Molecular cloning and functional characterization of a type-I neurotensin receptor (NTR) and a novel NTR from the bullfrog brain

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Abstract

Neurotensin (NT) is a tridecapeptide that functions as a neurotransmitter and neuromodulator in the nervous system. To date, three different types of NT receptor (NTR), NTR1, NTR2 and NTR3, have been identified only in mammalian species. In the present study we isolated the cDNAs for an NTR1 and a novel NTR in the bullfrog brain, designated bfNTR1 and bfNTR4 respectively. bfNTR1 and bfNTR4 encode 422- and 399-amino acid residue proteins respectively. bfNTR1 has a 64% amino acid identity with mammalian NTR1, and 34–37% identity with mammalian NTR2. bfNTR4 exhibits 43% and 45–47% identity with mammalian NTR1 and NTR2 respectively. Both receptors are mainly expressed in the brain and pituitary. bfNTR1 triggers both CRE-luc, a protein kinase A (PKA)-specific reporter, and c-fos-luc, a PKC-specific reporter, activities, indicating that bfNTR1 can activate PKA- and PKC-linked signaling pathways. However, bfNTR4 appears to be preferentially coupled to the PKA-linked pathway as it induces a higher CRE-luc activity than c-fos-luc activity. bfNTRs exhibit different pharmacological properties as compared with mammalian NTRs. Mammalian NTR1 but not NTR2 responds to NT, whereas both bfNTR1 and bfNTR4 show a high sensitivity to NT. SR 48692 and SR 142948A, antagonists for mammalian NTR1 but agonists for mammalian NTR2, function as antagonists for both bfNTR1 and bfNTR4. In conclusion, this report provides the first molecular, pharmacological and functional characterization of two NTRs in a non-mammalian vertebrate. These data should help to elucidate the phylogenetic history of the G protein-coupled NTRs in the vertebrate lineage as well as the structural features that determine their pharmacological properties.

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Introduction

Neurotensin (NT) is a brain and gastrointestinal tridecapeptide that was originally isolated from the bovine hypothalamus (Carraway & Leeman 1973) and subsequently from bovine intestine (Kitabgi *et al.* 1976). NT exhibits a dual function as a neurotransmitter or neuromodulator in the central nervous system and as a local hormone at the periphery. Central administration of NT induces various effects including modulation of dopaminergic transmission, naloxone-independent analgesia, inhibition of food intake and modulation of pituitary hormone release (Rostène & Alexander 1997, Kitabgi 2002). There is also increasing evidence that NT plays a role in neuropsychiatric diseases, notably in schizophrenia and Parkinson's disease (McMahon *et al.* 2002). At the periphery, NT induces hypotension, decrease in gastric acid secretion,

lipid digestion, proinflammatory response and proliferation of gut and cancer cells (Kitabgi 2002, Somai *et al.* 2002).

NT exerts its action through its own membrane receptors. To date, three distinct types of NT receptor, termed NTR1 (Tanaka *et al.* 1990, Vita *et al.* 1993), NTR2 (Chalon *et al.* 1996, Mazella *et al.* 1996) and NTR3 (Vita *et al.* 1998), have been identified in mammals. NTR1 and NTR2 have seven transmembrane helices (TMHs), a typical structure of G protein-coupled receptors (GPCRs), while NTR3 possesses a single transmembrane domain and belongs to a Vps10p domain receptor family (Mazella *et al.* 1998). Compared with NTR1, NTR2 has a relatively short extracellular N-terminal region and a long third intracellular loop (ICL3) (Vincent *et al.* 1999). Moreover, in NTR2, Asp at position 2·50 in TMH2 and Asn at position 7·49 in TMH7, the most conserved residues

among rhodopsin-like GPCRs, are replaced by Ala/Gly and Thr respectively (Vincent *et al.* 1999). NTR1 exhibits a high affinity for NT but is insensitive to levocabastine, a non-peptide H1 histamine antagonist (Tanaka *et al.* 1990, Vita *et al.* 1993), while NTR2 has a very low affinity for NT and is sensitive to levocabastine (Chalon *et al.* 1996, Mazella *et al.* 1996). The non-peptide NTR antagonist SR 48692 has a higher affinity to NTR1 than to NTR2 (Gully *et al.* 1993). A second-generation antagonist, SR 142948A, exhibits a similar binding affinity to mammalian NTR1 and NTR2 (Gully *et al.* 1997, Betancur *et al.* 1998). However, it is known that SR 48692 and SR 142948A act as agonists for NTR2 when expressed in *Xenopus* oocytes (Botto *et al.* 1997, Mazella *et al.* 1998). Recently, β -lactotensin has been suggested as a specific agonist for NTR2 (Yamauchi *et al.* 2003). However, the functional relevance of NT as a ligand of NTR2 is still controversial. NT acts as an agonist for mouse NTR2 (mNTR2) when expressed in *Xenopus* oocyte (Mazella *et al.* 1996) and rat cerebellar granule cells (Sarret *et al.* 2002), while NT is unable to activate any second messenger systems from mNTR2 expressed in human embryonic kidney 293 (HEK293) cells, although radiolabeled NT can bind mNTR2 (Botto *et al.* 1998). Further, it has been suggested that NT acts as a natural antagonist for human NTR2 (hNTR2) when expressed in COS7 and CHO cells (Vita *et al.* 1998, Richard *et al.* 2001).

The signal transduction pathways associated with NTRs have been studied in various cell lines. Activation of rat NTR1 (rNTR1) induces an increase in inositol trisphosphate (IP_3) and Ca^{2+} mobilization through an activation of $G_{q/11}$ (Watson *et al.* 1992, Chabry *et al.* 1994, Hermans *et al.* 1994). It has also been reported that stimulation of NTR1 evokes the production of arachidonic acid and cAMP through $G_{i/o}$ and G_s activation respectively (Yamada *et al.* 1993, Carraway & Mitra 1998, Gailly *et al.* 2000). Furthermore, levocabastine and SR 48692 trigger Ca^{2+} mobilization, IP production, arachidonic acid release and mitogen-activated protein kinase activity in CHO cells expressing rNTR2 or hNTR2 (Vita *et al.* 1998, Yamada *et al.* 1998).

Although the three types of NTRs have now been cloned, the physiological roles of these NTRs are still poorly understood. Using an NTR1-knockout mice model, it has been found that NTR1 is involved in the control of food consumption and body weight, but not in NT-induced analgesia (Remaury *et al.* 2002). On the other hand, NTR2 seems to account for the SR 48692-insensitive analgesic effect (Dubuc *et al.* 1999, Maeno *et al.* 2004). Recent studies have shown that NTR3 may control proliferation of human cancer cells (Dal Farra *et al.* 2001), migration of human microglia (Martin *et al.* 2003) and proNGF-induced neuronal cell death (Nykjaer *et al.* 2004).

In non-mammalian species, the primary structure of NT has been characterized in chicken (Carraway & Bhatnagar 1980), alligator (Bello *et al.* 1993), python (Conlon *et al.* 1997) and two species of frog (Shaw *et al.* 1992, Desrues *et al.* 1998). It has also been reported that frog NT (fNT) stimulates the secretion of corticosteroids from perfused frog adrenal slices (Sicard *et al.* 2000) and the release of α -melanocyte-stimulating hormone from perfused frog pars intermedia cells (Desrues *et al.* 1998). The latter process appears to be mediated by a receptor which shares pharmacological characteristics with both mammalian NTR1 and NTR2 (Belmeguenai *et al.* 2000). However, to date, NTRs have not been characterized in any non-mammalian vertebrate species. In the present study using the bullfrog, we have cloned and characterized an NTR1 like receptor (bfNTR1) and a novel NTR (bfNTR4) that is structurally and functionally different from mammalian NTR2.

