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Short communication

# Subchronic haloperidol administration decreases aminopeptidase N activity and [Met$^5$]enkephalin metabolism in rat striatum and cortex

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**Abstract**

Previously we have shown that subchronic intraperitoneal (i.p.) administration of haloperidol decreases the degradation of [Met$^5$]enkephalin by regional brain slices (Waters et al., 1995, J. Pharmacol. Exp. Ther. 274, 783). In the present study, subchronic (7-day i.p.) administration of haloperidol (1 mg/kg) decreased the accumulation of aminopeptidase-derived fragments Tyr and Gly-Gly-Phe-Met on cortical and striatal slices. The accumulation of Tyr-Gly-Gly, however, was not altered by haloperidol treatment on slices from either region. Further, aminopeptidase N activity was decreased in $P_2$ membranes isolated from either the cortex or striatum of haloperidol-treated animals. These data suggest that the haloperidol-induced decrease in [Met$^5$]enkephalin metabolism results, at least in part, from a reduction in the activity of aminopeptidase N.

*Keywords:* Haloperidol; Enkephalin; Aminopeptidase N; Neuroleptic; Neuropeptide

## 1. Introduction

[Met$^5$]Enkephalin (Tyr-Gly-Gly-Phe-Met), a product of enzymatic cleavage of the proenkephalin precursor, elicits biological responses by binding with high affinity to both $\delta$- and to $\mu$-opioid receptors (Cox, 1982). Receptor autoradiography has shown that both $\delta$ and $\mu$ binding sites are abundant in cortex and striatum (Goodman et al., 1980) while radioimmunoassay of brain has demonstrated high levels of [Met$^5$]enkephalin immunoreactivity in striatum and lower levels in cortex (Hughes et al., 1977). Both dopamine deafferentation and dopamine antagonists alter the expression of proenkephalin, as well as the levels and release of [Met$^5$]enkephalin (Augood et al., 1993). These data provide evidence for an interaction between [Met$^5$]enkephalin and midbrain dopaminergic pathways and suggest that alterations in enkephalinergic activity may be responsible for some of the therapeutic and/or untoward effects of neuroleptic treatment.

Selective peptidase inhibitors have been shown to prevent the degradation of [Met$^5$]enkephalin-like immunoreactivity released from depolarized brain slices. Such an approach has established the importance of neprilysin (EC 3.4.24.11) and membrane-bound/associated aminopeptidase(s) in the degradation of [Met$^5$]enkephalin (De La Baume et al., 1983). Recently, we have shown that aminopeptidase activity is primarily responsible for the metabolism of [Met$^5$]enkephalin on frontal cortical slices whereas multiple peptidases act on [Met$^5$]enkephalin on caudate-putamen slices (Konkoy and Davis, 1995). We have also shown that subchronic administration of haloperidol decreases the degradation of [Met$^5$]enkephalin on slices isolated from these brain regions (Waters et al., 1995). The present study examines the effect of subchronic treatment with haloperidol on the regional accumulation of [Met$^5$]enkephalin fragments and activity of aminopeptidase N (aminopeptidase M, EC 3.4.11.2), a membrane-bound metallopeptidase implicated in the inactivation of endogenously released enkephalins (Giros et al., 1986). Our results suggest that subchronic, but not acute, administration of haloperidol alters met-enkephalin metabolism by altering the activity of aminopeptidase N.

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## 2. Materials and methods

### 2.1. Reagents

[Met$^5$]Enkephalin and L-leucine-*p*-nitroanilide were purchased from Bachem California (Torrance, CA, USA). Haloperidol, amastatin, puromycin and *p*-nitroaniline were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Drug treatment

Adult, male Harlan Sprague-Dawley rats (200–300 g) were housed, four animals per cage, in an air-conditioned room with a 12-h light-dark cycle. Rats had free access to laboratory chow (Teklad pellets) and water. Animals were injected intraperitoneally (i.p.) once daily between 9 a.m. and 10 a.m. with haloperidol (1 mg/kg, $n = 4–5$) or vehicle (0.3% tartaric acid, $n = 5$) for 7 days. Acutely treated animals ($n = 3$) received a single injection. Rats were killed 24 h after the final injection.

