Journal of Neurochemistry
Raven Press, New York
© 1987 International Society for Neurochemistry

Rapid Degradation of Neurotensin by Intact Murine Neuroblastoma Cells (Clone N1E-115)

Judith A. Gilbert, Peter D. Hanson, *Daniel J. McCormick, and Elliott Richelson

*Departments of Psychiatry and Pharmacology, *Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota, U.S.A.*

Abstract: Murine neuroblastoma clone N1E-115, which possesses receptors for neurotensin mediating the formation of intracellular cyclic GMP and the stimulation of inositol phospholipid hydrolysis, exhibited only partial desensitization to neurotensin. This result led to the observation that neurotensin was very rapidly degraded by intact N1E-115 cells. In experiments measuring the time course of [^{3}H]neurotensin degradation, a minimum of six major tritiated products were found, with the breakdown peptides formed and the degree of proteolysis of [^{3}H]neurotensin being dependent upon the length of incubation and the concentration of cells. Clone N1E-115 degraded [^{3}H]-neurotensin in an apparently sequential fashion; the primary initial cleavage of intact neurotensin was at the peptide bond between residues Arg^8 and Arg^9. Initial degradation peptides from the active carboxyl-terminal portion of neurotensin were more rapidly degraded, after formation, than were the peptides from the inactive amino-terminal half of neurotensin. The final two degradation products found were tyrosine, from the carboxyl-terminal portion of neurotensin, and an as yet unidentified peptide from the amino-terminal half of neurotensin. [^{3}H]Neurotensin(8–13) was more rapidly hydrolyzed under identical conditions than was [^{3}H]neurotensin itself. A combination of the protease inhibitors 1,10-phenanthroline and Z-Pro-Prolinal was able to inhibit almost completely the degradation of neurotensin by clone N1E-115. **Key Words:** Neurotensin degradation—Neuroblastoma cells—Clone N1E-115. **Gilbert J. A. et al.** Rapid degradation of neurotensin by intact murine neuroblastoma cells (clone N1E-115). *J. Neurochem.* **49,** 1845–1852 (1987).

The tridecapeptide neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu; NT), first isolated and sequenced from bovine hypothalamus (Carraway and Leeman, 1973 and 1975), is known to have widespread distribution in the central nervous system and gastrointestinal tract (Carraway and Leeman, 1976). Specific binding sites for NT are distributed throughout the brain of mammals (see review of Nemeroff, et al., 1983*b*). Furthermore, evidence exists that binding sites in N1E-115 murine neuroblastoma cells and in rat brain are in fact functional receptors which mediate the formation of intracellular cyclic GMP and the stimulation of inositol phospholipid hydrolysis (Gilbert and Richelson, 1984; Gilbert, et al., 1984; Goedert et al., 1984; Snider et al., 1986). However, the ability of NT receptors to effect cyclic AMP formation is still in question. Reports exist that NT has no effect on basal levels of cyclic AMP production in rat brain slices (Quik et al., 1978; Goedert et al., 1984), hypothalamic nuclei (Redgate et al., 1986), pituitary cells (Memo et al., 1986), and striatal and nucleus accumbens homogenates (Nemeroff et al., 1983*a*). Furthermore, NT has been reported to have no effect on other receptor-stimulated cyclic AMP levels in rat brain slices (Goedert et al., 1984), rat striatal and nucleus accumbens homogenates (Nemeroff et al., 1983*a*), or murine neuroblastoma clone N1E-115 (Gilbert et al., 1986). These are in contrast to a report that NT inhibits other receptor-stimulated cyclic AMP levels in clone N1E-115 (Bozou et al., 1986).

The existence of functional receptors in neural tissue is supportive of the hypothesis that NT acts as a neurotransmitter (see review of Bissette et al., 1978). Another criterion considered important to establishing a neurotransmitter role for a molecule is that a mechanism exists for its rapid inactivation near the receptor site.

Received June 24, 1987; accepted July 7, 1987.
Address correspondence and reprint requests to Dr. E. Richelson at Guggenheim 7, Mayo Foundation, Rochester, MN 55905, U.S.A.

Abbreviations used: ACN, acetonitrile; BOC, *t*-butyloxycarbonyl; NT, neurotensin; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid.

In initial experiments designed to characterize the desensitization of the functional NT receptors on neuroblastoma clone N1E-115 following lengthy exposures to NT, we found that at best only partial desensitization could be achieved. This led us to the observation and study of the degradation of NT by intact N1E-115 cells. We will present evidence here that intact N1E-115 cells rapidly degraded radiolabeled NT in an apparently sequential fashion, with the initial degradation peptides from the active carboxyl-terminal portion of NT being more rapidly degraded, in turn, than the peptides formed from the inactive amino-terminal half of NT. These data have previously been presented (Gilbert and Richelson, 1986).

MATERIALS AND METHODS

Cell culture

Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco-Vogt's modification of Eagle's medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) without antibiotics and supplemented with 10% fetal bovine serum (Grand Island Biological Co.).

Clone N1E-115 (passage number <22) was grown in 20 ml of medium in 75-cm^2 Corning flasks (Corning Glass Works, Corning, NY, U.S.A.) in a humidified atmosphere of 10% CO_2/90% air at 37°C. Flasks were routinely inoculated with 0.5 to 1.0×10^6 cells which were fed daily beginning day 6 after subculture by removal of 10 ml of growth medium and replacement with 10 ml of fresh medium. Cells were harvested during the stationary phase of growth, 10–22 days after subculture.