Materials and methods

Materials

Oligonucleotides were purchased from GenoTech Co. (Seoul, Korea). The pcDNA3 expression vector was purchased from Invitrogen. All constructs of mammalian NTRs were a generous gift from Dr J Mazella (CNRS, Valbonne, France). The pCMV β -gal was purchased from Clontech. The CRE-luc vector that contains four copies of the cAMP response element (CRE, TGACGTCA) was obtained from Stratagene (La Jolla, CA, USA). The c-fos-luc vector containing the $-711 \sim +45$ sequence of the human c-fos promoter constructed in the pFLASH vector, was a kind gift from Dr R Prywes (Columbia University, NY, USA). Taq DNA polymerase was purchased from Promega. Vent polymerase was obtained from New England Biotech (Beverly, MA, USA). Frog (*Rana ridibunda*) NT (Table 2) was synthesized by the solid phase methodology on an Applied Biosystem model 432 synthesizer (St Quentin en Yvelines, France), using an Fmoc-Leu-PEG-PS resin, by the standard Fmoc procedure as previously described (Desrues *et al.* 1998). hNT, porcine NMN (pNMN) and NT(8–13) were obtained from Sigma. The NT analogs JMV 431, JMV 457, JMV 458 and JMV 510 (Table 2) were kindly provided by Dr Jean Martinez (CNRS UMR 5810, University of Montpellier 2, France). Levocabastine was a generous gift from Janssen Pharmaceutica (Beerse, Belgium). SR 48692 and SR 142948A (Table 2) were generously given by Sanofi-Synthelabo (Montpellier, France).

Animals and tissue preparation

Adult bullfrogs were purchased from a local supplier (BCPC, Taein, Korea) and housed in flow-through tanks

Table 1 Primers used for cloning bullfrog NTRs

Application	Primers	Sequences
NTR1 partial	NTR-F4	5'-CA[C/T][C/T]A[C/T]CCCTGGG[C/T]CTT[T/C]GG[G/C]GA
	NTR-R1	5'-C[A/G]TG[G/A]TA[G/C]GGCA[G/A]CCA[G/A]CAGA
NTR4 partial	dNTR4-F1	5'-GA[A/G][C/T]TGTG[C/T]GCNTA[C/T]GCNAC
	NTR-R3	5'-GCGGTGGTCA[C/G]CATGTAGAA[G/A]TAGTG
NTR4 partial (nest PCR)	dNTR4-F2	5'-CCNATGGCNGT[C/T]AT[A/T/C]ATGGG
	NTR-R2	5'-GGG[T/G]A[C/T][A/G]TAGCAG[T/A]ACATGAG
NTR1 5' RACE	bfNT1-5'GSP1	5'-TTGAAGGTGGACATCTCCAGAGTCCCTCGTG
	bfNT1-5'GSP2	5'-TGCAGACCAGGTGATCTGGGTCTTCCA
NTR1 3' RACE	bfNT1-3'GSP1	5'-TGCCAGCCTCAGCATGGAGAGGTACATG
	bfNT1-3'GSP2	5'-AGGCCAAAAGCATCATGTCAAGGAGCAGGAC
	bfNT1-3'GSP3	5'-CCGATGATTGCAATCTCCGTTCTCAACACC
NTR4 5' RACE	bfNT4 5'GSP1	5'-GCTGTGTTGCAGTGACTGTATACGGTGGG
	bfNT4 5'GSP2	5'-GCTGTGTTGCAGTGACTGTATACGGTGGG
	bfNT4 5'GSP3	5'-CCGGCTCTCCATCTGGGCTCATGTTCTC
NTR4 3' RACE	bfNT4 3'GSP1	5'-CAAGTATGAGAACATGAGCCCAGATGGAGAGC
	bfNT4 3'GSP2	5'-CCGGATCCCTCATCACTTATCTGCACTAGTC
NTR1 ORF	bfNTR1-F	5'-CGGAATTC ACCACCATG AACCCCGGCTCTGCAGCTGAA
	bfNTR1-R	5'-GCTCTAGAT TCAGT ATGGAGTTCCCTGGTGAT
NTR4 ORF	bfNTR4-F	5'-CCCAAGCTT ACCACCATG CTGGCACTACAGAATCCACC
	bfNTR4-R	5'-GCTCTAGACAT TCAGT CCGGTCTGATGTGC

Restriction enzyme sites are underlined and Kozak and stop codons are in bold.

under simulated natural conditions. Frogs were killed by decapitation. The tissues of interest were quickly dissected and immediately frozen in liquid nitrogen, and stored at -80°C until use. Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals at Chonnam National University.

RNA isolation

Total RNA was extracted from frozen tissues by Tri reagent (MRC, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. RNA quality was examined by electrophoresis on 1% formaldehyde agarose gels. The RNA, which showed two sharp bands of 18S and 28S, was stored at -80°C until use. Poly (A)⁺ RNA was prepared by using an RNeasy mini kit (Qiagen).

Isolation of partial fragments for bfNTR1 and bfNTR4

One microgram of total RNA isolated from the brain was reverse transcribed at 42°C for 1 h followed by an incubation at 52°C for 30 min in 20 μl of reaction buffer containing 1 mM of each dNTP, 200 U of AMV reverse transcriptase (Promega), 20 U of RNAGard Rnase Inhibitor (Amersham Pharmacia Biotech) and 0.5 μg oligo-deoxythymidine primer. Synthesized cDNA was amplified with the primer sets NTR-F4 and NTR-R1 (for bfNTR1) or dNTR4-F1 and NTR-R3 (for bfNTR4) (Table 1) under 35 cycles of denaturation at 94°C for

30 s, annealing at 50°C for 50 s and extension at 72°C for 1 min. For bfNTR4, a nest PCR was performed with primers dNTR4-F and NTR-R2 in the same PCR condition. PCR products were cloned into the pGEM-T easy vector (Promega). Positive clones were sequenced by a sequenase version 2.0 DNA sequencing kit (USB Corporation, Cleveland, OH, USA).

5'- and 3'-rapid amplification of cDNA ends (RACE)

A cDNA library was constructed from brain Poly (A)⁺ RNA using a Marathon cDNA amplification kit (Clontech) according to the manufacturer's instruction. 5'-RACE was performed by using adapter primer 1 (Ap1) combined with the gene-specific primers bfNT1-5'GSP1 (bfNTR1) or bfNT4-5'GSP1 (bfNTR4) (Table 1). The PCR products were diluted and further amplified with adapter primer 2 (Ap2) combined with the gene-specific primers bfNT1-5'GSP2 (bfNTR1) or bfNT4-5'GSP3 (bfNTR4) (Table 1). 3'-RACE was performed by using Ap1 combined with the gene-specific primers bfNT1-3'GSP1 (bfNTR1) or bfNT4-3'GSP1 (bfNTR4) (Table 1). The PCR products were diluted and further amplified with Ap2 combined with the gene-specific primers bfNT1-3'GSP3 (bfNTR1) or bfNT4-3'GSP2 (bfNTR4) (Table 1). RACE products were cloned into the pGEM-T easy vector and transformed into DH5 α competent cells. White colonies containing the right sized PCR product were sequenced by using an automatic ABI prism 377 DNA sequencer (Perkin-Elmer, Wellesley, MA, USA).

