Neuronal Cell Death in Alzheimer's Disease and a Neuroprotective Factor, Humanin

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Abstract: Brain atrophy caused by neuronal loss is a prominent pathological feature of Alzheimer's disease (AD). Amyloid β (Aβ), the major component of senile plaques, is considered to play a central role in neuronal cell death. In addition to removal of the toxic Aβ, direct suppression of neuronal loss is an essential part of AD treatment; however, no such neuroprotective therapies have been developed. Excess amount of Aβ evokes multiple cytotoxic mechanisms, involving increase of the intracellular Ca^{2+} level, oxidative stress, and receptor-mediated activation of cell-death cascades. Such diversity in cytotoxic mechanisms induced by Aβ clearly indicates a complex nature of the AD-related neuronal cell death. We have identified a 24-residue peptide, Humanin (HN), which suppresses *in vitro* neuronal cell death caused by all AD-related insults, including Aβ, so far tested. The anti-AD effect of HN has been further confirmed *in vivo* using mice with Aβ-induced amnesia. Altogether, such potent neuroprotective activity of HN against AD-relevant cytotoxicity both *in vitro* and *in vivo* suggests the potential clinical applications of HN in novel AD therapies aimed at controlling neuronal death.

Key Words: Alzheimer's disease, neuronal death, amyloid β, amyloid precursor protein (APP), presenilin (PS), Humanin (HN), neuroprotection.

INTRODUCTION

Alzheimer's disease (AD) is the most prominent aging-dependent neurodegenerative disorder. The clinical manifestation of AD is considered to correlate with the degree of neuronal loss in brain, particularly in hippocampus and cerebral neocortex. In addition to neuronal loss, two pathognomonic changes are commonly observed in AD brains: extracellular senile plaques and intraneuronal neurofibrillary tangles (NFT) [50, 69]. Amyloid β (Aβ), a major constituent of senile plaques, is produced from amyloid precursor protein (APP) by enzymatic cleavage with β- and γ-secretases [31,50,69,84]. While β-secretase works as a single protein, presenilin (PS) is a component of a protein complex that functions as γ-secretase [14,31, 36,75,84]. Although certain genetic mutations in the *APP, PS1,* or *PS2* genes cause dominantly inherited familial AD (FAD), the majority of AD cases are sporadic whose cause still remains unclear. Importantly, clinical and pathological features of FAD are virtually the same as those of sporadic AD [69]. Based on this observation, AD models obtained utilizing these FAD mutant genes have been justified for both *in vitro* and *in vivo* AD studies [34,35,58,69].

Currently, only neurotransmitter modulator is clinically available as an approved medication against AD. Cholinergic neurons are most damaged in AD brain. Decline of acetylcholine (ACh) concentration in AD brain is also known. Based on 'choline hypothesis', cholinesterase inhibitors have been established as AD drugs and are currently used in worldwide. In 2002, non-competitive inhibitor of NMDA receptor has been proven as AD drug aiming at the inhibition of excitotoxicity by excess glutamate. Since such neurotransmitter-based therapeutics is a symptomatic therapy, however, it is not expected that they completely cure patients from AD. It is, therefore, evident that the fundamental cause(s) and mechanism(s) behind the pathological changes in AD need to be identified in order to find a way toward complete cure from AD. This review will focus on discovery of a neuroprotective peptide, Humanin, as a potential AD therapy targeting neuronal protection by this peptide.

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NEURONAL LOSS IN AD AND STRATEGIES FOR THERAPY

Aβ Hypothesis and Anti-Aβ Therapies

The extracellular Aβ deposition has attracted major attention as a cause of cytotoxicity in AD. The original 'amyloid hypothesis' argues that Aβ deposition is the initiator for AD pathogenesis (Fig. **1**), based on the following observations: (1) Aβ is a major component of the amyloid plaques [69], (2) the deposition of Aβ occurs prior to other pathological events such as NFT formation and neuronal loss [52], and (3) synthetic Aβ peptides, particularly Aβ1-42/43, induce neuronal death *in vitro* [11,16,43]. Once APP is cleaved by β- and γ-secretases, Aβ peptides with different sizes ranging from 39 to 43 residues are produced. The major Aβ species are 40- and 42-residue peptides. The latter (Aβ42) has two additional residues at the C-terminal of Aβ40 and is more hydrophobic and more prone to aggregate than Aβ40. In a normal condition, Aβ42 is less abundant than Aβ40, about 10% of the latter. FAD-linked mutations in *APP* and *PS* genes increase the ratio of cytotoxic Aβ42/43 to non- or less-toxic Aβ40 [5,9,12,56,72]. Some genetic mutations in FAD cases such as Swedish-type APP mutant enhance the production of total Aβ as well [9]. It results in

