US 20140011890A1

(19) United States

(12) Patent Application Publication

(10) Pub. No.: US 2014/0011890 A1

(43) Pub. Date: Jan. 9, 2014

Milbrandt et al.

(54) METHODS AND COMPOSITIONS FOR TREATING NEUROPATHIES

(71) Applicants: **Jeffrey Milbrandt**, Clayton, MO (US); **Biplab Dasgupta**, St. Louis, MO (US)

(72) Inventors: **Jeffrey Milbrandt**, Clayton, MO (US); **Biplab Dasgupta**, St. Louis, MO (US)

(73) Assignee: **WASHINGTON UNIVERSITY**, St. Louis, MO (US)

(21) Appl. No.: **13/952,183**

(22) Filed: **Jul. 26, 2013**

Related U.S. Application Data

(63) Continuation of application No. 12/524,718, filed on Oct. 20, 2009, now abandoned, filed as application No. PCT/US08/01085 on Jan. 28, 2008.

(60) Provisional application No. 60/886,854, filed on Jan. 26, 2007.

Publication Classification

(51) **Int. Cl.**

G01N 33/50 (2006.01)

A61K 31/05 (2006.01)

(52) **U.S. Cl.**

CPC ***G01N 33/5058*** (2013.01); ***A61K 31/05*** (2013.01)

USPC **514/734**; 435/29

(57) **ABSTRACT**

Methods of treating or preventing axonal degradation in neuropathic diseases and neurological disorders in mammals are disclosed. The methods can comprise administering to the mammal an effective amount of an agent that acts at least in part by increasing sirtuin AMPK activity, LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons. The methods can also comprise administering to the mammal an effective amount of an agent that acts by increasing NAD activity in diseased and/or injured neurons, alone or in combination with agents that act by other mechanisms. Also disclosed are methods of screening agents for treating a neuropathies and recombinant vectors for treating or preventing such neuropathies.

FIG. 1A

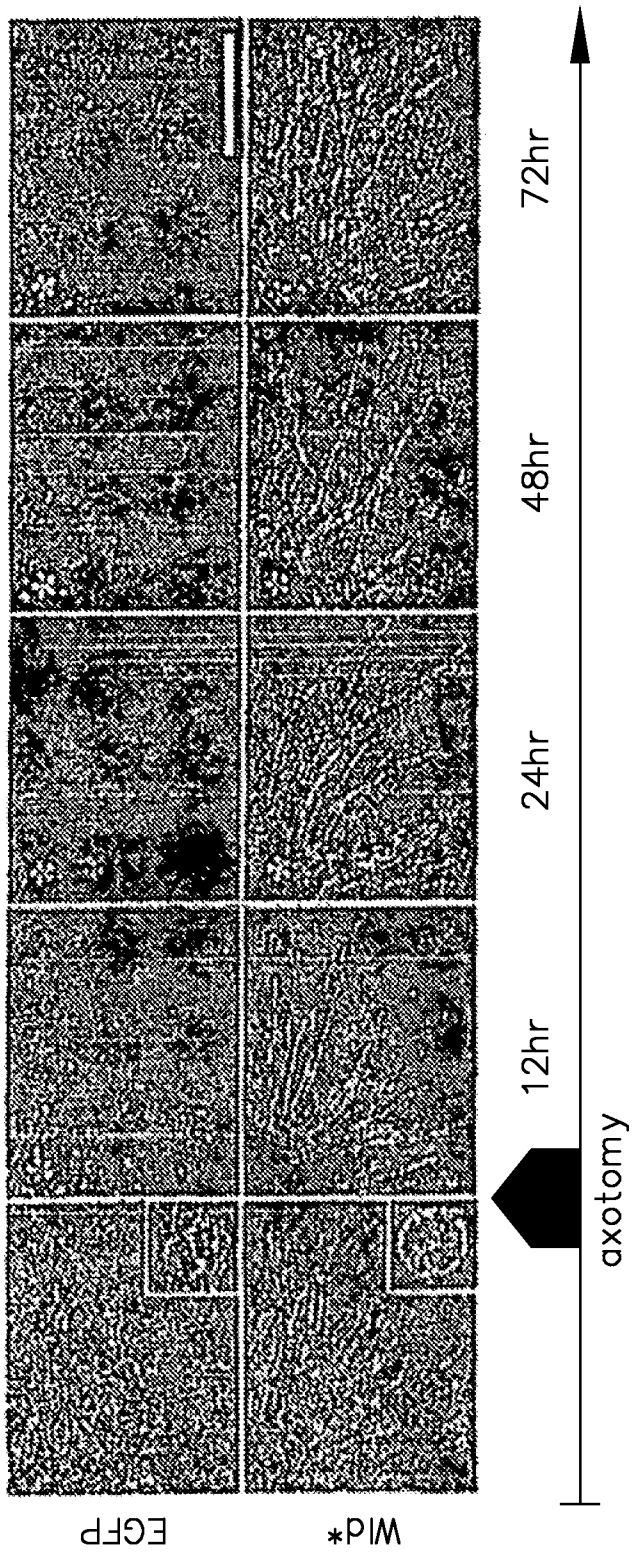


FIG. 1B

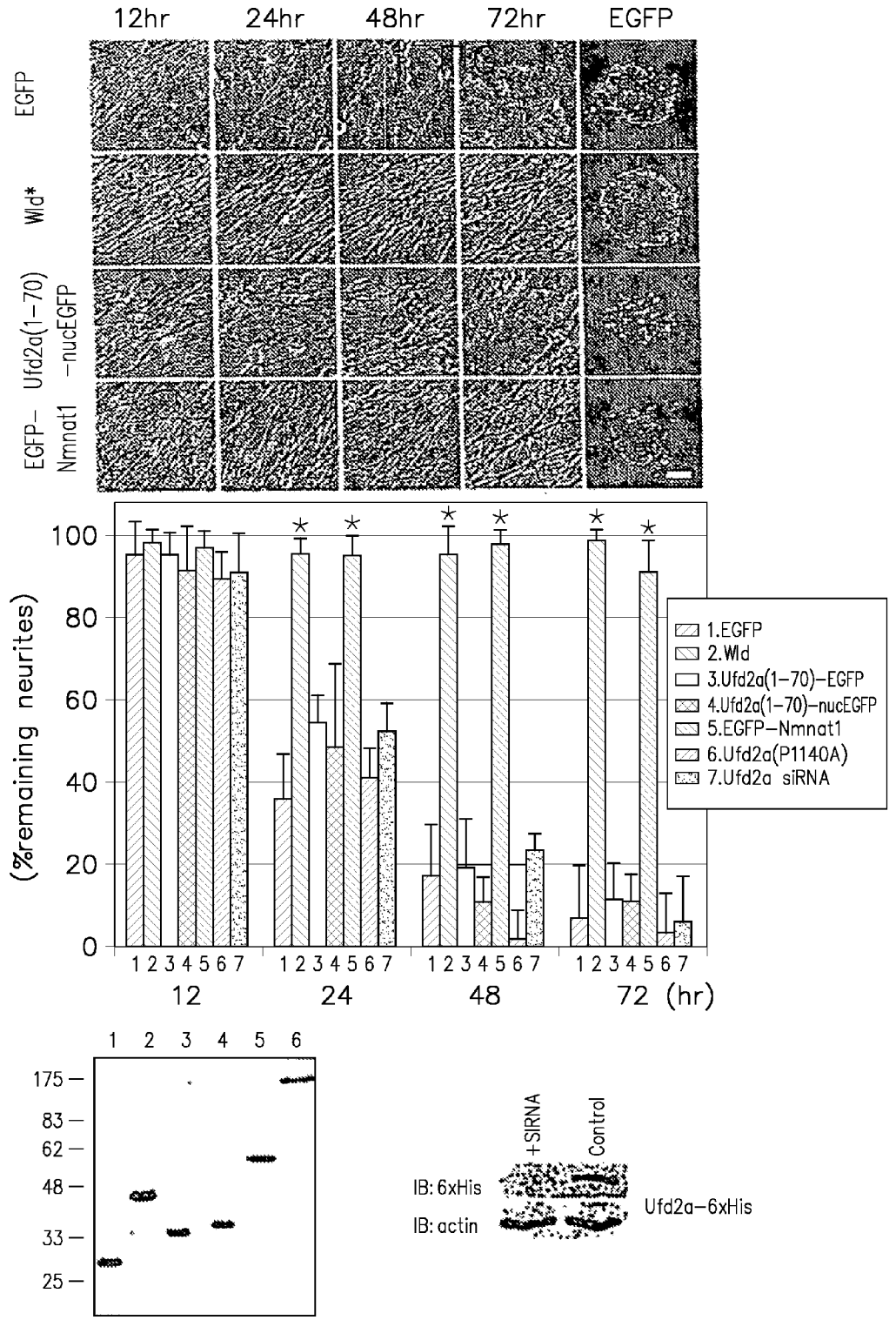

12hr
24hr
48hr
72hr
EGFP
EGFP
Wld*
Ufd2a(1-70) -nucEGFP
EGFP-Nmnat1
(%remaining neurites)
100
80
60
40
20
0
1.EGFP
2.Wld
3.Ufd2a(1-70)-EGFP
4.Ufd2a(1-70)-nucEGFP
5.EGFP-Nmnat1
6.Ufd2a(P1140A)
7.Ufd2a siRNA
1 2 3 4 5 6 7
12
24
48
72 (hr)
1 2 3 4 5 6
175
83
62
48
33
25
+SIRNA
Control
IB: 6xHis
IB: actin
Ufd2a-6xHis

FIG. 2A

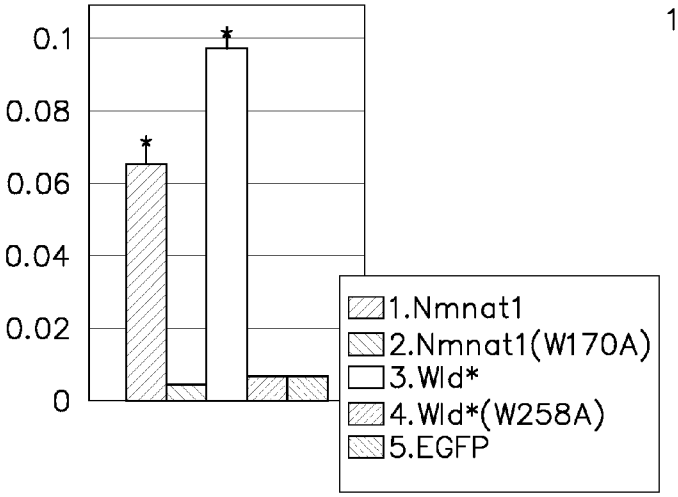


FIG. 2C

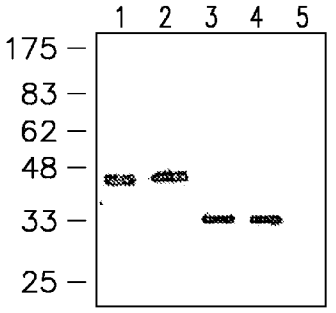


FIG. 2B

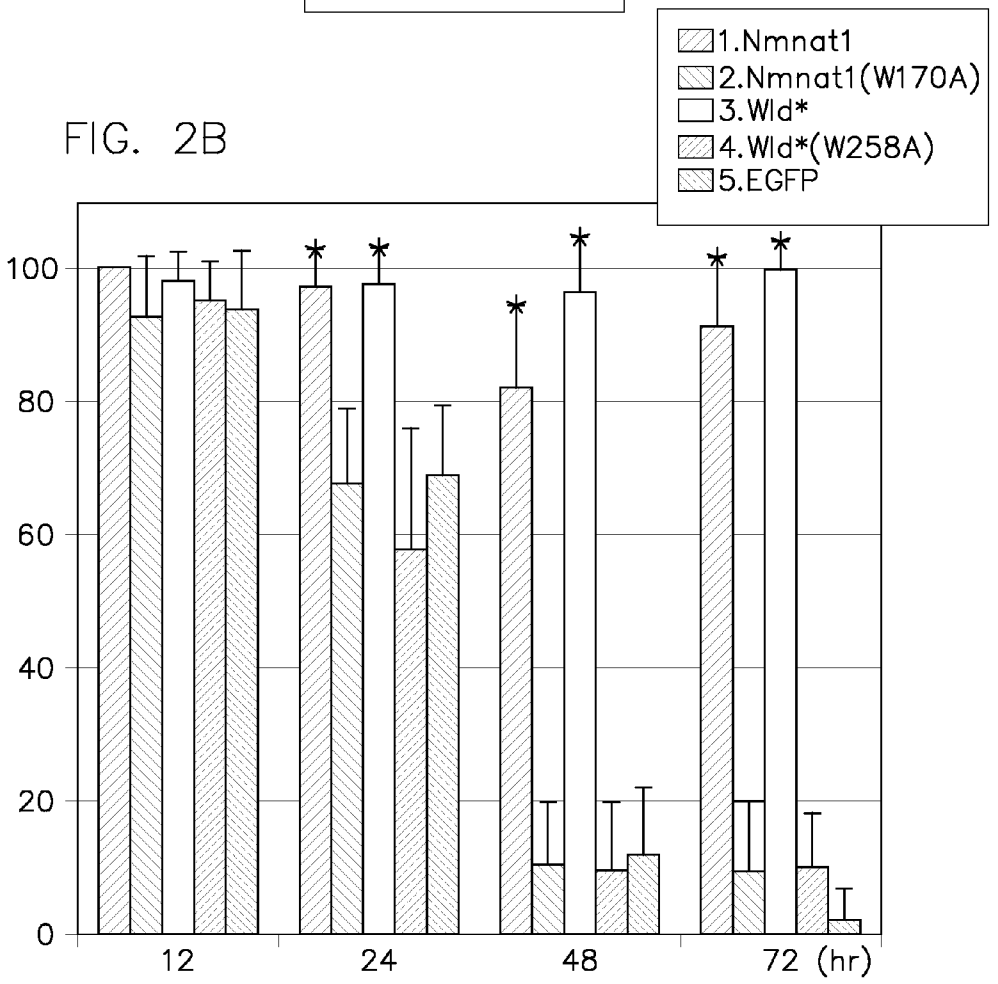
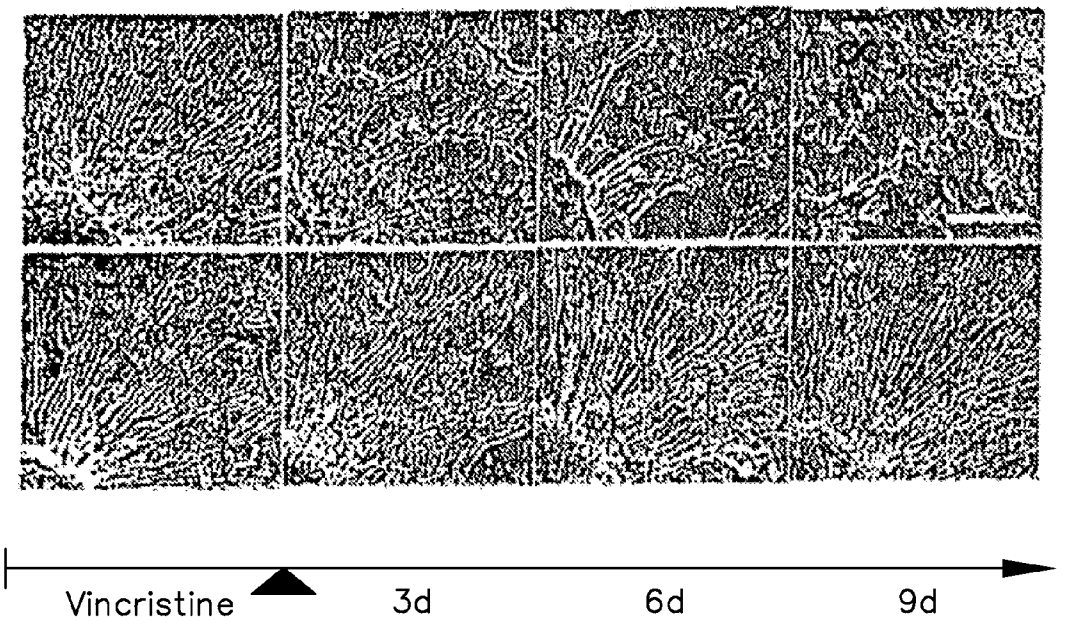


FIG. 2D



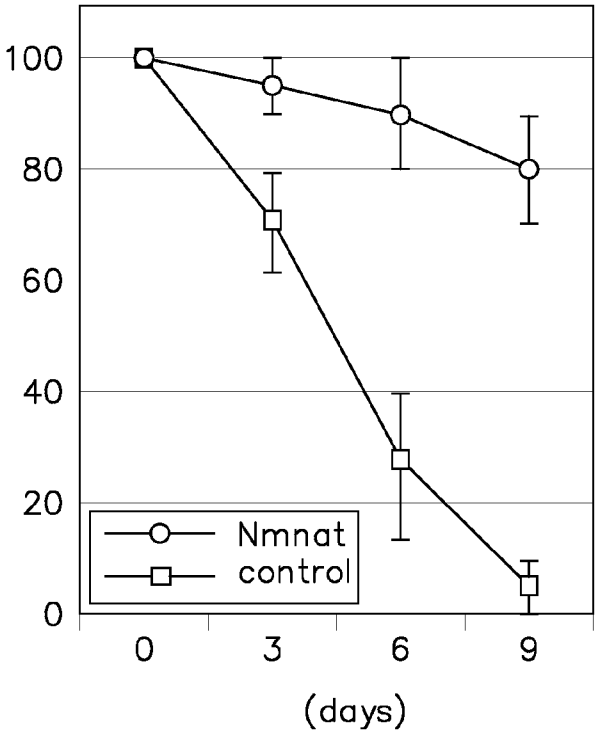
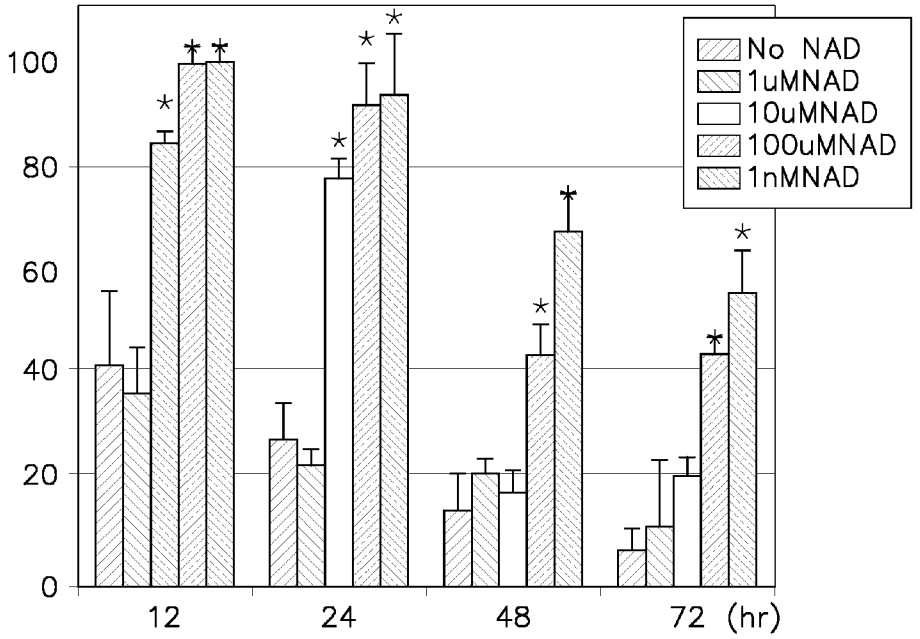


