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NEUROTENSIN(8–13): COMPARISON OF NOVEL ANALOGS FOR STIMULATION OF CYCLIC GMP FORMATION IN NEUROBLASTOMA CLONE N1E-115 AND RECEPTOR BINDING TO HUMAN BRAIN AND INTACT N1E-115 CELLS

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Abstract—Neurotensin(8–13), the carboxyl-terminal portion of neurotensin, is 4–50 times more potent than native neurotensin in binding to intact neuroblastoma N1E-115 cells and human brain tissue and in stimulation of intracellular cyclic GMP production and inositol phospholipid hydrolysis in clone N1E-115 (Gilbert JA and Richelson E, *Eur J Pharmacol* **99**: 245–246, 1984; Gilbert JA *et al.*, *Biochem Pharmacol* **35**: 391–397, 1986; Kanba KS *et al.*, *J Neurochem* **46**: 946–952, 1986; and Kanba KS and Richelson E, *Biochem Pharmacol* **36**: 869–874, 1987). A series of novel analogs of neurotensin (8–13) was synthesized, and a structure–activity study was done comparing the abilities of these peptides to stimulate intracellular cyclic GMP production in intact neuroblastoma clone N1E-115 and to inhibit the binding of [^{3}H]neurotensin to these cells and to membranal preparations from human brain. A direct correlation was found for each analog between its EC_{50} for biochemical activity and its K_D for binding ability in studies with clone N1E-115. Furthermore, a strong correlation existed for each peptide between its K_D for binding to neurotensin receptors on these cells and its K_D for binding to neurotensin receptors in human brain tissue. In this study, the residues that were important to the biochemical and binding activities of neurotensin (8–13) proved to be identical to the amino acids that are necessary for the functional integrity of native neurotensin (Gilbert JA *et al.*, *Biochem Pharmacol* **35**: 391–397, 1986).

The tridecapeptide neurotensin (NT‖), first isolated from bovine hypothalamus [1], is considered a neurotransmitter in the central nervous system. Neurotensin produces a variety of physiological activities upon peripheral or central administration. One focus of research has been the interaction of neurotensin with dopaminergic systems, specifically the mesolimbic dopamine pathway, in the mammalian central nervous system (see reviews in Refs 2 and 3). Studies have suggested that centrally-administered neurotensin induces many of the behavioral effects of systemically-administered neuroleptics. In addition, prolonged administration of neuroleptics to rats selectively increases the content of neurotensin immunoreactive material in certain areas of the brain [4–7], and neuroleptics that have a low incidence of inducing extrapyramidal side effects apparently fail to maintain increased neurotensin levels upon long-term treatment [8]. Chronic treatment with the neuroleptic haloperidol increases neurotensin receptor binding in human and rat substantia nigra [9], although not in striatum and nucleus accumbens [8]. Lengthy exposure to the long-acting neuroleptic pipotiazine palmitic ester increases the number of neurotensin binding sites in the lateral and median parts of the prefrontal cortex, the entorhinal cortex, the nucleus accumbens, and the central striatum of rat, although not in the lateral striatum [10]. These results suggest that the neuropharmacology of neuroleptics is importantly related to neurotensin and its receptors.

Because our interest is in the mechanism of action of psychiatric drugs, we are studying the functional biochemistry of neurotensin, particularly as it applies to neurotensin receptors in human brain. We have found, using neuronal tissue, that neurotensin (8–13), the carboxyl-terminal portion of neurotensin, is 4–50 times more potent than native neurotensin in binding to intact murine neuroblastoma N1E-115 cells and human brain and in stimulation of intracellular cyclic GMP production and inositol phospholipid hydrolysis in clone N1E-115 [11–14]. Here we report that we have synthesized and tested for activity a series of novel analogs of neurotensin(8–13).

MATERIALS AND METHODS

Cell culture. Murine neuroblastoma clone N1E-115 was cultured as described by Gilbert *et al.* [15].

Measurement of relative changes in cyclic GMP production. The assay used for measurement of intracellular cyclic GMP formation was essentially that of Richelson *et al.* [16], in which cyclic [^{3}H]GMP produced in clone N1E-115 was isolated chromatographically from cells labeled with the radio-

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‖ Abbreviations: NT, neurotensin; HPLC, high pressure liquid chromatography; and K_D, equilibrium dissociation constant.

active precursor [^{3}H]guanosine prior to receptor stimulation.

