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Neuroprotection Against Neurodegenerative Diseases

Development of a Novel Hybrid Neuroprotective Peptide Colivelin

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Abstract

Neuronal death is directly implicated in the pathogenesis of neurodegenerative diseases (NDDs). NDDs cannot be cured because the mechanisms underlying neuronal death are too complicated to be therapeutically suppressed. Neuroprotective factors, such as neurotrophins, certain growth factors, neurotrophic cytokines, and short neuroprotective peptides, support neuronal survival in both physiological and pathological conditions, suggesting that these factors may be good drug candidates for NDDs. We recently generated a novel neuroprotective peptide named Colivelin by attaching activity-dependent neurotrophic factor (ADNF) to the N-terminus of a potent Humanin derivative, AGA-(C8R)HNG17. HN was originally identified from an Alzheimer's disease (AD) brain as an endogenous neuroprotective peptide that suppresses AD-relevant toxicity. Colivelin protects neurons from death relevant to NDDs by activating two independent prosurvival signals: an ADNF-mediated Ca^{2+}/calmodulin-dependent protein kinase IV pathway and an HN-mediated STAT3 pathway. The neuroprotective effect of Colivelin provides novel insights into therapy for NDDs.

Index Entries: Colivelin; humanin; ADNF; neuronal death; neuroprotection; neurodegenerative diseases.

Introduction

Neurodegenerative diseases (NDDs) are a group of disorders in the central nervous system (CNS) characterized by progressive loss of neurons. Most NDDs are incurable and fatal; there is no effective therapy. Signs and symptoms of NDDs are the loss of function of the affected neuronal systems. For example, Alzheimer's disease (AD), the most common NDD, is characterized by memory deficit and learning disability, the result of neuronal loss in the cerebral cortex. Amyotrophic lateral sclerosis (ALS), the most

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common motor neuron disease, is characterized by progressive impairment of the voluntary motor system, which is caused by loss of both upper and lower motor neurons.

Because most NDDs occur in the middle to late stages of life, it is hypothesized that there may be endogenous regulatory factors protecting neurons from degeneration and that the aging-mediated reduction of these activities may contribute to the disease onset (we termed this idea the "defense hypothesis"). For example, serum levels of insulin-like growth factor (IGF)-1, which acts as a neuroprotective factor, are inversely correlated with the risk of AD *(1)*, suggesting that IGF-1 may be a factor that antagonizes neuronal death relevant to AD. It is also possible that several neuroprotective factors other than IGF-1, with multiple different protective mechanisms, may mediate the endogenous defense mechanism. This article reviews neuronal death mechanisms and neuroprotective factors in NDDs. It also discusses the relevance of neuroprotective therapies for NDDs by focusing on Colivelin, which we recently developed as a new-generation neuroprotective factor.

Mechanisms Underlying Neuronal Death in Neurodegenerative Diseases

Pioneering research on NDDs has morphologically revealed various types of abnormally aggregated protein deposits in addition to neuronal loss: neuritic plaques and neurofibrillary tangles for AD, Lewy bodies for Parkinson's disease (PD), nuclear inclusions for Huntington's disease (HD), Lewy body-like hyaline inclusions for ALS, and so forth. Although most NDD cases occur sporadically, certain cases are inherited and accumulate in specific pedigrees (familial NDDs). Biochemical analysis of the abnormal aggregates, together with the genetic identification of causative genes for familial NDDs, has clarified the initial pathogenic event relevant to each NDD (the initial insults of neuronal death). Accumulating evidence has also suggested that subsequent neuronal death processes (the executive phase of neuronal death) are largely common to NDDs. Therefore, molecular events underlying neuronal death in NDDs can be divided into two categories: an initial trigger to activate death machinery, which appears to be specific for each NDD, and an executive process to complete neuronal death, which is shared among NDDs.

Initial Triggers of Neuronal Death in Neurodegenerative Diseases

Initial triggers of neuronal death in NDDs can be classified into several categories: (a) cellular metabolic disturbance; (b) overload on protein quality control or degradation systems; and (c) direct activation of executive cell death mechanisms. Mutations in causative genes result in protein misfolding or protein processing errors, leading to neuronal metabolic distress, such as overproduction of reactive oxygen spiecies (ROS) (or oxidative stress), disturbed Ca^{2+} homeostasis, mitochondrial dysfunction, and dysregulation of axonal transport. An accumulation of abnormal proteins also results in an overload on the protein quality control system, such as the endoplasmic reticulum (ER)-associated protein quality control system, the ubiquitin (Ub)-proteasome system (UPS), and the autophagical degradation system, leading to increase in pro-apoptotic proteins and triggering cell death machinery. Additionally, endogenous cell death machinery can be directly activated by mutant products encoded by disease-causative genes. It is consequently believed that various combinations of these initial insults may be involved in the onset of each NDD.

AD is pathologically characterized by senile plaques, neurofibrillary tangles, and massive neuronal death *(2)*. About 1% of AD cases occur familially (familial AD [FAD]). Three genes have been identified as causes of dominantly inherited FAD: amyloid precursor protein (APP)-, presenilin (PS)1-, and PS2-encoding genes (Table 1; ref. *3*). APP, a receptor-like type 1 transmembrane protein, is a precursor of

Table 1
Causative Genes for Hereditary Neurodegenerative Diseases

Gene	Loci	Pattern	Gene product	Function
Alzheimer's disease				
APP	*21q21.3-q22.05*	Dominant	APP	Precursor of Aβ, neurite outgrowth
PS1	*14q24.3*	Dominant	PS1	Catalytic domain of γ-secretase
PS2	*1q31-q42*	Dominant	PS2	γ-Secretase?, regulate cell death
Parkinson's disease				
SNCA	*4q21 (PARK1 and PARK4)*	Dominant	α-Synuclein	Constituent of LB, synaptic function?
PARK2	*6q25.2-q27 (PARK2)*	Recessive	Parkin	Ubiquitin E3 ligase
PINK1	*1p35-p36 (PARK6)*	Recessive	PINK1	Mitochondrial (CaMK-like) kinase
DJ1	*1p36 (PARK7)*	Recessive	DJ1	ThiJ/Pfpl family molecular chaperone
SPR	*2p13 (PARK3)*	Dominant	SPR	Tetrahydrobioprotein-producing enzyme
LRRK2	*12p12 (PARK8)*	Dominant	LRRK2	RIP kinase family protein
UCHL1	*4p14 (PARK5)*	Sporadic	UCHL1	Ub hydrolase and ligase activity
Poly-glutamine disease				
IT15	*4p16.3*	Dominant	Huntingtin	Inhibit caspase-9, upregulate BDNF
DRPLA	*12p13.31*	Dominant	Atrophin1	Transcriptional co-repressor
SCA3/MJD	*14q32.1*	Dominant	Ataxin3	
Amyotrophic lateral sclerosis				
SOD1	*21q22.1 (ALS1)*	Dominant	SOD1	Scavenger of ROS
ALS2	*2q33*	Recessive	Alsin	GEF (Rho, Rag5)
SETX	*9q34 (ALS4)*	Dominant	SETX	RNA helicase like structure
VAPB	*20q13.3 (ALS8)*	Dominant	VAPB	Vesicle trafficking

SPR, sepiapterin reductase (230); UCHL1, Ub carboxyl-terminal esterase L1; DRPLA, dentatonubral-pallidoluysian atrophy; SCA, Spinocerebellar ataxia type 3; MJD, Machado-Joseph disease; SETX, senetaxin; VAPB, vesicle-associated protein/synaptobrevin-associated membrane protein B

amyloid β (Aβ), the main component of senile plaques *(4–6)*. Aβ is implicated in the pathogenesis of both sporadic and familial AD *(7,8)*. PS are assumed to constitute the catalytic domain of the γ-secretase complex, which is involved in generation of Aβ by cleaving APP *(9–11)*. It has been demonstrated that mutations in the three FAD genes increase toxic Aβ levels *(12–14)*. Neurotoxicity of Aβ is mediated by multiple mechanisms, including (a) induction of oxidative stress *(15)*, (b) disturbed Ca^{2+} homeostasis *(16)*, (c) mitochondrial dysfunction *(17)*, (d) increase in ER stress *(18)*, (e) inhibition of the UPS *(19)*, (f) activation of the macroautophagic death mechanism *(20)*, (g) phosphorylation of microtubule-associated protein τ *(21)*, and (h) activation of cell death machinery via Aβ receptors, such as the p75 neurotrophin receptor (p75NTR) (ref. *22*; Fig.1A).