FIG. 3A


No NAD
1uMNAD
10uMNAD
100uMNAD
1nMNAD
100
80
60
40
20
0
12
24
48
72 (hr)

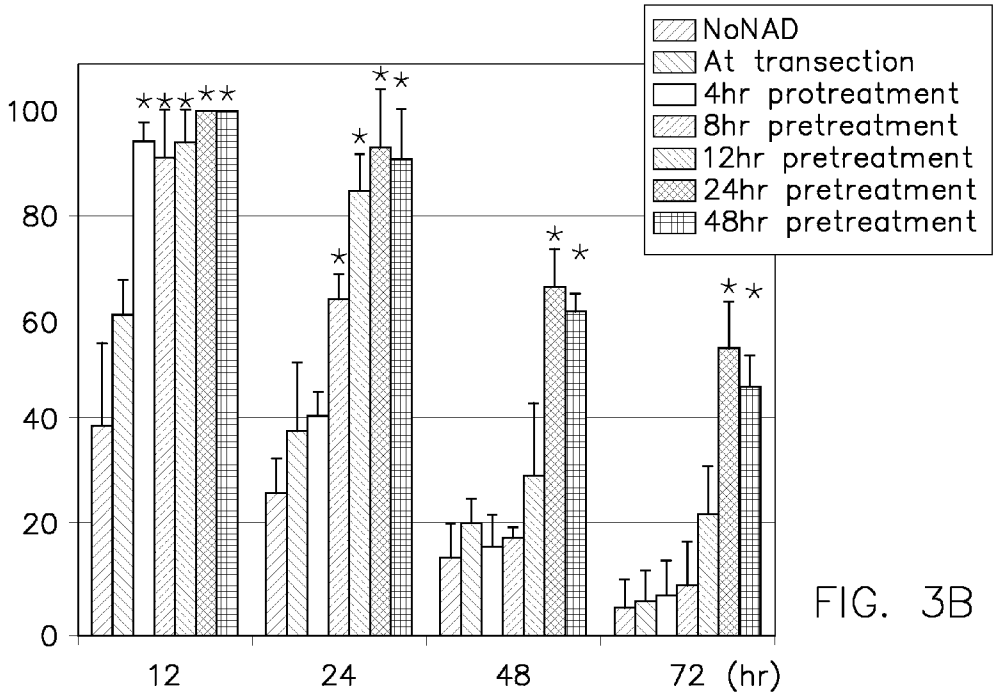

NoNAD
At transection
4hr protreatment
8hr pretreatment
12hr pretreatment
24hr pretreatment
48hr pretreatment
100
80
60
40
20
0
12
24
48
72 (hr)

FIG. 3B

FIG. 4A

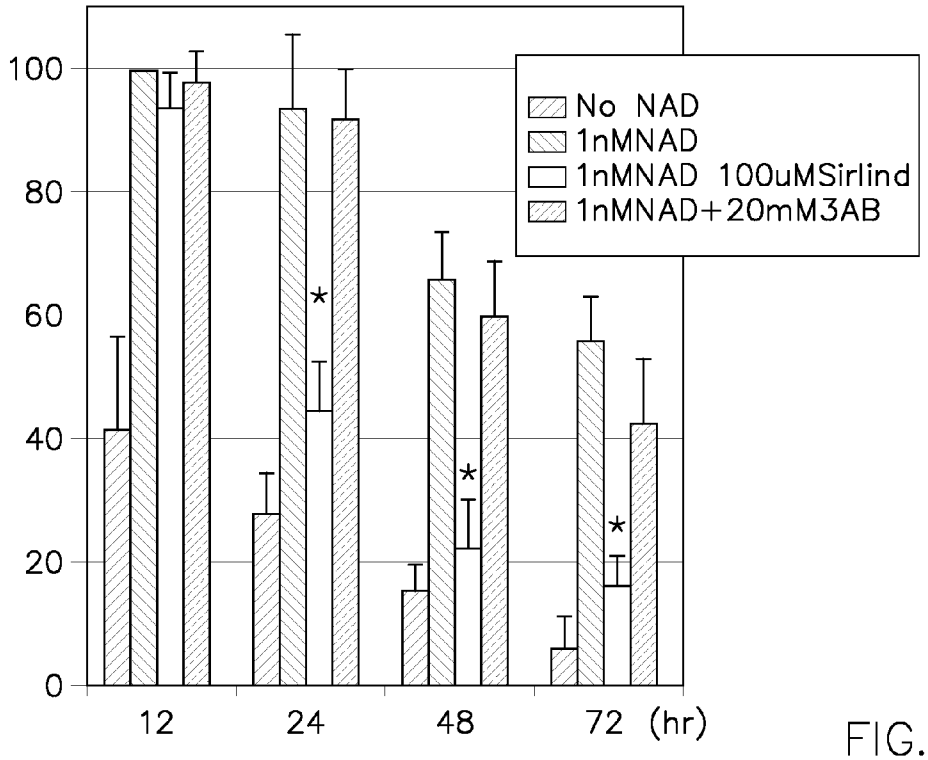

100
80
60
40
20
0
12
24
48
72
(hr)
No NAD
1nMNAD
1nMNAD 100uMSirlind
1nMNAD+20mM3AB
*
*
*

FIG. 4B

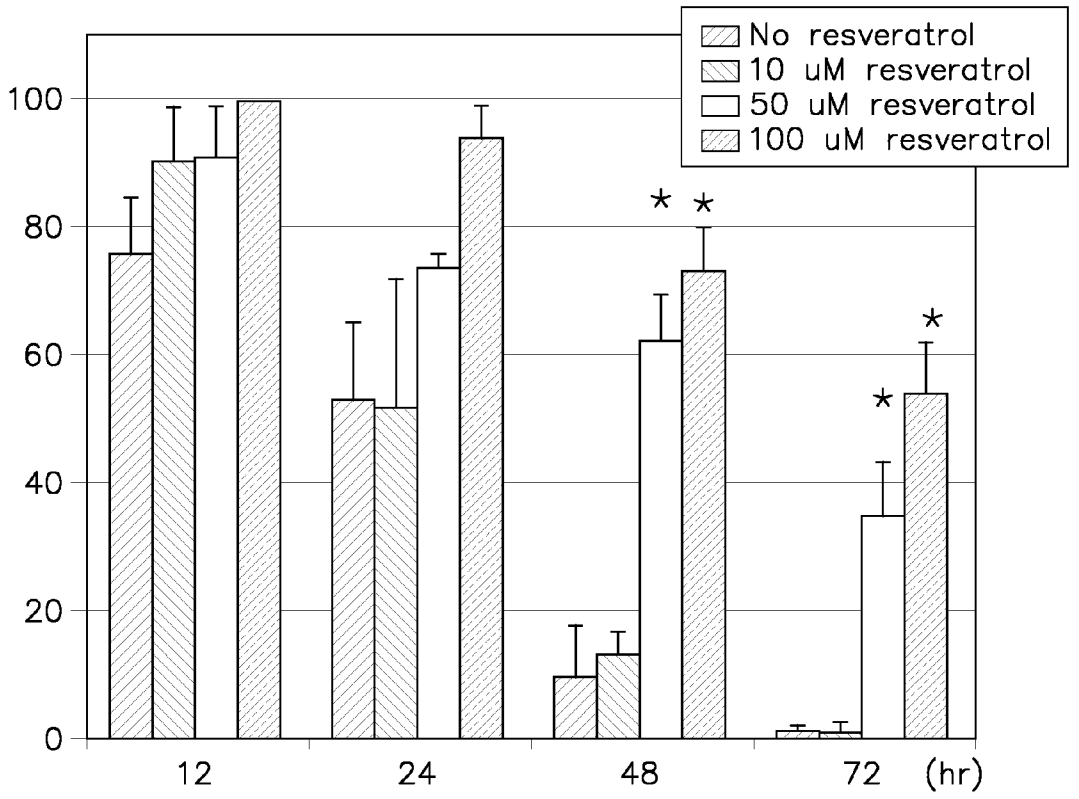

100
80
60
40
20
0
12
24
48
72
(hr)
No resveratrol
10 uM resveratrol
50 uM resveratrol
100 uM resveratrol
* *
*
*

FIG. 4C

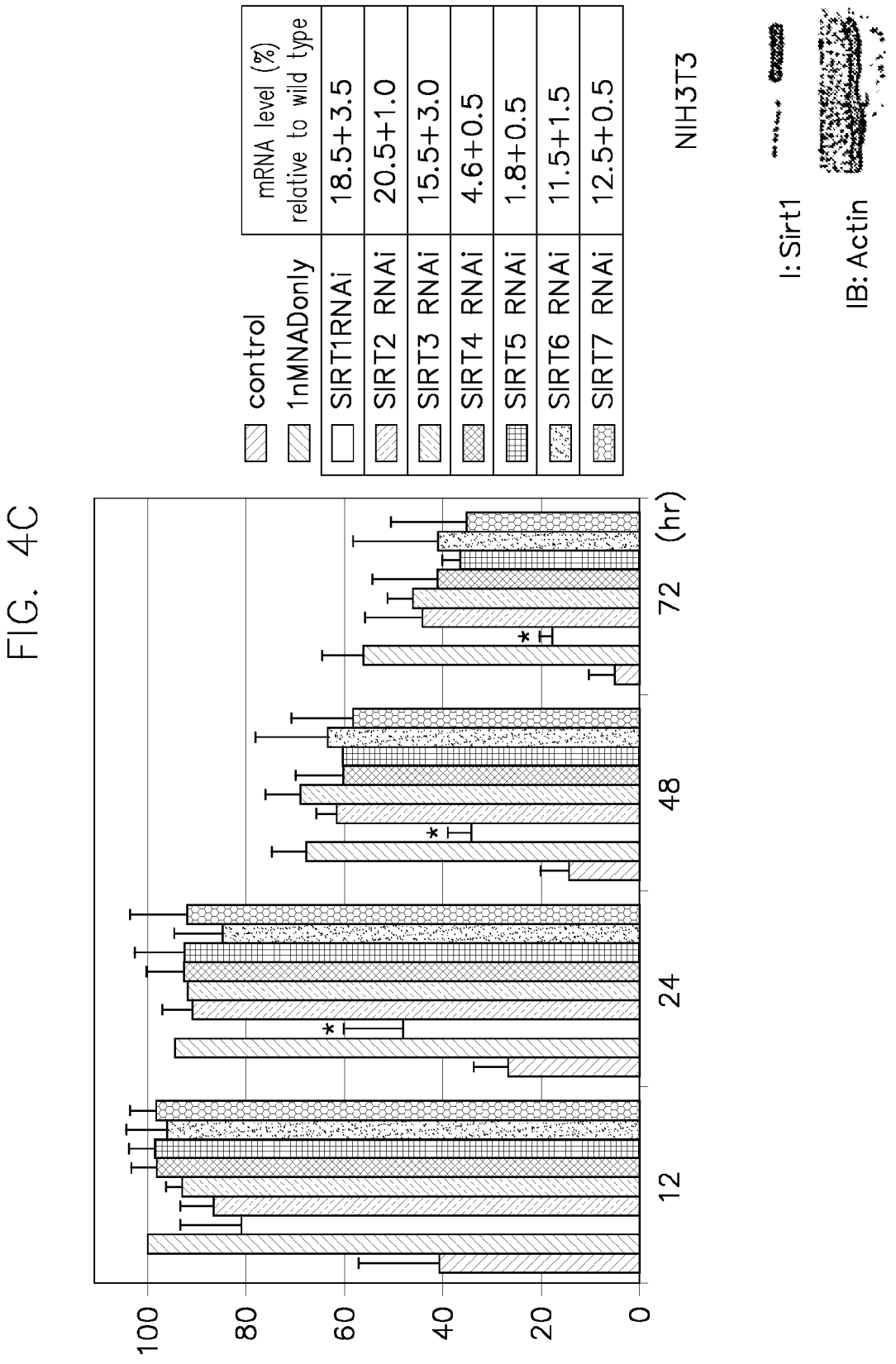

control
1nMNADonly
mRNA level (%) relative to wild type
SIRT1RNAi 18.5+3.5
SIRT2 RNAi 20.5+1.0
SIRT3 RNAi 15.5+3.0
SIRT4 RNAi 4.6+0.5
SIRT5 RNAi 1.8+0.5
SIRT6 RNAi 11.5+1.5
SIRT7 RNAi 12.5+0.5
NIH3T3
I: Sirt1
IB: Actin
100
80
60
40
20
0
12
24
48
72 (hr)
*

FIG. 5

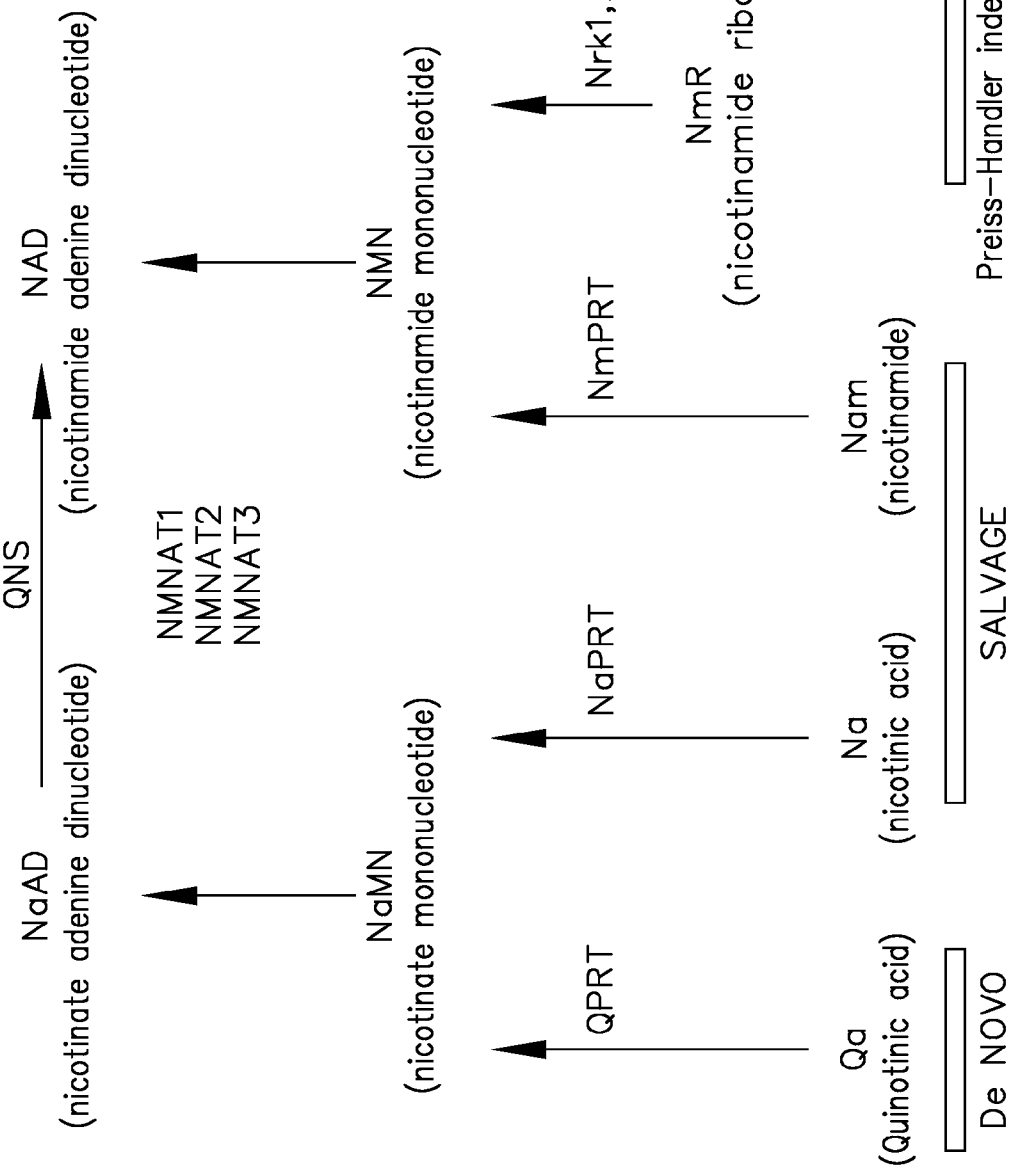

NaAD
(nicotinate adenine dinucleotide)
QNS
NAD
(nicotinamide adenine dinucleotide)
NMNAT1
NMNAT2
NMNAT3
NaMN
(nicotinate mononucleotide)
NMN
(nicotinamide mononucleotide)
QPRT
NaPRT
NmPRT
Nrk1,2
NmR
(nicotinamide riboside)
Qa
(Quinotinic acid)
Na
(nicotinic acid)
Nam
(nicotinamide)
De NOVO
SALVAGE
Preiss-Handler independent