Degradation of [^{3}H]NT

N1E-115 cells were harvested for use by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37°C in 10 ml of modified Puck's D_1 solution without antibiotics and phenol red (Honegger and Richelson, 1976), disruption of the layer by agitation of the flask, and collection of the cells by centrifugation at $300 \times g$ for 1 min at 4°C in a CRU-5000 centrifuge (Damon/IEC Division, Needham Heights, MA, U.S.A.). The cellular pellet was resuspended in 10 ml of phosphate-buffered saline solution (solution I) consisting of 110 m*M* NaCl, 5.3 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 2.0 m*M* Na_2HPO_4, 25 m*M* glucose, and 70 m*M* sucrose (pH 7.35; 340 mOsmol), and the washed cells were collected by centrifugation. The cellular pellet was resuspended in several milliliters of solution I, and 20 μl of the suspension was removed for enumeration of cells (Coulter Electronics, Hialeah, FL, U.S.A.). The solution was then adjusted, in most cases, to a concentration of 1×10^6 cells/ml.

Typically, a final concentration of 0.1 μM of [*tyrosyl*^{3,11}-^{3}H]NT (New England Nuclear, Boston, MA, U.S.A.; specific activity = 41–85 Ci/mmol; 4–8 μCi incubated per 1×10^6 N1E-115 cells) was incubated with 1×10^6 N1E-115 cells/ml for varying lengths of time in a 20-ml plastic scintillation vial (Research Products International, Mount Prospect, IL, U.S.A.) at 37°C and 60 oscillations/min (GCA/Precision Scientific, Chicago, IL, U.S.A.). To stop the reaction, a 300-μl aliquot of the cellular suspension was removed, placed into a 1-ml micro tube (Walter Sarstedt, Inc., Princeton, NJ, U.S.A.), and centrifuged at $8,700 \times g$ in a Microfuge B (Beckman Instruments, Palo Alto, CA, U.S.A.) for 20 sec. The supernatant was then poured into a 2-ml micro tube containing 15 μl of 1.0 *M* HCl and placed on ice. Measurement of the radioactivity remaining in each pellet following centrifugation of samples from a time course (two experiments) indicated that at most 7% of the total radioactivity used in the incubation was taken up by the cells or trapped during centrifugation. The purity of the [^{3}H]NT employed for degradation was a minimum of 90% with no radiolabeled impurities eluting during HPLC of standard [^{3}H]NT in the majority of experiments. In some degradation studies, [*tyrosyl*^{11}-^{3}H]NT(8–13) (New England Nuclear; specific activity = 11 Ci/mmol; purity = 98%; 1 μCi incubated per 1×10^6 N1E-115 cells) was employed under identical experimental conditions.

HPLC

For all of the degradation studies reported here, 20 μl of experimental supernatant or control [^{3}H]NT was analyzed by HPLC on a Hewlett-Packard 1090 integrated liquid chromatograph (Palo Alto, CA, U.S.A.) composed of a variable-volume auto-injector, autosampler, diode-array detector, DPU multichannel integrator, HP-85 system controller, HP 2225A Thinkjet printer, and HP 9153A disc drive. A μ-Bondapak C_{18} column (0.39×30 cm; Waters, Milford, MA, U.S.A.) with a guard column containing Bondapak C_{18}/Corasil (Waters) was employed at 35°C with a buffer flow rate of 1 ml/min and a series of linear gradients of acetonitrile (ACN; Burdick and Jackson, Muskegon, MI, U.S.A.) and 10 m*M* trifluoroacetic acid (TFA; J. T. Baker Chemical Company, Phillipsburg, NJ, U.S.A.). Due to the short lifetime of the Waters μ-Bondapak C_{18} column under the elution conditions employed, a total of three columns were used during the course of the experiments described here. The first column was employed with a 28-min series of linear gradients from 10% ACN/90% 10 m*M* TFA to 40% ACN/60% 10 m*M* TFA. To achieve a comparable separation of standard NT peptides, the second and third columns were used with a 26-min series of linear gradients from 10% ACN/90% 10 m*M* TFA to 50% ACN/50% 10 m*M* TFA. The first and second columns were used for analysis of all of the experiments except the protease inhibitor studies reported here, for which the third column was employed. During each analysis, 30-sec fractions were collected with either a Cygnet fraction collector (Isco, Inc., Lincoln, NE, U.S.A.) or a FOXY fraction collector (Isco, Inc.).

Following HPLC of each experimental sample, the fractions were transferred to plastic scintillation vials with 7 ml of Safety-Solve Counting Cocktail (Research Products International), and the radioactivity was measured in an Isocap/300 liquid scintillation counter (Searle, Des Plaines, IL, U.S.A.). Ninety to one hundred percent of the radioactivity in the applied sample was routinely recovered following HPLC.

Solid-phase peptide syntheses

The syntheses of the standard peptides NT(2–9), NT(2–10), NT(1–7), and NT(9–11) were carried out in an Applied Biosystems 430A peptide synthesizer employing the coupling schedules provided by the manufacturer. Peptides were synthesized by solid-phase methods described by Erickson and Merrifield (1976) as modified by McCormick and Atassi (1985). A detailed description of the methodol-

ogy has been published by Stewart and Young (1984). Briefly, each peptide was synthesized with *t*-butyloxycarbonyl (BOC) L-amino acids on phenylacetamidomethyl (PAM) resins (copolystyrene/1% divinylbenzene). The first C-terminal BOC-amino acid was coupled to the PAM resins by the condensation of BOC-aminoacyl-4-(oxymethyl)phenylacetic acids to aminomethyl polystyrene resin using dicyclohexylcarbodiimide in dichloromethane (Mitchell et al., 1978). The side-chain protecting groups γ-benzyl, tosyl, *O*-2-bromobenzyloxy carbonyl and ϵ-2-chlorobenzyloxy carbonyl were used for the BOC-amino acids glutamate, arginine, tyrosine, and lysine, respectively. The BOC-amino acids asparagine, isoleucine, leucine, and proline were used without side-chain protection. The coupling of each amino acid to the resin (0.5 mmol) employed their corresponding BOC-aminoacyl anhydrides (1 mmol). The amino acids asparagine and arginine were coupled as their *N*-hydroxybenzotriazole esters in dimethylformamide. The ninhydrin method of Kaiser et al. (1970) was used to check the completion of the coupling for each BOC-amino acid.