Independently of neurotoxicity mediated by Aβ, whose levels are upregulated by FAD-linked mutations, mutations in the three FAD genes may cause neurotoxicity by directly activating the intracellular death signaling machinery consisting of Go, c-Jun N-terminal kinase (JNK), and caspases (refs. *7,23–25*; Fig.1A). In

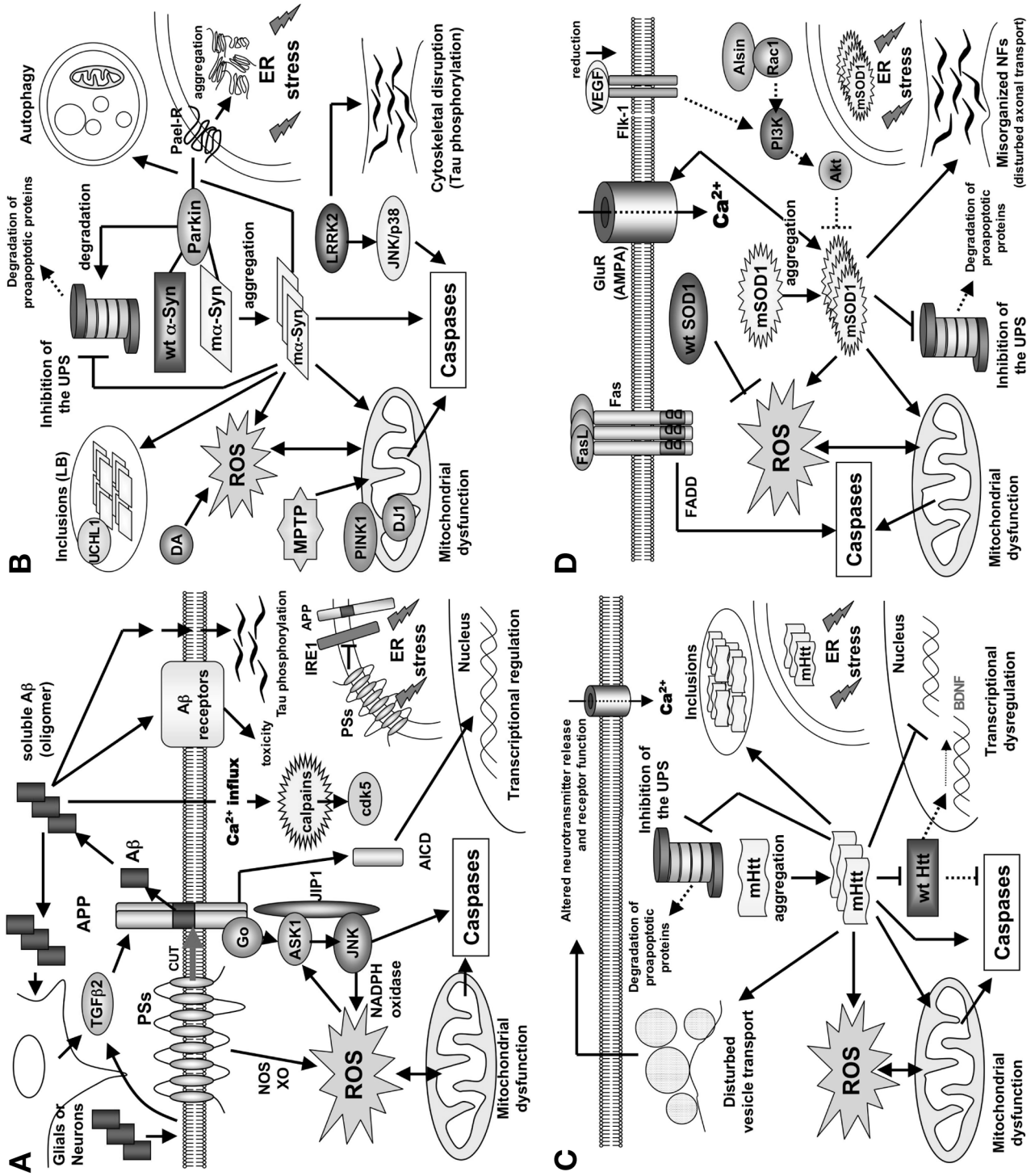

A
Glials or Neurons
TGFβ2
APP
PSs
CUT
Aβ
soluble Aβ (oligomer)
Aβ receptors
Tau phosphorylation
toxicity
Ca2+ influx
calpains
cdk5
IRE1
APP
ER stress
PSs
Nucleus
Transcriptional regulation
Go
ASK1
JNK
JIP1
AICD
NOS
XO
ROS
NADPH oxidase
Caspases
Mitochondrial dysfunction
B
Degradation of proapoptotic proteins
degradation
Parkin
Pael-R
aggregation
ER stress
Autophagy
Inhibition of the UPS
wt α-Syn
mα-Syn
aggregation
mα-Syn
LRRK2
JNK/p38
Cytoskeletal disruption (Tau phosphorylation)
Inclusions (LB)
UCHL1
DA
ROS
MPTP
PINK1
DJ1
Mitochondrial dysfunction
Caspases
C
Altered neurotransmitter release and receptor function
Ca2+
Inclusions
mHtt
ER stress
Nucleus
Inhibition of the UPS
Degradation of proapoptotic proteins
mHtt aggregation
mHtt
wt Htt
BDNF
Transcriptional dysregulation
Disturbed vesicle transport
ROS
Caspases
Mitochondrial dysfunction
D
reduction
VEGF
Flk-1
Alsin
Rac1
PI3K
Akt
mSOD1
ER stress
GluR (AMPA)
Ca2+
wt SOD1
mSOD1 aggregation
mSOD1
Degradation of proapoptotic proteins
Misorganized NFs (disturbed axonal transport)
Fas
FasL
FADD
ROS
Inhibition of the UPS
Caspases
Mitochondrial dysfunction

Fig. 1. Neurotoxic mechanisms in NDDs. **(A)**, AD-relevant neurotoxicity. AD-relevant neurotoxicity includes both Aβ-dependent and -independent types. Aβ causes abnormal Ca^{2+} influx and mediates neurotoxicity via Aβ receptors, such as p75NTR *(21)*, APP *(215)*, FPRL1 (formylpeptide receptor-like-1) *(216)*, β7 nicotinic acetylcholine receptor *(217)*, and BBP-1 (Aβ -binding protein-1) *(218)*. Aβ is also reported to induce hyperphosphorylation of tau, which results in NFT formation and neuronal death. A series of studies have demonstrated that FAD-linked APP mutants constitutively triggers death signals via Go (a heterotrimeric GTP-binding protein)/JNK/NADPH oxidase/caspases, in this order *(10,22,23,184)*. The APP-mediated death signal can also be autocrinally or paracrinally activated by TGFβ2 in a ligand-receptor fashion *(27)*, whose expression is induced by Aβ). Additionally, the APP intracellular domain (AICD), cleaved from APP by secretases, and its interactors, such as Fe65 and APP-BP1 *(219-221)*, exert neurotoxicity by transcriptionally regulating expression of various genes. Mutations in PSs result in (i) Aβ over-production, (ii) NOS or xanthine oxidase (XO)-mediated ROS production, (iii) increase in ER stress caused by inhibition of IRE1 processing, and (iv) perturbed Ca^{2+} homeostasis. **(B)**, PD-relevant neurotoxicity. MPTP causes selective dopaminergic neuron death via up-regulation of ROS and Bax. Mutant a-synuclein (ma-Syn) aggregates and induces neurotoxiciy, such as ROS overproduction *(34)*, mitochondrial dysfunction *(35)*, ER stress *(36)*, inhibition of the UPS *(37,38)*, caspase activation, and autophagic death. Mutations in the RIP kinase family proteins, leucine-rich repeat kinase 2 (LRRK2), which is another causative gene for dominantly inherited FPD *(222,223)*, might induce death via JNK/p38 MAPK activation *(224)*. Parkin degrades a-synuclein and suppresses neuronal death *(41)*. Impairment in degradation of the Pael receptor (Pael-R), a substrate of parkin, results in increased ER stress leading to neuronal death *(42)*. Gene knockdown of another causative gene for recessive FPD, PINK1 (PTEN-induced kinase 1), a putative mitochondrial kinase, resulted in apoptosis in a human dopaminergic cell line *(225)*. DJ1, another recessive FPD gene, is a member of the ThiJ/PfpI family of mitochondrial molecular chaperones induced under oxidative stress. DJ1 deficiency results in vulnerability to MPTP and induction of oxidative stress with impaired PI3K/Akt prosurvival signals *(226)*. Ubiquitin carboxyl-terminal esterase L1 (UCHL1) has Ub hydrolase and ligase activity and is a component of LB. **(C)**, HD-relevant neurotoxicity. An expansion of poly-Q repeats in huntingtin (Htt) results in misfolding and aggregation of Htt, which induces inclusion body-formation and multiple neurotoxic insults, such as (i) ROS overproduction *(43)*, (ii) mitochondrial dysfunction *(46)*, (iii) disturbed vesicle transport *(45)*, (iv) inhibition of the UPS *(43)*, (v) caspase activation *(50,51)*, and (vi) transcriptional dysregulation or inhibition *(47,48)*. Notably, wt-Htt inhibits caspase activation and upregulates BDNF expression, which is antagonized by mutant Htt (mHtt) *(52)*. **(D)**, ALS-relevant neurotoxicity. Mutation-induced conformational changes in SOD1 generate multiple neurotoxic events including glutamate-mediated excitotoxicity *(59)*, mitochondrial dysfunction *(60)*, cytoskeletal abnormality (neurofilament) *(61)*, ER stress *(62)*, inhibition of the UPS *(63)*, and activation of caspases *(64)*. Mutant SOD1 (mSOD1)-induced neuronal death is antagonized by Alsin-mediated activation of the Rac1/PI3K/Akt pathway *(65,66)*. Downregulation of the VEGF (vascular endothelial growth factor)-mediated PI3K/Akt signal enhances the risk of ALS *(107)*.

support of the relevance of this type of neurotoxicity in the pathogenesis of AD, recent studies have revealed that transforming growth factor (TGF)-β2 acts as a neuronal-death-inducing ligand for APP *(23)*. Toxic Aβ upregulates expression levels of TGF-β2 *(26)*, which in turn binds to APP and activates APP-mediated death signals in a ligand-receptor fashion *(27)*. Mutations in PS1 also directly activate toxic mechamisms, such as ROS production and increase in ER stress *(28–31)*. It has also been reported that FAD-linked PS2 mutants exert neurotoxicity by ROS production, as do APP mutants *(32)*. Genetic analysis has further established that an apolipoprotein E (Apo E) gene subtype is a risk factor of late-onset SAD: the inherited Apo E4 allele worsens the loss of neuronal function in patients with AD and lowers the age of disease onset *(3)*.

PD is characterized by akinesia, rigidity, and tremor, caused by loss of dopaminergic neurons in the substantia nigra *(33)*. Although pathogenesis of most PD cases is elusive, initial insults that cause two types of PD have been identified: drug-induced PD (or parkinsonism) and familial PD (FPD). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a chemical byproduct of synthetic heroin that can induce parkinsonism.