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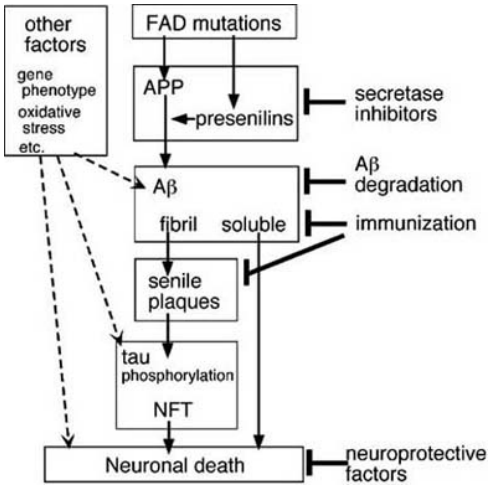

Fig (1). AD pathomechanism based on amyloid hypothesis and therapeutic strategies.

Aβ is produced from APP by enzyme cleavage. Soluble and fibril Aβ species directly or indirectly cause neuronal death. Several anti-Aβ therapies are under development: inhibition of secretase activity, degradation of Aβ, and elimination of Aβ by immune response. In addition, direct inhibition of neuronal death by neuroprotective factors is another potential strategy for AD therapy.

enhanced aggregation and accumulation of Aβ peptides followed by the formation of extracellular amyloid plaque. Accumulated Aβ induces multiple cytotoxic effects, including oxidative stress, and alternation of ionic homeostasis, on neuronal cells [10,40,93]. Aβ also alters the activities of various kinases, including GSK3β, cdk5, and PKA, and causes hyperphosphorylation of tau protein, leading to NFT formation [7,42,52,53,57,78]. These Aβ-initiated toxicities directly or indirectly induce neuronal cell death.

Although this classical Aβ hypothesis does explain some of the mechanisms underlying establishment and progression of AD, there is evidence against this hypothesis. For instance, Aβ deposits do not correlate with clinical features, as senile plaques are found in brains of aged, non-demented subjects [54]. Degrees of plaque formation do not necessarily correlate with synaptic loss [46,55]. Aβ fibril formation does not link to clinical manifestations [80]. Several lines of transgenic mice harboring human FAD mutant genes show severe Aβ deposits in brain, but do not always show other AD-specific pathological features or behavioral abnormality. On the other hand, accumulating evidence indicates the toxic role of soluble Aβ. The amount of soluble Aβ correlates with cognitive impairment, though such soluble Aβ is undetectable in brain by immunohistochemical method [18]. Concentration of soluble Aβ alone, but neither concentration of insoluble Aβ nor degree of Aβ deposition, distinguishes AD brains from control brains with high levels of Aβ deposition but without clinical dementia [46]. The amount of water soluble Aβ42 in AD cortex is 12-fold higher than the amount found in normal subjects by ELISA [39]. These data suggest that soluble Aβ plays more significant role in AD pathogenesis than fibril Aβ. Protofibril Aβ and Aβ-derived diffusible ligands (ADDLs) constitute soluble Aβ. The protofibril is a soluble intermediate found in the process of amyloid fibril formation and conforms β-sheet structure [37]. ADDLs are composed mainly of trimers-hexamers and formed with Aβ42, but not Aβ40, under the condition that suppresses Aβ fibril formation; for instance, at low temperature and in the presence of apolipoprotein J [37,80]. Both protofibrils and ADDLs are neurotoxic *in vitro*. However, since these soluble Aβs are heterogeneous Aβ multimers in size ranging 3~6 up to 24-peptides, it is unclear about the exact Aβ structure which is most neurotoxic.

