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AGONIST PROPERTIES OF A STABLE HEXAPEPTIDE ANALOG OF NEUROTENSIN, N^{α}MeArg-Lys-Pro-Trp-tLeu-Leu (NT1)

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Abstract—The major signal transduction pathway for neurotensin (NT) receptors is the G-protein-dependent stimulation of phospholipase C, leading to the mobilization of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and the stimulation of cyclic GMP. We investigated the functional actions of an analog of NT(8-13), N^{α}MeArg-Lys-Pro-Trp-tLeu-Leu (NT1), and other NT related analogs by quantitative measurement of the cytosolic free Ca^{2+} concentration in HT-29 (human colonic adenocarcinoma) cells using the Ca^{2+}-sensitive dye fura-2/AM and by effects on cyclic GMP levels in rat cerebellar slices. The NT receptor binding affinities for these analogs to HT-29 cell membranes and newborn (10-day-old) mouse brain membranes were also investigated. Data obtained from HT-29 cell and mouse brain membrane preparations showed saturable single high-affinity sites and binding densities (B_{max}) of 130.2 and 87.5 fmol/mg protein, respectively. The respective K_D values were 0.47 and 0.39 nM, and the Hill coefficients were 0.99 and 0.92. The low-affinity levocabastine-sensitive site was not present ($K_I > 10{,}000$) in either membrane preparation. Although the correlation of binding betwen HT-29 cell membranes and mouse brain membranes was quite significant ($r = 0.92$), some of the reference agents had lower binding affinities in the HT-29 cell membranes. The metabolically stable compound NT1 plus other NT analogs and related peptides [NT, NT(8-13), xenopsin, neuromedin N, NT(9-13), kinetensin and (D-Trp^{11})-NT] increased intracellular Ca^{2+} levels in HT-29 cells, indicating NT receptor agonist properties. The effect of NT1 in mobilizing $[Ca^{2+}]_i$ blocked by SR 48692, a non-peptide NT antagonist. Receptor binding affinities of NT analogs to HT-29 cell membranes were positively correlated with potencies for mobilizing intracellular calcium in the same cells. In addition, NT1 increased cyclic GMP levels in rat cerebellar slices, confirming the latter findings of its NT agonist action. These results substantiate the *in vitro* NT agonist properties of the hexapeptide NT analog NT1.

Key words: neurotensin; peptide; receptor binding; agonist; intracellular calcium; cyclic GMP

NT§ is a tridecapeptide that displays a wide spectrum of biological actions both in the central and peripheral nervous systems of different mammalian species [1]. These actions have led to the proposal that this peptide fulfills a dual function as a neurotransmitter/neuromodulator in the brain and as a hormone/cellular mediator in peripheral tissues [2]. NT and related analogs display a number of activities similar to that of known antipsychotics [3]. Biochemical and behavioral data in animals, as well as data from clinical studies, have suggested the existence of a close functional relationship between NT and dopamine systems in the central nervous system [4, 5]. Thus, the development of a centrally acting metabolically stable NT receptor agonist would be highly desirable and of potential use in the treatment of schizophrenia and possibly other disorders [3].

Specific NT binding sites have been localized and characterized in various species. Two distinct sites are found in the rat and mouse brain [6, 7] but only one in the newborn mouse brain, rabbit and various cell lines (neuroblastoma N1E-115 and HT-29 cells) [see Ref. 8 for a review]. The latter possess NT receptors functionally coupled to intracellular second messenger systems [9]. A lower affinity NT site that is sensitive to both NT, its analogs, and the histamine-1 antagonist levocabastine has been considered a recognition site for NT and named the NT levocabastine-sensitive acceptor site [4, 6]. A second high-affinity NT binding site sensitive to NT and its analogs but not to levocabastine is considered to be the physiological NT receptor.

A functional cDNA clone for the rat NT receptor coding for a 424 amino acid protein was identified by combining molecular cloning in an RNA

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§ Abbreviations: NT, neurotensin, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu; NT1, N^{α}MeArg-Lys-Pro-Trp-tLeu-Leu [NT(8-13) stable analog]; NT(8-13), Arg-Arg-Pro-Tyr-Ile-Leu; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxy methyl ester; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PMSF, phenylmethylsulfonyl fluoride; cyclic GMP, guanosine 3′,5′-cyclic phosphate; cyclic AMP, adenosine-3′,5′-cyclic phosphate; G-proteins, guanine nucleotide-binding regulatory proteins; and TFA, trifluoroacetic acid.

expression vector with an electrophysiological assay in *Xenopus oocytes* [10]. Moreover, Vita *et al.* [11] have published the complementary DNA (cDNA) sequence for the human NT receptor derived from HT-29 colonic cells. It was shown that the NT receptor belongs to the fast-growing superfamily of G-protein-coupled receptors with seven putative transmembrane spanning domains. The diverse functions of NT have been ascribed to interaction with its specific receptors and subsequent modulation of certain intracellular second messengers including inositol phosphates, cyclic AMP and cyclic GMP [12–14]. For example, regulation of the NT receptor-inositol phosphate-$[Ca^{2+}]_i$ pathway has been reported in HT-29 cells [12]. Formation of intracellular cyclic GMP associated with NT receptors was first reported in a neuronal cell type (N1E-115) and in rat cerebellar slices [7].

