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Humanin

After the Discovery

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Abstract

Humanin (HN) is a novel neuroprotective factor that consists of 24 amino acid residues. HN suppresses neuronal cell death caused by Alzheimer's disease (AD)-specific insults, including both amyloid-β (βAβ) peptides and familial AD-causative genes. Cerebrovascular smooth muscle cells are also protected from Aβ toxicity by HN, suggesting that HN affects both neuronal and non-neuronal cells when they are exposed to AD-related cytotoxicity. HN peptide exerts a neuroprotective effect through the cell surface via putative receptor(s). HN activates a cellular signaling cascade that intervenes (at least) in activation of c-Jun N-terminal kinase. The highly selective effect of HN on AD-relevant cell death indicates that HN is promising for AD therapy. Additionally, a recent study showed that intracellularly overexpressed HN suppressed mitochondria-mediated apoptosis by inhibiting Bax activity.

Index Entries: Humanin; Alzheimer's disease; amyloid β; AβPP; neuroprotection; cell death.

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder that causes dementia. AD is pathologically characterized by massive neuronal loss, senile plaques, and neurofibrillary tangles in the brain. Most approaches for AD therapies have focused on the modulation of synthesis or degradation of amyloid-β (Aβ) peptides, which are the major component of senile plaques. However, neuronal loss caused by cell death accounts for most—if not all—clinical manifestations of AD. Therefore, prevention of neuronal death should be an important target in developing therapeutics. If the agent that directly inhibits neuronal cell death can be combined with an Aβ-based remedy, then a complete cure of AD can be expected.

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Elucidation of the mechanisms of neuronal death is necessary to establish therapy for AD. Such efforts have unveiled the existence of several different toxic pathways, all of which lead to AD-related neuronal death. For example, we have found that the mutant Aβ precursor protein (AβPP), a familial AD (FAD)-causative gene product, induces distinct intracellular toxic mechanisms, depending on mutation types *(1).* Presenilin (PS)1 and -2, other known as FAD gene products, activate different intracellular machinery to elicit cell death; however, these two PS proteins share high homology in amino acid sequence (for review, *see* refs. *2–4*). These findings have led us to assume that the therapeutic agent that targets a specific molecule in a single toxic pathway is not omnipotent for suppression of neuronal death in all patients with AD. Therefore, we attempted a functional screening to isolate antagonistic gene(s) against AD-relevant neuronal death, regardless of the toxic mechanisms. Using a modified "Death Trap" method originally described by D'Adamo's group *(5),* we searched for complementary DNA (cDNA) that suppresses neuronal cell death caused by London-type FAD mutant of AβPP, V642I-AβPP, in a cDNA library constructed from the occipital lobe of an AD brain (for details, *see* ref. *4*). We expected neuronal surviving factor(s) to be abundant in the occipital lobe, because this region remains intact in most AD brains. Among several genes obtained by this screening, we found one cDNA that encoded a novel 24 amino acid polypeptide and named it Humanin (HN) *(6).* This article focuses on recent findings that have further uncovered AD-related functions and other potentials of this unique factor.

Structure–Function Relationship

HN has two major functions: a signal peptide-like function and a neuroprotective function. Residues that are responsible for each function have been determined, as demonstrated in Fig. 1.

Signal Peptide-Like Activity

When full-length HN cDNA (>1000 base) or the open reading frame (ORF) region of HN cDNA (75 basepairs) under the control of promoters for mammalian expression is introduced into cells, the HN peptide is translated and secreted from the cells *(6,7).* This is not surprising, because multiple programs have predicted that nearly the entire region of HN functions as a signal peptide for secreting proteins. The signal sequence-like activity of HN has also been experimentally proven. When enhanced green fluorescent protein (EGFP) is fused to HN at the C-terminus, EGFP is secreted from cells into the culture medium *(7).*

Substitution of Ala for each amino acid (from 3rd Pro to 23rd Arg) does not affect secretion of full-length HN. When one of the amino acids in Leu9–Leu11, Pro19, or Val20 is changed to Arg, these mutants are no longer secreted effectively, suggesting that two structures, Leu9–Leu11 and Pro19–Val20, are essential for the secretion. Among Leu9–Leu11, only the substitution of Asp for Leu10 completely abolished secretion, indicating that Leu10 plays a central role in the signal sequence-like activity of full-length HN. Generally, signal sequence is characterized by the N-terminal positively charged region, the central hydrophobic region, and the C-terminal-charged region *(8).* These findings indicate that Leu9–Leu11 in HN plays a role for the central hydrophobic core and demonstrate the basis for HN as a signal peptide for protein secretion. Furthermore, extracellular secretion is necessary when intracellularly expressed HN peptide exerts its neuroprotective function.

Neuroprotective Activity

The core domain required for rescue activity in the HN sequence contains the 17 residues located from Pro3 to Pro19 (Fig. 1). Pro3, Cys8, Leu9, Leu12, Thr13, Ser14, and Pro19 are essential amino acids within this neuroprotection core domain (NPCD) *(9).* Substitution of Ala for any one of these residues abolishes protective activity. Although they are essential, some of these residues can be substituted for

