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(54) Title: HUMANIN PROTECTION OF DOPAMINERGIC NEURONS

(57) Abstract: Methods are provided for treating Parkinson's disease, protecting dopaminergic neurons from neurotoxicity and for increasing DJ-1 levels in neurons. Methods are also provided for identifying candidate treatments for Parkinson's disease.
HUMANIN PROTECTION OF DOPAMINERGIC NEURONS

This application claims benefit of U.S. Provisional No. 61/554,660 filed November 2, 2011, the contents of which are hereby incorporated by reference.

[0001] This invention was made with government support under grant numbers R-01 AG035114-02; K-08 AG 027462; and 3K08 AG027462-03S1, awarded by the National Institutes of Health. The government has certain rights in the invention.

[0002] Throughout this application various publications are referred to by number in parentheses. The disclosures of these publications are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

BACKGROUND OF THE INVENTION

[0003] Parkinson's disease (PD) is a significant health concern, and is second only to Alzheimer's disease (AD) in prevalence of neurodegenerative diseases. More than a million Americans suffer from Parkinson's disease, double the estimated number of cases twenty years ago (1). Aging, genetic predisposition and diabetes increase the risk for PD (2-5). Unfortunately, available therapies have limited benefits. Conventional pharmacological dopamine replacement strategies are only palliative and they may exacerbate neuronal loss (6). In addition, over time many patients become insensitive to medication or develop debilitating dyskinesias. Mechanical lesions or electrical override of pathways in the basal ganglia offer clinical improvement, but are not viable strategies for the majority of patients. Cyclooxygenase inhibitors may attenuate the inflammation associated with neurodegeneration (7), but they also appear to increase the risk of ischemic stroke and cardiovascular complications (8) and may worsen memory deficits (9). Experimental treatments range from neural transplantation to gene therapy, but unfortunately, none have offered significant and safe clinical promise. The lack of any neuroprotective treatment at the present time underscores a critical need for research leading to new therapeutic targets to increase neuronal survival in PD.

[0004] Effective therapies for treating Parkinson's disease and for mitigating dopaminergic neuron injury are still sought, and the current invention provides such treatments.
SUMMARY OF THE INVENTION

[0005] A method is provided of treating Parkinson’s disease or of protecting dopaminergic neurons from neurotoxicity in a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to treat Parkinson’s disease or protect dopaminergic neurons.

[0006] Also provided is a method of increasing DJ-1 expression in a neuron of subject or of activating STAT-3 in a neuron of a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to increase DJ-1 expression in a neuron or activate STAT-3 in a neuron.

[0007] A humanin analog is also provided for treating Parkinson’s disease or for protecting dopaminergic neurons from neurotoxicity in a subject.

[0008] Also provided is a method of identifying a candidate treatment for Parkinson’s disease, the method comprising a) modeling in silico the 3-dimensional form of the humanin analog comprising SEQ ID NO:2, b) testing in silico if a compound from a library of small molecule compounds mimics the modeled 3-dimensional form, and c) determining in vitro if the small molecule identified in b) is chemically stable, thereby identifying the candidate treatment.

[0009] Additional objects of the invention will be apparent from the description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1: Rattin and its receptor, CNTFR-α are present in rat midbrain and dopaminergic MN9D cells. Western blot of Fischer/Brown Norway rat midbrain homogenates from 4 to 24 months and MN9D-cell lysates with anti-RN antibody. Note that the intensity of the rattin signal appears to decrease in the 24-month animal.

[0011] Figure 2A-2D: HNG, a potent analog of humanin, protects dopaminergic MN9D cells from neurotoxicity. Replicate cultures were treated in the following manner: A) Hydrogen peroxide: 1h pre-treatment with HNG before and during overnight exposure to H$_2$O$_2$; B) serum deprivation: 1h pre-treatment with HNG before and during overnight incubation in serum-free medium; C) Rotenone: 1h HNG pre-treatment before and during overnight incubation with 250nM rotenone; D) Rotenone: 24h HNG pre-treatment before and during overnight incubation with 250 nM rotenone. Results demonstrate that HNG
increased neuronal survival for all three types of toxicity. For rotenone, longer (24-h) pre-treatment of cultures with HNG was required for neuroprotection.