Among the various soluble Aβ species, small molecular weight complexes, particularly Aβ dimer and trimer, are called 'Aβ oligomers' [18,37,81]. Conditioned medium (CM) from the cells expressing London-type APP mutant, V642I-APP [33], contains the naturally produced Aβ oligomers, whose concentration is similar to that detected in normal human cerebrospinal fluid [81]. Although such CM does not cause cell death in cortical and hippocampal neurons [2], it inhibits hippocampal long-term potentiation (LTP) *in vivo* by injection in rat brains. It is also confirmed that the suppression of LTP is attributed to the Aβ oligomer, but not to the monomer or fibrils [81]. It is almost certain that soluble Aβ species have pivotal roles in establishment and progress of AD, particularly prior to the increase of Aβ deposition [18]. Possibly, Aβ oligomers may be the major cause of the synaptic dysfunction in the early phase of AD, and protofibrils may contribute to the neuronal death in the late phase. To prevent the Aβ-induced synaptic dysfunction, therapeutic approaches for removal of soluble Aβ by anti-Aβ antibody or by up-regulating presynaptic neprilysin, which is the most potent endogenous Aβ-degrading enzyme, are under development [for review, see 29,41,68].

The 'Aβ hypothesis' proposes that Aβ triggers most of the subsequent AD pathogenesis. Therefore, number of anti-Aβ therapeutics are under development. One approach is the suppression of Aβ production by inhibiting activity of secretases. Since (1) γ-secretase probably regulates production of Aβ40 and Aβ42 by cleaving C-terminus of Aβ, (2) FAD-linked mutations in APP locate at close proximity to the γ-secretase cleavage site, and (3) presenilins are FAD-responsible gene products and also involved in γ-secretase activity, the inhibition of γ-secretase, including peptidic inhibitors, attracts a particular attention for therapeutic application. Another approach of anti-Aβ therapy aims at the removal of excess Aβ. There are two distinct ideas to achieve this goal. One is to accelerate the natural degradation process of Aβ by a proteolytic enzyme, e.g., neprilysin present in the brain. It was revealed that this enzyme is responsible for the overall speed of the degradation process of Aβ [30]. Furthermore, reduced level of neprilysin in sporadic AD brains relative to normal control was reported [88]. Various approaches to increase neprilysin activity in the AD brain are under development. Another approach to remove Aβ from brain is anti-Aβ immunization. Production of anti-Aβ antibody by active immunization with Aβ1-42 reduced amyloid deposit in mice [67]. Unfortunately, inflammation reaction was experienced as a side effect in human clinical trials. To overcome this problem, alternative immunization technologies with reduced immune responses

and passive immunization with humanized anti-Aβ antibody are under progress as the practical methods.

Is Neuroprotection Necessary as a Component of Potential AD Therapy?

Abnormal increase of Aβ triggers subsequent neuronal dysfunction and neuronal cell death based on the Aβ hypothesis as discussed above. If the neuronal cell death solely depends on the quantity of neurotoxic insults, the neuronal cell death happens shortly after the insults and thereof AD symptoms appear. However, there is evidence that the increase and accumulation of Aβ begin in the middle age and plateau around 70 years old [52]. This observation implies that the onset of clinical manifestation of AD is prevented over decades even in the existence of neurotoxic insults including mainly Aβ deposition. Therefore, it is hypothesized easily that a mechanism exists to counteract the neurotoxic Aβ insults which is probably neuroprotection. The clinical onset of AD seems to be the outcome of the combined effect of reduced level of the hypothesized neuroprotection and the increased insults as depicted in Fig. **2A**. The anti-Aβ therapy aims to reduce the causative insults and prevent neuronal loss. However, the certain amount of neuronal loss progress during the therapy unfortunately because it takes time to remove Aβ deposit with the currently developed methodologies and the existing Aβ deposit continues to affect neuronal cells (Fig. **2B**). On the other hand, it would be possible to suppress the increase of neuronal cell death by enhancing neuroprotective function (Fig. **2C**). In optimistic scenario, if the neuroprotective function could be maintained at a high level, the neuronal death might be prevented even with the existence of on-going neurotoxic insults (Fig. **2D**). In this model, though, once the balance between the neuroprotective function and the insults is disturbed, by increased insults for example, neuronal death will resume (Fig. **2D**, far right). More realistic scenario, therefore, would probably be combining approaches to decrease the insults while maintaining a high level of the neuroprotective function (Fig. **2E**).