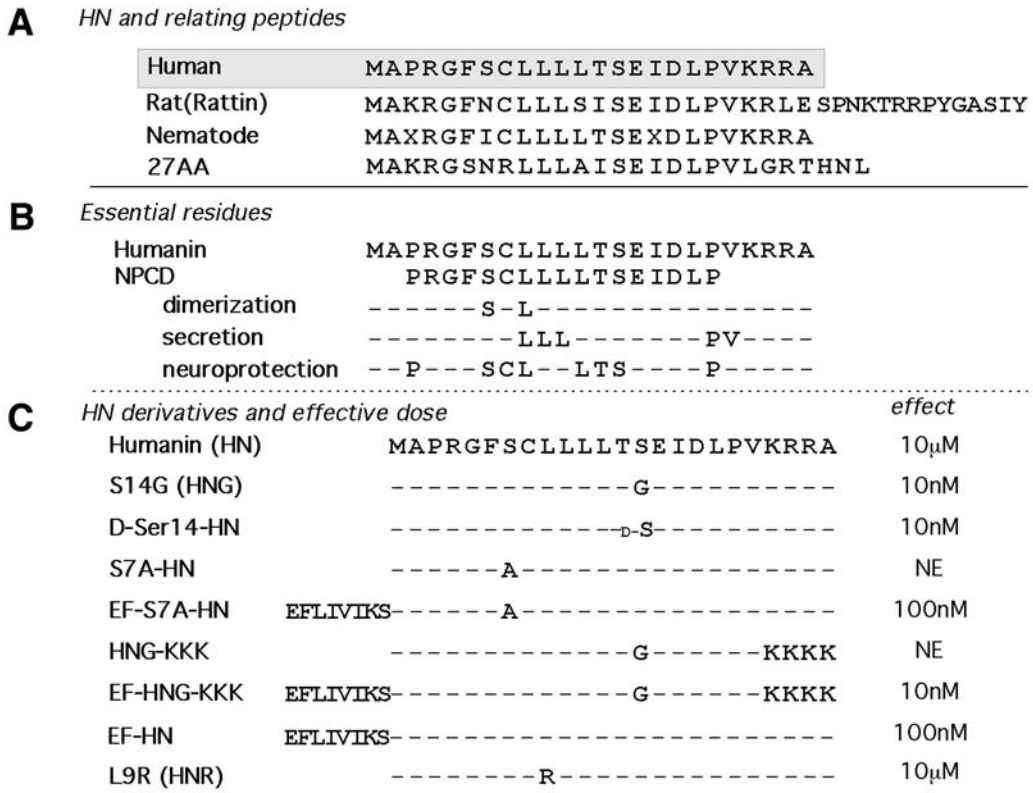


Fig. 1. Structural analysis of HN and related peptides. **(A)** Sequence alignment of HN and related peptides. HN (human) sequence is boxed. HN-like peptides are from rat EST (Rattin) *(10),* nematode EST *(32),* and deduced sequence from rat mitochondria 16S rRNA region (27 AA) *(23).* **(B)** Essential residues in HN sequence for its function. Humanin and NPCD sequences are listed. Essential amino acid residues for secretion, dimerization, and neuroprotection in full-length HN are shown. Residues shown as "–" can be replaced with Ala. **(C)** HN derivatives and effective doses. Names of HN derivatives are shown in the left column, and those sequences are shown in the middle. Changed amino acid residues are shown and "–" indicates unchanged residue. The effective dose of each HN derivative is shown in the right column. The dose indicates the minimal concentration to completely protect neuronal F11 cells from V642I-AβPP-induced cell death. NE, not effective at 10 μ*M.*

other amino acids without affecting neuroprotective activity. For example, Ser7 and Cys8 can be replaced with Pro and Lys/Arg, respectively. Additionally, Rattin (RN), the rat homolog of HN, contains natural variations of Lys for Pro3, Asn for Ser7, Ser for Leu12, and Ile for Thr13 but shows neuroprotective activity to a degree that is similar to HN *(10).*

Interestingly, Ala substitution analysis in full-length HN revealed that Ser7 is also essential for rescue activity *(7,11)*—that is, the substitution of Ala for Ser7 in NPCD did not affect its rescue effect, but the same substitution in full-length HN (S7A-HN) completely abolished its activity. A similar discrepancy was noted when the four C-terminal residues were replaced by Lys residues (HN-KKKK), which totally abolished the activity *(11).*

These findings prompted us to examine a hypothesis stating that HN requires a certain process before binding to its putative receptor and that these amino acid substitutions influence this process and result in functional loss. Using this hypothesis, we have demonstrated

that homodimerization of HN is necessary for its rescue activity *(11).* When S7A-HN and HN-KKKK were fused with N-terminal EF-tag (EFLIVIKS; which homodimerizes at low concentrations), EF-tagged S7A-HN (EF-S7A-HN) and EF-tagged HN-KKKK (EF-HN-KKKK) restored full activity, thus inhibiting neuronal cell death. An excessive amount of the EF-tag added to EF-S7A-HN or EF-HN-KKKK completely blocked the neuroprotective function of these fusion peptides. These results suggest that these mutants lost rescue function because of a deficiency in homodimerization. In vitro pull-down assay showed that EF-S7A-HN does not bind to HN, which also strongly suggests that S7A-HN is a dimerization-defective mutant. No HN mutant has been detected that is deficient in dimerization but protective of neurons. For example, HN with substitution of Ala for Leu9 (L9A-HN) is defective in both dimerization and neuroprotection, whereas HN with substitution of Arg for Leu9 (L9R-HN; HNR) is effective in both. Therefore, dimerization is an essential process for HN's neuroprotective activity.

Another unique feature in the structure–function relationship of HN is that the substitution of Gly for Ser14 (S14G-HN; HNG) enhances neuroprotective activity of HN 1000-fold *(6).* A similar enhancement of action potency was observed with the replacement of Ser14 with D-form Ser residue (D-Ser) *(11).* We assume that a certain conformational change of the HN peptide results in the enhancement of neuroprotective potency. Gly substitution may mimic D-isomerization of Ser14. In glia cells, serine racemase (an L- to D-Ser converting enzyme) has been identified *(12).* Thus, it is possible that authentic HN may be produced in a less active form and may be converted to a more active form by D-isomerization in vivo.

