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Non-opioid antinociceptive effects of supraspinal histogranin and related peptides: possible involvement of central dopamine D_2 receptor

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Abstract

The antinociceptive effects of intracerebroventricular (ICV) administration of histogranin (HN) and related peptides were assessed in the mouse writhing and tail-flick assays. In the writhing test, the peptides displayed dose-dependent analgesic effects with an AD_{50} of 23.9 nmol/mouse for HN and the following order for other peptides: HN-(7–15) < histone H4-(86–100) ≈ HN ≈ HN-(7–10) < [Ser[1]]HN < osteogenic growth peptide (OGP) ≈ HN-(1–10). HN-(6–9) and HN-(8–10) did not show any significant analgesic activity at 50 nmol/mouse. The importance of the C- and N-terminal amino acids in the analgesic activity of the peptides was demonstrated by the prolonged effects of HN and [Ser[1]]HN (≈ 30 min) compared with those of HN fragments (HN-(7–15), HN-(1–10) and HN-(7–10): 5–10 min). The analgesic activity of [Ser[1]]HN (50 nmol/mouse) was not affected by the coadministration of opioid (naloxone, 1 nmol/mouse), NMDA (CPP, 0.3 and MK-801, 0.3 nmol/mouse) and D_1 (SCH-23390, 0.5 nmol/mouse) receptor antagonists, but it was significantly antagonized by the coinjection of the D_2 receptor antagonist raclopride (0.5 nmol/mouse). In the mouse tail-flick assay, HN and related peptides (50 nmol/mouse) also showed significant analgesic activity (15–35% MPE). The analgesic effect of [Ser[1]]HN was dose-dependent and, at 75 nmol/mouse, lasted for up to 45 min, and was partially blocked by the coadministration of raclopride (1 nmol/mouse), but not naloxone (2 nmol/mouse). In the mouse rotarod assay, relative high doses (75–100 nmol/mouse) of HN and related peptides did not significantly affect motor coordination. These results indicate that supraspinal administration of HN and related peptides induce significant non-opioid analgesic effects devoid of motor activity by a mechanism that involves the participation of central dopamine D_2 receptors. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Histogranin; Analgesia; Dopamine receptor; Mouse writhing; Tail flick

1. Introduction

Histogranin (HN; H-Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-COOH), a pentadecapeptide whose structure presents 80% homology with that of fragment-(86–100) of histone H4, was first isolated in our laboratory from extracts of bovine adrenal medulla [24]. In the adrenal medulla, the peptide was concentrated in chromaffin granules, and it was released from perfused adrenal glands upon cholinergic receptor stimulation [24]. The immunoreactive peptide was also detected in various rat tissues, including the pituitary, adrenal glands, heart, spleen, lungs, and brain [23]. A possible physiological role for the peptide was suggested by its blockade of *N*-methyl-D-aspartate (NMDA)-induced convulsions in mice [23,24,40]. Recently, [Ser[1]]HN, a chemically stable analog of HN [40], was shown to attenuate hyperalgesia and allodynia caused by sciatic nerve injury [41] and intrathecal (IT) administration of NMDA [18]. In addition, [Ser[1]]HN suppressed the late (NMDA-dependent) phase but not the early phase of pain in the formalin pain assay, suggesting that HN could be an endogenous blocker of tonic or persistent pain [43]. Because NMDA receptors in the dorsal horn of the spinal cord are predominantly involved in the mediation of prolonged nociceptive information [12,30,36,49], the antinociceptive effects of [Ser[1]]HN at this level were then proposed to be due to its ability to modulate the activity of the NMDA receptor.

In in vitro binding studies with rat brain membranes, [^{125}I][Ser[1]]HN displayed a high affinity site with characteristics (saturability, reversibility, sensitivity to trypsin, and

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heat treatments) of a membrane-bound receptor [37]. However, the binding of [^{125}I][Ser^1]HN was unaffected by NMDA and competitive (CPP) and noncompetitive (AP5, MK-801) NMDA antagonists. Polyamines, including spermine and spermidine, were the only NMDA regulatory agents that affected the binding of [^{125}I][Ser^1]HN, although [Ser^1]HN itself was unable to displace the binding of [^{3}H]spermidine [37]. Therefore, it was suggested that HN may bind to its own site in the brain and affect NMDA receptor-mediated activity through an allosteric interaction with the NMDA receptor [35,37]. On the other hand, we have recently observed that [Ser^1]HN was a potent inhibitor of the binding of the σ_1/D_2 receptor ligand [^{3}H](+)3-PPP [48] to rat brain membranes (unpublished observation). The insensitivity of the binding of the specific σ_1 ligand [^{3}H](+)pentazocine to [Ser^1]HN [24] led us to hypothesize that HN could specifically interact with the central dopamine D_2 receptor.

