIleLysValSerSerTyrAspGluLeuGluLeuLysThrThrArgPheHisPro  394

AAACCGGCAAAGCAGTATGTACACCGTGACCAGAATGGAGTCCATGACAGTCGTGTTTGACCCCAACGATGCAGACACCACCAGGTCCAGTCGGAAGAAA
 AsnArgGlnSerSerMetTyrThrValThrArgMetGluSerMetThrValValPheAspProAsnAspAlaAspThrThrArgSerSerArgLysLys  427

AGAGCAACGCCAAGAGACCCAAGTTTCAATGGCTGCTCTCGCAGGAATTCCAAATCTGCCTCCGCCACTTCAAGTTTCATAAGCTCACCCTATACCTCTG
ArgAlaThrProArgAspProSerPheAsnGlyCysSerArgArgAsnSerLysSerAlaSerAlaThrSerSerPheIleSerSerProTyrThrSerVal  461

                                        <------ PRIMER --------<
TGGATGAATATTCTTAATTCCATTTCCTGAGGTAAAAGATTAGTGTGAGACCATCATGGTGCCAGTCTAGGACCCCATTCTCCTATTTATCAGTCCTGTC
  AspGluTyrSer                                                                               465

CTATATACCCTCTAGA
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←

Fig. 1. (A) Schematic diagram for the isolation of NK-3 exon containing fragments. NK-3 exon 1 was isolated on a 3000 bp *Xba*I fragment. Exons 2 and 3 were isolated from the same phage as 1300 bp *Sal*I and 2000 bp *Xba*I fragments, respectively. Exons 4 and 5 were isolated together on a 3500 bp *Xba*I fragment. The chimeric NK-3 gene was constructed by ligating a *Pst*I-*Bst*XI fragment from exon 1 (hatched region) to a cDNA fragment via the *Bst*XI site. Arrows denote PCR primer positions used in the analysis in Fig. 4. (B) Human NK-3 genomic sequence. Dideoxy sequencing was carried out on double-stranded plasmid template and exon–intron boundaries were assigned by comparison with NK-3 cDNA as well as other NK genes. All sequence shown comes from genomic DNA with the exception of the initial 17 bp on exon 2 (underlined) which was obtained from cDNA.

used as an NK-3 selective radioligand and saturation binding indicated a single class of binding site with a K_d of 9.6 nM and expression of approximately 17,000 receptors/cell (Fig. 3A). Specific [^{3}H]Senktide binding was >90% of total and this was displaced by a range of neurokinin agonists with the relative rank-order of potency senktide=NKB>Eledoisin>NKA=SP (Fig. 3B) reflecting the pharmacological characteristics expected of a NK-3 receptor subtype [2,11,12,28]. Concentrations of neuropeptide inhibiting specific [^{3}H]senktide binding by 50% were: senktide, 26.9±7.4 nM (n=4); NKB, 32±8.9 nM (n=4); eledoisin, 424.8±160.6 nM (n=4); NKA, 2729±1111 nM (n=3), and SP, 3097±253 nM (n=3). In addition to binding, this NK-3 expressing clone was found to respond to neurokinin stimulation with an immediate (<2 s) but transient increase in cytosolic Ca^{2+} concentration with a maximum 6-fold increase detected within 20 s. Basal levels were re-established after 2–3 min (data not shown). This response also displayed pharmacological characteristics expected of an NK-3 receptor and mirrored the binding data with the rank-order senktide=NKB>Eledoisin>NKA=SP (Fig. 3C). As reported previously for the bovine NK-2 receptor expressed in CHO cells [20], the human NK-3 receptor also mediated a 2–3-fold increase in [^{3}H]arachidonic acid release and this response showed identical pharmacological characteristics as obtained by radioligand binding and Ca^{2+} mobilization (data not shown).