Cleavage of the finished peptide and removal of all side-chain protecting groups was done by treating the peptide resin with 88% liquid hydrofluoric acid containing 10% anisole and 2% dimethylsulfide at 0°C for 60 min. The crude peptide was extracted with anhydrous TFA. The TFA-extracted peptide was reprecipitated from cold diethyl ether three times, dissolved in aqueous 5% acetic acid and lyophilized overnight.

Purification and characterization of synthetic peptides

Crude peptide was purified by gel filtration on Sephadex G-15 resin (Pharmacia, Piscataway, NJ, U.S.A.; 1.6 × 88 cm) in 0.1 *M* pyridine-acetate, pH 4.80. NT(2–9) and NT(2–10) were each further purified by HPLC on a semipreparative μ-Bondapak C_{18} column (Waters) employing the same instrument and the first elution system described in the HPLC section above, with a flow rate of 3 ml/min. NT(9–11) was further purified by chromatography on DEAE-Sephadex A-25-120 (Sigma Chemical Co., St. Louis, MO, U.S.A., 2.3 × 20 cm) in 0.05 *M* pyridine-acetate, pH 4.80.

Following complete purification of each peptide, 2 μg of the peptide was examined by analytical HPLC as described. Each peptide analysis was monitored at 210, 254, and 280 nm to check for the presence of impurities. To obtain sequence information and molecular weight for purified NT(9–11), fast atom bombardment mass spectrometry was performed on a Kratos MS50 high resolution mass spectrometer (Manchester, England) in the laboratory of Dr. Ian Jardine, Dept. of Pharmacology, Mayo Foundation, Rochester, MN, U.S.A. Amino acid analyses of purified NT(1–7), NT(2–9), and NT(2–10) were performed on triplicate hydrolysates (6 *M* HCl, 110°C, 22 h) on a Beckman 7300 amino acid analyzer by Dr. Stephen Powers, Gastroenterology Research Unit, Mayo Foundation.

Materials

NT was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). NT(8–13) and NT(9–13) were supplied by Bachem Biochemicals (Torrance, CA, U.S.A.). Bacitracin, bestatin, pepstatin, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, L-tyrosine, NT(1–11), and NT(1–8) were from Sigma Chemical Co. NT(1–6) was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY, U.S.A.); NT(11–13) was custom synthesized by Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.); and NT(10–11) was supplied by Research Plus Inc. (Bayonne, NJ, U.S.A.). Pyridine was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Z-Pro-Prolinal was generously donated by Dr. Sherwin Wilk, The Mount Sinai Medical Center, New York, NY, U.S.A. Captopril was a gift from Dr. Richard Weinshilboum, Department of Pharmacology, Mayo Foundation. Thiorphan was kindly provided by Dr. J.-C. Schwartz, INSERM, Paris, France.

RESULTS

HPLC analysis of NT and its fragments

Figure 1 demonstrates a typical HPLC separation of synthetic NT and 12 of its fragments, with the second elution system described in Materials and Methods. Two peptides from the carboxyl-terminal region of NT coelute in this system—NT(9–11) and NT(10–11). The radiolabeled degradation products in the studies reported here were identified by comparing their elution times with the retention times of these standard peptides.

Degradation of radiolabeled NT

[^{3}H]NT, at a concentration of 0.1 μM, was exposed to intact N1E-115 cells in solution I for 20 min at 37°C, and the reaction was terminated by removal of the cells by centrifugation. The supernatant was then analyzed by HPLC. The amount of radiolabeled NT degraded was dependent upon the number of cells present during the incubation, with essentially no degradation seen using 1,000 cells/ml and almost

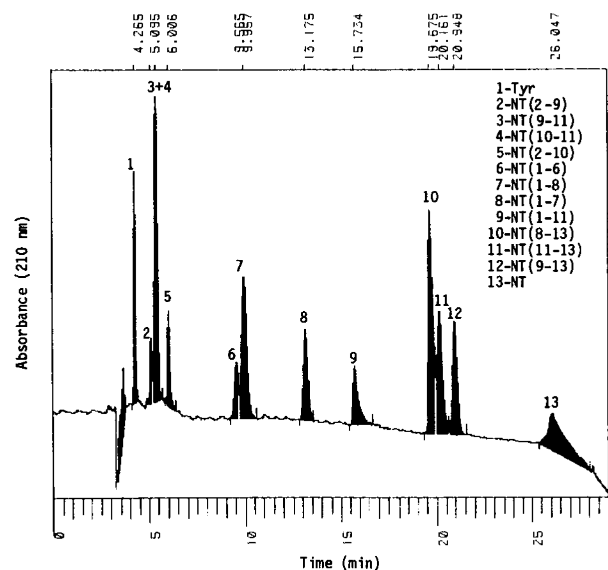

FIG. 1. Elution profile from HPLC of NT and 12 of its fragments. A standard mixture of 1.5 μg each of synthetic NT and 12 of its fragments was analyzed by the second HPLC system described in Materials and Methods. This standard mixture was one of the samples fractionated during HPLC of each degradation experiment in order to identify the radiolabeled breakdown products.

complete breakdown observed at a level of 1×10^6 cells/ml (Table 1). No degradation products of [^{3}H]NT were formed when the peptide was incubated under experimental conditions at 37°C over a period of 20 min in the absence of cells (n = 5; data not shown).