Spectrum of Rescue Action

AD-Related Neuronal Death

Through the analyses of cytotoxic mechanisms of FAD-linked mutant-induced cell death, we have discovered that different types of mutant proteins activate different intracellular molecules to cause neuronal death. V642I-AβPP evokes apoptotic cell death through a mechanism that is sensitive to a caspase inhibitor, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-aldehyde (DEVD), as well as to nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase inhibitors *(1,3).* The Swedish type AβPP mutant K595N/M596L-AβPP (NL-AβPP) and the PS2 mutant N141I-PS2 both activate the same cell death mechanism as V642I-AβPP at a low endogenous level of expression. However, overexpression (more than fivefold the endogenous expression level) of these mutant proteins in neuronal cells causes nonapoptotic cell death, which is resistant to DEVD. The mechanisms underlying the cell death caused by overexpression of these two mutant proteins appear to be different. Cell death by NL-AβPP overexpression is resistant to the antioxidant glutathione ethyl ester (GEE). In contrast, N141I-PS2 overexpression-induced cell death remains sensitive to GEE and is mediated by xanthine oxidase *(3).* Mutations in PS1 induce DEVD-resistant and GEE-sensitive death, as in the case of overexpression of N141I-PS2. However, in PS1 mutant-induced cytotoxicity, nitric oxide synthase plays the central role in the production of reactive oxygen species, which are directly responsible for the cell death *(2).* Therefore, although the amino acid sequences of PS1 and -2 are highly similar and they are considered to share the same functions, they may evoke different cell death pathways. Surprisingly, HN suppresses all these types of FAD mutant-induced neuronal death, despite the differences in the cell death mechanisms.

In addition to suppressing toxicity of FAD mutants, HN inhibits toxicity via authentic Aβ in primary cortical neurons. Although the mechanism of Aβ cytotoxicity has not been completely elucidated, multiple groups have postulated the presence of death receptors for Aβ (for review, *see* ref. *14*). The 75-kDa neurotrophin receptor (p75 NTR) is one such candidate *(15,16).* HN

completely suppresses this Aβ toxicity in the neuronal cell line via p75 NTR *(17).*

To our knowledge, HN is the sole neuroprotective factor that is capable of antagonizing all AD-relevant insults. In contrast to HN, the antiapoptotic neuroprotective factors insulin-like growth factor (IGF)-I and basic fibroblast growth factor (bFGF) cannot protect neurons from nonapoptotic cytotoxicity by FAD mutants, although in primary neurons, they certainly antagonize apoptotic cytotoxicity by Aβ *(9,18,19).* The broad spectrum of HN's action may imply that HN is a multipotential neuroprotective factor that is effective on both apoptotic and nonapoptotic cell death mechanisms in AD.

AD-Related Non-Neuronal Death

Cerebral amyloid angiopathy (CAA) is observed in more than 90% of AD brains *(20).* Aβ deposition in cerebrovascular vessels induces degeneration of human cerebrovascular smooth muscle (HCSM) cells in vivo and in vitro. Jung and Van Nostrand *(21)* demonstrated that extracellular treatment of HN inhibits apoptosis of HCSM cells in vitro. HN has no effect on Aβ fibril formation or Aβ binding to the cell surface. Presumably, HN counteracts Aβ toxicity in HCSM cells by activating an intracellular anticytotoxic mechanism. This is the first report demonstrating that the target cells of HN are not limited to neurons when the insult is related to AD. We must emphasize that no other known rescue factors suppress the cytotoxicity of Aβ on HCSM cells.

Non-AD-Related Cell Death

Mutation in *superoxide dismutase-1 (SOD1)* is a genetic cause of familial amyotrophic lateral sclerosis, which is characterized by specific degeneration of motor neurons *(22).* Mutant SOD1 induces cell death in F11 neuronal cells. Even at high concentrations, neither HN nor HNG displays any effect on this cell death. Similarly, HN cannot rescue F11 neuronal cells from death caused by polyglutamine repeats Q79, which is implicated in Huntington's disease and spinocerebellar ataxia *(6).* Furthermore, in primary neurons, HN does not antagonize toxicity by the prion peptide PrP106–126, which is responsible for Creutzfeldt–Jakob disease *(9).* To date, no report has suggested that HN antagonizes disease-related neurotoxic insults other than those relevant to AD. Therefore, HN appears to distinguish AD-related insults from other neurodegenerative disorders when it executes its neuroprotective effect.

RN has been shown to inhibit excitotoxicity by *N*-methyl-D-aspartate on primary neurons, whereas HN scarcely antagonizes this insult *(10).* This functional difference may reflect the difference between HN and RN in amino acid sequences. If the difference between RN and HN in amino acid sequences actually allows RN to have wider targets, then HN derivatives that have a wider range of target action (including excitotoxicity) may be designed by amino acid substitutions.

Kariya et al. *(23)* reported that HN and HNG suppress apoptosis caused by trophic factor deprivation in phenochromocytoma (PC12h) cells. Inhibition of caspase 3-activation and nuclear DNA degradation accompany this suppression of apoptosis. As in the case of HN's effect on neuronal cells, HNG shows a 10-fold greater potential than HN in this case. Because V642I-AβPP-induced apoptosis is inhibited by trophic factor IGF-I as well as HN *(9,24),* HN-sensitive apoptosis caused by trophic factor deprivation may be mediated by the same cytotoxic pathway as apoptosis that is induced by V642I-AβPP.

