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The Cloned Neurotensin Receptor Mediates Cyclic GMP Formation when Coexpressed with Nitric Oxide Synthase cDNA

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SUMMARY

Rat neurotensin (NT) receptor (NTR) cDNA was subcloned into the pRC-CMV expression vector and transfected into 293 cells, and cellular clones that stably expressed the NTR were isolated and characterized. [^{3}H]NT binding to membranes prepared from the NTR cDNA-transfected cells displayed specificity and saturability, with an apparent K_d of 1.25 nM and a B_{max} of 43.4 pmol/mg of protein (~3.5 × 10^6 binding sites/cell). NT stimulated an increase in [^{3}H]inositol phosphate levels in the NTR-expressing cells up to 2500% of basal levels. The response was time and dose dependent, with an EC_{50} of 10.4 nM. NT also stimulated cAMP formation in these cells, with an EC_{50} of 27.0 nM. In addition, NT evoked an increase in the level of intracellular calcium. Approximately 60% of the calcium rise was attributable to the release of intracellular stores and 40% was attributable to calcium influx. Although NTR occupancy has been shown to stimulate cGMP formation in several brain preparations and cell lines, NT was unable to mediate cGMP synthesis in the NTR-expressing 293 cells. We found that 293 cells have guanylate cyclase activity but have undetectable levels of nitric oxide synthase (NOS) activity. Because it was possible that the production of nitric oxide is required as the mediator of NT-induced cGMP synthesis, we subcloned NOS cDNA into the pCEP4 expression vector and transiently expressed it in the NTR cells. We report that NT increased cGMP levels up to 375% of basal levels when NOS cDNA was coexpressed and that the increase was completely inhibited by the NOS inhibitor N^{ω}-nitro-L-arginine. NT-induced cGMP accumulation was time and dose dependent, with an EC_{50} of 1.7 nM. To our knowledge, this is the first report of NT mediating cGMP formation with a cloned receptor and the first evidence that NT-induced cGMP accumulation requires the production of nitric oxide.

The tridecapeptide NT is a putative neurotransmitter that is heterogeneously distributed in the central nervous system, enriched in synaptosomes, localized to nerve terminal vesicles, and released from brain membranes in a calcium-dependent manner. NT produces a large array of biological activities, which are mediated by specific membrane receptors (for review, see Refs. 1 and 2). The properties of these receptors have been characterized extensively using rat and human membrane preparations and neuronal cell lines. It has been demonstrated that NTR occupancy modulates intracellular levels of inositol phosphates (3–8), calcium (4, 5, 9), cGMP (6, 8, 10, 11), and cAMP (12) and the phosphorylation of various protein substrates (13, 14).

Recently, the rat and human NTRs have been cloned (15, 16). The NTR consists of 424 (rat) or 418 (human) amino acids, with seven putative transmembrane domains, and belongs to the family of G protein-coupled receptors. The cloned rat NTR has been expressed and characterized in Chinese hamster ovary cells. In Chinese hamster ovary cells, NT mediates cAMP formation (17), phospholipase C activation (18), and generation of inositol phosphates (19).

In this report, we have derived a cell line, from transformed human embryonic kidney cells (293 cells), that stably expresses the high affinity NTR. We demonstrate that the cloned NTR expressed in 293 cells is pharmacologically similar to the NTR expressed endogenously in brain and that it mediates PI hydrolysis, cAMP formation, and intracellular calcium mobilization. In addition, we demonstrate that the cloned NTR mediates cGMP formation in 293 cells and we suggest that this linkage is dependent on the production of NO and the functional expression of NOS.

Materials and Methods

Cloning and stable expression of the NTR. Poly(A)$^+$ mRNA from rat ventral tegmental area was reverse transcribed and subjected to nested polymerase chain reaction using specific NTR primers (15). The resulting 1.3-kilobase cDNA was isolated, cloned into a Bluescript II SK+ phagemid vector (Stratagene) using *Hind*III and *Xba*I restric-

ABBREVIATIONS: NT, neurotensin; NTR, neurotensin receptor; NOS, nitric oxide synthase; NO, nitric oxide; CSS, controlled salt solution; HCSS, HEPES-buffered controlled salt solution; PI, phosphatidylinositol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Lant 6, [Lys^8, Asn^9] Neurotensin (8–13).

tion enzymes, and sequenced to ensure that it was devoid of mutations. The NTR cDNA was then subcloned into the eukaryotic expression vector pRC-CMV (Invitrogen), using *Hind*III and *Not*I restriction enzymes. The resulting construct was transfected into 293 cells (American Type Culture Collection, Rockville, MD) using a mammalian calcium phosphate transfection kit (Stratagene). Transfected cells were selected for their ability to grow in the presence of aminoglycoside G-418 (600 μg/ml). Resistant cell populations were subjected to limited dilutions to isolate cellular clones. Forty-two clones were isolated and screened for [^{3}H]NT binding. The clone demonstrating the highest level of specific binding (293-rNTR-#39) was further characterized and used for all subsequent experiments.

