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European Journal of Pharmacology 390 (2000) 107–111

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Highly potent neurotensin analog that causes hypothermia and antinociception

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Received 19 July 1999; received in revised form 11 November 1999; accepted 3 December 1999

Abstract

The tridecapeptide neurotensin has long been proposed as an endogenous neuroleptic. However, for neurotensin [or neurotensin(8–13) [NT(8–13)], the active fragment] to cause its effects, it must be administered centrally. Here, we report on an analog of NT(8–13), (*N*-methyl-Arg),Lys,Pro,L-*neo*-Trp,*tert*-Leu,Leu (NT69L), which contains a novel amino acid, L-*neo*-tryptophan and exhibits high affinity for the rat and human neurotensin receptor, subtype 1. After intraperitoneal (i.p.) injection (1 mg/kg), NT69L induced hypothermia of > 5°C (rectal), with a significant effect persisting for over 7 h. NT69L also caused a rapid (within 15 min) and persistent (for over 5 h) antinociceptive effect, as determined by the hot plate test. NT69L was overall the most potent and longest lasting neurotensin analog that has been reported. These studies provide the background for further testing of a stable, potent and long lasting neurotensin analog as a potential neuroleptic.

Keywords: NT(8–13); NT69L; Hot plate test

1. Introduction

Many studies show that neurotensin is a peptide neurotransmitter capable of exerting potent effects in animals, including hypothermia and antinociception, and that only the last six amino acids of the parent neurotensin are needed for this biological activity (Bissette et al., 1976; Elliott and Nemeroff, 1986; Al-Rodhan et al., 1991; Kasckow and Nemeroff, 1991). However, for neurotensin or neurotensin(8–13) [NT(8–13)] to exert these pharmacological and physiological effects, it must be delivered directly into the brain, due in part to its rapid degradation by peptidases upon systemic administration (Clineschmidt and McGuffin, 1977). Neurotensin mediates its effects through its receptors, which are distributed heterogeneously in the central nervous system with both high (NTS1 receptor) and low (NTS2 receptor) affinity sites (Goedert et al., 1984: Moyse et al., 1987; Kanba et al., 1988). A third neurotensin binding site has also been cloned and described, gp95/sortilin (Mazella et al., 1998).

Centrally administered neurotensin causes a variety of effects similar to those exhibited by neuroleptics including potentiation of sedatives and hypothermia (Bissette and Nemeroff, 1995). Clinically useful typical antipsychotics (e.g., haloperidol) cause an increase in neurotensin levels in both the caudate nucleus and nucleus accumbens, while clozapine, an atypical antipsychotic, causes an increase in neurotensin mRNA in the nucleus accumbens but not the striatum (Merchant et al., 1992). Lastly, we showed that haloperidol, but not clozapine elevates NTS1 receptor mRNA levels in the substantia nigra/ventral tegmental region of rat (Bolden-Watson et al., 1993).

In clinical studies, schizophrenic patients with the lowest levels of cerebrospinal fluid neurotensin suffered from higher levels of pretreatment psychopathology (Sharma et al., 1997). Improvements in overall psychopathology were correlated with increases in cerebrospinal fluid neurotensin concentrations during antipsychotic treatment. Similarly, post-mortem studies of schizophrenic patients show reduced levels of binding sites for neurotensin in the caudate, cingulate, and prefrontal cortices as compared to

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0014-2999/00/$ - see front matter
PII: S0014-2999(99)00877-8

normal controls (Lahti et al., 1998). Therefore, there is a great deal of biochemical and clinical evidence suggesting that neurotensin plays an integral role in the pathology and therapeutic treatment of schizophrenia.

Because of this potentially important correlation, we have been developing a series of neurotensin analogs that would (1) have a high affinity at the human and rat neurotensin receptors, (2) have in vivo (and central) activity as assessed by body temperature and antinociception, and (3) retain in vivo activity upon systemic administration. We measure antinociception because it is a central effect of neurotensin. Also, historically, many anti-schizophrenic drugs cause hypothermia. In fact, chlorpromazine was first used as hypothermic agent (Deniker, 1970).