Neurotensin receptor binding assay. Competition studies measuring the inhibition in binding of [^{3}H]neurotensin to intact N1E-115 cells by increasing concentrations of unlabeled neurotensin(8–13) analogs were performed by the method of Gilbert *et al.* [17].

Assays to determine the competitive binding of neurotensin(8–13) analogs to neurotensin receptors in membranal preparations of tissue from human brain were done with the technique of Kanba *et al.* [13]. Neurotensin and NT(8–13) were tested for binding with homogenates from human frontal cortex [12]; [Nle^{12}]NT(8–13), [Val^{12}]NT(8–13), [Nva^{12}]NT(8–13), and [Val^{13}]NT(8–13) were assayed with preparations from human frontal cortex and from human amygdala; [D-Leu^{13}]NT(8–13) was tested with homogenates from human frontal cortex and from human orbital cortex; and all other peptides were assayed with preparations from human frontal cortex, amygdala, and orbital cortex.

Synthetic peptides. All of the neurotensin(8–13) analogs except [Tyr^{11}-O-Me]NT(8–13) were synthesized by solid-phase methods as described by McCormick *et al.* [18]. Each crude synthetic peptide was purified by gel filtration on Sephadex G-15 resin (Pharmacia, Piscataway, NJ; 1.6 × 88 cm) in 0.1 M ammonium bicarbonate, 0.02% sodium azide buffer, pH 7.8. Following chromatography of each analog, the peptide was examined for purity by analytical high pressure liquid chromatography (HPLC) and characterized by fast atom bombardment mass spectrometry as described in Gilbert *et al.* [15].

Materials. The peptide [Tyr^{11}-O-Me]-neurotensin(8–13) was synthesized by the Regis Chemical Co. (Morton Grove, IL) under contract to the National Institute of Mental Health; Dr J. Steven Kennedy of the Neurosciences Research Branch therein provided this analog to the authors. Neurotensin was supplied by Boehringer Mannheim (Indianapolis, IN), and neurotensin(8–13) was purchased from Bachem Biochemicals (Torrance, CA). [^{3}H]Guanosine was from ICN Radiochemicals (Irvine, CA), and [^{3}H]neurotensin was obtained from New England Nuclear (Boston, MA). Polypropylene or polyethylene plasticware was used for all experiments. All other reagents were analytical grade.

RESULTS

Stimulation of intracellular cyclic GMP production and inhibition of [^{3}H]*neurotensin binding by neurotensin(8–13) analogs with intact* N1E-115 *cells.* Results of the investigation of a number of novel neurotensin(8–13) analogs (see Table 1) for their abilities to stimulate the intracellular formation of cyclic GMP in clone N1E-115 are presented in Table 2. Typical concentration–response curves from a given cyclic GMP experiment are illustrated in Fig. 1. Table 2 also provides data obtained when the same peptides were assessed for their potencies in inhibiting radioligand binding to the single class of non-cooperative neurotensin receptors on intact N1E-115 cells [12]. Representative competition curves obtained in the neurotensin binding assay are shown in Fig. 2. Furthermore, when the potency for each of the neurotensin(8–13) peptides in stimulating intracellular cyclic GMP formation was compared to its ability to compete for neurotensin receptors on intact N1E–115 cells, a direct correlation between each EC_{50} and corresponding K_D was found to exist (Fig. 3). The EC_{50} values for NT(8–13) and its analogs were an average of 7-fold lower than the corresponding K_D values, confirming previous results [12] that fewer than 50% of the neurotensin receptors need to be occupied for production of 50% of the maximal biochemical activity in clone N1E-115.

Inhibition of [^{3}H]*neurotensin binding to human brain tissue by neurotensin(8–13) analogs.* Data obtained when a number of neurotensin(8–13) analogs were assessed for their potencies in inhibiting radioligand binding to the single class of non-cooperative neurotensin receptors found by this laboratory in human brain tissue [13] are also presented in Table 2. When the K_D for each of the neurotensin(8–13) peptides for binding to neurotensin receptors on intact N1E-115 cells was compared to its K_D for binding to neurotensin receptors in human brain tissue, a direct correlation was found to exist (Fig. 4). The K_D values for binding of NT(8–13) and its analogs to human brain tissue were an average of 6-fold lower than the corresponding K_D values found with intact N1E-115 cells, possibly a result of the use of homogenized versus intact tissue.