MPTP causes selective dopaminergic neuron death by upregulation of ROS and pro-apoptotic protein Bax. It also promotes the formation of Lewy-like intracellular aggregates (Fig. 1B). The latter PD is caused by mutations in a certain single causative gene. Genetic linkage analyses have identified seven causative genes for FPD or parkinsonism (Table 1), all of which have been demonstarated to be involved in PD-related dopaminergic neuronal death (Fig. 1B). Dominantly inherited FPD is caused by mutations in the *SNCA* gene, which encodes α-synuclein, the main component of Lewy bodies. Mutant α-synuclein forms abnormal aggregates and exhibits multiple types of neurotoxicities similar to those in AD pathogenesis, as depicted in Fig.1B *(34–38)*. Mutations in the *parkin* gene were found to cause autosomal-recessive juvenile parkinsonism in Japanese families *(39)*. Parkin, which is assumed to function as an E3-Ub-ligase, suppresses mutant α-synuclein-induced neuronal death through UPS-mediated degradation *(40,41)*. It has been also reported that impaired ubiquitination of Pael receptor (Pael-R), a substrate of parkin, results in accumulation of the unfolded Pael-R in ER and induces neuronal cell death *(42)*.

Huntington's chorea (HD) is an autosomal-dominant disease caused by expansion of a trinucleotide (CAG) repeat in the gene encoding huntingtin (Htt) that results in Htt with expanded polyglutamine (poly-Q) *(43)*. Expanded poly-Q in Htt misfolds and aggregates in the inclusion bodies. Although misfolding of Htt is certainly involved in HD neurotoxicity, inclusion body formation has been reported to be rather neuroprotective *(44)*. Mutant Htt initiates multiple neurotoxic events similar to other NDDs (Fig. 1C; refs. *45–51*). Notably, expression of brain-derived neurotrophic factor (BDNF) is directly regulated by wild-type-Htt and is disturbed in HD *(52)*.

About 10% of ALS cases occur familially (familial ALS [FALS]), and four causative genes have been identified *(53,54)*. Point mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) are associated with autosomal-dominant ALS, which accounts for about 20% of FALS cases (Table 1; ref. *55)*. An autosomal-recessive juvenile ALS is caused by loss of the *ALS2* gene, which encodes a protein named Alsin, with three GDP/GTP exchanging factor (GEF)-homologous domains *(56,57)*. Because SOD1 physiologically acts as a scavenger of ROS, it was suggested that loss-of-function of SOD1 might lead to neurodegeneration as a result of increased ROS levels. However, this hypothesis is now assumed not to be the case because SOD1-deficient mice did not develop ALS-like motor neuron phenotypes *(58)*. Consequently, SOD1 mutants are currently believed to gain neurotoxic function (gain-of-function). Actually, mutations in the *SOD1* gene result in protein misfolding and conformational changes in SOD1 that cause multiple types of neurotoxicity through various mechanisms similar to other NDDs and increased vulnerability to glutamate toxicity (Fig. 1D; refs. *59–64*). Interestingly, mutant SOD1-induced neuronal death is antagonized by the Rho GEF domain in alsin through activation of a Rac 1/phosphatidyl inositol-3-phosphate kinase (PI3K)/Akt pathway, which might be directly involved in the pathogenesis of ALS2 *(65,66)*. Pathogenic mechanisms of other two causative genes, RNA helicase-like protein senetaxin and vesicle-associated membrane protein/synaptobrevin-associated membrane protein B, are still enigmatic.

Executive Mechanisms Underlying Neuronal Death in Neurodegenerative Diseases

The initial triggers described above activate executive neuronal-death processes, including programmed cell death (PCD) *(67,68)*. Classically, PCD is assumed to be equal to apoptotic death, which is executed by activation of a series of aspartyl proteases or caspases (caspase-dependent or canonical PCD), and to be a sole common death pathway in NDDs. Presently, however, this idea is believed to be incorrect because (a) some portion of apoptosis is mediated by factors other than caspases (caspase-independent apoptotic PCD [C^I-a-PCD]); (b) some portion of nonapoptotic death is also

endogenously programmed (caspase-independent nonapoptotic PCD [C^I-na-PCD]); and (c) multiple PCD pathways can be simultaneously activated in neuronal death.

The canonical PCD pathways are activated by two distinct pathways: one initiated by cell surface receptors containing death domains (DDs) such as Fas and tumor necrosis factor (TNF) receptor 1 (the "death-receptor-mediated pathway") and the other initiated through mitochondria (the "mitochondrial pathway"). The former pathway, initiated upon ligand-binding to cell-surface receptors, is followed by activation of death effector domain (DED)-containing initiator procaspases (caspases-8 and -10) (Fig. 2A). Activated initiator caspases then activate effector procaspases (caspases-3, -6, and -7) and induce chromatin condensation, nuclear fragmentation, and death by mechanisms such as caspase-mediated DNase activation. The mitochondrial pathway is initiated by various insults, such as oxidative stress, disturbed Ca^{2+} homeostasis, inhibition of the UPS, and JNK activation *(69)*. These insults induce release of cytochrome *c* and Smac/Diablo from the mitocondria *(67)*, which activates initiator procaspase-9 (Fig. 2A). Effector procaspases are then cleaved to induce apoptosis. Recent evidence has revealed an additional pathway originating from ER that results in the activation of initiator procaspases (caspases-12 and -9) *(18)*.

The C^I-a-PCD pathway is initiated by poly-ADP-ribose polymerase (PARP)-1-mediated recognition of nuclear DNA damages, which is caused by ROS *(67,68,70)*. PARP-1-induced poly-ADP-ribosylation somehow triggers translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus. AIF then causes chromatin condensation and large-scale DNA fragmentation by activation of endonucleases such as cyclophilin A (Fig. 2B).

The C^I-na-PCD pathway includes all cell death pathways that progress via organelles other than mitochondria, such as lysosomes, ER, and nucleus (Fig. 2C; refs. *68* and *71*). C^I-na-PCD is mediated by proteases other than caspases, such as calpains and cathepsins. Calpains are a family of Ca^{2+}-dependent cysteine proteases, whereas cathepsins are lysosomal aspartyl (cathepsin D) or cysteine-type (cathepsin B, H, and L) proteases. Autophagy can also induce C^I-na-PCD (autophagic degeneration) *(72,73)*. Another type of C^I-na-PCD, characterized by cytoplasmic vacuolation (Paraptosis), is initiated by IGF-1 receptor (IGF-1R)-mediated activation of MEK2 and JNK *(74)*.

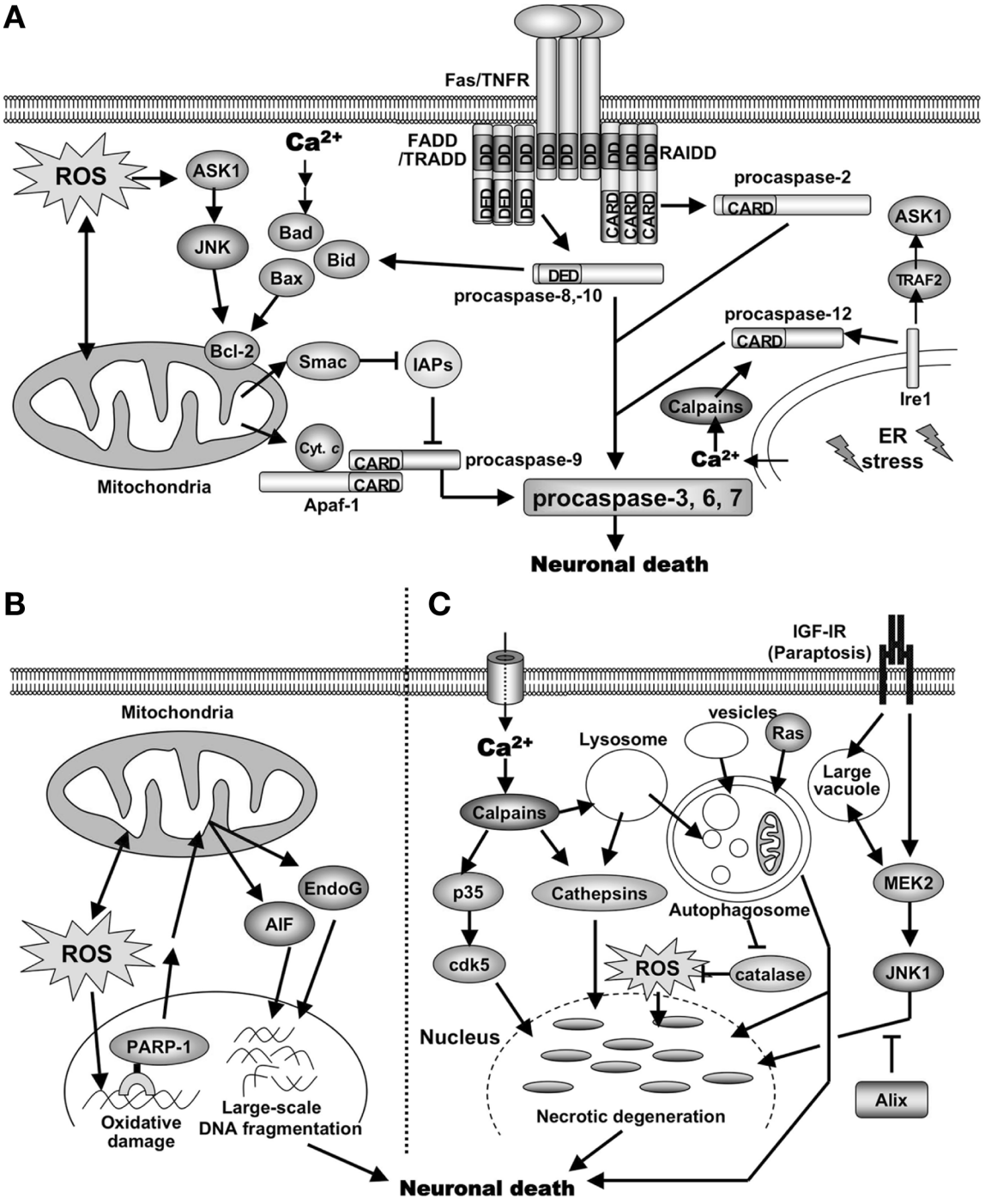
These PCD pathways can be simultaneously activated and can crosstalk. For example, oxidative stress can activate the canonical mitochondrial PCD pathway, the C^I-a-PCD pathway, and the autophagic C^I-na-PCD pathway. Mitochondrial dysfunction results in activation of the cytochrome *c*-mediated canonical PCD pathway and the AIF-mediated C^I-a-PCD pathway *(70)*. Cysteine-type cathepsins cleave and activate pro-apoptotic Bid, whereas calpain and cathepsin D activate Bax to induce mitochondrial canonical PCD in addition to the C^I-na-PCD pathway *(71,73)*. Additionally, PARP-1 is a substrate for active caspase-3, and cleavage of PARP-1 has been reported to modulate cell death *(70)*.