NT1 is an analog of NT(8-13), which has been reported to be metabolically stable and shown to produce central nervous system effects upon systemic administration [15, 16]. The present studies characterized the receptor binding affinities of NT1 to NT receptors and its functional effects on $[Ca^{2+}]_i$ and cyclic GMP.

MATERIALS AND METHODS

Chemicals. [^{3}H]NT (sp. act. 107.2 Ci/mmol) and [^{125}I]Tyr^3-NT (sp. act. 2200 Ci/mmol) were obtained from Dupont-New England Nuclear (Boston, MA, U.S.A.). NT analogs, preset TRIZMA crystals, BSA and polyethylenimine (PEI) at 50% free base in water were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The peptidase inhibitors bacitracin and leupeptin were from Aldrich (Milwaukee, WI, U.S.A.) and Cambridge Research Biochemicals Inc. (Wilmington, DE, U.S.A.), respectively. Beckman Ready Gel/Safe liquid scintillation fluid was from Beckman (Fullerton, CA, U.S.A.). DMSO was purchased from Aldrich. Fura-2/AM dye was purchased from Molecular Probes (Junction City, OR, U.S.A.). N^{α}Boc-N^{α}MeArg(Tos) and N^{α}Boc-Ile were obtained from Bachem Bioscience (Philadelphia, PA, U.S.A.). All other N^{α}-t-Boc-protected amino acids and peptide resin were obtained from Applied Biosystems Inc. (Foster City, CA, U.S.A.). TFA was obtained from Halocarbon (River Edge, NJ, U.S.A.) and hydrogen fluoride (HF) from Matheson Gas Products (East Rutherford, NJ, U.S.A.). All other reagents, solvents and chemicals were purchased from commercial sources and were of reagent grade. Frozen brains from newborn male Swiss mice were obtained from ABS Inc. (Wilmington, DE, U.S.A.) and male Long-Evans rats were purchased from Harlan Laboratories (Indianapolis, IN, U.S.A.). HT-29 cells were obtained from the American Type Culture Collection (ATCC) No. HTB 3 (Rockville, MD, U.S.A.).

*Preparation of NT*1. NT1 was prepared on an Applied Biosystems 430A peptide synthesizer using standard solid phase techniques for N^{α}-t-butyloxycarbonyl (Boc) protected amino acids on an N^{α}-Boc-Leu-PAM resin [17]. Amino acid side chains were protected using 2-chlorocarbobenzyloxyl for (Lys) and tosyl for (N^{α}MeArg). Individual amino acids were coupled sequentially to the resin as their symmetrical anhydrides or their hydroxybenzotriazole activated esters. After the incorporation of each amino acid and resin cleavage, the peptide resin was N^{α}-t-Boc deprotected with TFA in dichloromethane (DCM) (1:1) and, subsequently neutralized with 10% diisopropylethylamine (DIEA) in DCM. Simultaneous side chain deprotection and cleavage from the resin were accomplished by treatment with 90% anhydrous HF, 10% anisole and 3-methylindole (100 mg) at 0° for 60 min. The peptide resin was then washed with anhydrous diethyl ether, and the peptide was extracted with 10% aqueous acetic acid, concentrated under reduced pressure, diluted with H_2O, and lyophilized. The crude peptide was purified by preparative reversed-phase HPLC (Vydac C18, 218TP1022; 2.2 × 25.0 cm, 15 mL/min) with a linear gradient of 0.1% TFA in H_2O to 0.1% TFA in acetonitrile, concentrated and lyophilized. The purity and structural integrity of the peptide were assessed by analytical HPLC, capillary electrophoresis, proton nuclear magnetic resonance, amino acid analysis, and fast atom bombardment mass spectrometry.

Tissue preparation. The method of Mazella *et al.* [18] was used with some modifications. Briefly, seven frozen newborn mouse brains were thawed and the cerebella were discarded. Membranes were homogenized with a Polytron (Beckman Instruments Co., Westbury, NY, U.S.A.) at setting No. 6 for 20 sec in 20 vol. of ice-cold 5 nM TRIZMA buffer, pH 7.5 (buffer A). The homogenates were centrifuged at 110,000 *g* for 15 min at 4° in a Beckman Ultracentrifuge, XL-90 (SW41TI rotor). The resulting pellets were resuspended twice in the same volume of buffer and recentrifuged. The final pellets were suspended in buffer B (50 mM TRIZMA, pH 7.4, 0.1% BSA, 0.2 mM bacitracin, 1 mM 1,10-phenanthroline, 0.3 mM PMSF, 1 mM EDTA), at 1 g wet weight/mL and used for assay. The remaining membranes were distributed in 200-μL aliquots into microcentrifuge tubes and stored at −80° for no more than 1 month.