[0012] Figure 3A-3B: DJ-1 expression is induced by oxidative stress and potentiated by HNG. Expression of DJ-1 was assessed by Western blot (A) and RT-PCR (B) at 4h after treatment with H₂O₂. HNG treatment significantly increased protein levels. RNA levels decreased with H₂O₂ at 4h, which could result in a subsequent decline in DJ-1 protein at a later time. This decrease was attenuated by HNG (p≤0.05).

[0013] Figure 4: HNG potentiates AMPK activation: Incubation for 4h with 1nM HNG increased AMPK activation over H₂O₂ alone.

[0014] Figure 5: Humanin activates STAT-3 in primary hypothalamic neurons. Significant induction (increase in p-STAT-3/total STAT-3), was apparent at 2 minutes, peaking at 5 min to 1 h, and remained elevated at 4h.

[0015] Figure 6: DJ-1 protein levels remain constant over the lifespan of FBN rats. Representative lanes of a western blot depicting DJ-1 signals. Bars represent the mean ± SEM for groups of 4.

[0016] Figure 7: HNG potentiates AMPK activation: Incubation for 4h with 1nM HNG increased AMPK activation over H₂O₂ alone.

DETAILED DESCRIPTION OF THE INVENTION

[0017] As used herein, “treating” Parkinson’s disease means ameliorating an extant Parkinson’s disease, or reducing or preventing progression of Parkinson’s disease. Treatment is effected when one or more symptoms of the Parkinson’s disease are ameliorated, stabilized, reduced or reversed.

[0018] As used herein, “Parkinson’s disease” is a neurodegenerative medical condition well-known in the art in which subjects usually display tremor, rigidity, slowness of movement (bradykinesia), and postural instability. Neuropsychiatric symptoms, such as disturbances of speech, cognition, mood, behaviour and thought may also occur. Parkinson’s disease (PD) is usually idiopathic, though some subjects show an apparent genetic component. The main pathological characteristic of PD is cell death in the substantia nigra and, more specifically, in the ventral part of the pars compacta. Pro-dromal symptoms associated with Parkinsons’ disease are known, and the treatment methods described herein can be applied to a subject exhibiting a plurality of pro-dromal symptoms.
associated with Parkinson's disease, for example a combination of more than one of constipation, loss of smell, executive dysfunction, fluctuating cognition, sleep disturbances, and visuospatial dysfunction. Specifically, with regard to prodromal symptoms associated with Parkinson's disease, treating the disease means either or both of delaying onset of Parkinson's disease and slowing progression of Parkinson's disease.

[0019] As used herein, humanin (HN) is a peptide having the sequence: MAPRGFSCLLLLTSEIDLPVKRA (SEQ ID NO:1). An active humanin analog is a peptide or peptidomimetic based on humanin and having equivalent or improved activity thereof with regard to DJ-1 and/or STAT-3 as described hereinbelow. Such activities are readily determined, for example using the assays described in the experimental section hereinbelow. As used herein, unless otherwise indicated, “humanin analog” and “active humanin analog” are used interchangeably. In a preferred embodiment, the analog is a peptide. In an embodiment, the humanin or humanin active analog is 17-50 amino acids in length. In a preferred embodiment the humanin or humanin active analog is 20-25 amino acids in length. In a particularly preferred embodiment the humanin or active humanin analog is 24 amino acids in length. Analogs of humanin are known in the art (e.g. see WO/2008/153788 A2). In a preferred embodiment, the humanin analog is HNG, which has the sequence MAPRGFSCLLLLTGEIDLPVKRA (SEQ ID NO:2). Analogs of humanin can also be created by substitution of conservative amino acids into humanin.

[0020] Other humanin analogs, including those containing D-amino acids, which can be used in the methods described include:

C8A-HN (HNA) MAPRGFSALLLLLTSEIDLPVKRA
D-Ser14-HN - MAPRGFSCLLLLT*EIDLPVKRA
AGA-HNG - MAPAGASCLLLLTGEIDLPVKRA
AGA-(D-Ser14) - MAPAGASCLLLLT*EIDLPVKRA
HN AGA-(D-Ser14) - PAGASCLLLLT*EIDLP
HN17 AGA-(C8R) - PAGASRLLLLTGEIDLP
HNG17 EF-HN - EFLIVIKSMAPRGFSCLLLLTSEIDLPVKRA
EF-HNA - EFLIVIKSMAPRGFSALLLLLTSEIDLPVKRA
EF-HNG - EFLIVIKSMAPRGFSCLLLLTGEIDLPVKRA
EF-AGA-HNG - EFLIVIKSMAPAGASCLLLLTGEIDLPVKRA
Colivelin - SALLRSPIPA-PAGASRLLLLTGEIDLP
P3R-HN - MARRGFSCLLLSTTATDLPKRRT

As used herein, “DJ-1” is the protein known in the art as product of the DJ-1 gene. In an embodiment, the DJ-1 has the sequence set forth in NCBI Reference Sequence NP_009193.2 (human DJ-1).