Neuroprotective Factors

Neuronal death in NDDs can not be completely inhibited by antagonizing a certain single molecular target in the death pathways because complicated death mechanisms are involved, as described earlier. Neuroprotective factors, defined as endogenous secretory molecules, antagonize neuronal death in both physiological and pathological conditions. Because they exhibit multiple protective activities on neurons and appear to regulate the onset and the progression of NDDs in vivo, they are good drug candidates for treatments of NDDs.

Neurotrophins and Growth Factors With Neuroprotective Activity

Neurotrophins are well-characterized "trophic" factors that promote survival and sustain normal function in the nervous system *(75,76)*.



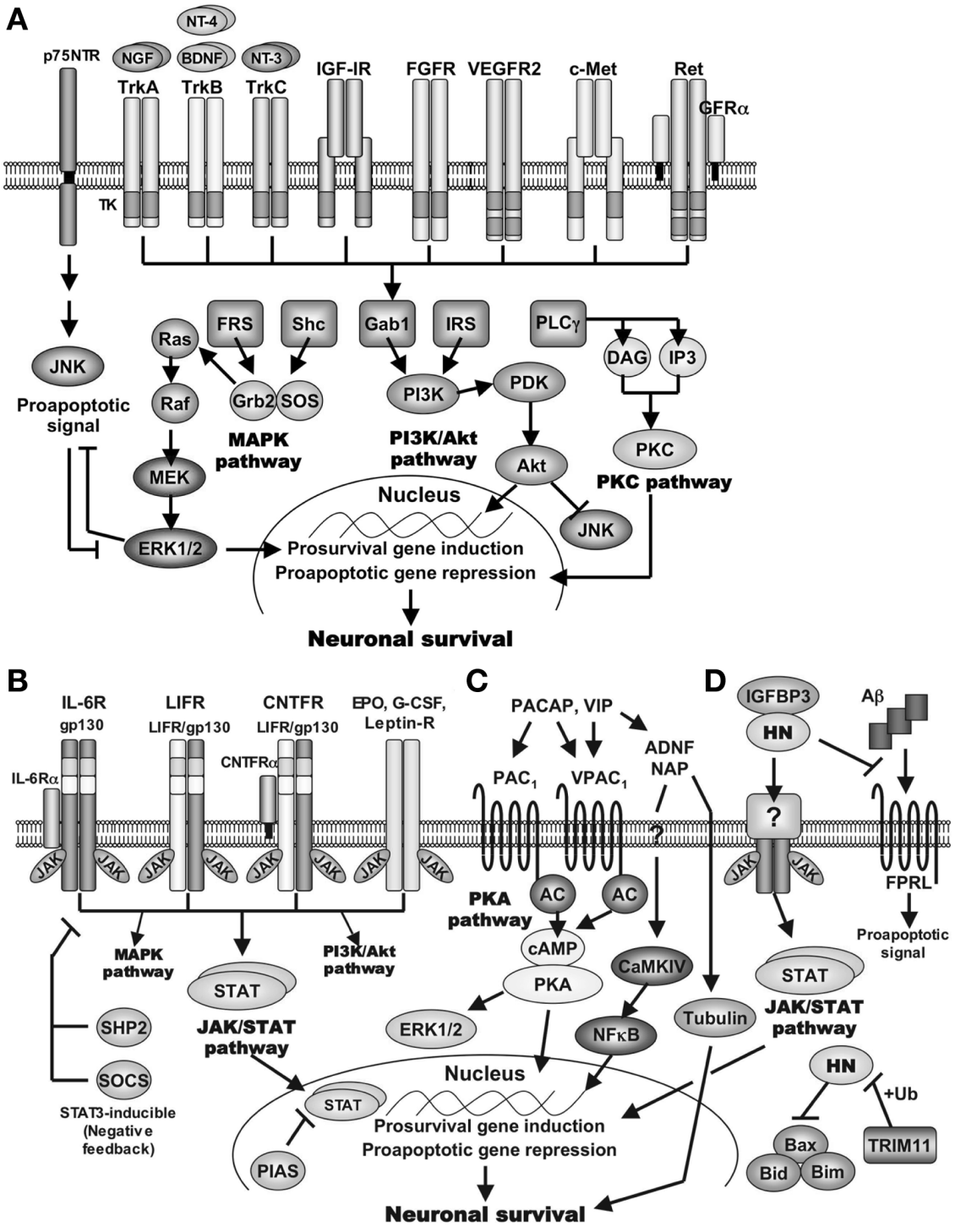
There are four neurotrophins in mammals: nerve growth factor (NGF), BDNF, neurotrophin (NT)-3, and NT-4. NGF, discovered in the 1950s to be the first neurotrophic factor *(77)*, acts on sympathetic neurons as well as on sensory neurons that are related to nociception and temperature sensation in the peripheral nervous system (PNS). In the CNS, NGF plays

Fig. 2. PCD pathways. **(A),** Canonical PCD pathway. Death-receptor-mediated pathways are initiated upon ligand binding, which recruits adaptor proteins with "DED," such as FADD and TRADD, or ones with "CARD (caspase recruit domain)," such as RAIDD, to the DD of the death receptors *(227,228)*. Adoptor proteins then activate DED-containing initiator procaspases (caspase-8, caspase-10) or a CARD-containing initiator procaspase-2. Activated initiator caspases then activate effector procaspases (caspase-3, caspase-6, and caspase-7) to induce chromatin condensation, nuclear fragmentation, and death. The mitochondrial pathway is initiated by various insults, including translocation of pro-apoptotic Bcl-2 family proteins (Bax, Bad, Bid, and Bim) to the mitochondria, which results in inhibition of Bcl-2 to increase mitochondrial membrane permeability. As a result, cytochrome *c* and Smac/Diablo are released from mitochondria to the cytosol *(67)*. Cytosolic cytochrome *c* subsequently forms a complex with Apaf1 and initiator procaspase-9 to active caspase-9. Cytosolic Smac/Diablo inhibits the antagonistic effect of IAPs (inhibitors of apoptosis) on initiator procaspase-9. Effector procaspases are then cleaved to induce apoptosis. Initiator procaspases (caspase-12 and caspase-9) are also activated on ER *(18)*. **(B),** C^{I}-a-PCD is initiated by PARP-1-mediated recognition of nuclear DNA damages caused by ROS *(68,70)*. Subsequent poly(ADP ribosyl)ation triggers release of AIF and endonuclease G (EndoG) from mitochondria, which causes death via chromatin condensation and large-scale DNA fragmentation. **(C),** C^{I}-na-PCD is generally initiated in lysosomes, ER, and nucleus *(68,71)*. C^{I}-na-PCD is mediated by calpains and cathepsins. Calpain cleaves p35 to generate p25, which activates cdk5 (cycline-dependent kinase 5) to induce death *(10)*. Autophagy also induces C^{I}-na-PCD upon activation of mutated Ras *(72)* via catalase degradation *(73)*. Paraptosis with large vacuole formation is mediated by IGF-IR via activation of MEK2 and JNK1 and is inhibited by Alix *(74)*.

a key role in stimulation, maintenance, and survival of basal forebrain cholinergic neurons *(78,79)*, which are destroyed in AD. BDNF was purified from porcine brain homogenates in 1982 *(80)*. Conversely to the PNS-dominant expression of NGF, BDNF is highly expressed in cortical and hippocampal structures *(81)*. BDNF has been reported to support neuronal survival, induce neurite outgrowth, and enhance synaptic plasticity *(82,83)*. A single nucleotide polymorphism in the *BDNF* gene that results in Met substitution of Val 66 in the pro-domain (V66M-BDNF or $BDNF_{Met}$) causes dysregulation in BDNF secretion *(84)*. This polymorphism has been demonstrated to be linked to memory impairment as well as to altered susceptibility to neuropsychiatric disorders, such as AD, PD, depression, eating disorder, and bipolar disorder *(76,85)*.

