Bioactive Peptidic Analogues and Cyclostereoisomers of the Minimal Antinociceptive Histogranin Fragment-(7−10)

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Novel analogues of the minimal antinociceptive histogranin (HN) fragment Gly[7]-Gln-Gly-Arg[10], in which amino acids in positions 8, 9, and 10 were replaced by lipophilic amino acids and corresponding D-amino acid residues in combination with N- to C-terminal cyclization, were synthesized and tested in various animal models of pain. All synthetic compounds were potent and efficacious analgesics in the mouse writhing test. Cyclic [-Gly-Ala-Tyr-D-Arg-] (**9**) and cyclic [-Gly *p*-Cl-Phe-Tyr-D-Arg-] (**10**) were the most potent analgesics, being 17 and 135 times as potent as HN, respectively (AD_{50} of 1.37 and 0.17 nmol/mouse icv, as compared with 23 nmol/mouse for HN). The times of action of compounds **9** and **10** were also much improved with half-maximal effects still being observed 60 min and >90 min after their administration, respectively, as compared with 8.1 min for the parent peptide HN-(7−10) and 22.1 min for HN. At analgesic doses, compounds **9** and **10** were devoid of motor effect as assessed by the mouse rotarod assay. As already observed with HN, compounds **9** (10 nmol/rat; i.t.) and **10** (0.5 nmol/rat; i.t.) were effective in blocking persistent inflammatory pain in the formalin test and hyperalgesia induced by intraplantar administration of complete Freund adjuvant. In addition, the analgesic effects evoked by compounds **9** (10 nmol/mouse; icv) and **10** (1 μmol/kg; i.v.) in the mouse writhing test and compound **9** (10 nmol/mouse; icv) in the mouse tail flick assay were similarly antagonized by the dopamine D_2 receptor antagonist raclopride (1 nmol/mouse; icv) but not the opiate antagonist naloxone (1 nmol/mouse; icv). Finally, the various cyclic compounds competed with the binding of [^{3}H]raclopride in rat brain membrane preparations. Their ability to compete with the binding of the D_2 ligand correlated well with their potency in alleviating pain in the mouse writhing test ($r = 0.95$). These results indicate that the analgesic activity of the minimal active core in HN can be improved by changes that favor its interaction with the dopamine D_2 receptor.

Introduction

The pentadecapeptide histogranin (HN[1]; MNYALKGQGRTLYGF) was first discovered in the bovine adrenal medulla.[2] Thereafter, the peptide was also found to be present in various rat tissues including the pituitary, adrenal glands, lungs, heart, and brain.[3] In vivo, HN and related peptides display analgesic activity in various mouse and rat models of pain. Supraspinal (icv, 0.5−50 nmol) administration of HN and related peptides in mice produced strong and moderate analgesia in the AcOH-induced writhing and radiant heat-induced tail flick assays, respectively.[4] Such effects were insensitive to the coadministration of the opiate antagonist naloxone. A high affinity binding site for the stable HN analogue [^{125}I][Ser[1]]HN was characterized in rat brain membranes.[5] The binding characteristics of HN and related peptides correlated to their abilities to inhibit *N*-methyl-D-aspartate (NMDA; icv)-induced convulsions in mice.[6] In agreement with this finding, Sagen and colleagues[7] reported that i.t. administration of [Ser[1]]-HN in rats caused analgesia in rat pain models where the NMDA receptor is known to play a role.[8] Thus, the second phase (tonic) of pain in the formalin test[7a] and hyperalgesia and allodynia consecutive to sciatic nerve injury[7b] were suppressed with low doses (0.1−0.5 nmol) of [Ser[1]]HN. In addition, [Ser[1]]HN (i.t.) dose-dependently blocked hyperalgesia and allodynia induced by i.t. administration of NMDA[7c] and hindpaw administration of complete Freund's adjuvant (CFA).[7d] Therefore, the antinociceptive effects of HN and related peptides in these tests were attributed to their abilities to modulate NMDA receptor functions.[2,3]

However, a large body of evidence suggests that the antinociceptive effects of HN and related peptides may also involve the participation of the dopamine D_2 receptor. First, dopamine D_2 agonists are potent modulators of NMDA receptor functions.[9] Second, high concentrations of immunoreactive HN were observed in the striatum, medulla, and hippocampus,[3] three brain areas rich in dopamine D_2 receptor.[10] Third, HN and related peptides have a pattern of analgesic activity that resembles that of D_2 agonists,[11] alleviating pain more efficiently in the mouse writhing assay than in the mouse tail flick assay[4] and being effective analgesics in the formalin test only during the second (tonic) phase of pain.[7a] Fourth, the analgesic effects of HN and related peptides in the mouse writhing and tail flick assays are blocked by the D_2 receptor antagonist raclopride.[4]

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Table 1. Physicochemical Characteristics of Synthetic HN and Related Linear and Cyclic Tetrapeptides