Figure 2 illustrates the products formed during a typical time course of the degradation of 0.1 μM [^{3}H]NT incubated in solution I with 1×10^6 cells/ml at 37°C. HPLC analyses demonstrated that a minimum of six major tritiated products were formed over a period of 20 min, with the degradation peptides produced and the degree of proteolysis of [^{3}H]NT being dependent upon the length of incubation. At these concentrations, an average of 97% ± 1% (mean ± SEM) of the [^{3}H]NT was degraded after 5 min of exposure to intact cells (n = 4). Two of the initial products formed eluted with the same retention times as the standards NT(9–13) and NT(11–13). These peptides, in turn, were each rapidly degraded such that they had completely disappeared by the end of the 20-min time course. Another early degradation product eluted with the same retention time with which NT(9–11) and NT(10–11) coelute, and so could not be positively identified. However, this peptide was not rapidly degraded after formation. One major tritiated product with the elution time of standard tyrosine was formed initially and continued to accumulate over the 20-min time course. Two peptides with the retention times of standard NT(1–8) and NT(1–7) accumulated during the first 5 min of the incubation, while they decreased significantly in amount by 20 min. Finally, an as yet unidentified peptide appeared at 5 min and had increased dramatically in quantity by 20 min.

Figure 2 also demonstrates that as [^{3}H]NT was degraded, tritiated NT(9–13) and NT(11–13) were quickly formed and then rapidly degraded, whereas NT(1–7) and NT(1–8) were quickly formed but more slowly degraded. The two major, final products of radiolabeled NT degradation were tyrosine and the as yet unidentified, late-forming peptide.

TABLE 1. *NT degradation as a function of N1E-115 cell number*

Cell number/ml	Percent [^{3}H]NT degradation
10^3	1
10^4	12
10^5	86
10^6	98

Intact cells were incubated in solution I with 0.1 μM [^{3}H]NT for 20 min at 37°C. The incubation was stopped, and the analyses of [^{3}H]NT by HPLC were performed as described in Materials and Methods. Recovery of [^{3}H]NT in each sample was calculated as percent of the total counts eluted from the HPLC column. Number of independent experiments = 2.

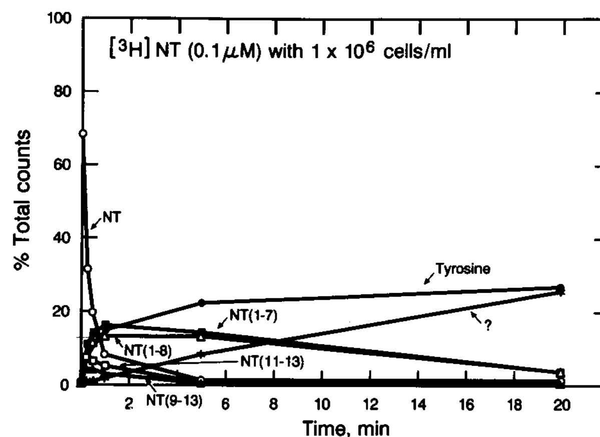

FIG. 2. Time course of the degradation of [^{3}H]NT and the formation of breakdown products during incubation with intact N1E-115 cells. [^{3}H]NT (0.1 μM) was incubated in solution I with 1×10^6 intact N1E-115 cells/ml (passage number 11, 21 days after subculture) for 20 min at 37°C as described in Materials and Methods. At various times, a sample was taken and the reaction was terminated upon removal of the cells by centrifugation, with the supernatant then being acidified and chilled. Twenty microliters of the supernatant from each time point was analyzed directly by the second HPLC system described in Materials and Methods. The HPLC buffer flow rate was 1 ml/min, and 0.5-min fractions were collected and subsequently transferred to scintillation vials for measurement of radioactivity. The radiolabeled degradation products were identified by comparing their elution times with the retention times of the synthetic peptides in the standard mixture seen in Fig. 1. These data were obtained from HPLC profiles and were from one of four independent experiments.

Degradation of radiolabeled NT(8–13)

NT(8–13) is the active carboxyl-terminal fragment of NT and has 20 times more binding ability than NT and as much as 50 times more biological activity than NT in intact clone N1E-115 (Gilbert et al., 1986). When tritiated NT(8–13) was incubated with intact N1E-115 cells under the same conditions as was radiolabeled NT, two of the tritiated products formed were peptides which eluted with the retention times of NT(11–13) and NT(9–13) (see Fig. 3). The single, final product found after 5 min of degradation was radiolabeled tyrosine. As illustrated in Fig. 3, as [^{3}H]NT(8–13) was degraded, radiolabeled NT(9–13) and NT(11–13) were quickly formed and then rapidly degraded, with the simultaneous increase in the quantity of labeled tyrosine present. Interestingly, radiolabeled NT(8–13) was more rapidly hydrolyzed under identical conditions than was intact [^{3}H]NT itself. [^{3}H]NT(8–13) was degraded to an average of 11% of its original level upon only 60 sec of exposure to intact N1E-115 cells at 37°C (n = 2).

