Pharmacological Reports
2010, 62, 767–777
ISSN 1734-1140

Review

Humanins, the neuroprotective and cytoprotective peptides with antiapoptotic and anti-inflammatory properties

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Abstract:
Humanin (HN) is a newly discovered 24-amino acid peptide, which may suppress neuronal cell death. HN cDNA includes an open reading frame (HN-ORF) of 75 bases located 950 bases downstream of the 5' end of the HN cDNA. It has been demonstrated that HN cDNA is 99% identical to the mitochondrial DNA (mtDNA) sequence. HN homologs have been identified as expressed sequence tags (ESTs) in both rats and nematodes. Certain regions that are homologous to the HN cDNA exist on human chromosomes. HN forms homodimers and multimers and this action seems to be essential for peptide function. HN acts as a ligand for formyl peptide receptor-like 1 (FPRL1) and 2 (FPRL2). It has been demonstrated that HN plays a protective role through its antiapoptotic activity that interferes with Bax activation, which suppresses Bax-dependent apoptosis. HN has also been shown to suppress the c-Jun N-terminal kinase (JNK) and ASK/JNK-mediated neuronal cell death. Several studies have also confirmed that HN could be important in the prevention of angiopathy-associated Alzheimer's disease dementia, diseases related to mitochondrial dysfunction (MELAS), and other types of β-amyloid accumulation-associated neurodegeneration. A very recent study demonstrated a pluripotent cytoprotective effect and mechanisms of HNs in cells not from the CNS, such as germ cells or pancreatic β-cells, and the potent physiological consequences that result from HN interaction with IGFBP3 and STAT3. *In vivo* studies suggest that HN may also protect against cognitive impairment due to ischemia/reperfusion injury.

Key words:
humanin (HN), HN derivatives, cytoprotection, Alzheimer's disease, mitochondrial function, apoptosis, inflammatory response, ischemia, cognitive impairment

Abbreviations: AD – Alzheimer disease, ADNF – activity-dependent neurotrophic factor, ADP – adenosine diphosphate, Ala – alanine, APP – amyloid precursor protein, Arg – arginine, ASK1 – apoptosis signal-regulating kinase 1, ATP – adenosine triphosphate, Aβ – amyloid-β, Aβ40D – Dutch variant β-amyloid, AβPP – amyloid β protein precursor, CA1 – Cornus Ammonis 1, CAA – cerebral amyloid-β protein angiopathy, cAMP – cyclic A mononucleotide, cDNA – complementary DNA, CNS – central nervous system, CNTFR – ciliary neurotrophic factor receptor, Cys – cysteine, DNA – deoxyribonucleic acid, DRPLA – dentatorubral-pallidoluysian atrophy, D-Ser – D-form serine residue, ER – endoplasmic reticulum, ESTs – expressed

sequence tags, FAD – familial Alzheimer's disease, fEPSPs – field excitatory postsynaptic potentials, ERK – extracellular signal-regulated kinase, Fas – Fas-ligand receptor, fM – femtomolar, FPRL1, -2 – formyl peptide receptor like 1 and 2, FPR2-formyl peptide receptor 2, GFAP – glial fibrillary acidic protein, HCSM – human cerebrovascular smooth muscle, HFS – high-frequency stimulus, HN – humanin, HN-ORF – humanin open reading frame, HNA – C8A- HN, HNG – S14G-HN, [Gly^{14}]-humanin, I/R – ischemia/reperfusion, IGF-1 – insulin-like growth factor 1, IGFBP3 – insulin- like growth factor-binding protein 3, IL-6 – interleukin-6, JNK – c-Jun N-terminal kinase, Leu – leucine, LTP – long-term potentiation, Lys – lysine, MAP – mitogen-activated protein, MCAO – middle cerebral artery occlusion, MELAS – mitochondrial encephalomyopathy with lactic acidosis & stroke-like episodes, mRNA – messenger RNA, mtDNA – mitochondrial DNA, MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, NOD – non-obese diabetic mice, ORF – open reading frame, PARP – poly (ADP-ribose) polymerase, Phe – phenylalanine, PI3 – phosphatidylinositol 3, polyQ – polyglutamine, PPF – paired-pulse facilitation, Pro – proline, PrPc – cellular prion protein, PS1, -2 – presenilin 1 and 2, PTX – pertussin toxin, QNB – 3-quinuclidinyl benzilate, RNA – ribonucleic acid, rRNA – ribosomal RNA, Ser – serine, SOD-1 – superoxide dismutase 1, STAT – signal transducer and activator of transcription, STAT3 – signal transducer and activator of transcription 3, T2DM – type 2 diabetes mellitus, TGFβ – transforming growth factor β, Thr – threonine, TNFα – tumor necrosis factor α, TRIM11 – tripartite motif protein 11, TUNEL – TdT-dependent dUTP-biotin nick end labeling, V642I-AβPP – London-type familial Alzheimer's disease mutant of amyloid β protein precursor, Val – valine, β-AP – β-amyloid