no.	compd	structure	purification[a] yield (mg/%)	purity[b] (%)	TLC-Rf[c]	K′ (HPLC)	ES-MS or FAB-MS
1	HN	Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-OH	167 (30)	95	0.45	3.80[d]	1720.1 $(M + H)^+$
2	HN-(7−10)	Gly-Gln-Gly-Arg-OH	60 (28)	95	0.16	0.51[e]	417.2 $(M + H)^+$
3	Ala^9HN-(7−10)	Gly-Gln-Ala-Arg-OH	65 (30)	95	0.14	0.52[e]	431.2 $(M + H)^+$
4	cyclic [Ala^9]HN-(7−10)	cyclic[Gly-Gln-Ala-Arg]	65 (20)	98	0.24	2.25[e]	412.4 $(M + H)^+$
5	cyclic [Ala^9, D-Arg^{10}] HN-(7−10)	cyclic[Gly-Gln-Ala-D-Arg]	50 (15)	98	0.44	2.46[e]	412.4 $(M + H)^+$
6	cyclic [Tyr^9]HN-(7−10)	cyclic[Gly-Gln-Tyr-Arg]	36 (10)	98	0.56	2.70[e]	505.0 $(M + H)^+$
7	cyclic [Tyr^9, D-Arg^{10}] HN-(7−10)	cyclic[Gly-Gln-Tyr-D-Arg]	45 (11)	97	0.53	2.69[e]	505.0 $(M + H)^+$
8	cyclic [D-Gln^8, Tyr^9, D-Arg^{10}] HN-(7−10)	cyclic[Gly-D-Gln-Tyr-D-Arg]	40 (10)	97	0.60	2.73[e]	505.0 $(M + H)^+$
9	cyclic [Ala^8, Tyr^9, D-Arg^{10}] HN-(7−10)	cyclic[Gly-Ala-Tyr-D-Arg]	40 (11)	98	0.75	2.95[e]	448.3 $(M + H)^+$
10	cyclic [*p*-Cl-Phe^8, Tyr^9, D-Arg^{10}]HN-(7−10)	cyclic[Gly *p*-Cl-Phe-Tyr-D-Arg]	50 (11)	98	0.77	4.47[e]	558.3 $(M + H)^+$
11	cyclic [*p*-NH_2-Phe^8, Tyr^9, D-Arg^{10}]HN-(7−10)	cyclic[Gly *p*-NH_2-Phe-Tyr-D-Arg]	50 (11)	98	0.52	4.19[d]	539.2 $(M + H)^+$

[a] Product was purified as described under the Experimental Section. [b] By analytical HPLC on μ-Bondapak C-18. [c] B:A:W:P (*n*-butanole:AcOH:water:pyridine; 15:3:10:12). [d] Analytical RP-HPLC (Bondpak C-18) with the gradient of 15−65% water−acetonitrile, 0.1% TFA over 60 min. [e] Analytical RP-HPLC (Bondpak C-18) with the gradient of 0−50% water−acetonitrile, 0.1% TFA over 50 min.

Finally, HN and related peptides are potent competitors of the binding of [^{3}H]raclopride to rat brain membranes and a good correlation was observed between their abilities to cause analgesia in the mouse writhing test and inhibit [^{3}H]raclopride binding.[12] The present study was aimed at investigating the role of pharmacophores in HN, which confer to the peptide its efficacy as analgesic in various animal models of pain as well as its ability to interact with the dopamine D_2 receptor. The introduction in the HN minimal active core HN-(7−10), Gly-Gln-Gly-Arg, of aromatic amino acids present in the C-terminal portion of the most potent HN fragment HN-(7−15),[4] combined with D-amino acid substitutions and N- to C-terminal cyclization, provided HN-like cyclic tetrapeptides with improved analgesic potency, efficacy, and times of action and no motor effect at analgesic doses.

Chemistry

HN (**1**), HN-(7−10) (**2**), and [Ala^9]HN-(7−10) (**3**) were prepared by the solid phase peptide synthesis methodology as described previously.[6] The cyclic analogues of HN-(7−10) (**4**−**11**) were constructed via manual solid phase synthesis on Kaisers' 4-nitrobenzophenone oxime resin using standard Boc protection strategy.[13] Cyclization was made on the resin to take advantage of a self-diluting effect, which favors unimolecular reactions over the formation of oligomeric byproducts via a bimolecular mechanism.[14] The simultaneous cyclization and cleavage method on the oxime resin provided convenient chemical manipulations for obtaining the products with moderate yield and high purity (95−98%).[14,15] In addition, the yield of products significantly depended on the sequence of the linear peptide assembled on the resin. Because Arg(Tos)−oxime resin is unstable and undergoes spontaneous cleavage with the formation of a lactam,[16] Boc-Gly was chosen as the first amino acid for coupling on the oxime resin. The peptide chain was elongated by using PyBOP/HOBt as coupling agents, and after the final coupling step, deprotection of Boc-Xaa^8-Yaa^9-(L/D)Arg(Tos)-Gly oxime resin was achieved with 25% TFA in CH_2Cl_2 (DCM). Then, DIEA and AcOH (2.0 mol equivalent, respectively) in DMF were added to the peptide−resin. An excess of DIEA was employed to complete the liberation of the free amino group from the TFA salt, allowing the simultaneous cyclization and cleavage of the peptides from the resin. AcOH was used to activate the oxime group by keeping the reaction media in a neutral to slightly acidic condition. The completion of the cyclization procedure was indicated by the lack of reaction with ninhydrin.[17] The cleaved cyclic peptides were then treated with liquid HF to remove the remaining protecting group(s) and purified by preparative RP-HPLC (Table 1). The purity and identity of the synthetic compounds were assessed by analytical RP-HPLC, TLC, and MS (Table 1).