The dopamine D_2 receptor is a G_i protein-bound receptor that shows great structural similarities with the D_3 and D_4 receptors, two other G_i protein-bound receptors [29]. Previous studies have indicated that drugs such as bromocriptine, quinpirole, apomorphine, cocaine, and amphetamine can alleviate pain in various animal models by a mechanism that involves the participation of central dopamine D_2 receptor [9,15,22,25,31]. Their analgesic activity was mainly observed in the writhing [15,17], formalin [2,31] and hot plate [22] assays, the tail-flick assay being somewhat less sensitive to these compounds [17,28]. Herein, we investigated the effects of HN and related peptides in the mouse writhing and tail-flick assays. The results suggest that HN and related peptides can produce, first in the writhing but also in the tail-flick assays, dose-, structure-, and time-dependent non-opioid analgesic effects that are blocked by the D_2 receptor antagonist raclopride with no significant effect on motor function.

2. Materials and methods

2.1. Animals

Mice (male 20–25 g, Swiss–Webster) obtained from Charles River (Canadian Breeding Farm, St. Constant, Quebec) were housed five per cage in a room with controlled temperature (22 ± 2°C), humidity, and artificial light (06:30–19:00 h). The animals had free access to food and water, and were used after a minimum of 4 days of acclimation to housing conditions. Experiment were carried out between 10:00 and 16:00 h in an air-regulated and soundproof laboratory (23 ± 1°C, 40% humidity). Mice were habituated at least 30 min before experiment. The experiments were authorized by the Animal Care Committee of the University of Ottawa in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. Drugs and peptides

(±)3-(2 Carboxypiperazine-4-yl)-propyl-propionic acid (CPP) was purchased from Tocris Neuramin (Buckhurst Hill, Essex, UK). (+)5-Methyl-10, 11-dihydro 5*H*-dibenzo[*a*,*d*]cyclohepten-5, 10-imine hydrogen maleate (MK-801), raclopride and SCH-23390 were obtained from Research Biochemical Inc. (Natick, MA). Naloxone was the product of ENDO laboratory (Garden City, NY). HN and related analogues and fragments were synthesized in our laboratory by the solid-phase procedure as described previously [35]. The purity of the synthetic peptides was assessed by analytical HPLC on μ-Bondapack C18 (Waters) and by thin-layer chromatography on silica gel plates (60 F 254; BDH chemical, Darmstadt, Germany) in the following solvent system (v/v): 1-butanol/acetic acid/water/pyridine (15/3/10/12). Their composition and molecular weight were determined by amino acid analysis of acid (HCl) hydrolysates and fast atom bombardment mass spectrophotometry (FAB MS). Peptides were dissolved in double-distilled sterile water (vehicle), and 10 μl peptide solutions and vehicle were delivered gradually through intracerebroventricular (ICV) injection. Free hand ICV injections into the lateral ventricles of the conscious mouse were made using a No. 27 gauge, 0.25-in. needle attached to 500-μl Hamilton syringe and an automatic dispenser (PB 600; Hamilton Col, Reno, NV). The needle was filled with polyethylene tubing, leaving 3 mm of the needle tip exposed. The site of injection confirmed by injecting Indian ink in preliminary experiments was on an imaginary line drawn through the anterior lobe of the ears and from an imaginary midsagittal line. The whole injection procedure was completed within 10–15 s, so that the animals should suffer minimal discomfort and pain.

2.3. Mouse writhing test

The antinociceptive activity of HN and its related peptides was evaluated using the acetic acid-induced writhing test according to a modification [39] of the method of Hayashi and Takemori [19]. Male Swiss–Webster mice were injected intraperitoneally (IP) with 1.0% acetic acid (10 ml/kg) 5 min (or the indicated time) after ICV injection of 0 (distilled water), or three to five effective doses (between 0.5 and 100 nmol) of HN or related peptides. The number of writhes displayed by each mouse was counted for a period of 10 min after the injection of the acetic acid solution. An abdominal stretch is characterized by the contraction of the abdominal muscles, the arching of the back ventrally such as the abdomen touches the bedding surface, and the extension of one or both hind limbs. Mice were used only once and killed immediately. Groups of 10 mice were used per dose and the average number of writhes per mouse was compared with that of the control group. Antinociceptive activity was expressed as percentage an-

algesia as calculated by the formula: [(mean number of writhes in control group − mean number of writhes for the test group)/(mean number of writhes in control group) × 100]. The percentage analgesia for various effective doses was then used to calculate the AD_{50} by the method of Litchfield and Wilcoxon [27] using the procedure 47 of the computer program of Tallarida and Murray [45]. The times of action of HN and related peptides were determined by injection of 1% acetic acid at various times after the administration of the peptides. The results represent the mean percent analgesia ± SE in groups of 10 mice, and statistical analysis of the analgesic effect of the peptides were carried out by comparison of the number of writhes displayed in peptide-treated groups with that in vehicle-treated group at each time point, using repeated-measures ANOVA, followed by Newman–Keuls multiple comparison test. For antagonism experiment, selective antagonists of opioid, NMDA, D_2 or D_1 receptors were administered ICV in groups of 20 mice in an aliquot of 10 μl, alone or in combination with [Ser^1]HN as indicated. The results are expressed as the mean number of writhes per mouse ± SE. Repeated-measures ANOVA, followed by the Newman–Keuls multiple comparison test, was used to compare the effect of drug combination. The criterion for statistical significance in all tests was $p < 0.05$.