Introduction

Humanin (HN) is a recently discovered 24-amino acid cytoprotective peptide. It was identified by a Japanese group in 2001 in the brain of a patient diagnosed with Alzheimer's disease (AD) [18]. AD is one of the most common neurodegenerative diseases in the aging population [1]. This form of dementia leads to the progressive loss of mental capacity and ability to learn [56]. One of the main neuropathological features of AD is increased cellular synthesis of the amyloid precursor protein (APP), which is a precursor of the amyloid-β (Aβ) peptide, a component of senile plaques that characterize AD [2, 9]. APP also acts as a single transmembrane receptor-like protein that adopts the transporting kinesin mechanism [25, 32]. Multiple studies revealed that the β-amyloid protein (β-AP) is neurotoxic *in vitro* and plays a central role in the neuronal death pathway, the development of disability and dementia [3].

Several types of APP gene mutations pathognomonic for AD have been discovered [10]. One of them, the London-type APP mutant, was characterized as the first familial AD-linked mutation [10]. Well-documented mutations in one of three protein genes, APP, presenilin 1 (PS1), and presenilin 2 (PS2), are most frequently involved in the early-onset of familial AD (FAD) [57].

Hashimoto et al. [19] used the modified functional expression screening named "death-trap" (developed by D'Adamio and coworkers [7]) to identify molecules that might suppress FAD gene-induced death in the brains of patients diagnosed with Alzheimer's disease. They constructed a cDNA library from the occipital cortex of a patient with AD to identify cDNA fragments that suppressed the neuronal cell death induced by the London-type FAD mutant of APP (V642I-AβPP) [18]. Expression of certain antiapoptotic genes that would protect against AD-related neurotoxicity in the occipital lobe was previously suggested [1]. Thus, it was hypothesized that there were

A Humanin and related peptides

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Human:      MAPRGFSCLLLLTSEIDLPVKRRA
Rat:        MAKRGFNCLLLSISEIDLPVKRLESPNKTRRPYGASIY
Nematode:   MAXRGFICLLLLTSEXDLPVKRRA
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B Essential residues

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Humanin:            MAPRGFSCLLLLTSEIDLPVKRRA
NPCD:                  PRGFSCLLLLTSEIDLP
Dimerization:       - - - - - - S–L - - - - - - - - - - - - - - -
Secretion:          - - - - - - - - LLL- - - - - - - PV - - - -
Neuroprotection:    - - P - - - SCL- -LTS- - - - P - - - - -
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Fig. 1. Recent known sequences and the origin of humanins (figure adapted from Niikura et al. [47]). (**A**) The sequence of HN. The HN-like peptides are from the rat expressed sequence tag (EST) (Rattin) [4], nematode EST [13], and deduced sequence from the rat mitochondria 16S rRNA region (27 AA) [22]. (**B**) The structure-function relationship of HN. Essential amino acid residues for secretion, dimerization, and neuroprotection in full-length HN are shown. Residues shown as "–" can be replaced with Ala

some neuronal survival factor(s) abundant in this region of the brain [50]. Further screening resulted in the discovery of a cDNA consisting of 1,567 bases that encoded a short polypeptide (MAPRGFSCLLLLTSEIDLPVKRRA) (Fig. 1A). This molecule was named humanin (HN) and was found to suppress the neuronal cell death induced by proteins that resulted from expression of the familial Alzheimer's disease genes: neurotoxic Aβ peptides and an anti-amyloid precursor protein antibody [18].

The sequence and origin of humanin

Following the identification of HN, multiple structure-activity studies were undertaken [12, 18, 47]. It is presently known that the 1,567-base HN cDNA includes an open reading frame (HN-ORF) of 75 bases, which is located 950 bases downstream of the 5' end of the HN cDNA. Moreover, in the long HN sequence, at least seven potential in-frame ORFs are included in the 5'region of the HN-ORF, and there is no signal peptide sequence at the N terminus [60]. Within the HN sequence, there is a hydrophobic core region, GFSCLLLLTSEIDL, flanked by a C-terminal region, PVKRRA, and an N-terminal region, MAPR [18] (Fig. 1A). Other studies have revealed that HN cDNA containing the humanin open reading frame (HN-ORF) is 99% identical to positions 1680–3231 of mitochondrial DNA (mtDNA). This region of mtDNA corresponds to 16s ribosomal RNA (rRNA) (Fig. 1A) [60].