Neurotrophins, initially generated as precursors, are processed either inside (by furin or pro-hormone convertases) or outside the cell (by plasmin or matrix metalloproteinases) to be mature homodimerized forms. Pro-NGF, pro-NT-3, and pro-NT-4 are secreted via constitutive vesicles, whereas pro-BDNF is mainly secreted via regulated vesicles in an activity-dependent manner *(75,86)*. Neurotrophins transduce signals via two distinct receptors: tyrosine kinase receptors (Trks) and p75NTR (Fig.3A). Upon ligand binding, Trks are activated via tyrosine-transphosphorylation and further activates a mitogen-activated protein kinase (MAPK) pathway, a PI3K/Akt pathway *(87,88)*, and a protein kinase C (PKC) pathway *(89)* to promote neuronal survival (Fig. 3A). The MAPK pathway appears to suppress death by upregulation of Bcl-2, whereas the PI3K/Akt pathway may suppress death by inhibiting Bax and pro-apoptotic forkhead domain transcriptional factors *(75)*. p75NTR is a member of the TNFR/Fas/CD40 superfamily of receptors. All neurotrophins are equal in binding affinity to p75NTR, and proneurotrophins show higher affinity to p75NTR. Upon ligand binding, p75NTR activates JNK and nuclear factor (NF)κB, which are assumed to induce cell death *(21,90)*. Notably, however, in some cases, p75NTR is required in Trk-mediated neuronal survival *(91)*.

IGF-1 exhibits a neuroprotective effect *(92)*. IGF-1, mainly produced in the liver upon stimulation by growth hormone (GH), enters CNS by passing the blood-brain barrier (BBB) through a



transportation system mediated by IGF-1 binding proteins. IGF-1 is also synthesized in non-liver tissues, including brain, independently of regulation by GH *(93)*. Upon ligand binding, IGF-1 receptor, a Trk receptor, associates with insulin receptor substrate (IRS) and transduces

Fig. 3. Receptors and prosurvival signals of neuroprotective factors. **(A),** Neurotrophins and neurotrophic growth factors. Neurotrophins trigger intracellular signals via Trks and p75NTR. The Trks, consisting of TrkA, TrkB, and TrkC, are relevant to neuroprotection. Ligands for TrkA, TrkB, and TrkC are NGF, BDNF and NT-4, and NT-3, respectively. Upon binding of the neurotrophins, Trks are homodimerized and transphosphorylated. Phosphorylated tyrosine residues are recognized by Gab1, insulin receptor substrate, Src homology 2-containing protein, fibroblast growth factor receptor substrate 2, and phospholipase C-γ, which further activates a Ras/Raf/MAPK pathway, a PI3K/Akt pathway *(87,88)*, and a PLC-γ/inositol 1,4,5-triphosphate/diacylglycerol/PKC pathway *(89)* to promote neuronal survival. IGF-I receptor is also transphosphorylated upon ligand binding and recruits insulin receptor substrate to transduce prosurvival signals. Fibroblast growth factor receptor, VEGFR2 (or Flk-1), GFRα/RET, and c-Met are also tyrosine kinase receptors. **(B),** Neuroprotective cytokines. Type I cytokine receptors are characterized by the WSXWS motif, four conserved cysteine residues, and immunoglobulin-like domains. IL-6 receptor consists of ligand-binding nonsignaling IL-6Rα and two signal-evoking gp130 subunits. LIF binds to LIFR/gp130 heterodimer. CNTF binds to CNTFRα/LIFR/gp130 heterotrimer. Upon ligand binding, gp130 or LIFR/gp130 signaling subunits are tyrosine phospholyrated by intracellular JAK tyrosine kinases. STATs are then recruited to the phospho-tyrosine residues of the receptor and are also phosphorylated by JAKs. Phosphorylated STATs subsequently translocate to the nucleus and promote survival. SHP-2 and suppressor of cytokine signaling 3, which is translationally upregulated by STATs, negatively regulate the receptor activity in a negative feedback manner. Protein inhibitors of activated STAT also inhibit phosphorylated STATs in the nucleus. JAKs can also activate the MAPK and the PI3K/Akt pathways. Receptors of EPO, G-CSF, and leptin are also members of the type I cytokine receptor family. **(C),** Neuroprotective short peptides. PACAP, VIP, and PHI bind to specific GPCRs, such as PAC_1 type receptors (for PACAP) and $VPAC_1$ receptor (for VIP and PACAP). Upon ligand binding, the receptors trigger the PKA pathway and promote survival. PKA-mediated neuroprotection may involve activation of MAPKs, such as extracellular signal-regulated protein kinase 1/2 *(229)*. VIP-mediated neuroprotection is also exhibited indirectly via releasing glia-derived trophic molecules, such as ADNF-9 and NAP. The ADNF-mediated prosurvival signal has been reported to involve activation of CaMKIV, NFkB, etc. It is elusive how ADNF activates intracellular signaling molecules such as CaMKIV and NFkB. At least NAP and its relative peptides somehow enter into cells and bind tubulin to stabilize, which promotes survival. It is unknown whether there may be cell-surface receptors or transporters for ADNF/NAP. **(D),** HN. Eight HN interactors have been identified: (i) a cell surface receptor activating STAT3, (ii) Aβ receptor, FPRL, (iii) Bax, (iv) Bid, (v) Bim (BimEL), (vi) E3-Ub-ligase like protein TRIM11, (vii) IGFBP3, and (viii) α-actinin 4. The neuroprotective effect of HN is principally mediated by cell surface receptor-mediated STAT3 activation. HN may also competitively antagonize Aβ binding to its receptor FPRL and inhibit Aβ neurotoxicity. It is also reported that HN binds and antagonizes intracellular proapoptotic Bcl-2 family proteins, such as Bax, Bid, and Bim.

signals to the PI3K/Akt, the MAPK, and the PKC pathway similarly to that of Trks. Multiple studies have suggested that the major prosurvival pathway in IGF-1-mediated-neuroprotection appears to be the PI3K/ Akt pathway *(87,88)*. IGF-1 has been reported to protect neurons from AD- and ALS-relevant neurotoxicity *(94-96)*. In agreement, serum IGF-1 levels inversely correlate with the risk of AD, ALS, and other NDDs *(1,97)*, as mentioned earlier.

Basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), glial-cell-line-derived neurotrophic factor (GDNF), and hepatocyte growth factor (HGF) are also considered to be neuroprotective factors whose receptors belong to the Trk receptor family. bFGF (or FGF-2) has been reported to be upregulated after nerve injury at the lesion site and has been reported to be involved in axonal regeneration *(98)*. Prosurvival signals of bFGF are transduced via its high-affinity Trk receptors, FGFRs 1–4. FGFR1 and 2 are constitutively expressed, whereas expression of FGFR3 is upregulated at nerve injury. bFGF exhibits a neuroprotective effect against Aβ neurotoxicity *(21,94)*, PD-relevant MPTP *(99)*, and poly-Q *(100)*. VEGF (or VEGF-A), originally identified as a tumor-secreted vascular permeability factor *(101,102)*, has at least six splicing variants in human, among which $VEGF\text{-}A_{165}$ is the most abundant and biologically active form. VEGF-A-mediated neuroprotection is exerted

mainly via a Trk receptor VEGFR2 (or Flk-1). VEGF-A protects against ALS-relevant insults *(103–105)* and PD-relevant insults *(106)*. Genetic variations in the VEGF gene modifies risks of ALS *(107)* and AD *(108)*. GDNF was originally purified as a survival factor for midbrain dopaminergic neurons whose loss is closely related to the onset of PD *(109,110)*. GDNF has a neuroprotective effect on spinal cord motor neurons *(111)* and central noradrenergic neurons *(112)*. Prosurvival signals by GDNF are mediated by a receptor complex consisting of RET Trk receptor and a ligand-binding subunit termed GDNF family receptor (GFR)α *(113,114)*. HGF was identified as a protein triggering motility, proliferation, and morphogenesis in various cell types *(115,116)*. HGF is generated by proteolytic cleavage of pro-HGF by proteases such as urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). It binds HGF receptor c-Met, a Trk receptor, and shows a neuroprotective effect on dopaminergic neurons *(117)*, motor neurons *(118)*, and sympathetic neurons *(119)*.

Neuroprotective Cytokines

Interleukin (IL)-6 family cytokines, including IL-6, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophin (CT)-1, belong to another group of neuroprotective factors *(120,121)*. These cytokines show limited sequence homology but share a common tertiary structure (i.e., four antiparallel α-helices). IL-6 family cytokines bind to their specific cell-surface type 1 cytokine receptors that consist of two or three subunits featured by an extracellular tryptophan-serine-X-tryptophan-serine (WSXWS) motif. A single-spanning transmembrane protein gp130 is a major signal-evoking subunit of the IL-6 receptor family (Fig. 3B; ref. *122*). Upon ligand binding, gp130 (or gp130 with LIF receptor) is tyrosine phospholyrated by Janus kinases (JAKs), which subsequently activate signal transducers and activators of transcription (STATs) to promote neuronal survival (the JAK/STAT pathway) (Fig. 3B). JAKs also activate the MAPK pathway and the PI3K/Akt pathway, which might be involved in neuroprotection. IL-6 *(123)*, CNTF *(124)*, LIF *(125)*, and CT-1 *(126)* have been reported to exhibit a neuroprotective effect against various NDD-related toxicities. Notably, mice deficient in LIF and CNTF ($LIF^{-/-}$, $CNTF^{-/-}$) show significant functional motor deficits, suggesting the relevance of these cytokines in physiological motoneuronal survival *(127)*.

Receptors of erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), and leptin are also members of the type 1 cytokine receptor family with the WSXWS motif. These cytokines have a neuroprotective effect as well. EPO is a 34-kDa glycoprotein produced in various tissues, including liver, kidney, and nervous system *(128)*. The *EPO* gene in the brain is upregulated by hypoxia under the control of hypoxia-inducible factor (HIF)-1 *(129)*. Binding of EPO to EPOR triggers prosurvival pathways, including the JAK/STAT, PI3K/Akt, and MAPK pathways, to suppress neurotoxicity *(130)*. CREB-mediated transcriptional upregulation of BDNF is also implicated in EPO neuroprotection *(131)*. G-CSF plays roles in the proliferation, survival, and differentiation of neutrophic progenitor cells *(132,133)*. Both G-CSF and its receptor (GCSF-R) are widely expressed in neurons. G-CSF has also exhibited a neuroprotective effect through activation of GCSF-R in a stroke model *(134)* and a PD model *(135)*. Leptin, another type 1 cytokine, is identified as the *ob* gene product and a blood-circulating satiety factor of adipocyte origin *(136)*. Leptin is expressed in white adipose tissue, placenta, stomach, pituitary, and hypothalamus *(137)*. Leptin protects neurons from *N*-methyl-D-aspartate (NMDA) toxicity by the leptin receptor (Ob-R)-mediated JAK2/STAT3 activation *(138)*. Additionally, leptin has been also reported to regulate Aβ production *(139)*.