A method is provided of treating Parkinson’s disease or of protecting dopaminergic neurons from neurotoxicity in a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to treat Parkinson’s disease or protect dopaminergic neurons. In an embodiment, the neurotoxicity results from oxidative stress. In an embodiment, the neurotoxicity results from metabolic stress.

Also provided is a method of increasing DJ-1 expression in a neuron of subject or of activating STAT-3 in a neuron of a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to increase DJ-1 expression in a neuron or activate STAT-3 in a neuron. As used herein, “STAT-3” is Signal transducer and activator of transcription 3 also known as STAT3, and is a transcription factor which in humans is encoded by the STAT-3 gene. In an embodiment, the STAT-3 is encoded by the sequence set forth in GenBank: DQ039079.1. Without being bound by theory, it is advantageous to increase DJ-1 expression because DJ-1 is a known mediator of neuronal survival through its role in receptor-mediated signal transduction. In addition, STAT3 signaling is known to promote protection against oxidative stress under various pathophysiological conditions in other systems.

In an embodiment of the methods, the humanin or active humanin analog is administered in an amount and manner effective to treat Parkinson’s disease or protect dopaminergic neurons or to treat a subject exhibiting a plurality of pro-dromal symptoms associated with Parkinson’s disease. In an embodiment of the methods, the humanin or active humanin analog is administered in an amount and manner effective to increase DJ-1
expression in a neuron or activate STAT-3 in a neuron. In an embodiment, the neuron is a dopaminergic neuron. In an embodiment, the neuron is a substantia nigra neuron. In an embodiment, the neuron is a striatal neuron.

[0026] In an embodiment of the methods, the humanin or active humanin analog is administered in an amount and manner effective to enter the midbrain of the subject. In an embodiment of the methods, the humanin or humanin analog is administered parenterally. In an embodiment of the methods, the humanin or humanin analog is administered into the central nervous system of the subject. In an embodiment of the methods, the humanin or humanin analog is administered into the cerebrospinal fluid of the subject. In an embodiment of the methods, the humanin or humanin analog is administered via an implant in the central nervous system of the subject. In a further embodiment of the methods, the implant comprises a polymer matrix. In an embodiment of the methods, the humanin or humanin analog is administered intraventricularly or intrathecally. In an embodiment of the methods, the humanin or humanin analog is administered intranasally. In an embodiment of the methods, the humanin or humanin analog is administered in a manner effective to enter the substantia nigra of the subject and/or the striatum of the subject. In an embodiment of the methods, the humanin or humanin analog is administered directly into the substantia nigra of the subject and/or the striatum of the subject. In an embodiment, the humanin or humanin analog is administered directly into the pars compacta of the subject. Such direct administration can be effected by any method known in the art, including cannulation, catheterization, injection and via an implant in the vicinity or, or within, the substantia nigra and/or striatum.

[0027] In an embodiment, the humanin or humanin analog is administered by gene therapy, e.g. using a suitable vector. For a choice of vector and delivery conditions see Gene Therapy of the Central Nervous System: From Bench to Bedside (Kaplitt et al.), Academic Press, (2005), the contents of which are hereby incorporated by reference. For example, a lentiviral, adenoviral, or adeno-associated viral vector comprising a nucleic acid encoding the humanin or humanin analog can be administered to the subject. Incorporation of a site-specific promoter into the vector, such as a mammalian CNS-specific promoter, or a substantia-nigra and/or striatum specific promoter is a preferred embodiment. In an embodiment, the vector encodes the humanin analog comprising SEQ ID NO:2.

[0028] In an embodiment of the methods, it is the active humanin analog which is administered, and the humanin analog comprises SEQ ID NO:2.
[0029] In an embodiment of the methods, the subject has been identified as suffering from Parkinson’s disease. In an embodiment of the methods, the subject has been identified as exhibiting a plurality of pro-dromal symptoms associated with Parkinson’s disease.