Table 2. Comparison of AD_{50}, Potency Ratio, and Half-Maximal Response Decays of HN and Related Linear and Cyclic Tetrapeptides in the Mouse Writhing Pain Assay

compd	AD_{50} (nmol/mouse)[a] (95% CL)[b]	potency ratio (95% CL)[b]	time of half-maximal response decay (min) [dose, in nmol]
1	23.0 (12.5−47.0)	1.0	22.1 [50]
2	25.9 (5.9−112)	0.90 (0.11−7.92)	8.1 [50]
3	3.42 (0.80−14.6)	6.72 (0.86−58.7)[c]	8.2 [10]
4	13.3 (1.54−113)	1.73 (0.11−30.5)	20.0 [10]
5	7.05 (2.55−19.5)	3.26 (0.64−18.4)[c]	
6	4.21 (1.79−9.79)	5.46 (1.27−26.2)[c]	15.0 [10]
7	3.65 (1.31−10.2)	6.30 (1.22−35.8)[c]	24.1 [10]
8	2.26 (1.47−3.49)	10.2 (3.58−32.0)[c]	75.0 [10]
9	1.35 (0.56−3.22)	17.0 (3.88−83.9)[c]	60 [10]
10	0.17 (0.06−0.46)	135 (27.2−783)[c]	>90 [2.5]
11	2.52 (2.02−3.50)	9.12 (3.57−23.2)[c]	60 [10]

[a] For comparison, HN-(7−15) had an AD_{50} value of 8.5 nmol per mouse and a potency ratio of 2.7 as compared with HN (compound **1**).[4] [b] 95% confidence limit. [c] $P \leq 0.05$ as compared with HN.

Results

HN, HN-(7−10) and related linear and cyclic peptides were first evaluated for their ability to inhibit writhing in response to i.p. administration of AcOH in mice.[18] The introduction of Ala (compound **3**) at position 9 of HN-(7−10) (compound **2**) caused a marked increase in its antinociceptive potency (AD_{50} of 3.24 nmol/mouse as compared with 25.9 and 23.0 nmol/mouse for compound **2** and HN, respectively; Table 2). However, the analgesic effect of the peptide did not last long since a 50%

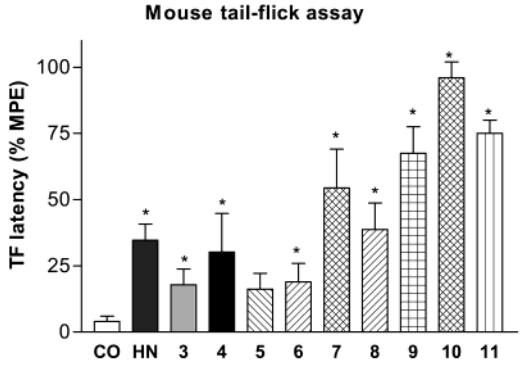

Figure 1. Analgesic effects of HN and related linear and cyclic tetrapeptides in the mouse tail flick assay. Peptides were administered icv at 50 nmol for compound **1**, 2.5 nmol for compound **10**, and 10 nmol for the other compounds 30 min prior to the tail flick assay as described in the Experimental Section. For each value, $n = 10$ and $^*P \leq 0.05$ is considered significant as compared with the control test (CO).

decrease in the activity was observed at 8.2 min after its administration. In contrast, the head to tail cyclization of linear compound **3** gave the cyclic compound **4**, which was less potent analgesic than the precursor peptide but demonstrated a prolonged activity with a half-maximal response decay 2.4 times longer than that of compound **3** (Table 2).

Accounting for the observation that the most potent fragment in HN, e.g., HN-(7−15),[4] contains the aromatic amino acids Tyr and Phe, the amino acid in position 9 of cyclic [Ala^9]HN-(7−10) (**4**) and cyclic [Ala^9, D-Arg^{10}]-HN-(7−10) (**5**) was replaced by Tyr to provide compounds **6** and **7**, respectively, whereas Gln at position 8 of cyclic [Tyr^9, D-Arg^{10}]HN-(7−10) (**7**) was replaced by the lipophilic amino acids D-Gln, Ala, *p*-Cl-Phe, and *p*-NH_2-Phe to provide compounds **8**−**11**, respectively. As shown in Table 2, the starting compounds **4** and **5** were more potent analgesics than HN in the mouse writhing pain assay with AD_{50} values of 13.3 and 7.05 nmol/mouse, respectively, as compared with 23.0 nmol/mouse for HN. All following derived cyclic tetrapeptides displayed improved potency and/or times of action, the most potent peptides being compounds **9** and **10** with AD_{50} values of 1.35 and 0.17 nmol/mouse, respectively. Replacement of *p*-Cl-Phe in compound **10** by *p*-NH_2-Phe markedly diminished the potency and time of action of the peptide (reflected by an increase of the AD_{50} value from 0.17 to 2.52 nmol/mouse and a decrease in the times of half-maximal response decays from >90 to 60 min). Replacement of Gln in cyclic [-Gly-Gln-Tyr-D-Arg-] (**7**) by D-Gln(**8**), Ala (**9**), *p*-Cl-Phe (**10**), and *p*-NH_2-Phe (**11**) markedly increased the times of action of the compounds with half-maximal response decays observed at times >60 min as compared with 24.1 min for the parent compound **7** and 22.1 min for HN (Table 2).

The analgesic activity of the synthetic peptides was also determined using the radiant heat tail flick assay (a thermal pain assay).[19] This test was less sensitive to HN and related peptides than the mouse writhing test.[4] However, at 50 nmol for HN, 2.5 nmol for compound **10**, and 10 nmol for all other linear and cyclic tetrapeptides (icv), all compounds, except compound **5**, displayed significant analgesic activity, the highest effects being observed with compounds **9** and **10** (Figure 1). The two latter compounds were also evaluated for their ability