The most interesting observation from these studies is that while eledoisin is approximately 15-fold less potent than either senktide or NKB at the human NK-3 receptor (Fig. 3B and C), all three neuropeptides display a similarly high affinity for the NK-3 receptor in some animal tissue preparations such as guinea pig ileum [5]. This potentially distinct pharmacological property of human NK-3 may account in part for the reported inability to detect this receptor in human brain using

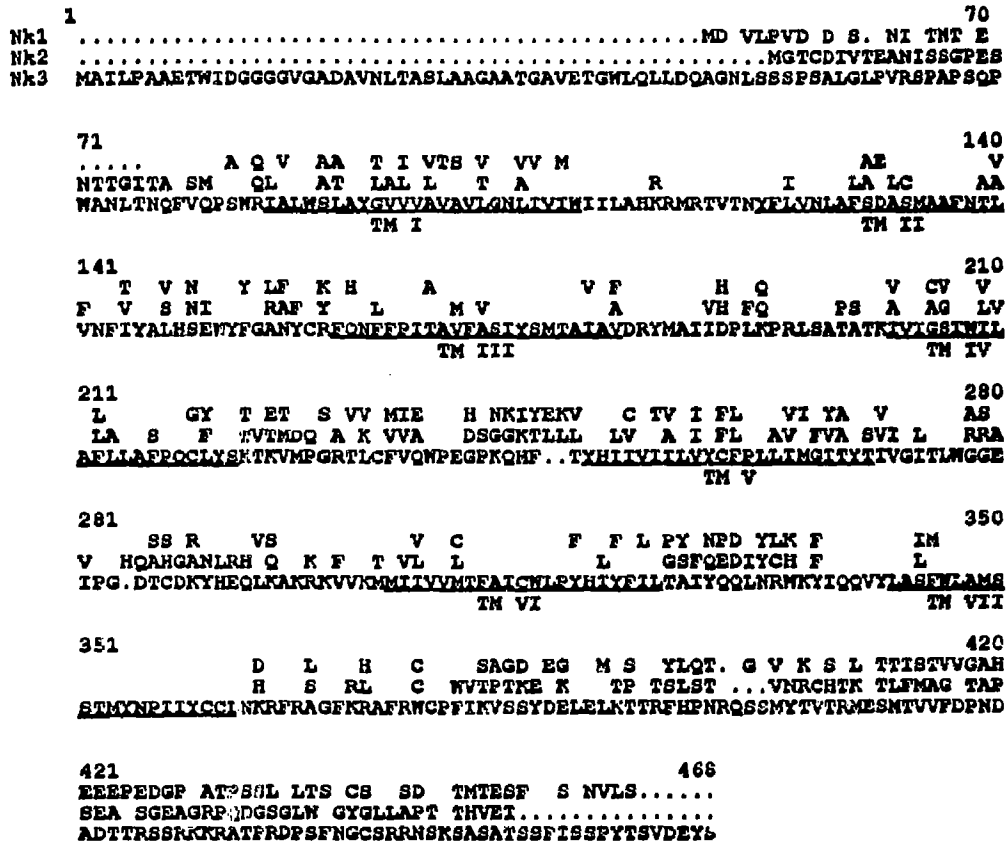

Fig. 2. Comparison of the amino acid sequences for the three human NK genes. Human NK-1 [22] and NK-2 [23] amino acid sequences were aligned with NK-3 for maximal sequence homology. A dot indicates no amino acid in that position whereas an open space in NK-1 or NK-2 indicates the same amino acid as in NK-3.