Short Peptides With Neuroprotective Activity

Three structurally related short peptides—pituitary adenylyl cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide

(VIP), and peptide histidine-isoleucine (PHI)—are active in neuroprotection *(140–145)*. PACAP was originally isolated from sheep hypothalamic extracts as a 27- or 38-amino acid peptide with a potent adenylyl cyclase (AC)-stimulating effect on rat pituitary cells, *(146)*. VIP was isolated from porcine duodenum as a 28-amino acid peptide with vasodilatating activity *(147)*. PHI was isolated from porcine upper intestinal tissue as a peptide with 27 to 42 amino acid residues with structural similarity to VIP *(148)*. PACAP, VIP, and PHI are widely expressed in a similar distribution pattern. They are expressed in tissues, including the cerebral cortex, pituitary, adrenal gland, gastrointestinal tract, and reproductive system.

These peptides bind specific membrane receptors with seven-transmembrane domains, belonging to the G protein-coupled receptors (GPCRs) (Fig. 3C; ref. *140*). VIP/PACAP receptors are classified into two types: the PAC_1 type (eight variants) and the VPAC type ($VPAC_1$ and $VPAC_2$). PACAP binds PAC_1-type receptors, whereas both VIP and PACAP bind the $VPAC_1$ receptor. The intracellular signaling pathway downstream of VIP/PACAP receptors consists of AC/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA). The $VPAC_1$ receptor also activates a PLC-mediated PKC pathway.

PACAP and VIP protect neurons from multiple types of toxicities, such as ethanol, oxidative stress, ceramides, Aβ, Prion protein, electrical blockade by tetrodotoxin (TTX), and gp120 of the human immunodeficiency virus *(140)*. VIP-mediated neuroprotection is exerted both directly via VPAC receptors and indirectly by promotion of the release of glia-derived trophic molecules, such as IL-1, IL-6, chemokine RANTES, MIP, and a short peptide with the most potent neuroprotective activity. This short peptide is termed activity-dependent neurotrophic factor (ADNF) *(149)*.

ADNF-9 or SAL (SALLRSIPA) is an active core domain of ADNF that antagonizes various types of neurotoxicity, such as TTX, oxidative stress, NMDA, Aβ, and gp120 *(149–151)*. ADNF-9 exerts a neuroprotective effect at extremely low concentrations, such as hundred femtomolar concentrations, whereas it loses its neuroprotective effect at 1 n*M* or greater concentrations *(94,150)*. The reasons for the inefficiency of ADNF-9 at higher concentrations remain elusive. However, several explanations have been proposed: (a) downregulation of its receptor, (b) self-aggregation, or (c) additional activation of low-affinity receptors with an opposing effect. Notably, we found that ADNF-9 inhibited neuronal death induced by FALS-linked SOD1 mutants in a dose-dependent manner, without attenuation in neuroprotective activity, at high concentrations *(152)*. Although ADNF receptors have not been identified, the ADNF-mediated prosurvival signal has been reported to involve activation of (a) CREB *(153)*, (b) NFκB *(154)*, (c) CaMKIV *(152)*, (d) hsp60 against Aβ *(155)*, (e) transcriptional upregulation of IGF-1 *(156)*, and (f) poly-ADP-ribosylation *(157)*.

Through expression screening for proteins recognized by antiserum against ADNF, a gene-encoding, activity-dependent neurotrophic protein (ADNP) was identified *(158)*. Because ADNP contains zinc finger domains, a proline-rich region, a nuclear bipartite localization signal, and a homeobox domain, it has been assumed to be involved in gene expression. It also contains a glutaredoxin-active site and a leucine-rich nuclear export signal, alternatively suggesting that ADNP might act as a secretory factor. ADNP-deficient mice die as a result of neural tube closure defects *(159)*. An eight-amino acid sequence termed NAP (NAPVSIPQ) in ADNP shows homology with ADNF and is recognized by antiserum against ADNF. NAP exhibits ADNF-like neuroprotective activity against various insults *(158–162)*. NAP and its relative peptides (such as ADNF-9, D-SAL, and D-NAP) have been reported to bind with tubulin *(163,164)*. NAP suppressed zinc-mediated microtubule depolymerization in astrocytes by promoting microtubule assembly and reorganization.

ADNF and NAP ameliorate learning and memory performance in cholinotoxin (etylcholine aziridium)-treated mice *(165)* and

ApoE-deficient mice *(158)*. In these cases, treatment with NAP, but not ADNF, protected against loss of cholineacetyl transferase (ChAT) activity. Both ADNF and NAP also exhibit neuroprotection in fetal alcohol syndrome *(166)*. NAP has been reported to exert neuroprotection in a closed head injury model *(167)* and in a stroke model *(168)*. ADNF, NAP, and their derivatives can be delivered into the CNS by intranasal administration, oral administration, or systemic administration *(166–168)*.

Humanin As an Alzheimer's Disease-Relevant Neuronal Death-Suppressing Factor

HN, a 24-amino acid peptide, has been identified as an endogenous factor that antagonizes V642I-APP-induced neuronal death. Complementary DNA (cDNAs) encoding HN were cloned from a cDNA library constructed with an occipital lobe of a patient with AD through a functional expression screening *(169–171)*. The primary sequence of the N-terminal portion of HN resembles a signal peptide, and consistently, HN is secreted from cells *(169)*. Immunoblot analysis demonstrated that HN or an HN-like peptide was expressed in colon and testis in young mice *(172)*. Reactive glial cells and neurons in the remaining cortex of an AD brain were also immunostained with anti-HN polyclonal antibody, whereas glial cells and neurons in the cortex of an age-matched non-AD brain were not *(172)*. HN or its relatives were also immunologically detected in the Leydig cells of the rat testis *(173)*, in the human glial cell line *(174)*, and in human skeletal muscles affected with mitochondrial encephalopathy with lactic acidosis and stroke-like episodes *(175)*.

HN suppresses neurotoxicity by all the AD-relevant insults tested, such as Aβ and mutants of APP, PS1, and PS2. Additionally, HN antagonizes some non-AD-related neurotoxicity: HN has been reported to suppress death caused by (a) serum deprivation in neuronal *(176)* and non-neuronal cells *(177,178)*, (b) PrP(118-135) in cortical neurons *(179)*, and (c) DRPLA protein with expanded poly-Q in PC12 cells *(180)*. Conversely, HN does not antagonize other non-AD-related insults, such as etoposide, poly-Q (Q79), and glutamate *(94,169,181)*. Notably, a rat homolog of HN, termed Rattin (RN), elicits neuroprotective action against glutamate toxicity as well as the HN-like anti-AD activity *(182)*. RN, a 38-amino acid peptide, is composed of the N-terminal HN-homologous domain and an additional 14-amino acid domain in the C-terminus that may exert neuroprotective activity against glutamate toxicity.

Eight HN interactors (or receptors) have been identified (Fig. 3D). HN appears to bind to a cell surface receptor and to trigger the HN-mediated prosurvival signal by activation of STAT3 *(169,183)*. A JAK/STAT pathway, the putative major prosurvival pathway triggered by HN, antagonizes JNK-mediated death signals triggered by AD-relevant insults *(184)*. It has been demonstrated that HN also binds to G protein-coupled formylpeptide receptor-like-1 (FPRL-1) and FPRL-2 *(185,186)*, which is believed to behave as a functional receptor of Aβ. It has been suggested that HN inhibits Aβ neurotoxicity in PC12 cells by competitively binding to FPRL-1 *(185)*. Three Bcl-2 family pro-apoptotic proteins have been also identified as intracellular HN receptors. HN interacts with Bax and suppresses apoptosis by preventing translocation of Bax from cytosol to mitochondria *(174)*. HN also binds with the BH3-only Bcl-2 family proteins Bid and Bim (BimEL) and inhibits Bid/Bim-induced release of Smac and cytochrome *c* from mitochondria *(187,188)*. Two HN interactors that may regulate HN activity have been identified. An E3-Ub-ligase-like protein, a tripartite protein TRIM11, binds to and destabilizes HN *(189)*. IGFBP3, a serum carrier protein of IGF-1, was co-immunoprecipitated with HN from mouse testes. The interaction between these proteins causes inhibition of IGFBP-3-induced apoptosis in human glioblastoma cells, whereas it enhances HN activity against Aβ *(190)*. Podocyte α-actinin 4, an essential component of the glomerular filtration barrier, also interacts with HN through spectrin-like repeats *(191)*.

In vivo HN activity has been demonstrated with animal AD models. Intracerebroventricular injection of an HN derivative, HNG (S14G-HN), suppresses scopolamine-induced spatial working memory impairment in mice *(192)*. HNG and des-Leu-PAGA, another HN derivative, reverse memory impairment caused by 3-quinuclidinyl benzilate (3-QNB) *(193)*. Based on the fact that administration of HN derivatives less than 30 min before treatment with scopolamine and 3-QNB reverses spatial memory impairment, it is suggested that HN has short-term trophic activity on synaptic function of cholinergic neurons in vivo in addition to long-term activation of the Jak2/STAT3 prosurvival pathway. Intracerebroventricular injection of HNG also suppresses spatial working memory impairment induced by bolus intracerebroventricular. injection of Aβ25-35 *(194)*. Combined with the fact that HN expression is upregulated in AD brains *(172)*, HN may be an endogenous defense factor suppressing the onset of AD.