[0030] In embodiments, the methods further comprise identifying the subject as suffering from Parkinson’s disease prior to administering the humanin or humanin analog. In embodiments, the methods further comprise identifying the subject as as exhibiting a plurality of pro-dromal symptoms associated with Parkinson’s disease.

[0031] In an embodiment the patient does not have Alzheimer’s disease. In an embodiment the patient is diagnosed as not having Alzheimer’s disease.

[0032] A humanin analog is also provided for treating Parkinson’s disease or for protecting dopaminergic neurons from neurotoxicity in a subject. In an embodiment, the humanin analog comprises SEQ ID NO:2. In an embodiment, the neurotoxicity results from oxidative stress. In an embodiment, the neurotoxicity results from metabolic stress.

[0033] The humanin and active humanin analogs described herein can be administered to the subject in a pharmaceutical composition comprising a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier used can depend on the route of administration. As used herein, a “pharmaceutically acceptable carrier” is a pharmaceutically acceptable solvent, a suspending vehicle, for delivering the instant agents to the animal or human subject. The carrier may be liquid or solid and is selected with the planned manner of administration in mind. Liposomes are also a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known in the art, and include, but are not limited to, additive solution-3 (AS-3), saline, phosphate buffered saline, Ringer’s solution, lactated Ringer’s solution, Locke-Ringer’s solution, Krebs Ringer’s solution, Hartmann’s balanced saline solution, and heparinized sodium citrate acid dextrose solution. In an embodiment the pharmaceutical carrier is acceptable for administration into the central nervous system of a mammal.

[0034] The inhibitors, active fragments, active analogs of fragments, and agents can be administered together or independently in admixtures with suitable pharmaceutical diluents, extenders, excipients, or carriers (collectively referred to herein as a pharmaceutically acceptable carrier) suitably selected with respect to the intended form of administration and as consistent with conventional pharmaceutical practices.

[0035] Techniques and compositions for making dosage forms useful in the invention are described in the following references: Modern Pharmaceutics, Chapters 9 and 10
Dosing can be any method or regime known in the art. For example, daily, twice daily, weekly, bi-weekly, monthly, as needed, and continuously. Implants are advantageous for continuous administration, but are not the only means of continuous administration.

In an embodiment the humanin or humanin analog is conjugated to an entity that improves the half-life and/or stability of the humanin or humanin analog. Non-limiting examples include PEG or derivatives thereof.

The peptide humanin and peptide humanin analogs can be synthesized by any technique known in the art including solid-phase synthesis, liquid-phase synthesis, and expression of appropriate encoding DNA in a host cell and recovery therefrom.

In accordance with the methods of the present invention, the subject is a mammal. Preferably, the subject is a human.

Also provided is a pharmaceutical composition comprising humanin or an active analog of humanin for treating Parkinson's disease. Also provided is a pharmaceutical composition comprising humanin or an active analog of humanin for activating STAT-3 in a neuron of a subject or for increasing DJ-1 expression in a neuron of a subject.

Also provided is a method of identifying a candidate treatment for Parkinson's disease, the method comprising a) modeling in silico the 3-dimensional form of the humanin analog comprising SEQ ID NO:2, b) testing in silico if a compound from a library of small molecule compounds mimics the modeled 3-dimensional form, and c) determining
in vitro if the small molecule identified in b) is chemically stable, thereby identifying the candidate treatment.

[0042]  In silico modeling of 3-D binding sites for rational drug design is known in the art. For example, see Computational Resources for Protein Modelling and Drug Discovery Applications, Infectious Disorders - Drug Targets (2009), 9, 557-562, B. Dhaliwal and Y. W. Chen, the contents of which are hereby incorporated by reference.

[0043]  All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0044]  This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0045]  The unique mitochondrial peptide called humanin (HN) was first isolated from the surviving neurons of a patient with Alzheimer's Disease (AD) (10). Since then, its role has been characterized in offering protection from multiple AD related insults (11-14). In addition to AD-type neurodegeneration, HN and its potent analog HNG have been shown to increase cell survival in response to a variety of insults including serum deprivation, prion induced apoptosis and in an ischemia/reperfusion model of stroke (15-17). This laboratory has shown that HN and its analogs influence glucose metabolism by improving insulin sensitivity (18) and, in addition, have shown that HNG improves survival in cardiomyocytes when exposed to ischemia/reperfusion (19), an improvement that occurs through the activation of metabolic pathways involving cAMP-activated protein kinase (AMPK) and attenuation of apoptosis. Thus, HN is at the crossroads of cell survival, metabolism, oxidative stress, and aging. It improves cell survival in response to many stressors related to age, nutritional deprivation, ischemia, and oxidative stress that translate to improved outcomes. Strengthening this link between HN and aging and implicating a role for HN in many age-related diseases is this laboratory's observations that HN levels decline with age in human serum, CSF, and brain tissue, and in rodent and human hypothalamus (18).
Results