DISCUSSION

A summary of the effects on biochemical and binding activities observed following substitutions at different residues of neurotensin(8–13) is presented in Table 2. The results of this structure–activity study indicated that the residues that were important to the biological and binding activities of NT(8–13) were identical to the amino acids that are necessary for the functional integrity of native neurotensin [12]. In experiments measuring the activity of neurotensin analogs and fragments with N1E-115 cells [12], substitution of D-Arg into position 8 of NT did not change significantly the biochemical potency although it halved the binding affinity. Similarly, in this structure–activity study employing neuroblastoma clone N1E-115, replacement of Arg^8 in NT(8–13) with the D isomer of the basic amino acid lysine had no significant effect on the biological potency; however, the binding activity was decreased 15-fold. As in studies with N1E-115 cells in which NT analogs having D-amino acid substitutions for Pro^{10} or Tyr^{11} demonstrated drastically decreased biochemical and binding abilities, the ring structures in positions 10 and 11 of NT(8–13) were important to the functional ability of this peptide in the present report. Neurotensin(8–13) analogs with nonpolar, aliphatic residues in place of Pro^{10}, e.g. [Gly^{10}]NT(8–13), or Tyr^{11}, e.g. [Ala^{11}]NT(8–13), were 4300- and 7500-fold, respectively, less active biologically than NT(8–13) and had a 2500- and 3700-fold lower binding affinity. A selective modification of Tyr^{11}, i.e. the methylation of the hydroxyl oxygen, greatly affected the ability of NT(8–13) to function; [Tyr^{11}-O-

Table 1. Structures of neurotensin(8–13) and its analogs

Peptide	Sequence												
	1	2	3	4	5	6	7	8	9	10	11	12	13
NT(8–13)								Arg	Arg	Pro	Tyr	Ile	Leu
[D-Lys^8]NT(8–13)								D-Lys	Arg	Pro	Tyr	Ile	Leu
[Nva^{12}]NT(8–13)								Arg	Arg	Pro	Tyr	Nva	Leu
NT	<Glu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu
[Nle^{12}]NT(8–13)								Arg	Arg	Pro	Tyr	Nle	Leu
[Val^{12}]NT(8–13)								Arg	Arg	Pro	Tyr	Val	Leu
[Val^{13}]NT(8–13)								Arg	Arg	Pro	Tyr	Ile	Val
[D-Arg^9]NT(8–13)								Arg	D-Arg	Pro	Tyr	Ile	Leu
[D-Lys^9]NT(8–13)								Arg	D-Lys	Pro	Tyr	Ile	Leu
[Tyr^{11}-O-Me]NT(8–13)								Arg	Arg	Pro	Tyr-O-Me	Ile	Leu
[Gly^{10}]NT(8–13)								Arg	Arg	Gly	Tyr	Ile	Leu
[Ala^{11}]NT(8–13)								Arg	Arg	Pro	Ala	Ile	Leu
[D-Leu^{13}]NT(8–13)								Arg	Arg	Pro	Tyr	Ile	D-Leu

Table 2. Potencies of neurotensin(8–13) and its analogs in biochemical and binding activities*