Some of the neurotrophic factors, which play critical roles in the neuronal development, show neuroprotective action including suppression of neuronal cell death caused by Aβ. In search of other neuroprotective factors effective in AD-related neuronal cell death, we discovered a novel neuroprotective peptide, Humanin.

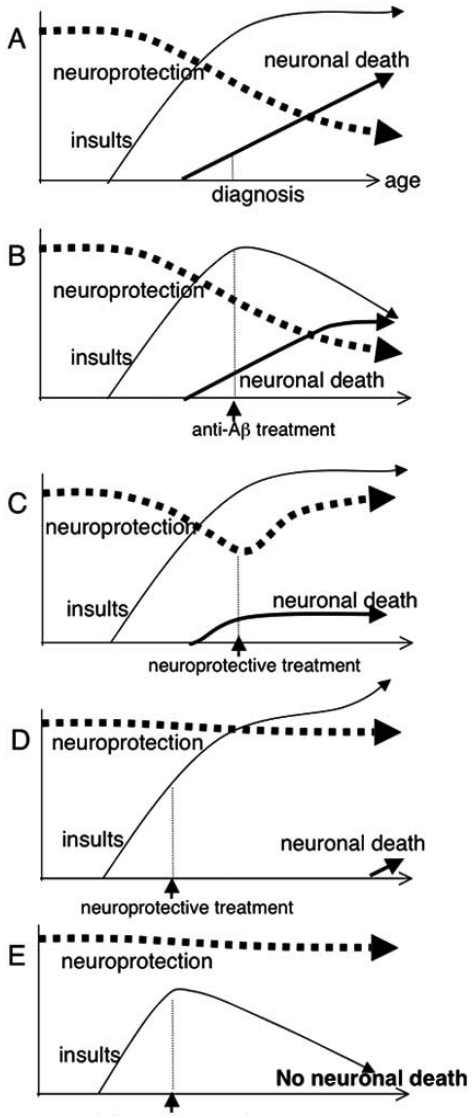

Fig (2). Progression of neuronal death in AD in various models.
The relationship between neurotoxic insults (thin line) and neuronal cell death (bold line) is influenced by the neuroprotective function (dotted line) counteracting to neurotoxic insults (A). The degree of neuronal death is reduced reflecting the degree of insults or neuroprotection by anti-Aβ or neuroprotective treatment at the time of diagnosis (B, C). When treatments start at earlier time point, neuronal death may be prevented (D, E). X- and Y-axis indicate age and degree of each phenomenon, respectively. See text for details.

HUMANIN

Humanin is a Neuroprotective Factor

While working on the pathomechanisms of neuronal loss in AD, an observation of AD brains caught out attention. In most of AD brains, occipital lobe remains rather intact comparing to other vulnerable regions such as hippocampus and parietal lobe. We then speculated that a certain neuroprotective molecule(s) might be expressed at high level in occipital lobe relative to other regions. Based on this hypothesis, we attempted to isolate antagonistic gene(s) that suppress neuronal death caused by AD-specific insults, from a cDNA library that is constructed from mRNA in the occipital lobe of an AD patient. We utilized a screening method termed 'Death trap' that selects genes from a cDNA library on the basis of survival of cDNA-transfected cells, despite of cytotoxic mechanism, under a certain cell death-causing insult [79]. We used the London-type APP mutant, V642I-APP as the AD-specific insult [59], since the expression of V642I-APP induces cell death in neuronal cell lines and primary cortical neurons *in vitro* [15,23,47,59, 60,64,85,87,92]. As the result of the functional screening, we isolated a group of cDNA clones that encoded a novel 24-

residue polypeptide (MAPRGFSCLLLLTSEIDLPVKRRA), named Humanin (HN) [25,57,58,61].