Cell culture. HT-29 cells were grown in and subcultured in McCoy's 5A medium (90%) containing 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) without antibiotics. HT-29 cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°. The medium was changed every 2–3 days and at least 18 hr before harvesting the cells. Confluent cultures were harvested by replacement of medium with cold phosphate-buffered saline containing 0.02% EDTA followed by centrifugation at 1000 *g* for 2 min at 4°. The pellets obtained were suspended in an appropriate volume of buffer and homogenized for 5 sec with a Polytron at setting No. 5 for use in the assay.

Ligand binding assay. To label the NT receptor, [^{125}I]Tyr^3-NT (0.1 nM, final concentration) or [^{3}H]-NT (0.2 nM) was used. The optimal binding condition for our assay was established from a modification of the protocol by Kitabgi *et al.* [7]. Briefly, 50 μL of [^{3}H]ligand, 50 μL of either drug or buffer, and 400 μL of brain membranes or HT-29 cell membranes (all diluted with ice-cold buffer B) were added to polypropylene microtubes (96-well microplate

format; total vol., 500 μL). The incubation proceeded for 20 min at 25° and was terminated by rapid filtration through Whatman GF/B glass fiber filters (pre-soaked for about 1 hr in 0.5% polyethylenimine) using a Brandel MR48 cell harvester. Radioactivity remaining on the filters was counted either with an LKB gamma counter, with an efficiency of 80% ([^{125}I]Tyr^3-NT) or with a Beckman LS 6800 liquid scintillation counter following the addition of liquid scintillation fluid, with 50% efficiency ([^{3}H]NT). Specific binding, defined as total binding minus binding in the presence of 1 μM NT, was about 96% for [^{125}I]Tyr^3-NT and about 90% for [^{3}H]NT.

Saturation binding curves were determined for [^{125}I]Tyr^3-NT using newborn mouse brain membranes, and for [^{3}H]NT using HT-29 cell membranes. Competition studies were conducted using these membranes as well as adult rat cerebellum membranes. Data from saturation plots were analyzed by Scatchard [19] transformation of binding data with the LIGAND program [20] and the Lundon ReceptorFit Saturation program (Lundon Software Inc., Chagrin Falls, OH) [21] to determine the binding density and the equilibrium dissociation constant. Statistical significance between one- and two-site binding models was also determined by using the *F* test analysis of both the ligand and Lundon software programs.

Intracellular calcium measurements. Changes in $[Ca^{2+}]_i$ were determined with the Ca^{2+}-sensitive dye fura-2/AM and a Proton Technology International (South Brunswick, NJ, U.S.A.) Photoscan-2 dual excitation spectrofluorometer. The protocol followed was essentially that of Turner *et al.* [12]. Briefly, a suspension of HT-29 cells pelleted by centrifugation at 1000 *g* was washed in a balanced salt solution consisting of 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 15 mM glucose, 10 mM Tris-HEPES, 0.1% BSA, 1.0 mM $MgCl_2$ and 0.2 mM $CaCl_2$ at pH 7.4 (Solution 1). The pellet obtained was suspended in a different balanced salt solution containing 1.0 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 0.1% BSA (Solution 2). To load cells, cell suspensions (3×10^6 cells/mL) were incubated with 2 μM fura-2/AM for 40 min at 37° in a shaking incubator. After washing and resuspension in Solution 2 at the original density, the cell suspensions were further incubated for 40 min to allow for deesterification. After centrifugation, cells were washed twice in Solution 2. The final pellet was resuspended in a balanced salt solution containing 1.0 nM $MgCl_2$, 1.0 mM $CaCl_2$ and 0.1% BSA. Samples were stored temporarily in a water bath maintained at 37°, which was placed on top of a stir plate. For $[Ca^{2+}]_i$ measurements, 1-mL aliquots of fura-2/AM loaded cells were transferred to quartz cuvettes, and the latter were placed in the spectrophotometer to establish a stable baseline. Measurement of autofluorescence was conducted in an equal aliquot of cell suspension taken through the same steps but without dye added. Compounds were then added to the cell suspension. A magnetic stir bar was always placed in each cuvette to allow proper mixing of drugs and to prevent cells from settling to the bottom.

The $[Ca^{2+}]_i$ mobilized was determined using a PTI Deltascan II spectrofluorometer by measuring the ratio of fluorescence changes at excitation wavelengths of 340 and 380 nm (emission at 505–510 nm). This was calibrated using the equation developed by Grynkiewicz *et al.* [22]: $[Ca^{2+}]_i = K_D \cdot Sf_2/Sb_2(R\text{-}R_{min}/R_{max}\text{-}R)$, where $K_D = 224$ nM, Sf_2 is the signal of the free fura-2/AM at 380 nm, SB_2 is the signal of the bound fura-2/AM at 380 nm and R is the ratio of the fluorescence intensities. Values for R_{min} were obtained from the fluorescence ratio with a final concentration of 500 nM EGTA (pH balanced to 7.4 with TRIZMA base) added to the cuvette containing a 2-mL sample of cells (calcium-free fura-2/AM sample), while R_{max} was determined in a different sample of cells after addition of 0.1% Triton-X (calcium-saturated fura-2/AM sample). The 340 and 380 nm excitation wavelengths for a sample of unloaded cells were determined to correct for tissue or background autofluorescence. The percent of maximal calcium mobilized for each agent was measured and the EC_{50} values were determined.