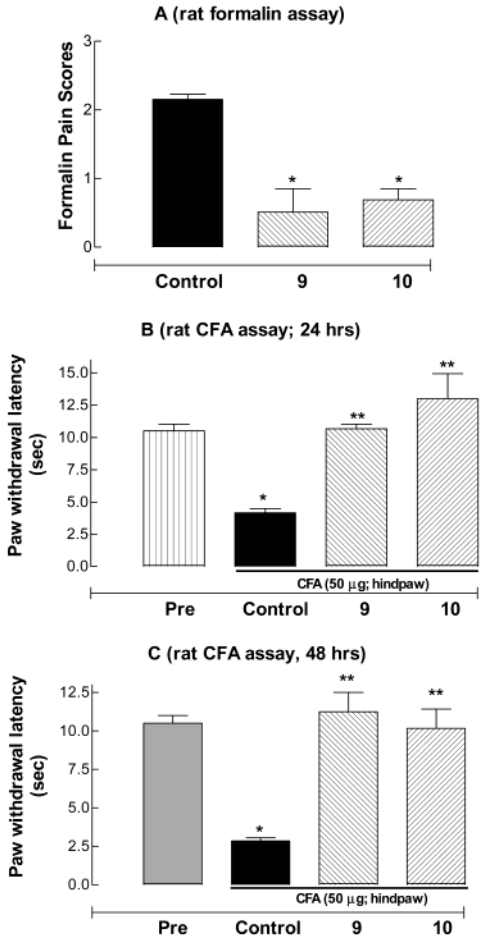

Figure 2. Analgesic effects of compounds **9** (10 nmol; i.t.) and **10** (0.5 nmol; i.t.) in the second (tonic) phase of pain of the rat formalin test (A) and antihyperalgesic effects of compounds **9** (10 nmol; i.t.) and **10** (0.5 nmol; i.t.) in the rat CFA assay 24 h (B) and 48 h (C) after the administration of CFA into one hind paw. Formalin and CFA assays in rats are performed as described in the Experimental Section. For each value, $n = 7$ and $^*P \leq 0.05$ are considered significant as compared with the preadministration test (Pre). $^{**}P \leq 0.05$ is considered significant as compared with the control test.

to modulate persistent inflammatory pain in the second phase of the rat formalin test[7a] and to reverse thermal hyperalgesia induced by intraplantar administration of CFA[7d] (Figure 2). Intrathecal administration of compounds **9** and **10** in rats decreased formalin pain in a dose-dependent fashion although the dose−response relation was bell-shaped (not shown). The maximal analgesic effects were obtained with 10 nmol for compound **9** and 0.5 nmol for compound **10** (Figure 2A). In the rat CFA test, compounds **9** (10 nmol; i.t.) and **10** (0.5 nmol; i.t.) significantly blocked CFA-induced hyperalgesia at 24 (Figure 2B) and 48 h (Figure 2C). Finally, compounds **9** and **10** were also monitored for possible effect on motor function. Optimal analgesic doses of the compounds in the writhing test (10 and 2.5 nmol/mouse, icv, respectively), did not significantly affect the performance of mice in the mouse rotarod assay as compared with control groups administered with vehicle (Table 3).

The nature of the receptor involved in the analgesic activity of compounds **9** and **10** was verified by preadministration of specific receptor antagonists. As already observed with HN,[4] 5 min of preadministration of the

Table 3. Lack of Motor Effect of Compounds **9** and **10** as Assessed by the Mouse Rotarod Assay

	rotarod performance[a]		
treatment	15 min[b]	30 min[b]	60 min[b]
vehicle	103.0 ± 7.3	117.3 ± 2.7	118.2 ± 2.8
compd **9**	107.7 ± 8.6	120.0 ± 0.0	120.0 ± 0.0
compd **10**	104.1 ± 8.2	115.8 ± 4.2	110.2 ± 6.6

[a] Mean times ± SE for the mice to remain on the rotarod. [b] Times interval after icv administration of compounds **9** (10 nmol) and **10** (2.5 nmol) or vehicle.

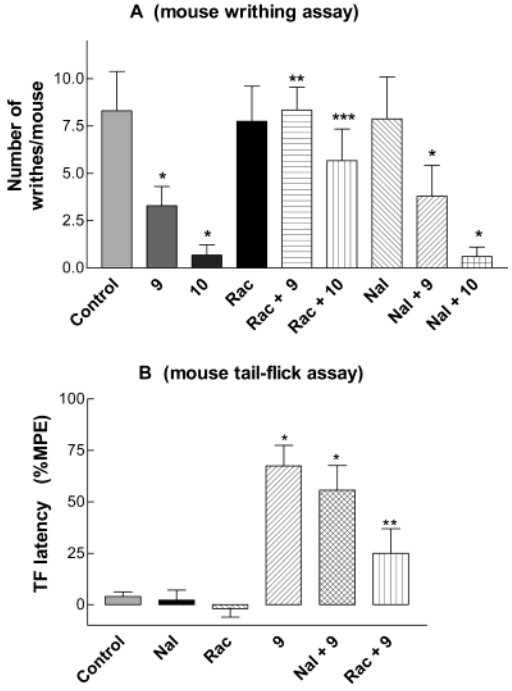

Figure 3. Effects of preadministration (5 min) of the opiate antagonist naloxone (Nal ;1 nmol/mouse, icv) and dopamine D_2 antagonist raclopride (Rac; 1 nmol/mouse; icv) on the analgesic activity of compounds **9** (10 nmol/mouse; icv) and **10** (1 μmol/kg; i.v.) in the mouse writhing (A) and tail flick (B) assays. For each value, $n = 10$ and $*P \leq 0.05$ is considered significant as compared with the control test. $**P \leq 0.05$ is considered significant as compared with the animals treated with compound **9**. $***P \leq 0.05$ is considered significant as compared with the animals treated with compound **10**.