We previously described two neurotensin agonists, NT66L and NT67L, that meet these criteria (Tyler et al., 1999), but here present some data on a more potent and longer lasting analog, (*N*-methyl-Arg),Lys,Pro,L-*neo*-Trp,*tert*-Leu,Leu (NT69L). Additionally, the role of NT69L as a potential neuroleptic is supported by our observations that it blocked apomorphine-induced climbing behavior in rats (Cusack et al., 2000). In other experiments, it did not induce catalepsy but blocked or reversed the cataleptic effects of haloperidol (Cusack et al., 2000).

2. Materials and methods

2.1. Materials

2.1.1. Synthesis of NT69

L-*neo*-Trp[(2*S*)-2-amino-3-(1*H*-4-indolyl)propanoic acid], a novel analog of tryptophan (Fauq et al., 1998) was synthesized as previously described (Cusack et al., 2000). The amino acid, *N*-methyl-L-arginine was obtained from Bachem (Torrance, CA) as its BOC-*N*-methyl-L-arginine (MTR)–OH derivative for peptide synthesis. Peptides (Table 1) were synthesized by the Mayo Protein Core Facility (Rochester, MN). Briefly, NT(8–13) analogs were synthesized using Fmoc chemistry, with *t*-butyl-protected side chains, on an automated peptide synthesizer (431A, Applied Biosystems, Foster City, CA). Activation, coupling times, amino acid dissolution, coupling solvents, and synthesis scale were followed according to protocols developed in the Protein Core Facility. All peptides were purified by reverse-phase high performance liquid chromatography using a C_{18} column (2.2 × 25 cm, Vydac, Hesperia, CA) in 0.1% TFA/water and a gradient of 10–60% acetonitrile in aqueous 0.1% TFA with a gradient of 10–60% acetonitrile. A combination of analytical high performance liquid chromatography and mass spectrometry (PE Sciex 165B, Foster City, CA) was used to analyze peptide purity and confirm peptide mass weight, respectively.

Table 1
Structure of NT(8–13) and analogs
Table lists the amino acid substitution for various analogs at positions 8–13.

Peptide	8	9	10	11	12	13
NT(8–13)	L-Arg	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu
NT66L	D-Lys	L-Arg	L-Pro	L-*neo*-Trp	*tert*-Leu	L-Leu
NT67L	D-Lys	L-Arg	L-Pro	L-*neo*-Trp	L-Ile	L-Leu
Eisai	*N*-methyl-Arg	L-Lys	L-Pro	L-Trp	*tert*-Leu	L-Leu
NT69L	*N*-methyl-Arg	L-Lys	L-Pro	L-*neo*-Trp	*tert*-Leu	L-Leu

2.2. Methods

2.2.1. Measurement of antinociception and hypothermia

NT69L, related peptide (Table 1), or saline (vehicle) was injected intraperitoneal (i.p.) into male Sprague–Dawley rats (200 g; Harlan, Prattville, AL). Independent groups of animals were used for each dose of NT69L, as well as for each dosing of the related peptides. Animals were maintained on a normal light/dark cycle (12 h each) in our accredited animal facility and testing occurred during the light cycle. The baseline hot plate and body temperature measures were obtained immediately prior to the experiment as described (Tyler et al., 1998a). Hot plate measurements were taken on a metal surface maintained at a temperature of 52.0 ± 0.15°C. Latency was measured as the time that the rat took to lick a hind paw after being placed on the hot plate. Failure to respond in 30 s resulted in ending of the trial and assignment of that latency. Body temperatures were taken using a thermistor probe inserted 3 cm into the rectum.

At various times after injection, the rat was placed on the hot plate and latency was measured. Immediately after each body temperature measurement, the hot plate trial was conducted. Hot plate tests were scored as the percent of maximum possible effect (% MPE) and calculated using the following equation: % MPE = [(post-drug latency − pre-drug latency)/(cut-off − pre-drug latency)] × 100; where the cut-off was 30 s. For these behavioral and physiological measures, $n \geq 5$ in all experiments.

2.2.2. Statistical analysis

Statistical analysis was performed using the rank sum test with $P < 0.05$ being considered significant.