FIG. 6A

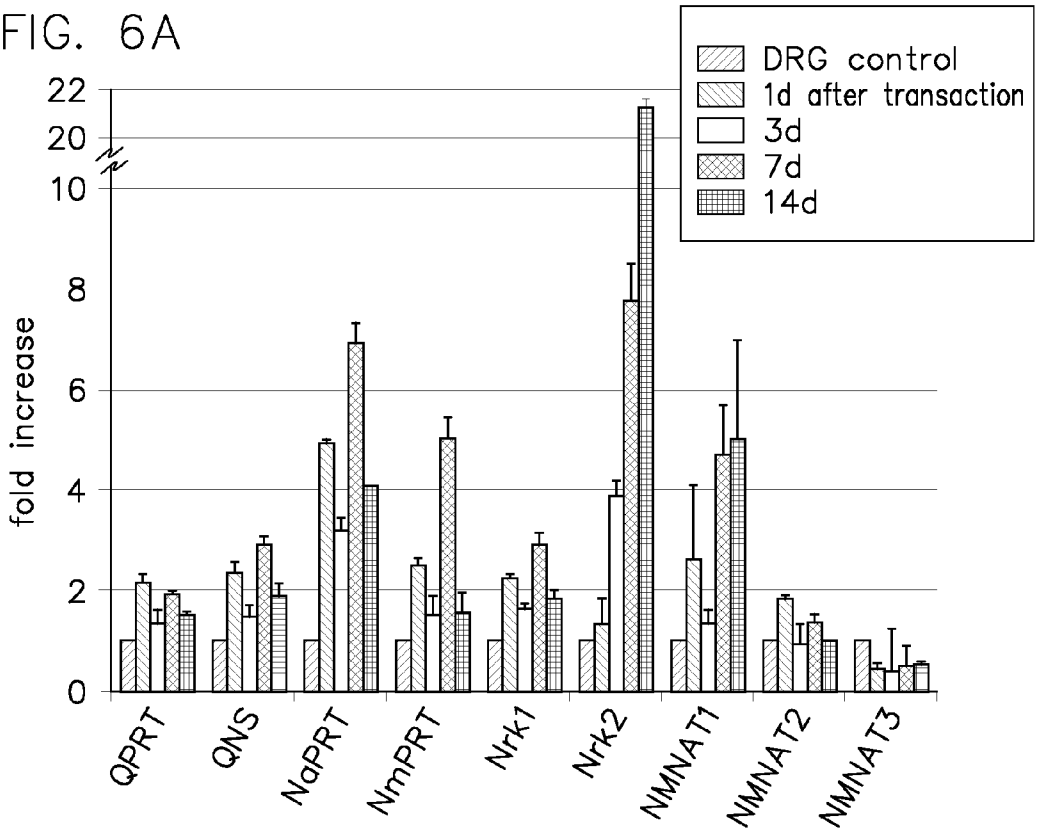

DRG control
1d after transaction
3d
7d
14d
fold increase
22
20
10
8
6
4
2
0
QPRT
QNS
NaPRT
NmPRT
Nrk1
Nrk2
NMNAT1
NMNAT2
NMNAT3

FIG. 6B

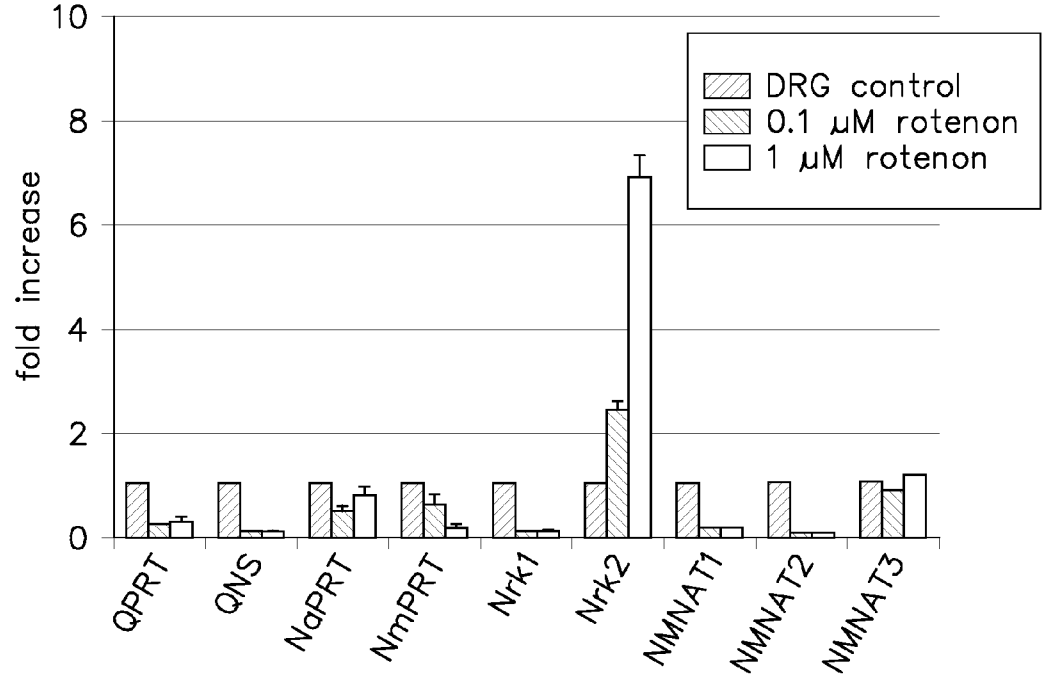

DRG control
0.1 μM rotenon
1 μM rotenon
fold increase
10
8
6
4
2
0
QPRT
QNS
NaPRT
NmPRT
Nrk1
Nrk2
NMNAT1
NMNAT2
NMNAT3

FIG. 7A

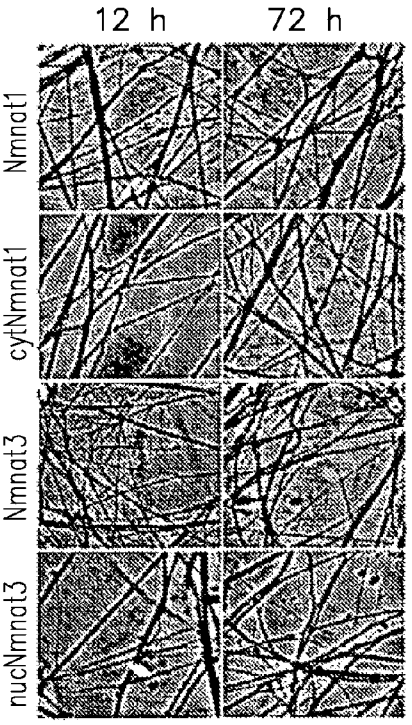


FIG. 7B

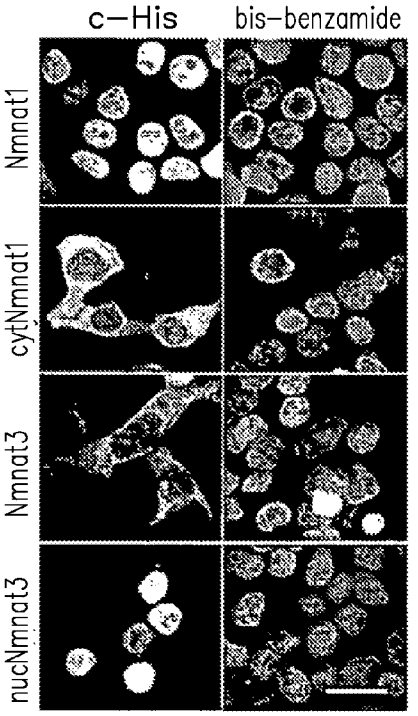


FIG. 7C

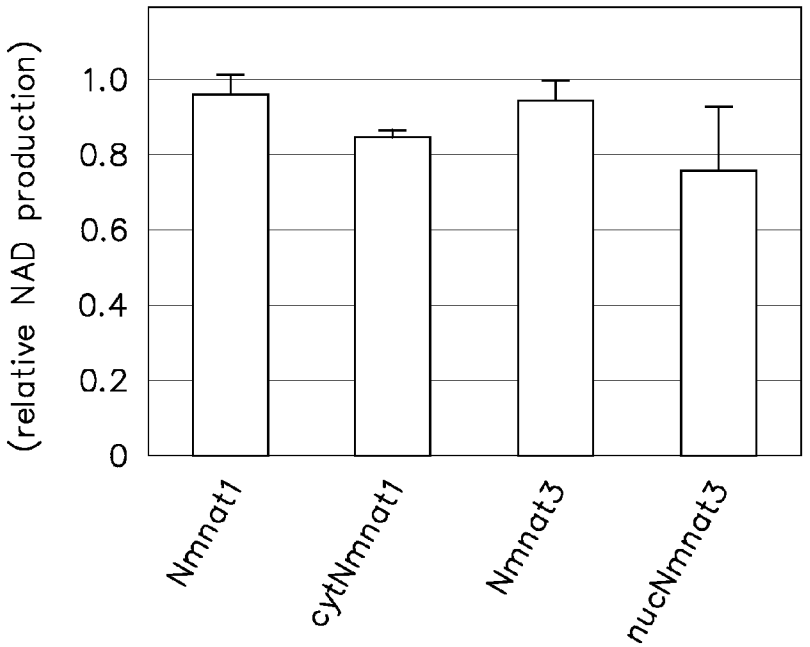


FIG. 7D

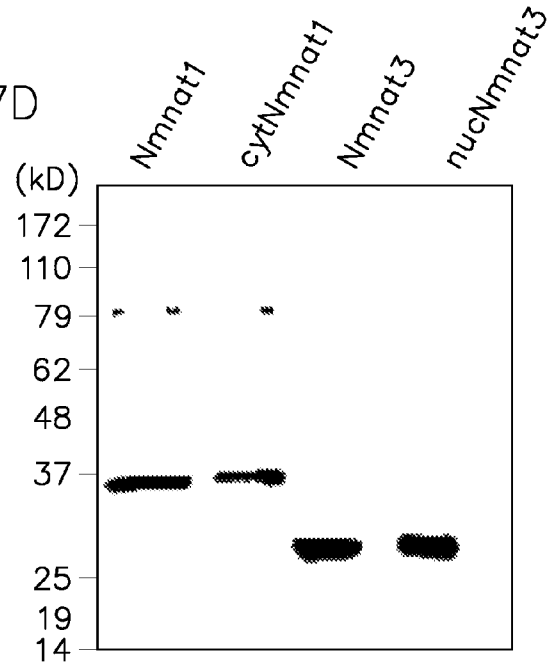

Nmnat1
cytNmnat1
Nmnat3
nucNmnat3
(kD)
172
110
79
62
48
37
25
19
14

FIG. 7E

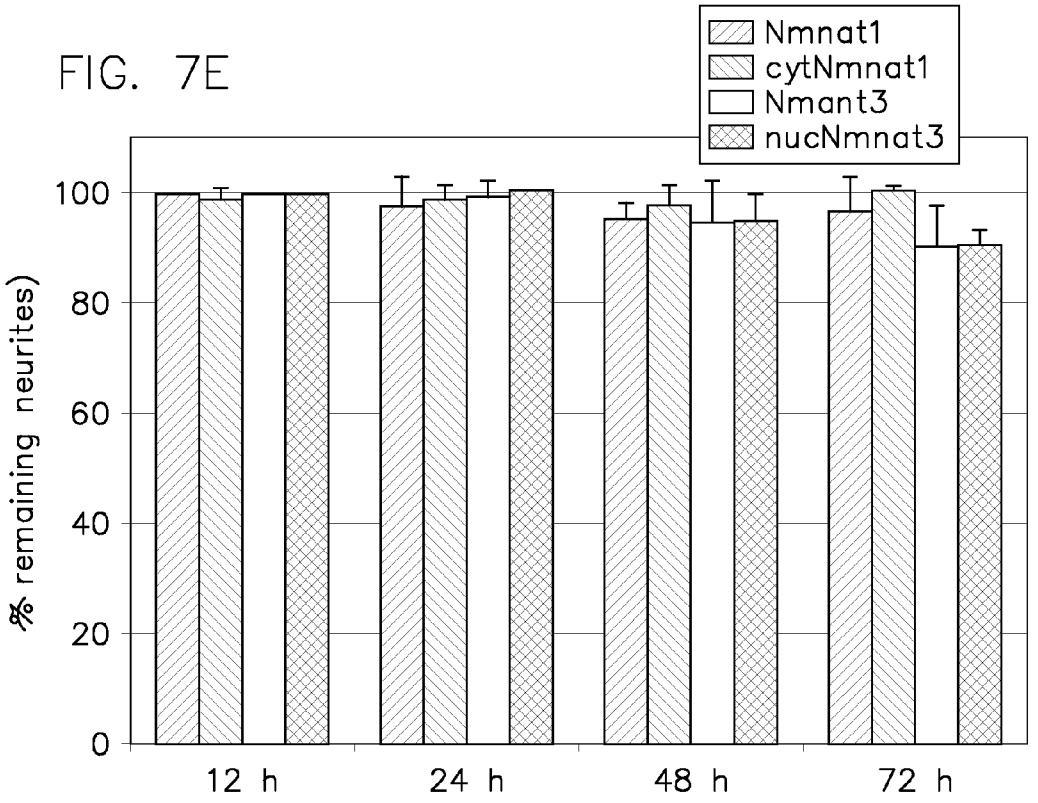

Nmnat1
cytNmnat1
Nmant3
nucNmnat3
% remaining neurites)
100
80
60
40
20
0
12 h
24 h
48 h
72 h

FIG. 8A

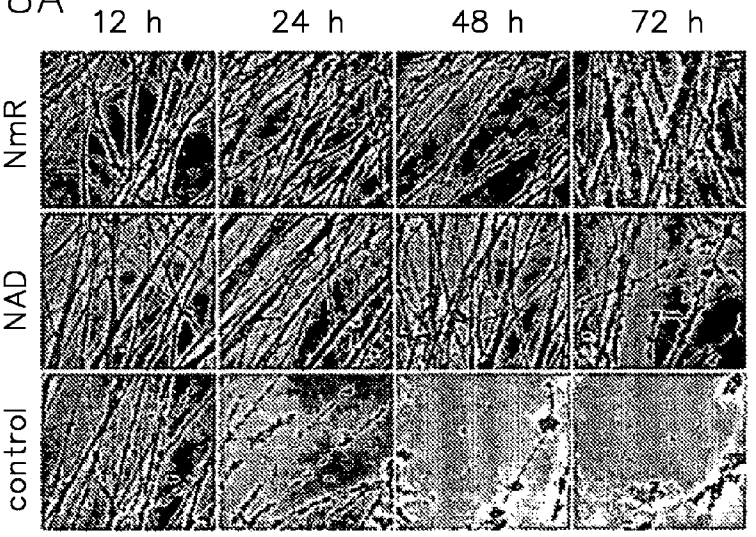

12 h
24 h
48 h
72 h
NmR
NAD
control

FIG. 8B

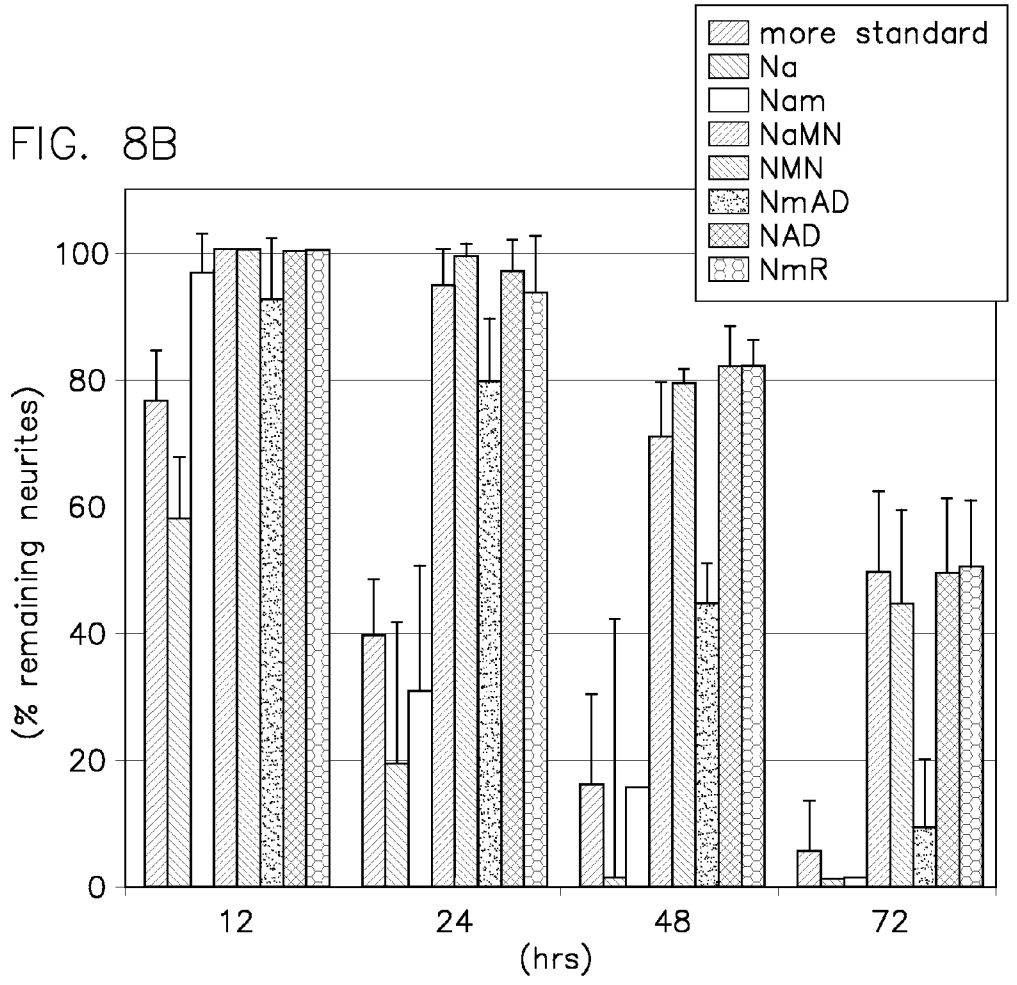
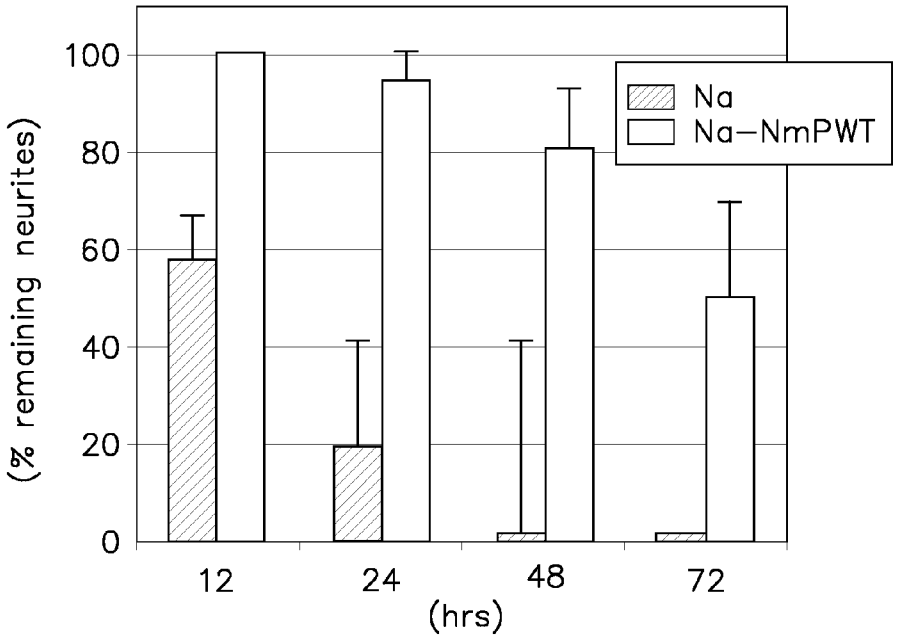

more standard
Na
Nam
NaMN
NMN
NmAD
NAD
NmR
(% remaining neurites)
0
20
40
60
80
100
12
24
48
72
(hrs)