D_2 receptor antagonist raclopride (1 nmol/mouse; icv), but not the opioid antagonist naloxone (1 nmol/mouse; icv), blocked the analgesic effects of compound **9** (10 nmol/mouse; icv) in the mouse writhing (Figure 3A) and tail flick (Figure 3B) assays. The analgesic activity of compound **10** (1 μmol/kg; i.v.) in the mouse writhing test was also significantly affected by icv preadministration of the D_2 receptor antagonist raclopride (Figure 3A). The various cyclic tetrapeptides were also tested for their abilities to compete with the binding of [^{3}H]raclopride to rat brain membranes (Table 4). All cyclic tetrapeptides in the micromolar range caused marked (>60%) inhibitions of the binding of [^{3}H]raclopride with IC_{50} correlating well to the AD_{50} of the compounds in the mouse writhing assay ($r = 0.95$; Figure 4).

Discussion

Previous studies indicated that HN and related peptide analogues and fragments are potent analgesics in the mouse writhing and tail flick assays by a mechanism

Table 4. Relative Potencies of HN and Related Cyclic Tetrapeptides in Inhibiting the Binding of [^{3}H]Raclopride to Rat Brain Membranes

compd	[^{3}H]raclopride (IC_{50}, μM)	potency ratio	compd	[^{3}H]raclopride (IC_{50}, μM)	potency ratio
1	0.6 ± 0.3	1.0	**8**	4.91 ± 0.90	0.12
5	12.5 ± 6.2	0.035	**9**	2.33 ± 0.25	0.26
6	6.75 ± 1.67	0.088	**10**	0.89 ± 0.50	0.67
7	4.12 ± 0.91	0.14	**11**	3.52 ± 2.45	0.17

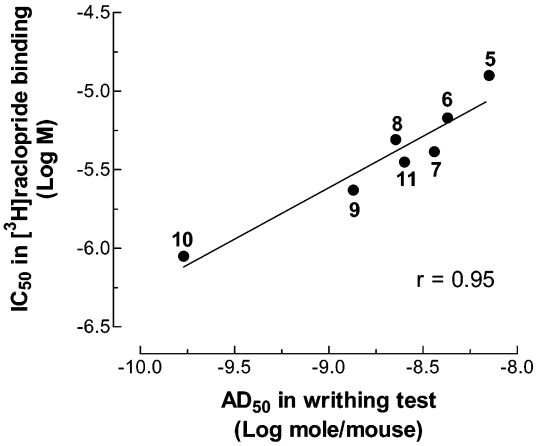

Figure 4. Correlation between potencies of cyclic tetrapeptides in inhibiting [^{3}H]raclopride binding to rat brain membranes and causing analgesia in the mouse writhing test.

that involves the participation of the dopamine D_2 receptor.[4] While HN-(7−10) was shown to be the minimal active fragment, HN-(7−15) was significantly more potent than the parent pentadecapeptide HN. In the C-terminal fragment HN-(7−15), the side chains of the two aromatic amino acids Tyr and Phe, not present in HN-(7−10), protrude outside of the backbone of the molecule and therefore are likely to be involved in its interaction with the D_2 receptor and particular high analgesic efficacy.[4] Because Ala-scanning experiments with the minimal analgesic peptide HN-(7−10) indicated that Ala can be substituted for Gly[9] (Table 1) with some increase in the analgesic potency, the position 9 was chosen to introduce Tyr in structurally constrained cyclic analogues of HN-(7−10). On the other hand, the Arg amino acid at position 10 of HN-(7−10) was not changed, besides being converted into its D-isomer, due to the importance of the basic amino group in dopamine D_2 agonists.[20] These initial changes in the structure of HN-(7−10) conferred to the peptide some similarity with the structures of dopamine D_2 agonists, compounds **6**−**11** containing two pharmacophores present in prototypic dopamine D_2 agonists, e.g., a phenolic oxygen and a basic amine.[20,21] In addition, the replacement of Gln[8] by D-Gln or Ala in compounds **8** and **9**, respectively, provided marked increases in binding and analgesic potencies as well as time of action in the mouse writhing test as compared with the parent compound **7**. Thus, position 8 was chosen to introduce two selected derivatives of Phe (the C-terminal amino acid in HN-(7−15)). While the introduction of *p*-Cl-Phe in position 8 provided a compound (**10**) with the highest analgesic potency and time of action in the mouse writhing test combined with the highest ability to compete with the binding of the D_2 ligand [^{3}H]raclopride to rat brain membranes, the introduction of *p*-NH_2-Phe (**11**) was less efficient pos-

sibly due to the addition of a cationic nitrogen atom on the phenyl group.

Compound **10** has a biological profile, which, in many respects, is similar to that of HN. First, it competes with the binding of [^{3}H]raclopride at submicromolar concentrations. Its affinity for the D_2 site is somewhat lower than that of HN (potency ratio of 0.67 as compared with HN; Table 4), but its increased potency and time of action in the various pain assays may be due to its high lipophilicity and resistance to the action of peptidases, allowing the compound to reach its action site(s) more efficiently without being degraded by proteolytic enzymes. Its content of an halogenated phenyl group and its cyclization are structural characteristics that are expected to be responsible for such increases in biological activity.[22,23] In this regard, compound **10** is the sole compound tested herein that displays analgesic activity after both peripheral (i.v., Figure 3A; i.p. and oral, data not shown) and central (icv; i.t.) administrations. All of the other compounds, including HN, were effective analgesics only after central administration. Second, like HN,[4] compound **10** is a more efficient analgesic in the writhing test than the tail flick assay. Third, its antinociceptive effects in the mouse writhing test are sensitive to the D_2 receptor antagonist raclopride but not the opiate antagonist naloxone. Fourth, its analgesic effects in the rat formalin assay are observed within a small range of concentrations and only during the second (tonic) phase of pain. Finally, compound **10** (i.t.) completely abolishes hyperalgesia in rats 24 or 48 h after hindpaw administration of CFA, e.g., at times when hyperalgesia is firmly established, and its antihyperalgesic effects last for more than 80 min after the administration of the peptide (not shown). All the abovementioned properties of compound **10** are shared by HN.[4,7]