Analysis of amino acid sequence of HN showed that the full length of HN sequence is qualified as a signal sequence by prediction programs of signal peptide region in proteins. This prediction was experimentally proven as upon transfection of HN cDNA in neuronal cells, HN peptide was detected in the culture medium of the transfected cells [25,86]. This indicates that HN is a self-secretive peptide, i.e., the entire sequence of HN serves as a signal peptide to transport itself into the extracellular medium. Interestingly, the culture medium containing secreted HN peptide exhibited neuroprotective action. Consistent with this result, synthetic HN peptide, when added into the culture medium, suppresses cell death caused by V642I-APP in neurohybrid cells and primary cortical neurons [25,60]. These results confirmed that HN is a neuroprotective peptide.

Action Spectrum of HN

In addition to V642I-APP, Swedish-type APP mutant (K595N/M596L-APP, NL-APP) and FAD-linked PS mutants, M146L-PS1 and N141I-PS2, induce neuronal cell death *in vitro* [1,3,19,22,23,28,32,76,83,85]. Besides, anti-APP antibodies that recognize the extracellular region of APP function as a putative ligand of APP and induce apoptosis in wild-type APP-expressing neuronal/non-neuronal line cells and primary neurons (Table **1**) [6,27,51,62,64,66,70,71]. Moreover, Aβ appears to serve as an APP ligand although more specific APP ligand may exist, since APP was demonstrated as one of Aβ-binding proteins on cell surface [44]. In fact, Aβ treatment of neuronal cells expressing V642I-APP increases cell mortality *in vitro* [23]. HN suppresses neuronal cell death caused by these FAD-linked mutants and anti-APP antibody [1,20,21,24,25,28]. More importantly, HN blocks cell death induced by cytotoxic Aβ peptides, Aβ1-42, Aβ1-43, and Aβ25-35, in primary cortical neurons [24,25,77]. Thus, HN suppresses neuronal death caused by all AD-related insults so far tested. It should be noted that various types of cytotoxic mechanisms are evoked by both Aβ and FAD-linked mutants [11,34,35,40,58]. Considering these findings, it is presumed that target of HN's neuroprotective action is not a single signaling molecule or a specific pathway. In other words, HN appears to be a neuroprotective factor that can suppress a broad range of toxic insults related to AD pathogenesis.

Neurotrophic factors, basic fibroblast growth factor (bFGF) [49], activity-dependent neurotrophic factor (ADNF) [8], and insulin-like growth factor-I (IGF-I) [13], have been reported to protect neurons from Aβ-induced cytotoxicity. These factors also suppress V642I-APP-induced cell death in neurohybrid cells [24]. However, ADNF blocks the neuronal death caused by overexpression of NL-APP only partially and shows no effect on the cell death caused by M146L-PS1 or N141I-PS2. Neither IGF-I nor bFGF is effective in suppressing neuronal death caused by these three mutants. IGF-I evokes anti-apoptotic signaling pathway through the activation of receptor tyrosine kinase and PI3 kinase [82]. Therefore, IGF-I may suppress only apoptotic cell death caused by APP and PS mutants. In any case, HN is the only factor that is effective in suppressing various types of AD-related neuronal death so far. Because of this uniquely wide action spectrum, it is also inferred that the mechanism of HN action is probably different from the mechanisms of other neurotrophic factors.

Table 1. Action Spectrum of HN

Stimuli	Cell*	Effect	References
AD-related			
Aβ1-42	PCN	yes, extracellular	[74]
Aβ1-43	PCN	yes, extracellular	[71]
Aβ25-35	PCN	yes, extracellular	[74]
FAD-linked APP mutants	F11, PCN	yes, extracellular	[50, 63, 71]
FAD-linked PS1 mutants	F11	yes, extracellular	[69,71]
FAD-linked PS2 mutants	F11	yes, extracellular	[65,71]
anti-APP antibody	PCN	yes, extracellular	[62,74]
Apoptosis-related			
etoposide	PCN	no, extracellular	[71]
anti-Fas antibody	Jurkat	no, extracellular	[71]
staurosporine	CSM14.1	yes, intracellular	[80]
Bax overexpression	CSM14.1	yes, intracellular	[80]
BimEL overexpression	HEK293 T	yes, intracellular	[90]
BimEL overexpression	HEK293 T	no, extracellular	[90]
tBit overexpression	HEK293 T	yes, intracellular	[89]
tBit overexpression	HEK293 T	no, extracellular	[89]

*PCN: mouse primary cortical neuron, F11: mouse/rat neurohybrid cell line, Jurkat: human T-cell leukemia cell line, CSM14.1: rat neuronal cell line, HEK293T: human embryonic kidney cell line.