FIG. 8C


100
80
60
40
20
0
(% remaining neurites)
Na
Na−NmPWT
12
24
48
72
(hrs)

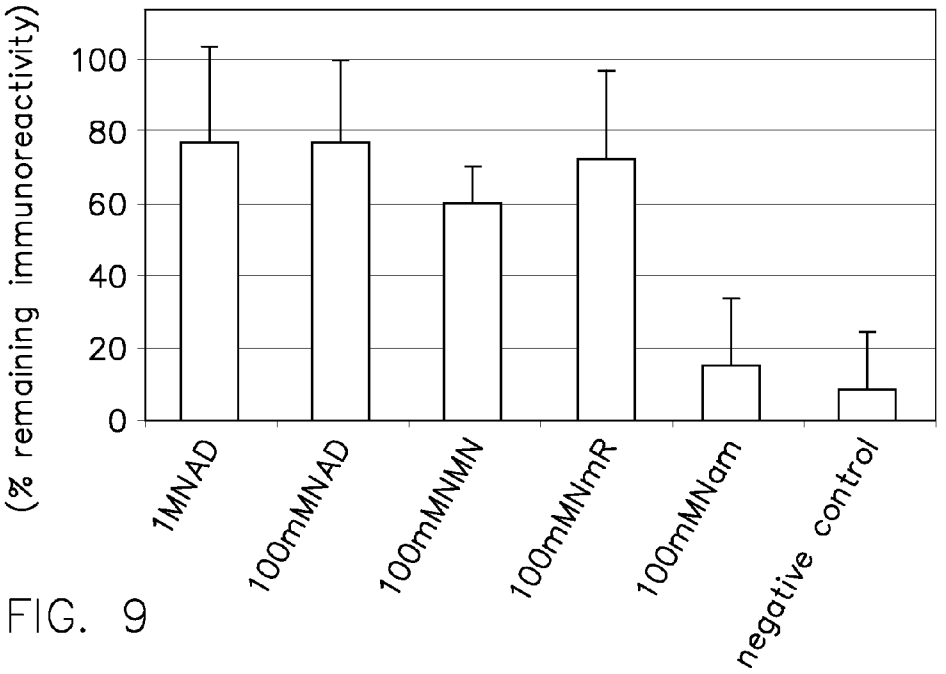

100
80
60
40
20
0
(% remaining immunoreactivity)
1MNAD
100mMNAD
100mMNMN
100mMNmR
100mMNam
negative control

FIG. 9

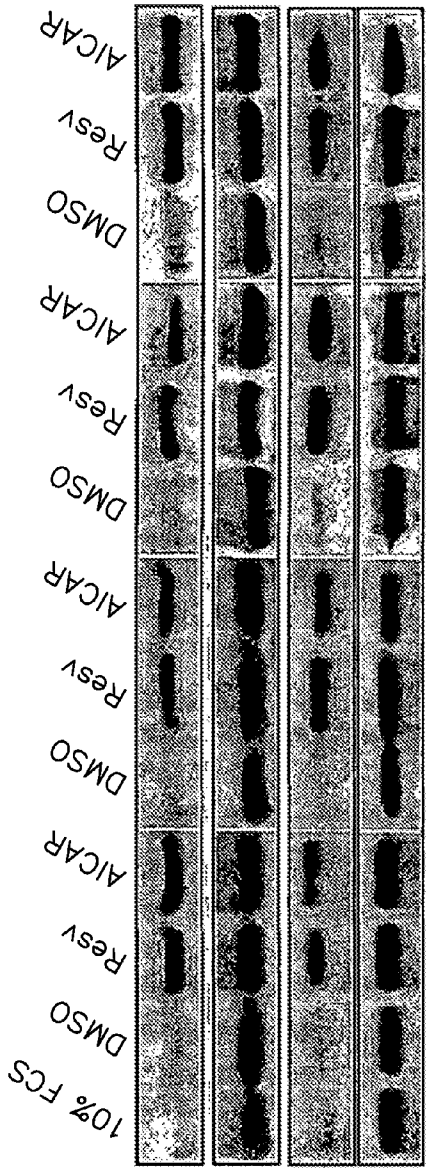


FIG. 10A

FIG. 10B

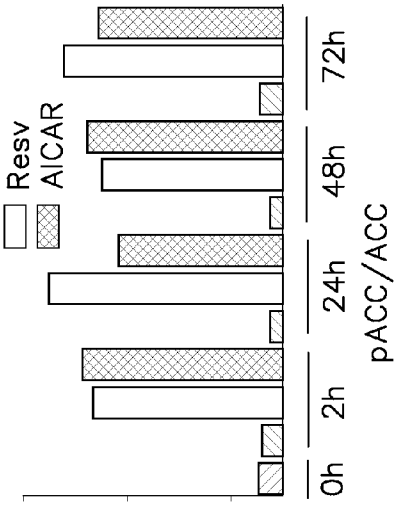


FIG. 10D

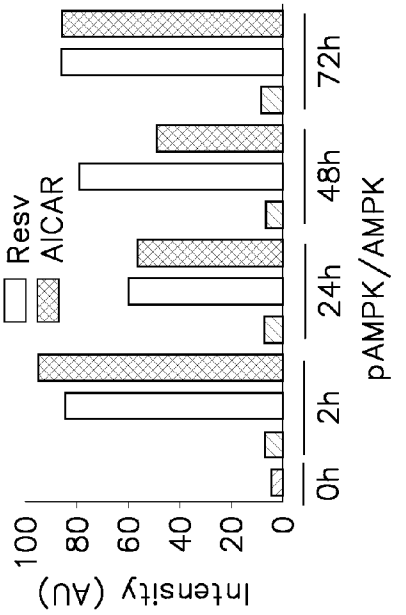


FIG. 10C

FIG. 11A

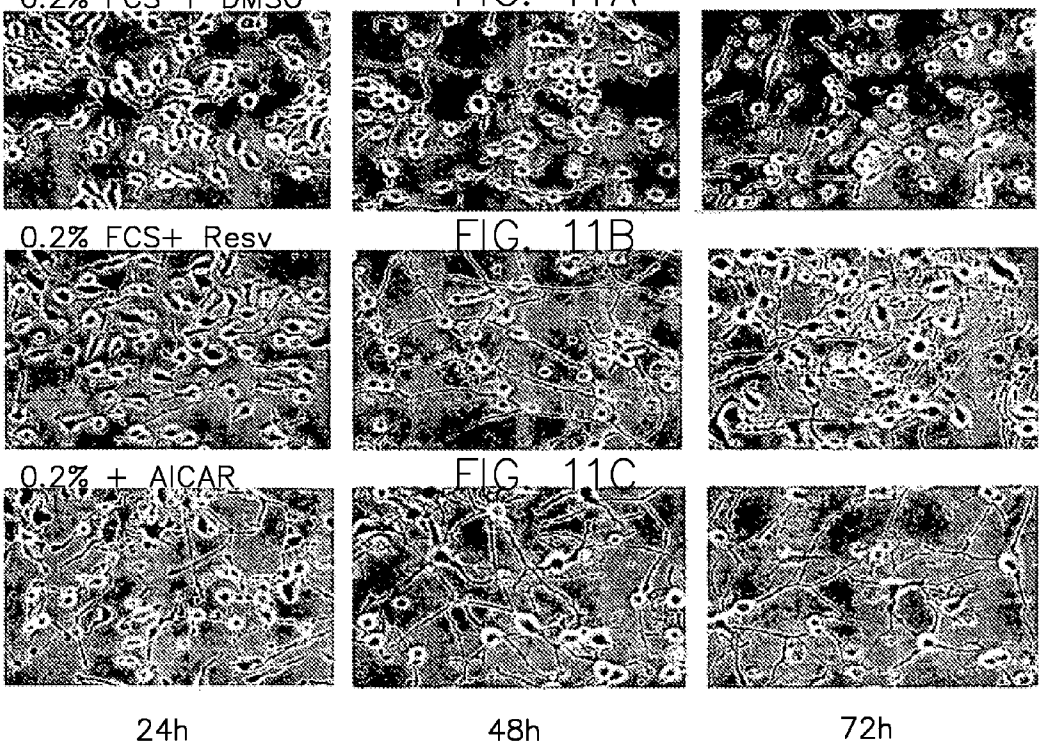


FIG. 11D

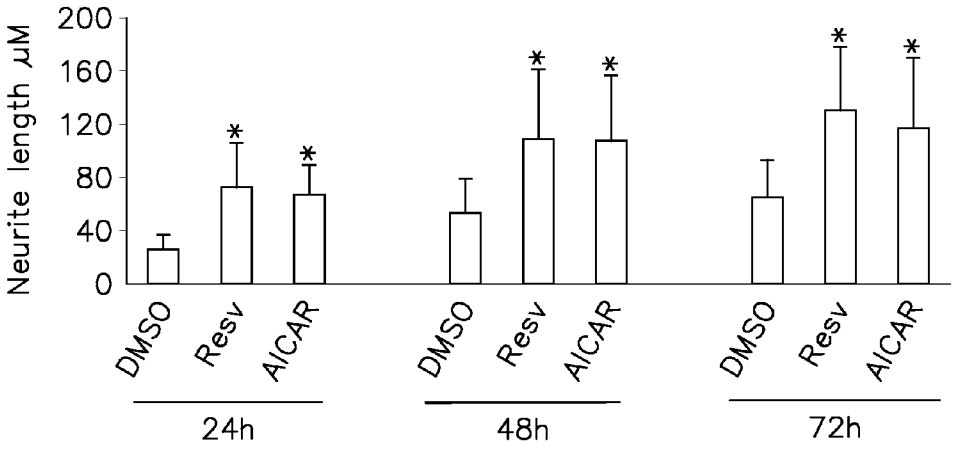


FIG. 12A FIG. 12B FIG. 12C FIG. 12D

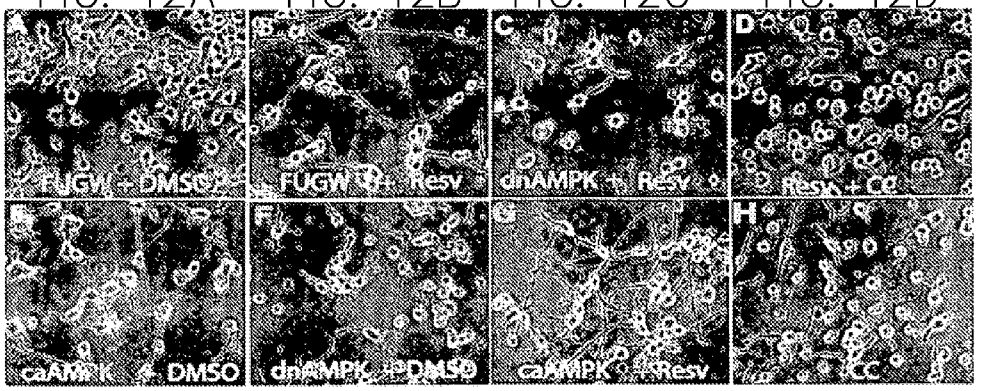


FIG. 12E FIG. 12F FIG. 12G FIG. 12H

FIG. 12I

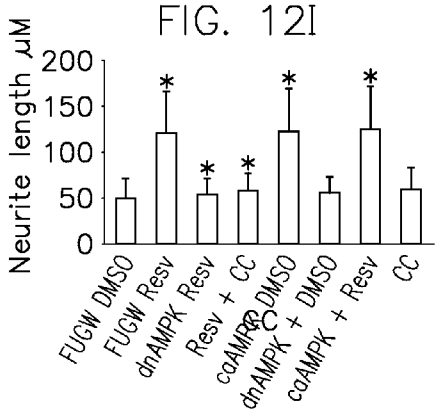


FIG. 12J

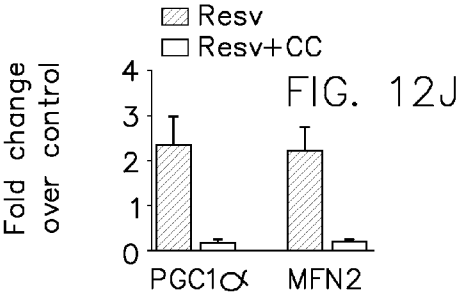


FIG. 12K

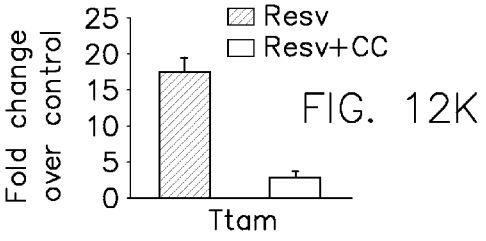


FIG. 12M

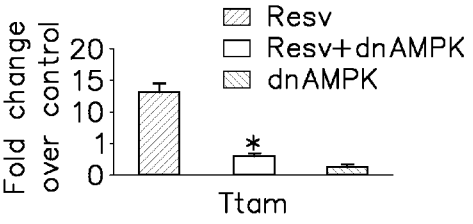
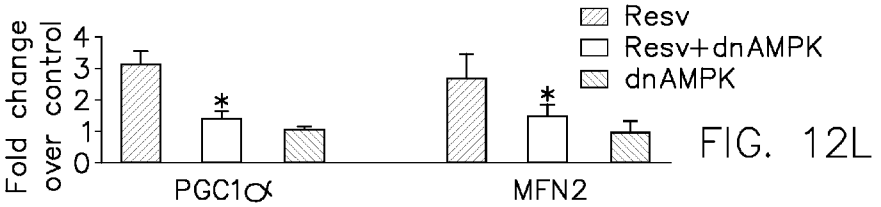