A large body of biological evidence indicates that spinal[24−26] and supraspinal[27−29] dopamine D_2 receptors are involved in the modulation of pain. HN and related peptides interact as agonists on the dopamine D_2 receptor.[12] Hence, their action on the D_2 site may be direct or indirect. A direct interaction of HN and related peptides with the D_2 receptor is supported by the finding that the potent compounds **6**−**11**, as well as HN, contain two important D_2 pharmacophores, e.g., a phenolic oxygen and a basic amine. In addition, the marked increase in the biological activities of compound **10** as compared with compound **9** suggests that Phe at position 15 in HN may be considered as a third pharmacophore, although the contribution of *p*-Cl-Phe to the activity of compound **10** may be more relevant to its increased ability to pass different membrane barriers than to bind to a specific receptor. On the other hand, the presence of the phenolic oxygen of Tyr in HN and compounds **6**−**11** may allow them to share a hydrogen atom with Ser at positions 193 or 197 of the D_2 site,[20] whereas the cationic guanidino group at position 10 (Arg or D-Arg) of HN and HN-(7−10)-derived cyclic peptides may associate with Asp at position 114 of the D_2 site.[21] The substitution of D-Arg for L-Arg in compound **7** does not affect much its binding and analgesic activities as compared with compound **6** (Tables 2 and 4), although such modification is expected to produce a major change in the orientation of the guanidino group. The change of orientation of the guanidino group may be allowed for biological activity due to the versatility of association of the oxygen of Tyr^9 with either Ser^{193} or Ser^{197} in the D_2 site.[20] Finally, the action of HN and related compounds on the D_2 receptor may also be indirect, first by acting on some HN specific receptor and thereafter causing an allosteric change (agonist-like change) in the conformation of the D_2 receptor. This latter possibility would be supported by the observation that the binding of [^{125}I][Ser^1]HN itself to rat brain membranes is not affected by the D_2 receptor antagonist sulpiride (unpublished data). On the other hand, like calmodulin,[30] HN and related compounds may interact with the D_2 receptor on a binding motif that is distinct from that of D_2 ligands and evoke a D_2 agonist-like activation of $G_{i/o}$. The mechanism by which the D_2 receptor is involved in the analgesic activity of HN and related compounds remains to be established.

Conclusion

We have described key aspects for the design of cyclic tetrapeptides with antinociceptive and D_2 binding profiles similar to those of the pentadecapeptide HN. The close correlation observed between the analgesic potencies of the various synthetic compounds and their abilities to compete with the binding of the D_2 receptor ligand [^{3}H]raclopride indicates that the antinociceptive effects of such compounds can be improved by ameliorating their ability to compete with the binding of specific radiolabeled ligands to the D_2 receptor without losing the physicochemical properties of the key amino acids Arg, Tyr, and Phe present in HN and its potent antinociceptive fragment HN-(7−15).

Experimental Section

General Methods. Chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Boc-protected amino acids derivatives, oxime resin or Merrifield resin, and all the reagents for solid phase peptide synthesis were from Bachem Bioscience Inc. (King of Prussia, PA) or Calbiochem-Novabiochem (San Diego, CA). Side chain protecting groups employed for peptide synthesis were as follows: Arg, tosyl (Tos); Lys, benzyloxycarbonyl (Z); Thr, benzyl (Bzl); Tyr, 2,6-dichloro benzyl (2,6-Cl_2-Bzl). Other high purity solvents suitable for peptide synthesis or biosynthesis were obtained from VWR Canlab (Mississauga, ON). Analytical HPLC separations were performed on a Waters model 600E, operating at a flow rate 1 mL/min, using a μ-Bondapak C18 (125 Å, 10 μm) column (3.9 mm × 300 mm) and monitoring at 280 and 230 nm with a Waters 484 variable wavelength. The crude peptides were purified by gel filtration using Sephadex G-10 (Amersham Pharmacia Biotech, QC) and then on a preparative μ-Bondapak C18 (125 Å, 10 μm) column (25 mm × 100 mm), at a flow rate of 5 mL/min. The HPLC solvent systems were (i) a linear gradient of 0−50% water−acetonitrile with 0.1% TFA over 50 min, (ii) a linear gradient of 0−60% water−acetonitrile with 0.1% TFA over 65 min, (iii) 15−65% water−acetonitrile with 0.1% TFA over 60 min. TLC was performed on precoated silica gel plates 60 F_{254}, (Merck KGaA, Darmstadt, Germany) with the solvent system, (v/v) 1-butanol−AcOH−water−pyridine (15:3:10:12). Peptides were visualized by three of the following procedures: (i) UV, (ii) Ninhydrin spray reagent (amino group),[31] or (iii) Pataki spray reagent (amide function).[32] Mass spectra were recorded by the Centre Régional de Spectrométrie de Masse (Chemistry Department, University of Montreal) and the University of Ottawa Mass Spectrometry Centre (Ottawa, ON) using fast atom bombardment mass spectrometry (FAB-MS) and the electrospray ionization (ESI) technique. Amino acid analyses

were obtained from the Biotechnology Service Centre, Department of Laboratory Medicine & Pathobiology, University of Toronto (Toronto, ON).