Cell culture. 293-rNTR-#39 cells were grown in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum and 4 mM glutamine. Cultures were grown in 80-cm^2 tissue culture flasks (Nunc) containing 20 ml of medium and were maintained at 37° in a 5% CO_2 humidified atmosphere. Stock cultures were passaged every 7 days (1:20 split) and fed twice weekly. Confluent cells (passage number, 10–25) were used in this study.

NTR ligand binding. NTR ligand binding assays were performed according to the method of Goedert (20). In brief, assays were carried out at 0° for 90 min in 1.0 ml of 50 mM Tris·HCl, pH 7.4, containing 0.1% bovine serum albumin, 40 mg/liter bacitracin, and 1 mM EDTA. Each assay contained 20 μg of membrane protein from 293-rNTR-#39 cells, various concentrations of [^{3}H]NT (saturation experiments) or 0.5 nM [^{3}H]NT (displacement experiments), and various concentrations of unlabeled NT analogs. The reactions were terminated by rapid filtration over presoaked Whatman GF/B filters. Radioactivity on the filter was determined by scintillation counting. Nonspecific binding was defined as the binding activity in the presence of 1 μM NT and was subtracted from total binding to determine specific binding. Each experiment was carried out at least three times in triplicate.

Measurement of PI hydrolysis. 293-rNTR-#39 cells cultured in 24-well plates were incubated with 0.5 μCi/ml [^{3}H]inositol (Amersham) for 18–20 hr at 37°. The cells were then washed twice with 1 ml of lithium (10 mM)-containing CSS (114 mM NaCl, 12 mM KCl, 2.5 mM $CaCl_2$, 800 mM $MgCl_2$, 20 mM HEPES, pH 7.4) and incubated for >10 min at 37°. The assay was initiated by the addition of agonist to the cultures. The reaction was terminated by the addition of perchloric acid to a final concentration of 4.5%. Total [^{3}H]inositol phosphates, including inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, were isolated by anion exchange chromatography using Bio-Rad AG 1-X8 resin, as described previously (21). Each experiment was carried out at least three times in triplicate.

Measurement of adenylyl cyclase. NTR-expressing 293 cells cultured in 24-well plates were washed twice with 1 ml of cAMP buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM D-glucose, 1 mM 1,10-phenanthroline, 0.2% bovine serum albumin, 25 mM Tris·HCl, pH 7.4) and incubated for >10 min at 37°. The assay was initiated by the addition of various concentrations of NT, incubated for 5 min at 37°, and terminated by removal of the medium and addition of 0.5 M perchloric acid. The cells were homogenized by sonication and were centrifuged at 27,500 × *g* for 15 min, and the supernatant was removed and lyophilized. The lyophilized material was reconstituted in 200 μl of sodium acetate buffer, pH 6.2, and evaluated for cAMP content using an ^{125}I-cAMP radioimmunoassay kit (NEN Research Products).

Measurement of intracellular calcium levels. 293-rNTR-#39 cells cultured in 80-cm^2 tissue culture flasks were washed three times with HCSS (120 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.62 mM $MgSO_4$, 10 mM HEPES, 6 mM D-glucose, pH 7.4), centrifuged at 1000 × *g* for 2 min, washed with HCSS, and resuspended in HCSS containing 5 μM levels of the calcium-sensitive dye fura-2/acetoxymethyl ester (Molecular Probes). After dye loading for 30 min at 37°, cells were washed and divided into ten 1-ml aliquots. Immediately before assay, aliquots were microfuged and resuspended in HCSS containing either 2 mM calcium or 1 mM EGTA. Fluorescence was measured in a fluorescence spectrophotometer (Photon Technologies Inc.), with excitation wavelengths of 340 and 380 nm and emission at 510 nm.

Transient expression of NOS. NOS cDNA (22) cloned into a Bluescript II SK− phagemid vector (Stratagene) was generously supplied by Dr. Solomon Snyder (Johns Hopkins University, Baltimore, MD). The NOS cDNA was subcloned into the eukaryotic expression vector pCEP4 (Invitrogen) using *Pvu*II restriction enzyme and *Eco*RI/*Not*I adapters. The resulting NOS/pCEP4 plasmid (0.00014–1.4 μg of NOS plasmid/10^6 cells) was transiently transfected into 293-rNTR-#39 cells using a calcium phosphate transfection kit (BRL). All assays were performed 48–52 hr after transfections.

Tissue preparation and measurement of NOS activity. NOS activity was quantitated in both membrane-bound and cytosolic fractions of 293-rNTR-#39 cells and 293-rNTR-#39 cells that had been transiently transfected with NOS cDNA (0.07 μg of NOS plasmid/10^6 cells). NOS activity was quantified using a citrulline assay, which monitors the production of [^{3}H]citrulline from L-[^{3}H]arginine, as described previously (23). Cells were homogenized at 20% (w/v) in 50 mM triethanolamine buffer containing 30% glycerol, 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 1 μM pepstatin A, 2 μM leupeptin, and 1 mM phenylmethylsulfonyl flouride, pH 7.4, and were centrifuged at 20,000 × *g* for 30 min at 4°. The resulting supernatant was assayed for NOS activity. The resulting pellet was rehomogenized in buffer containing 1 M KCl and was centrifuged at 20,000 × *g* for 30 min. The supernatant was then discarded and the pellet was rehomogenized and assayed for NOS activity. Protein concentrations were determined by the Bradford Coomassie Brillant Blue method, as described by Bio-Rad, using bovine serum albumin as standard.