Table 2 NTR ligands used in this study

Compounds	Amino acid sequence or chemical formula
Frog NT	pGlu Ala His Ile Ser Lys Ala Arg Arg Pro Tyr Ile Leu
Human NT	pGlu Leu Tyr Glu Asn Lys Pro Arg Arg Pro Tyr Ile Leu
Porcine NMN	Lys Ile Pro Tyr Ile Leu
NT(8–13)	Arg Arg Pro Tyr Ile Leu
Boc-[Trp ¹¹]NT*8–13) (JMV 458)	Boc Arg Arg Pro Trp Ile Leu
Boc-[D-Trp ¹¹]NT(8–13) (JMV 457)	Boc Arg Arg Pro D-Trp Ile Leu
Boc-[Lys ^{8,9} ,Nal ¹¹]NT(8–13) (JMV 510)	Boc Lys Lys Pro Nal Ile Leu
Boc-[psi ^{11,12}]NT(8–13) (JMV 431)	Boc Arg Arg Pro Tyr psi (CH ₂ NH) Ile Leu
SR 48692	2-[(1-(7-chloro-4-quinolinyl)-5-[2,6-dimethoxyphenyl]pyiazol-3-yl)]carbonylamina]ticyclo-(3.3.1.1)decan-2-carboxylic acid)
SR 14948A	2-[(5-[2,6-dimethoxyphenyl]-1-[4-(N-[3-dimethylamina propyl]-N-methylcarbamoyl)2-isopropylphenyl]-1H-pyrazole-3-carbonyl)amino]adamantane-2-carboxylic acid hydrochloride

Cloning of the full-length open-reading frame (ORF) of bfNTRs

The ORFs of bfNTR1 and bfNTR4 were amplified with Vent DNA Taq polymerase using primer sets bfNTR1-F (including EcoRI and Kozak site) and bfNTR1-R (including XbaI) or bfNTR4-F (including HindIII and Kozak site) and bfNTR4-R (including XbaI) (Table 1). The PCR products of anticipated size were digested with the corresponding restriction enzymes and ligated into pcDNA3. The selected colonies were analyzed by PCR with their corresponding primer sets and further confirmed by sequencing.

Tissue distribution of bfNTRs mRNA

One microgram of total RNA prepared from various tissues was reverse transcribed as described above. One microliter out of 20 µl RT samples was subjected to PCR. For bfNTR1 and bfNTR4, the following pairs of primers were used respectively: bfNT1–3'GSP1 and bfNT1–5'GSP1; bfNT4–3'GSP1 and bfNT4–5'GSP1 (Table 1). The PCR conditions were set as follows: denaturation at 95 °C for 3 min followed by 35 cycles at 95 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s. As an internal control, bullfrog GAPDH-specific primers, GAPDH-F (5'-CAATCCAATGGGGAGCTTCTG-3') and GAPDH-R (5'-CAATCCAATGGGGAGCTTCTG-3'), were used. The PCR products and 100 bp DNA size marker (Elpis Biotech, Daejeon, Korea) were analyzed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml).

Cell culture, transfection and luciferase assay

CV1 cells were maintained as described (Oh *et al.* 2003, Seong *et al.* 2003). CHO-K1 cells were maintained at 37 °C in DMEM/F-12 medium with 10% heat-

inactivated fetal bovine serum (FBS), 100 U penicillin and 100 µg/ml streptomycin. Cells were seeded in 24-well plates and transfection was performed using the SuperFect transfection kit (Qiagen). For each transfection, 100 ng construct cDNA and 200 ng c-fos-luc (or CRE-luc) along with 200 ng of the internal control plasmid pCMVβ-gal were used. The empty vector pcDNA3 was used to adjust the total amount of transfected DNA. Cells were incubated in serum-free DMEM for 18 h before treatment with NT, NT analogs and/or NT antagonists (Table 2). Six hours after drug treatment, cells were harvested and the luciferase activity in the cell extract was determined as previously described (Oh *et al.* 2003). The luciferase activities were normalized using the β-galactosidase values. Transfection experiments were performed in triplicate and repeated three to five times.

Establishment of stable cell lines expressing bfNTRs

The pcDNA3 containing the ORF of bfNTR1 or bfNTR4 was transfected into CHO-K1 cells using the SuperFect transfection kit. After 24 h, cells were treated with 400 µg/µl G418 (Clontech) for approximately 2 weeks. The large healthy colonies were selected and expanded. The presence of the stably expressing genes was confirmed by RT-PCR and CRE-luc reporter assay. CHO-K1 cells stably expressing bfNTR1 or bfNTR4 were stored in liquid nitrogen until use.

IP assay

Forty-eight hours before assay, CHO-K1 cells stably expressing bfNTR1 or bfNTR4 were seeded into 12-well plates (1 × 10⁵ cells/well). The next day, the cells were labeled for 16–24 h in 1 ml inositol-free special medium (Life Technologies, Rockville, MD, USA) containing

1 μCi *myo*[^3H]inositol (Amersham Pharmacia Biotech) per well. Cells were incubated in 0.45 ml buffer (140 mM NaCl, 20 mM Hepes, 4 mM KCl, 8 mM D-Glucose, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mg/ml fatty acid free BSA, pH 7.2) containing 20 mM LiCl at 37 °C for 25 min. Then, NT was added and incubated at 37 °C for 25 min. The reaction was terminated by replacing the solution with 0.5 ml ice-cold formic acid (10 mM). IPs were extracted by incubating at 4 °C for 30 min and separated using a Dowex ion exchange resin. Total IPs were eluted with buffer containing 1 M ammonium formate and 0.1 M formic acid. The radioactivity was counted using a liquid scintillation counter.

Measurement of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

CHO-K1 cells stably expressing bfNTR1 or bfNTR4 were grown on cover slip slides for 48 h. The medium was removed and washed with Ca^{2+} buffer (138 mM NaCl, 6 mM KCl, 1 mM MgSO_4 , 2 mM CaCl_2 , 1 mM Na_2HPO_4 , 5 mM NaHCO_3 , 5 mM glucose, 10 mM Hepes, pH 7.4, 0.1% BSA) and then incubated with buffer containing 3 μM Fura-2/AM (Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. The cells were then washed and bathed in Ca^{2+} buffer at room temperature for at least 20 min before measurement of $[\text{Ca}^{2+}]_i$. The fluorescence emission of Fura-2, induced by excitation at 510 nm, was monitored at two wavelengths (340 nm and 380 nm) by a photomultiplier-based system, linked to an Olympus IX-70 microscope equipped with a $\times 40$ objective (Fluor). Three signals (340, 380 and 340/380 nm ratio) were continuously recorded with the MetaFluor Program (Downingtown, PA, USA) at 500 ms intervals.