FIG. 12L



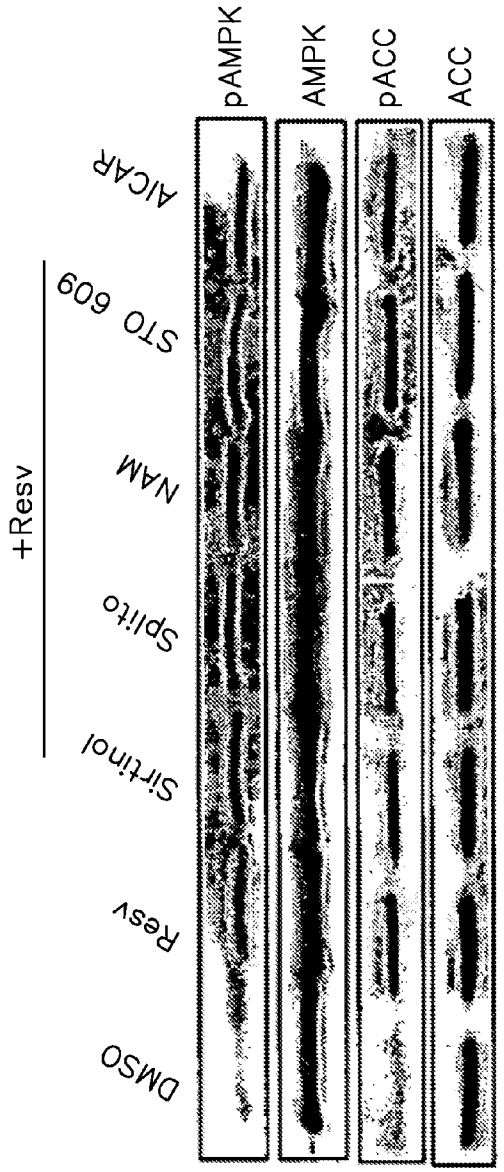


FIG. 13A

FIG. 13B

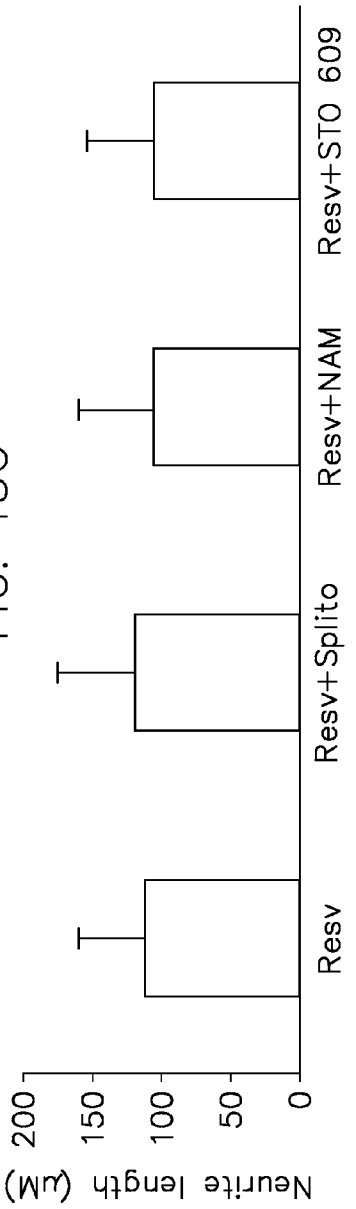


FIG. 13C

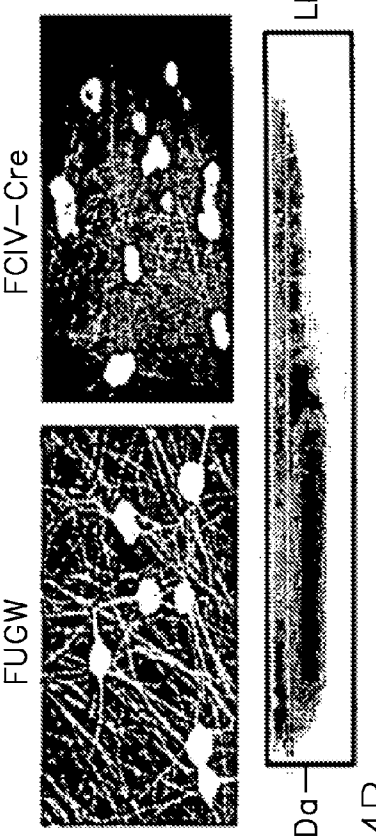


FIG. 14A

FIG. 14B

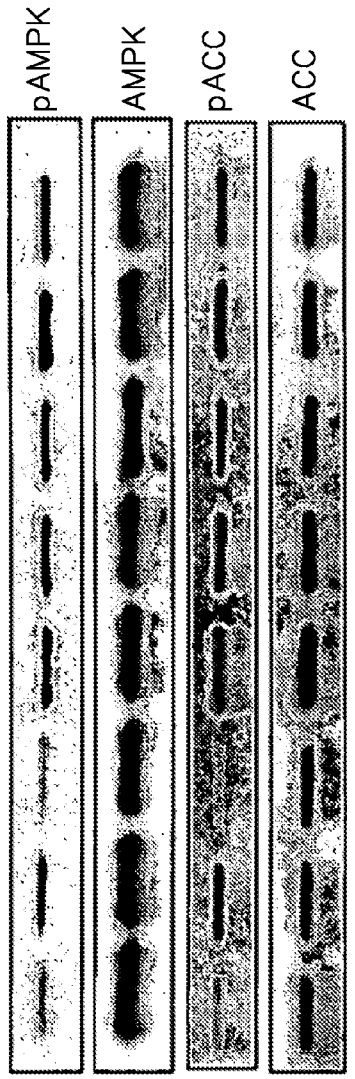


FIG. 14C

FIG. 14D

FIG. 14E

FIG. 14F

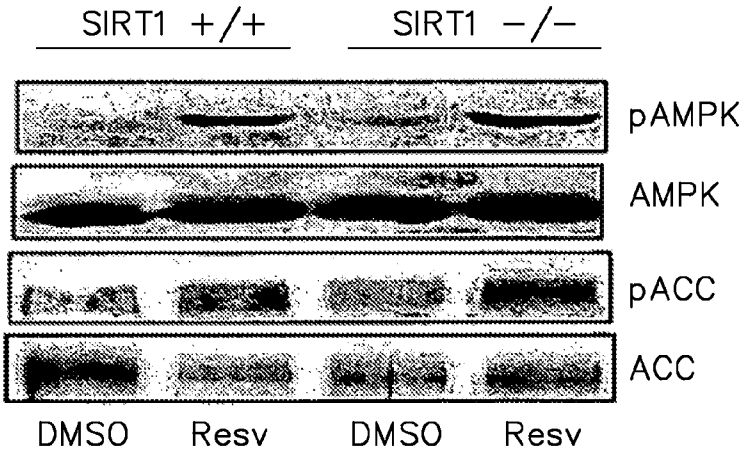


FIG. 14G

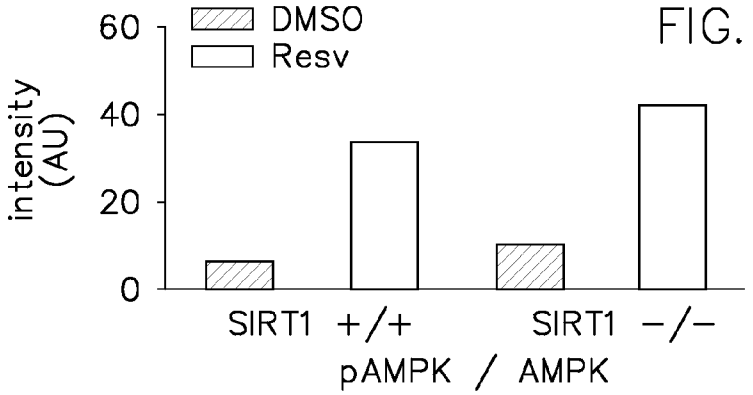
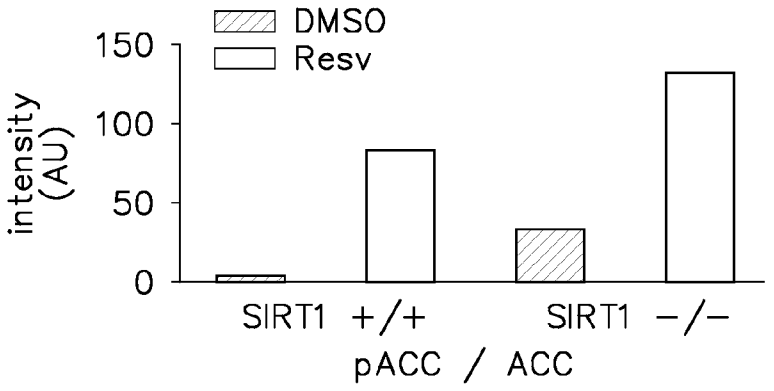


FIG. 14H



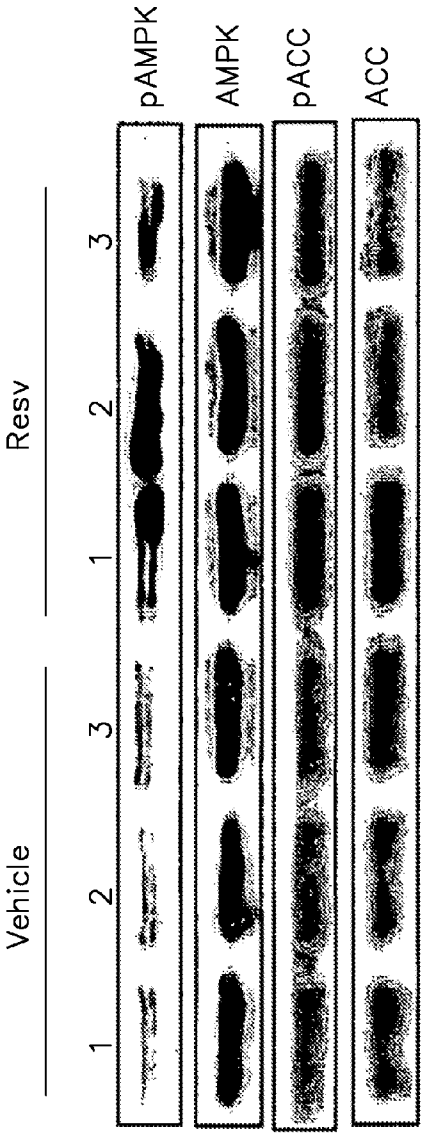


FIG. 15A

FIG. 15B

FIG. 15C

FIG. 15D

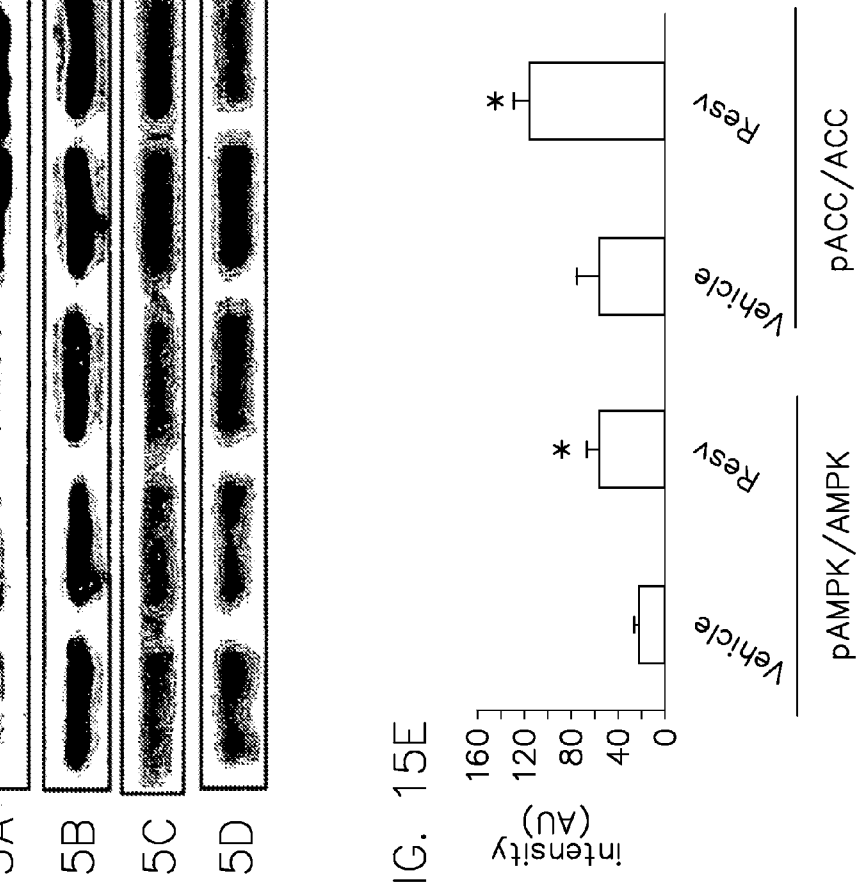


FIG. 15E

FIG. 16A

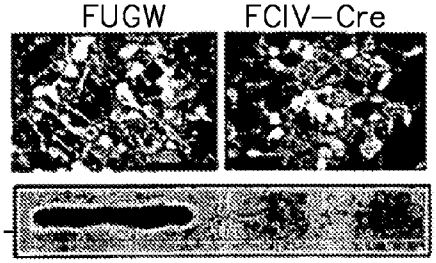


FIG. 16B

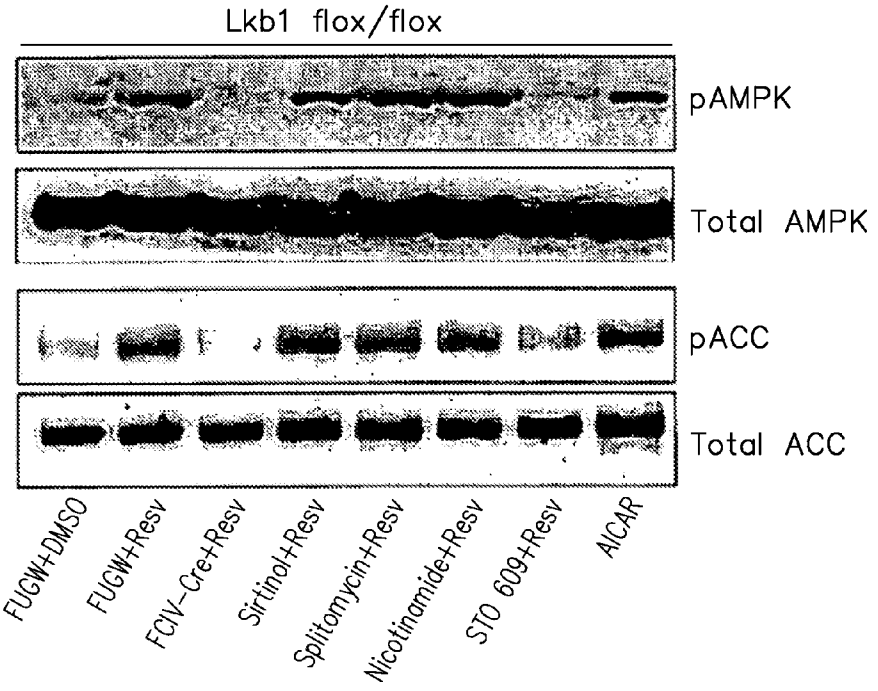


FIG. 16C

FIG. 16D

FIG. 16E

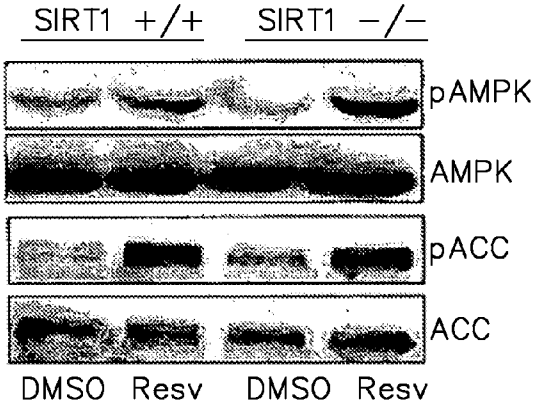


FIG. 16F

FIG. 17

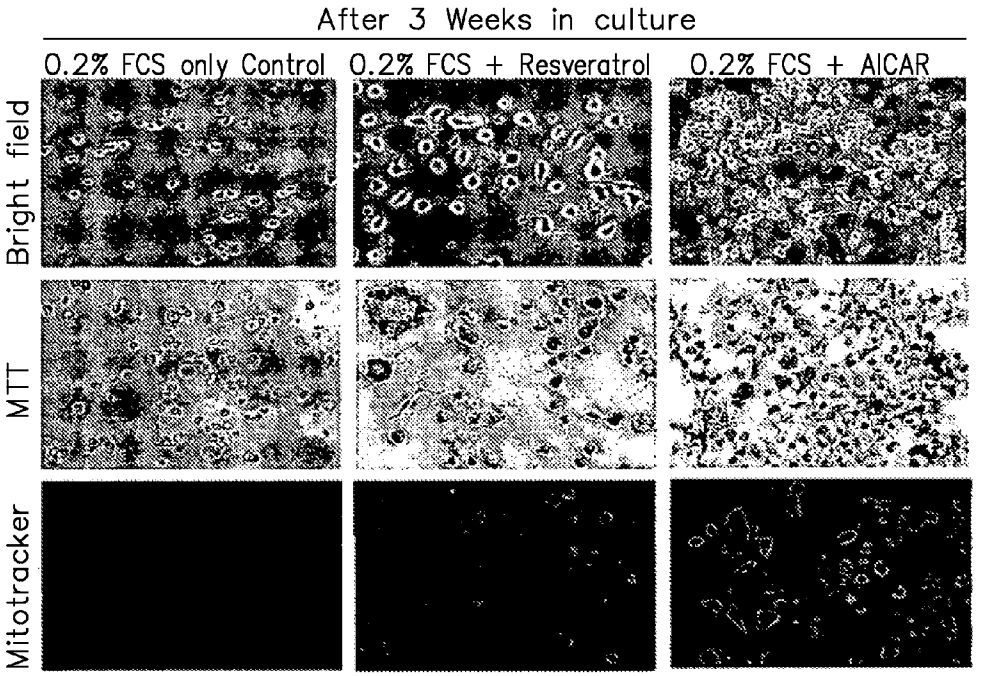
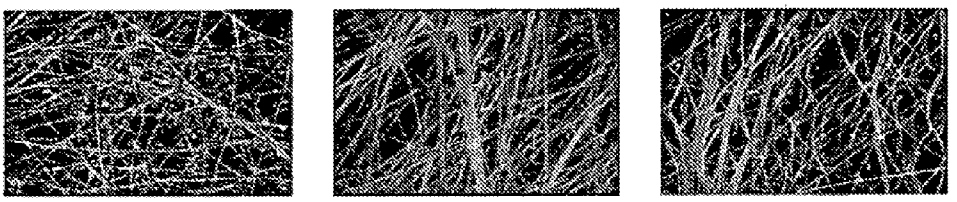

After 3 Weeks in culture
0.2% FCS only Control
0.2% FCS + Resveratrol
0.2% FCS + AICAR
Bright field
MTT
Mitotracker

FIG. 18


Hypoxia + DnAMPK
Hypoxia + AICAR
Hypoxia + CaAMPK

METHODS AND COMPOSITIONS FOR TREATING NEUROPATHIES

RELATED APPLICATION DATA

[0001] This application is a continuation of U.S. Non-Provisional application Ser. No. 12/524,718, filed on Oct. 20, 2009, which is a National Stage Entry of and claims benefit to PCT/US08/01085 with an international filing date of Jan. 28, 2008 and claims benefit under 35 U.S.C. §119(e) to U.S. Provisional Application Ser. No. 60/886,854 filed Jan. 26, 2007. The disclosures of the applications above are incorporated herein by reference in their entirety.