2.4. Mouse tail-flick assay

The nociceptive thresholds of all mice treated with HN and related peptides were also assessed using a noxious reflexive pain model by the heat tail-flick test [11], using a specially designed apparatus (TAILFLICK 7360, UGO-Basile, Varese, Italy). Experiments were performed between 10:00 and 15:00 h. Mice were lightly restrained under paper wadding, and their tails were placed gently on a beam radiation window. Noxious stimulation was provided by a beam of high-density light focused on the tail. The light intensity was set at 40 to give a control reading of about 3 s. The response time latency was measured automatically, and was defined as the interval between the onset of the thermal stimulus and the abrupt flick of the tail. Each determination was performed in at least 10 animals. The mean score was taken as the response latency. A cutoff latency of 12 s was employed to prevent the possibility of tissue damage. The antinociceptive effects in this assay were expressed as the percentage of the maximum possible effect (% MPE), which was calculated by the formula:

$$\% \text{ MPE} = \frac{(\text{postinjection latency} - \text{baseline latency})}{(\text{cutoff latency} - \text{baseline latency})} \times 100$$

The use of % MPE takes into account differences in baseline nociceptive latencies so that these differences will not bias the measurement of antinociception. The significance of the possible analgesic effects of HN and related peptides (ICV) alone or in combination with antagonists were determined using repeated-measures ANOVA followed by Newman–Keuls multiple comparison test at each time point.

2.5. Effects of motor movements

The ability of HN and its related peptides to interfere with the coordinated motor movements was evaluated using a rotarod assay. Impairment of coordinated motor movements was defined as the inability of the mice to remain on a rotarod for a 2-min test period [13]. The rotarod apparatus (UGO-Basile, Varese, Italy) consisted of a rotating bar above a series of plates connected to an automatic counter. Mice were placed upon the bar and the time to fall onto the plate was immediately registered. Two hours prior to evaluation of drug action, mice underwent a condition selection. Rotation velocity was set at 20 rpm. After two trials, mice that remained on the bar for over 120 s (cutoff time) were used for evaluating the effects of peptides. Rotarod assays were conducted 5 and 10 min after the ICV administration of vehicle (distilled water) or peptides at a dose that provided maximal analgesic response in the writhing test. Rotarod performance (up to 120 s), which represented the mean time of mice remaining on the rotating bar was compared between the vehicle-treated group and the peptide-treated groups. Each group consisted of 15–20 animals. Statistical significance was determined using repeated-measures ANOVA followed by Newman–Keuls multiple comparison test.

3. Results

3.1. Dose and structure dependency of HN and related peptides in mouse writhing test

ICV administration of HN and related peptides in mice induced dose- and structure-dependent analgesic activities as assessed by their ability to inhibit writhing in response to IP administration of acetic acid (Fig. 1, Table 1). The chemically stable HN analogue, [Ser^1]HN, displayed an analgesic potency similar to that of HN (AD_{50} of 31.7 nmol/mouse compared with 23.9 nmol/mouse for HN). The C-terminal fragment of HN, HN-(7–15), displayed a shallow dose–response curve with an AD_{50} 2.5 times lower than that of HN, whereas the N-terminal peptide HN-(1–10) showed a sharp dose–response curve with an AD_{50} of 54.8 nmol/mouse. The minimal core for producing the analgesic effect appeared to reside in sequence 7–10 of HN (H-Gly-Gln-Gly-Arg-COOH), because HN-(7–10) had a potency comparable to that of HN itself and the shorter peptides HN-(6–9) and HN-(8–10) did not produce significant analgesic activity at a dose of 50 nmol/mouse (Table 1). The AD_{50} of histone H4-(86–100) [23] and osteogenic growth peptide (OGP) [5], two unmodified

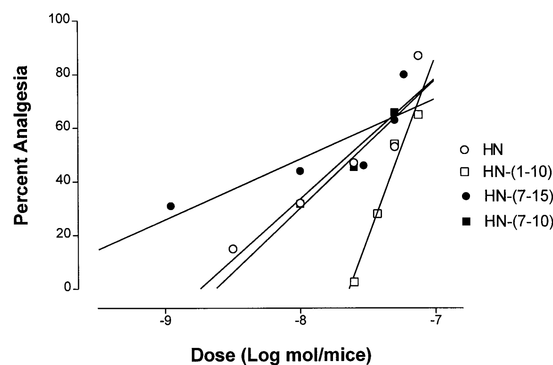

Fig. 1. Dose-dependent analgesic effects of HN and its fragments in the mouse writhing pain assay. Peptides were administered ICV at the indicated doses 5 min prior to IP administration of 1% acetic acid and their analgesic effects were monitored as indicated in Section 2. Results are expressed as the mean percent analgesia (n = 10).