Effect of protease inhibitors on NT degradation

To assess what enzymes might be involved in the breakdown of NT and, more importantly, to find experimental conditions for preventing this degradation and thus obtaining maximal desensitization of

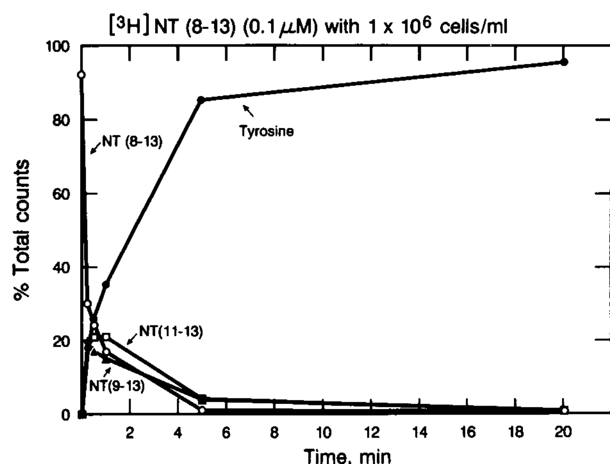

FIG. 3. Time course of the degradation of [^{3}H]NT (8–13) and the formation of breakdown products during incubation with intact N1E-115 cells. [^{3}H]NT (8–13) (0.1 μM) was incubated in solution I with 1×10^6 intact N1E-115 cells/ml (passage number 15, 13 days after subculture) for 20 min at 37°C as described in Materials and Methods. At various times, a sample was taken and the reaction was terminated upon removal of the cells by centrifugation, with the supernatant then being acidified and chilled. Twenty microliters of the supernatant from each time point was analyzed directly by the first HPLC system described in Materials and Methods. The HPLC buffer flow rate was 1 ml/min, and 0.5-min fractions were collected and subsequently transferred to scintillation vials for measurement of radioactivity. The radiolabeled degradation products were identified by comparing their elution times with the retention times of the synthetic peptides in the standard mixture seen in Fig. 1. These data were obtained from HPLC profiles and were from one of three independent experiments.

the NT receptor, preliminary experiments were done to examine various protease inhibitors for their ability to inhibit breakdown of [^{3}H]NT by clone N1E-115. Thus, intact cells (0.2×10^6/ml) were incubated for 20 min at 37°C with 0.1 μM [^{3}H]NT in the presence of specific inhibitors. Inhibitors specific for pepsin and other acidic proteases (pepstatin, 1 μg/ml), exopeptidases in general (bacitracin, 140 μg/ml), some aminopeptidases (bestatin, 100 μM), angiotensin-converting enzyme (captopril, 1 μM), serine proteases (phenylmethylsulfonyl fluoride, 1 μg/ml), and enkephalinase (thiorphan, 10 μM) each showed little or negligible inhibition of [^{3}H]NT degradation upon analysis by thin layer chromatography (data not shown). However, Z-Pro-Prolinal, a specific inhibitor of prolyl endopeptidase, and 1,10-phenanthroline, a metallopeptidase inhibitor, were each more effective at inhibiting the breakdown of radiolabeled NT, although their inhibition, individually, was not significant statistically.

The incubation of 1 μM of Z-Pro-Prolinal (Dr. Sherwin Wilk, personal communication) with 10^{-8} M [^{3}H]NT and 100,000 intact cells/ml resulted in the level of inhibition of degradation seen in Table 2. By 20 min, 79% of the [^{3}H]NT had been degraded, compared to the breakdown of 95% of [^{3}H]NT with no inhibitor present. In HPLC analyses of these studies, the major degradation products found were NT(1–8), NT(9–13), and the peptide identified as NT(9–11)/NT(10–11). The formation of free tyrosine was greatly slowed, and no NT(1–7) or final, unidentified peptide was found (data not shown).

The inclusion of 1 mM of 1,10-phenanthroline (Snider and Richelson, 1984) with the same concentrations of radiolabeled NT and intact cells was as effective as Z-Pro-Prolinal at decreasing the breakdown of [^{3}H]NT (Table 2). By 20 min, 75% of the [^{3}H]NT had been degraded compared to the breakdown of 95% of [^{3}H]NT with no inhibitor present. The major degradation products seen in this case were NT(11–13), NT(1–7), and a never-before-seen, unidentified peptide; little free tyrosine was found (data not shown).

Incubation of a combination of Z-Pro-Prolinal and 1,10-phenanthroline with [^{3}H]NT and intact cells at the previously described concentrations almost completely inhibited the degradation of [^{3}H]NT over a period of 20 min (see Table 2). In fact, this combination of Z-Pro-Prolinal and 1,10-phenanthroline has been successfully used for studies of NT receptor desensitization (Gilbert and Richelson, 1987).

DISCUSSION

The degradation of [^{3}H]NT by intact murine neuroblastoma cells (clone N1E-115) was demonstrated to be a rapid process, with the tritiated products formed and the degree of proteolysis of [^{3}H]NT being dependent upon the length of incubation and the concentration of cells. The experiments described here indicated that [^{3}H]NT was degraded in an apparently sequential fashion by intact N1E-115 cells, with the initial degradation peptides from the active carboxyl-terminal portion of NT being more rapidly degraded, in turn, than the peptides formed from the

TABLE 2. *Inhibition of [^{3}H]NT degradation*

Incubation condition	Percent [^{3}H]NT degradation ± SEM
No cells or inhibitor	0
No inhibitor	95 ± 1
Z-Pro-Prolinal (1 μM)	79 ± 4
1,10-Phenanthroline (1 mM)	75 ± 7
1,10-Phenanthroline (1 mM) + Z-Pro-Prolinal (1 μM)	7 ± 1[a]

10^{-8} M [^{3}H]NT was incubated for 20 min at 37°C in solution I with the described concentrations of inhibitors and, except for the Control, with 10^5 intact cells/ml. The incubation was stopped, and the analyses of [^{3}H]NT by HPLC were performed as described in Materials and Methods. Recovery of [^{3}H]NT in each sample was calculated as percent of the total counts eluted from the HPLC column. Number of independent experiments = 3.

[a] Significant difference, $p < 0.05$ from cell-containing control with no inhibitor present, using two-tailed t test for two means.