The data presented here show a decrease in levels of endogenous HN with age in the rat midbrain (Fig. 1), a region where significant numbers of dopaminergic neurons are lost in humans in PD. CNTFR-α, a component of the HN receptor, is also present in the rat midbrain, implicating a physiologic role for this peptide in dopaminergic neurons. Together, these data show that HNG improves survival of dopaminergic neurons in response to multiple stressors. The results demonstrate a common element that links metabolism to dopaminergic neuronal survival and serves as a unifying mechanism to increase the overall understanding of how aging increases the risk of a number of diseases that currently appear unrelated mechanistically. Considering that diseases of aging such as Type II diabetes mellitus (T2DM), PD and AD are associated with mitochondrial dysfunction (20), it is especially compelling that a mitochondria-associated peptide such as HN can modulate their course.

Rattin is present in the rat midbrain and may decline with age. Humanin has been shown to offer protection relevant to a variety of age-related diseases such as Alzheimer’s disease, stroke, insulin resistance, and myocardial infarction in addition to other insults such as prion-induced apoptosis and amyotrophic lateral sclerosis (10, 16-19, 21). The common thread among these diseases is the presence of cellular stress in the form of metabolic defects, cellular toxins, ischemia or reactive oxygen species. Although protection has been demonstrated in a variety of cell types, including neurons and PC-12 cells, effects on midbrain dopaminergic neurons or models of PD have not been reported. The results here from Western blots demonstrate that rattin (RN), the rat homolog of HN, is present in the rat midbrain and in MN9D, a dopaminergic cell line. The results also demonstrate that CNTFR-α is present in MN9D cells and in rat midbrain and others have shown that it is present in the basal ganglia of primates (22).

HN is neuroprotective in a dopaminergic cell line. In the presence or absence of HNG, cultures were challenged by serum withdrawal, H₂O₂, a free radical, and rotenone, a specific and potent inhibitor of mitochondrial complex I. Clinical and experimental evidence suggest that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of Parkinson’s disease. Using MN9D, a dopaminergic cell line, it was demonstrated that HNG, a potent HN analog with activity in rodents, offers protection to dopaminergic neurons to serum withdrawal (Fig. 2A), hydrogen peroxide (Fig. 2B) and rotenone (Fig. 2C-D). This shows that humanin confers resistance to metabolic stress,
generalized oxidative stress, and to a pesticide that mimics the mitochondrial oxidative stress seen in PD.

[0049] HNG increased expression of DJ-1 in MN9D cells at the RNA and protein levels after serum deprivation and in response to H₂O₂ (Fig. 3). DJ-1 is an evolutionarily conserved multi-functional transcriptional co-activator and the product of the Park7 gene associated with PD. Oxidative insults induce an up-regulation and redistribution of DJ-1 from the nucleus to mitochondria (23) and DJ-1 protects dopaminergic neurons in response to rotenone, H₂O₂, MPTP, and 6-OHDA toxicity (24, 25). DJ-1 is also activated as part of physiological receptor-mediated signal transduction. In addition to cell-autonomous protection, DJ-1 may also act indirectly, through astrocytes, where it is upregulated in neurodegenerative diseases and in stroke (26). Cells deficient in DJ-1 are exquisitely sensitive to damage from both rotenone and MPTP in flies and in mice (26). In addition to neurons themselves, astrocytic DJ-1 has been shown to protect neurons in stroke and inflammation and importantly, DJ-1 knockdown in astrocytes impaired neuroprotection against rotenone (27). Importantly, levels of DJ-1 are low in cerebrospinal fluid in Parkinson’s disease and therefore this protein has been regarded as a biomarker (28). Genetic mutations in PARK7/DJ-1 can cause the rare form of autosomal recessive hereditary Parkinson’s disease.