Several genomic regions and expressed sequence tags (ESTs) have shown a high degree of similarity with HN cDNA. Moreover, HN homologs have been identified as ESTs in rat and other species, such as nematode (Fig.1A) [47]. There is also evidence that certain regions of over 1000 bases located in human chromosomes 5, 11 and X show 92–95% homology with HN cDNA [18].

Despite many studies, it is still not clear whether the DNA source of the tissue/cellular HN peptide is of mitochondrial or nuclear origin [11, 47]. One possibility is that HN mRNA is transcribed from the mitochondrial DNA and translocated to the cytoplasm before its translation into the peptide [11, 50]. Another possibility is that HN messenger RNA (mRNA) is transcribed from the nuclear genomic DNA only [50]. It has also been suggested that HN mRNA is transcribed from the mitochondrial DNA and translated into the peptide in the mitochondria [50]. Future studies are essential to define this pathway.

The function, structural requirements and humanin analogs

HN can act as both a signal peptide and a neuroprotective factor [50]. Molecular studies on the HN family peptides have revealed that there is a strong structure-function relationship (Fig. 1B). The entire sequence of HN may function as a signal peptide for secreting proteins [50]. Introduction of full-length HN cDNA or HN-ORF into cells results in translation and secretion of the HN peptide [18, 68]. Such a signal sequence contains the positively charged N-terminal region, the central hydrophobic region and the negatively charged C-terminal region [50]. Mutagenesis studies demonstrated also no effect of Ala substitution for any of the amino acids located between the third Pro and twenty-third Arg on the secretion of full-length HN. However, when one of the following amino acids including Leu^{9} to Leu^{11}, Pro^{19} or Val^{20} is changed to Arg, these HN mutants are no longer effectively secreted [50] (Fig. 1B). There is also evidence that the lipophilic region of $Leu^{9}Leu^{10}Leu^{11}$, Pro^{19} and Val^{20} are essential for HN secretion [50, 68] (Fig. 1B). Within the lipophilic region of $Leu^{9}Leu^{10}Leu^{11}$, substitution of Leu^{10} with Asp abolished the secretion of HN, indicating that Leu10 plays a crucial role in the secretion of full-length HN [50].

Several studies have revealed that a Pro^{3}-Pro^{19} region in the HN sequence acts as a core domain and is required for the neuroprotective activity of HN (Fig. 1B). In this domain, Pro^{3}, Cys^{8}, Leu^{9}, Leu^{12}, Thr^{13}, Ser^{14}, and Pro^{19} were shown to be essential residues [50]. Substitution of any of the residues listed above with Ala led to elimination of the HN protective activity [50]. However, it was also reported that Cys^{8} could be substituted with Lys and Arg without impairing HN function, whereas His substitution significantly, but not completely, attenuated HN activity. Any other substitution resulted in the loss of the protective function of HN [17]. It was also suggested that only Ser^{7} could be substituted with Ala in the core domain without a negative effect on the neuroprotective activity of the Pro^{3}-Pro^{19} region [18, 51]. Also, a number of the

leucine residues in the middle region of various N- or C-terminally truncated HN analogs may affect the neuroprotective role of HN [62].

A conformational change in HN peptide structure may also be linked to the neuroprotective activity of HN [18]. Substitution of Gly for Ser^{14} in the mutant $S^{14}G$-HN (HNG) enhances its cytoprotective activity by 1000-fold [18]. Also, the exchange of Ser^{14} to the D-form Ser residue (D-Ser) resulted in the similar enhancement of HN-mediated neuroprotection [62].

Biochemical and biophysical studies based on the HN protein sequence reveal that HN forms homodimers and multimers, which are essential for HN activity [1, 49] (Fig. 1C). Dimer and multimer formation may be also connected to the solubility and tissue distribution of HN [1].

Arakawa et al. [1] classified known HN analogs into 6 groups (Tab. 1). In accordance with this classification, L9R-HN belongs to class 1 and is characterized by the loss of its ability to be secreted. HNG, class 2 analog, contains a mutation of Ser^{14} to Gly or D-Ser. HNA and $S^{7}A$-HN are members of class 3 and are considered inactive mutants. The most active HN analog is AGAC8R-HNG17, which constitutes class 4. The HN analog EF-S7A-HN from class 5 was shown to reactivate the inactive HN mutant. Colivelin (class 6) is a HN analog, which was synthesized to enhance the chemical and biological stability of HN. Colivelin was produced by fusion of a neurotrophic factor (activity-dependent neurotrophic factor 9 (ADNF9)) and AGAC8R-HNG17, and is characterized by activity at very low (fM) concentrations [1].