GOVERNMENT INTERESTS

[0002] This invention was made with government support under U.S.P.H.S. 5RO1 NS40745; NIH Neuroscience Blueprint Core Grant NSO57105; and NIH Grants AG13730 and NS39358. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0003] The Sequence Listing, which is a part of the present disclosure, includes a computer readable form comprising nucleotide and/or amino acid sequences of the present invention submitted via EFS-Web. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD

[0004] This invention relates generally to diseases and conditions involving neurons and, more particularly, to methods and compositions for treating or preventing neuropathies and other diseases and conditions involving neurodegeneration. Also included are methods of identifying agents for treating or preventing neuropathies.

BACKGROUND

[0005] Axon degeneration occurs in a variety of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases as well as upon traumatic, toxic or ischemic injury to neurons. Such diseases and conditions are associated with axonopathies including axonal dysfunction. One example of axonopathy is Wallerian degeneration (Waller, Philos Trans R. soc. Lond. 140:423-429, 1850), which occurs when the distal portion of the axon is severed from the cell body. The severed axon rapidly succumbs to degeneration. Axonopathy can, therefore, be a critical feature of neuropathic diseases and conditions and neurological disorders and axonal deficits can be an important component of a patient's disability.

SUMMARY

[0006] Accordingly, the present inventors have succeeded in discovering that axonal degeneration can be diminished or prevented by increasing, separately or in combination, NAD activity, sirtuin activity, AMP activated kinase (AMPK) activity, LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons. These discoveries can also be used in various combinations with treatments employing other known mechanisms.

[0007] It is believed that the increased NAD activity can act to increase sirtuin activity which then produces a decrease in axonal degeneration of injured neuronal cells. Thus, one approach to preventing axonal degeneration can be by activating sirtuin molecules, i.e. SIRT1 in injured mammalian axons. The activation of SIRT1 can be through direct action on the SIRT1 molecule or by increasing the supply of nicotinamide adenine dinucleotide (NAD) which acts as a substrate for the histone/protein deacetylase activity of SIRT1. The activation of SIRT1 results in a decrease in severity of axonal degeneration or a prevention of axonal degeneration. It is also believed possible that the increase in NAD activity could act through other mechanisms not involving sirtuin. Thus, increasing NAD activity, which may act through increasing SIRT1 activity or through one or more other mechanisms or both can diminish or prevent axonal degeneration in injured mammalian axons.

[0008] In addition, the inventors found that axonal degeneration can be prevented or decreased in severity by increasing AMPK activity, LKB1 activity and/or calcium/calmodulin-dependent protein kinase β (CaMKKβ) activity in diseased or injured neurons. In addition, the inventors demonstrated that the polyphenol compound resveratrol is a potent stimulator of AMPK and that activity of LKB1, an upstream regulator of AMPK, is required for this AMPK stimulation. In addition, the inventors found that increased AMPK activity is neuroprotective, and furthermore promotes axonal growth.

[0009] Thus, in various aspects, the present teachings disclose methods of treating or preventing a neuropathy or axonopathy in a mammal and, in particular, in a human in need thereof. In various configurations, the methods can comprise administering an effective amount of an agent that acts to increase AMPK activity and/or LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons. In some other configurations, the methods can comprise selecting an agent on the basis of having a property of effecting an increase in AMPK activity and/or LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons upon administration, and administering an effective amount of the agent.

[0010] Thus, in various embodiments, the present teachings disclose methods of treating or preventing a neuropathy in a mammal and, in particular, in a human in need thereof. These methods can comprise administering an effective amount of an agent that acts to increase sirtuin activity and, in particular, SIRT1 activity in diseased and/or injured neurons.

[0011] In various embodiments, the agent can increase SIRT1 activity through increasing NAD activity. It is believed that increasing NAD activity can increase sirtuin activity because NAD can act as a substrate of SIRT1. Such agents can include NAD or NADH, a precursor of NAD, an intermediate in the NAD salvage pathway or a substance that generates NAD such as a nicotinamide mononucleotide adenylyltransferase (NMNAT) or a nucleic acid encoding a nicotinamide mononucleotide adenylyltransferase. The nicotinamide mononucleotide adenylyltransferase can be an NMNAT1 protein.

[0012] In various embodiments, the agent can also act to directly increase SIRT1 activity and as such, the agent can be a sirtuin polypeptide or a nucleic acid encoding a sirtuin polypeptide or, to increase SIRT1 activity or to increase AMPK and/or LKB1 and/or CaMKKβ activity, a substance such as a stilbene, a chalcone, a flavone, an isoflavanone, a flavanone or a catechin. Such compounds can include a stilbene selected from the group consisting of resveratrol, piceatannol, deoxyrhapontin, trans-stilbene and rhapontin; a chal-

cone selected from the group consisting of butein, isoliquiritigenin and 3,4,2',4',6'-pentahydroxychalcone; a flavone selected from the group consisting of fisetin, 5,7,3',4', 5'-pentahydroxyflavone, luteolin, 3,6,3,4'-tetrahydroxyflavone, quercetin, 7,3',4',5'-tetrahydroxyflavone, kaempferol, 6-hydroxyapigenin, apigenin, 3,6,2',4'-tetrahydroxyflavone, 7,4'-dihydroxyflavone, 7,8,3',4'-tetrahydroxyflavone, 3,6,2', 3'-tetrahydroxyflavone, 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 5,7-dihydroxyflavone, morin, flavone and 5-hydroxyflavone; an isoflavone selected from the group consisting of daidzein and genistein; a flavanone selected from the group consisting of naringenin, 3,5,7,3',4'-pentahydroxyflavanone, and flavanone or a catechin selected from the group consisting of (−)-epicatechin, (−)-catechin, (−)-gallocatechin, (+)-catechin and (+)-epicatechin. In some configurations, the compound can be a resveratrol, fisetin, butein, piceatannol or quercetin. In some configurations, the compound is a resveratrol.

[0013] In various aspects, the present teachings also include methods of treating a neuropathy. In some configurations, these methods include administering to a mammal, such as a human in need of treatment, an effective amount of an agent that acts by increasing NAD activity in diseased and/or injured neurons and/or supporting cells such as, for example, glia, muscle cells and/or fibroblasts.

[0014] In some configurations, an agent of these aspects can be NAD or NADH, nicotinamide mononucleotide, nicotinic acid mononucleotide or nicotinamide riboside or derivatives thereof; an enzyme that generates NAD such as a nicotinamide mononucleotide adenylyltransferase; a nucleic acid encoding an enzyme that generates NAD such as a nucleic acid encoding a nicotinamide mononucleotide adenylyltransferase; an agent that increases expression of a nucleic acid encoding an enzyme in a pathway that generates NAD or an agent that increases activity and/or stability of an enzyme in a pathway that generates NAD or an agent that increases NAD activity. The nicotinamide mononucleotide adenylyltransferase can be an NMNAT1 protein.

[0015] In various aspects, the present teachings include methods of treating or preventing an optic neuropathy in a mammal in need thereof. In various configurations, the mammal can be a human, and the methods can comprise administering to the mammal an effective amount of an agent that acts at least in part by increasing AMPK activity, and/or LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons. In some aspects, the administering to the mammal can comprise administering to an eye. In some aspects, the administering can comprise administering the agent using a sustained-release delivery system, such as, without limitation, administering to the eye a sustained-release pellet comprising the agent.

[0016] In various aspects, the present teachings also include methods of treating or preventing an optic neuropathy in a mammal in need thereof. These methods can comprise administering to the mammal an effective amount of an agent that acts by increasing NAD activity in diseased and/or injured neurons. Administering to the mammal can comprise administering to the eye, in particular by administering the agent with a sustained release delivery system or by administering a sustain release pellet comprising the agent to the eye.

[0017] In various configurations, the agent can be NAD, NADH, nicotinamide mononucleotide, nicotinic acid mononucleotide or nicotinamide riboside; or an enzyme that generates NAD such as a nicotinamide mononucleotide adenylyltransferase; or a nucleic acid encoding an enzyme that generates NAD such as a nucleic acid encoding a nicotinamide mononucleotide adenylyltransferase or an agent that increases NAD activity. The nicotinamide mononucleotide adenylyltransferase can be an NMNAT1 protein or an NMNAT3 protein.

[0018] In various aspects of methods of the present teachings, the neuropathy associated with axonal degradation can be any of a number of neuropathies such as, without limitation, a disease that is hereditary, a congenital disease, Parkinson's disease, Alzheimer's disease, Herpes infection, diabetes, amyotrophic lateral sclerosis, a demyelinating disease such as multiple sclerosis, a seizure disorder, ischemia, stroke, chemical injury, thermal injury, or AIDS.

[0019] In various embodiments, the present invention is also directed to methods of screening agents for treating a neuropathy in a mammal. These methods can comprise administering a candidate agent to neuronal cells in vitro or in vivo, producing an axonal injury to the neuronal cells and detecting a decrease in axonal degeneration of the injured neuronal cells. In some aspects, the candidate agent can be an agent that acts at least in part by increasing AMPK activity, LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons and/or supporting cells. In various aspects, methods of screening agents can comprise detecting an increase in AMPK activity. LKB1 activity, CaMKKβ activity and/or activity of AMPK downstream effector acetyl Co-A carboxylase (ACC) following administration of a candidate agent to cells, tissues and/or organisms in vitro or in vivo, in particular, to one or more neuronal cells in vitro or in vivo. In other aspects, a method can comprise detecting an increase in NAD activity produced by a candidate agent, in one or more cells and, in particular, in one or more neuronal cells, in vitro or in vivo. In some configurations, an increase in NAD activity can be an increase in nuclear NAD activity.

[0020] Methods are also provided for screening agents that increase sirtuin activity in neurons as well as for screening agents that increase NAD biosynthetic activity in neurons. The methods can comprise administering to mammalian neuronal cells in vitro or in vivo a candidate agent, producing an axonal injury to the neuronal cells and detecting a decrease in axonal degeneration of the injured neuronal cells. Such methods can further comprise, in some aspects, secondary assays which further delineate AMPK activity, LKB1 activity and/or CaMKKβ activity, with sirtuin activity, NAD and enzymes or components of NAD biosynthetic or salvage pathways, or various combinations thereof:

[0021] In various configurations of the screening methods of the present teachings, axonal injury can be produced by various methods of injuring neuronal cells, such as chemical injury, metabolic injury, genetic impairment, altering mitochondrial activity, thermal injury, oxygen-deprivation, and/or mechanical injury.

[0022] A recombinant vector is also provided in various aspects of the present teachings. A vector of these aspects can comprise a promoter operatively linked to a sequence encoding a mammalian NMNAT1 protein or NMNAT3 protein, such as a human NMNAT1 protein or NMNAT3 protein. In various aspects of such embodiments, the recombinant vector can be a lentivirus or an adeno-associated virus.

[0023] Also provided in various aspects, is a recombinant vector comprising a promoter operatively linked to a

sequence encoding a SIRT1 protein. In various configurations of these aspects, a recombinant vector can be a lentivirus or an adeno-associated virus.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] FIG. **1** illustrates that NMNAT1 activity of the Wlds fusion protein produces a delayed degeneration of injured axons showing: A) in vitro Wallerian degeneration in lentivirus-infected dorsal root ganglia (DRG) neuronal explant cultures expressing Wlds protein or EGFP wherein tubulin βIII-immunoreactive neurites are shown before transection and 12, 24, 48, and 72 hr after transection (Scale Bar=1 mm and the "*" denotes the location of the cell bodies prior to removal; and B) in vitro Wallerian degeneration in lentivirus-infected DRG neurons expressing EGFP only, Wlds protein, Ufd2a portion (70 residues) of Wlds protein fused to EGFP (Ufd2a(1-70)-EGFP), Ufd2a(1-70)-EGFP with C-terminal nuclear localization signal, NMNAT1 portion of Wlds protein fused to EGFP, dominant-negative Ufd2a (Ufd2a(P1140A)), or Ufd2a siRNA construct in which representative images of neurites and quantitative analysis data of remaining neurite numbers (percentage of remaining neurites relative to pre-transection±S.D.) at the indicated time-point with each construct (bottom left) are shown and the "*" indicates significant difference ($p<0.0001$) with EGFP-infected neurons; also showing EGFP signal before transection confirming transgene expression (bottom row; Scale bar=50 μm) and immunoblot analysis confirming protein expression by lentiviral gene transfer and siRNA downregulation of Ufd2a protein (bottom right panels).

[0025] FIG. **2** illustrates that increased NAD supply protects axons from degeneration after injury showing: A) Enzymatic activity of wild type and mutant Wlds and NMNAT1 proteins in which lysates were prepared from HEK293 cells expressing the indicated protein were assayed for NAD production using nicotinamide mononucleotide as a substrate and the amount of NAD generated in 1 h was converted to NADH, quantified by fluorescence intensity, and normalized to total protein concentration showing that both mutants have essentially no enzymatic activity; and B) In vitro Wallerian degeneration in lentivirus-infected DRG neurons expressing NMNAT1 or Wlds protein, mutants of these proteins that lack NAD-synthesis activity NMNAT1(W170A) and Wlds (W258A), or EGFP wherein the bar chart shows the quantitative analysis data of the number of remaining neurites at indicated time-point for each construct (percentage of remaining neurites relative to pre-transection±S.D.) and the "*" indicates significant difference ($p<0.0001$) with EGFP-infected neurons; C) Protein expression in lentivirus-infected cells detected by immunoblot analysis using antibodies to the 6×His tag; and D) DRG neuronal explant expressing either NMNAT1 or EGFP (control) cultured with 0.5 μM vincristine wherein representative images of neurites (phase-contrast; Bar=1 mm) are shown at the indicated times after vincristine addition and quantification of the protective effect at the indicated time points is plotted as the area covered by neurites relative to that covered by neurites prior to treatment.

[0026] FIG. **3** illustrates that axonal protection requires pre-treatment of neurons with NAD prior to injury showing: A) in vitro Wallerian degeneration using DRG explants cultured in the presence of various concentrations of NAD added 24 hr prior to axonal transection; and B) DRG explants pre-incubated with 1 mM NAD for 4, 8, 12, 24, or 48 h prior to transection wherein the bar chart shows the number of remaining neurites in each experiment (percentage of remaining neurites relative to pre-transection±S.D.) at each of the indicated time points and the "*" indicates significant axonal protection compared to control ($p<0.0001$).

[0027] FIG. **4** illustrates that NAD-dependent Axonal Protection is mediated by SIRT1 activation showing: A) In vitro Wallerian degeneration using DRG explant cultures preincubated with 1 mM NAD alone (control) or in the presence of either 100 μM Sirtinol (a Sir2 inhibitor) or 20 mM 3-aminobenzimide (3AB, a PARP inhibitor); B) in vitro Wallerian degeneration using DRG explant cultures incubated with resveratrol (10, 50 or 100 μM); and C) left: in vitro Wallerian degeneration using DRG explant cultures infected with lentivirus expressing siRNA specific for each member of the SIRT family (SIRT1-7) wherein the bar chart shows the quantitative analysis of the number of remaining neurites (percentage of remaining neurites relative to pre-transection±S.D.) at indicated time-point for each condition and the "*" indicates points significantly different than control (<0.0001); middle table: The effectiveness of each SIRT siRNA (expressed as % of wild type mRNA level) using qRT-PCR in infected NIH3T3 cells; and right: immunoblot using antibodies to SIRT1 to show decreased expression of SIRT1 in the presence of SIRT1 siRNA which effectively blocked NAD dependent axonal protection.

[0028] FIG. **5** illustrates the mammalian NAD biosynthetic pathway in which predicted mammalian NAD biosynthesis is illustrated based on the enzymatic expression analysis and studies from yeast and lower eukaryotes (Abbreviation used; QPRT, quinolinate phosphoribosyltransferase; NaPRT, nicotinic acid phosphoribosyltransferase; NmPRT, nicotinamide phosphoribosyltransferase; Nrk, nicotinamide riboside kinase; NMNAT, nicotinamide mononucleotide adenylyltransferase; QNS, NAD synthetase)

[0029] FIG. **6** illustrates expression analysis of NAD biosynthetic enzymes in mammal showing (A) NAD biosynthesis enzyme mRNA levels after 1, 3, 7, and 14 days after nerve transection in rat DRG were determined by qRT-PCR in which the expression level was normalized to glyceraldehydes-3-phosphate dehydrogenase expression in each sample and is indicated relative to the expression level in non-axotomized DRG; (B) neurite degeneration introduced by incubation DRG in 1 or 0.1 μM rotenone for indicated time and NAD synthesis enzyme mRNA levels were determined by qRT-PCR as described in the text.

[0030] FIG. **7** illustrates the subcellular localization of NMNAT enzymes and their ability to protect axon showing (A) in vitro Wallerian degeneration assay using lentivirus infected DRG neuronal explant cultures expressing NMNAT1, cytNMNAT1, NMNAT3, or nucNMNAT3 in which representative pictures taken at 12 and 72 hours after transaction are shown; (B) Subcellular localization of NMNAT1, cytNMNAT1, NMNAT3, or nucNMNAT3 in HEK 293T cells using immunohistochemistry with antibody against 6×His tag to detect each proteins and staining of the cells with the nuclear marker dye (bisbenzimide) for comparison to determine the nuclear vs. cytoplasmic location of each protein (Scale bar=25 μm); (C) enzymatic activity of wild type and mutant NMNAT1 and NMNAT3 in which 6×His tagged each protein was purified from lysate of HEK293T cells expressing NMNAT1, cytNMNAT1, NMNAT3, nucNMNAT3 in which the amount of NAD generated after 1 hour at 37 deg was converted NADH, quantified and normalized to protein concentration; (D) protein expres-

sion of NMNAT1, cytNMNAT1, NMNAT3, and nucNMNAT3 by lentivirus gene transfer confirmed by immunoblot analysis of HEK293T cells infected with each of the virus and (E) in vitro Wallerian degeneration assay using lentivirus infected DRG neuronal explant cultures expressing NMNAT1, cytNMNAT1, NMNAT3, or nucNMNAT3 showing quantitative analysis data of remaining neurite numbers at 12, 24, 48, and 72 hours after axotomy.