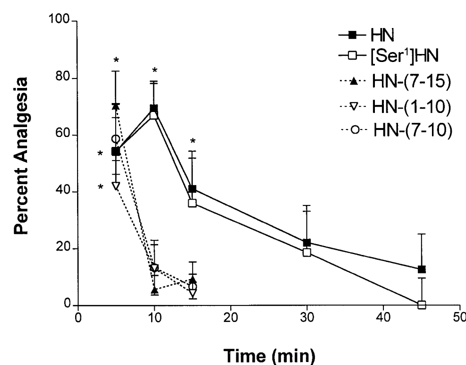

Fig. 2. Time-dependent analgesic effects of HN, [Ser[1]]HN, HN-(7–15), HN-(1–10) and HN-(7–10) in the mouse writhing test. Peptides (50 nmol/mouse, ICV) were administered at various times prior to the administration of 1% acetic acid, and analgesia was monitored as described in Section 2. Results are expressed as the mean percent analgesia ± SE (n = 10). * $p < 0.05$ compared with the control group (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test).

C-terminal fragments of histone H4, were comparable to those of HN and HN-(1–10), respectively.

3.2. Time of action

The time responses of these analgesic effects induced by HN, [Ser[1]]HN, and selective HN fragments are illustrated in Fig. 2. Measured at 5 min after ICV administration, all these five peptides displayed significant analgesia ($p < 0.05$, Newman–Keuls comparison test). HN (50 nmol/mouse) produced an analgesic effect that lasted for about 30 min, with the maximal effect (67 ± 12% analgesia) being observed 10 min after the administration of the peptide. The full-length HN analogue, [Ser[1]]HN, had a time response profile that was comparable to that of HN. The lack of either C-terminal or N-terminal portions of HN resulted in HN fragments with reduced times of action. At 5 min, HN-(7–15) (50 nmol/mouse) produced a stronger analgesic effect than HN itself (70% analgesia compared with 54% analgesia for HN); but its analgesic effect rapidly decreased with only 5.6% analgesia remaining 10 min after the administration of the peptide. Likewise, the HN fragments, HN-(1–10) and HN-(7–10), displayed rapid losses in their analgesic activities.

3.3. Non-involvement of opiate and NMDA receptors

The possible involvement of opiate and NMDA receptors in the analgesic effect of HN in the mouse writhing test was verified by coadministration of [Ser[1]]HN (50 nmol/mouse) with specific opiate (naloxone, 1 nmol/mouse) or NMDA (CPP or MK-801, 0.3 nmol/mouse) receptor antagonists (Fig. 3). Analysis of the mean number of writhes

Table 1
Comparison of AD_{50} dose of HN (ICV) and related peptides in producing analgesia in the mouse writhing pain assay

Peptides	AD_{50} (nmol/mouse) (95% CL)[a]	AD_{50} ratio[b]
HN	23.9 (12.5–45.5)	1.0
[Ser[1]]HN	31.7 (19.2–52.3)	1.33
HN-(7–15)	8.5 (1.9–15.4)	0.35
HN-(1–10)	54.8 (34.8–80.2)	2.29
HN-(7–10)	25.8 (5.9–112)	1.07
HN-(6–9)	NA[c]	–
HN-(8–10)	NA[c]	–
H4-(86–100)	23.5 (14.0–39.4)	0.98
H4-(89–102) (OGP)	52.6 (35.7–77.7)	2.20

[a] 95% Confidence limit.

[b] As compared with HN.

[c] NA: Not active at 50 nmol/mouse, the mean number of writhes was not significantly different from that in the control group (6.5 writhes/mouse for HN-(6–9) and 6.1 writhes/mouse for HN-(8–10) compared with 7.2 writhes/mouse in the control group). OGP: osteogenic growth peptide. H4: histone H4.

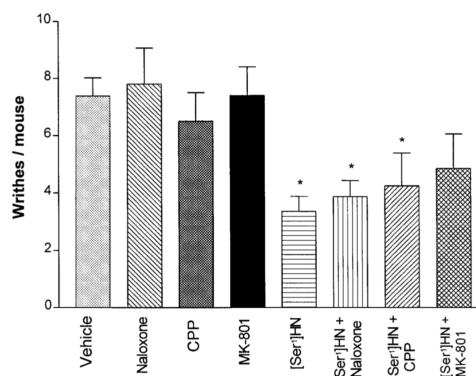

Fig. 3. Effects of opioid (naloxone) and NMDA (CPP, MK-801) receptor antagonists on the analgesic effect of [Ser[1]]HN in the mouse writhing test. [Ser[1]]HN (50 nmol/mouse, ICV) was administered alone or in combination with naloxone (1 nmol/mouse), CPP or MK-801 (0.3 nmol/mouse) 5 min prior to IP administration of 1% acetic acid, as indicated in Section 2. Results are expressed as the mean number of writhes ± SE (n = 20). * $p < 0.05$ is considered significant as compared to the control group (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test).