Tab. 1. Suggested classification of HN analogs (table adapted from Arakawa et al. [1])

Class	Peptide Name	Mutation
	HN	MAPRGFSCLLLLTSEIDLPVKRRA
Class 1	L9R-HN	Leu 9 → Arg
Class 2	HNG	Ser 14 → Gly
Class 2	D-Ser 14	L-Ser 14 → D-Ser
Class 3	HNA	Cys 8 → Ala
Class 3	S7A-HN	Ser 7 → Ala
Class 4	AGA-HNG	Arg 4 → Ala, Phe 6 → Ala, Ser 14 → Gly
Class 4	AGAC8R-HNG17	Deletion of N-terminal MA Arg 4 → Ala, Phe 6 → Ala Cys 8 → Arg, Ser 14 → Gly Deletion of C-terminal VKRRA
Class 5	EF-S7A-HN	Ser 7 → Ala N-terminal addition of EFLIVIKS
Class 6	Colivelin	ADNF9-AGAC8R-HNG17

Regulation of humanin expression

The exact location of HN expression and whether it is transcribed from the genomic or mitochondrial DNA has not yet been clarified [51]. Immunohistochemical studies demonstrated HN immunoreactivity in intact large neurons of the occipital lobe in patients with AD [60]. However, HN expression was not observed in the neurons of other brain regions or in the age-matched control brains [60]. Moreover, HN immunoreactivity was found in the hippocampal glial cells of AD patients [11].

Immunoblot analysis of normal mouse tissues revealed an immunoreactive band of 3 kDa (the supposed molecular weight of HN) in the testis and colon of 3-week-old mice, and then only in the testis of 12-week-old mice [11]. These findings may suggest maturation-dependent expression of the HN peptide and indicate that HN may also be produced in the other tissues.

HN release from the neuronal cells can be blocked by (+)-brefeldin A, an inhibitor of ER-Golgi transport [18]. In the same cells, the release of HN was greatly augmented by elevation of cytosolic calcium and cAMP, which suggests an important role for activation/stress conditions [18].

The regulatory mechanism of HN biosynthesis is also not well clarified. Niikura et al. [48] have presented the first evidence suggesting that the intracellular HN level is regulated by the tripartite motif protein 11 (TRIM11). TRIM11, a member of the TRIM/RBCC protein family, was identified as a novel HN-interacting protein, which targeted HN to an E3 ligase and regulated HN level through the proteasomal degradation pathway [48].

Evidence of the protective effect of humanin (*in vitro* models)

Several recent *in vitro* studies have confirmed the protective effect of HNs against different stress models using tissue culture of neuronal F11 cells [14, 18, 16], PC12 cells [30, 28], and cortical neurons [58], but also lymphocytes [29, 27], erythroleukemia K562 cells [65] and cerebrovascular smooth muscle cells [24].

The neuroprotective effect of HN peptides against Aβ1-42-induced neuronal death has been demonstrated in F11 cells, hybrid cells of the rat day 13 (E13) embryonic primary neurons and mouse neuroblastoma. Similar positive effects were also demonstrated in the presence of low concentrations of synthetic HNG (10 nM), as opposed to HNA even at concentrations as high as 10 μM. The abovementioned observations were corroborated by cell viability assays using calcein [16]. HN and HNG, but not HNA, demonstrated cytoprotective effects by suppressing the dystrophic changes induced by the anti-APP antibody 22C11, Aβ1-43, Aβ1-42, Aβ25-35, FAD mutants of APP (V642I-APP, NL-APP, A617G-APP, L648P-APP), FAD mutants of PS1 (M146L-PS1, A246E-PS1, L286V-PS1, C410Y-PS1, H163R-PS1) and N1411-PS2 [16].

The neuroprotective activity of HN appears to be somewhat selective as neither HN nor HNG protects neuronal cells from neurotoxicity induced by free radicals (multiple superoxide dismutase 1 (SOD-1) mutants), Fas activators, or etoposide [18].

Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder linked to disruption of polyglutamine (polyQ) segment length (the polyglutamine repeat) [34, 61]. Intracellular aggregates of polyglutamine induce activation of apoptosis signal-regulating kinase 1 (ASK1) and cellular death [33, 35, 43, 45, 52]. In undifferentiated neuronal PC12 cells, it was demonstrated that there was no direct interaction between HN and polyQs but HN could prevent activation of the proapoptotic kinase ASK1 [28]. Irregular modulation of prion-peptide PrP^c biogenesis at the ER could induce neurodegeneration as a result of the accumulation of incorrect topologies of PrP^c [59].