[0031] FIG. **8** illustrates exogenous application of NAD biosynthetic substrates and their ability to protect axon showing (A) in vitro Wallerian degeneration assay using DRG neuronal explant cultures after exogenous application of NAD, NmR with representative pictures taken at 12, 24, 48, and 72 hours after transaction are shown; (B) in vitro Wallerian degeneration assay using DRG neuronal explant cultures after exogenous application of Na, Nam, NaMN, NMN, NaAD, NAD, and NmR showing quantitative analysis data of remaining neurite numbers at 12, 24, 48, and 72 hours after axotomy are shown; (C) DRG neuronal explants infected with NaPRT expressing lentivirus and incubated with or without 1 mM of Na for 24 hours before axotomy, in in vitro Wallerian degeneration assay showing quantitative analysis data of remaining neurite numbers at 12, 24, 48, and 72 hours after axotomy.

[0032] FIG. **9** illustrates optic nerve transection after intravitreal injection of NAD biosynthetic substrates NAD, NMN, NmR, or Nam was injected into intravitreal compartment of left rat eye and allowed to incorporate retinal ganglion cells for 24 hours after which, left optic nerve was transected by eye enucleation and right and left optic nerves were collected at 4 days after transection and analyzed by Western blotting in which optic nerves transected from mice without any treatment prior to axotomy were used for negative control; showing in the figure, the quantitative analysis data of percentage of remaining neurofilament immunoreactivity from transected optic nerve relative to non-transected±S.D.

[0033] FIG. **10** illustrates resveratrol activation of AMP kinase in Neuro2a cells. Neuro2a cells were switched to serum-starvation medium (medium containing 0.2% FCS) and treated with DMSO (vehicle control), resveratrol (10 μM) or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (1 mM). (A) Both resveratrol and AICAR stimulated AMPK phosphorylation by 2 hr and maintained the phosphorylated state through the 72 hr test period. (B) Both resveratrol and AICAR promoted increased phosphorylation of the AMPK downstream effector acetyl Co-A carboxylase (ACC) at all time points tested. Very low levels of phosphorylated AMPK and ACC were detected in DMSO treated control cells at all time points tested. (C) Densitometric analysis of changes in phosphorylated AMPK and (D) phosphorylated ACC under different conditions is shown.

[0034] FIG. **11** illustrates the resveratrol and AICAR stimulation of neurite outgrowth in Neuro2a cells. Neuro2a cells were switched to serum-starvation medium (medium containing 0.2% FCS) and treated with DMSO (vehicle control), resveratrol (10 μM) or AICAR (1 mM). (A) Serum deprivation of Neuro2a cells results in growth of short neurites that increase in length over 72 h. (B) Resveratrol induced rapid neurite outgrowth resulting in elaborate neurite network formation by 48 h. (C) AICAR stimulated extensive neurite outgrowth similar to that observed with resveratrol. (D). Quantification of neurite length showed significantly longer neurites in resveratrol or AICAR treated cells compared with Neuro2a cells grown in 0.2% serum alone ($p<0.001$).

[0035] FIG. **12** illustrates the dependency of resveratrol-induced neurite outgrowth and mitochondrial biogenesis on AMPK. Neuro2a cells were infected with lentivirus expressing GFP only (FUGW, control), dominant negative AMPK (dnAMPK) or constitutively active AMPK (caAMPK). Three days later cells were shifted to serum-starvation medium containing DMSO (control) or resveratrol (10 μM). In addition, uninfected Neuro2a cells in serum-starvation medium were treated with resveratrol alone, the AMPK inhibitor Compound C(CC, Biomol International L.P. (Plymouth Meeting, Pa.)) (10 μM) alone or with resveratrol and CC together. Images of the cultures captured in bright field and green fluorescence were overlaid to visualize neurite outgrowth. Resveratrol treated cells demonstrated robust neurite outgrowth (B) that was blocked by inhibition of AMPK using dominant-negative AMPK (dnAMPK) (C) or Compound C (D). Conversely, cells infected with constitutively active AMPK (caAMPK) demonstrated increased neurite outgrowth in the absence of resveratrol (compare E vs. A). dnAMPK alone (F) or CC alone (H) did not inhibit neurite out growth by themselves. Neurite length in cells treated with caAMPK+resveratrol is also shown (G). Quantitative analysis of average neurite length (I) showed significant (denoted by asterisks) neurite outgrowth inhibition by AMPK inhibition ($p<0.001$) and neurite outgrowth promotion by caAMPK ($p<0.005$). Quantitative RT-PCR analysis of markers of mitochondrial biogenesis demonstrated that resveratrol treatment resulted in an 18-fold increase in Tfam (K) and 2-fold increases in PGC-1α and MFN2 mRNA levels (J), Values were normalized to the 18S transcript. Data shown is representative of two independent experiments.

[0036] FIG. **13** illustrates that resveratrol-mediated AMPK activation and neurite outgrowth is independent of SIRT1 and CaMKKβ in Neuro2a cells. Phospho-specific antibodies were used to assess activation of AMPK and ACC in lysates of Neuro2a cells treated with DMSO (control) or resveratrol (10 μM) in the presence or absence of three SIRT1 inhibitors (Sirtinol, splitomycin, nicotinamide) or the CaMKKβ inhibitor STO 609 for 2 hr. Resveratrol induces rapid activation of AMPK (A) that occurs in concurrence with phosphorylation of ACC (B) and is not prevented by SIRT1 or CaMKKβ inhibitors. AICAR is included as a positive control for AMPK and ACC phosphorylation. Total AMPK and ACC are shown in the lower panels of (A) and (B). In (C), Neuro2a cells were allowed to differentiate in scrum-starvation medium containing resveratrol in the absence or presence of the SIRT1 inhibitors splitomycin (10 μM), nicotinamide (10 mM), sirtinol (10 μM, data not shown) or STO 609 (2.5 μM). No inhibition of neurite outgrowth was observed in the presence of either SIRT1 or CaMKKβ inhibitors.

[0037] FIG. **14** illustrates that AMPK activation by resveratrol in dorsal root ganglia sensory neurons requires Lkb1.

(A) Embryonic DRG neurons from Lkb1 flox/flox mice were infected with lentivirus expressing Cre recombinase (FCIV-Cre) or GFP only (FUGW control). Lentiviral infection was monitored by GFP fluorescence (A). A western blot using antibody against LKB1 demonstrates complete loss of Lkb1 in Cre-expressing neurons (B). Lkb1 flox/flox DRG neurons were infected with FUGW or FCIV-Cre and treated as indicated. AMPK or ACC was immunoprecipitated from neuronal lysates and western blots were probed with the respective phospho-specific antibodies. Resveratrol-mediated AMPK (C) and ACC phosphorylation (D) was significantly reduced upon Lkb1 excision by FCIV-Cre. No inhibition of

AMPK or ACC phosphorylation was observed by treatment with SIRT1 or CaMKKβ inhibitors. Lysate from AICAR treated DRG neurons was included as a positive control. (E, F) Embryonic DRG neurons were cultured from wild type and SIRT1-deficient littermates derived from SIRT1 heterozygous matings. Western blot analysis with phospho-specific antibodies revealed that resveratrol stimulated AMPK (E) and ACC phosphorylation (F) equivalently in wild type and SIRT1-deficient neurons. Levels of total AMPK and ACC are shown in the bottom panels of C, D, E and F. Densitometric analysis of changes in levels of phospho AMPK (6) and phospho ACC(H) in SIRT1+/+ and SIRT1−/− DRG neurons in presence and absence of resveratrol is shown. Note: Resv, resveratrol.

[0038] FIG. **15** illustrates that resveratrol treatment causes AMPK phosphorylation in the brain. Two-month-old male mice were injected intraperitoneally with resveratrol (20 mg/kg body weight) or DMSO (vehicle) (n=3, for each treatment). Two hr after treatment, the animals were sacrificed and brain lysates were prepared. Western analysis with AMPK and ACC phospho-specific antibodies showed increased levels of phosphorylated AMPK (A) and ACC (C) in brains of resveratrol treated animals. Total AMPK and ACC levels are shown as loading controls in B and D. (E) Densitometry was used to quantify the increased level of AMPK and ACC phosphorylation in the brain of resveratrol treated animals (*p<0.005).

[0039] FIG. **16** illustrates that resveratrol activates AMP kinase in cortical neurons through Lkb1 and CaMKKβ (A) Cortical neuron cultures were established from E 13.5 Lkb1 flox/flox embryos and infected with lentivirus expressing Cre recombinase (FCIV-Cre) or GFP only (FUGW control). Lentiviral infection of cortical neurons was monitored by GFP fluorescence (A). A western blot using LKB1 antibody demonstrates complete loss of Lkb1 in Cre-expressing cortical neurons (B). Lkb1 flox/flox cortical neurons were infected with FUGW or FCIV-Cre and treated as indicated. AMPK or ACC was immunoprecipitated from neuronal lysates and western blots were probed with the respective phospho-specific antibodies. Resveratrol-mediated AMPK (C) and ACC phosphorylation (D) was significantly reduced upon Lkb1 excision by FCIV-Cre and in neurons treated with the CaMKKβ inhibitor STO 609 (2.5 μM). No inhibition of resveratrol-stimulated AMPK or ACC phosphorylation was observed in neurons treated with SIRT1 inhibitors. Lysates from AICAR treated cortical neurons is included as a positive control. (E, F) Embryonic cortical neurons were cultured from wild type and SIRT1-deficient littermates derived from SIRT1 heterozygous matings. Western blot analysis with phospho-specific antibodies revealed that resveratrol-stimulated AMPK (E) and ACC phosphorylation (F) equivalently in wild type and SIRT1-deficient cortical neurons. Levels of total AMPK and ACC are shown in the bottom panels of C, D, E and F.

[0040] FIG. **17** illustrates that activation of the AMPK pathway by resveratrol or AICAR promotes the survival of neuronal cells in nutrient-deprived conditions. When Neuro2A cells are grown in culture in presence of 10% FCS, cells proliferate and ultimately die if cells are not passaged. If grown in the presence of 0.2% FCS, neuro2A cells differentiate and project neurites but ultimately die within one week. But when neuro2A cells are treated with either resveratrol or AICAR, regardless of the FCS concentration, these cells are resistant to death for a prolonged period of time. A subset of the surviving cells also exhibit robust neurite projections. These cells maintain mitochondrial integrity as observed by MTT cell viability assay (middle row) and by MitoTracker® (Invitrogen Corporation, Carlsbad Calif.) staining (bottom row). Mitochondrial movement was also observed in these 'saved' cells, indicating that several aspects of mitochondrial function are preserved by AMPK activators.

[0041] FIG. **18** illustrates that activation of AMPK protects axons while inhibition of AMPK function enhances axonal injury during oxygen deprivation. To elucidate AMPK function in dorsal root ganglia (DRG) sensory neurons during hypoxic metabolic stress, AMPK function was genetically inhibited by expressing a dominant negative AMPK (DnAMPK; which perturbs endogenous AMPK function) in DRG neurons by lentiviral transduction or was pharmacologically inhibited by pre-incubating DRG neurons with the AMPK inhibitor Compound C for 45 min prior to hypoxia (0.1% O2). Hypoxia for either 8 or 16 hrs was followed by 20 hrs of reoxygenation and this caused severe axonal injury (significant increase in axonal beading) in DRG neurons pre-incubated with Compound C or expressing DnAMPK (FIG. **18**). However no Caspase positive cells were observed suggesting that inhibition of endogenous AMPK function during hypoxia severely enhances axonal injury but does not cause apoptotic cell death.

[0042] To examine whether AMPK activation prior to hypoxic stress protects axons from injury, either constitutively active AMPK (CaAMPK) was expressed in DRG neurons, or neurons were preincubated with the AMPK activator AICAR for 45 min prior to hypoxia. CaAMPK or AICAR significantly reduced axonal injury after hypoxia (FIG. **18**). Thus, treatments that increase AMPK activity (e.g. resveratrol, AICAR) can block the axonal damage and are neuroprotective.

DETAILED DESCRIPTION

[0043] The present invention involves methods and compositions for treating neuropathies, neurodegenerative diseases, and other neurological disorders in which axonal degeneration is a component. The methods, in various embodiments, comprise administering to a mammal such as a human an effective amount of a substance that increases AMPK activity, LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons or supporting cells. In other embodiments, the methods can comprise administering to a mammal an effective amount of an agent that effects an increase in NAD activity in diseased and/or injured neurons or supporting cells. Without being limited by theory, it is believed that an increase in NAD activity can act to increase sirtuin activity which then produces a decrease in axonal degeneration of injured neuronal cells compared to axonal degeneration that occurs in injured neuronal cells not treated with the agent. Such a decrease in axonal degeneration can include a complete or partial amelioration of the injury to the neuron. In addition, it is also possible that an increase in NAD activity could act through mechanisms not involving sirtuin molecules to produce or to contribute to the production of a decrease in axonal degeneration. Moreover, an agent effective for treatment of diseased and/or injured neurons or supporting cells may act via several mechanisms, such as, for example, in the case of resveratrol, as shown below.

[0044] Seven known sirtuin molecules referenced as SIRT's make up the Sir2 family of histone/protein deacetylases in mammals and all such sirtuin molecules are included

within the scope of the present teachings. The seven human sirtuins, SIRT1-SIRT7, are NAD-dependent histone/protein deacetylases which are described more fully in connection with NCBI LocusLink ID Nos. 23411, 22933, 23410, 23409, 23408, 51548 and 51547, respectively (see http://www.ncbi.nlm.hih.gov/LocusLink/). Said NCBI LocusLink reference sites are hereby incorporated by reference. Amino acid sequences of human SIRT1-SIRT7 are set forth herein as SEQ ID NO: 1-SEQ ID NO: 7, respectively. In various embodiments, the methods and compositions of the present invention can increase activity of any one or more of the sirtuins and, in particular, various methods of the present teachings can lead to an increase of activity of SIRT1.

[0045] As used herein, activity of a particular substance can depend upon the concentration of the substance and the functional effectiveness of the substance. Activity of a substance can be increased by numerous factors including, for example, increasing synthesis, decreasing breakdown, increasing bioavailability of the substance or diminishing binding of the substance or otherwise increasing the available amount of free substance. Increasing functional effectiveness can result, for example, from a change in molecular conformation, a change in the conditions under which the substance is acting, or a change in sensitivity to the substance. Increasing activity with respect to sirtuin molecules is intended to mean increasing concentration or enhancing functional effectiveness or increasing the availability of NAD, increasing the flux through one or more biosynthetic pathways for NAD or any combination thereof. Reference to an agent or substance acting "at least in part" by a certain activity or mechanism indicates that the activity or mechanism represents at least one effect of administration of the agent or substance.

[0046] Neuropathies in various aspects of the present teachings can include any disease or condition involving neurons and/or supporting cells, such as, for example, glia, muscle cells, or fibroblasts. In some aspects of the present teachings, neuropathies include diseases or conditions involving axonal damage, i.e., axonopathies. Axonal damage can be caused by traumatic injury or by non-mechanical injury due to diseases or conditions and the result of such damage can be degeneration or dysfunction of an axon and loss of functional neuronal activity. Disease and conditions producing or associated with such axonal damage are among a large number of neuropathic diseases and conditions. Such neuropathies can include peripheral neuropathies, central neuropathies, and combinations thereof. Furthermore, peripheral neuropathic manifestations can be produced by diseases focused primarily in the central nervous systems, and central nervous system manifestations can be produced by essentially peripheral or systemic diseases.

[0047] Peripheral neuropathies involve damage to the peripheral nerves and such can be caused by diseases of the nerves or as the result of systemic illnesses. Some such diseases can include diabetes, uremia, infectious diseases such as AIDs or leprosy, nutritional deficiencies, vascular or collagen disorders such as atherosclerosis, and autoimmune diseases such as systemic lupus erythematosus, scleroderma, sarcoidosis, rheumatoid arthritis, and polyarteritis nodosa. Peripheral nerve degeneration can also result from traumatic, i.e. mechanical damage to nerves as well as chemical or thermal damage to nerves. Such conditions that injure peripheral nerves include compression or entrapment injuries such as glaucoma, carpal tunnel syndrome, direct trauma, penetrating injuries, contusions, fracture or dislocated bones; pressure involving superficial nerves (ulna, radial, or peroneal) which can result from prolonged use of crutches or staying in one position for too long, or from a tumor; intraneural hemorrhage; ischemia; exposure to cold or radiation or certain medicines or toxic substances such as herbacides or pesticides. In particular, the nerve damage can result from chemical injury due to a cytotoxic anticancer agent such as, for example, a vinca alkaloid such as vincristine. Typical symptoms of such peripheral neuropathies include weakness, numbness, paresthesia (abnormal sensations such as burning, tickling, pricking or tingling) and pain in the arms, hands, legs and/or feet. The neuropathy can also be associated with mitochondrial dysfunction. Such neuropathies can exhibit decreased energy levels, i.e., decreased levels of NAD and ATP.

[0048] A peripheral neuropathy can also be a metabolic and endocrine neuropathy which includes a wide spectrum of peripheral nerve disorders associated with systemic diseases of metabolic origin. Some non-limiting examples of these diseases include diabetes mellitus, hypoglycemia, uremia, hypothyroidism, hepatic failure, polycythemia, amyloidosis, acromegaly, porphyria, disorders of lipid/glycolipid metabolism, nutritional/vitamin deficiencies, and mitochondrial disorders, among others. The common hallmark of these diseases is involvement of peripheral nerves by alteration of the structure or function of myelin and axons due to metabolic pathway dysregulation.

[0049] Neuropathies also include optic neuropathies such as glaucoma; retinal ganglion degeneration such as those associated with retinitis pigmentosa and outer retinal neuropathies; optic nerve neuritis and/or degeneration including that associated with multiple sclerosis; traumatic injury to the optic nerve which can include, for example, injury during tumor removal; hereditary optic neuropathies such as Kjer's disease and Leber's hereditary optic neuropathy; ischemic optic neuropathies, such as those secondary to giant cell arteritis; metabolic optic neuropathies such as neurodegenerative diseases including Leber's neuropathy mentioned earlier, nutritional deficiencies such as deficiencies in vitamins B12 or folic acid, and toxicities such as due to ethambutol or cyanide; neuropathies caused by adverse drug reactions and neuropathies caused by vitamin deficiency. Ischemic optic neuropathies also include non-arteritic anterior ischemic optic neuropathy.