### 2.3. Tissue preparation

#### 2.3.1. Regional brain slices

Brain slices were prepared as previously described (Waters et al., 1995). Rats were decapitated and the brains quickly removed. The brains were placed into an ice-cold rat brain matrix (Activational Systems, Warren, MI, USA) and cut into 2 mm slabs. The slabs were punched using a stainless-steel, 2 mm tissue corer (Fine Science Tools, Foster City, CA, USA) and placed in cold, sterile Hepes buffer (consisting of 141 mM NaCl, 4.80 mM KCl, 0.95 mM $KH_2PO_4$, 11.1 mM glucose, 2.00 mM $CaCl_2$, 1.20 mM $MgSO_4$, and 10 mM Hepes free acid adjusted to pH 7.4 with NaOH). Cortical and striatal punches were hand-sliced using a custom-designed apparatus (Vitron, Tucson, AZ, USA). Slices (230 $\mu$m thickness) were placed in micro weighing dishes (VWR Scientific) and rinsed with cold, sterile Hepes buffer to remove tissue debris and damaged slices (Konkoy et al., 1994).

#### 2.3.2. Membrane homogenates

Membranes ($P_2$ fraction) were prepared by a technique modified from the method of Gray and Whittaker (1962). Briefly, brain regions were isolated and cored as described above. Tissues were homogenized in Tris-sucrose buffer (0.5 mM Tris, 0.32 M sucrose, pH 7.4 with HCl) using 10 strokes of a Potter-Elvehjhem tissue homogenizer. Homogenates were centrifuged at $3000 \times g$ for 10 min and the nuclear membrane pellet discarded. The supernatant was centrifuged at $16\,500 \times g$ for 10 min. The resulting pellet was resuspended in assay buffer (0.2 M Tris, pH 7.0) and protein concentration was determined from an aliquot via the method of Bradford (1976).

### 2.4. Brain slice incubation

The slices were rinsed twice with room temperature, sterile Hepes buffer, pH 7.4 and gently agitated on an orbital shaker (Hoefer Scientific Instruments, San Francisco, USA) for 10 min to remove cytosolic enzymes from the cut surfaces. Slices (4) were transferred to sterile polypropylene tubes containing 160 $\mu$l of sterile Hepes buffer. [Met$^5$]Enkephalin (20 $\mu$l of a 3600 $\mu$M stock solution in sterile Hepes buffer) was added to the tubes resulting in a final volume of 180 $\mu$l. Thus, the starting concentration of met-enkephalin was 400 $\mu$M. The tubes were placed in a $37 \pm 0.5$°C rotating incubator for 45 min. Incubations were terminated by boiling for 5 min. Control tubes were prepared by boiling for 5 min prior to the addition of the peptide stock solution or by adding [Met$^5$]enkephalin to tubes which lacked slices. Following centrifugation, the supernatant was separated from the slices and frozen until HPLC analysis. All samples were conducted in duplicate. The protein content of sample tubes was analyzed by the method of Lowry et al. (1951).

### 2.5. HPLC analysis

Supernatants were thawed and centrifuged to precipitate any remaining tissue debris. Samples were analyzed by automatic injection (TosoHaas TSK 6080, Philadelphia, PA, USA) onto a Vydac (Hesperia, CA, USA) $C_{18}$ small pore column ($0.46 \times 25$ cm) as previously described (Konkoy and Davis, in press). [Met$^5$]Enkephalin fragments were separated using isocratic phosphate buffer (100 mM $NaH_2PO_4$ adjusted with $H_3PO_4$ to pH 2.4) over a 10 min time period, followed by a linear gradient from 0–20% acetonitrile versus phosphate buffer over a 40 min time period. Sample components were detected with a Perkin-Elmer model LC-95 detector at 210 nm and integrated with a Perkin-Elmer model LCI-100 integrator. [Met$^5$]Enkephalin fragments were identified by coinjection with authentic standards and quantified against such standards injected in 1 $\mu$g amounts.

### 2.6. Aminopeptidase N assay

Aminopeptidase N activity was determined as previously described (Gillespie et al., 1992). Membrane protein ($P_2$ fraction, 25 $\mu$g) was added to 4 tubes. All assay tubes received puromycin (10 $\mu$M) to inhibit the activity of puromycin-sensitive aminopeptidase (aminopeptidase M II). Amastatin (6 $\mu$M), an inhibitor of aminopeptidase N, was added to duplicate sample con-

trol tubes. The total volume of each tube was adjusted to 100 μl with assay buffer (0.2 M Tris, pH 7.0). After 30 min 500 μl L-leucine-*p*-nitroanilide (2.4 mM in assay buffer) was added to each tube. After mixing, tubes were immediately placed in a 37 ± 0.5°C waterbath for 4 h. Samples were boiled for 2 min to terminate enzyme activity, cooled on ice and spun in a microcentrifuge (Beckman Instruments, Fullerton, CA, USA) for 7 min. Sample absorbance was measured at 405 nm in a Beckman model 25 spectrophotometer. Product formation was quantified against the absorbance of 10–100 μM *p*-nitroaniline. The activity of each sample was expressed as pmol *p*-nitroaniline produced/mg protein/min.