Radiolabeled ligand binding assay

hNT was radioiodinated by the chloramine-T method and purified by chromatography on a Sephadex G-25 column as described previously (Sadoul *et al.* 1984). Cell membranes were prepared from CHO-K1 cells stably expressing bfNTR1, bfNTR4 or rNTR1 and suspended in binding buffer (50 mM Tris, 2 mM MgCl_2 , 1 mM dithiothreitol, 0.1% BSA and protease inhibitor, pH 7.4). The membrane preparation (20 μg) was incubated at 16 °C for 1 h with increasing concentrations of ^{125}I -hNT in the presence (non-specific binding) or absence (total binding) of 10 μM unlabeled hNT. Specifically bound ligand was separated by rapid filtration through GF/C filters (Brandel, Gaithersburg, MD, USA) presoaked in binding buffer containing 0.01% polyethylenimine (Sigma) and washed twice with ice-cold washing buffer (40 mM Tris, 2 mM MgCl_2 , 0.1% BSA, pH 7.4). Competition binding assay was carried out with intact cells in 12-well plates at 16 °C for

1 h. Cells stably expressing bfNTR1 or bfNTR4 were incubated with 100 000 c.p.m. ^{125}I -hNT and increasing concentrations of unlabeled competitor in 500 μl DMEM containing 0.1% BSA. Non-specific binding was determined in the presence of 10 μM unlabeled hNT.

Data analysis

All data are expressed as means $\pm$ S.E.M. of three independent experiments. Data were analyzed using non-linear regression and plotted as sigmoid dose-response curves. Ligand concentrations inducing half-maximal stimulation (EC_{50}), half-maximal inhibition (IC_{50}) and/or maximal fold increase (E_{max}) were calculated using GraphPad PRISM3.0 software (GraphPad, San Diego, CA, USA). Statistical analysis was performed by one-way ANOVA followed by a Bonferroni test. Data were considered as significant at $P < 0.05$.

Results

Primary structure of bfNTRs

Using degenerate PCR combined with 5'- and 3'-RACE, we isolated full-length cDNAs for bfNTR1 (1467 nucleotides accession No. AY613326) and bfNTR4 (1779 nucleotides accession No. AY613327). The bfNTR1 and bfNTR4 cDNAs were predicted to encode putative receptors with 422 and 400 amino acids respectively (Fig. 1). bfNTR1 and bfNTR4 exhibited the typical characteristics of GPCRs with seven hydrophobic TMHs (Fig. 1). Sequence alignment showed that bfNTR1 had a 64% amino acid identity with mammalian NTR1 and a 34–37% identity with mammalian NTR2. bfNTR4 had a 45–47% identity with mammalian NTR2 and a 43% identity with mammalian NTR1. bfNTR1 and bfNTR4 showed 43% sequence identity to each other. bfNTR1 contained two potential N-glycosylation sites in the N-terminal domain, one potential protein kinase A (PKA) phosphorylation site in ICL1 and two potential protein kinase C (PKC) phosphorylation sites in ICL3 and the intracellular C-terminal tail (Fig. 1). While mammalian NTR2 does not possess N-glycosylation sites, bfNTR4 had two potential N-glycosylation sites in the N-terminal domain (Fig. 1). bfNTR4 contained multiple potential PKC phosphorylation sites in ICL2, ICL3 and the C-terminal tail (Fig. 1).

Tissue distribution of bfNTRs mRNA

The tissue distribution of bfNTR1 and bfNTR4 mRNAs was determined by RT-PCR using the primer pairs bfNT1–3'GSP1 and bfNT1–5'GSP1 (for bfNTR1) or bfNT4–3'GSP1 and bfNT4–5'GSP1 (for bfNTR4) (Table 1). The mRNA for bfNTR1 was abundant in various brain regions including the olfactory lobe,

		TMH1	
bNTR1	MNPGSAEILHQPPIMR Q ATPQVPLFQEVQDFWLSTTSVM Q LS Q VSTRHVSEGLD V NTDIYSKVLVTVIYLF F VV		80
rNTR1	MHLNSSVP---QGTPGEFDAQFFSGPQSEMEATFLALSLSNGSGNTSESDTAGPNSDL V NTDIYSKVLVTAVIYLF F VV		77
hNTR1	MRLNSSAP-----GTPGTPAADPFQRAQAGLEALLAPGFGNASGNASERVLAAPSS E LDVNTDIYSKVLVTAVIYLF F VV		76
bNTR4	-----MLALQ Q STWGLEVR Q ATRTDTSHP L GVNTNLLSKVFITVLYILIF F V		47
rNTR2	-----METSSPPWPPRPS S PAGLSLEARLGVDTRLWAKVLF T ALYSLIF F AF		45
hNTR2	-----METSSPPRPPR S SNPGLSLDARLGVDTRLWAKVLF T ALYALIWAL		45
		TMH2	TMH3
bNTR1	GCTGNSITAYTL L RKK S MONLQSTVHYHLASL S LDLLILL L SMPIETYNFIWVHHPWAFGNVICKGY F FLRD A CTYATA		160
rNTR1	GTVGNSVTAFTLARKKSLQSLQSTVHYHLGSL S LDLLILL L AMPVELYNFIWVHHPWAFGDAGCRGY F FLRD A CTYATA		157
hNTR1	GTVGNTVTAFTLARKKSLQSLQSTVHYHLGSL S LDLLILL L AMPVELYNFIWVHHPWAFGDAGCRGY F FLRD A CTYATA		156
bNTR4	GTTGNVLTIQVLVKQSLHGLQGTVHYHLVSL S LDILILVISIPIELYNFIWFHYPWVFGDAVCRGY F FFRD I CSYATV		126
rNTR2	GTAGNALS V HVV L KARA--GRPGRLRYHVL S LSALL L LVSMPELYNFVW S HPWVFGDLGCRGY F FVREL C AYATV		123
hNTR2	GAAGNALS V HVV L KARA--GRAGRLRHV L SLALAGLL L LVGVPELYSFVWFHYPWVFGDLGCRGY F FVHEL C AYATV		123
		TMH4	
bNTR1	LNIASLSMERYMAMCHPFKA S IMSRSR T KKFISFIWVASLL L ATPMIFAMGEIYGLGV-E---DPDHLVCTRT V EMSTF		240
rNTR1	LNVASLSVERYLAICH P FAKTLMSRSR T KKFISAIWLASALLA I PMFLTMGLQNRSGDGT---HPGGLVCTPIV D TATV		237
hNTR1	LNVASLSVERYLAICH P FAKTLMSRSR T KKFISAIWLASALLA V PMFLTMGEQNR S ADGQ---HAGGLVCTPTI H TATV		236
bNTR4	LNIASLSCEERYLAICH P MAKRVMSKMR Q KKLLAALWVSSLI F FALPMAFIMGKIYENMSPDGEDPSS L ICTSLVSTATL		206
rNTR2	LSVASLSAERCLAVCQPLRARLLTPRRTRRL L SVVWVASLGLALPMAVIMGQKHEVESADGEPEPASRVCTVLVSRATL		203
hNTR2	LSVAGLSAERCLAVCQPLRARLLTPRRTRWL V ALSWAASLGLAMPMAVIMGQKHELETADGEPEPASRVCTVLVSRATL		203
		TMH5	
bNTR1	KTVIQVNAFLSFI F PMIAISVLNTIIANQLIIMVKQA H QENQVCTIGG---Q-----QTVLSM		316
rNTR1	KVVIQNTFMSFL P MLVISILNTVIANKLTVMVHQA E QGRVCTVGT H NGL-----HSTFNM		314
hNTR1	KVVIQNTFMSFI F PMVVISVLNTIIANKLTVMVRQA E QGVCTVGG---E-----HSTFSM		313
bNTR4	KIFIQVNAFVSFL P VLPTLIGLNCITVSHLV S LRSSSLIVH S CK S PRASKELRKSQSSPSNLSRI P VGGT S WKKNLTV		286
rNTR2	QVFIQNVNLSFALPLALTAFLNGITVNHLMALYSQVPSASAQVSSIP-SRLELLSEEGLLGFTTWRKTL S LGVQASLVR		283
hNTR2	QVFIQNVNLSFVLPLALTAFLNGVTVSHLLALCSQVPS T STPGSS T P-SRLELLSEEGLLSFIVWKKTFIQGGQVSLVR		283
		TMH6	TMH7
bNTR1	SMEPSRIQSLKHGVRVLRVVVIAFVVCWLPYHVRRLMFCYVPEELW T DELNDFYHYFMYLNTNLFYVSS T VNPILYNLVS		371
rNTR1	TIEPGRVQALRHGV L VRVIAFVVCWLPYHVRRLMFCYISDEQW T TF L DFYHYFMYLTN A LFYVSSAINPILYNLVS		373
hNTR1	AIEPGRVQALRHGV L RAVIAFVVCWLPYHVRRLMFCYISDEQW T PF L YDFYHYFMYMTN A LFYVSS T INPILYNLVS		368
bNTR4	SSEP N RIQSLQHSIYMLRAIVIAIVCWLPYHARRLMFCYIPDD D WSGS L YTFYHYFMYLTNTLFYISSAVNPVLYNVVS		366
rNTR2	HKDASQIRSLQ S HAQVLR A IVAVVICWLPYHARRLMFCYIPDDGWTNELYDFYHYFMYMTNTLFYVSSAV T PILYNAVS		362
hNTR2	HKDVRIRSLQ S RVQLRAIVVMYVICWLPYHARRLMFCYV P DDA W TDP L YNFYHYFMYMTNTLFYVSSAV T PLLYNAVS		362
bNTR1	ANFRQIEVSTLSFLCPPWR-RKKKHGPGFS S SKSNSISSTNHTFSTQITRETPY	422	
rNTR1	ANFRQVFLSTLACLCPGWRHRRKKRPTFSRKPN S MS--NHAFTSATRETLY	424	
hNTR1	ANFRHIFLATLACLCPPWR--RRKRPAFSRKADSVSS--NHTLSSNATRETLY	418	
bNTR4	SSFRKLFLETLSPSCSKQNSN S FIKSSSSHIRPD	400	
rNTR2	SSFRKLFLESLGSLCGEQHSLVPLPQEAPESTTSTYSFRLWGS P RNP S LG E IQV	416	
hNTR2	SSFRRLFLEAVSSLCGEHHPMKRLPPK-PQSPTLMDTASGFGDPPE T RT	410	