Two Distinct Rescue Mechanisms of HN

We have demonstrated that HN peptide is secreted and stimulates cells extracellularly (Fig. 2A). In addition to this functional model, a recent report demonstrated that HN plays important intracellular roles in suppression of an apoptosis cascade (Fig. 2B).

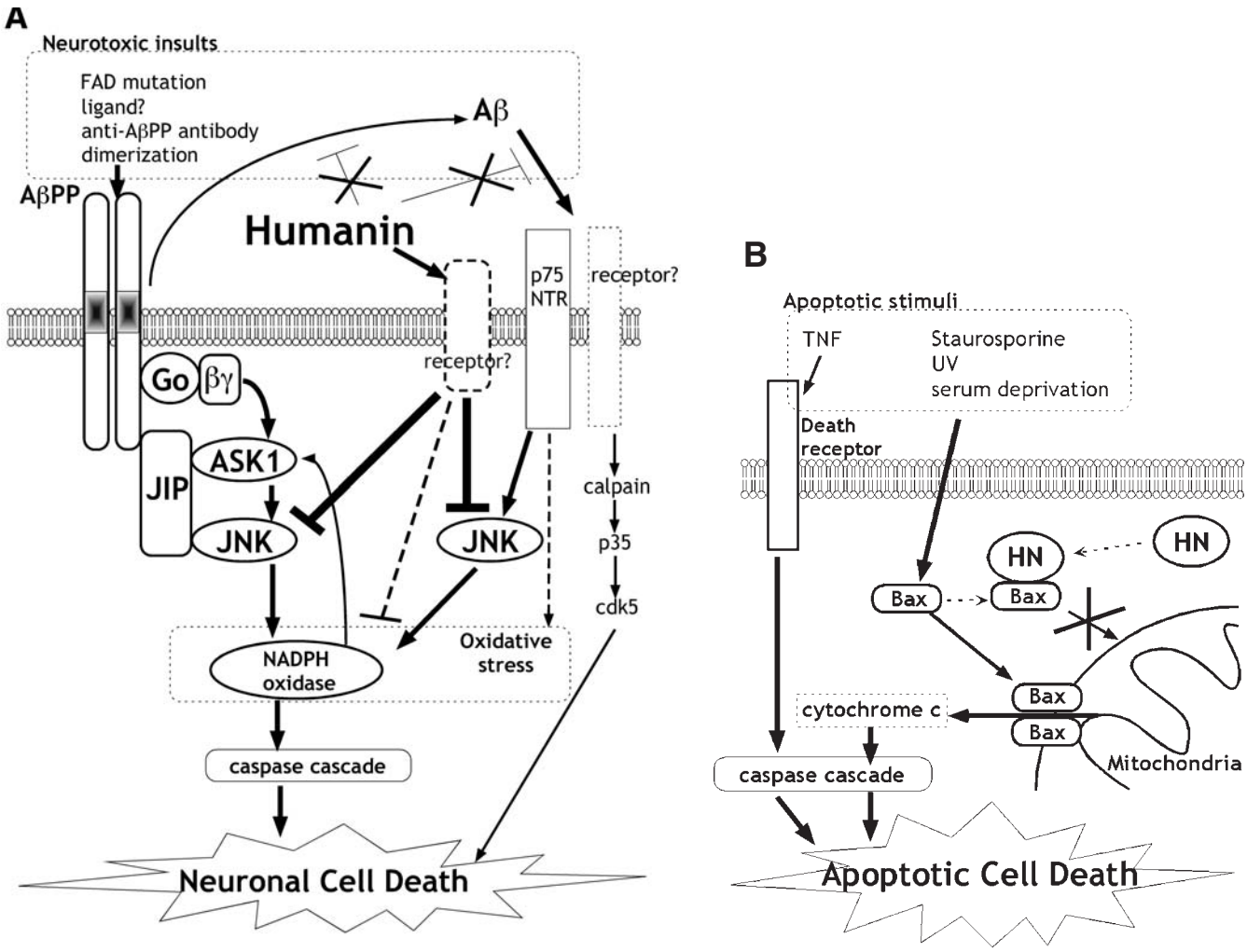


Fig. 2. Mechanisms of cell death and inhibition by HN. **(A)** Mechanisms of neuronal cell death by AβPP and Aβ and suppression by HN. Stimuli of neurotoxic insults activate intracellular cytotoxic pathway, resulting in neuronal cell death. HN binds its putative receptor, activates intracellular signaling cascade, and inhibits JNK activation to suppress cell death. **(B)** Mechanisms of apoptotic cell death and inhibition by HN. Apoptotic stimuli induce Bax to translocate to mitochondria and release cytochrome-c, followed by caspase activation and cell death. Intracellular HN binds to Bax and prevents its translocation, resulting in suppression of apoptosis. HN has no effect on direct activation of caspase cascade by death receptor. *See* text for details.

Extracellular Neuroprotective Mechanism

The extracellular neuroprotective effect of HN has been demonstrated on cell death caused by all AD-related insults. In the search for the target of HN's neuroprotective action, it was hypothesized that the rescue action of HN against FAD mutants might be a direct suppression of toxicity that is induced by augmented Aβ42/43. In fact, the common feature of FAD mutants is that all the toxic FAD mutations in AβPP and PS increase either the absolute amount of cytotoxic Aβ42/43 or a relative ratio of Aβ42/43 to nontoxic Aβ40. NL-AβPP pro-

duces increased absolute amounts of both Aβ40 and Aβ42/43. HN does not affect the amount of Aβ42/43 or Aβ40 that is produced from NL-AβPP cDNA-transfected cells, whereas HN completely suppresses nonapoptotic cell death caused by NL-AβPPΔ41/42, which is a modified AβPP with deletions at the 41st and 42nd amino acids in the Aβ region *(13).* NL-AβPPΔ41/42 does not produce Aβ42/43. Therefore, the neuroprotective activity of HN against FAD mutants is not directly connected to the augmented Aβ42/43. HN also does not inhibit Aβ fibril formation *(21),* further suggesting that Aβ is not the direct target of HN.