in animals treated with [Ser¹]HN alone (3.3 ± 0.5) compared with that of the control group (7.4 ± 0.6) revealed a significant effect corresponding to 54.5 ± 7.4% analgesia ($p < 0.05$, Newman–Keuls multiple comparison test). The mean numbers of writhes in mice treated with naloxone (7.8 ± 1.3), CPP (6.5 ± 1.0), or MK-801 (7.4 ± 1.0) alone did not differ significantly from that of vehicle-treated mice. Coadministration of naloxone with [Ser¹]HN did not modify the antinociceptive activity of the peptide (4.2 ± 0.5 writhes compared with 3.3 ± 0.5 for [Ser¹]HN alone; $p > 0.05$). Similarly, the coadministration of the NMDA antagonists CPP or MK-801 with [Ser¹]HN did not affect the analgesic activity of the peptide (4.5 ± 0.9 or 4.9 ± 1.2 writhes; $p > 0.05$).

3.4. Specific blockade by the D_2 receptor antagonist raclopride

Fig. 4 shows the effects of D_2 (raclopride) and D_1 (SCH-23390) receptor antagonists on the antinociceptive activity of [Ser¹]HN (50 nmol/mouse) in the mouse writhing test. Raclopride (Fig. 4A) and SCH-23390 (Fig. 4B) administered alone (0.5 nmol/mouse, ICV) did not affect the pain response. Coadministration of raclopride with [Ser¹]HN significantly and almost completely reversed the antinociceptive activity of the peptide, increasing the mean number of writhes from 3.2 ± 0.9 (mice receiving [Ser¹]HN alone) to 6.9 ± 1.3 ($p < 0.05$) (Fig. 4A). In contrast, coadministration of SCH-23390 with [Ser¹]HN failed to significantly affect the analgesic activity of the peptide (3.0 ± 1.05 writhes compared with 2.4 ± 0.9 in [Ser¹]HN-treated group, $p > 0.05$) (Fig. 4B).

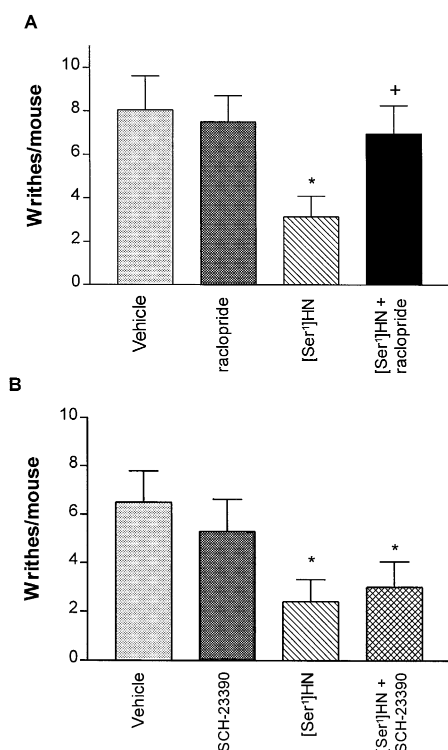

Fig. 4. Effects of D_2 and D_1 receptor antagonists on the analgesic effect of [Ser¹]HN in the mouse writhing test. [Ser¹]HN (50 nmol/mouse, ICV) was administered alone or in combination with raclopride (0.5 nmol/mouse): (A) or SCH-23390 (0.5 nmol/mouse), (B) 5 min prior to IP administration of 1% acetic acid. Results are expressed as the mean number of writhes ± SE (n = 20). * $p < 0.05$ is considered significant compared with the control group; $^{+}p < 0.05$ is considered significant compared with the group of mice treated with [Ser¹]HN alone (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test).

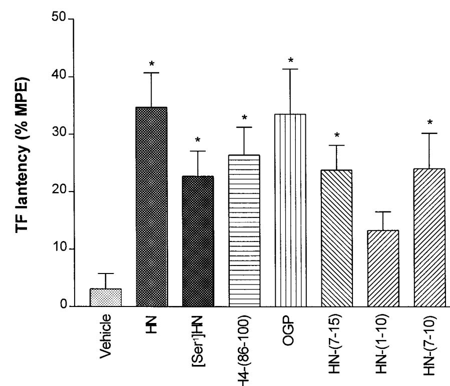

Fig. 5. Analgesic effects of HN and related peptides in the tail-flick assay. Peptides were administered ICV at 50 nmol/mouse 10 min prior to the tail-flick assay as described in Section 2. Results are expressed as the mean percentage of maximal possible effect (% MPE) ± SE (n = 10). * $p < 0.05$ is considered significant compared with that of the vehicle-treated group (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test).