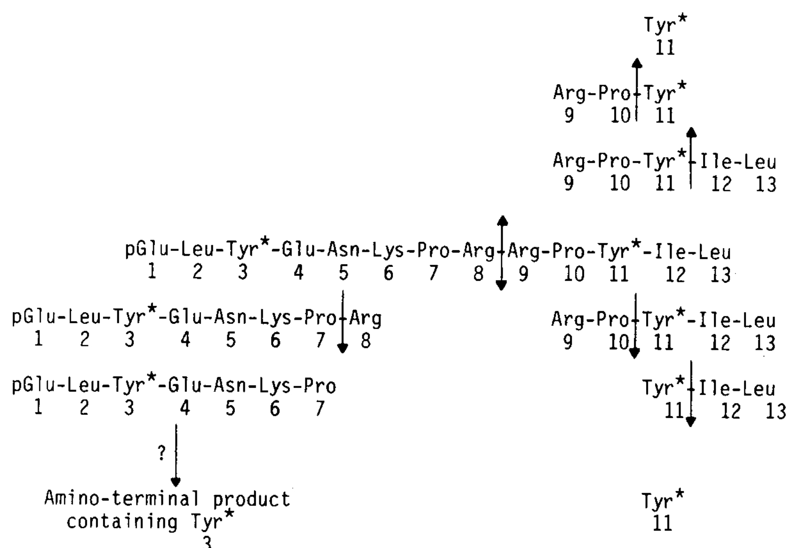

FIG. 4. Proposed scheme for the usual degradation process of [^{3}H]NT by intact N1E-115 cells. Data from experiments employing specific protease inhibitors and from studies of the time course of degradation suggested the occurrence of the indicated cleavages. It should be noted, however, that NT(9–11) and NT(10–11) could not be differentiated in the HPLC analyses. The asterisks indicate the tritiated residues.

inactive amino-terminal half of neurotensin. The final two degradation products found were tyrosine, from the carboxyl-terminal portion of NT, and an as yet unidentified peptide, from the amino-terminal half.

Figure 4 presents what we are hypothesizing to be the usual degradation sequence of [^{3}H]NT by intact N1E-115 cells. Our data suggest that the primary initial cleavage of the intact peptide was at the bond between residues Arg^8 and Arg^9. In studies measuring the degradation of [^{3}H]NT as a function of cell number, at $\frac{1}{10}$ the number of cells typically employed, NT(9–13) and NT(1–8) were the primary products formed. Furthermore, the inclusion of the metallopeptidase inhibitor 1,10-phenanthroline in the incubation mixture, which inhibited NT(9–13) formation, completely prevented the formation of the peptide NT(9–11)/NT(10–11). Thus, our data indicated that NT(9–13) was the initial carboxyl-terminal product formed, and that this peptide was then rapidly degraded, by different pathways, into NT(11–13) and the peptide NT(9–11)/NT(10–11). These products were then apparently degraded to give free tyrosine.

As previously stated, NT(1–8) was apparently the initial amino-terminal fragment formed from degradation of [^{3}H]NT. The inclusion of the specific inhibitor Z-Pro-Prolinal in the incubation mixture slowed the formation of NT(1–7) and also totally prevented the formation of the late-forming, final, and as yet unidentified peptide. Thus, our evidence indicated that, after formation, NT(1–8) was subsequently cleaved by prolyl endopeptidase to produce NT(1–7), and that NT(1–7) was in turn degraded to a final, and as yet unidentified, amino-terminal product.

Finally, when the radioactivity measured in all of the sequentially formed carboxyl-fragments [NT(9–13), NT(9–11)/NT(10–11), NT(11–13), and tyrosine)], was summed at any given time during the degradation process, the total quantity equaled the combined amounts found in all of the amino-terminal products [NT(1–8), NT(1–7), and the late-forming, unidentified peptide]. During the experimental time courses, 97% of the intact [^{3}H]NT was degraded by the end of 5 min, while breakdown of some of the degradation products continued for another 15 min.

With NT receptors on N1E-115 cells, as well as in most assay systems, the activity of NT resides in the carboxyl-terminal portion, while the amino-terminal half of the peptide has no binding or biological activity. Specifically, this laboratory has demonstrated that NT(8–13) has 10–20 times more binding ability to both intact N1E-115 cells and human brain tissue and as much as 50 times more biological activity in clone N1E-115 than does NT itself; NT(9–13) has at least fivefold less activity than native NT (Gilbert et al., 1986; Kanba et al., 1986). Thus, it was of interest to note from these degradation experiments that NT(8–13) was not one of the peptides synthesized during the proteolysis of [^{3}H]NT, suggesting that the degradation of neurotensin by clone N1E-115 served solely as an inactivation mechanism for the active molecule. [^{3}H]NT(8–13), as the active portion of the NT peptide, was more quickly hydrolyzed under identical experimental conditions than was radiolabeled NT itself; furthermore, the same sequence of carboxyl-terminal fragments was formed during degradation.

Experiments performed with the protease inhibitors 1,10-phenanthroline and Z-Pro-Prolinal were intended primarily to find conditions under which NT degradation was prevented sufficiently to enable the performance of NT receptor desensitization studies. In fact, a combination of these two inhibitors almost completely prevented the breakdown of NT, and has now been successfully employed for NT desensitization studies (Gilbert and Richelson, 1987). In addi-

tion, these inhibitor studies provided useful information about the mechanism of NT degradation itself. Thus, the cleavage of the bond between residues Arg^8 and Arg^9 was inhibited by 1,10-phenanthroline, and therefore was apparently due to the action of a metallopeptidase. Cleavage of the bond between residues Pro^7 and Arg^8 and the bond between residues Pro^{10} and Tyr^{11} was inhibited by Z-Pro-Prolinal, and thus could be attributed to the action of prolyl endopeptidase.