On the other hand, it has been shown that the neuroprotective effect of HN works only from the outside of cells: a secretion-defective mutant of HN, HNR, can suppress neurotoxicity when added extracellularly but cannot suppress neuronal death by intracellular overexpression. Additionally, specific binding of HN to neuronal F11 cells has been demonstrated. The dose of HN for complete neuronal protection is low—10 nM and 100 to 300 pM for HNG and AGA-HNG (R4A, F6A, S14G-HN), respectively. All these findings suggest that the binding of HN to putative receptor(s) is necessary to exert its rescue effect. It has been proven that HN activates intracellular signaling molecules. Tyrosine kinase inhibitors completely abolish HN's rescue effect; however, PI3 kinase and mitogen-activated protein (MAP) kinase inhibitors do not, indicating that certain types of tyrosine kinases play a critical role in the neuroprotective signaling cascade activated by HN *(6).* Furthermore, all features of the rescue action of HN that have been revealed by the structure–function analyses are common when acting against all AD-relevant insults, suggesting that HN activates a common neuroprotective pathway against these insults. Because HN suppresses both apoptotic and nonapoptotic AD-related cell death, it is conceivable that multiple signaling cascades are employed to inhibit cell death in an orchestrated manner.

Several AD-related insults (the V642I mutation of AβPP, dimerization of AβPP, stimulation by anti-AβPP antibody, and stimulation by Aβ via p75NTR) commonly activate the cytotoxic cascade Go->JNK->NADPH oxidase->caspase *(25,27–31).* We have found that JNK activation is attenuated by HN's signaling cascade *(25)* (Fig. 2A). This finding suggests that HN inhibits the upstream signaling pathway of JNK. It was recently demonstrated that apoptosis signal-regulating kinase (ASK)1 is involved in the upstream pathway of JNK and the downstream pathway of Go in the AβPP-induced neurotoxic mechanism *(32).* Because HN inhibits JNK activation by constitutively active ASK1, one target of HN should be located in the pathway between ASK1 and JNK. Additionally, HN suppresses cell death caused by constitutively active JNK, indicating that HN also blocks the downstream pathway of JNK *(25).* Therefore, HN may target two different molecules, both upstream and downstream of JNK, among signaling molecules in the AβPP-induced cytotoxic pathway. Alternatively, HN may induce/activate JNK phosphatases and attenuate JNK phosphorylation.

IGF-I also targets JNK to antagonize Aβ toxicity. However, the intracellular neuroprotective mechanism that is evoked by IGF-I appears to be different from that evoked by HN. IGF-I inhibits JNK phosphorylation by activating Akt through PI3 kinase *(26),* whereas the PI3 kinase inhibitor wortmannin does not interfere with the rescue activity of HN *(6).* HN suppresses neuronal death caused by FAD-linked PS mutants, whereas IGF-I does not *(9).* Presently, only a part of the molecular mechanism of HN has been revealed; in particular, the way that HN protects neurons from nonapoptotic death caused by such FAD mutants as in PS1 has not been understood. Further clarification of the action mechanism of HN should provide key information to obtain a holistic view of AD pathomechanisms.

Intracellular Antiapoptotic Mechanism

Guo et al. *(33)* isolated HN by yeast-two-hybrid screening using Bax (a proapoptotic member of Bcl-2 family proteins) as the bait. HN specifically binds to Bax but not to other

Bcl-2 family proteins, including Bak and Bcl-2. Overexpressed green fluorescent protein (GFP)-fused or FLAG-tagged HN binds to Bax intracellularly, interferes with translocation of Bax to mitochondria, and, consequently, inhibits release of cytochrome c. As a result, HN attenuates mitochondria-mediated apoptosis caused by staurosporine and ultraviolet irradiation but does not attenuate death receptor-induced apoptosis in which Bax is not involved (Fig. 2B). When extracellularly applied, HN does not rescue primary neurons from cell death that is induced by etoposide, which is known as a typical inducer of mitochondria-mediated apoptosis. This is predictable, because extracellularly applied HN cannot bind directly to Bax in cytoplasm. The extracellular treatment of HN shows no protective effect against cell death that is induced by Fas, a well-known death receptor *(6).* It has been reported that Bax is involved in Aβ-induced cell death; intracellular Aβ42, but not Aβ40, causes cell death in primary neurons *(34),* and the fibrillar Aβ40 induces Bax-dependent neuronal death at high concentrations—particularly over 40 μM *(35).* These findings imply that intracellular HN can inhibit Aβ toxicity by directly interfering with the Bax activity. However, intracellular HN is undetectable in neuronal cell lines, neurons of normal brain, or majority neurons of AD brain. Moreover, HN that is added extracellularly is effective at low concentrations. Thus, it is highly likely that the extracellular mechanism described previously plays the major role in the neuroprotection of HN against Aβ toxicity.