Action Mechanism of HN

An active form of HN may be multimers. By structure-function analyses, we found that S7A-HN, a HN derivative with a substitution of alanine for 7th serine, does not form multimer either with itself or with wild-type HN (wt-HN) in *in vitro* binding assay, and this mutant has no neuroprotective activity (Table **2**) [77,86]. When a self-dimerizing tag-sequence (EFLIVIKS) is fused to the N-terminal of the S7A-HN, such fusion peptide forms multimer complex and suppresses cell death by Aβ in primary neurons [77]. These results suggest that self-multimerization, most likely dimerizaion, of HN peptide is necessary for the neuroprotective action.

Table 2. HN and Derivatives

Name	Sequence	Comments	Effective dose
HN	MAPRGFSCLLLLTSEIDLPVKRRA		1~10 μM
S7A-HN	MAPRGFACLLLLTSEIDLPVKRRA	dimerization defective	Not effective
L9R-HN	MAPRGFSCRLLLTSEIDLPVKRRA	secretion defective Bax-binding defective	1~10 μM (extracellular treatment)
S14G-HN	MAPRGFSCLLLLTGEIDLPVKRRA	highly potent	1~10 nM
R4A/F6A/S14G-HN	MAPAGASCLLLLTGEIDLPVKRRA	highly potent	100~300 pM

HN with a substitution of arginine for 9th leucine (L9R-HN) is a secretion-defective HN mutant. L9R-HN shows no neuroprotective effect on V642I-APP-induced cell death when L9R-HN-expressing plasmid construct is co-transfected with V642I-APP-expressing plasmid to the cells. When L9R-HN synthetic peptide is added to the culture medium of V642I-APP-expressing cells, however, L9R-HN peptide does suppress cell death [25]. These results indicate that extracellular action is necessary and sufficient for the neuroprotective function of HN in this system. This leads to a hypothesis that a cell surface molecule, probably a receptor, mediates HN's action. A series of evidence support the presence of HN receptor (Fig. **3**): HN and HN derivatives are effective at low concentrations (pM level in case of certain HN derivatives) [24,77]; specific binding of radiolabeled HN peptide to the surface of neurohybrid cells was demonstrated [25]; intracellular signaling molecule(s) are activated by the treatment of HN [25]. Ying *et al*. [89] proposed that formylpeptide receptor-like-1 (FPRL-1), one of the Aβ-binding receptors, is the HN receptor. They presumed that HN functions by competitively inhibiting the association of Aβ to FPRL-1 and blocking Aβ-induced cytotoxicity. However, it has also been demonstrated that HN suppresses neuronal death by FAD mutant or by antibody stimulation independently of Aβ toxicity [20,21, 27]. HN, thus, may elicit alternative neuroprotective action(s) *via* FPRL-1 in addition to competitively antagonize Aβ toxicity. Another possibility is that other receptor(s) exist to mediate stimulation of HN for its functionally unique output, a broad range of neuroprotection.

Neuroprotection by HN is blocked by a tyrosine kinase inhibitor, genistein, but not by inhibitors of PI3 kinase or MEK [25], indicating the involvement of certain tyrosine kinase(s) in HN-induced signaling pathway. Recently, Hashimoto *et al*. [26] demonstrated that STAT3, one of the principal molecules in tyrosine kinases-mediated signaling cascades, plays an essential role in HN-induced neuroprotection. This finding suggested that STAT3-mediated transcriptional regulation is involved in the neuroprotective action. A major question for the understanding of mechanisms behind HN's neuroprotective function would be which molecules in the cells HN affects when HN exerts its neuroprotective function. It was revealed that both V642I-APP and anti-APP antibodies activate a cytotoxic cascade constituted with a chain of molecules, Go-ASK1-JNK-NADPH oxidase-caspases and induce apoptotic cell death [15,21,23,27,58-60,64,65,85,87]. Among the molecules involved in this APP-induced cell death cascade, it has been demonstrated so far that HN inhibits the activation of JNK and caspase 3 [25,27]. HN also suppresses cell death caused by constitutively active ASK1, which is the upstream kinase of JNK [21]. These suggest that HN-evoked intracellular signaling cascade(s) probably inhibits upstream molecule(s) of JNK in this pathway.

