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Proneurotensin 1–117, a stable neurotensin precursor fragment identified in human circulation

A. Ernst [a,*], S. Hellmich [a], A. Bergmann [a,b]

[a] SphingoTec GmbH, Tulpenweg 6, D-16556 Borgsdorf, Germany
[b] BRAHMS AG, Neuendorfstraße 25, D-16761 Hennigsdorf, Germany

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ABSTRACT

Proneurotensin/neuromedin N (pro NT/NMN) is the common precursor of two biologically active peptides, neurotensin (NT) and neuromedin N (NMN). We have established antibodies against peptide sequences of the NT/NMN precursor and developed a sandwich immunoassay for the detection of pro NT/NMN immunoreactivity in human circulation. Endogenous pro NT/NMN immunoreactivity was enriched by affinity chromatography using antibodies against two different pro NT/NMN epitopes, and further purified by reversed phase HPLC. Mass spectrometry analysis revealed pro NT/NMN 1–117 as major pro NT/NMN immunoreactivity in human circulation. Pro NT/NMN 1–117 is detectable in serum from healthy individuals (n = 124; median 338.9 pmol/L). As known for NT, the release of pro NT/NMN 1–117 from the intestine into the circulation is stimulated by ingestion of an ordinary meal. Investigation of the pro NT/NMN 1–117 in vitro stability in human serum and plasma revealed that this molecule is stable for at least 48 h at room temperature. Since pro NT/NMN 1–117 is theoretically produced during precursor processing in stoichiometric amounts relative to NT and NMN, it could be a surrogate marker for the release of these bioactive peptides.

1. Introduction

Neurotensin (NT) and neuromedin N (NMN) are two related peptides, primarily localized in the central nervous system [7,27] and the gastrointestinal tract [23], where they excert neurotransmitter/neuromodulator [24,34] and endocrine/paracrine functions, respectively. Moreover, expression of NT was demonstrated in the heart [32] and the adrenals [19].

Intestinal NT is produced and stored by specific enteroendocrine mucosal cells (N-cells) in the jejunum and ileum and to a lesser extent in the colon and duodenum [21,31,39]. The release of NT into the circulation is triggered after ingestion of food [25], and fat has been shown to be the strongest stimulus [33,16,40]. NT exhibits various digestive functions: stimulation of pancreatic and biliary secretions, inhibition of gastric acid secretion and motility, stimulation of colon motility, and inhibition of jejuno-ileum motility (for review see [17]). In the central nervous system NT excerts diverse effects including hypothermia [5], antinociception [12], stimulation of anterior pituitary hormone secretion [26,36], and modulation of the dopaminergic transmission [30,34].

The human prepro NT/NMN precursor molecule, containing the peptide sequences of NT and NMN, consists of 170 amino acids [2] and its structure is illustrated in Fig. 1. Cleavage of the N-terminal signal sequence by specific signal peptidases [3] results in the formation of pro NT/NMN (147 amino acids) [14]. The N- and C-termini of NT and NMN are flanked by Lys–Arg-sequences which represent potential

* *Corresponding author*. Tel.: +49 3302 883 740; fax: +49 3303 29 50 92.
E-mail address: a.ernst@sphingotec.de (A. Ernst).
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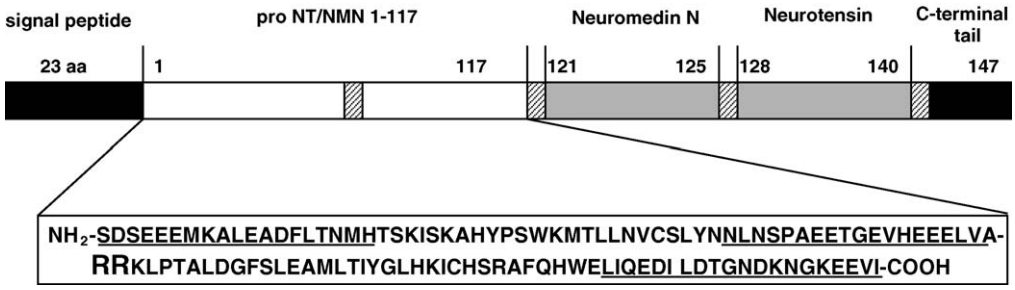