Peptide	Stimulation of cyclic [^{3}H]GMP formation in intact N1E-115 cells	Competition with [^{3}H]neurotensin binding to: Intact N1E-115 cells	Competition with [^{3}H]neurotensin binding to: Human brain tissue
	EC_{50} (nM)	K_D (nM)	K_D (nM)
NT(8–13)	0.32 ± 0.04† (8)‡	0.61 ± 0.02§ (3)	0.16 ± 0.03‖ (3)
[D-Lys^8]NT(8–13)¶	0.30 ± 0.04 (5)	8.9 ± 0.4 (3)	0.4 ± 0.10 (3)
[Nva^{12}]NT(8–13)¶	1.3 ± 0.40 (6)	9.0 ± 0.8 (3)	0.9 ± 0.40 (3)
Neurotensin	1.5 ± 0.6§ (11)	11 ± 1§ (10)	2.0 ± 0.3‖ (9)
[Nle^{12}]NT(8–13)	1.6 ± 0.4 (6)	12 ± 2 (3)	2.8 ± 1.4 (3)
[Val^{12}]NT(8–13)	2.9 ± 0.6 (7)	18 ± 1 (3)	3.8 ± 0.9 (3)
[Val^{13}]NT(8–13)	10 ± 2 (8)	80 ± 1 (3)	22 ± 5 (3)
[D-Arg^9]NT(8–13)	32 ± 7 (5)	120 ± 40 (4)	15 ± 6 (3)
[D-Lys^9]NT(8–13)	140 ± 30 (5)	430 ± 10 (3)	88 ± 17 (3)
[Tyr^{11}-O-Me]NT(8–13)	120 (2)	1,400 ± 200 (3)	ND
[Gly^{10}]NT(8–13)¶	1,380 ± 320 (5)	1,530 ± 330 (3)	510 ± 150 (3)
[Ala^{11}]NT(8–13)¶	2,400 ± 310 (3)	2,240 ± 630 (4)	1,260 ± 130 (3)
[D-Leu^{13}]NT(8–13)	6,100 ± 300 (3)	16,000 ± 8,000 (4)	3,500 (2)

* Values are means ± SEM; K_D = apparent equilibrium dissociation constant; and EC_{50} = concentration inducing 50% of the maximal response. ND = not determined.

† From Gilbert and Richelson [19].

‡ Number of independent experiments performed.

§ From Gilbert *et al.* [12].

‖ From Kanba *et al.* [13].

¶ From Al-Rodhan *et al.* manuscript submitted for publication.

Me]NT(8–13) had 380 and 2300 times, respectively, less biochemical and binding activity.

The importance of Arg^9, Ile^{12}, and Leu^{13} to the functional integrity of NT(8–13) was also studied. Arg^9 appeared to be more critical to the activity of NT(8–13) than was Arg^8. Replacement of Arg^9 with the D form of Arg or Lys decreased the biological activity of NT(8–13) 100 or 440 times, respectively, and lowered the binding affinity 200 or 700 times. The ability of NT(8–13) to function was not greatly affected by the substitution for Ile^{12} and Leu^{13} of similar aliphatic amino acids having different side chain branches. The biochemical activity of NT(8–13) was somewhat reduced, i.e. 4- and 5-fold, respectively, if isoleucine^{12}, with two different branches at the β carbon, was replaced with norvaline having an unbranched 3-carbon side chain, or norleucine, with an unbranched 4-carbon side chain; binding affinity was decreased 15- and 20-fold. Substitution of Ile^{12} with valine, an amino acid with a ramification at the β carbon, reduced slightly more the biochemical and binding activities of NT(8–13), to 9- and 30-fold the original values. NT(8–13) was more sensitive to replacement with a valine residue

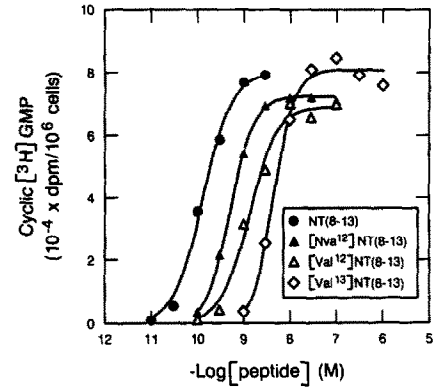

Fig. 1. Effect of concentration on stimulation of intracellular cyclic GMP synthesis in clone N1E-115 by analogs of neurotensin(8–13). The data presented are from one experiment and are representative of the concentration–response curves obtained when increasing concentrations of analogs of neurotensin(8–13) were assayed for their ability to induce cyclic GMP formation in intact cells at 37° with the procedure described under Materials and Methods. Each point is the average of triplicates from which the average of triplicate basal values has been subtracted.