Figure 1 Sequence alignment of the bullfrog (bf), human (h) and rat (r) NTRs. Amino acid sequences of the indicated NTRs were aligned using MacVector Software (Acce lrys, San Diego, CA, USA). The conserved amino acid residues are shaded. The numbers on the right side indicate the position of amino acid residues. The putative N-glycosylation sites, PKC and PKA phosphorylation sites are indicated by diamonds, circles and squares respectively. The predicted seven transmembrane helices (TMHs) are indicated above the aligned sequences. Asp in TMH2 and Asn in TMH7 that are highly conserved in rhodopsin-like GPCRs are indicated by dots. The two Cys residues suggested to form a disulfide bridge between ECL1 and ECL2 are indicated by solid triangles. Two conserved motifs D/ERY and NPXXY are indicated by asterisks. GenBank accession Nos are hNTR1: P30989; hNTR2: Q95665; rNTR1: P20789; rNTR2: Q63384.

cerebral cortex and hypothalamus, as well as in the spinal cord (Fig. 2). The mRNA for bfNTR4 was abundant in the cerebral cortex and hypothalamus, but weakly expressed in the olfactory lobe and spinal cord (Fig. 2). In the distal and neurointermediate lobes of the pituitary, bfNTR1 mRNA was highly expressed, while bfNTR4 mRNA was moderately expressed. At the periphery, weak signals for bfNTR1 were detected in the heart, kidney and small intestine. An intense signal for bfNTR4 was observed in the ovary (Fig. 2).

Differential signal transduction for mammalian and bullfrog NTRs

To investigate the signaling pathways of bfNTRs, CV-1 cells or CHO-K1 cells were co-transfected with either bfNTR1, bfNTR4, rNTR1 or hNTR2 in combination with the c-fos-luc (or CRE-luc) reporter vector. We have previously shown that c-fos-luc and CRE-luc reporter are sensitive to phospholipase C (PLC)/PKC- and adenylate cyclase (AC)/PKA-linked signaling pathways

Figure 2 RT-PCR analysis of bullfrog (bf) NTRs in a variety of tissues. Total RNAs isolated from the distal lobe (DL) and neurointermediate lobe (NIL) of the pituitary, olfactory lobe (OL), cerebral cortex (CC), hypothalamus (HT), optic tectum (OT), spinal cord (SC), heart, liver, kidney (Kid), adrenal gland (AG), small intestine (SI), large intestine (LI), skin and ovary were subjected to RT-PCR.

respectively (Oh *et al.* 2003, Seong *et al.* 2003). Activation of bfNTR1 by hNT strongly increased both c-fos-luc and CRE-luc reporter activity with similar strength. bfNTR4 slightly induced c-fos-luc and CRE-luc activity when expressed in CV1 cells. When expressed in CHO-K1 cells, bfNTR4 was able to induce a 9-fold increase in CRE-luc activity but only a 4-fold increase in c-fos-luc activity (Fig. 3). rNTR1 induced both CRE-luc and c-fos-luc reporter activity in CV1 and CHO-K1 cells. In contrast, no significant increase in CRE-luc and c-fos-luc reporter activity was observed in either CV1 or CHO-K1 cells expressing hNTR2 (Fig. 3) or mNTR2 (data not shown).

NT-induced IP production and $[Ca^{2+}]_i$ elevation were determined in CHO-K1 cells stably expressing bfNTR1 or bfNTR4. In agreement with the c-fos-luc

assay results, hNT induced a 3-fold increase in IP production in cells expressing bfNTR1 and only a 1.5-fold increase in cells expressing bfNTR4 (Fig. 4). Exposure of CHO-K1 cells expressing bfNTR1 or bfNTR4 to hNT provoked an immediate elevation of $[Ca^{2+}]_i$ and the Ca^{2+} signal lasted for at least 45 s (Fig. 5A and B). In mock-transfected cells, hNT did not induce $[Ca^{2+}]_i$ increase although thapsigargin, an inhibitor of sarcoendoplasmic Ca^{2+} ATPase, was able to increase $[Ca^{2+}]_i$, indicating that Fura-2 was successfully loaded (Fig. 5C).

Effect of peptide agonists on NTRs

Ligand selectivity was examined using the c-fos-luc and CRE-luc reporter systems. In the c-fos-luc assay system, bfNTR1 and rNTR1 exhibited similar patterns of ligand

Figure 3 hNT-induced CRE-luc and c-fos-luc reporter activity. CV1 or CHO-K1 cells were transfected with 200 ng CMV β -gal and 200 ng c-fos-luc or CRE-luc plus 100 ng individual pcDNA3, rat (r) NTR1, human (h) NTR2, bullfrog (bf) NTR1 or bfNTR4. One day after transfection, cells were serum-starved for 24 h followed by 6 h treatment with 1 μ M hNT. Results shown are the mean of duplicate observations from a single representative experiment. (* P < 0.05; ** P < 0.01).