3.5. Antinociceptive activity of HN and related peptides in mouse tail-flick assay

HN and some related peptides, which were previously shown to be effective in the mouse writhing test, were also tested in the mouse tail-flick assay, an animal model of acute reflex type of pain. When examined at 10 min after administration, all tested peptides (50 nmol/mouse, ICV), except for HN-(1–10), induced small but significant increases (22.7–34.7% MPE, $p < 0.05$) in the TF latency compared with the vehicle-treated group (3.4% MPE), the most efficient peptides being HN and OGP (Fig. 5). [Ser¹]HN was chosen to assess the dose and time dependence of this increase in pain reflex latency (Fig. 6A). Significant analgesia was observed up to 45 min after ICV administration of 75 nmol/mouse of the peptide. At a lower dose (50 nmol/mouse), the peptide produced a smaller analgesic effect that reached significance only at 10 min after the administration. Finally, the analgesic effect of [Ser¹]HN (75 nmol/mose) was significantly reversed by the raclopride (1 nmol/mouse) at 20, 30, and 45 min after combination treatment ($p < 0.05$, Newman–Keuls multiple comparison test), but not by naloxone (2 nmol/mouse) (Fig. 6B).

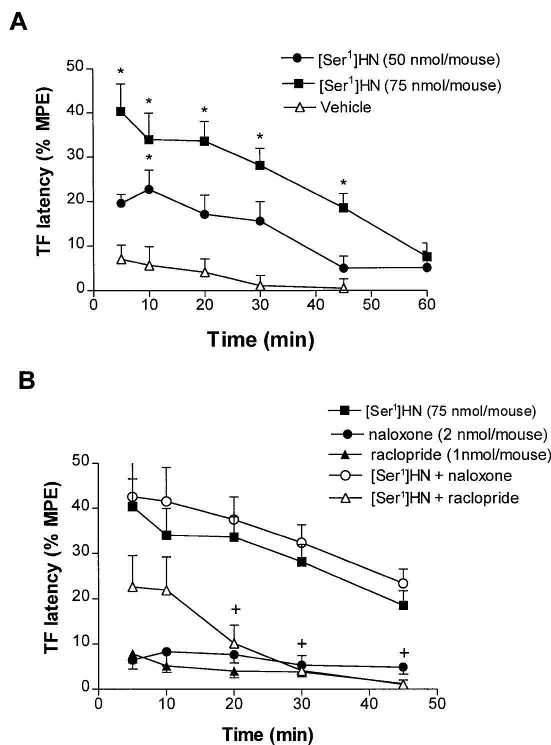

Fig. 6. (A) Time- and dose-dependent analgesic effects of [Ser[1]]HN in the mouse tail-flick assay. Mice were administered with [Ser[1]]HN (50 or 75 nmol ICV) or vehicle at the indicated times prior to the measurement of analgesia. Results are expressed as the mean percentage of maximal possible effect (% MPE) ± SE ($n = 10$). $^{*}p < 0.05$ is considered significant compared with the vehicle-treated group (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test). (B) Effect of D_2 (raclopride, 1 nmol/mouse) and opioid (naloxone, 2 nmol/mouse) receptor antagonists on [Ser[1]]HN (75 nmol/mouse) induced-analgesia in the mouse tail-flick assay. $^{+}p < 0.05$ is considered significant compared with the [Ser[1]]HN-treated group (repeated-measures ANOVA followed by the Newman–Keuls multiple comparison test).

3.6. *Effects of HN and related peptides in the mouse rotarod assay*

The possibility that HN and related peptides affect motor coordination was evaluated by the mouse rotarod assay (Table 2). At the highest doses tested in the mouse writhing test, none of the HN peptides or fragments significantly affected the abilities of mice to stay on the rotarod as assessed by the percentage of mice falling and the mean performance in each group of animals (p>0.05 compared with vehicle-treated animals, Newman–Keuls multiple comparison test).

4. Discussion

HN was first isolated from the bovine adrenal medulla [24], a tissue recognized to contain various pain-reducing substances, including the endogenous opioid peptides Met- and Leu-enkephalins and catecholamines [7,26]. Recently, Sagen et al. [38,42,44] have developed a technique of transplantation of adrenal medullary tissue or chromaffin cells into the spinal subarachnoid space for the alleviation of pain in various rat models of tonic and chronic pain. While most of the analgesic effects of adrenal transplants were attributed to opioid peptides and catecholamines released from adrenomedullary chromaffin cells, the alleviation of the noxious response in the second phase of the formalin assay was not completely antagonized by naloxone and phentolamine, alone or in combination [44], suggesting that other factor(s) were involved in the mediation of the analgesic activity of the adrenal transplants. Recently, the same group of investigators found that IT administration of [Ser[1]]HN, a stable synthetic analogue of the adrenal medullary peptide HN [40], can mimic the beneficial effects of adrenal transplants [18,41,43]. The analgesic activity of [Ser[1]]HN was then ascribed to its property to inhibit the NMDA receptor [23,35,40] on the basis that the spinal NMDA receptor plays an important role in the mediation of prolonged nociceptive transmission [12,30,36,49]. However, no attempt was made to define the nature of the receptor involved in the actions of the HN peptide at this level.