Measurement of guanylyl cyclase. NTR- or NTR/NOS-expressing 293 cells cultured in 24-well plates were washed twice with 1 ml of cGMP buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM D-glucose, 1 mM 1,10-phenanthroline, 0.2% bovine serum albumin, 10 nM L-arginine, 25 mM Tris·HCl, pH 7.4), with or without 10 μM N^{ω}-nitro-L-arginine (Sigma), and were incubated for >10 min at 37°. The assay was initiated by addition of agonist and was terminated by removal of the medium and addition of 0.5 M perchloric acid. cGMP content was measured with an ^{125}I-cGMP radioimmunoassay kit (NEN Research Products).

Guanylyl cyclase activity was also measured in olfactory bulb slices dissected from male Sprague Dawley rats (150–200 g). The olfactory bulbs were sectioned into slices (400 × 400 μm) using a McIlwain tissue chopper (Brinkman Instruments) and were equilibrated in oxygenated (95% O_2/5% CO_2) Krebs buffer (120 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM KH_2PO_4, 25 mM $NaHCO_3$, 12 mM glucose) for 1 hr at 37°. The assay was initiated by addition of agonist and terminated by addition of 1.0 M perchloric acid. Slices were homogenized by sonication and centrifuged at 27,500 × *g* for 15 min. cGMP content was measured in the supernatant as described above.

Results

NTR binding assays. No specific [^{3}H]NT binding was detected in the parent 293 cells. Of the 42 colony clones from the NTR cDNA-transfected cells that were screened, 16 were strongly positive. The most positive clone, 293-rNTR-#39, was characterized further. Membranes from 293-rNTR-#39 cells bound [^{3}H]NT with an apparent K_d of 1.25 nM and a B_{max} of 43 pmol/mg of protein (~3.5 × 10^6 binding sites/cell). The binding was specific and saturable (Fig. 1). We compared several NT peptide analogs for their ability to inhibit 0.5 nM [^{3}H]NT binding. The IC_{50} values of binding for NT-8–13, [Gln-4]NT, NT, NT pseudopeptide H-[Ψ8,9] (24), xenopsin, acetylated NT-8–13, neuromedin N, LANT-6, [D-Trp-11]NT, [D-Phe-11]NT, and [D-Tyr-11]NT were 2.9 nM, 3.2 nM, 4.1 nM, 5.2 nM, 5.2 nM, 6.1 nM, 15.4 nM, 40.7 nM, 5000 nM, >10,000 nM, and >10,000 nM, respectively. NT-1–8, NT-1–11, and levocabastine (1 mM) had no effect on [^{3}H]NT binding (Table 1).