Substitution of Pro for Cys8 (C8P-HN) results in the loss of both Bax binding and antiapoptotic effect. Even when added extracellularly, C8P-HN does not show the neuroprotective effect *(9),* despite the apparently different action mechanism. The core domain for the Bax binding and the antiapoptotic effect is Pro3-Asp17, which is close to the NPCD; this suggests a similar structural requirement because HN has an effect on both anti-Bax and the neuroprotective activities. L9R-HN; HNR helps to explain the mechanisms underlying these two distinct effects. Intracellularly expressed HNR is defective in cell protection against both Bax-mediated apoptosis *(33)* and V642I-AβPP-induced neuronal cell death *(6).* The former deficiency is owing to the lack of Bax-binding capability of this mutant. The latter deficiency in cell protection results from the deficiency of secretion—not the rescue function. In fact, when synthetic HNR peptide is extracellularly applied, it definitely suppresses cell death caused by V642I-AβPP *(6),* indicating that HNR is not a rescue function-defective mutant and that extracellular action is necessary for the neuroprotective function of HN. The discrepancy between the ineffective anti-Bax and extracellular neuroprotective action of HNR clearly illuminates how HN can suppress cell death through two distinct mechanisms. The new anti-Bax effect of HN implies that the antiapoptotic effect of HN is not limited to neuronal cells or to AD-related cell death.

Expression and Regulation of HN

HN cDNA is 1567 bp in length, with the ORF following a long 934 bp of 5′ untranslated region (UTR). Despite this long UTR, HN peptide is efficiently translated in mammalian cells once it is transfected as a full-length HN cDNA *(36).* Northern blot analysis revealed several polyA RNAs of different sizes that were hybridized to an oligonucleotide probe encoding the HN ORF region. Several genomic regions and expressed sequence tags (ESTs) show high (more than 95%) identity with HN cDNA *(6).* HN homologs have been identified as ESTs in rat and other species, such as the nematode (Fig. 1). These findings suggest that HN polypeptide originates in the genome that is conserved from nematode to human. HN cDNA is also 99% identical to a portion of mitochondrial 16S rRNA, suggesting that the HN gene is transcribed from mitochondrial DNA. Guo et al. *(33)* speculated that HN might be produced in mitochondria. They demonstrated that HN polypeptide translated by mitochondrial codon usage (MAPRGFSCLLLLTSEMDLPVK) has robust activity in preventing cell death, similarly to that of

authentic HN translated by mammalian codon usage (MAPRGFSCLLLLTSEIDLPVKRRA). Therefore, there are three possibilities for the origin of HN peptide: (a) HN messenger RNA (mRNA) is transcribed from genomic DNA and translated to the peptide in cytoplasm; (b) HN mRNA is transcribed from mitochondrial DNA, translocates to cytoplasm if such a mechanism exists, and is translated to the peptide in cytoplasm; and (c) HN mRNA is transcribed from mitochondrial DNA and translated to the peptide in mitochondria.

Immunohistological analyses by anti-HN antibody *(36)* exhibited immunoreactivity in intact large neurons in an occipital lobe of AD brain but did not exhibit this effect in an age-matched control. This is consistent with the finding that HN cDNA was isolated from a cDNA library from the occipital lobe of an AD brain, suggesting that the expression of HN in occipital lobe neurons is augmented in patients with AD. Glia cells are also immunoreactive throughout the AD brain, mainly in hippocampus. Because a few glia cells are also immunopositive in the normal brain, these cells could be major HN-producing sites in the brain in normal physiological condition. Concomitantly, relatively higher expressions of HN were detected in glial cell lines *(33).* Immunoblot analyses of normal mouse tissues detected HN in testis and colon in 3-wk-old mice and in testis only in 12-wk-old mice, suggesting that HN expression is modulated in an age-dependent manner *(36).* Because the expression of HN peptide was undetectable in other tissues (including brain) in the immunoblot analysis, it appears that in the physiological condition, HN peptide is predominantly produced in nonbrain organs.

Although the transcriptional regulation of HN remains obscure, one aspect of posttranslational regulation of HN peptide has been elucidated in detail. Tripartite motif protein 11 (TRIM11) was reported as the first binding partner of HN *(37).* TRIM11 binds to HN, mediates ubiquitination, and accelerates degradation of HN through the ubiquitin–proteasome pathway. Because the RING finger motif functions as a ubiquitin ligase in the TRIM family of proteins, disruption of the RING finger motif in TRIM11 significantly increases the intracellular level of HN peptide. The impaired activity of proteasome in AD brains *(38,39)* may contribute to the enhanced expression of HN in the glias and neurons of patients with AD. Ubiquitous expression of TRIM11 mRNA suggests that TRIM11 may also be involved in the regulation of HN expression levels throughout the body.

Another binding partner of HN provides an attractive hypothesis for in vivo regulation of HN. IGF-binding protein 3 (IGFBP3), a major binding protein for IGF-I/II in blood, also specifically binds to HN without losing interaction with IGF-I *(40).* Because IGFBP3 functions as a carrier protein to distribute IGF-I throughout the body, it is possible that IGFBP3 may also distribute HN to the central nervous system from nonbrain organs where HN is normally produced. Furthermore, neuroprotective activity of HNG against Aβ toxicity is enhanced in the presence of IGFBP3. IGFBP3 may stabilize HN or increase the interactive affinity between HN and its putative receptor. These findings imply that IGFBP3 also may regulate localization and action potency of HN in vivo.