The present data show that supraspinal administration of HN and related peptides induce marked antinociceptive activity in the mouse-writhing and tail-flick assays. The

Table 2
The effects of HN and related peptides in the mouse rotarod assay

		% of mice falling[a]		Rotarod performance[b]	
Treatment	Dose (nmol/mouse)	5 min[c]	10 min[c]	5 min[c]	10 min[c]
Vehicle	–	8.8	11.1	112.1 ± 4.5	110.7 ± 6.6
HN	75	10	20	114.8 ± 5.2	102.9 ± 11.4
[Ser[1]]HN	75	0	0	120.0 ± 0.0	120.0 ± 0.0
HN-(1–10)	75	13.3	6.7	110.7 ± 6.3	115.1 ± 4.9
HN-(7–15)	75	11.1	0	109.4 ± 10.6	120.0 ± 0.0
H4-(86–100)	75	26.7	20	96.8 ± 10.1	99.9 ± 10.8
H4-(89–102) (OGP)	100	20	20	105.8 ± 7.8	103.5 ± 10.5

[a] Percentage of mice falling during the 2-min assay.
[b] Mean times ± SE for the mice to remain on the rotarod.
[c] Time interval after ICV administration of the peptides or vehicle.

nonreversal of the analgesic effects of [Ser[1]]HN by naloxone in both tests confirms their nonopioid nature. While a large body of evidence supports the potential utility of NMDA antagonists (IT) for the treatment of chronic pain [10,12,36,49], the supraspinal administration (ICV) of competitive (CPP, AP5) and noncompetitive (MK-801) NMDA antagonists has been shown not to affect nociception [32]. These data are consistent with our results, which show the non-effectiveness of the competitive (CPP) and noncompetitive (MK-801) NMDA antagonists (ICV) in the mouse writhing test (Fig. 3). Therefore, the analgesic activities of HN and related peptides in the current assays cannot be ascribed to the NMDA receptor antagonist properties of the peptides [23,35,40].

On the other hand, there exists a descending pain control pathway [14], the major components being the midbrain periaqueductal gray (PAG), several nuclei of the rostral ventral medulla (RVM) and the spinal dorsal horn. The excitation of the RVM by the PAG projection is mediated partly by excitatory amino acids (EAA) [1,14]. EAA agonists injected into the PAG and some of the nuclei of RVM suppressed nociceptive responses in the spinal dorsal horn [1,21,50]. Therefore, the analgesic effects of supraspinal HN and related peptides could be due to the stimulation of EAA receptors. Such a possibility was also suggested by the observation that high doses of [Ser[1]]HN (IT) produce NMDA agonist-like behavior [41,43]. However, the inability of the NMDA antagonists (CPP and MK-801) to block the analgesic activity of [Ser[1]]HN in the mouse writhing test (Fig. 3) indicated that such effect of the peptide does not involve the stimulation of the NMDA receptor.

The observation that the D_2 selective antagonist raclopride markedly antagonizes the analgesic effects of [Ser[1]]HN in the mouse writhing and tail-flick assays suggests that HN and related peptides produce their effects by an interaction with some central dopamine D_2-like receptor(s) either directly or through a modulation of dopaminergic transmission. Several studies have indicated that both D_2 agonists and indirect dopamine receptor stimulants produce analgesia in different animal models of pain [15,17,22,31]. Their analgesic effects were suggested to be mediated through enhanced dopamine D_2 receptor transmission in terminals of the mesolimbic pathway. In support of this concept, Altier and Stewart [2] have recently reported that the analgesic effects of amphetamine infused into the nucleus accumbens septi are prevented by raclopride.

The supraspinal sites responsible for the analgesic effects of dopamine D_2 agonists have not yet been clearly identified, but it was recently proposed that activation of mesolimbic dopamine neurons arising from the cell bodies of the ventral tegmental area and projecting to the nucleus accumbens may result in an inhibition of tonic pain either directly at the level of the nucleus accumbens where D_2 receptors are present or indirectly through fibers projecting from the nucleus accumbens to the medial thalamus and/or amygdala [3]. On the other hand, the dopamine D_2 receptors located in the nucleus raphe magnus have also been reported to be involved in the analgesic effects of dopamine agonists [34]. Stimulation and inhibition of descending inhibitory [34] and facilitatory [46] pathways, respectively, in the RVM would explain the analgesic effects of D_2 agonists at this level. Even though we may anticipate that the above-mentioned supraspinal sites could be involved in the D_2-like analgesic activity of HN and related peptides, the central sites of action of the peptides remain to be established.

While some D_2 receptor-mediated behaviors such as locomotion and stereotypy are expressed only when the D_1 receptor system is costimulated [4,8,20], the analgesic effects of direct D_2 receptor agonists or indirect dopamine receptor stimulants generally do not involve the activation of the D_1 receptor [6,9,15,33]. The non-involvement of the D_1 receptor in the analgesic response to [Ser[1]]HN was evidenced by its insensitivity to the D_1 receptor antagonist SCH-23390. Thus, the analgesic activities of HN and related peptides, like those of other analgesics acting through the D_2 receptor, did not involve the participation of the D_1 receptor.