NTR stimulation of PI hydrolysis. NT (1 μM) stimulated

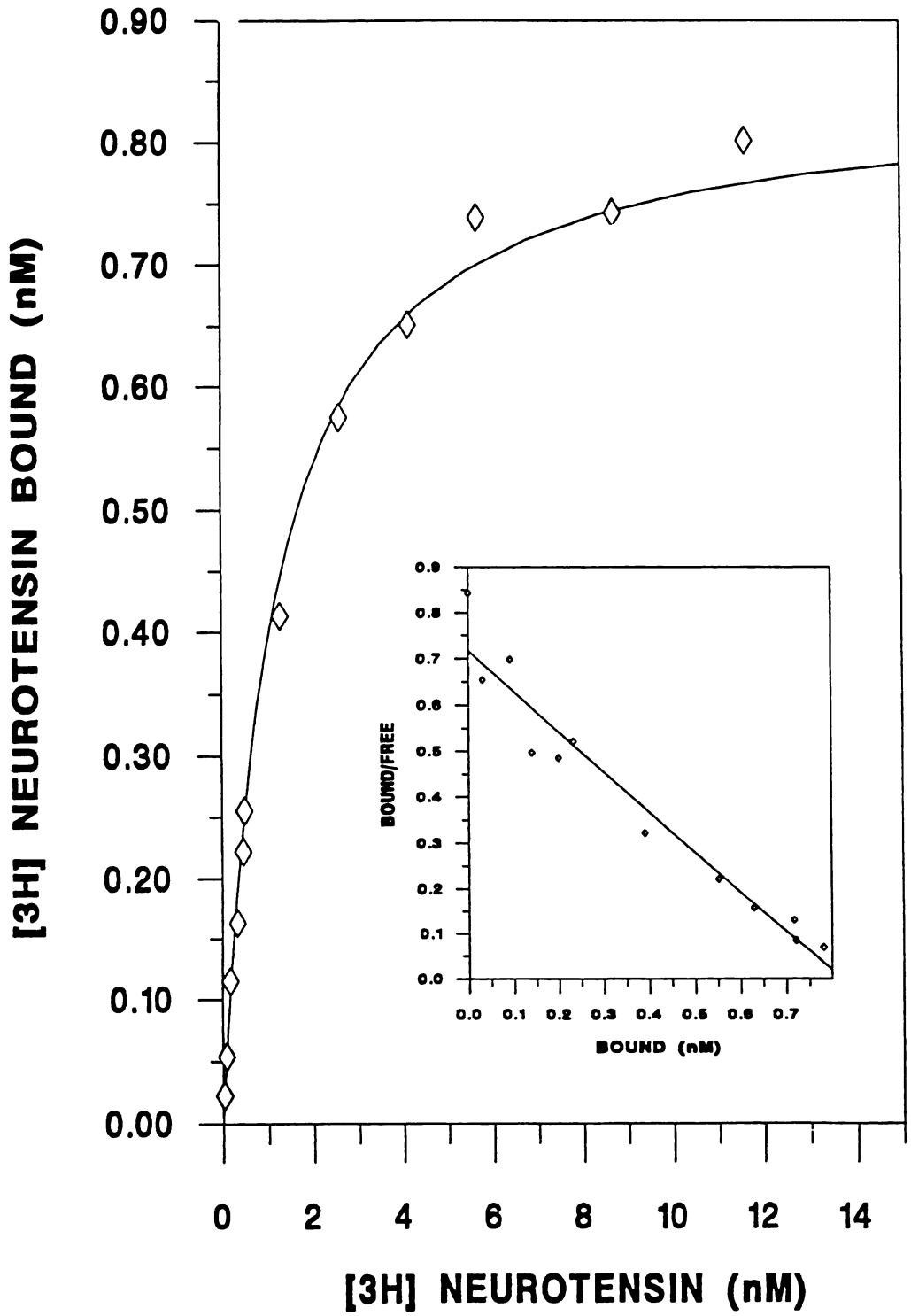

Fig. 1. Saturation isotherm and Scatchard plot (*inset*) of the specific binding of [^{3}H]NT to 293 cells stably expressing the rat NTR cDNA. Each *point* represents the mean of at least three separate experiments.

an increase in [^{3}H]inositol phosphate levels in the 293-rNTR-#39 cells up to 2500% of basal levels; no stimulation was observed in nontransfected cells. The response was dose dependent, with an EC_{50} of 10.4 nM, and was linear up to 30 min (Fig. 2). We tested several NT peptide analogs for their ability to stimulate PI hydrolysis. The EC_{50} values for PI hydrolysis for NT 8–13, [Gln-4]NT, NT, NT pseudopeptide H-[Ψ8,9] (24), xenopsin, acetylated NT-8–13, neuromedin N, LANT-6, [D-Trp-11]NT, [D-Phe-11]NT, and [D-Tyr-11]NT were 7.1 nM, 9.4 nM, 10.4 nM, 10.6 nM, 6.4 nM, 9.5 nM, 7.7 nM, 12.2 nM, 986 nM, 1778 nM, and 802 nM, respectively. NT-1–8, NT-1–11, and levocabastine (1 mM) had no effect on [^{3}H]inositol phosphate levels (Table 1).

NTR activation of adenylyl cyclase. We found that NT was able to stimulate cAMP formation in the 293-rNTR-#39 cells; no response was observed in untransfected control cells. The effect was dose dependent, with an EC_{50} of 27.0 nM (Fig. 3).

TABLE 1

Potencies of NT and related peptides in biological and binding activities with clone 293-r NTR-#39

Values are means ± standard errors of three separate experiments performed in triplicate.

Peptide	Competition with [^{3}H]NT binding, IC_{50}	Stimulation of release of [^{3}H]inositol phosphates, EC_{50}
	nM	nM
NT-8-13	2.9 ± 0.45	7.1 ± 0.8
[Gln-4]NT	3.2 ± 0.35	9.4 ± 1.6
NT	4.1 ± 0.4	10.4 ± 1.1
NT pseudopeptide H-[Ψ8,9]	5.2 ± 0.72	10.6 ± 0.6
Xenopsin	5.2 ± 0.12	6.4 ± 0.9
Acetylated NT-8-13	6.1 ± 0.14	9.5 ± 1.1
Neuromedin N	15.4 ± 0.62	7.7 ± 0.4
LANT-6	40.7 ± 6.44	12.2 ± 1.8
[D-Trp-11]NT	~5,000	986.4 ± 180.5
[D-Phe-11]NT	>10,000	1,778.2 ± 104.4
[D-Tyr-11]NT	>10,000	801.9 ± 33.0
NT-1-8	>100,000	No effect
NT-1-11	>100,000	No effect
Levocabastine	>100,000	No effect