The effects of SR 48692, a non-peptide antagonist [23], on the NT1-induced mobilization of $[Ca^{2+}]_i$ were evaluated. The basal level of Ca^{2+} was determined, and the maximal effect of $[Ca^{2+}]_i$ produced by NT1 was also measured. Fura-2/AM-loaded cells were then pretreated with various concentrations of SR 48692 followed by 1 μM NT1, and then the amount of intracellular calcium mobilized was measured. Consequently, the IC_{50} (concentration of SR 48692 required to inhibit 50% of the maximal $[Ca^{2+}]_i$ mobilized by 1 μM NT1) was estimated.

Cyclic GMP assay. The levels of cyclic GMP in rat cerebellum were measured essentially according to the method of Bredt and Snyder [24]. Male Long-Evans rats weighing between 130 and 150 g were killed by decapitation; the cerebellum of each rat was dissected out and slices of 200 μM square were cut using a McIlwain tissue chopper. The slices were dispersed in Krebs Henseleit buffer (118 mM NaCl; 4.7 mM KCl; 2 mM $CaCl_2$; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 25 mM $NaHCO_3$ and 11 mM glucose) also containing 1 mM EDTA, 0.2 mM bacitracin, 1 mM 1,10-phenanthroline and 0.3 mM PMSF to prevent breakdown of the NT. Cerebellar slices from several rats were pooled and incubated for 1 hr in buffer continuously gassed with 95% oxygen/5% carbon dioxide at 37°. After a 1-hr preincubation, slices were distributed in incubation wells such that each well contained slices equivalent to 0.40 mg of original tissue. Preliminary studies indicated that exposure of slices to NT for 30 sec gave optimal stimulation of cyclic GMP content. Therefore, test compounds and NT were added 30 sec before the assay was terminated by the addition of 250 μL of absolute ethanol. Following lyophilization, the cyclic GMP content was determined by scintillation proximity assay (Amersham, Arlington Heights, IL). Results are reported as mean cyclic GMP levels per mg of protein and expressed as a percentage of control levels (±SEM).

Protein assays. In all these studies, protein concentration was determined by the Bio-Rad assay [25].

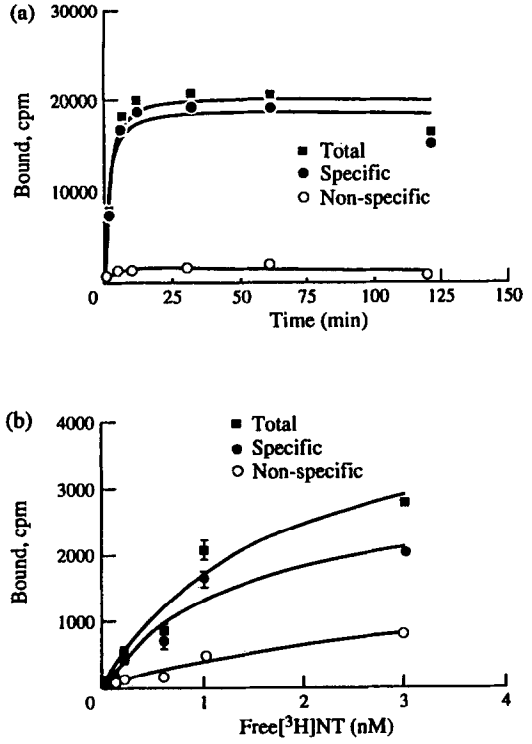

Fig. 1. Binding to NT receptors in membrane preparations. (A) Time course to determine optimal incubation time for NT receptors. This representative plot is for [^{125}I]Tyr^3-NT binding in newborn mouse brain membranes. (B) A representative saturation study of [^{3}H]NT binding to HT-29 cell membranes. Eight different concentrations of the radioligand were used. Values are means ± SEM of three experiments performed in triplicate. Specific binding was calculated as the difference between total binding and non-specific binding (defined by the presence of 1 μM NT)