Animals. Male albino Sprague−Dawley rats (225−250 g) were obtained from Charles River, St. Constant, Quebec. Rats were housed six or seven per cage upon arrival. Mice (male 20−25 g, Swiss Webster) were obtained from Charles River (Canadian Breeding Farm, St. Constant, Quebec). They were housed five per cage in a room with controlled temperature (22 ± 2 °C), humidity, and artificial light (06.30−19 h). The animals had free access to food and water and were used after a minimum of 4 days of acclimation to housing conditions. Experiments were carried out between 10:00 a.m. and 4:00 p.m. in an air-regulated and soundproof laboratory (23 ± 1 °C, 40% humidity), in which mice were habituated at least 30 min before each 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.

Peptide Synthesis. HN, H4-(7−10), and [Ala^9]HN-(86−100) were synthesized by the solid phase procedure as described previously.[6] For the synthesis of cyclic tetrapeptides, Boc-Gly-oxime-resin was first prepared by mixing the oxime−resin (Novabiochem, 1.5 g, 0.53 mmol/g) with Boc-Gly-OH (1.3 g, 9 equiv) in the presence of 9.9 mL of DCC 8% in DCM (4.5 equiv), DMAP (0.3 g, 3 equiv), and HOBt (0.4 g, 3 equiv) in DCM at room temperature for 12 h. The resin was submitted to two washes with DCM, one wash with propanol-2, and one wash with DCM. The free oxime groups were capped by acetylation with acetic anhydride (0.4 mL, 5 equiv) for 30 min. The peptide chain was then assembled according to the following coupling steps (i) one wash with 25% (v/v) TFA in DCM; (ii) deprotection with 25% TFA in DCM (30 min); (iii) two washes with DCM; (iv) one wash with propanol-2; (v) three washes with DCM; (vi) one wash with DMF); (vii) coupling of Boc-amino acids (Boc-L- or D-Arg(Tos)OH (1.0 g, 3 equiv), Boc-Ala-OH (0.45 g, 3 equiv), Boc-Tyr(2,6-di-Cl-Bzl)OH (1.05 g, 3 equiv), Boc-L- or D-Gln-OH (0.6 g, 3 equiv), Boc-Phe(*p*-Cl)OH (0.7 g, 3 equiv). or Boc-*p*-amino-Phe(Z)-OH (1.0 g, 3 equiv)) in the presence of PyBOP (1.25 g, 3 equiv), HOBt (0.4 g, 3 equiv), and DIEA (0.9 mL, 6.5 equiv) in DMF (1−2 h); (viii) three washes with DMF; (ix) two washes with DCM. Solvent volume was 15 cm^3 g^{-1} resin. Coupling efficiency was checked at each coupling cycle by the Kaiser test.[17] The peptide was cleaved from the resin by intrachain aminolysis in the presence of AcOH (0.09 mL, 2 equiv) and DIEA (0.27 mL, 2 equiv) in 30 mL of DMF at room temperature for 24 h. The product was obtained from the solution phase by filtration. DMF was removed in vacuo, and the residue was dried in a desiccator (24 h) before final deprotection with liquid HF containing 10% anisole at 0 °C for 30 min. After the removal of HF in vacuo, the crude product was precipitated and washed with diethyl ether (50 mL) and extracted with DMF (100 mL). Cyclic peptide was obtained by passage through Sephadex G-10 resin (1.5 cm × 30 cm column) followed by purification on a preparative RP-HPLC. The purity and identity of the synthetic peptides were confirmed by TLC, analytical HPLC, MS, and amino acid composition analysis. The physicochemical data of the HN analogues are detailed in Table 1.

Administration of Peptides. The icv administration of the peptides in mice was performed as described previously.[4] Peptides were dissolved in double-distilled sterile water (vehicle), and 10 μL of the peptide solution or vehicle was delivered gradually within approximately 3 s. The administration site was confirmed by injecting Indian ink in preliminary experiments.

Mouse Writhing Test. Male swiss webster [(SW)f BR] mice were injected i.p. with 1.0% AcOH (10 mL/kg) 5 min after icv (or i.v., as indicated) injection of 0 (vehicle), 0.25, 0.5, 1, 10, 25, and 50 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 AcOH solution. An abdominal stretch was 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 once and then killed immediately. Groups of 10 mice were used for each dose. Antinociceptive activity was expressed as % analgesia as calculated by the formula: [(mean number of writhes in control group − mean number of writhes in the test group)/(mean number of writhes in control group)] × 100. The percent analgesia for various effective doses was then used to calculate the AD_{50} and potency ratio by the method of Lichfield and Wilcoxons,[33] using procedure 47 of the computer program of Tallarida and Murray.[34] The times of action of the compounds were determined by injection of 1% AcOH at various times after the administration of the peptides. The experiments for the assessment of the peripheral antinociceptive activity of the peptides were performed by i.p. administration of 5 μmol/kg of the tested compounds 10 min prior to the injection of the AcOH solution. Data were analyzed by the Wilcoxon's paired nonparametric test. The criterion for statistical significance was $P < 0.05$.

Mouse Tail Flick Assay. The latency to withdraw the tail from a focused light stimulus was determined using a photocell.[19] The light intensity was set to give a control reading of about 3 s. Baseline latencies were determined before experimental treatment as the mean of two trials, and a maximal latency of 12 s was used to minimize tissue damage. Post-treatment latencies were determined 5 min after icv injection. The antinociceptive effect was expressed as the percentage of the maximum possible effect, as calculated by the formula: %MPE = [(postinjection latency − baseline latency)/(cut off latency − baseline latency)] × 100. The use of %MPEs takes into account differences in baseline latencies so that these differences do not bias the quantification of antinociception. Group %MPE means were compared using one way analysis of variance (ANOVA), and $P \leq 0.05$ was considered significant.