Another approach to elucidate the site of action of HN and related peptides was provided by the structure–activity relationship (SAR) study of selected HN analogues and fragments. Analysis of SAR revealed several interesting characteristics for the analgesic effects of HN and related peptides. First, in accordance with previous findings [35,37], [Ser[1]]HN, a chemically stable analog of HN, displayed the same range of potency as HN in the mouse writhing test, suggesting that the synthetic analogue possesses the structural requirements of the natural peptide for biological activity. However, in contrast with previous findings, which indicated that the removal of a single amino acid from either the N- or C-terminals provides HN fragments with dramatic losses of activities in the [^{125}I][Ser[1]]HN binding [37] and anticonvulsant [35] assays, the analgesic potencies of the N-terminal (HN-(1–10)) and C-terminal (HN-(7–15)) fragments were largely retained and even improved compared with that of HN (Fig. 1, Fig. 5, and Table 1). These data indicate that the mechanism underlying the analgesic activities of supraspinal HN and related peptides in the mouse writhing and tail-flick assays is distinct from that involved in their binding to the high affinity HN site in the brain [37] and inhibition of NMDA-induced convulsions [35].

The non-parallelism of the dose–response curves obtained with HN and its C- and N-terminal fragments in the mouse writhing test (Fig. 1) may be explained either by the involvement of more than one receptor site or by different rates of metabolism for the different peptides. However, the dose–response curves were established at 5 min after the administration of the peptides when highest or close to highest analgesic activity was observed with all peptides. Therefore, the time factor should not have influenced much

the shape of the dose–response curves. On the other hand, HN and related peptides may bind to a still uncharacterized low-affinity central HN receptor and produce analgesia through an allosteric modulation of D_2 receptor conformation. Such indirect effects of the peptides could account for the non-parallelism of the dose–response curves. The involvement of one or multiple receptor sites in the analgesic effects of HN and related peptides remains to be established.

The minimal active core for inhibition of mouse writhing in response to visceral pain appears to reside in the middle portion (amino acid sequence 7–10) of HN, because HN-(7–10) displayed an AD_{50} comparable to that of HN, while HN-(8–10) and HN-(6–9) were inactive at the dose of 50 nmol/mouse. Even though both C- and N-terminal portions of HN were not essential for production of the analgesic activity, their presence markedly prolonged the action of the peptides (Fig. 2). Thus, HN-(1–10), HN-(7–15) and HN-(7–10), possibly due to their reduced resistance to the action of peptidases, displayed short times of action compared with the full-length peptides, HN and [Ser^1]HN. Fragment-(86–100) of histone H4, a pentadecapeptide in histone H4 that shows 80% structural homology with HN [23], displayed an AD_{50} comparable to that of HN (Table 1). OGP [5], another C-terminal fragment of histone H4, was less potent than HN with an AD_{50} 2.2 times greater than that of HN (Table 1). The possible processing of histone H4 into its C-terminal fragment, H4-(86–100), has not yet been demonstrated, but the recent finding of the presence of histone immunoreactivity on the cell surface of lymphocytes [47], the existence of C-terminal histone H4 related peptides in blood plasma (OGP) [5] and HN in the adrenal medulla [24] are compatible with the hypothesis that histone H4 or some variant of histone H4 such as H4-v.1 [16] may generate C-terminal histone H4 fragments with extranuclear and/or extracellular function(s).

ICV administration of HN and related peptides in mice significantly but less effectively attenuated the pain response in the tail-flick assay (a thermal acute pain assessed by a reflex response) than the writhing test. The tail-flick assay was recognized to be less sensitive to the analgesic effects of non-narcotic analgesics [10]. Although the analgesic effects of drugs acting through the D_2 receptor were well pronounced in animal models of abdominal pain [15], hyperalgesia associated with peripheral nerve injury [31] and the late phase of pain due to formalin [25], the antinociceptive effects of D_2 agonists in the tail-flick assay were less prominent [17,28]. Therefore, the observation that HN and related peptides are less potent and efficient analgesics in the tail-flick than writhing assays correlates with the previous findings, which indicated that the tail-flick assay is less sensitive than other tonic pain assays to D_2 agonists. On the other hand, the possibility that the analgesic effects of HN and related peptides in both writing and tail-flick assays resulted from an impairment of motor functions was weakened by the observation that the peptides (ICV) at doses that produced maximal analgesic activities, did not impair motor function as assessed by the mouse rotarod assay (Table 2).

In conclusion, these results, along with previous findings, indicate that HN and related peptides are potent and effective analgesics in various animal models of pain. While the analgesic properties of the HN and related peptides in the mouse writhing and tail-flick assays most likely involve the participation of dopamine D_2-like receptor(s), the possible involvement of this (these) receptor(s) in other pain-relieving activities (tonic pain induced by formalin [43], hyperalgesia induced by sciatic nerve injury [41], and IT administration of NMDA [18]) remains to be established.

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