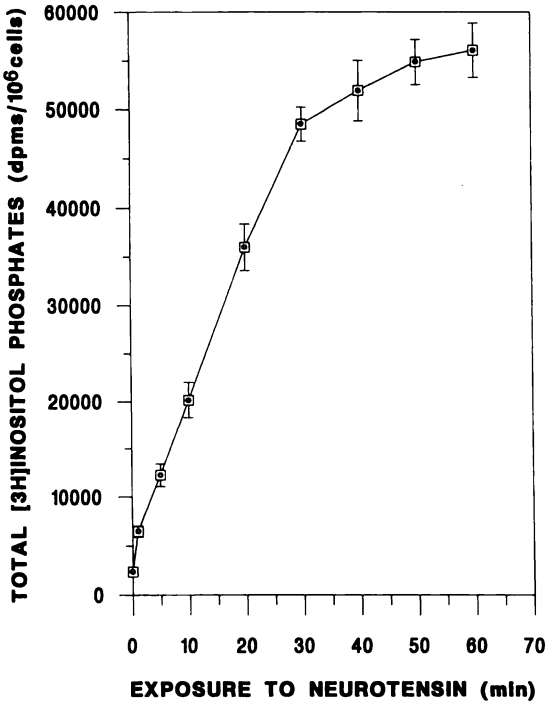

Fig. 2. Time course of NT-induced PI hydrolysis in the NTR-expressing 293 cells. The NTR-expressing cells were incubated with 1 μM NT for the times indicated, and formation of [^{3}H]inositol phosphates was assayed as described in Materials and Methods. Each *point* represents the mean of at least three separate experiments.

NTR activation of intracellular calcium mobilization. We analyzed NT-triggered changes in the levels of intracellular calcium. The addition of NT (1 μM) to the 293-rNTR-#39 cells evoked a rapid and marked increase in the level of intracellular calcium; no response was observed in untransfected control cells. More than half of this response persisted when extracellular calcium was chelated with 1 mM EGTA (Fig. 4). After integration of the area under the peaks, it was determined that 40% of the NT-induced calcium rise was dependent on the presence of extracellular calcium and approximately 60% was independent of extracellular calcium. The carboxyl-terminal peptide fragment NT-8–13 (1 μM) also evoked a marked increase in intracellular calcium levels, whereas the amino-terminal octapeptide fragment NT-1–8 (1 μM) had no effect (data not shown).

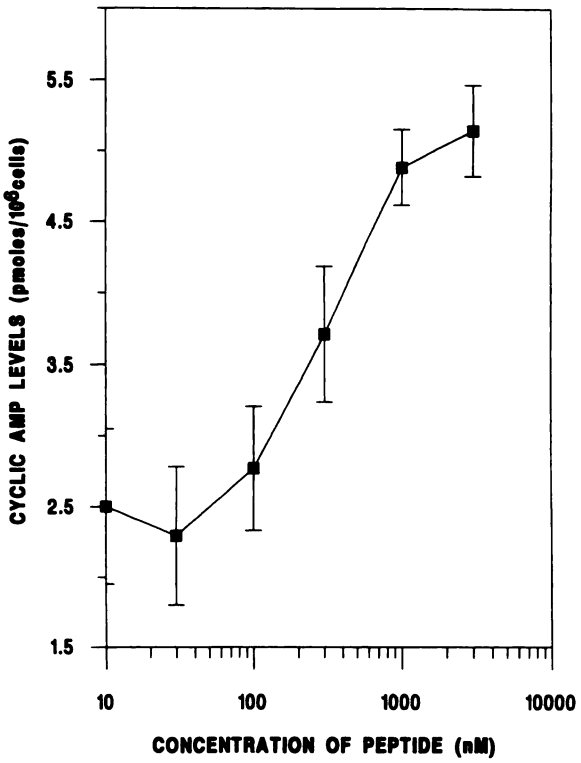

Fig. 3. Dose-response curve for cAMP accumulation in the NTR-expressing 293 cells. The NTR-expressing cells were incubated with various concentrations of NT for 5 min, and formation of cAMP was assayed as described in Materials and Methods. This is a representative graph of three separate determinations.

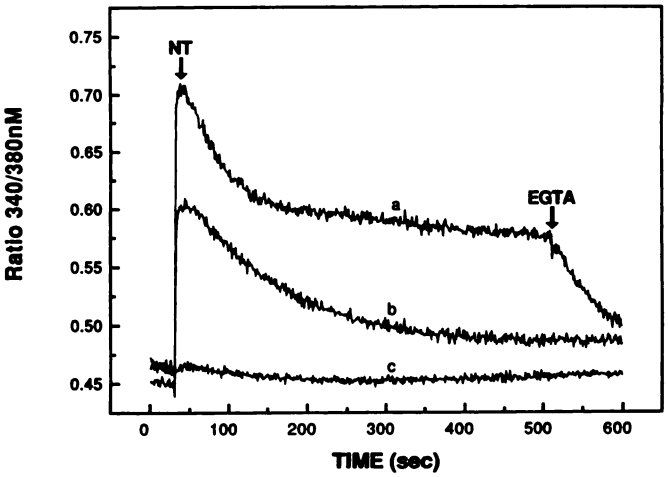

Fig. 4. Increase in intracellular calcium concentration in NTR-expressing 293 cells in response to NT. Cells were assayed in the presence of 2 mM extracellular calcium (*trace a*) or 1 mM extracellular EGTA (*trace b*). Nontransfected 293 cells were used as control (*trace c*). NT (1 mM) and EGTA (2 mM) were added as indicated.