### 2.7. Data analysis

All samples were assayed in duplicate and results are expressed as means ± S.E.M. unless otherwise indicated. Statistical significance was evaluated with a Student's *t*-test for unpaired data.

## 3. Results

Administration of haloperidol to rats produced catalepsy and sedation for approximately 4 h. Vehicle- and haloperidol-injected animals exhibited similar gains in weight over the course of the treatment period. Acute administration of haloperidol (1 mg/kg) had no effect on the degradation of [Met$^5$]enkephalin (% recovery ± S.E.M.) on either cortical (52.0 ± 1.9, control vs. 50.3 ± 3.8, haloperidol-treated) or striatal (30.0 ± 0.5, control vs. 30.2 ± 1.5, haloperidol-treated) slices. In agreement with a previous study (Waters et al., 1995), subchronic (7-day) treatment with haloperidol (1 mg/kg) decreased [Met$^5$]enkephalin degradation on cortical slices by approximately 17% vs. control, and on striatal slices by approximately 28% vs. control. Subchronic haloperidol treatment significantly ($P < 0.01$) decreased the accumulation of met-enkephalin fragments Tyr and Gly-Gly-Phe-Met on cortical slices (18% and 16%, respectively, vs. control), and striatal slices (28% and 31%, respectively, vs. control; Table 1). Haloperidol had no effect on the accumulation of Tyr-Gly-Gly in either region.

Table 2
Effect of subchronic (7-day) administration of haloperidol (1 mg/kg) on aminopeptidase N (3.4.11.2) activity in $P_2$ brain membranes

| Brain region | Vehicle | Haloperidol |
|---|---|---|
| Cortex | 2198 ± 57 ($n = 4$) | 1763 ± 43 [a] ($n = 4$) |
| Striatum | 1639 ± 86 ($n = 5$) | 1299 ± 68 [b] ($n = 5$) |

Activity (pmol/mg protein/min) is expressed as means ± S.E.M. [a] Significantly different than vehicle-treated samples ($P < 0.01$) by Student's unpaired *t*-test. [b] Significantly different than vehicle-treated samples ($P < 0.05$) by Student's unpaired *t*-test.

The activity of aminopeptidase N was measured in the $P_2$ fraction of membranes isolated from cortex and striatum of rats after subchronic administration of either haloperidol (1 mg/kg) or vehicle. Haloperidol significantly ($P < 0.01$, cortex; $P < 0.05$, striatum) decreased aminopeptidase N activity by approximately 20% in both regions (Table 2).

## 4. Discussion

Previous work in our laboratory has provided evidence for both structural and regional specificity in the metabolism of neuropeptides by intact, brain microslices (Davis et al., 1992). [Met$^5$]Enkephalin, in particular, is rapidly degraded on caudate-putamen slices and more slowly on nucleus accumbens and frontal cortical slices. Moreover, aminopeptidase activity is primarily responsible for the degradation of [Met$^5$]enkephalin in frontal cortex whereas multiple peptidases participate in the degradation of [Met$^5$]enkephalin in caudate-putamen and nucleus accumbens (Konkoy and Davis, 1995).

Hong et al. (1978) were the first to describe dose- and time-dependent increases of 50–90% in striatal [Met$^5$]enkephalin content after the chronic administration of neuroleptic drugs, an effect which is accompanied by increases in striatal proenkephalin mRNA (Tang et al., 1983). As neuroleptic drugs are characterized pharmacologically as dopamine $D_2$ receptor

Table 1
Effect of subchronic (7-day) intraperitoneal administration of haloperidol on [Met$^5$]enkephalin fragment accumulation by regional brain slices

| Brain region | Tyrosine | | Gly-Gly-Phe-Met | | Tyr-Gly-Gly | |
|---|---|---|---|---|---|---|
| | Vehicle | Haloperidol | Vehicle | Haloperidol | Vehicle | Haloperidol |
| Cortex | 1880 ± 36 | 1550 ± 57 [a] | 1540 ± 29 | 1290 ± 63 [a] | 70 ± 4 | 68 ± 3 |
| Striatum | 1610 ± 60 | 1160 ± 70 [a] | 930 ± 33 | 640 ± 40 [a] | 1340 ± 46 | 1260 ± 50 |