The effect of HN, HNA and HNG on apoptosis induced by the PrP has been studied in cortical neurons [58]. HN and HNG attenuated PrP(118-135)-induced neuronal apoptosis, and HNG exhibited 500-fold higher protective properties in this model. These results suggest that the HN peptide may also protect neurons from other injuries that are not Alzheimer's disease-related [58]. This protective potential of HN was found to be dependent on primary structure of the peptide – especially Cys^8 and Ser^{14} [14].

The cytoprotective properties of HN were also examined in the serum-deprived human lymphocyte model. The metabolic activity measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay demonstrated a reduction in metabolism under serum-deprived conditions to nearly 85% of the control value. Treatment with HN provides significant protection of the metabolic activity in cells [29]. These findings support the hypothesis that HN may augment mitochondrial energy production and hence prolong the survival of these cells. HN-induced ATP concentration augmentation is also observed in the lymphocytes of patients harboring A3243G mutant mtDNA [27].

Additionally, in human rhabdomyosarcoma TE671 cells cultured under serum-free conditions, the presence of HN increased the biosynthesis of ATP, but not of pyruvate [26]. These results point to a possible role for HNs in diseases related to mitochondrial dysfunction such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) or brain ischemia [26, 27, 29].

The antiapoptotic effect and potential mechanism of HN in hematopoietic cells can be explored using serum-free culture of human chronic myelogenous leukemia K562 cells [65]. Serum deprivation caused a decrease in HN expression, as well as inhibition of the ERK pathway and activation of p38 kinase signaling. HN transfection of K562 cells does not increase cell viability, but only delays the onset of apoptosis and suppresses p38 activation during the first 24 h. Overexpression of HN decreases the number of cells arrested at the G2/M phase of the cell cycle, and precludes them from apoptosis. Thus, the ability of HN to reduce cell death may also include the downregulation of p38 activity, which is supposed to be involved in both cell cycle arrest and apoptosis in K562 cells under serum deficient conditions. Transfection with HN decreased the differentiation of K562 cells into megakaryocytic cells. HN could thus be involved in the preservation of undifferentiated progenitor hematopoietic stem cells *in vivo*, which, combined with HN antiapoptotic properties, may prove important in the understanding of bone marrow maturation [65].

The HN peptide is also known to protect cultured human cerebrovascular smooth muscle (HCSM) cells from the cytotoxic influence of amyloid-β [24]. Cell degeneration caused by the accumulation of Aβ is frequently present in cerebral Aβ protein-induced angiopathy (CAA), [31, 64]. Incubation of the HCSM cells with Aβ40D causes a nearly 60% decrease in their vitality [24]. Application of HN or HNG prior to Aβ significantly reduces the extent of cell death. The Aβ-induced HCSM α-actin degeneration is partially blocked by humanin. HN does not directly interfere with the Aβ precursor protein or Aβ accumulation, or fibril formation on the HCSM cell surface. These findings indicate that HN peptides could also be important in the prevention of angiopathy associated with AD (CAA) [24].

Cytoprotective mechanisms of humanin and humanin receptors

HN was first identified as a neuroprotective factor that suppressed the neuronal cell death induced by proteins generated by the expression of familial Alzheimer's disease-related genes and Aβ accumulation [18]. In the series of transfections, it has been demonstrated that HN acts in the extracellular environment because certain, nonsecreted point mutants of HN retained in the cytoplasm do not exert any neuroprotective activity [18, 68]. Also, the extracellular L9R-HN peptide suppresses neurotoxicity, whereas intracellular L9R-HN is not neuroprotective against an insult induced by Aβ peptides [18]. These findings suggest that there must be some putative cellular surface receptor(s) for HN [48]. In 2004, Ying et al. demonstrated for the first time that FPRL-1 acts as a functional receptor for HN [70]. They found that HN may bind to pertussin toxin (PTX)-sensitive G-protein-coupled human formyl peptide receptor-like-1 (FPRL-1), which was originally identified as a receptor for Aβ (1–42) [38]. In the same study, HN interacted with FPRL-1 in PC12 pheochromocytoma cells by competing with Aβ (1–42) for binding to FPRL-1 and inhibiting Aβ (1–42)-induced neurotoxicity [70]. This interaction activates signal transduction pathways mediated by ERK in the neuronal cells [70]. Another study revealed that HN may act as a ligand for the formyl peptide receptor-like 1 (FPRL1) and 2 (FPRL2) receptors [13]. FPRL1 and FPRL2 are also Aβ-binding receptors. These results may indicate that HN competes with Aβ for binding to these receptors and thereby decreases Aβ-induced toxicity [13]. Moreover, it was later discovered that FPR2 (formyl peptide receptor 2), the mouse ortholog of FPRL-1, was not required to mediate HN-induced neuroprotection against AD-related insults in F11 neurohybrid cells. These findings suggested that HN activity is not exclusively mediated by human FPRL-1 and FPRL-2 or mouse FPR receptors, but that several mechanisms could exist, which may participate HN neuroprotective activity in different cells [20, 49].