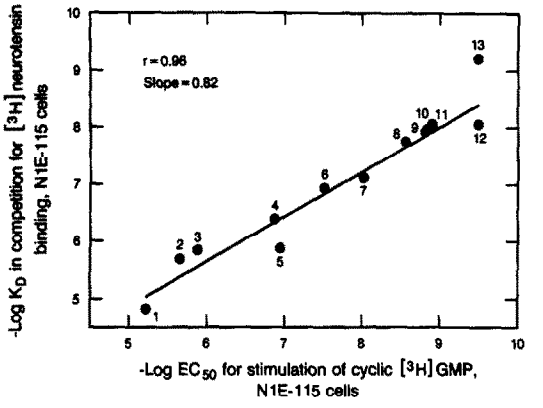

Fig. 3. Correlation between the K_D for binding to neurotensin receptors and the EC_{50} in stimulating intracellular cyclic GMP formation for neurotensin(8–13) analogs with neuroblastoma clone N1E-115. The peptides employed were: 1, [D-Leu^{13}]NT(8–13); 2, [Ala^{11}]NT(8–13); 3, [Gly^{10}]NT(8–13); 4, [D-Lys^{9}]NT(8–13); 5, [Tyr^{11}-O-Me]NT(8–13); 6, [D-Arg^{9}]NT(8–13); 7, [Val^{13}]NT(8–13); 8, [Val^{12}]NT(8–13); 9, [Nle^{12}]NT(8–13); 10, NT; 11, [Nva^{12}]NT(8–13); 12, [D-Lys^{8}]NT(8–13); and 13, NT(8–13).

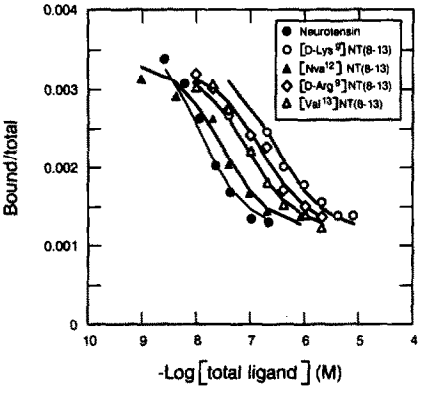

Fig. 2. Effect of concentration on inhibition of [^{3}H]neurotensin binding to clone N1E-115 by analogs of neurotensin(8–13). The data presented are representative of the competition curves obtained when increasing concentrations of analogs of neurotensin(8–13) were assessed for their ability to compete with [^{3}H]neurotensin for binding to intact cells, employing the technique described under Materials and Methods. Binding experiments were performed at 0° to inhibit uptake of radiolabel by the intact cells and to prevent proteolysis of the ligand during the incubation. Each point is the average of triplicates.

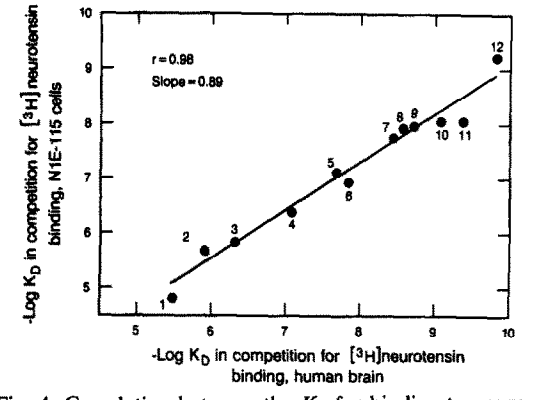

Fig. 4. Correlation between the K_D for binding to neurotensin receptors on intact N1E-115 cells and the K_D for binding to neurotensin receptors in human brain tissue for neurotensin(8–13) analogs. The peptides employed were: 1, [D-Leu^{13}]NT(8–13); 2, [Ala^{11}]NT(8–13); 3, [Gly^{10}]NT(8–13); 4, [D-Lys^{9}]NT(8–13); 5, [Val^{13}]NT(8–13); 6, [D-Arg^{9}]NT(8–13); 7, [Val^{12}]NT(8–13); 8, [Nle^{12}]NT(8–13); 9, NT; 10, [Nva^{12}]NT(8–13); 11, [D-Lys^{8}]NT(8–13); and 12, NT(8–13).

for Leu^{13}, an amino acid with a ramification at the γ carbon; [Val^{13}]NT(8–13) had a 31-fold higher EC_{50} and a 130-fold larger K_D than did NT(8–13). Finally, substitution with the D isomer of Leu^{13} resulted in a neurotensin(8–13) analog with functional capabilities only at very high concentrations. All of the NT(8–13) analogs studied induced the same maximal stimulation of intracellular cyclic GMP production as NT(8–13) itself except for [D-Leu^{13}]NT(8–13), which had an efficacy that was routinely less than that of NT(8–13).