Brain slices were prepared from rats ($n = 4$–5) treated with either haloperidol (1 mg/kg/day) or tartaric acid vehicle as described in Materials and methods. Brain slices were incubated with [Met$^5$]enkephalin (400 μM) for 45 min and supernatants analyzed by HPLC. Values are expressed as picomoles of metabolite recovered per sample ± S.E.M. [a] Significantly different than vehicle-treated samples ($P < 0.01$) by Student's unpaired *t*-test.

antagonists, these data suggest that activation of dopaminergic neurons tonically inhibits the biosynthesis of enkephalins in striatum. Furthermore, chronic administration of haloperidol increases the content of met-enkephalin in ventricular perfusates, suggesting that dopaminergic agents modify the release of striatal enkephalins (Herman et al., 1991). In the present study, haloperidol decreased the accumulation of [Met$^5$]enkephalin metabolites Tyr and Gly-Gly-Phe-Met, cleavage products of aminopeptidase activity, but had no effect on the accumulation of Tyr-Gly-Gly, a product of neprilysin activity, on cortical and striatal slices. In addition to providing an additional mechanism whereby a decrease in the metabolism of [Met$^5$]enkephalin may contribute to neuroleptic-induced increases in [Met$^5$]-enkephalin levels, our data suggest that subchronic treatment with haloperidol alters enzymatic cleavage of the peptide primarily at one site, i.e. the Tyr$^1$-Gly$^2$ bond.

Aminopeptidase N is an integral membrane, Zn-peptidase which preferentially cleaves neutral $NH_2$-terminal amino acids from small peptides. The activity of aminopeptidase N is high in cortex and striatum (Dauch et al., 1993), and, like other ectoenzymes, its catalytic site is oriented toward the extracellular space (Louvard et al., 1975). Aminopeptidase N degrades [Met$^5$]enkephalin on regional brain slices (Giros et al., 1986; Konkoy and Davis, 1995) and has been implicated in the physiological inactivation of enkephalins (Giros et al., 1986). Subchronic administration of haloperidol decreased the activity of aminopeptidase N in cortical and striatal $P_2$ membranes in parallel with the decrease in Tyr and Gly-Gly-Phe-Met accumulation measured on brain slices from these regions. Several possible mechanisms might account for the reduction in aminopeptidase N activity observed after treatment with haloperidol. Inasmuch as aminopeptidase N activity was measured at a saturating substrate concentration, it is unlikely that this reduction was due to a decrease in substrate affinity. A similar argument can be applied to the reduction by haloperidol of metabolite accumulation on brain slices since previous work suggests that the initial concentration (400 $\mu$M) of [Met$^5$]enkephalin chosen was sufficient to saturate aminopeptidase N throughout the time course of incubation (Konkoy and Davis, 1995). A more likely possibility is that chronic exposure to haloperidol resulted in a lower $V_{MAX}$ value for aminopeptidase N-catalyzed hydrolysis, possibly as a consequence of an alteration in the brain regional expression of the enzyme. Whether treatment with neuroleptics modulates levels of aminopeptidase N mRNA is currently under investigation in our laboratory.

Although the activities of other ectoenzymes may be influenced by subchronic administration of haloperidol, a selective reduction in aminopeptidase N activity may account for the selective decrease in the accumulation of aminopeptidase-catalyzed [Met$^5$]enkephalin fragments on brain slices from cortex and striatum. In view of neuroanatomical and pharmacological studies which posit a close association between dopaminergic and enkephalinergic neurons, perturbation by neuroleptics, such as haloperidol, of enkephalin metabolism may have important clinical implications. For example, alterations in the activity of enkephalin-containing, striatopallidal neurons have been implicated in the production of extrapyramidal symptoms after neuroleptic administration (Mercugliano and Chesselet, 1992). Together with previous studies that have demonstrated neuroleptic-induced alterations in the metabolism of other neuropeptides known to interact with mesocorticolimbic dopamine, e.g. neurotensin and substance P (Konkoy et al., 1994; Waters et al., 1995), the present study suggests that neuropeptide-degrading enzymes may represent important regulatory sites in the modulation of neuropeptide levels by dopaminergic activity.

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