[0050] Humanin protects dopaminergic neurons from oxidative stress by inducing mitochondrial stress response genes. MN9D cells were exposed to 200 µM H₂O₂ in the presence or absence of a neuroprotective concentration of HNG. DJ-1 protein and RNA were quantified in cell lysates harvested at 4 hours after treatment. Results demonstrate that DJ-1 protein levels increased after H₂O₂ treatment, and increased further with toxin and HNG together. Interestingly, DJ-1 RNA levels decreased at this time point after challenge with H₂O₂ alone, suggesting that perhaps at longer intervals, DJ-1 protein levels would be lower. In contrast, the addition of HNG restored levels of DJ-1 mRNA to baseline (control levels). In addition to its direct antioxidant role as a free radical scavenger, DJ-1 regulates redox signaling kinase pathways and acts as a transcriptional regulator of antioxidative gene batteries. DJ-1 enhances the activity of the transcription factor, Nuclear-factor-E2-related factor (Nrf)-2, a master regulator of antioxidant genes and up-regulates response genes such as hemeoxygenase-1 (HO-1), thioredoxin (Trx), thioredoxin reductase (TrxR), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), and catalase (29, 30-33) by binding to the antioxidant responsive element (ARE) found in their
promoter (34). In addition, DJ-1 increases superoxide dismutase (SOD) activity (35). Predicted stimulation of these enzymes would increase resistance to oxidative stress, resulting in higher resistance to oxidative challenges.

[0051] Humanin influences metabolic signaling in dopaminergic neurons. Previous work from this laboratory and others suggests that HN is positioned at a critical interface between cell survival and cell metabolism. This laboratory has shown that HN offers cardio-protection following myocardial ischemia-reperfusion injury through activation of cAMP-activated protein kinase (AMPK) (19). In addition, HN and analogs improve insulin sensitivity and increase glucose uptake (18). AMPK is a metabolic sensor and important for the adaptive responses of energetically stressed cells (36). Activation of AMPK is especially relevant for neurons, which are very metabolically active and have a poor capacity for nutrient storage, rendering them highly sensitive to energy fluctuations. Recent reports demonstrate that AMPK can be activated by resveratrol, a polyphenol found in red wine, but it can also be activated by metabolic stressors, such as ischemia/hypoxia and glucose deprivation (36-38). AMPK activation in rat brain protected neurons after energy deprivation, whereas mutations in the AMPK subunit in Drosophila led to progressive neurodegeneration (39). Thus AMPK links metabolic control and neuronal survival (40). Relevant to the present work, inhibition of AMPK increased MPP+-induced cell death and over-expression increased survival in human neuroblastoma cells (41).

[0052] In order to examine the effects of HN on activating metabolic pathways in dopaminergic neurons, MN9D cultures were exposed to a neuroprotective concentration of HNG for specified intervals (2 min, 5 min, 15 min, 30 min, 1 hr, 4 hr, 12 hr and 24 hr). Protein lysates were collected and processed for western blotting to quantify phosphorylated and total amounts of AMPK using phospho-specific and pan-antibodies (Cell Signaling; Beverly, MA). The ratio of phosphorylated to total AMPK serves as an index of activation.

[0053] Herein, it is demonstrated that AMPK can be activated in dopaminergic MN9D cells in response to HNG. Thus, it is possible that activation of AMPK will prevent the death of midbrain dopaminergic neurons and influence neuronal vulnerability in PD (41). In addition, HN can activate Jak/STAT, MAPK, and PI3K/Akt signaling pathways in the hypothalamus, skeletal muscle and liver (42-44) and it is known that HN-mediated protection of non-dopaminergic neurons involves the activation of tyrosine kinases and STAT-3 (13). In an experiment using primary cultures of hypothalamic neurons, Western blots were probed with antibodies to total and phosphorylated STAT-3 protein. It was found
that HNG could activate STAT-3 in these neurons within 5 minutes of treatment (Fig. 5). Other groups have demonstrated HN mediated neuroprotection by other molecules involved in metabolic signaling. For example, in non-neuronal K562 cells, HN delayed apoptosis by down-regulating P38 and MAPK (15) and prevented cell death when Jun N-terminal kinase (JNK) was constitutively activated, suggesting that an important mechanism of cell protection could be to interrupt JNK activity (45). JNK can mediate the cytotoxic effects of MPTP (24). The data here shows that HNG protects dopaminergic neurons from rotenone. Conversely, ERK1/2 signaling was shown to mitigate neurotoxicity in nigral dopaminergic neurons (46), but it is not known whether HN or AMPK activate ERK1/2 in these cells. Activation of metabolic signaling through AMPK should lead to an increase in cellular glucose uptake, which may influence cell survival. HNG has also been shown to increase Akt phosphorylation and increase survival in primary cortical neurons (16). Akt is also important to survival in dopaminergic neurons (47).