As is seen with intact neuroblastoma N1E-115 cells, neurotensin(8–13) is more potent at inhibiting [^{3}H]neurotensin binding to human brain tissue than is native neurotensin [13]. Several studies have reported the existence of two neurotensin binding sites of high and low affinity in mammalian (including human) brain [see Table 3 in Ref. 13]. We have consistently found one class of neurotensin receptors in human brain, regardless of the region studied or whether the radioligand employed was [^{3}H]neurotensin [13] or [^{3}H]neurotensin(8–13) [20]. Other binding studies, however, have shown that the two reported populations of neurotensin binding sites clearly correspond to a low affinity, high capacity,

levocabastine-sensitive site and a high affinity, low capacity, levocabastine-insensitive site [21]. The low affinity site may be an acceptor or recognition site due to its existence only in murine brain and the fact that it is evenly distributed in all areas of the brain. The high affinity site is considered to be the true receptor site [22]. Neither neuroblastoma N1E-115 cells nor human brain tissue has been reported to bind levocabastine in significant amounts [21, 22].

Six neurotensin analogs and fragments were compared in our earlier report [13] for binding activity at the neurotensin receptors in human frontal cortical membranes and on intact N1E-115 cells. There was a significant correlation between the K_D values for binding to the two different tissues for neurotensin, [D-Trp^{11}]neurotensin, [D-Tyr^{11}]neurotensin, [Gln^4]neurotensin, neurotensin(8–13), and neurotensin(9–13). This strong correlation between the characteristics of neurotensin binding sites in human brain and those of the receptors on intact N1E-115 cells was also indicated in the experiments with NT(8–13) analogs reported here (see Fig. 4). From these data, one may infer that these binding sites in human brain have physiological relevance and that clone N1E-115 provides a very useful model system for studying human brain neurotensin receptors.

Two of the novel NT(8–13) analogs were more potent than neurotensin in binding and biochemical activities with clone N1E-115 as well as in binding affinity for neurotensin receptors in human brain tissue: [D-Lys^8]NT(8–13) and [Nva^{12}]NT(8–13). These analogs are currently undergoing further study. [D-Lys^8]NT(8–13), in preliminary experiments, appeared to be more resistant to rapid degradation by intact N1E-115 cells than was NT(8–13) itself, presumably because of the substitution of Arg^8 with the D isomer of lysine. [^{3}H]NT(8–13) (0.1 μM) incubated with 1×10^6 intact N1E-115 cells/ml at 37° is degraded to an average of 11% of its original level upon only 60 sec of exposure [15, 23]. In initial studies, we compared the degradation rate of unlabeled NT(8–13) with that of [D-Lys^8]NT(8–13) when a peptide concentration of 10^{-5} M was incubated with 3×10^6 cells/ml at 37°, and supernatant was subjected to trichloroacetic acid precipitation and extraction [24] prior to HPLC analysis. Under these conditions, NT(8–13) and its peptide degradation products were totally degraded by the end of the 20-min time course, whereas [D-Lys^8]NT(8–13) appeared to be entirely resistant to proteolysis. Thus, [D-Lys^8]NT(8–13) might be considered a long-lasting, as well as a more active, analog of neurotensin.

In other studies*, [Nva^{12}]NT(8–13) was found to be potent at mediating antinociception upon microinjection into the periaqueductal gray region of the rat brainstem as measured by the hot plate assay. In that investigation, neurotensin was two times more potent than morphine as an analgesic, whereas [Nva^{12}]NT(8–13) was essentially equipotent. Thus, the possibility exists*, aside from a likely involvement of neurotensin and NT(8–13) in the pharmacology of neuroleptics, that analogs of NT(8–13) might, in the future, prove to be powerful analgesics free of some of the undesirable side effects of opiates.

The fact that neurotensin(8–13), in most *in vitro* studies, is equipotent or more potent than native neurotensin, as well as the fact that a number of naturally occurring analogs of NT(8–13) have been isolated and identified, e.g. xenopsin [25], LANT-6 [26], and neuromedin N [27], suggest that the neurotensin(8–13)-like family of peptides may be of physiological importance [19] and, therefore, merits further study, particularly in regard to its role in the human brain.

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