Figure 4 IP accumulation in cells expressing bullfrog (bf) NTRs. CHO-K1 cells stably expressing each bfNTR or mock-transfected cells were seeded in 12-well plates (1×10^5 per well). One day after seeding, cells were grown in inositol-free DMEM containing 1 μ Ci *myo*-[3 H]inositol/well and 2% FBS for 24 h and then treated with 1 μ M hNT for 6 h. IP production was expressed as fold-induction over basal. Data shown are the means $\pm$ S.E.M. of three independent experiments. (* $P < 0.01$, ** $P < 0.001$).

sensitivity toward NTs and different pseudopeptide analogs. fNT showed the highest potency for both bfNTR1 and rNTR1, followed by hNT $\approx$ JMV 458 $\approx$ NT(8–13) $>$ pNMN $>$ JMV 510 $\approx$ JMV 457 $\approx$ JMV 431 (Fig. 6 and Table 3). Compared with rNTR1 and bfNTR1, bfNTR4 showed slightly lower sensitivity to these compounds (Fig. 6 and Table 3). In the CRE-luc assay system, rNTR1 and bfNTR1 exhibited a similar pattern of ligand sensitivity as observed in the c-fos-luc assay system. It is of interest to note that bfNTR4 showed a slightly higher sensitivity to all NT analogs, except pNMN, than bfNTR1 (Fig. 7 and Table 3).

Effect of non-peptide ligands on NTRs

SR 48692 and SR 142948A are known to function as antagonists for mammalian NTR1 and agonists for

mammalian NTR2 (Botto *et al.* 1997, Gully *et al.* 1997, Vita *et al.* 1998). Indeed, SR 142948A and SR 48692 showed antagonistic activity to rNTR1, the former having a higher potency than the latter (Fig. 8A and Table 4). SR 142948A completely inhibited hNT-induced CRE-luc activity in bfNTR1-expressing cells, while SR 48692 marginally inhibited hNT-induced CRE-luc activity in these cells (Fig. 8B and Table 4). Both SR 142948A and SR 48692 showed an antagonistic property for bfNTR4 (Fig. 8C). In particular, SR 142948A was a potent antagonist ($IC_{50} = -9.1 \pm 0.2$) for bfNTR4. Levocabastine, at high concentrations, induced CRE-luc activity in cells expressing bfNTR1 and bfNTR4, but not in rNTR1-transfected cells (Fig. 8D and Table 4).

Binding affinity of bfNTR1 and bfNTR4

The binding properties of bfNTR1 and bfNTR4 were examined using 125 I-hNT as a radioligand. Specific binding to membranes prepared from CHO-K1 cells expressing bfNTR1, bfNTR4 or rNTR1 was observed while only background binding occurred in mock-transfected CHO-K1 cells. Saturation experiments revealed that hNT had a high affinity to each NTR (Fig. 9A and B): $K_d = 0.88 \pm 0.12$ nM for bfNTR1, 1.29 ± 0.23 nM for bfNTR4 and 0.83 ± 0.14 nM for rNTR1. Maximum binding (B_{max}) was as follows: 7.61 ± 0.49 , 12.24 ± 1.15 and 10.6 ± 0.84 pmol/mg protein for bfNTR1, bfNTR4 and rNTR1 respectively.

Displacement experiments showed that hNT could efficiently inhibit the binding of 125 I-hNT to bfNTR1 and to bfNTR4, indicating hNT has a high affinity for both receptors. In bfNTR1-expressing cells, SR 142948A showed a 100-times lower affinity than hNT, while in bfNTR4-expressing cells, SR 142948A had similar affinity to hNT. Levocabastine exhibited very low affinity for both bfNTR4 and bfNTR1 (Fig. 9C and D and Table 4).

Figure 5 Effect of NT on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in CHO-K1 cells expressing bullfrog (bf) NTRs. CHO-K1 cells stably expressing bfNTR1 (A) or bfNTR4 (B) and mock-transfected cells (C) were challenged with 1 μ M hNT. Data show the means $\pm$ S.E.M. of ~ 15 individual recordings of $[Ca^{2+}]_i$ during 100 s. Cells were exposed to thapsigargin (1 mM) to confirm Fura-2 loading (C).

Figure 6 Differential ligand selectivity of rat (r) NTR1, bullfrog (bf) NTR1 and bfNTR4. CV1 cells were transfected with 200 ng CMV β -gal and 200 ng c-fos-luc plus 100 ng rNTR1, bfNTR1 or bfNTR4. One day after transfection, cells were serum-starved for another 24 h, then treated for 6 h with graded concentrations of various agonists and luciferase activity was measured. Data are means $\pm$ S.E.M. of one representative experiment from three to five experiments.

Table 3 Ligand selectivity of NTRs. Cells were transfected with NTR in combination with c-fos-luc reporter (CV1 cells) or CRE-luc reporter (CHO-K1 cells). EC₅₀ (log M) and E_{max} (fold-increase over basal) values are obtained from c-fos-luc or CRE-luc reporter assay systems. Each value indicates the mean $\pm$ S.E.M. from three independent experiments

	rNTR1		bfNTR1		bfNTR4	
	EC ₅₀	E _{max}	EC ₅₀	E _{max}	EC ₅₀	E _{max}
c-fos-luc activity (CV1 cells)						
fNT	-9.6 $\pm$ 0.1	31.4 $\pm$ 0.8	-9.1 $\pm$ 0.1	9.3 $\pm$ 0.2	-8.0 $\pm$ 0.4	1.9 $\pm$ 0.1
hNT	-9.1 $\pm$ 0.1	25.3 $\pm$ 0.8	-8.8 $\pm$ 0.2	7.4 $\pm$ 0.3	-8.5 $\pm$ 0.2	2.4 $\pm$ 0.1
pNMN	-7.2 $\pm$ 0.1	35.0 $\pm$ 1.1	-7.8 $\pm$ 0.2	5.9 $\pm$ 0.2	-5.6 $\pm$ 0.3	2.0 $\pm$ 0.2
NT8-13	-8.9 $\pm$ 0.1	39.0 $\pm$ 1.0	-8.8 $\pm$ 0.1	6.4 $\pm$ 0.2	-8.7 $\pm$ 0.3	1.8 $\pm$ 0.1
JMV457	-6.8 $\pm$ 0.1	35.3 $\pm$ 2.0	-7.1 $\pm$ 0.2	5.6 $\pm$ 0.3	ND	ND
JMV458	-9.5 $\pm$ 0.2	27.7 $\pm$ 1.1	-8.4 $\pm$ 0.2	7.0 $\pm$ 0.3	ND	ND
JMV431	-6.2 $\pm$ 0.0	44.2 $\pm$ 1.2	-6.8 $\pm$ 0.1	9.2 $\pm$ 0.4	ND	ND
JMV510	-7.5 $\pm$ 0.3	34.2 $\pm$ 3.4	-6.7 $\pm$ 0.4	3.4 $\pm$ 0.4	ND	ND
CRE-luc activity (CHO-K1 cells)						
fNT	-8.8 $\pm$ 0.2	53.7 $\pm$ 2.3	-8.4 $\pm$ 0.1	20.3 $\pm$ 0.7	-9.1 $\pm$ 0.2	6.7 $\pm$ 0.3
hNT	-9.0 $\pm$ 0.2	55.7 $\pm$ 2.2	-8.3 $\pm$ 0.2	19.8 $\pm$ 0.8	-9.3 $\pm$ 0.1	7.4 $\pm$ 0.2
pNMN	-7.3 $\pm$ 0.1	29.2 $\pm$ 1.2	-7.2 $\pm$ 0.1	13.2 $\pm$ 0.6	-6.0 $\pm$ 0.1	10.0 $\pm$ 0.6
NT8-13	-8.3 $\pm$ 0.1	33.2 $\pm$ 1.3	-7.9 $\pm$ 0.1	12.4 $\pm$ 0.4	-8.8 $\pm$ 0.3	8.4 $\pm$ 0.5
JMV457	-6.5 $\pm$ 0.2	23.2 $\pm$ 1.7	-5.7 $\pm$ 0.1	9.1 $\pm$ 0.5	-6.4 $\pm$ 0.1	7.3 $\pm$ 0.4
JMV458	-9.0 $\pm$ 0.1	8.3 $\pm$ 0.3	-8.1 $\pm$ 0.2	9.8 $\pm$ 0.6	-9.0 $\pm$ 0.2	6.4 $\pm$ 0.3
Levocabastine	ND	ND	-6.4 $\pm$ 0.2	3.3 $\pm$ 0.3	-5.2 $\pm$ 0.5	4.1 $\pm$ 1.2