NTR activation of guanylyl cyclase. Under all assay conditions examined, NT (up to 10 μM) was unable to stimulate cGMP formation in the 293-rNTR-#39 cells. We found that these 293 cells have guanylate cyclase activity, which could be directly stimulated by the NO-releasing molecule sodium nitroprusside (500% of basal levels at 0.1 mM; data not shown).

However, it has been reported that 293 cells have undetectable levels of NOS (22). Because it was possible that the production of NO is required as the mediator of NT-induced cGMP synthesis, we transiently expressed NOS cDNA in the 293-rNTR-#39 cells.

We found that NT (1 μM) was able to stimulate cGMP formation up to 375% of basal levels in the 293-rNTR-#39 cells when NOS cDNA was coexpressed (0.07 μg of plasmid transfected/10^6 cells). In the NTR/NOS-expressing cells, NT-induced cGMP accumulation was time dependent and increased linearly up to 3 min (Fig. 5). The effect was dose dependent, with an EC_{50} of 1.7 nM, and was completely abolished by the NOS inhibitor N^{ω}-nitro-L-arginine (10 μM). The carboxyl-terminal fragment NT-8–13 also stimulated cGMP formation (EC_{50} of 1.6 nM), whereas NT-1–8 had no effect (Fig. 6).

We observed that the ability of NOS to mediate cGMP accumulation was dependent on the quantity of NOS plasmid transfected in the 293-rNTR-#39 cells. To examine this systematically, we transiently transfected 0.00014–1.4 μg of NOS plasmid/10^6 cells and then measured basal cGMP levels and NT-induced changes in cGMP formation (Fig. 7). Transfection of ≤0.0014 μg of NOS plasmid had no effect on either basal or NT-induced cGMP levels. Transfection of 0.007–1.4 μg of NOS plasmid caused a dose-dependent increase in both basal and NT-induced cGMP accumulation. At the higher NOS transfection levels examined (0.14–1.4 μg), we observed a large increase in basal cGMP levels, up to 1200% of levels in untransfected control cells. It is possible that overexpression of NOS generates high levels of NO that, in turn, lead to constitutive stimulation of guanylate cyclase activity. Such findings of overexpression have been described for other systems (25–27). Because the optimal ratio between basal and stimulated cGMP

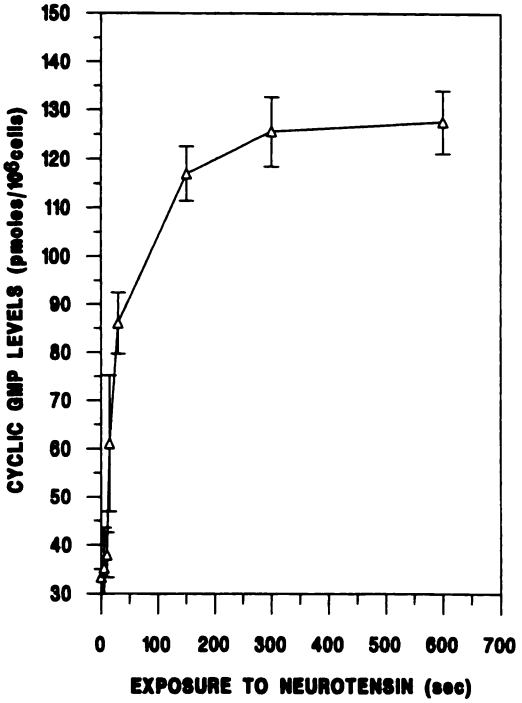

Fig. 5. Time couse of NT-induced cGMP accumulation in 293-rNTR-#39 cells transiently expressing NOS cDNA. 293 cells coexpressing rat NTR and NOS cDNAs were incubated with 1 μM NT for the times indicated, and formation of cGMP was assayed as described in Materials and Methods. Each *point* represents the mean of at least three separate experiments.

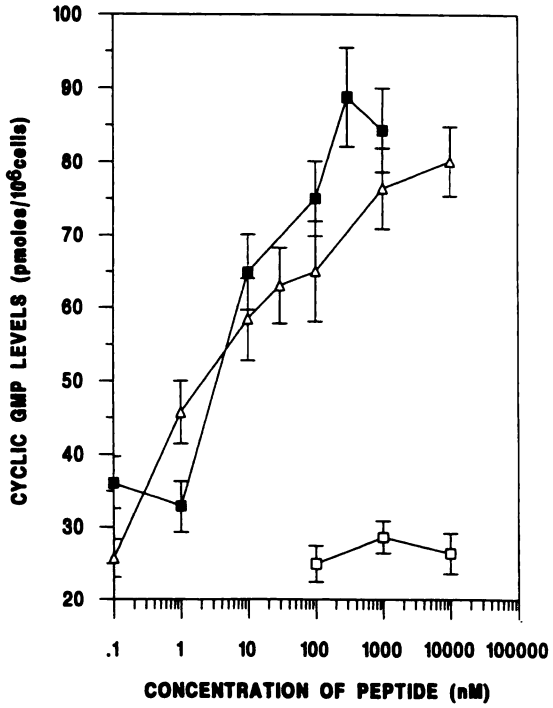

Fig. 6. Dose-response curve for cGMP accumulation in 293-rNTR-#39 cells transiently expressing NOS cDNA. 293 cells coexpressing rat NTR and NOS cDNAs were incubated for 2.5 min with NT (■), NT-8–13 (△), or NT-1–8 (□), as indicated, and formation of cGMP was assayed as described in Materials and Methods. Each *point* represents the mean of at least three separate experiments.