3. Results

NT69L was injected i.p. at a variety of doses and body temperature change, Fig. 1, and was monitored periodically from 10 min post-injection to over 7 h. Saline (vehicle) controls were also tested along with the treated animals. Significant hypothermia was seen at all doses, even at the lowest dose of 0.05 mg/kg, at 15 min post-injection ($P < 0.001$ vs. saline). At 1 mg/kg, a profound hypothermia of greater than 5°C was seen at 90 min

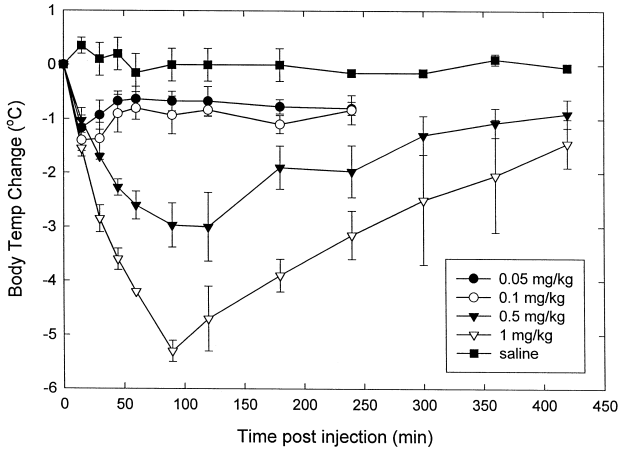

Fig. 1. Hypothermic response to NT69L in rats. Rats received varying concentrations of NT69L injected i.p. (baselines were measured prior to injection). Mean change in body temperature (°C) ± S.E.M. was determined with a thermistor probe inserted 3 cm into the rectum at various times post-injection.

post-injection. This hypothermia was not only large in magnitude, but also very prolonged, since at 7 h post after a single i.p. injection (1 mg/kg), a decrease of 2°C was still evident. This duration of the hypothermic effect was evident at all doses tested and at 4 h post-injection, all groups were significantly different from the saline controls ($P < 0.01$).

Immediately following each body temperature test, animals were also tested for antinociception as scored by the hot plate test. Significant antinociception was found at only the two highest doses, 0.5 and 1 mg/kg (Fig. 2). The antinociceptive effect reached its maximum effect at 90 min post-injection and remained significant in both groups for 5 h post-injection ($P < 0.01$ vs. saline). At 90 min post-injection, dose–response experiments for hypothermia (Fig. 3A) and antinociception (Fig. 3B) revealed similar

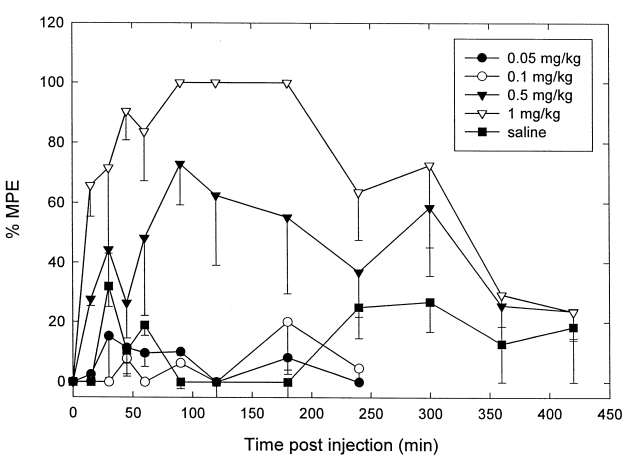

Fig. 2. Antinociceptive response to NT69L in rats. Rats received varying concentrations of NT69L injected i.p. Animals were tested for hot plate antinociceptive response at the times indicated post-injection and % MPE ± S.E.M. was calculated, as described in the Methods section. % MPE is the percent of maximum possible effect.

