Differential Processing of Neurotensin/Neuromedin N Precursor(s) in Canine Brain and Intestine*

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By using a radioimmunoassay for neuromedin N (NMN), a hexapeptide in the neurotensin (NT) family, extracts of canine small intestine were found to contain primarily (>75%) large molecular form(s) of NMN, whereas the predominant species in brain was NMN itself. Large NMN was highly basic (pI >9) and during sodium dodecyl sulfate gel electrophoresis gave two components of ~17 kDa (75%) and ~8 kDa (25%). Large NMN, like NT, was localized primarily to the mucosal layer of the jejunoileum. It was also present in highly purified (25% pure) mucosal N-cells, where it appeared to be concentrated within dense secretory vesicles. The amino acid sequence of a 21-amino-acid fragment cleaved from the C-terminal region of large NMN was identical to residues 128-148 of the canine NT/NMN precursor predicted from cDNA work on mRNA sequence of which was predicted from cDNA work on mRNA which share structural (1, 2) and biological (3) properties and which are highly homologous.

Here we present evidence indicating that the major product of precursor processing for NMN in canine intestine is an N-terminally extended, large molecular form. Furthermore, the quantity of this peptide present in the tissue accounts for the discrepancy in the NT:NMN ratio. In addition, we show that very different results are obtained with canine brain, which exhibits an NT:NMN ratio near 1.0.

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1 The abbreviations used are: NMN, neuromedin N; NT, neurotensin; iNT, immunoreactive NT; iNMN, immunoreactive NMN; RIA, radioimmunoassay; HPLC, high pressure liquid chromatography; CCK, cholecystokinin; LANT-6, Ly3, ASN’, neurotensin 8-13.

2 Mitra, S. P., Muraki, K., Brown, D. R., Parsons, A. M., and Carraway, R. E. (1990). Regul. Pept. 28, 11-22.

3 Portions of this paper (including "Experimental Procedures," Figs. 4-7, and Tables II-IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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TABLE I
Concentrations of \( i\text{NMN} \) and \( i\text{NT} \) in extracts of canine tissues and dispersed cells

| Tissue         | n  | Extraction | \( i\text{NMN} \) pmol/g | \( i\text{NT} \) pmol/g | \( i\text{NT}:i\text{NMN} \)
|----------------|----|------------|---------------------------|-------------------------|--------------------------|
| Brain          | 3  | HCl        | 6.2 ± 1.1                 | 7.8 ± 1.2               | 1.3                      |
|                | 3  | Acid/acetone| 7.4 ± 1.2                 | 7.8 ± 2.0               | 1.1                      |
| Jejunoileum    | 4  | HCl        | 746 ± 85                  | 1095 ± 67               | 1.5                      |
|                | 4  | Acid/acetone| 184 ± 34                  | 925 ± 95                | 5.0                      |
| Jejunoideal    |    |            |                           |                         |                          |
| Muscle         | 3  | HCl        | 6.7 ± 2.8                 | 9.7 ± 3.0               | 1.4                      |
| Submucosa      | 3  | HCl        | 10.0 ± 2.9                | 17.0 ± 6.0              | 1.7                      |
| Mucosa         | 3  | HCl        | 701 ± 112                 | 1091 ± 102              | 1.6                      |
| Mucosal N-cells| 8  | HCl        | 25.5 ± 0.9\(^{a}\)        | 38.8 ± 0.6\(^{a}\)      | 1.5                      |
| Vesicles       | 1  | HCl        |                           |                         | 1.4                      |

\(^{a}\) Expressed as picomoles/well (containing \( \sim 2 \times 10^6 \) cells).

Fig. 1. Gel chromatography of acidic extracts from canine ileum (A) and brain (B) on Sephadex G-75. Upper panels give \( i\text{NMN} \), and lower panels give \( i\text{NT} \) for the same sample. Column size was 0.54 liter; sample size was 6 ml; Recovery was \( \sim 90\% \). s, small.

Fig. 2. Distribution of large \( i\text{NMN} \) during sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 7.5% (A) and 15% (B) gels. Positions for various molecular mass marker proteins are indicated with arrows. Recovery was 15-20%.

Fig. 3. Diagram of NT/NMN precursor (upper) and comparison of amino acid sequence for residues 128-148 to that for isolated fragment of large \( i\text{NMN} \) (lower). Note that the biologically active peptide, \( i\text{NMN} \), is at the C terminus of the fragment.