Subsequent to our first presentation of these data on the degradation of NT by clone N1E-115 (Gilbert and Richelson, 1986), another report on this clone was published (Checler et al., 1986*c*). However, our results differed from that report, presumably because our identification of nearly all of the NT fragments formed and our taking of very early time points during the degradation process resulted in our identification of the sequential nature of the formation of NT fragments by clone N1E-115. Checler et al. (1986*b,c*) have concluded from their studies with primary cultured neurons, neuroblastoma N1E-115 cells, and HT29 cells and with samples beginning at 5 min of incubation, that three primary cleavages occur during NT degradation: the bond between the residues Pro^7-Arg^8 [giving NT(1–7) and NT(8–13)], Arg^8-Arg^9 [giving NT(1–8) and NT(9–13)], and Pro^{10}-Tyr^{11} [giving NT(1–10) and NT(11–13)]. However, they have yet to show data demonstrating the existence of the product NT(8–13); in addition, with N1E-115 cells and HT29 cells, they did not demonstrate the formation of the product NT(9–13). Our interpretation from our clone N1E-115 studies is that NT(1–7) is formed from NT(1–8); and, as we have demonstrated, NT(9–13) is rapidly degraded after formation and is completely hydrolyzed within 5 min.

Our results suggest that the pattern of NT degradation by intact clone N1E-115 is different from that of rat brain membranes. Detailed studies of degradation of tritiated NT by purified rat brain synaptic membranes (Checler et al., 1983, 1984, and 1985) have indicated that the major cleavages of the intact peptide are the bonds Arg^8-Arg^9, Pro^{10}-Tyr^{11}, and Tyr^{11}-Ile^{12}, with the major degradation products being $tyrosine^{11}$, NT(1–8), NT(1–10), NT(11–13), NT(1–11), and, in one study (Checler et al., 1985), barely detectable levels of NT(9–13). These authors reported an initial lag time of 5 min prior to the appearance of any degradation products, and demonstrated that, as NT was degraded, tyrosine and NT(1–8) accumulated during the course of the 60 min incubation, while NT(1–10), NT(11–13), and NT(1–11) were degraded after formation and reached barely detectable levels by 60 min.

At least one enzyme involved in the degradation of NT by neuroblastoma N1E-115 cells was different from those present in membranes isolated from rat brain. While prolyl endopeptidase acted in clone N1E-115 to cleave the bond between residues Pro^{10} and Tyr^{11} in NT(9–13) during neurotensin metabolism, this bond is cleaved in neurotensin to form NT(1–10) and NT(11–13) by a neutral metallopeptidase which has been isolated from rat brain synaptic membranes (Checler et al., 1986c).

The differences between the pattern of $[^3H]$NT degradation by murine neuroblastoma clone N1E-115 and that of rat brain synaptic membranes may reflect dissimilarities in the properties of these two tissues. Intact neuroblastoma N1E-115 cells, as an established clone derived from a tumor, may degrade NT differently from membranes isolated from normal tissue such as rat brain. However, using clone N1E-115 as a model for the intact neuron has numerous advantages for studying the degradation of the putative neurotransmitter NT, not the least of which is the fact that NT is encountering degrading enzymes on the same non-disrupted, intact cells with which it also interacts to perform receptor binding and induce receptor-mediated, intracellular, biochemical activities. Differences may also exist in the degradation of peptides between intact cells and membranal preparations. Thus, Palenker et al., (1984) studying degradation of $[Leu^5]$enkephalin by N1E-115 cells, found marked differences in the pattern of degradation between intact cells and a crude membranal preparation from these cells. In agreement with our results with intact N1E-115 cells, McDermott et al., (1986) demonstrated that when tritiated neurotensin was superfused over rat hypothalamic slices and the superfusates were analyzed by HPLC, the major cleavage point of the intact peptide was the Arg^8-Arg^9 bond.

Acknowledgment: The authors thank Carol Cooper for typing this manuscript. This work was supported by the Mayo Foundation and Grant MH 27692.

REFERENCES

Bissette G., Manberg P., Nemeroff C. B., and Prange A. J. Jr. (1978) Neurotensin, a biologically active peptide. *Life Sci.* **23,** 2173–2182.

Bozou J.-C., Amar S., Vincent J.-P., and Kitabgi P. (1986) Neurotensin-mediated inhibition of cyclic AMP formation in neuroblastoma N1E-115 cells: Involvement of the inhibitory GTP-binding component of adenylate cyclase. *Mol. Pharmacol.* **29,** 489–496.

Carraway R. and Leeman S. C. (1973) The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. Biol. Chem.* **248,** 6854–6861.

Carraway R. and Leeman S. E. (1975) The amino acid sequence of a hypothalamic peptide, neurotensin. *J. Biol. Chem.* **250,** 1907–1911.

Carraway R. and Leeman S. E. (1976) Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. *J. Biol. Chem.* **251,** 7045–7052.

Checler F., Vincent J.-P., and Kitabgi P. (1983) Degradation of neurotensin by rat brain synaptic membranes: Involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzyme, and other unidentified peptidases. *J. Neurochem.* **41,** 375–384.

Checler F., Emson P. C., Vincent J.-P., and Kitabgi P. (1984) Inactivation of neurotensin by rat brain synaptic membranes. Cleavage at the pro^{10}-tyr^{11} bond by endopeptidase 24.11 (enkephalinase) and a peptidase different from proline-endopeptidase. *J. Neurochem.* **43**, 1295–1301.

Checler F., Vincent J.-P., and Kitabgi P. (1985) Inactivation of neurotensin by rat brain synaptic membranes partly occurs through cleavage at the arg^{8}-arg^{9} peptide bond by a metalloendopeptidase. *J. Neurochem.* **45**, 1509–1513.