Rat Surgery. After surgery, rats were housed individually. The housing room was maintained at 23 ± 0.5 °C with a 12 h light/dark cycle. Food and water were available ad libitum and for the first 3 days after surgery, wet food was provided on the floor of the cage. Rats were premedicated with atropine sulfate injected intramuscularly (i.m.) in a dose of 0.05 mg/kg. Thirty minutes later, they were anesthetized with a mixture of ketamine (90 mg)/xylazine (6 mg) i.m. Animals were placed into the stereotaxic apparatus to isolate the caudal part of the skull and to pierce the occipital membrane. Once removed from the stereotaxic apparatus, the animal was bent forward on the bench and a polyethylene tubing (PE 10, 11 cm) was slid down 7.5 cm (to the level of the lumbar enlargement). The tubing was secured onto the skull by a drop of dental cement that extends around two screws. The incision was closed using wound clips and a topical antibacterial cream was applied onto the wound. A piece of tubing (PE50, 1 cm) was inserted onto the extremity of the catheter to avoid clogging. Rats were allowed at least 7 days to recover from the surgery before testing began.

Rat Formalin Assay. To avoid stress-induced analgesia, rats were habituated to the formalin boxes and testing environment 30 min per day for four consecutive days. On testing day, rats were brought to the formalin testing room, and the protective tube at the end of the i.t. catheter was removed. Saline or one of the analogues were administered i.t. in a volume of 10 μL using a 50 μL Hamilton syringe attached to a polyethylene tubing (PE20) through a 30 g needle. Five minutes later, 50 μL of diluted formalin (2.5%) was injected subcutaneously into the plantar surface of the hindpaw using a 0.3 mL disposable syringe. Testing started 25 min after formalin injection, and animals were observed for a period of 30 min. Behaviors were rated using a BASIC program, which calculates the time spent in four mutually exclusive categories of behavior (BASIC program K. B. J. Franklin, McGill University). Categories of behaviors were defined according to the description of Dubuisson and Dennis.[35] The four behaviors rated were as follows: 0, normal weight bearing on the injected paw; 1, favoring, resting the paw lightly on the floor or limping during locomotion; 2, elevation of the

affected paw, with nails touching the floor at the most; 3, licking, biting, or grooming the affected paw.

Rat CFA Assay. Animals were allowed to freely wandered on the unheated hot plate for two sessions of 30 min before testing began. Animals were brought to the testing room, and baseline paw withdrawal latencies from the hot plate were obtained (two trials per rat). The hot plate consisted of a 30 cm × 30 cm metal plate heated at 49 °C. This low intensity of thermal stimulation was chosen to allow the observation of the hyperalgesic effects induced by CFA. Once baseline latencies were obtained, 100 μL of CFA (50 μg) was administered into the plantar surface of one hind paw and animals were returned to their home cage. Twenty-four hours later, animals were brought to the testing room. Baseline latencies were obtained again to verify that CFA injection had effectively induced a hyperalgesic response. Each rat received an i.t. injection of saline or HN analogue as described above for the formalin assay. Testing started 10 min after injection. Paw withdrawal latencies were obtained at 10 min intervals for 70 min. A cutoff of 20 s was imposed in order to avoid tissue damage. At the end of the testing, catheters were flushed with 10 μL of saline and animals were put back to their home cage. The experiments were repeated at 48 h, using the same animals. Data were analyzed by means of ANOVA followed by the planned post-hoc comparisons using the Fisher procedure. In all cases, differences were considered significant if they had a probability of random occurrence of less than 5%.

Mouse Rotarod Assay. The mouse rotarod assay was performed on a rotary apparatus as indicated by Dunham and Miya.[36] The apparatus was constituted of a rod with a diameter of 2.5 cm suspended horizontally 50 cm above a plane working area. The rod was turning at a speed of 8 revolutions per min. Circular perpex separators were placed at regular intervals along the rod so that five mice could be tested at the same time. Before administering any compound, all animals were placed on the turning rod for 1 min in two consecutive rounds. Mice that fell from the rod during these conditioning experiments were excluded from the assay. For the assay, the test compounds were administered icv, and the animals were placed on the turning rod for 2 min. The % of mice in groups of 10 mice that fell during this latter 2 min experiment was recorded as the % of mice showing motor effects. Rotarod assays were conducted at different times (up to 60 min) after the administration of peptides. Statistical calculations were made using Student's *t*-test.

[^{3}H]Raclopride Binding to Rat Brain Membranes. Brain membrane preparations of male Wistar rats (250−300 g, Charles Rivers) were obtained according to the procedure already described.[5] They were suspended in 5 mM Tris-HCl, pH 7.4 (buffer A). The binding assays with [^{3}H]raclopride (2.5 nM) to rat brain membranes (0.8 mg protein) were carried out at room temperature (22 °C) for 60 min in a total volume of 2 mL as described.[12] Specific binding of [^{3}H]raclopride was defined as the difference between the total radiolabel bound and that bound in the presence of 10 μM (+)butaclamol. Competition experiments were performed with 2.5 nM [^{3}H]-raclopride in the presence of 9−12 increasing concentrations (10^{-10}−10^{-4} M) of the peptides or competing ligands. All values from competition (IC_{50}) curves were generated by the iterative nonlinear least-squares curve-fitting procedure using the computer program PRISM (Graphpad Software Inc, San Diego, CA). All data represent the mean ± SE of at least three experiments conducted in duplicate.

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(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−379. The other abbreviations are as follows: AcOH, acetic acid; AD_{50}, the concentration that produces 50% analgesia; Boc, *tert*-butyloxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N*,*N*′-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; HN-(7−10), H-Gly-Gln-Gly-Arg-OH; HF, hydrogen fluoride; HOBt, N-hydroxybenzotriazole; icv, intracerebroventricular; i.t., intrathecal; i.v., intravenous; i.p., intraperitoneal; PyBOP, benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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