ND, not detected.

Figure 7 Differential ligand selectivity of rat (r) NTR1, bullfrog (bf) NTR1 and bfNTR4. CHO-K1 cells were transfected with 200 ng CMV β -gal and 200 ng CRE-luc plus 100 ng rNTR1, bfNTR1 or bfNTR4. One day after transfection, cells were serum-starved for another 24 h, then treated for 6 h with graded concentrations of various agonists, and luciferase activity was measured. Data are means $\pm$ S.E.M. of one representative experiment from three to five experiments.

Discussion

This report has described for the first time the identification and functional characterization of two non-mammalian NTRs. As previously described for mammalian NTRs, bfNTR1 and bfNTR4 were mainly expressed in the brain and pituitary. bfNTR1 has a high degree of sequence identity with mammalian NTR1 while bfNTR4 exhibits a relatively low similarity to either mammalian NTR1 or NTR2. They differed in signal transduction pathways and ligand affinity from each other. Further, they exhibited distinct pharmacological characteristics from those of the mammalian receptors.

bfNTR1 exhibited a high degree of sequence identity and structural similarity with the corresponding mammalian receptor NTR1. Like mammalian NTR1, bfNTR1 has a long extracellular N-terminal domain and a short extracellular loop 3 (ECL3). A Thr-X-Tyr motif in the C-terminal domain, which is critical for internalization of mammalian NTR1 (Chabry *et al.* 1995), was also found in bfNTR1. bfNTR4 has a short extracellular N-terminal domain and a relatively long ECL3, characteristics of mammalian NTR2. bfNTR4, however, showed some structural differences with mammalian NTR2. First, mammalian NTR2 does not

contain glycosylation sites in the extracellular N-terminal domain while bfNTR4 possesses two putative glycosylation sites in the N-terminal region as found in mammalian NTR1. Secondly, the sequence of ICL1 in bfNTR4 is different from that of mammalian NTR2 but similar to that of mammalian NTR1 and bfNTR1. As the ICLs are involved in G protein coupling and signal transduction, it could be anticipated that differences in the signaling pathways occur between mammalian NTR2 and bfNTR4. Thirdly, the two conserved residues Asp²⁵⁰/Asn⁷⁴⁹ in TMH2/TMH7, which are characteristic of the rhodopsin-like GPCR family, are absent in the mammalian NTR2 but present in bfNTR4. The presence of Asp²⁵⁰/Asn⁷⁴⁹ residues is important for receptor conformation as they are involved in a hydrogen bond interaction responsible for the close association of TMH2 and TMH7 (Sealfon *et al.* 1997). It has been reported that the conserved Asp²⁵⁰ residue in TMH2 accounts for the Na⁺ sensitivity and/or receptor activation in rNTR1 as in other GPCRs (Kong *et al.* 1993, Martin *et al.* 1999, Belmeguenai *et al.* 2000). Indeed, the mutation on these residues critically affects receptor activation (Awara *et al.* 1996). Further, the Asn⁷⁴⁹ residue followed by the Pro-X-X-Tyr motif is known to be involved in coupling with the small G protein ARF and RhoA (Mitchell *et al.*

Figure 8 Effects of antagonists on rat (r) NTR1, bullfrog (bf) NTR1 and bfNTR4. CHO-K1 cells were transfected with 200 ng CMV β -gal and 200 ng CRE-luc plus 100 ng rNTR1 (A), bfNTR1 (B) or bfNTR4 (C). One day after transfection, cells were serum-starved for another 24 h, then treated for 6 h with graded concentrations of SR 48692 (SR) or SR 142948A (SRA) in the presence of 1 nM hNT. (D) Effect of graded concentrations of levocabastine on bfNTR1-, bfNTR4- and NTR1-expressing cells. Data are means $\pm$ S.E.M. of one representative experiment from three to five experiments

1998). Thus, the presence of the Asp²⁵⁰/Asn⁷⁴⁹ residues in bfNTR4 may be responsible for differential signal transduction coupling and ligand sensitivity, as com-

Table 4 IC₅₀ of SR 48692, SR 142948A and levocabastine to various NTRs. For the CRE-luc assay, CHO-K1 cells transiently expressing NTRs together with CRE-luc reporter were used. For the binding assay, CHO-K1 cells stably expressing NTRs were used. IC₅₀ (log M) more values obtained from the CRE-luc assay and competition binding assay respectively. Values are the mean $\pm$ S.E.M. from three independent experiments

	IC ₅₀ (log M)		
	rNTR1	bfNTR1	bfNTR4
CRE-luc assay			
SR 48692	-7.2 $\pm$ 0.2	PI	-7.2 $\pm$ 0.2
SR 142948A	-8.6 $\pm$ 0.1	-6.1 $\pm$ 0.2	-9.1 $\pm$ 0.2
Binding assay			
NT	—	-9.1 $\pm$ 0.1	-9.2 $\pm$ 0.2
Sr 142948A	—	-6.6 $\pm$ 0.1	-8.8 $\pm$ 0.2
Levocabastine	—	ND	-5.8 $\pm$ 0.3

PI, partial inhibition; ND, not determined.

pared with mammalian NTR2. However, we do not exclude the possibility that bfNTR4 and mammalian NTR2 originate from the same ancestral gene as they show a global similarity in structure to some extent.