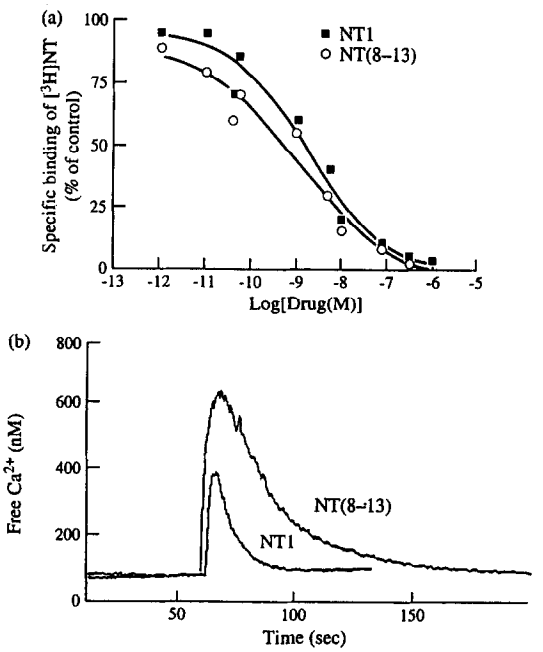

Fig. 2. (A) Representative curves for inhibition of [^{3}H]NT specific binding to HT-29 cell membranes by NT1 and NT(8-13). Each point is the mean of triplicate observations and the data for each competition curve are from one of three independent experiments. (B) Representative curves for intracellular calcium stimulated by 100 nM NT1 and by 50 nM NT(8-13). $[Ca^{2+}]_i$ levels were determined in HT-29 cells loaded with fura-2/AM dye and resuspended in a balanced salt solution containing 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ and 0.1% BSA. The $[Ca^{2+}]_i$ mobilized was measured by a PTI spectrophotofluorometer Deltascan program, and calcium concentration was determined according to Grynkiewicz *et al.* [22] using a K_D of 224 nM. The percent of maximal calcium mobilized for each agent was measured to determine the EC_{50} values. The basal level of $[Ca^{2+}]_i$ was 85 ± 5 nM.

RESULTS

Saturation binding of NT receptors in membrane preparations. Preliminary equilibrium studies indicated that steady state was achieved for specific binding of [^{125}I]Tyr^3-NT to newborn mouse brain membranes within 10 min (Fig. 1A). On the basis of this study, the total incubation time chosen for all studies was 20 min. Using newborn mouse brains and [^{125}I]Tyr^3-NT as the radioligand, the data obtained showed a saturable, single high-affinity binding site (Hill coefficient of 0.92) with a B_{max} of 87.5 ± 5.5 fmol/mg protein and a K_D of 0.39 ± 0.07 nM (N = 3) (Table 1). Similarly, a single high-affinity site was obtained in HT-29 cell membranes using [^{3}H]NT with a B_{max} of 130.2 ± 40 fmol/mg protein and a K_D of 0.47 ± 0.10 nM (see Table 1) with a Hill coefficient of 0.99. Figure 1B is a representative saturation study of [^{3}H]NT binding to HT-29 cell membranes. Eight different concentrations of the radioligand were used. Levocabastine was inactive at these sites ($K_I > 10\ \mu$M), thus indicating only the presence of physiologically relevant high-affinity NT receptors [9].

Inhibition constants for neurotensin receptor by neurotensin analogs. Figure 2A shows representative curves of NT1 and NT displacements of [^{3}H]NT

Table 1. Equilibrium binding parameters of specific binding for NT receptors

	K_D (nM)	n_H*	B_{max} (fmol/mg protein)
Newborn mouse	0.39 ± 0.07	0.92	87.5 ± 5.5
HT-29 cells	0.47 ± 0.10	0.99	130.2 ± 40

Saturation binding studies were carried out in newborn mouse brain membranes with [^{125}I]Tyr^3-NT and in HT-29 cell membranes with [^{3}H]NT. Each curve was made up of eight different concentrations of the radioligand. Non-specific binding was determined with 1 μM NT. Results are means ± SEM of three experiments, and each data point was performed in triplicate.

* n_H = Hill coefficient.

Table 2. Potencies of NT and its analogs to inhibit the binding of [^{3}H]NT to HT-29 cell and newborn mouse brain membranes and to mobilize intracellular calcium in HT-29 cell membranes

Compounds	HT-29 cells		Mouse brain		HT-29 cells
	K_I (nM)	n_H*	K_I (nM)	n_H	EC_{50} (nM)
NT(8-13)	0.09 ± 0.02	0.60	0.22 ± 0.06	0.70	4.21 ± 0.41
NT	0.15 ± 0.04	1.0	0.17 ± 0.02	0.90	11.8 ± 1.8
NT1	1.60 ± 0.10	0.70	0.64 ± 0.02	0.90	112.1 ± 1.1
Xenopsin	2.4 ± 0.50	0.91	0.12 ± 0.06	0.92	79.4 ± 14.4
Neuromedin N	2.8 ± 0.67	0.99	0.63 ± 0.16	1.1	79.0 ± 10.8
NT(9-13)	5.8 ± 1.6	1.20	4.1 ± 1.9	1.1	2089 ± 112
SR 48692	11.3 ± 1.7	0.78	3.9 ± 0.13	0.85	—
Kinetensin	1396 ± 108	0.71	149.6 ± 14.8	0.58	>10000
(D-Trp^{11})-NT	1422 ± 146	1.1	187.5 ± 17.5	0.53	1244 ± 108
Levocabastine	>10,000		>10,000		>10,000
NT(1-8)	>10,000		>10,000		>10,000

For binding studies, tissues were incubated for 20 min with 0.2 nM [^{3}H]NT for determining specific binding to NT receptors. K_I values represent the geometric mean ± SEM from at least three independent experiments using eight concentrations of various ligands. Mobilization of free intracellular calcium was measured by a PTI spectrophotofluorometer Deltascan program for fluorescence ratio determinations using fura-2/AM. $[Ca^{2+}]_i$ was determined according to Grynkiewicz *et al.* [22] using a K_D of 224 nM, and the percent of maximal calcium mobilized for each agent was measured to determine the EC_{50} values. Values represent the geometric mean ± SEM of at least three experiments. The basal level of $[Ca^{2+}]_i$ was about 85 nM.