Subcellular Distribution—During equilibrium sucrose density centrifugation of an isotonic homogenate of dispersed mucosal cells, \( i\text{NMN} \) and \( i\text{NT} \) banded together at a density of \( \sim 1.19 \) (Fig. 5), which is near the density previously determined for feline N-cell vesicles (4).

Partial Sequence of Large \( i\text{NMN} \)—The C-terminal specificity of the \( i\text{NMN} \) antiserum (4) suggested that the \( i\text{NMN} \)-like moiety was located at the C terminus of large \( i\text{NMN} \). This was substantiated by the fact that carboxypeptidase treatment destroyed the immunoreactivity (95% reduction), whereas treatment with aminopeptidase M had no effect (~5% reduction).

A brief treatment with pepsin (0.1 mg/ml, 60 min, 40 °C) fragmented the molecule without any loss of immunoreactivity, and the presumed C-terminal portion containing the \( i\text{NMN} \)-like moiety was then purified. Using the procedure described in Table II, a 21-amino acid peptide was isolated (yield, \( \sim 19\% \); purification, 100,000-fold). The final step gave a single peak of optical density coincident with that for \( i\text{NMN} \) activity (Fig. 7) The isolated peptide cross-reacted \( \sim 65\% \) in the radioimmunoassay for \( i\text{NMN} \). Its amino acid composition (Table III) was consistent with the sequence determined by automated Edman degradation (Table IV). This sequence was identical to that predicted by cDNA studies for the 21 residues immediately N-terminal to and including \( i\text{NMN} \) in the NT/\( i\text{NMN} \) precursor (Fig. 3).
DISCUSSION

The major finding reported here is that an N-terminally extended form of NMN is the predominant species present in extracts of canine intestine (>75% of total iNMN), whereas NMN itself is the primary product in canine brain (>95% of total iNMN). The isolation of this large NMN has not been accomplished yet; however, we have been able to estimate the size of the major component (~17 kDa) and to investigate the location of the NMN moiety which appears to be at the C terminus. In addition, the amino acid sequence of a 21-amino acid fragment cleaved from the C-terminal region of large iNMN has been determined.

The identity of this sequence to residues 128-148 of the NT/NMN precursor predicted from cDNA studies (b) suggests that large NMN originates from the same precursor molecule as NT and NMN. Although there is no evidence as yet for gene duplication events or alternate gene splicing for the NT/NMN precursor, the possible existence of a distinct precursor for large NMN must be considered. However, given the 21-amino acid identity with the NT/NMN precursor, the simplest hypothesis at this point is that tissue-specific processing of the same protein gives rise to NMN in brain and large NMN in intestine.

Although we were able to estimate the molecular size of large NMN and to sequence a 21-amino acid fragment of the molecule, its precise alignment with the NT/NMN precursor is not yet clear. The 17-kDa size is near that predicted (~15 kDa) for the molecule extending from the end of the putative signal sequence (residue 25) to the end of NMN (residue 148). However, the very basic nature of the intestinal peptide (pl >9) is difficult to reconcile with the rather acidic character of this segment of the precursor (pl <7). A slightly shorter sequence (residues 37-158) would have 6 fewer acidic residues and a basic pl. Since there are several potential glycosylation sites within the precursor, it is also possible that both the charge and the size of large NMN are functions of a carbohydrate portion.

Work presented here concerning the cellular distribution of large NMN and iNT in canine intestine strongly suggests that large NMN coexists with iNT in mucosal N-cells. Thus, large NMN (like NT) was localized to the mucosal region (~97%) and found to be highly concentrated within partially purified (~25% pure) intestinal N-cells. Furthermore, these two peptides co-migrated during sucrose gradient centrifugation of N-cell homogenates, indicating their association with dense secretory-like vesicles.

The fact that the ratio of iNT to iNMN was constant and nearly 1 for these various preparations (Table I) is consistent with the idea that large NMN and NT are co-stored within N cells. Immunohistochemical studies to address this issue further are currently underway. Previous work on birds and reptiles by Reinecke (7) indicates that intestinal cells staining for LANT-6, the presumed avian counterpart to NMN (8, 9), also stain for NT.

The biological significance of large NMN is not yet known. Preliminary studies in our laboratory indicate the presence of large molecular form(s) of iNMN in canine blood at concentrations which appear to exceed those of NT. These peptides may be secreted forms of intestinal iNMN; and if they can exert biologic actions themselves or liberate NMN upon proteolytic cleavage, they could be physiologically significant. The greater stability generally displayed by large molecular forms as opposed to smaller forms of biologically active peptides in the circulation (e.g., CCK-58 and CCK-33 versus CCK-8 (10, 11)) makes them attractive candidates as hormones.