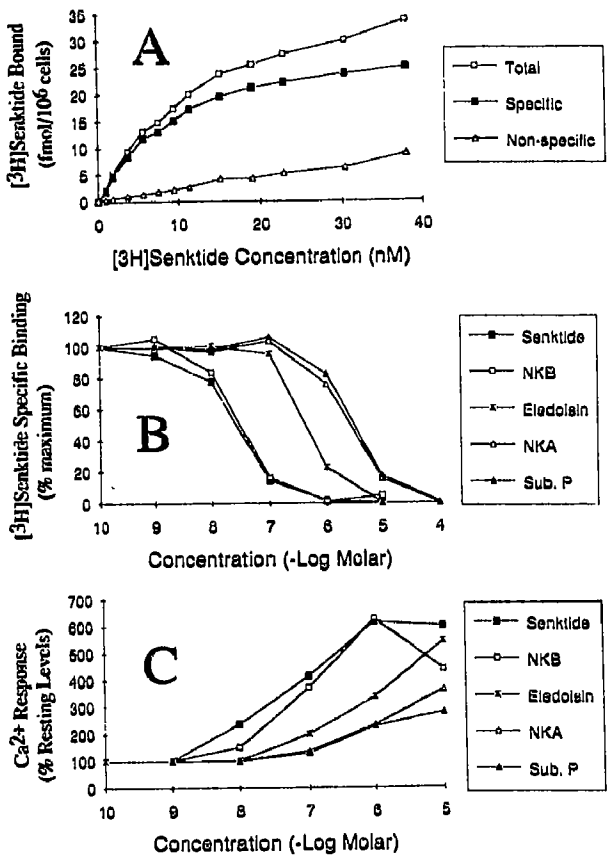

Fig. 3. Pharmacological characteristics of human NK-3 receptor expressed in CHO cells. (A) [^{3}H]Senktide saturation binding to CHO cells. Non-specific binding was determined in the presence of 1 μM NKB. Points are the mean of triplicate determinations and is representative of 3 experiments. (B) Competition by several neurokinin agonists for specific [^{3}H]senktide binding which was employed at concentrations between 2.5 and 3.8 nM. Points are the means of 3 (NKA and SP) or 4 (senktide, NKB and eledoisin) determinations each performed in triplicate. IC_{50} values were calculated and are reported in text. (C) Intracellular Ca^{2+} concentrations were measured using *fura2* levels increased as a function of agonist concentration. Resting levels were normally 80–120 nM. Points are the means of 2 (NKA), 3 (SP and NKB), 4 (eledoisin) or 6 (senktide) determinations.

[^{125}I]eledoisin [6]. Indeed, although NK-3 receptors were undetectable in primate brain or spinal cord using [^{125}I]eledoisin [6], [^{3}H]senktide has been used recently to map successfully NK-3 receptors in monkey tissue [2].

To establish the pattern of NK-3 receptor expression in human tissues we performed PCR analysis on mRNA extracted from brain and some peripheral tissues. Using NK-3 specific oligonucleotide primers 2 and 3 (Fig. 1), all brain regions that were examined showed the presence of NK-3 mRNA (Fig. 4). We also observed lesser amplification of the NK-3 specific fragment with mRNA from some peripheral tissues, notably kidney with longer film exposures revealing a very weak signal from lung and colon (not shown). Our inability to detect NK-3 receptor mRNA by Northern blotting of the RNA from all of the above tissues suggests an extremely low abundance for this mRNA (data not shown).

In summary, we have isolated and characterised the human NK-3 gene and shown that while this receptor exhibits expected pharmacological characteristics towards the physiological neurokinins NKB, SP and NKA, it exhibits an unexpectedly low affinity for eledoisin. We also show that the human NK-3 receptor gene is expressed in many areas of human brain and selectively in some peripheral tissues.

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REFERENCES

[1] Maggio, J.E. (1988) Annu. Rev. Neurosci. 11, 13–28.
[2] Guard, S. and Watson, S.P. (1991) Neurochem. Int. 18, 149–165.
[3] Helke, C.J., Krause, J.E., Mantyh, P.W., Couture, R. and Bannon, M.J. (1990) FASEB J. 4, 1606–1615.
[4] Lefkowitz, R.J., Kobilka, B.K. and Caron, M.G. (1989) Biochem. Pharmacol. 38, 2941–2948.
[5] Guard, S., Watson, S.P., Maggio, J.E., Phon Too, H. and Watling, K.J. (1990) Br. J. Pharmacol. 99, 767–773.
[6] Dietl, M.M. and Palacios, J.M. (1991) Brain Res. 539, 211–222.
[7] Bergstrom, L., Torrens, Y., Saffroy, M., Beaujouan, J.C., Lavielle, S., Chassaing, G., Morgat, J.L., Glowinski, J. and Marquet, A. (1987) J. Neurochem. 49, 125–133.
[8] Guard, S., Watling, K.J. and Watson, S.P. (1988) Br. J. Pharmacol. 94, 148–154.
[9] Hanani, M., Chorev, M., Gilon, C. and Selinger, Z. (1988) Eur. J. Pharmacol. 153, 247–253.
[10] Laufer, R., Wormser, U., Friedman, Z.Y., Gilon, C., Chorev, M. and Selinger, Z. (1985) Proc. Natl. Acad. Sci. USA 82, 7444–7448.
[11] Wormser, U., Laufer, R., Hart, Y., Chorev, M., Gilon, C. and Selinger, Z. (1986) EMBO J. 5, 2805–2808.