* n_H = Hill coefficient.

binding to HT-29 cells. Results presented in Table 2 are geometric means, which more appropriately describe the responses of such agonists. NT(8-13) was the most potent in HT-29 cells with a K_I of 0.09 nM, although it was slightly less potent (K_I = 0.22 nM) than NT (K_I = 0.17 nM) and xenopsin (K_I = 0.12 nM) in the mouse membrane preparation. This observation is in agreement with other reports [9, 15, 25, 26], which show that the carboxy-hexapeptide terminal end of NT(8-13) is the biologically active portion of the peptide. Furthermore, NT1, which is an NT analog with increased metabolic stability [15], exhibited binding affinities of 1.6 and 0.64 nM for HT-29 cell and newborn mouse brain membranes, respectively (Table 2). Other NT-related analogs [xenopsin, neuromedin N, NT(9-13), kinetensin and (D-Trp^{11})-NT] had lower affinities for [^{3}H]NT binding in both membrane preparations. As expected, levocabastine and NT(1-8) were inactive in this assay. All the compounds tested exhibited Hill coefficients close to unity except for kinetensin. However, statistical analysis indicated that all the data fit a one-site model better than a two-site model.

Mobilization of intracellular calcium. This study was designed to evaluate the agonist properties of NT and related analogs by their abilities to mobilize $[Ca^{2+}]_i$ in HT-29 cells. Compounds were tested at 1, 5, 10, 50, 100, 500, 1000 and 10,000 nM. Figure 2B represents the time-dependent effects of NT1 and NT(8-13) on the mobilization of $[Ca^{2+}]_i$. The results indicate that NT(8-13) was slightly more potent than NT (4.2 vs 11.8 nM, respectively, Table 2). In contrast, NT(1-8) was inactive in this test, which reflects its inactivity in binding to the NT receptors (Table 2). NT1, which is the stable analog of NT(8-13), exhibited an EC_{50} value of 112 nM in this

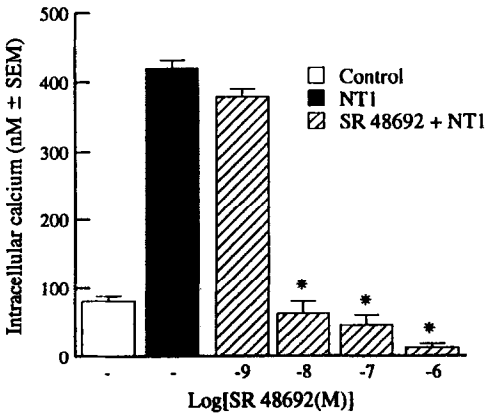

Fig. 3. Effects of SR 48692 on the NT1-induced mobilization of intracellular calcium in HT-29 cells. Cells were preincubated with various concentrations of SR 48692, and the amount of $[Ca^{2+}]_i$ mobilized by 1 μM NT1 (added to all groups except for control) was determined as described in Materials and Methods. Values of the basal and maximal $[Ca^{2+}]_i$ mobilized by NT1 were (mean ± SEM) 85 ± 5 and 425 ± 55 nM, respectively. The IC_{50} (concentration of SR 48692 required to inhibit 50% of the maximal $[Ca^{2+}]_i$ mobilized by 1 μM NT1) was 44 nM. Each value is the mean ± SEM of three determinations. Key: * $P < 0.05$ vs NT1 (Student's *t*-test).

test (Table 2). The maximal level of $[Ca^{2+}]_i$ mobilized by NT1 was 425 ± 55 nM. Other NT-related analogs [xenopsin, neuromedin N, NT(9-13), kinetensin and (D-Trp^{11})-NT that had lower affinities for [^{3}H]NT binding in both membrane preparations also had lower potencies for mobilizing intracellular calcium

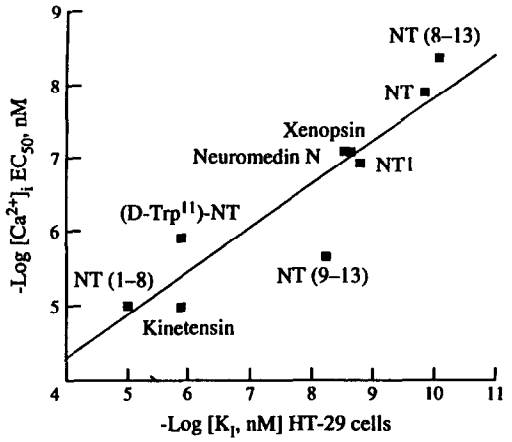

Fig. 4. Correlation of binding affinities to intracellular calcium mobilized in HT-29 cell membranes by various reference agents. Values were derived from Table 2. The correlation coefficient (r) was 0.855.