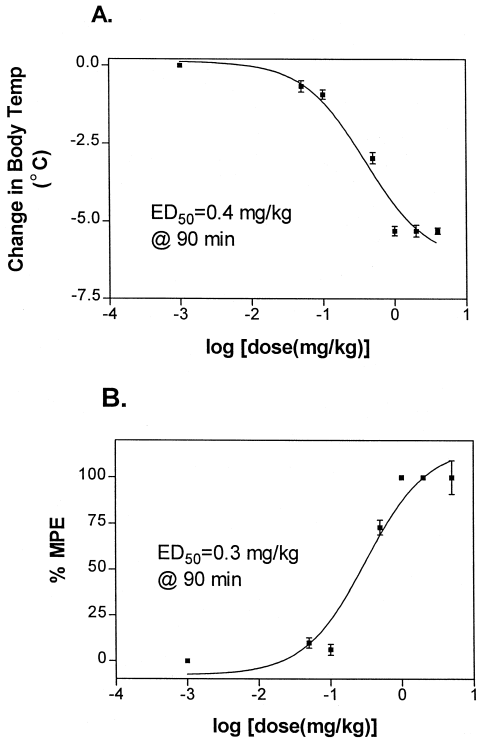

Fig. 3. Dose response curve of NT69L (A) hypothermic response, rats received varying concentrations of NT69L injected i.p. and change in body temperature (°C) was determined with a thermistor probe inserted 3 cm into the rectum at 90 min post i.p. injection, ED_{50} values were calculated by plotting the response vs. log[dose(mg/kg)]; (B) antinociception as scored by % MPE, rats received varying concentrations of NT69L injected i.p. and were tested for hot plate latency 90 min post i.p. injection, ED_{50} values were calculated by plotting the response vs. log[dose(mg/kg)].

ED_{50} values, 0.4 mg/kg (95% confidence interval = 0.10–1.4; $R = 0.98$) and 0.3 mg/kg (95% confidence interval = 0.08–1.28; $R = 0.98$), respectively. There was no statistical difference between the ED_{50} values for these responses ($P = 0.75$). Additional gross observations of animals at all doses included a transient but rapid change (within 30 s of injection) in ear color to bright red. Animals at 1 and 0.5 mg/kg dose also showed a change in the coloration of their feet and tails to pale blue. This discoloration lasted for up to 10 min before normal color returned. Preliminary results suggest that NT69L has oral activity as well, but it seemed to be more potent at inducing hypothermia rather than antinociception via this route at the dose tested 20 mg/kg.

4. Discussion

There are now at least four neurotensin analogs (Eisai compound, NT66L, NT67L, and NT69) that are known to cross the blood brain barrier and cause hypothermia and antinociception. As discussed previously (Tyler et al., 1999), the Eisai compound has a high affinity for the rat

NTS1 receptor but is only about 1/20th as potent at the human receptor. NT69L had a high affinity at both the rat and human cloned NTS1 receptor with K_d's of 0.82 ± 0.18 and 1.6 ± 10.3 nM, respectively (Cusack et al., 2000). Others have shown that patients suffering from schizophrenia show improvement of psychopathology with increased levels of neurotensin in the cerebrospinal fluid (Sharma et al., 1997). Therefore, since one of the ultimate goals of producing a neurotensin analog would be for the treatment of humans, the potency of a compound at the human NTS1 receptor could be a critical factor. Of additional consideration is the magnitude and duration of the effects of a neurotensin analog. NT69L, described here, is overall the most potent and longest lasting neurotensin analog yet described.

Table 1 lists the sequences of the active neurotensin analogs as well as their parent compound, NT(8–13). At equal doses of 1 mg/kg (Fig. 4), NT69L induced almost a twofold greater hypothermic effect than the next most potent compound, NT66L (Tyler et al., 1999). Not only was the hypothermic effect of NT69L larger in magnitude, it persisted at significant levels for far longer (up to 7 h) than any of the other three neurotensin analogs. Interestingly, while the Eisai compound ranked third in potency at causing hypothermia, its duration was also significant for 7 h post-injection. Structurally, there is only one difference between the Eisai compound and NT69L. NT69L contains our novel tryptophan analog, L-neo-Trp (Fauq et al., 1998), at position 11, while the Eisai compound has L-Trp at this position. Thus, the positioning of the side chain has a tremendous impact on the in vivo activity of these compounds. The differences between NT69L and NT66L are at the amino-terminus of the peptide with *N*-methyl-Arg[8] and L-Lys[9] vs. D-Lys[8] and Arg[9], respectively. Clearly, conservative and stereoselective changes within this peptide have a marked effect on the potency and duration of causing this behavioral effect.