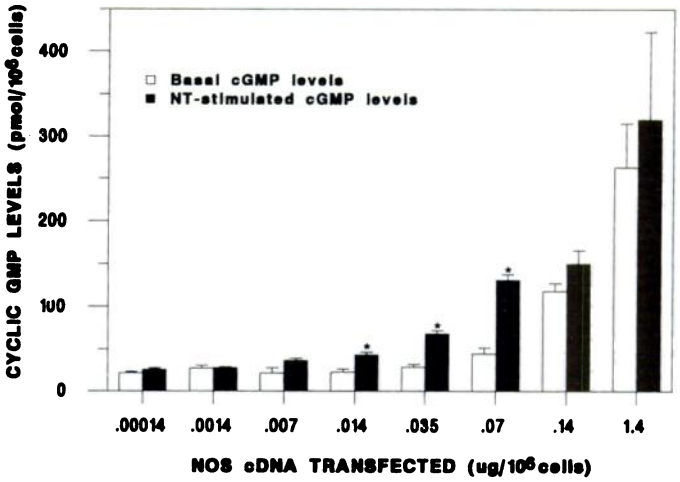

Fig. 7. Evidence that the quantity of NOS cDNA transfected affects both basal and NT-induced cGMP levels. Various amounts (0.0014–1.4 μg/10^6 cells) of NOS plasmid were transiently transfected into 293-rNTR-#39 cells, as described in Materials and Methods. Forty-eight to 52 hr later, both basal cGMP levels and NT-induced changes in cGMP levels were assayed. □, Basal cGMP levels; ■, increase in cGMP levels induced by exposure to 1 μM NT for 2.5 min. Values were calculated as percentage of basal levels in untranfected 293-rNTR-#39 cells. Each *point* represents the mean of at least three experiments. Because the optimal ratio between basal and stimulated cGMP levels occurred using 0.07 μg of NOS plasmid, this amount of plasmid was used for all characterization experiments. *, $p < 0.01$, compared with basal cGMP control for each quantity of NOS cDNA transfected.

levels occurred using 0.07 μg of NOS plasmid, this amount of plasmid was used for all subsequent experiments.

Quantification of NOS activity in 293-rNTR-#39 cells. NOS activity in the 293-rNTR-#39 cells was not signif-

icantly above background levels. The specific activity of NOS in 293-rNTR-#39 cells transiently transfected with NOS plasmid (0.07 μg of NOS plasmid/10^6 cells) was found to be 8.24 ± 0.32 pmol/mg of protein/min. The NOS activity was confined to the cytosolic fraction and was not membrane bound to any appreciable extent. The properties of the NOS were those of the constitutive neuronal isoform rather than the inducible isoform, because calcium-chelating agents (0.1 mM EGTA plus 1 mM EDTA) and a calmodulin antagonist (100 μM calmidazolium) were found to inhibit NOS activity (23) (data not shown).

NTR activation of guanylyl cyclase in olfactory bulb slices. To determine whether NOS activity is also important in NT-induced cGMP formation in endogenous brain tissue, we characterized guanylyl cyclase activity in rat olfactory bulb slices. Olfactory bulb was chosen because the NTR is known to be abundant in this brain region and the bulb is easy to excise. We found that NT was capable of stimulating cGMP production in olfactory bulb slices (210 ± 14% of control) and that this accumulation was completely inhibited by the NOS inhibitor N^{ω}-nitro-L-arginine (10 μM) (65 ± 5% of control; data not shown).

Discussion

Because this is the first report on the cloned NTR expressed in 293 cells, we extensively characterized its pharmacology and second messenger associations. We report that [^{3}H]NT bound to membranes prepared from the NTR cDNA-transfected 293 cells with an apparent K_d of 1.25 nM and a B_{max} of 43.4 pmol/mg of protein (Fig. 1). The K_d value is similar to what has been reported in other systems (9, 11, 15, 16, 18). The B_{max} value, however, is 1000–3000-fold larger than the B_{max} reported for rat brain (20, 28) and accounts for approximately 3.5×10^6 NT binding sites/cell. Such high expression levels are not uncommon in transfected cells, presumably due to the strong transcription promoters in the plasmids used for cloning.