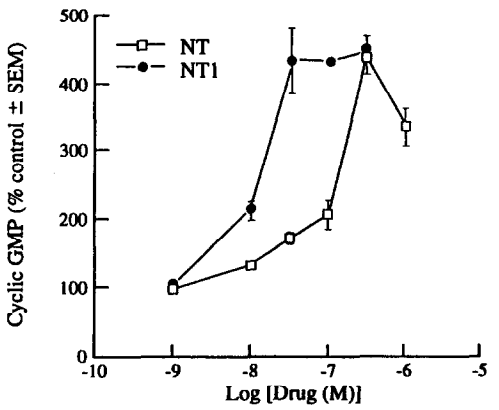

Fig. 6. Effects of NT and the metabolically stable NT(8-13) analog, NT1, on the stimulation of intracellular cyclic GMP synthesis in rat cerebellar slices. Each point is the mean ± SEM of 4–16 values and is expressed as a percentage of the basal cyclic GMP value, which averaged 2.18 ± 0.12 fmol/mg protein. Analysis of concentration–response curves revealed EC_{200} values of 90 and 9 nM for NT and NT1, respectively.

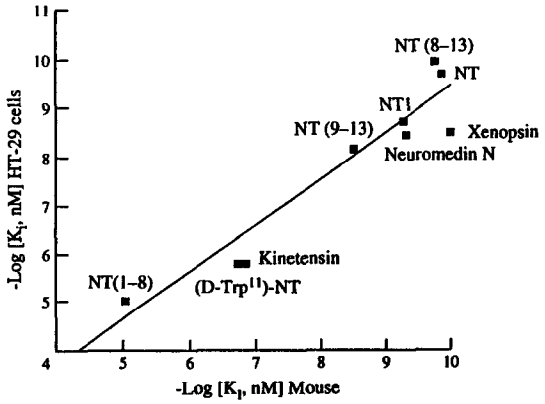

Fig. 5. Correlation of binding data for NT receptors in HT-29 cell membranes to that in newborn mouse brain membrane. K_I values were derived from data presented in Table 2. The points were fitted by linear regression analysis. The correlation coefficient (r) was 0.924.

(Table 2). As shown in Fig. 3, SR 48692, which is a non-peptide NT antagonist, concentration-dependently inhibited NT1 (1 μM)-induced Ca^{2+} mobilization with an IC_{50} of 43.5 nM. In a similar control study, the effect of NT1 was not blocked by atropine (data not shown), which is a muscarinic antagonist for the cholinergic receptor, indicating that the effect of NT1 in mobilizing intracellular Ca^{2+} was specific to NT receptors.

Binding affinities of NT analogs to HT-29 cell membranes were positively correlated with their potency for mobilizing $[Ca^{2+}]_i$ in HT-29 cells (r = 0.855, Fig. 4) and were also positively correlated with binding affinities found in newborn mouse brain membranes (r = 0.924, Fig. 5).

Intracellular cyclic GMP accumulation in rat cerebellar slices. An additional study comparing the potency of NT1 with that of NT was conducted by examining the effect on stimulating intracellular cyclic GMP formation in rat cerebellar slices. Both NT and NT1 maximally stimulated cyclic GMP in rat cerebellar slices in a concentration-dependent manner with EC_{200} (concentration causing a 200% increase in cyclic GMP) values of 90 and 9 nM, respectively (Fig. 6). Interestingly, both NT (K_I = 1.93 nM) and NT1 (K_I = 2.83 nM) competed for [^{125}I]Tyr^3-NT binding to adult rat cerebellum membranes with about equivalent affinity (Table 3). However, in contrast to newborn mouse brain and HT-29 cells, the rat cerebellum also contained the levocabastine-sensitive NT sites, as evidenced by the affinity of levocabastine (K_I = 112 nM) (Table 3). NT(1-8) was inactive in binding (K_I > 10,000 nM) and also did not stimulate cerebellar cyclic GMP accumulation (data not shown).

DISCUSSION

Data obtained from both the HT-29 cell membranes and newborn mouse brain membranes showed the existence of a saturable, single high-affinity NT binding site and indicate that the low-affinity levocabastine-sensitive site was not present. This is in agreement with previous reports using newborn mouse brain preparations [6]. The lower binding affinity for NT1 observed in rat cerebellum as compared with its affinity in newborn mouse brain or HT-29 cell membranes can be attributed to the additional binding of NT1 to the lower affinity levocabastine-sensitive sites present in adult rat membrane preparations.