In Vivo Function of HN

In vivo function of HN was first confirmed in a scopolamine-induced amnesia model *(41).* The impairment of short-term memory caused by scopolamine is dose-dependently reversed by HNG 15 min after an HNG intracerebroventricular (ICV) injection. Complete recovery is achieved at 1 nmol of HNG injection. Amnesia caused by ICV injection of Aβ25-35 is also prevented by HNG *(42)*, indicating that HN antagonizes Aβ toxicity in vivo as well as in vitro. It should be noted that Aβ toxicity becomes evident 3 wk after Aβ25-35 injection, and HNG suppresses this delayed effect. However, amnesia by scopolamine is observed 30 min after treatment, and HN attenuates this

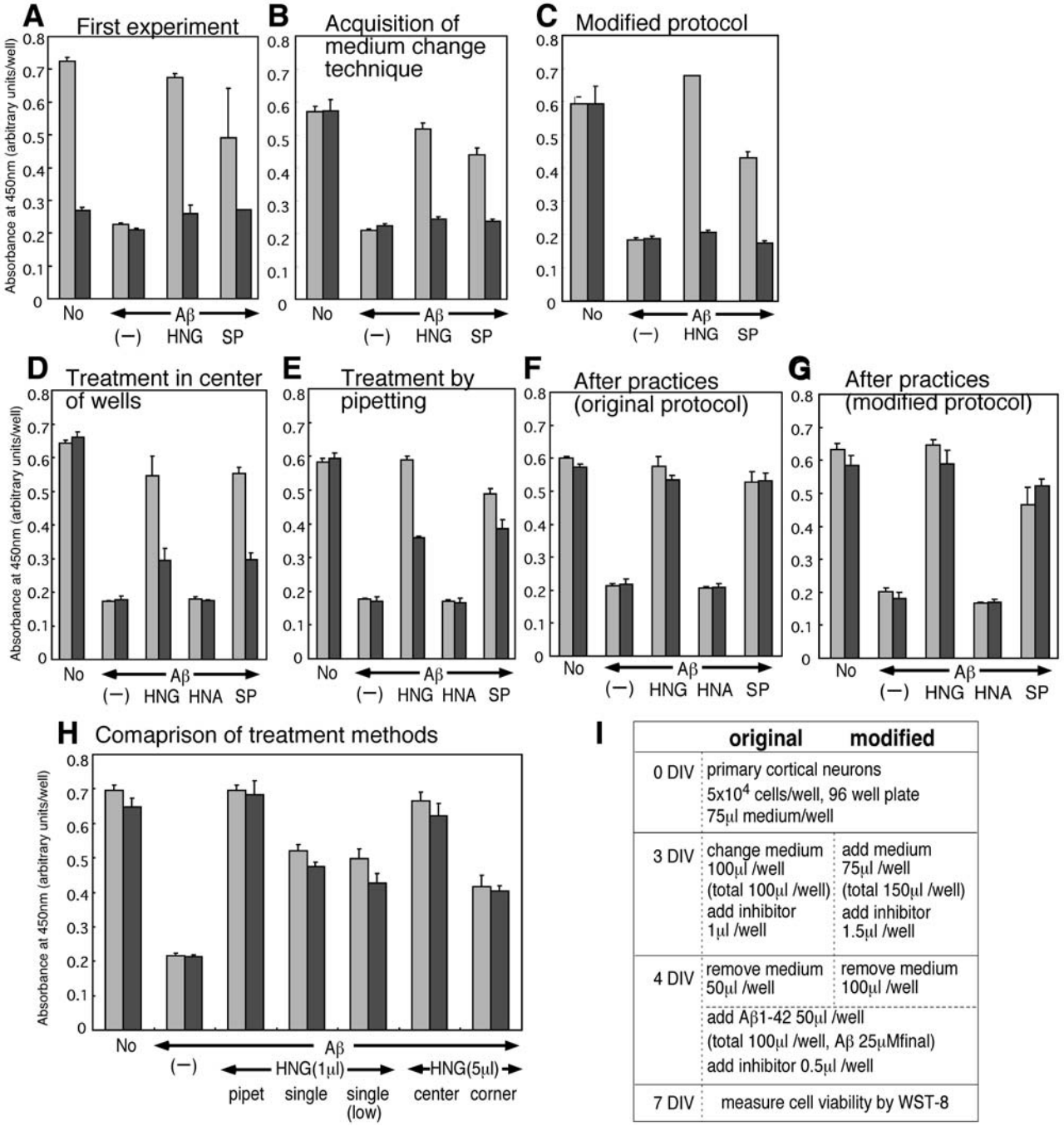
Fig. 3. Technical requirement for the examination of HN's rescue effect against Aβ-induced cytotoxicity. Results of the original (**A, B, F,** and **H**) and modified protocol **(C–E, G).** Details of each protocol are shown in **I.** Mouse primary cortical neurons were seeded at 5×10^4 cells/well in 96-well plates. On 3 d in vitro, medium was replaced by fresh medium **(A, B, F, H)** or fresh medium was added **(C–E, G)** and cells were treated with 10 n*M* of HNG, 10 μ*M* of HNA, 1 μ*M* of SP600125 (SP), or without treatment (–). On 4 d in vitro, Aβ1-42 was added to a final concentration of 25 μM (Aβ). On 7 d in vitro (after 72 h of Aβ treatment), cell viability was assessed by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disul-phonyl)-2H-terrazolium] assay (cell counting kit-8, Dojindo Laboratories, Japan (WST-8 assay). Results were obtained by measuring each absorbance of formazan produced from WST-8 at OD450. "No" indicates medium change alone. The *dark gray* bars indicate results obtained by a "beginner" for this type of experiments and *light gray* bars are results by an "experienced" person. **(A)** First experiment. **(B)** Result after the "beginner" has acquired medium change technique. **(C)** Result of modified protocol (details of modified protocol is shown in **I**). **(D)** Result by altered method to add inhibitor in center of wells. **(E)** Result by altered method to add inhibitor by pipetting. **(F)** Result in original protocol after several practices. **(G)** Result in modified protocol after several practices. **(H)** Result of comparing different methods for adding inhibitor. One microliter of 10 μ*M* of HNG (1μL) or total 5 μL (1 μL of 10 μ*M* of HNG plus 4 μL of medium: 5 μL) was applied in each well with 100 μL of medium. HNG was added by three or four pipettings (pipet), by a single push (single), by a single push using a low-affinity tip (low), in center of wells (center), or in corner of wells (corner). **(I)** Summary of original and modified protocols. *See* text for details.

effect in 15 min. This implies that HN can antagonize both long-term toxicity by Aβ and temporal functional deficiency on synapses.