NTR occupancy in these transfected cells led to large and dose-dependent increases in [^{3}H]inositol phosphate levels (Fig. 2). The rate of NT-stimulated PI hydrolysis in the 293-rNTR-#39 cells increased linearly up to 30 min. Unlike most systems in which the NTR is expressed endogenously (29), no rapid agonist-induced desensitization or down-regulation of this response was detected. This could be due to the inability of the cloned NTR to link to desensitization/down-regulation mechanisms in these cells. Alternatively, because of the extremely high NTR expression levels, it is possible that a significant portion of the NTRs could be down-regulated with no measurable effect on maximal PI hydrolysis.

Similarly to studies performed by Yamada *et al.* (17) using the cloned NTR expressed in Chinese hamster ovary cells, we found that NT dose-dependently stimulated cAMP formation in the 293-rNTR-#39 cells, with an EC_{50} of 27 nM (Fig. 3). These data are in contrast to studies recently reported by Bozou *et al.* (12), who found that NT mediates inhibition of cAMP formation in neuroblastoma N1E115 cells. In addition, several other researchers have reported that NTRs do not mediate cAMP accumulation in a variety of endogenous tissues (30–32). It is not unusual, however, for cloned receptors expressed in foreign cell lines that express large amounts of the receptor to couple to transduction systems that may not be relevant endogenously.

In the NTR-expressing 293 cells, NTR occupancy also evoked a rapid and marked increase in intracellular calcium mobilization (Fig. 4). More than half of this response persisted even in the absence of extracellular calcium and in the presence of EGTA (Fig. 4, *trace b*), suggesting that a significant portion of the NT-induced increase in intracellular calcium levels is due to mobilization of intracellular stores. NT-8–13 caused calcium mobilization similar to that produced by NT, whereas NT-1–8 was devoid of calcium-mobilizing activity (data not shown). These data support other findings describing the cloned NTR expressed in rat cortical cells and HT-29 cells (9, 33).

We further characterized the cloned NTR in 293 cells by determining the potencies of several NT peptide analogs for inhibition of [^{3}H]NT binding and for stimulation of [^{3}H]inositol phosphate release. The rank order of potencies for the 12 NT peptide analogs examined was similar to that found previously in other tissues and cell systems (8, 11, 15, 20). The carboxyl-terminal peptide NT-8–13, the reduced-bond NT pseudopeptide H-[Ψ8,9] (24), and the naturally occurring NT analogs xenopsin, LANT-6, and neuromedin N were all potent inhibitors of [^{3}H]NT binding and potent stimulators of PI release (Table 1). The amino-terminal peptide fragments NT-1–8 and NT-1–11 had no effect on binding or PI release, consistent with the observation that the carboxyl-terminal portion of NT contains the structural requirements for binding. Levocabastine also had no effect on binding or PI hydrolysis, suggesting that our cloned receptor is the high affinity NTR.

NT was unable to mediate cGMP formation in the NTR-expressing 293 cells. We found that 293 cells have guanylate cyclase activity but have undetectable levels of NOS activity. Bredt *et al.* (22) previously reported that, with an immunostaining assay, NOS was undetectable in 293 cells. In some systems, it has become apparent that NO is an essential mediator of neurotransmitter-induced stimulation of guanylate cyclase. For example, NO has been shown to be a mediator of *N*-methyl-D-aspartate-induced cGMP formation in rat cerebellum (34), glutamate- and norepinephrine-induced cGMP formation in neuronal cultures (35, 36), cytokine-induced cGMP synthesis in hepatocytes (37), and angiotensin II-induced activation of cGMP levels in neuroblastoma cells (38). In addition, it has been demonstrated that NO mediates *N*-methyl-D-aspartate-induced PI hydrolysis in rat cerebellum (39). Because it was possible that the production of NO is required as the mediator of NT-induced cGMP synthesis, we subcloned NOS cDNA into the pCEP4 expression vector and transiently expressed it in the 293-rNTR-#39 cells. We report that NT was able to increase the basal cGMP level by 375% when NOS cDNA was coexpressed. The effect was time dependent (Fig. 5) and dose dependent (Fig. 6) and was completely abolished by the NOS inhibitor N^{ω}-nitro-L-arginine. To our knowledge, this is the first report of NT mediating cGMP formation with a cloned receptor and the first direct evidence that NT-mediated cGMP formation requires the production of NO.

To evaluate whether this finding has relevance in an endogenous neuronal system, we characterized the NOS dependence of guanylyl cyclase activity in rat olfactory bulb slices. We found that NT was capable of stimulating cGMP production in olfactory bulb slices and that this accumulation was completely inhibited by the NOS inhibitor N^{ω}-nitro-L-arginine (data not shown). These data corroborate our cloned receptor data and suggest that NT-induced cGMP formation in this

tissue source is also dependent on NOS activity and the production of NO.

In summary, we have characterized for the first time the cloned NTR expressed in 293 cells. We demonstrate that the NTR expressed in these cells is pharmacologically similar to the NTR described in brain and mediates PI hydrolysis, cAMP formation, and calcium mobilization. In addition, we show that NTR occupancy mediates cGMP synthesis and we demonstrate that this linkage is dependent on the functional expression of NOS and the production of NO.

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