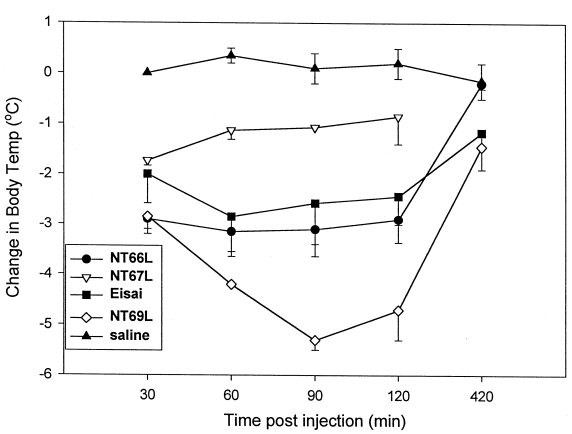

Fig. 4. Comparison of the hypothermic response of various NT analogs in rats. Rats received 1 mg/kg of indicated NT analog injected i.p. Change in body temperature (°C) was determined with a thermistor probe inserted 3 cm into the rectum at various times post-injection.

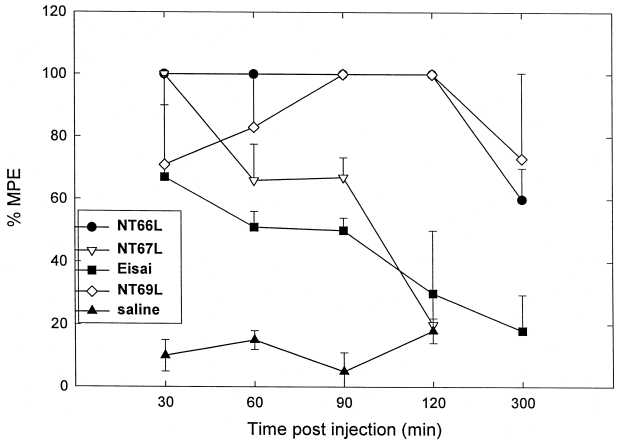

Fig. 5. Comparison of the antinociceptive response to NT analogs in rats. Rats received 1 mg/kg of indicated NT analog injected i.p. Animals were tested for hot plate antinociceptive response at the times indicated post-injection and % MPE ± S.E.M. was calculated, as described in the Methods section. % MPE is the percent of maximum possible effect.

In terms of antinociception, both NT66L (Tyler et al., 1999) and NT69L caused profound and long-lasting effects through 5 h post-injection (Fig. 5). At 1 mg/kg, NT66L, however, had a slightly quicker onset reaching 100% MPE within 15 min while at the same time NT69L produced a strong but not full effect of 70% MPE. Although the Eisai compound is structurally very similar to NT69L, the Eisai compound consistently failed to produce 100% MPE at any time. In addition, its duration was significantly shorter than that of NT69L. The fact that these various analogs had different rank orders of potency in causing antinociception and hypothermia supports our long held contention that there are different receptors and/or pathways that lead to each of these behavioral responses (Tyler et al., 1998b).

Further, supporting the notion of receptor subtypes is the fact that the differences in the magnitude and duration of the two behavioral responses is not explained by affinity at the NTS1 receptor or degradation rates. NT67L actually has the highest affinity for the rat NTS1 receptor, 0.21 nM, the Eisai compound the weakest 5.4 nM, with NT66L and NT69L being in between at 0.85 and 0.82 nM, respectively. Clearly, neither hypothermic nor antinociceptive effects follow this rank order of potency, indicating that other subtypes may be important in causing these two different behavioral effects. All of the compounds are very stable in our plasma degradation assays, with half-lives ranging from 130 to 350 h (Cusack et al., 2000). Thus, if the in vivo stability is even closely reflected by this in vitro plasma assay, degradation of the compounds does not provide an answer to the differences in duration and magnitude of the action of these neurotensin analogs.

Neurotensin behaves like an atypical neuroleptic, such as clozapine, in animal studies (Jolicoeur et al., 1981). We have demonstrated that NT69L has a high affinity at the human and rat NTS1 receptor, is a potent and long lasting

inducer of hypothermia and antinociception, and does not induce catalepsy, while at the same time it blocks and reverses haloperidol-induced catalepsy and blocks apomorphine-induced climbing behavior (Cusack et al., 2000). Since one of our ultimate goals was to produce a series of potential atypical neuroleptics for human use, we think that NT69L (and possibly NT66L and NT67L) may be a good candidate for additional experiments, including human trials in schizophrenic patients.

Acknowledgements

This work was supported by the Mayo Foundation and grants from the N.I.M.H. (ER) and N.I.N.D.S. (BMT).

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