Development of Colivelin As a New-Generation Neuronal-Death-Suppressing Peptide

To clinically use neuroprotective peptides as therapeutic agents, it is essential to enhance their neuroprotective effects in vitro and in vivo because their innate activating mechanisms may be impaired or their innate inactivating mechanisms may be upregulated in NDDs, causing inefficacy in their clinical usage. Therefore, several strategies may be employed: (a) increase of their specific neuroprotective activities (structural potentiation); (b) identification of small chemical compounds that mimic their activities by binding to the receptors directly; and (c) combinatorial administration with other neuroprotective drugs that have different action mechanisms (combination therapy).

There are two procedures to accomplish the first strategies: identifying more active mutants by changing their primary amino acid sequences and introducing chemical modification of their composite amino acids that trigger their activities. We have demonstrated the relevance of strategies of structural potentiation and combination therapy by successful development of an improved (second-generation) neuroprotective factor known as Colivelin, which consists of a potent HN derivative fused to ADNF-9 *(195)*.

Structural Potentiation of HN Activity

Through characterization of the structure-activity relationship of HN, we found its unique structural features (Table 2): (a) Gly substitution of Ser14 (HNG) or isomerization of Ser14 (D-Ser14-HN) potentiates the neuroprotective activity of HN 1000-fold *(169,196)*; (b) Ala substitution of both Arg4 and Phe6 (AGA-HNG) that disrupts sites for trypsin and chymotrypsin digestion augments the potency of HNG by 10 to 100 times *(196)*; (c) the active core domain of HN that mediates the neuroprotective activity is the 17-amino acid sequence from Pro3 to Pro19 (HN17) *(94)*; (d) Ser7 is necessary in self-dimerization *(196)*; (e) the modulation of the free thiol group in Cys8 negatively affects the neuroprotective activity of HN and Arg substitution of Cys8 (C8R) potentiates HN activity *(94)*. In agreement, structural analysis of HN by nuclear magnetic resonance suggested that HN has two bends at Ser7 and Ser14, which appears to be functionally important in dimerization and potency, respectively *(197)*. Based on these data, we assume that AGA-(C8R)HNG17 may exhibit potent neuroprotective activity. Realistically, AGA-(C8R)HNG17 exhibits neuroprotection against Aβ neurotoxicity at 10 p*M*, suggesting that AGA-(C8R)HNG17 is 10^6-fold as potent as authentic HN *(195)*.

Colivelin, A Hybrid Peptide Composed of Activity-Dependent Neurotrophic Factor and a Potent HN Derivative

HN-mediated neuroprotection appears to be highly specific to AD-relevant neurotoxicity. Effective concentrations of HNG and AGA-(C8R)HNG17 are 10 n*M* (or more) and 10 p*M* (or more) in vitro, respectively *(94,196)*. On the

Table 2
Structure/Activity Relationships of HN Derivatives

HN derivatives	Structure	Full effect (vs Aβ)
Humanin (HN)	**MAPRGFSCLLLLTSEIDLPVKRRA**	10 μ*M*
C8A-HN	MAPRGFS**A**LLLLTSEIDLPVKRRA	N.E.
S7A-HN	MAPRGF**A**CLLLLTSEIDLPVKRRA	N.E.
S14G-HN (HNG)	MAPRGFSCLLLLT**G**EIDLPVKRRA	10 n*M*
D-Ser7-HN	MAPRGF**(D-S)**CLLLLTSEIDLPVKRRA	10 μ*M*
D-Ser14-HN	MAPRGFSCLLLLT**(D-S)**EIDLPVKRRA	10 n*M*
D-Ser7/14-HN	MAPRGF**(D-S)**CLLLLT**(D-S)**EIDLPVKRRA	10 n*M*
HN17	PRGFSALLLLTSEIDLP	10 μ*M*
HNG17	PRGFSCLLLLT**G**EIDLP	10 n*M*
EF-HN	**EFLIVIKS**MAPRGFSCLLLLTSEIDLPVKRRA	100 n*M*
EF-HNA	**EFLIVIKS**MAPRGFS**A**LLLLTSEIDLPVKRRA	N.E.
EF-HNG	**EFLIVIKS**MAPRGFSCLLLLT**G**EIDLPVKRRA	1 n*M*
EFLIVIKS	**EFLIVIKS**	N.E.
AGA-HNG	MAP**A**G**A**SCLLLLT**G**EIDLPVKRRA	100-300 p*M*
AGA-(D-Ser14)HN	MAP**A**G**A**SCLLLLT**(D-S)**EIDLPVKRRA	100-300 p*M*
AGA-(D-Ser14)HN17	P**A**G**A**SCLLLLT**(D-S)**EIDLP	100 p*M*
AGA-(C8R)HNG17	P**A**G**A**S**R**LLLLT**G**EIDLP	10 p*M*
Colivelin	**SALLRSIPA**P**A**G**A**S**R**LLLLT**G**EIDLP	100 f*M*

other hand, ADNF-9 has more wide-spectrum neuroprotective activity against Aβ, TTX, oxidative stress, NMDA, gp120, and ALS-relevant SOD1 mutants *(149,150,152)*. Effective concentrations of ADNF-9 are about 100 f*M* to 1 n*M*. Unfortunately, for unknown reasons, it loses its neuroprotective activities at concentrations of more than 1 n*M*. Similarly, ADNF-9 shows neuroprotective activity against SOD1 mutants at concentrations of 100 f*M* and more. Notably, however, it never loses its activities at higher concentrations. A key molecule in the intracellular prosurvival signaling pathway of HN has turned out to be STAT3, whereas that of ADNF-9 is CaMKIV. Based on the difference in neuroprotective mechanisms between HN and ADNF-9, we hypothesized that HN-mediated neuroprotection is supplemented by that mediated by ADNF-9 and vice versa.

Dimer formation is one of the most essential processes in HN-mediated neuroprotection because (a) a dimerization-deficient HN derivative completely loses protective activity *(196, 198)*; (b) dimerizable HN derivatives without neuroprotective activity antagonize HN-mediated neuroprotection *(199)*; and (c) fusion of a dimerization tag sequence (EFLIVIKS) to the N-terminus of HN derivatives potentiates neuroprotective activity *(196)*. ADNF-9 is a highly lipophilic 9-amino acid peptide, which is likely to oligomerize as a dimerization tag. Therefore, it is assumed that attaching ADNF-9 to the N-terminus of AGA-(C8R)HNG17 may further potentiate its rescue activity. The resulting fusion peptide was named Colivelin *(195)*. Colivelin exhibits a neuroprotective effect on various types of insults, including glutamate toxicity, possibly through its ADNF portion (an

additive neuroprotective effect). It has more potent HN-like neuroprotective activity than that of AGA-(C8R)HNG17, presumably resulting from enhanced dimerizing ability (a synergic neuroprotective effect).

Effect of Colivelin on Alzheimer's Disease-Relevant Insults

Colivelin completely suppresses Aβ neurotoxicity in vitro at a concentration of 100 f*M*, which is 100 times as potent as AGA-(C8R)HNG17 *(195)*. Colivelin also completely antagonizes neuronal death induced by overexpression of V642I-APP or M146L-PS1 at a concentration of 100 f*M*, whereas AGA-(C8R) HNG17 does so at 10 p*M*. Considering that ADNF does not exhibit a protective effect on neurotoxicity caused by ectopic overexpression of M146L-PS1 *(94)*, it is assumed that potentiated neuroprotective activity of Colivelin against M146L-PS1-mediated neurotoxicity may result from N-terminally attached ADNF-enhanced dimerization of AGA-(C8R)HNG17. Notably, Colivelin also antagonizes neuronal death caused by glutamate-induced excitotoxicity in primary cortical neurons, on which HN does not exert any neuroprotective effect, suggesting that Colivelin also has a neuroprotective effect caused by the ADNF portion in addition to the HN derivative portion. Therefore, Colivelin-mediated neuroprotection is mediated by two distinct prosurvival pathways: the ADNF/CaMKIV pathway and the HN/STAT3 pathway, both of which are simultaneously and independently activated by Colivelin (Fig. 4).

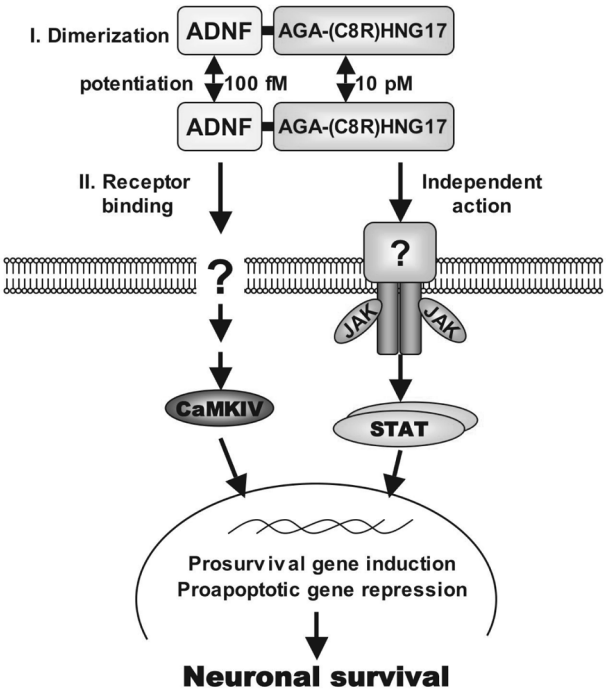


Fig. 4. Putative mechanisms of Colivelin neuroprotection. Colivelin-mediated neuroprotection involves two steps—that is, (i) dimerization (196) and (ii) binding to receptors. Colivelin exerts both anti-ALS and anti-AD neuroprotective activity by binding to both the putative ADNF receptor and HN receptor independently, which activates CaMKIV- and STAT3-mediated prosurvival pathways *(195)*. N-terminal ADNF-9 may have a greater dimerizing ability (dimerization of ADNF may take place at concentrations of less than about 100 f*M*) than that of C-terminal AGA-(C8R)HNG17 (about 10 p*M*). Consequently, it is likely that attachment of ADNF-9 potentiates HN-like neuroprotective activity of Colivelin by enhancing dimerization *(195)*.