Hashimoto et al. [20] suggested that HN-mediated neuroprotection may involve the activation of certain tyrosine kinases and the signal transducers and activators of transcription (STATs), specifically STAT3. Tyrosine kinases play important roles in several signal transduction pathways. STATs are known to be cytoplasmic proteins that play important roles in normal cellular responses to cytokines and growth factors [63]. Abnormal activity of certain STAT family members, especially STAT3, has been found in oncogenesis [63]. It is known that HN suppressed the cell death induced by familial Alzheimer's disease independently of Aβ-induced toxicity [18, 16, 48]. In summary, HN, which is able to interrupt both apoptotic and nonapoptotic AD-related cell death, might also be associated with oncogenesis through the STAT3-dependent antiapoptotic signal transduction cascade [20, 51].

The same group demonstrated that the HN-induced neuroprotective activity can be blocked by genistein, the tyrosine kinase inhibitor, while the PI3 kinase and mitogen-activated protein (MAP) kinase inhibitors did not abolish the protective effects of HN [18].

HN has also been reported to suppress the c-Jun N-terminal kinase (JNK) and ASK/JNK-mediated neuronal cell death [16]. Thus, HN blocked APP-induced neurotoxicity by the suppression of JNK activation as well as by inhibition of the downstream JNK pathway [16].

A very recent study provides new evidence for the "anti-inflammatory" effect of HN [15]. HN was shown to inhibit neuronal cell death and dysfunction by binding to a cytokine receptor complex or complexes involving ciliary neurotrophic factor receptor (CNTFR), IL-27 receptor WSX-1, and gp130. Gp130, a common subunit of receptors that belong to the IL-6 receptor family, and CNTFR have been found in neuronal cells [8]. WSX-1 is expressed in all neuronal cells including F11 cells, SH-SY5Y cells and primary cortical

neurons [8]. These findings suggested that hetero-oligomers composed of WSX-1/CNTFR/gp130 are involved in HN-induced signaling in neuronal cells and that gp130 and two gp130-related subunits, CNTFR and WSX-1, are required for cellular responsiveness to HN-mediated neuroprotection [15]. They also demonstrated that CNTFR and WSX-1 are essential to mediate HN binding to neuronal cells [15]. Taking together, these results demonstrated that HN protects neurons by binding to a complex or complexes involving CNTFR/WSX-1/gp130 [15]. Therefore, it could also be taken into consideration that HN inhibits neuronal cell death by interacting with two or three cytokine receptor dimers consisting of gp130/WSX-1, gp130/CNTFR or CNTFR/WSX-1 [15].

Guo et al. [40] demonstrated that the interaction of HN with Bax represents another potential HN receptor-mediated mechanism by which HN exerts its antiapoptotic activity. It has been demonstrated that HN interferes with and inhibits Bax activation [40]. Bax, a proapoptotic, Bcl-2-associated X protein, is an apoptosis-inducing protein that participates in cell death [54]. Apoptosis is genetically regulated programmed cell death, which plays a critical role in normal development, tissue homeostasis, the elimination of infected or damaged cells and aging [21].

Mitochondria are the main organelles for ATP generation, integration and execution of various stimuli responsible for cell death [55]. Integrity of the mitochondrial membranes is controlled by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the Bcl-2 family [11]. HN was found to selectively bind to Bax in the cytoplasm and prevent Bax translocation from the cytoplasm to the mitochondria, thus suppressing apoptosis [11]. It was demonstrated that the core domain, Pro^3-Asp^{17}, is essential for HN/Bax binding and the antiapoptotic effect of HN. The substitution of Pro for Cys^8 results in the loss of both Bax binding and the antiapoptotic effect [48].

Efficacy of humanin in *in vivo* cognition studies

In vivo studies, of which there have been relatively few, show that HN may protect against cognitive impairment, inflammatory response, apoptosis and stroke induced by different factors such as scopolamine [36], Aβ25-35 [10, 38], Aβ31-35 [10], 3-quinuclidinyl benzilate [36, 37] or brain ischemia caused by the middle cerebral artery occlusion [67] in mouse or rat models.