Checler F., Vincent J.-P., and Kitabgi P. (1986*a*) Purification and characterization of a novel neurotensin-degrading peptidase from rat brain synaptic membranes. *J. Biol. Chem.* **261**, 11274–11281.

Checler F., Mazella J., Kitabgi P., and Vincent J.-P. (1986*b*) High-affinity receptor sites and rapid proteolytic inactivation of neurotensin in primary cultured neurons. *J. Neurochem.* **47**, 1742–1748.

Checler F., Amar S., Kitabgi P., and Vincent J.-P. (1986*c*) Catabolism of neurotensin by neural (neuroblastoma clone N1E-115) and extraneural (HT29) cell lines. *Peptides* **7**, 1071–1077.

Erickson B. W. and Merrifield R. B. (1976) Solid-phase peptide synthesis, in *The Proteins: Vol. II* (Neurath H., Hill R. L., and Boeder C.-L., eds). pp. 255–257. Academic press, New York.

Gilbert J. A. and Richelson E. (1984) Neurotensin stimulates formation of cyclic GMP in murine neuroblastoma clone N1E-115. *Eur. J. Pharmacol.* **99**, 245–246.

Gilbert J. A. and Richelson E. (1986) Rapid degradation of neurotensin by neuroblastoma clone N1E-115. *Soc. Neurosci. Abstr.* **12**, 762.

Gilbert J. A. and Richelson E. (1987) Desensitization of neurotensin receptor-mediated cyclic GMP formation in neuroblastoma clone N1E-115. *Soc. Neurosci. Abstr.* **13**, in press.

Gilbert J. A., McKinney M., and Richelson E. (1984) Neurotensin stimulates cyclic GMP formation in neuroblastoma clone N1E-115 and rat cerebellum. *Soc. Neurosci. Abstr.* **10**, 378.

Gilbert J. A., Moses C. J., Pfenning M. A., and Richelson E. (1986) Neurotensin and its analogs—Correlation of specific binding with stimulation of cyclic GMP formation in neuroblastoma clone N1E-115. *Biochem. Pharmacol.* **35**, 391–397.

Goedert M., Pinnock R. D., Downes C. P., Mantyh P. W., and Emson P. C. (1984) Neurotensin stimulates inositol phospholipid hydrolysis in rat brain slices. *Brain Res.* **323**, 193–197.

Honegger P. and Richelson E. (1976) Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell culture. *Brain Res.* **109**, 335–354.

Kaiser E., Colescott R. L., Bossinger C. D., and Cook P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Analyt. Biochem.* **34**, 595–598.

Kanba K. S., Kanba S., Okazaki H., and Richelson E. (1986) Binding of [^{3}H]neurotensin in human brain: Properties and distribution. *J. Neurochem.* **46**, 946–952.

McCormick D. J. and Atassi M. Z. (1985) Antigenic structure of human hemoglobin: Delineation of the antigenic site (site 2) within region 41–65 of the alpha chain by immunochemistry of synthetic peptides. *J. Protein Chem.* **4**, 171–184.

McDermott J. R., Virmani M. A., Turner J. D., and Kidd A. M. (1986) Peptidases involved in the catabolism of neurotensin: Inhibitor studies using superfused rat hypothalamic slices. *Peptides* **7**, 225–230.

Memo M., Castelletti L., Missale C., Valerio A., Carruba M., and Spano P. F. (1986) Dopaminergic inhibition of prolactin release and calcium influx induced by neurotensin in anterior pituitary is independent of cyclic AMP system. *J. Neurochem.* **47**, 1689–1695.

Mitchell A. R., Kent S. B. H., Engelhard M., and Merrifield R. B. (1978) A new synthetic route to *tert*-butyloxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin, an improved support for solid-phase peptide synthesis. *J. Org. Chem.* **43**, 2845–2852.

Nemeroff C. B., Luttinger D., Hernandez D. E., Mailman R. B., Mason G. A., Davis S. D., Widerlov E., Frye G. D., Kilts C. A., Beaumont K., Breese G. R., and Prange A. J. Jr. (1983*a*) Interactions of neurotensin with brain dopamine systems: biochemical and behavioral studies. *J. Pharmacol. and Exp. Therapeut.* **225**, 337–345.

Nemeroff C. B., Luttinger D., and Prange A. J. Jr. (1983*b*) Neurotensin and bombesin. *Handbook of Psychopharmacol.* **16**, 363–466.

Palenker J., Lentzen H., and Brandt U. (1984) Enkephalin degradation by enkephalinergic neuroblastoma cells—Involvement of angiotensin-converting-enzyme. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **325**, 214–217.

Quik M., Iversen L. L., and Bloom S. R. (1978) Effect of vasoactive intestinal peptide (VIP) and other peptides on cAMP accumulation in rat brain. *Biochem. Pharmacol.* **27**, 2209–2213.

Redgate E. S., Deupree J. D., and Axelrod J. (1986) Interaction of neuropeptides and biogenic amines on cyclic adenosine monophosphate accumulation in hypothalamic nuclei. *Brain Res.* **365**, 61–69.

Snider R. M. and Richelson E. (1984) Bradykinin receptor-mediated cyclic GMP formation in a nerve cell population (murine neuroblastoma clone N1E-115). *J. Neurochem.* **43**, 1749–1754.

Snider R. M., Forray C., Pfenning M., and Richelson E. (1986) Neurotensin stimulates inositol phospholipid metabolism and calcium mobilization in murine neuroblastoma clone N1E-115. *J. Neurochem.* **47**, 1214–1218.

Stewart J. M. and Young J. D. (1984) *Solid Phase Peptide Synthesis* (Pierce, ed.), pp. 1–176. Rockford, Illinois.