In vivo experiments have further established the therapeutic effect of Colivelin *(195)*. We have developed a novel AD model mouse, a repetitive-injected Aβ model *(200,201)*. In this model, we intracerebroventricularly injected low-dose Aβ25-35 or Aβ1-42 every other day for 3 wk to recapitulate subacute Aβ neurotoxicity in vivo. Intracerebroventricular injection of Colivelin every 6 d at the total dose of 40 pmol/mouse almost completely inhibits spatial working memory impairment in a Y-maze (YM); this is caused by repetitive injection of Aβ25-35 at a total dose of 10 nmol/mouse. Immunohistochemical analysis further revealed that Colivelin treatment inhibited deteriorated expression of ChAT because of Aβ in the cholinergic neurons of the medial septum. To examine the death-suppressing effect of Colivelin in vivo, we utilized a hippocampal injection Aβ model in which 300 pmol Aβ1-42 was directly injected into the unilateral CA1 region of hippocampus. Aβ injection-induced neuronal loss in the

hippocampus was almost completely antagonized by intracerebroventricular injection of 100 pmol of Colivelin into the contralateral cerebral ventricle, performed 24 h before injection of Aβ. Because Aβ-mediated hippocampal neuronal loss is mediated by JNK activation (unpublished observation by T.C. and M.M.), Colivelin-mediated neuroprotection in vivo might involve the STAT3-linked prosurvival pathway, which antagonizes JNK activation. We further examined the neuroprotective effect of intraperitoneal injection of Colivelin on working memory impairment caused by muscarinic acetylcholine receptor antagonist 3-QNB. Intraperitoneal injection of Colivelin significantly reversed spatial working memory impairment in YM induced by 3-QNB, suggesting that Colivelin can pass through the BBB.

Effect of Colivelin on Amyotrophic Lateral Sclerosis-Relevant Insults

We reported that ADNF-9 protected neurons from toxicity caused by FALS-linked SOD1 mutants in vitro *(152)*. However, in vivo experiments using a G93A-SOD1 Tg mouse (a universally established model of ALS) revealed that intracerebroventricular injection of ANDF-9 was not potent enough to prolong survival, although it significantly improved motor function of the model. Poor delivery of ADNF-9 to motor neurons (e.g., rapid degradation or inactivation in vivo similarly to other small peptides) has been assumed to be the main reason for the inability of ADNF-9 to prolong survival. To test this hypothesis, we examined the neuroprotective effect of Colivelin on ALS mice, because Colivelin is longer than ADNF-9, which may result in enhanced in vivo stability. Therefore, we found that Colivelin dose-dependently prolonged survival of ALS mice as well as functional improvement of the motor system *(202)*. Histological analysis in the midphase of the disease progression confirmed increased motoneuronal survival in the ventral horn of the spinal cords of ALS mice. Although therapeutic mechanisms of Colivelin on ALS-relevant insults in vivo remain uncharacterized, it is highly likely that the ADNF motif in Colivelin is more stable than ADNF-9. Additionally, the HN moiety of Colivelin may show a neuroprotective effect on ALS mice, as supported by the earlier studies showing that STAT3-activating neuroprotective factors such as CNTF and LIF play important roles in motoneuronal survival in the embryonic spinal cords *(127,203)*.

Discussion and Future Perspectives

Neuronal death-inducing mechanisms in NDDs are too complicated to be suppressed easily because multiple PCD pathways are simultaneously activated by various initial insults. Accordingly, it is notable that neuroprotective factors somehow suppress NDD-related neuronal death in vitro and in vivo, indicating that death-suppressing mechanisms of neuroprotective factors also appear to involve multiple pathways, including one that attenuates initial insults and another that antagonizes the death-executive process. Such neuroprotective reactions appear to be actually mediated by intracellular prosurvival signaling pathways, such as the PI3K/Akt pathways, the MAPK pathways, and the JAK/STAT pathways.

Several lines of evidence have suggested that dysregulation in endogenous neuroprotective factors results in higher risk of NDDs *(1,85,96,97,107,108)*. It should be emphasized that even patients suffering from hereditary forms of NDDs are healthy in their youth for several decades, although disease-causative mutations already exist prenatally. The latter may be explained by the very slow progressive nature of neurotoxic events in NDDs. However, taking the former finding into account, we think it likely that there are endogenous protective mechanisms that can suppress NDD-related neuronal death. If this notion is true, then we could further hypothesize that an age-related decrease in endogenous neuroprotective activity may be involved in the onset and progression of NDDs ("defense hypothesis") and that neuroprotective factors may be good drug candidates for NDDs.

Clinical trials using neuroprotective factors have been performed in the treatment of NDDs. A pilot study involving 3-mo continuous intracerebroventricular treatment of NGF for three patients with AD was performed in Sweden *(204)*. Although [^{11}C]nicotine binding, as measured by positron emission tomography (PET), showed slight cognitive improvement, side effects (including pain and weight loss) outweighed the positive effect. Recently, a phase I clinical trial of NGF gene therapy for AD was performed *(205)*. Patients received the implantation of autologous fibroblasts, introduced with the NGF gene ex vivo into the forebrain, and cognitive improvement was observed without side effects. A double-blind trial of intracerebrovascular treatment of GDNF in PD was also performed *(206)*. The study failed to prove the clinical efficacy of GDNF. It also indicated severe side effects, such as nausea, anorexia, vomiting, and paresthesia. However, a phase I study on intraputamenal injection of GDNF showed some potential efficacy without obvious side effects *(207,208)*. Unfortunately, a subsequent phase II study failed to prove the clinical efficacy of GDNF *(209)*. In ALS, clinical trials of IGF-1, BDNF, and CNTF were performed. A phase III trial on subcutaneous treatment of CNTF and BDNF resulted in no efficacy with side effects such as anorexia and pain *(210,211)*. However, subcutaneous administration of recombinant human IGF-1 might slightly slow disease progression *(212–214)*.

Therefore, all clinical trials on neuroprotective factors indicated the difficulties in clinical application of neuroprotective factors. Insufficient drug delivery to the CNS may be a major reason for the inefficacy of neuroprotective factors on NDDs. The insufficiency is presumably caused by BBB-mediated obstruction of CNS transportation in systemic treatment, inactivation by serum binding proteins or antibodies, and degradation by proteases. Severe side effects also prevent the administration of a maximal dose of neuroprotective factors. To overcome these difficulties, drug-delivery systems (such as intrathecal or local administration) and some gene therapeutic approaches are being intensively investigated *(95,104,205)*.

This article proposes another way to realize neuroprotective therapies for NDDs—that is, development of Colivelin, a novel neuronal death-suppressing peptide *(152,195)*. To potentiate neuroprotective activity, we used two strategies: structural potentiation and combination treatment with other neuroprotective factors. Regarding the former strategy, we characterized the structure-activity relationship of HN in detail, which revealed the active core domain, putative protease-accessible sites, a potency-determining amino acid (Ser14), and an amino acid essential for dimerization (Ser7). Because many neuroprotective factors need to dimerize before binding their receptors, enhancement in the dimerizing ability potentiates the neuroprotective activity. As for the latter strategy, we chose ADNF-9 as a partner of HN because it is also a short peptide and activates CaMKIV to exert neuroprotective activity. Moreover, we came up with the idea that N-terminal attachment of ADNF-9 to an HN derivative would further potentiate HN-mediated neuroprotective activity by enhancing HN dimerization. The hybrid peptide, Colivelin, is a femtomolar acting neuroprotective peptide and passes through the BBB. Because neuroprotection by Colivelin is mediated by CaMKIV and STAT3, a combined treatment of Colivelin with other neuroprotective factors activating the MAPK pathway, the PI3K/Akt pathway, or the PKA pathway (a so-called "neuroprotection cocktail") may give rise to further, more potent neuroprotective activity against NDDs.

In clinical trials, CNTF showed side effects such as anorexia and pain, possibly caused by activating the CNTF receptor/STAT3-mediated signaling pathway *(210,211)*. Similarly, Colivelin might exhibit side effects by activating the HN receptor/STAT3 signaling pathway (receptor-mediated side effects). On the other hand, because Colivelin protects neurons from various neurotoxicity at extremely low concentrations (as low as 100 f*M*), Colivelin may antagonize neuronal death in NDDs without exhibiting major non-receptor-mediated side

effects because non-receptor-mediated side effects, to some extent, result from overload of the neuroprotective factors. CNS delivery by intrathecal and intranasal administration can further minimize systemic side effects. More reasonably, we have developed various HN derivatives that bind to HN and form an inactive heterodimer (HN antagonists) *(199)*. We are developing a series of HN antagonists that cannot pass through the BBB. Systemic co-administration of such HN antagonists reduces systemic HN receptor-mediated side effects caused by HN derivatives without lessening their CNS effects. Therefore, CNS delivery of Colivelin with systemic co-administration of HN antagonists may be an ideal powerful treatment with minimum systemic side effects.

In conclusion, neuroprotective factors are good candidate drugs for treatment of NDDs. Progress in neuroprotective therapies may be achieved by structural potentiation and combination strategies of neuroprotective factors, as exemplified by the successful development of Colivelin.

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