Although there was an excellent correlation for binding affinities of NT analogs to HT-29 cell membranes and newborn mouse brain membranes,

Table 3. Potencies of NT and its analogs to inhibit [^{125}I]-Tyr^3-NT binding to rat cerebellum membranes

Compounds	K_I (nM)
NT	1.93 ± 1.0
NT1	2.83 ± 0.8
Levocabastine	112 ± 119
NT(1-8)	>10,000

Rat cerebellar tissues were incubated for 20 min with 0.1 nM [^{125}I]Tyr^3-NT for determining specific binding to NT receptors. Non-specific binding was determined with 1 μM NT. K_I values are the means ± SEM from at least three independent experiments using eight concentrations of various ligands.

certain compounds (xenopsin, kinetensin, and others) exhibited weaker affinities in HT-29 cells than in mouse brain. A recent report indicated that UK-73,093, a non-peptide NT antagonist, displays an affinity for NT receptors of 4–6 μM in brain membranes from cows and mice but was inactive up to 100 μM in membranes from human frontal cortex [27]. In contrast, the non-peptide antagonist SR 48692 was reported to have high affinity for membranes from both the newborn mouse brain (K_I = 13.7 nM) and HT-29 cells (30.0 nM) [23], as was confirmed in the present study (Table 2). These differences in inhibition constants may be due to different species being studied or perhaps are indicative of the existence of NT receptor subtypes, as suggested by others [28, 29]. Although further studies are needed to clarify these observations, in the present study some of the NT analogs, such as xenopsin and kinetensin, displaced [^{3}H]NT binding with Hill slopes significantly less than 1.0 in partial support of the hypothesis that there may be NT receptor subtypes in the newborn mouse brain and HT-29 cell membranes.

The coupling of NT receptors to second messenger systems has been studied primarily in HT-29 and N1E-115 cells. In HT-29 cells, NT-induced receptor activation has been shown to increase production of inositol phosphates and the mobilization of $[Ca^{2+}]_i$ [12]. In addition, NT-induced changes in cyclic AMP [13] and cyclic GMP [14] have been reported in N1E-115 cells. NT also has been shown to increase cyclic GMP levels in rat cerebellar slices [23]. In this study, we examined the functional activity of NT1 together with NT and related analogs on both of these parameters. The results indicate that there was an excellent correlation between the binding of NT1 and related peptides to HT-29 cellular membranes and their potency in mobilizing $[Ca^{2+}]_i$ in the same cells. The maximum response for both NT1 and NT (data not included) did plateau at the same level in support of NT1 being a full agonist at NT receptors in this preparation.

In rat cerebellar slices, NT1, like NT, induced a concentration-dependent increase in cyclic GMP levels, consistent with a receptor-mediated agonist effect. Both NT1 and NT exhibited full agonist effects in stimulation of intracellular cyclic GMP synthesis in rat cerebellar slices as they did in mobilizing intracellular calcium in HT-29 cells. However, in contrast to the findings in HT-29 cells, NT1 was about 10-fold more potent than NT in this respect. The reasons for this discrepancy are not apparent. One possible explanation is that despite the presence of peptidase inhibitors, NT, in contrast to NT1, is broken down more rapidly in the slice preparation as compared with the cell preparation, thus accounting for the difference in potency in the two assays. This would agree with published findings that NT1 is more resistant to peptidase degradation than NT [15]. It is unlikely that the presence of the levocabastine-sensitive NT binding site contributed to the difference observed since this site has been reported to be an acceptor or recognition site without any physiological role [4, 6]. This difference could also stem from differences in receptor sensitivity and/or receptor–effector coupling in human cells versus rat cerebellar slices.

In general, EC_{50} values for NT1, NT and related analogs calculated from concentration–response curves of Ca^{2+} mobilization and stimulation of cyclic GMP were greater than the corresponding binding affinities. NT1 was about 27- and 10-fold less effective than NT(8-13) and NT, respectively. These discrepancies are most easily explained by the different experimental conditions used in each experiment. For example, binding data were generated after equilibrium was reached at 25° in the presence of peptidase inhibitors. By contrast, intracellular Ca^{2+} and cyclic GMP were relatively transient effects that were measured within 30 sec at 37° (without peptidase inhibitors) after addition of NT and related analogs to HT-29 cells or rat cerebellar slices. This very short time was not sufficient to reach binding equilibrium. Differences in absolute values of binding affinities and EC_{50} values could also be a reflection of the different membrane preparations used. For example, functional assays were conducted in intact systems possibly with intracellular elements such as GTP and other G-proteins present and may be affecting the estimated parameters differently compared with their effects on membrane preparations used in radioligand binding studies.

Previous findings have shown that NT1 administered systemically reverses methamphetamine-induced hypermotility in rats, does not induce catalepsy in rats, and inhibits a conditioned avoidance response in rats [15]. These results are similar to the effects of centrally administered NT [31, 31]. The present data showing that NT1 bound to both rodent and human NT receptors and was a functionally active full agonist in two *in vitro* functional assays, support the use of NT1 in studying the physiological and pharmacological importance of NT *in vivo*.

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