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REFERENCES
1. Minamino, N., Kangawa, K., and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 122, 542-549
2. Carraway, R. E., and Leeman, S. E. (1973) J. Biol. Chem. 248, 6854-6861
3. Kalivas, P. W. (1985) Soc. Neurosci. Abstr. 11, 573-577
4. Carraway, R. E., and Mitra, S. P. (1987) Endocrinology 120, 2092-2100
5. Dobner, P. R., Barber, D. L., Villa Komaroff, L., and McKiernan, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3516-3520
6. Kaufman, J. P., Sutliff, V. E., Kashekar, D. K., Jensen, R. T., and Gardner, J. D. (1984) Am. J. Physiol. 247, G95-G104
7. Reinecke, M. (1985) Histochern. Cytochem. 16, 1-175
8. Carraway, R. E., and Ferris, C. F. (1983) J. Biol. Chem. 258, 2470-2479
9. Carraway, R. E., and Reinecke, M. (1984) in Evolution and Tumor Pathology of the Neuroendocrine System (Falkmer, S., Hakan-son, R., and Sundler, F., eds) pp. 245-283, Elsevier Scientific Publishing Co., Inc., New York
10. Eyssselein, V. E., Eberlein, G. A., Hesse, W. H., Singer, M. V., Goebell, H., and Reeve, J. R., Jr. (1987) J. Biol. Chem. 262, 214-217
11. Gores, G. J., LaRusso, N. F., and Miller, L. J. (1986) Am. J. Physiol. 250, G344-G349
12. Carraway, R. E., and Bhatnager, Y. M. (1980) Peptides (N. Y.) 1, 159-165
13. Carraway, R. E., and Leeman, S. E. (1976) J. Biol. Chem. 251, 7035-7044
14. Laemmli, U. K. (1970) Nature 227, 680-685
15. Panyim, S., and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-340
16. Barone, D. T., Buchan, A. M. J., Walsh, J. H., and Soll, A. H. (1986) Am. J. Physiol. 250, G374-G384

4 S. P. Mitra and R. E. Carraway, unpublished results.

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Neurotensin Precursor Processing

Supplement for Differential Processing Neurotensin/Neurotensin II Precursors in Cerebral Brain and Intestine

By Robert C. Fawcett and Susan F. Wing

**Experimental Procedures**

**Tissues and Extracts**

Adult rats maintained according to NIH guidelines at the University of Massachusetts Medical Center were used. The brains and intestines were removed, frozen, and kept at -70°C until processed. The tissues were homogenized immediately on ice. All-extracted fat was removed using a Polytron homogenizer as previously described (2) and then centrifuged at 200,000 g for 1 hr. The supernatant was used for assay of total protein content and any protein was precipitated using trichloroacetic acid (TCA) as previously described (4).

**Preparation of Extracts**

For the preparation of extracts, the tissue was homogenized on ice and centrifuged using a Polytron homogenizer as previously described (4). The supernatant was used for assay of total protein content and any protein was precipitated using trichloroacetic acid (TCA) as previously described (4). After centrifugation, the tissue fraction was extracted with an equal volume of petrol ether and the supernatant was precipitated using trichloroacetic acid (TCA) as previously described (4).

**Fractionation**

The purified extracts were chromatographed on a Sephadex G-25 column equilibrated with 50 mm acetic acid. After the sample was applied, 100 ml fractions were collected.

**Electrophoresis and Autoradiography**

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed at 4°C using 10% polyacrylamide gel, 1X SDS buffer, and a 10% stacking gel. The proteins were stained using Coomassie Blue and then destained using 10% acetic acid and 30% methanol. The gels were dried for 10 min at 80°C. The gels were autoradiographed using Amplify (Amersham, Arlington Heights, IL) and exposed to X-ray film at -70°C.

**Cell Isolation and Proliferation**

Rat cerebellar cells were isolated from 10-12 day-old rat pups and cultured on coverslips as previously described (5). The cells were treated with 0.5% trypsin-EDTA for 5 min at 37°C and then washed with PBS before plating onto collagen-coated coverslips. After plating, the cells were cultured in DMEM containing 10% FCS and 1% penicillin-streptomycin for 24 hr. The cells were then processed for autoradiography as described above.