The effects of [Gly^{14}]-humanin (HNG) on the impairment of spontaneous alternation behavior in mice were investigated with regard to scopolamine-induced (muscarinic acetylcholine receptor antagonist) learning and memory deficits as assessed by the Y-maze test. HNG and scopolamine were administered 30 and 15 min before the Y-maze test, respectively. Scopolamine impairs spontaneous alternation behavior and this deficit was reversed by HNG treatment. These findings suggest that HN may be useful for the alleviation of certain types of memory disturbance. Additionally, HNG itself normalized the defecation rate during the Y-maze test, suggesting that HN may affect fear and anxiety [42].

Another model of Aβ25-35 (the neurotoxic domain of the full-length Aβ peptide [5]) injection was used to assess the effects of HNG on the impaired cognitive function in mice or rats [12, 44]. Intracranial injection of aggregated Aβ25-35 causes deficiencies in short-term memory as evaluated by spontaneous alternation behavior in the Y-maze and in long-term memory as monitored by "step-down type passive avoidance task" [44]. Treatment with Aβ25-35 biochemically led to an increase in activated astrocytes, an increased number of activated microglia and elevated local levels of IL-6 and TNFα in the brain as is typical for AD. Impairment of spatial working memory is significantly reduced when HNG is administered intraperitoneally (0.1 μg/0.5 ml in saline). The effect of HNG on attenuation of cognitive dysfunction can be confirmed by the increased retention latency of the passive avoidance test. HNG treatment reduces the number of GFAP (glial fibrillary acidic protein)-immunopositive astrocytes and CD11b-immunopositive microglia in the hippocampus and frontotemporal cortex. Treatment with HNG decreases the IL-6 and TNFα staining induced by Aβ25-35 and reduces the TUNEL-positive apoptotic cells in the wide region of frontal cortex [44]. Thus, the results of this study point toward the anti-inflammatory effects of humanin biological activity.

The aim of the other cognitive experimental study seems to be examination of the effect of intracerebroventricular injections of HNG on Aβ fragment-induced (Aβ25-35 or Aβ31-35) suppression of long-term potentiation (LTP) in the rat hippocampal CA1 region [12]. First, animals were surgically prepared for

acute LTP recordings *in vivo* (two electrodes: stimulating in the Schaffer-collateral/commissural pathway and recording in the stratum radiatum of CA1 area). Baseline field excitatory postsynaptic potentials (fEPSPs) are provoked by test stimuli with intensities that elicited 50% of the maximal response. LTP is induced by a high-frequency stimulus (HFS). The experiment demonstrates that Aβ31-35 inhibits hippocampal LTP with the same efficiency as Aβ25-35. Coadministration of HNG and Aβ31-35 effectively reverses the Aβ-induced LTP suppression at all recorded time points, but there is no effect on normal LTP. It was also observed that pretreatment with HNG and Aβ25-35, either separately or jointly, did not influence paired-pulse facilitation (PPF). The protective action of HNG against LTP inhibition is significantly reduced by intracerebroventricular injections of the tyrosine kinase inhibitors, genistein or butein, suggesting that tyrosine kinases may also be essential for the protective action of HNG [12].

Disturbances in spatial memory in the 3-quinuclidinyl benzilate (QNB) model [36, 66] have also been used for the investigation of HN activity; QNB is an anticholinergic drug. In this study, the neuroprotective role of HNG and the HN analog des-Leu-PAGA is evaluated by assessing the impairment of spatial orientation and memory in multiple T-maze tests [36]. Deterioration of T-maze performance is observed 15 min and 24 h after QNB application as evidenced by an elevated number of entries into wrong arms and prolonged passage times. Both peptides demonstrated important anti-amnesic effects. These findings confirm the neuroprotective potential of HNG and demonstrate the potential of des-Leu-PAGA in this respect [36]. Also, other members of the HN family peptides have been investigated regarding the QNB-induced memory deficit [37]. The results show that six HN analogs (des-Leu-[Tle7] PAGA, des-Leu-RG-PAGA, RG-PAGA, plus-Leu-RG-PAGA, colivelin and des-Leu-colivelin) presented some beneficial effects, which reversed the impairment of spatial orientation and memory in rats [37].

To examine the neuroprotective role of HNG elicited by brain ischemia, neurological deficits and middle cerebral artery occlusion (MCAO) and reperfusion (achieved by withdrawing the suture) of the middle cerebral artery were examined in mice [67]. HNG or saline were administered by a needle inserted into the left lateral ventricle over a period of 5 min [67]. Evaluation of neurological scores [69] after 75 min of MCAO and 24 h of reperfusion demonstrated subsequent neurological deficits including circling movements, postural abnormalities, severe paw flections and decreased spontaneous movements [67]. This neurological subsidence was significantly attenuated by HNG treatment. HNG reduces the infarct volume in the cerebral ischemia/reperfusion injury in mice. Additionally, this study demonstrates that HNG significantly decreases the levels of cleaved poly(ADP-ribose) polymerase (PARP) activity [67], an important marker for caspase-3 activity in apoptosis [39]. This effect was accompanied by decreased activity of the MAP kinase-activated protein kinase family, ERK. HNG did not influence the other members of the MAPK family, JNK and p38, in this model. These results suggest that the neuroprotective effect of HNG in brain ischemia is probably mediated by receptor-dependent inhibition of ERK activity and demonstrated that HNG protects against ischemia/reperfusion injury in the mouse MCAO model [67].