[0054] cAMP-activated protein kinase (AMPK) is a metabolic sensor important for the adaptive responses of energetically stressed cells (50). Activation of AMPK is especially relevant for neurons, which have high metabolic activity, but low capacity for nutrient storage, rendering them highly sensitive to energy fluctuations. AMPK activation in rat brain can protect neurons after energy deprivation (51), whereas mutations in AMPK led to progressive neurodegeneration in Drosophila (52). Thus AMPK links metabolic control and neuronal survival (53). Relevant to the present study, inhibition of AMPK increased MPP+ toxicity and over-expression increased survival in human neuroblastoma cells (54). Herein, it is demonstrated that AMPK can be activated in dopaminergic MN9D cells in response to HNG (Fig. 7). Thus, activation of AMPK will prevent the death of midbrain dopaminergic neurons and influence neuronal vulnerability in PD5.

[0055] Accordingly, it is disclosed that humanin analog HNG can protect against neurotoxicity of dopaminergic neurons, and is a viable therapy for treating Parkinson's disease.
REFERENCES


What is claimed is:

1. A method of treating Parkinson's disease or of protecting dopaminergic neurons from neurotoxicity in a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to treat Parkinson's disease or protect dopaminergic neurons.

2. A method of increasing DJ-1 expression in a neuron of subject or of activating STAT-3 in a neuron of a subject comprising administering to the subject an amount of humanin or an active humanin analog effective to increase DJ-1 expression in a neuron or activate STAT-3 in a neuron.

3. The method of Claim 1 or 2, wherein the humanin or humanin analog is administered in a manner effective to enter the midbrain of the subject.

4. The method of any of Claims 1-3, wherein the humanin or humanin analog is administered parenterally.

5. The method of any of Claims 1-3, wherein the humanin or humanin analog is administered into the central nervous system of the subject.

6. The method of any of Claims 1-5, wherein the humanin analog is administered into the cerebrospinal fluid of the subject.

7. The method of any of Claims 1-6, wherein the humanin or humanin analog is administered via an implant in the central nervous system of the subject.

8. The method of claim 7, wherein the implant comprises a polymer matrix.

9. The method of any of Claims 1-5, wherein the humanin or humanin analog is administered intraventricularly or intrathecally.
10. The method of any of Claims 1-4, wherein the humanin or humanin analog is administered intranasally.

11. The method of any of Claims 1-10 wherein the humanin analog is administered, and wherein the humanin analog comprises SEQ ID NO:2.

12. The method of any of Claims 1-11, wherein the subject has been identified as suffering from Parkinson’s disease or exhibits a plurality of pro-dromal symptoms associated with Parkinson’s disease.

13. The method of any of Claims 1-12, further comprising identifying the subject as suffering from Parkinson’s disease, or as exhibiting a plurality of pro-dromal symptoms associated with Parkinson’s disease, prior to administering the humanin or humanin analog.

14. The method of any of Claims 1-13, wherein the humanin or humanin analog is administered in a manner effective to enter the substantia nigra of the subject and/or the striatum of the subject.

15. The method of Claim 14, wherein the humanin or humanin analog is administered directly into the substantia nigra of the subject and/or the striatum of the subject.

16. A humanin analog for treating Parkinson’s disease, for treating a subject exhibiting a plurality of pro-dromal symptoms associated with Parkinson’s disease, or for protecting dopaminergic neurons from neurotoxicity in a subject.

17. The humanin analog of Claim 16, wherein the humanin analog comprises SEQ ID NO:2.

18. A method of identifying a candidate treatment for Parkinson’s disease, the method comprising a) modeling in silico the 3-dimensional form of the humanin analog comprising SEQ ID NO:2, b) testing in silico if a compound from a library of small molecule compounds mimics the modeled 3-dimensional form, and c) determining in vitro if the
small molecule identified in b) is chemically stable, thereby identifying the candidate treatment.
Fig. 1
Fig. 2A-2D
Fig. 3A-3B
Fig. 4

The diagram shows a comparison of AMPK activity between control, $H_2O_2$, and $H_2O_2 + HNG$. The control group shows the lowest AMPK activity, followed by the $H_2O_2$ group, and the highest AMPK activity is observed in the $H_2O_2 + HNG$ group.
Fig. 5
Fig. 7