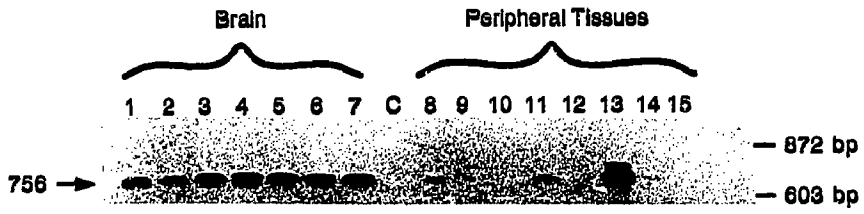

Fig. 4. Detection of human NK-3 mRNA by PCR from brain (lanes 1–7) and peripheral tissues (lanes 8–15). Nick-translated human NK-3 receptor cDNA probe was used to assess the level of amplification of a 756 bp fragment. Samples were: 1=frontal cortex; 2=temporal cortex; 3=parietal cortex; 4=hippocampus; 5=locus niger; 6=hypothalamus; 7=striatum; 8=kidney; 9=thymus; 10=adrenals; 11=total human embryo; 12=pancreas; 13=placenta; 14=lung; 15=colon; C=PCR control done in the absence of RNA. The left arrow indicates the size of the amplified fragment versus that of DNA molecular weight markers on the right.

[12] Mastrangelo, D., Mathison, R., Huggel, H.J., Dion, S., D'Orleans-Juste, P., Rhaleb, N.E., Drapeau, G., Rovero, P. and Regoli, D. (1986) Eur. J. Pharmacol. 134, 321–326.
[13] Stoessl, A.J., Dourish, C.T. and Iverson, S.D. (1988) Br. J. Pharmacol. 94, 285–287.
[14] Stoessl, A.J., Dourish, C.T. and Iverson, S.D. (1988) Regul. Peptides 22, 178.
[15] Buell, G., Reisman, D., Kintner, C., Crouse, G. and Sugden, B. (1980) J. Virol. 40, 977–982.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, New York.
[17] Devereux, Haeberlie and Smithies (1984) Nucleic Acids Res. 12, 387–395.
[18] Bertrand, D., Cooper, E., Valera, S., Rungger, D. and Ballivet, M. (1991) Methods Neurosci. 4, 174–193.
[19] Cate, R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., Fisher, R.A., Bertonis, J.M., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Manganaro, T.F., MacLaughlin, D.T. and Donahoe, P.K. (1986) Cell 45, 685–698.
[20] Eistetter, H.R., Church, D.J., Mills, A., Godfrey, P.P., Capponi, A.M., Brewster, R., Schulz, M.-F., Kawashima, E. and Arkinstall, S.J. (1991) Cell Regul. 2, 767–779.
[21] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[22] Shige noto, R., Yokota, Y., Tsuchida, K. and Nakanishi, S. (1990) J. Biol. Chem. 265, 623–628.
[23] Graham, A., Hopkins, B., Powell, S.J., Danks, P. and Briggs, I. (1991) Biochem. Biophys. Res. Commun. 177, 8–16.
[24] Takeda, Y., Chou, K.B., Takeda, J., Sachais, B.S. and Krause, J.E. (1991) Biochem. Biophys. Res. Commun. 179, 1232–1240.
[25] Hershey, A.D., Dykema, P.E. and Krause, J.E. (1991) J. Biol. Chem. 266, 4366–4374.
[26] Gerard, N.P., Eddy, R.L., Shows, T.B. and Gerard, C. (1990) J. Biol. Chem. 265, 20455–20462.
[27] Barish, M.E. (1983) J. Physiol. 342, 309–325.
[28] Foster, A.C. and Tridgett, R. (1988) Br. J. Pharmacol. 94, 602–608.