Optimization of Experimental Procedures for Reproducible Results

This section offers some tips to those who have become interested in HN after reading this article and who wish to explore the potential of this unique neuroprotective peptide. Having completed a large number of analyses, we have found that there are several small, but important, technical considerations to detect the maximum potential of HN—particularly in experiments using primary neurons. The protocol to examine HN's effects on Aβ neurotoxicity is relatively simple. Briefly, primary cortical neurons are split into 96-well plates. After 3 d in vitro, the medium is completely replaced by fresh DMEM medium with N2 supplement, and HN peptide (1 μL of 100-fold concentration into 100 μL of medium) is then added. After 16 h of HN treatment, half of the medium is replaced with fresh medium that contains 50 μ*M* Aβ1-42/43 (final concentration: 25 μ*M*) and this is supplemented with 0.5 μL of HN peptide to maintain its concentration. Cell viability is measured after 72 h of Aβ treatment. Although slight modifications of the procedure usually do not affect the result, some researchers do face difficulty in confirming the rescue effect of HN. Because reproducibility is crucial, we have attempted to clarify the technical points critical for success. We find that the following points are important.

First, a potential pitfall lies in the medium replacement. Because primary neurons at low density are notoriously vulnerable, simply changing the entire medium to a fresh one often leads to cell death, even in the absence of Aβ (Fig. 3A compares results of dark gray bars, which were obtained by a "beginner" for this type of experiment, with those of light gray bars, which were by an "experienced" person). A careful, but quick, medium replacement overcomes this problem (Fig. 3B). Alternatively, a modified protocol that omits a complete change of medium also works (Fig. 3C). In the modified protocol, fresh medium is added without removal of the seeding medium, and cells are treated by inhibitors on 3 d in vitro (*see* detailed protocol in Fig. 3I).



The next major pitfall concerns the inhibitor treatments. Under the same conditions, one researcher could demonstrate the complete rescue of cell death by HNG or a JNK inhibitor (SP600125), whereas another failed in both (Fig. 3A–C). We noted that addition of inhibitors into the center, rather than into the corner, of each well of a 96-well plate is preferable. Generally, this kind of small technical difference should not influence the overall result. Nevertheless,

changing this procedure led to an increase of cell viability (Fig. 3D). Furthermore, it is important to mix inhibitors in the medium by pipetting three to four times rather than adding them in a single push. Again, this kind of difference does not normally affect the result because inhibitors should spread into every corner of each well during the treatment period. Unexpectedly, however, the addition of inhibitors by three to four pipettings resulted in a further increase of cell viability (Fig. 3E). In fact, after establishing the pipetting technique in our laboratory, the effect of inhibitors (both HNG and SP600125), became perfectly reproducible in both original and modified protocols (Fig. 3F,G). Therefore, inhibitors must be added to the center of the well by pipetting three to four times in experiments using 96-well plates with primary neurons. Presumably, inhibitors need to be spread throughout the well immediately after the addition to achieve maximum activity.

To demonstrate how these small technical differences affect results, we compared several conditions for HNG treatment (Fig. 3H). When HNG was added by only one push, it did not exhibit the complete effect, even when performed with a low-affinity tip. Increasing the volume of inhibitor solutions (1 μL of HNG peptide and 4 μL of medium to make the total volume 5 μL/well) resulted in a complete rescue effect, without pipetting, when added to the center of the well but not to the corner of the well. Two researchers produced very similar results, indicating that the difference of rescue effect results from the difference in the methodology but not from individual skill. It should be noted that under other conditions—for example, using another type of plate or dish or changing medium volume or cell density—these points may not always be applicable.

Conclusion and Prospects

HN was discovered as a neuroprotective factor that specifically antagonizes AD-relevant insults. HN itself is promising for AD therapy, because HN is a short peptide and antagonizes all AD-related insults. Modulation of HN expression by controlling the function of TRIM11 may be another approach to establish HN-based AD therapies. Because HN directly targets neuronal cell death, it may be possible to combine it with anti-Aβ therapies to accomplish complete cure for AD. Furthermore, HN protects HCSM cells from Aβ toxicity, indicating that HN might be effective for the prevention of hemorrhagic stroke and other disorders caused by CAA.

Structure–function analyses have clarified that dimerization is an indispensable process for the neuroprotective function of HN. Studies of action mechanisms have revealed that HN targets JNK that is stimulated in AβPP-induced cell death and Aβ cytotoxicity. Because HN suppresses all AD-relevant neurotoxicity, further elucidation of the action mechanism of HN should lead to the understanding of the pathomechansim of neuronal cell death in AD.

Recent studies have uncovered further properties of HN. In particular, it is interesting that HN evokes an antiapoptotic effect that is totally independent of the originally identified neuroprotective effect. This antiapoptotic effect is specific to Bax-mediated apoptosis but is not limited to neurons, whereas the extracellular neuroprotective effect of HN is observed in both apoptotic and nonapoptotic cell death but is limited to AD-related insults. This intracellular antiapoptotic effect in non-neuronal cells may be the physiological function of HN because the expression of HN in normal mouse is predominantly detected in nonbrain organs. This unique multipotential polypeptide is opening a wide range of possibilities for understanding both diseased and physiological states as well as for developing therapeutics.

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