Other targets: HN improves insulin sensitivity and inhibits β-cell and germ cell apoptosis

HN was isolated from the surviving neurons of the human AD brain; it is thus believed to play a role in promoting the survival of neuronal cells [18]. Since its initial discovery several studies have demonstrated that HN is a wide-spectrum cellular survival molecule, which can be considered a therapeutic target not only for AD.

Very recent studies revealed that HN can also play an important role in the regulation of glucose homeostasis [46]. Insulin resistance and altered insulin release are associated with age-related diseases including Type 2 diabetes mellitus (T2DM) and AD [6, 46]. Because there is a link between AD and insulin resistance, it was hypothesized that HN may influence insulin sensitivity [46]. The Muzumdar group recently demonstrated the role of HN in glucose metabolism [46]. They showed that the intracerebroventricular infusion of HN significantly improves both hepatic and peripheral insulin sensitivity in rats [46]. Additionally, they demonstrated that the dimerization of HN is essential for its action involving hypothalamic STAT3 activation [46]. The same group demonstrated that the

effects of HN on glucose metabolism are tempered by competitive binding of HN to insulin-like growth factor-binding protein-3 (IGFBP-3) in the hypothalamus. Therefore, HN has been identified as the centrally acting peptide that may modulate glucose uptake in skeletal muscle [46].

The modulatory effect of HN on glucose metabolism also involves pancreatic β-cell cytoprotection. The recent study by Hoang et al. demonstrated that HN may be a potent survival factor for pancreatic endocrine β-cells and delay the onset of diabetes in non-obese diabetic (NOD) mice [22]. Pancreatic β-cell function and survival depend on multiple intrinsic and environmental factors, and pancreatic β-cell apoptosis plays an important role in the pathogenesis of diabetes mellitus [22, 53]. It was recently demonstrated that HN prolongs β-cell survival and insulin release by inhibiting apoptosis [22]. Therefore, it has been suggested that HN could also be a possible therapeutic target for diabetes [22].

Binding of HN to IGFBP-3 and modification of the insulin growth factor (IGF) bioactivity, i.e., cell growth and survival, were originally demonstrated by the Ikonen group [23]. IGFBP-3 is upregulated by proapoptotic signal transduction pathways, including tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), and the tumor suppressor protein p53 [23]. IGFBP-3 was found to inhibit cell growth and induce apoptosis by binding to IGF-1. HN binds to IGFBP3 with high affinity and blocks the IGFBP-3-induced cell death of glioblastoma cells [23].

IGFBP3 was found to be the carrier protein for IGF-1 and HN throughout the body [23]. Thus, IGFBP3 may regulate the blood level of HN [23], and the HN generated in peripheral tissues outside of the central nervous system may be transported by IGFBP3 to the CNS [48]. For CNS cells, IGFBP3 was found to enhance the neuroprotective activity of HNG against Aβ-induced toxicity [48]. Therefore, it has been hypothesized that IGFBP3 may regulate the distribution and tissue-specific action of HN *in vivo* [48].

There is a strong interaction between IGFPB3 and HN in the regulation of testicular germ cell homeostasis [41]. It has recently been discovered that IGFBP3 as a proapoptotic factor and HN with its antiapoptotic activity are important regulators of male germ cell apoptosis. Testicular hormonal deprivation by excess IGFBP3 induces germ cell apoptosis in the testis of mice and rats. The intratesticular administration of HN attenuates this type of germ cell death [41]. The findings described above point to the potent role of HN in the regulation of spermatogenesis and prevention of testicular malfunction [41].

In summary, the cytoprotective effects of HNs seem to be associated with antiapoptotic, metabolic (improving mitochondrial bioactivity) and possibly anti-inflammatory effects by the way of competition with hormone carrier proteins and cellular receptor agonists. To unravel the pluripotential mechanism(s) of action, bioavailability and regulation of expression and the fate of endogenous HN peptide(s), further studies are needed.

Acknowledgment:
This review was supported by Polish-Norwegian grant no. PNRF-104-AI-1/07.

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Received:
December 15, 2009; in revised form: April 13, 2010.