In addition to the extracellular neuroprotective action of HN, Guo *et al*. [17] demonstrated a distinct intracellular anti-apoptotic action of HN. In this function, HN binds to Bax, prevents its translocation to mitochondria, and inhibits the mitochondria-mediated apoptosis. Overexpressed HN can suppress Bax-mediated apoptosis induced by non-AD-related insults, such as staurosporine, in non-neuronal cells. However, the specificity of Bax-HN binding indicates that this intracellular action of HN is effective only in Bax-mediated apoptosis but not in apoptotic death irrelevant to Bax or non-apoptotic death caused by AD-related insults. The secretion-defective mutant, L9R-HN, is defective in Bax-binding, and hence no anti-apoptotic action. However, as described above, the extracellular adminis-tration of synthetic L9R-HN suppresses V642I-APP-induced apoptotic death that is also suppressed by Bcl-2 [87], suggesting that HN's action through Bax inhibition is distinguishable from extracellular neuroprotective action. Subsequent studies revealed that HN inhibits BimEL and Bid, BH3-only pro-apoptotic members of Bcl-2 family that function as agonist of Bax, by directly binding to them [45,90]. Extracellularly added HN peptide shows no antagonistic effect against BimEL and tBid, whereas HN fused with polyarginine does. Since fused peptides with poly arginines can penetrate the cells, this is an additional strong evidence for the requirement of intracellular presence of HN to induce its anti-apoptotic action through inhibition of such pro-apoptotic Bcl-2 family proteins. Thus, HN possesses at least two distinct mechanisms/functions depending on its localization.

How do these two distinct cell protection mechanisms contribute to the HN's function in antagonizing against Aβ-induced neuronal cell death? Extracellular Aβ treatment activates several cytotoxic signaling molecules including JNK. Aβ-induced neuronal death is blocked by JNK inhibitors and is attenuated in cortical neurons from JNK-knock-out mice [7,53,57,78]. These findings clearly indicate that JNK is involved in Aβ-induced cytotoxicity. Therefore, it is highly probable that the inhibition of JNK is involved in

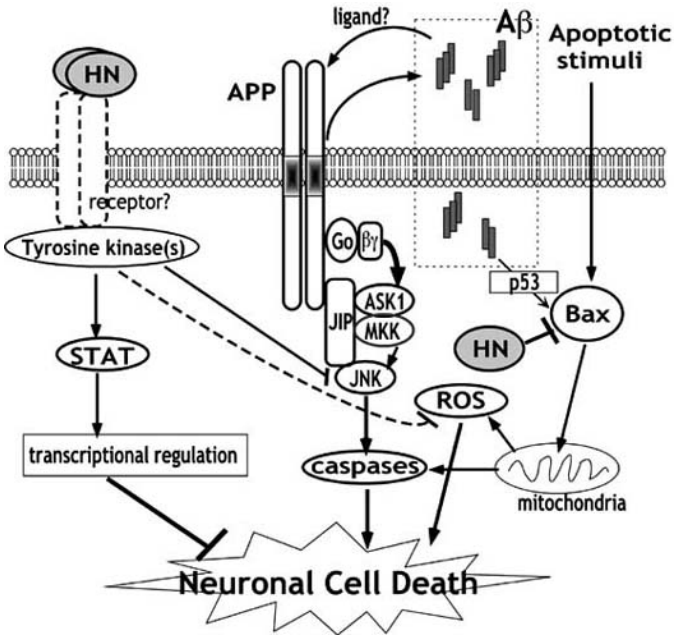

Fig (3). Neuroprotective mechanisms of HN.
Extracellular HN binds its putative receptor and activates intracellular signaling cascade(s). One of such effects inhibits JNK activity. Intracellular HN binds Bax and inhibits Bax-mediated apoptosis.