**Proliferation Assays**

The proliferating activity of neurotensin II was determined by counting the number of cells stained with 1% toluidine blue in a Nucleuscount (Coulter Electronics, Hialeah, FL) and expressed as mean ± SEM.

**Results**

The results of the experiments are shown in Table 1. The percentage of cells stained with 1% toluidine blue in the presence of 10 ng/ml neurotensin II was significantly greater than in the control group (P<0.05). The proliferation of cerebellar cells was inhibited by 30% in the presence of 10 ng/ml neurotensin II. The inhibitory effect of neurotensin II on cerebellar cell proliferation was dose-dependent, with the maximal inhibition occurring at 10 ng/ml. The proliferative activity of neurotensin II was also dose-dependent, with the maximal activity occurring at 10 ng/ml.

**Discussion**

The results of these experiments suggest that neurotensin II has a significant effect on cerebellar cell proliferation. The inhibitory effect of neurotensin II on cerebellar cell proliferation was dose-dependent, with the maximal inhibition occurring at 10 ng/ml. The proliferative activity of neurotensin II was also dose-dependent, with the maximal activity occurring at 10 ng/ml.

**Figure Legends**

Figure 1: Autoradiography of cerebellar cells treated with neurotensin II. The cells were incubated with [3H]-thymidine and then processed for autoradiography as described above. The percentage of cells stained with 1% toluidine blue in the presence of 10 ng/ml neurotensin II was significantly greater than in the control group (P<0.05). Figure 2: Proliferation of cerebellar cells in the presence of neurotensin II. The proliferation of cerebellar cells was inhibited by 30% in the presence of 10 ng/ml neurotensin II. The inhibitory effect of neurotensin II on cerebellar cell proliferation was dose-dependent, with the maximal inhibition occurring at 10 ng/ml. Figure 3: Proliferation of cerebellar cells in the presence of neurotensin II. The results of the experiments are shown in Table 1. The percentage of cells stained with 1% toluidine blue in the presence of 10 ng/ml neurotensin II was significantly greater than in the control group (P<0.05). Figure 4: Proliferation of cerebellar cells in the presence of neurotensin II. The proliferation of cerebellar cells was inhibited by 30% in the presence of 10 ng/ml neurotensin II. The inhibitory effect of neurotensin II on cerebellar cell proliferation was dose-dependent, with the maximal inhibition occurring at 10 ng/ml.
Table IV

| Cycle | Residue | Yield (%) |
|-------|---------|-----------|
| 1     | Asp     | 1.6       |
| 2     | Ala     | 3.2       |
| 3     | Gly     | 2.7       |
| 4     | Arg     | 2.1       |
| 5     | Asn     | 0.4       |
| 6     | Lys     | 2.3       |
| 7     | Asp     | 1.4       |
| 8     | Lys     | 1.4       |
| 9     | Val     | 1.4       |
| 10    | Glu     | 0.6       |
| 11    | Ile     | 1.0       |
| 12    | Leu     | 1.2       |
| 13    | Lys     | 1.5       |
| 14    | Arg     | 0.8       |
| 15    | Lys     | 0.6       |
| 16    | Leu     | 0.4       |
| 17    | Pro     | 0.3       |
| 18    | Tyr     | 0.3       |
| 19    | Ile     | 0.1       |

Yield of the major PTC-Arg-Acid obtained at each cycle is given.

Fig. 1
Profile of HPLC and absorbance at 270 nm obtained during the final HPLC step of the purification of pepstatin-cleaved large VMN. See text for details.

Table III
Molar Ratios of Amino Acids in Isolated Peptide-Generated Fragment from Large VMN

| Amino Acid | Ratio (Integer) |
|------------|-----------------|
| Asp        | 4.0             |
| Glu        | 2.28            |
| Arg        | 1.20            |
| Asn        | 1.22            |
| Lys        | 1.26            |
| Val        | 1.6             |
| Leu        | 1.66            |
| Fru        | 1.85            |
| Tyr        | 1.66            |
| Ile        | 0.00 (2-10)     |
| Total residues | 1.97 (26) |

Given are the molar ratios of the constituent amino acids of the acid hydrolyzable peptide (from fraction 2.8 mm) obtained when the final HPLC step. Spectrophotometric analysis was consistent with 1 Tyr and 0 Trp.

*Fig. 1-13 bond is known to resist hydrolysis.
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