Consistent with a previous report showing that mNTR2 stably expressed in HEK293 cells is able to bind radioiodinated NT but cannot induce any signaling cascade, including PLC and AC activation (Botto *et al.* 1998), we found that NT did not induce CRE-luc and c-fos-luc reporter activity in CV1 or CHO-K1 cells expressing hNTR2 or mNTR2. Thus, although mammalian NTR2 can bind NT and NT agonists, its ability to induce signal transduction is very low. Unlike mammalian NTR2, bfNTR4 was able to induce CRE-luc and c-fos-luc reporter activities as well as IP production and elevation of [Ca²⁺]_i. The fact that bfNTR4 has a better signaling activity than mammalian NTR2 is consistent with the structural differences described above. bfNTR4 could induce both CRE-luc and c-fos-luc reporter activity but their fold-increases depended on the cell line in which the receptor was transfected. Thus, in CV1 cells, bfNTR4 induced a

Figure 9 Binding assays. CHO-K1 cells stably expressing bullfrog (bf) NTR1, bfNTR4 (A) and rat (r) NTR1 (B) were applied to a saturation-binding assay using graded concentrations of ^{125}I -hNT. Non-specific binding was determined in the presence of 10 μM unlabeled hNT. Competitive inhibition of ^{125}I -hNT binding on cells expressing bfNTR1 (C) or bfNTR4 (D) by cold NT (circles), SR 142948A (squares), or levocabastine (triangles). The results are expressed as the percent specific binding. Data are means $\pm$ S.E.M. of one representative experiment from three to five experiments.

similar fold increase in CRE-luc and c-fos-luc reporter activity, while in CHO-K1 cells bfNTR4 produced a 2.0-fold higher increase in CRE-luc activity (9-fold) than in c-fos-luc activity (4-fold). Since bfNTR1 provoked a similar induction of c-fos-luc and CRE-luc reporter activity, it appears that bfNTR1 may trigger both the AC/PKA- and PLC/PKC-linked signaling pathways with similar strength, at least in CHO-K1 cells, while bfNTR4 may activate preferentially the AC/PKA-linked signaling pathway. Consistent with this observation, bfNTR4-expressing CHO-K1 cells exhibited only a 1.5-fold increase in IP production, while bfNTR1-expressing CHO-K1 cells showed approximately a 3-fold increase in IP production. However, results obtained from these cellular tools cannot simply be extrapolated to a normal physiological situation, which is very complicated as tissues contain the target

receptor but also its isoforms as well as a variety of splice variants. Further, the coupling efficiency to the effectors is largely dependent on the cellular context (Oh *et al.* 2003).

There is now clear evidence that NT is an important neuroendocrine regulator of the hypothalamo-pituitary system (McCann & Vijayan 1992, Rostène & Alexander 1997). In particular, in the frog, fNT stimulates the electrical and secretory activity of melanotrope cells through an activation of a receptor whose pharmacological profile shares similarities with mammalian NTR1 (Labbé-Jullié *et al.* 1994, Desrues *et al.* 1998, Belmeguenai *et al.* 2000). In this model, the most potent agonist is JMV 458 followed by JMV 457, JMV 510 and JMV 431 (Belmeguenai *et al.* 2000). The specific NTR1 antagonist SR 48692 does not inhibit fNT-evoked stimulation of melanotrope cells, while the

wide-spectrum antagonist SR 142948A completely blocks the response (Belmeguenai *et al.* 2000). Consistent with this observation, the present study showed that JMV 458 exhibited the highest potency to stimulate bfNTR1 followed by JMV 457, JMV 510 and JMV 431 and that SR 142948A but not SR 48692 completely blocked bfNTR1 activation induced by NT. These pharmacological data strongly suggest that fNT-induced stimulation of frog melanotrope cells is mediated through the NTR1 receptor subtype. In support of this hypothesis, we found that, in the bullfrog neurointermediate lobe, bfNTR1 mRNA was actively expressed whereas bfNTR4 was hardly detectable.

Strong evolutionary pressure has acted to conserve the structure of the C-terminal hexapeptide of NT(8–13) from amphibians to mammals (Table 2) and this region is known to bear the biological activity of the peptide (Rostène & Alexander 1997). In accord, NT(8–13) was found to have the same potency as hNT and fNT in inducing activation of bfNTR1 and bfNTR4. The low potency of pNMN, JMV 457, JMV 510 and JMV 431 might be ascribed to replacement of the Arg⁹ by an Ile or Lys residue and/or by substitution for a bulky amino acid at position 11, which are known to affect ligand binding (Pang *et al.* 1996). It is noteworthy that, in the c-fos-luc assay system, bfNTR1 showed a higher sensitivity than bfNTR4 to all agonists used in the present study whereas, in the CRE-luc reporter assay system, bfNTR4 responded better to these agonists, except for pNMN, than did bfNTR1. The reason for the differential sensitivity toward each signaling pathway is currently unknown. It may be due to differential coupling efficiency of the receptors or differential receptor trafficking (Kenakin 2001).

One serendipitous finding of this study is that bfNTRs exhibited distinct pharmacological properties as compared with mammalian NTRs. SR 142948A completely inhibited NT-induced rNTR1 activation with a higher potency than SR 48692, which is in good agreement with a previous report (Gully *et al.* 1997). SR 48692 and SR 142948A, however, showed a relatively low potency to inhibit the activity of bfNTR1, the bullfrog counterpart of mammalian NTR1. Surprisingly, SR 48692 and SR 142948A, which act as agonists on mammalian NTR2 (Botto *et al.* 1997), strongly inhibited NT-induced bfNTR4 activity. The residues Met²⁰⁸ in TMH4, Tyr³²⁴, Arg³²⁷ and Phe³³¹ in TMH6 and Tyr³⁵¹, Thr³⁵⁴, Phe³⁵⁸, Tyr³⁵⁹ in TMH7 of rNTR1 play a crucial role in SR 48692 binding (Labbé-Jullié *et al.* 1998). As these residues are conserved in bfNTR1 and bfNTR4, it is assumed that other nearby residues are involved in the differential selectivity of bfNTR1 and bfNTR4 for SR 142948A and SR 48692. Levocabastine, an agonist for mammalian NTR2 but not for NTR1, induced CRE-luc reporter activity in both bfNTR1- and bfNTR4-expressing cells. Altogether,

these data indicate that, despite the high sequence similarity among bfNTRs and mammalian NTRs, bfNTRs have different characteristics compared with mammalian NTRs in terms of ligand selectivity and signal transduction. As the agonists and antagonists for mammalian NTRs exhibit different functionalities on the amphibian NTRs, one should be careful when using these NT analogs to characterize NTRs in non-mammalian vertebrates.

The characterization of two fNTRs provides novel information regarding the phylogenetic history of G protein-coupled NTRs in the vertebrate lineage. The fact that bfNTR4 exhibits structural and functional similarities to mammalian NTR1 suggests that the ancestral gene from which the two receptors arose encoded an NTR1-like receptor. Duplication of the ancestral NTR gene, which probably occurred before the divergence of the amphibian and mammalian lineages, gave rise to two distinct genes that may have evolved at vastly different rates. Thus, while bfNTR1 closely resembles its rat and human counterparts, the structural and functional characteristics of amphibian NTR4 and mammalian NTRs are more divergent. Further, while bfNTR4 has clearly retained NT as a natural agonist, mNTR2 and hNTR2 are insensitive to NT, suggesting that, in the mammalian phylum, NTR2 may have adopted a novel, yet unknown, peptide ligand. Finally, comparison of the sequences of amphibian and mammalian receptors will provide important clues as to the structural determinants that are responsible for ligand selectivity and signal transduction coupling.

In conclusion, this report provides the first molecular, pharmacological and functional characterization of two NTRs in a non-mammalian vertebrate. The data show that bfNTRs exhibit differential ligand selectivity and signal coupling mechanisms from each other, as well as distinct functional characteristics as compared with their mammalian counterparts. These data should help to elucidate the evolutionary history of NTRs in the vertebrate lineage and to identify the structural motifs that determine their pharmacological and physiological properties.

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