the extracellular action of HN against Aβ-induced cell death as in the case of V642I-APP-induced toxic pathway. In addition, it has been reported that intracellular Aβ induced apoptosis through p53-dependent transcriptional upregulation of Bax [63,91]. This suggests that intracellular anti-Bax action of HN may also contribute to the suppression of Aβ-induced toxicity. It is also reported that both Aβ and some of the FAD mutants induce non-apoptotic cell death through increase in production of reactive oxygen species [93]. The mechanisms underlying the suppression of this type of cell death by HN remains obscure for now. Nonetheless, immunohistochemical analyses of human brains showed that the immunopositive neurons were detected only in occipital lobe of sporadic AD brain, but not other regions or age-matched control brain [74]. From these results, it can be speculated that high level of intracellular expression of HN may be necessary for neuronal survival in sporadic AD brain. Both intracellular and extracellular effects of HN or potentially synergistic effect of them might contribute to the neuronal cell survival in the occipital lobe of sporadic AD cases.

Potential of HN for AD Therapy

Amino acid substitutions of HN sequence change the effective dose for neuroprotection [24,25,77,86]. A replacement of 14th serine to glycine in HN peptide (S14G-HN) drastically increases the potency of HN, i.e., 1000-fold: wt-HN suppresses neuronal death at 1-10 μM of concentration, whereas S14G-HN exhibits complete suppressive action at 1-10 nM [25]. An additional substitution of alanines for 4th arginine and 6th phenylalanine that are potential cleavage sites of trypsin and chymotrypsin, respectively, (R4A/F6A/S14G-HN) results in further potentiation of neuroprotective activity by 10-fold. The R4A/F6A/S14G-HN is effective at 100-300 pM [24]. The 8th cysteine can be replaced by arginine or lysine without changing the effective dose [24,77]. These findings suggest a possibility that more potent and stable HN derivatives, which are applicable to clinical use, may be achieved by additional modification(s). Recently, Benaki *et al.* [4] analyzed the solution structure of HN, and found that HN acquires a helical conformation in hydrophobic environment. Such structural information should serve as a guide to design more effective HN derivatives.

In vivo effect of S14G-HN, a potent HN derivative, was first demonstrated by Mamiya & Ukai [48]. Intracerebroventricular (icv) injection of S14G-HN suppressed memory impairment caused by scopolamine, a muscarinic receptor antagonist, in mice. The effect of S14G-HN on scopolamine-induced amnesia is blocked by administration of genistein [73], suggesting that the action of S14G-HN is mediated by tyrosine kinase(s) *in vivo* as observed *in vitro*. Tajima *et al*. [73] demonstrated the neuroprotective action of HN against AD-related insult *in vivo*: Aβ25-35-induced amnesia was suppressed by icv administration of S14G-HN, but not by neuroprotection-defective HN mutant, in mice. Consisting with this behavioral observation, S14G-HN administration into Aβ25-35-injected mice retained the number of choline acetyltransferase-positive neurons nearly to the level of control mice, while they were significantly reduced in mice injected with Aβ25-35 alone. Furthermore, intraperitoneal administration of S14G-HN reverses memory impairment induced by 3-quinuclidinyl benzilate, a muscarinic receptor antagonist, in rats [38]. The results may imply that HN derivative(s) can be effective against AD-related insults through conventional administration methods for human use.

Thus, applications of such highly potent HN derivative in a clinical setting for AD seems promising.

CONCLUSION

Multiple approaches to understand the AD pathogenesis have unraveled the mechanism of neuronal death in AD. Aβ is considered as a principal player of AD pathogenesis. Aβ and other AD-related insults cause various types of toxic mechanisms that contribute to neuronal death, indicating the complex nature of AD pathogenesis. Despite such complexity of AD pathomechanism, HN can antagonize against all these insults. Unlike HN, other neuroprotective factors so far known protect neuronal cells only from certain AD-related insults. Thus, HN is the only factor that shows such a wide range of neuroprotective actions at present. Receptor-mediated signal should be a central for the unique neuroprotective action of HN, but detailed mechanism is still to be investigated. The fact that a potent HN derivative suppresses AD-related insults *in vivo* is highly encouraging for the clinical applications of HN as a promising candidate of AD therapy targeting the neuronal death. The combination of HN-based remedy and other therapeutics, such as anti-Aβ therapy, hopefully will read to a complete cure of AD.

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