Fig. 1 – Symbolic sequence structure of the human prepro-NT/NMN precursor. The signal sequence consists of 23 amino acids. Dibasic amino acids representing targets for proteolytic processing are shown as dashed boxes. The amino acid sequence of pro NT/NMN 1–117 is indicated. Peptide sequences pro NT/NMN 1–19, 44–62 and 98–117, used for antibody production, are underlined. The potential prohormone convertase cleavage site Arg^{63}–Arg^{64} is highlighted.

cleavage sites for prohormone convertases [15,35]. A fourth dibasic site consisting of an Arg-doublette is present within the N-terminal region of the precursor. Processing of the NT/NMN precursor is tissue-specific. In brain processing results in the formation of NT and NMN [10,13], whereas in the gut and in the adrenals processing leads to the production of NT and two other potentially bioactive peptides, large NT and large NMN, which are N-terminal extended peptides of NT and NMN, respectively [8–10].

However, the reliable measurement of both, mature NT and NMN as well as large NT and large NMN in biological fluids, is limited due to the instability of these molecules in vivo [1,11] as well as in vitro [18].

In this report, we describe the identification of a new neurotensin precursor fragment in human blood, characterized as pro NT/NMN 1–117. We developed an immunoassay for the detection of pro NT/NMN 1–117 and were able to show, that this molecule is completely stable in human serum and plasma for at least 48 h when stored at room temperature. In addtion, we demonstrate, that the secretion of pro NT/NMN 1–117 into the blood stream is stimulated by ingestion of an ordinary meal, as it was reported for neurotensin. Since pro NT/NMN 1–117 is produced in equimolar amounts to neurotensin and neuromedin N, released amounts of pro NT/NMN 1–117 should represent those of NT and NMN, respectively. The high stability of pro NT/NMN 1–117 is a significant advantage and a main prerequisite for its use in laboratory and clinical routine.

2. Materials and methods

2.1. Chemicals

If not stated otherwise, chemicals were obtained at p.a. grade from Merck (Darmstadt, Germany). Non-specific rabbit immune globulin, bovine serum albumin and horse serum H1270 were purchased from SIGMA (Deisenhofen, Germany).

2.2. Peptides

Three peptides chemically related to the NT/NMN precursor (1–147), pro NT/NMN 1–19 (SDSEEEMKALEADFLTNMH), pro NT/NMN 44–62 (NLNSPAEETGEVHEEELVA) and pro NT/NMN 98–117 (LIQEDILDTGNDKNGKEEVI), were supplied by JPT Peptide Technologies GmbH (Berlin, Germany). These peptides were synthesized with an additional N-terminal cystein-residue for conjugation of the peptides to Keyhole limpet hemocyanin (KLH) and covalent immobilization on Sulfolink coupling gel (Perbio Science, Bonn, Germany). The peptide pro NT/NMN 1–62 (SDSEEEM KALEADFLTNMHTSKI-SKAHVPSWKMTLLNVCSLVNNLNSPAEETGEVHEEELVA) was synthesized as standard material for the sandwich immunoassay using antibodies against the peptides pro NT/NMN 1–19 and 44–62.

2.3. Antibodies

Antibodies against the pro NT/NMN-peptides 1–19, 44–62 and 98–117 were raised in rabbits by InVivo BioTech Services GmbH (Hennigsdorf, Germany). Peptide-specific antibodies were purified from rabbit antisera by affinity chromatography using Sulfolink gel according to the suppliers instructions.

2.4. Serum and plasma samples

Serum and plasma samples (EDTA and heparin) from healthy blood donors used for determination of stability, frequency distribution and purification of pro NT/NMN, were obtained from InVent Diagnostica GmbH (Hennigsdorf, Germany). These samples were taken randomly without any information about eating behaviour or daytime. For the purification of pro NT/NMN immunoreactivity serum samples from healthy blood donors were pooled (final volume: 1000 mL), filtrated through a 0.22 μm Millipore filter. Subsequently, the serum pool was diluted 1:2 in PBS, pH 7.2 containing 10 mmol/L Na-EDTA and pro NT/NMN immunoreactivity was isolated as described later.

To determine the level of pro NT/NMN 1–117 in humans before and after an ordinary meal (about 1200 kcal), three men and three women, volunteer employees of a local biotechnology center, fasted overnight (14 h), consumed a 1-L water load the next morning and a standardized meal for lunch. Fifteen serum samples of each study participant obtained with peripheral venipuncture at several time points were subsequently centrifuged and frozen at −20 °C until measurement.