[0050] Neurodegenerative diseases that are associated with neuropathy or axonopathy in the central nervous system include a variety of diseases. Such diseases include those involving progressive dementia such as, for example, Alzheimer's disease, senile dementia, Pick's disease, and Huntington's disease; central nervous system diseases affecting muscle function such as, for example, Parkinson's disease; motor neuron diseases and progressive ataxias such as amyotrophic lateral sclerosis; demyelinating diseases such as, for example multiple sclerosis; viral encephalitides such as, for example, those caused by enteroviruses, arboviruses, and herpes simplex virus; and prion diseases. Mechanical injuries such as glaucoma or traumatic injuries to the head and spine can also cause nerve injury and degeneration in the brain and spinal cord. In addition, ischemia and stroke as well as conditions such as nutritional deficiency and chemical toxicity such as with chemotherapeutic agents can cause central nervous system neuropathies.

[0051] Additional manifestations within the scope of the neurological conditions which can be treated or ameliorated

by the methods of the present teachings include convulsions and seizures, e.g., those associated with epilepsy, migraine, syncope, bipolar disorder, psychosis, anxiety, a stress-inducing disorder, or other neuropsychiatric disorders having paroxysmal or periodic features.

[0052] The term "treatment" as used herein is intended to include intervention after the occurrence of neuronal injury. As such, a treatment can ameliorate neuronal injury by administration after a primary insult to the neurons occurs. Such primary insult to the neurons can include or result from any disease or condition associated with a neuropathy, "Treatment" also includes prevention of progression of neuronal injury. "Treatment" as used herein can include the administration of drugs and/or synthetic substances, the administration of biological substances such as proteins, nucleic acids, viral vectors and the like as well as the administration of substances such as neutraceuticals, food additives or functional foods.

[0053] The methods and compositions of the present invention can be useful in treating mammals. Such mammals include humans as well as non-human mammals. Non-human mammals include, for example, companion animals such as dogs and cats, agricultural animals such live stock including cows, horses and the like, and exotic animals, such as zoo animals.

[0054] Substances that can increase sirtuin activity in mammals can include polyphenols, some of which have been described earlier (see for example Howitz et al., Nature 425: 191-196, 2003 and supplementary information that accompanies the paper all of which is incorporated herein by reference). Polyphenol compounds of the present teachings can include stilbenes such as resveratrol, piceatannol, deoxyrhapontin, trans-stilbene and rhapontin; chalcones such as butein, isoliquiritigenin and 3,4,2,4',6'-pentahydroxychalcone and chalcone; flavones such as fisetin, 5,7,3',4',5'-pentahydroxyflavone, luteolin, 3,6,3',4'-tetrahydroxyflavone, quercetin, 7,3',4',5'-tetrahydroxyflavone, kaempferol, 6-hydroxyapigenin, apigenin, 3,6,2',4'-tetrahydroxyflavone, 7,4'-dihydroxyflavone, 7,8,3',4'-tetrahydroxyflavone, 3,6,2',3'-tetrahydroxyflavone, 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 5,7-dihydroxyflavone, morin, flavone and 5-hydroxyflavone; isoflavones such as daidzein and genistein; flavanones such as naringenin, 3,5,7,3',4'-pentahydroxyflavanone, and flavanone or catechins such as (−)-epicatechin, (−)-catechin, (−)-gallocatechin, (+)-catechin and (+)-epicatechin. In some aspects, a polyphenol can be resveratrol, fisetin, butein, piceatannol or quercetin.

[0055] In various aspects of the present teachings, based at least on the demonstration herein that resveratrol can increase AMPK activity in a LKB1- and/or CaMKKβ-dependent manner in neuronal tissue and in models of neuronal disease, resveratrol and other polyphenol compounds as discussed above can be used to treat or prevent a neuropathy or axonopathy in a mammal in need thereof. In some aspects of the disclosed methods, other substances which increase AMPK activity, LKB1 activity and/or CaMKKβ) activity can be used to treat or prevent a neuropathy or axonopathy in a mammal in need thereof. Hence, in some aspects, an activator of AMPK, such as AICAR, metformin or phenformin, can be used to treat or prevent a neuropathy or axonopathy. In some aspects, additional polyphenols or other substances that increase AMPK activity, LKB1 activity and/or CaMKKβ activity can be identified using the assay systems described herein, as well as in commercially available assays known to those skilled in the art. Additional assays are disclosed, e.g., in U.S. published patent applications 2005026233 (Carling et al.) and 20060035301 (Corvera et al.).

[0056] In some aspects of the present teachings, additional polyphenols or other substances that increase sirtuin deacetylase activity can be identified using assay systems described herein as well as in commercially available assays such as fluorescent enzyme assays (Biomol International L.P., Plymouth Meeting, Pa.). Sinclair et al., also disclose substances that can increase sirtuin and/or AMPK activity (Sinclair et al., WO2005/02672; and Sinclair et al., Publication No. 20060111435, which are incorporated in their entirety by reference).

[0057] In various further aspects, other substances can increase sirtuin activity indirectly by increasing NAD activity as a result of the particular sirtuin functioning through NAD-dependent histone/protein deacetylase activity. NAD activity can be increased by administration of NAD or NADH as well as by synthesizing NAD. NAD can be synthesised through three major pathways, the de novo pathway in which NAD is synthesized from tryptophan, the NAD salvage pathway in which NAD is generated by recycling degraded NAD products such as nicotinamide (Lin et al. Curent Opin. Cell Biol. 15:241-246, 2003; Magni et al., Cell Mol. Life Sci. 61:19-34, 2004) and the nicotinamide riboside kinase pathway in which nicotinamide riboside is converted to nicotinamide mononucleotide by nicotinamide riboside kinase (Bieganowski et al., Cell 117:495-502, 2004). Thus, administering to injured neurons, a precursor of NAD in the de novo pathway such as, for example, tryptophan or nicotinate and/or substances in the NAD salvage pathway such as, for example, nicotinamide, nicotinic acid, nicotinic acid mononucleotide, or deamido-NAD and/or substances in the nicotinamide riboside kinase pathway such as, for example, nicotinamide riboside or nicotinamide mononucleotide, could potentially increase NAD activity. As shown below, nicotinamide mononucleotide, nicotinic acid mononucleotide or nicotinamide riboside, in addition to NAD, can protect against axonal degeneration to a similar extent as NAD, however, nicotinic acid and nicotinamide do not. The increased NAD activity can then increase sirtuin histone/protein deacetylase activity in the injured neurons and diminish or prevent axonal degeneration. In addition, it is believed that other substances can act by increasing enzyme activity or by increasing levels of NAD, nicotinamide mononucleotide, nicotinic acid mononucleotide, nicotinamide riboside or sirtuin enzymes or by decreasing degradation of NAD, nicotinamide mononucleotide, nicotinic acid mononucleotide, nicotinamide riboside or sirtuin enzymes.

[0058] In various aspects. NAD can be increased in injured neurons by administering enzymes that synthesize NAD or nucleic acids comprising enzymes that synthesize NAD. Such enzymes can include an enzyme in the de novo pathway for synthesizing NAD, an enzyme of the NAD salvage pathway or an enzyme of the nicotinamide riboside kinase pathway or a nucleic acid encoding an enzyme in the de novo pathway for synthesizing NAD, an enzyme of the NAD salvage pathway or an enzyme of the nicotinamide riboside kinase pathway and, in particular, an enzyme of the NAD salvage pathway such as, for example, a nicotinamide mononucleotide adenylyltransferase (NMNAT) such as NMNAT1. Thus, in one non-limiting example, administration of an NMNAT such as NMNAT1 or NMNAT3 or a nucleic acid comprising a sequence encoding an NMNAT such as

NMNAT1 or NMNAT3 can diminish or prevent axonal degeneration in injured neurons.

[0059] The human NMNAT1 enzyme (E.C.2.7.7.18) is represented according to the GenBank Assession numbers for the human NMNAT1 gene and/or protein:NP_073624; NM_022787; AAL76934; AF459819; and NP_073624; AF314163. A variant of this gene is NMNAT-2 (KIAA0479), the human version of which can be found under GenBank Accession numbers NP_055854 and NM_015039.

[0060] As used herein, the term "percent identical" or "percent identity" or "% identity" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAR computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded amino acid sequences can be used to search both protein and DNA databases. Databases with individual sequences are described in Methods in Enzymology, ed. Doolittle, supra. Databases include Genbank, EMBL, and DNA Database of Japan (DD3BJ).

[0061] A "variant" of a polypeptide refers to a polypeptide having the amino acid sequence of the polypeptide in which is altered in one or more amino acid residues. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). A variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, LASERGENE software (DNASTAR).

[0062] The term "variant," when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to that of a particular gene or the coding sequence thereof. This definition may also include, for example, "allelic," "splice," "specics," or "polymorphic" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variation is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs) in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population, a disease state, or a propensity for a disease state.

[0063] An agent that can be used in treating or preventing a neuropathy in accordance with the methods and compositions of the present invention can be comprised by a nicotinamide mononucleotide adenylyltransferase (NMNAT) or a polynucleotide encoding an NMNAT. In particular, the agent can be an enzyme having NMNAT activity and at least 50% identity with a human NMNAT1 or at least 50% identity with a human NMNAT3, at least 60% identity with a human NMNAT1 or at least 60% identity with a human NMNAT3, at least identity with a human NMNAT1 or at least 70% identity with a human NMNAT3, at least 80% identity with a human NMNAT1 or at least 80% identity with a human NMNAT3, at least 90% identity with a human NMNAT1 or at least 90% identity with a human NMNAT3, at least 95% identity with a human NMNAT1 or at least 95% identity with a human NMNAT3. Moreover, the agent can be comprised by a human NMNAT1, a human NMNAT3 or a conservatively substituted variants thereof.

[0064] The agent can also be comprised by a polynucleotide having at least 50% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 50% identity with a nucleic acid encoding a human NMNAT3, a polynucleotide having at least 60% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 60% identity with a nucleic acid encoding a human NMNAT3, a polynucleotide having at least 70% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 70% identity with a nucleic acid encoding a human NMNAT3, a polynucleotide having at least 80% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 80% identity with a nucleic acid encoding a human NMNAT3, a polynucleotide having at least 90% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 90% identity with a nucleic acid encoding a human NMNAT3, a polynucleotide having at least 95% identity with a nucleic acid encoding a human NMNAT1 or a polynucleotide having at least 95% identity with a nucleic acid encod-

ing a human NMNAT3. The agent can also be a polynucleotide encoding a human NMNAT1, a human NMNAT3 or a variant thereof.

[0065] The agent can also be comprised by a sirtuin polypeptide or a nucleic acid encoding a sirtuin polypeptide. In particular, the agent can comprise an enzyme having SIRT activity and at least 50% identity with a human SIRT1, at least 60% identity with a human SIRT1, at least 70% identity with a human SIRT1, at least 80% identity with a human SIRT1, at least 90% identity with a human SIRT1, or at least 95% identity with a human SIRT1. Moreover, the agent can be comprised by a human SIRT1 or a conservatively substituted variants thereof. The agent can also be comprised by a polynucleotide having at least 50% identity with a nucleic acid encoding a human SIRT1, a polynucleotide having at least 60% identity with a nucleic acid encoding a human SIRT1, a polynucleotide having at least 70% identity with a nucleic acid encoding a human SIRT1, a polynucleotide having at least 80% identity with a nucleic acid encoding a human SIRT1, a polynucleotide having at least 90% identity with a nucleic acid encoding a human SIRT1 or a polynucleotide having at least 95% identity with a nucleic acid encoding a human SIRT1. Moreover, the agent can comprise a polynucleotide encoding a human SIRT1 or a variant thereof.

[0066] Administration can be by any suitable route of administration including buccal, dental, endocervical, intramuscular, inhalation, intracranial, intralymphatic, intramuscular, intraocular, intraperitoneal, intrapleural, intrathecal, intratracheal, intrauterine, intravascular, intravenous, intravesical, intranasal, ophthalmic, oral, otic, biliary perfusion, cardiac perfusion, priodontal, rectal, spinal subcutaneous, sublingual, topical, intravaginal, transermal, ureteral, or urethral. Dosage forms can be aerosol including metered aerosol, chewable bar, capsule, capsule containing coated pellets, capsule containing delayed release pellets, capsule containing extended release pellets, concentrate, cream, augmented cream, suppository cream, disc, dressing, elixer, emulsion, enema, extended release fiber, extended release film, gas, gel, metered gel, granule, delayed release granule, effervescent granule, chewing gum, implant, inhalant, injectable, injectable lipid complex, injectable liposomes, insert, extended release insert, intrauterine device, jelly, liquid, extended release liquid, lotion, augmented lotion, shampoo lotion, oil, ointment, augmented ointment, paste, pastille, pellet, powder, extended release powder, metered powder, ring, shampoo, soap solution, solution for slush, solution/drops, concentrate solution, gel forming solution/drops, sponge, spray, metered spray, suppository, suspension, suspension/drops, extended release suspension, swab, syrup, tablet, chewable tablet, tablet containing coated particles, delayed release tablet, dispersible tablet, effervescent tablet, extended release tablet, orally disintegrating tablet, tampon, tape or troche/lozenge.

[0067] Intraocular administration can include administration by injection including intravitreal injection, by eyedrops and by trans-scleral delivery.

[0068] Administration can also be by inclusion in the diet of the mammal such as in a functional food for humans or companion animals.

[0069] It is also contemplated that certain formulations containing the compositions that increase sirtuin activity of the invention can be administered orally. In some configurations, such formulations can be encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and promote absorption such as, for example, surface active agents.

[0070] The specific dose can be calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also depend upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity in assay preparations such as has been described elsewhere for certain compounds (see for example, Howitz et al., Nature 425:191-196, 2003 and supplementary information that accompanies the paper). Exact dosages can be determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

[0071] As the present teachings demonstrate several mechanisms (such as increased activity of AMPK, LKB1, CaMKKβ, NAD, and/or sirtuin) for treating neuropathies, and other mechanisms are known, included among the methods of the present teachings are methods of treating or preventing a neuropathy or axonopathy in a mammal in need thereof, involving administering to the mammal an effective amount, in combination, of two or more of: (a) an agent that acts at least in part by increasing AMPK activity, LKB1 activity and/or CaMKKβ activity in diseased and/or injured neurons and supporting cells; (b) an agent that acts at least in part by increasing sirtuin activity in diseased and/or injured neurons and supporting cells; (c) an agent that acts at least in part by increasing NAD activity in diseased and/or injured neurons and supporting cells; and (d) an agent that acts at least in part by another mechanism in diseased and/or injured neurons and supporting cells. While not intended to be limiting, three major classes of medications are commonly used in the treatment of pain or other neuropathic symptoms: antidepressants, e.g., tricyclics; anticonvulsants, e.g., gabapentin and carbamazepine; and sodium channel blockers, e.g. mexiletine, and are included in various combinations contemplated herein.

[0072] Some methods of the present teachings, in addition to a primary step of administering therapeutically effective amounts of an agent, or combination of agents, also include methods for treatment or prevention of a neuropathy which involve identifying a subject, in need of such administration. In various configurations, identification of such a subject can

be accomplished by diagnosing an individual as having, or being at risk of developing, a clinically diagnosable neurodegenerative disease or neurological condition wherein the disease or condition is believed to be treatable or preventable by increasing AMPK activity, LKB1 activity and/or CaMKKβ activity, as described herein. In various configurations, these methods can involve assessment of the levels of AMPK activity. LKB1 activity, CaMKKβ and/or ACC activity, assessment of the effects low levels of AMPK activity, LKB1 activity, CaMKKβ and/or ACC activity, or assessment of the neurological symptoms or effects associated therewith related to the disease or condition in question. In some configurations, the methods can also involve monitoring of the subject before, during or after a course of treatment to assess the effectiveness of the regimen to increase AMPK and/or LKB1 and/or CaMKKβ activity, other activities disclosed herein, or to determine the need for, or appropriate modifications to, further treatment or prophylaxis. Additionally, an assessment may include genetic analysis of mutations or alterations in a sample of a subject's DNA for AMPK, LKB1, CaMKKβ or related proteins. Assessment may be accomplished by various sequencing procedures well know to those in this art.

[0073] In various embodiments, the present teachings include methods of screening candidate agents. In such assay methods, agents can be tested for effectiveness in decreasing or preventing axonal degeneration of injured neuronal cells. In these methods, a candidate agent can be administered to neuronal cells subjected to injury; the injured neuronal cells can then be assayed for a decrease in axonal degeneration. In some configurations, a candidate agent can be added prior to producing the injury. In some other configurations, an injury can be introduced prior to addition of the candidate compound. The method can be performed in vitro or in vivo. The in vitro tests can be performed using any of a number of mammalian neuronal cells, or neuronal cell lines (e.g. Neuro2a), under a variety of experimental conditions in which injury is elicited. An example of mammalian neuronal cell-types that can be used are primary dorsal root ganglion cells injured by either transection and removal of the neuronal cell body or growth in media containing vincristine as described below. The in vivo tests can be performed in intact animals such as, for example, a mouse model of peripheral nerve regeneration (Pan et al., J. Neurosci. 23:11479-11488, 2003) or mouse model of progressive motor neuronopathy (Schmalbruch et al., J. Neuropathol. Exp. Neurol. 50:192-204, 1991; Ferri et al., Current Biol. 13:669-673, 2003).

[0074] Because an increase in AMPK activity, LKB1 activity and/or CaMKKβ activity can lead to a decrease or prevention of neuronal injury, assays which measure, directly or indirectly, increases in such activities can also be used in screens for therapeutic agents. Thus, in some aspects, methods described above can be used as part of a system to screen for agents that increase AMPK activity, LKB1 activity and/or CaMKKβ activity, or candidate agents can be screened directly for their impact on such activity, or some combination can be used.

[0075] Further, because, another mechanism of decreasing or preventing neuronal injury results from an increase in NAD-dependent histone/protein deacetylase activity of sirtuin molecules, the assay method can also be used as part of a primary screen for substances that either increase sirtuin activity directly or through increasing NAD activity. Thus the methods above also can be used to screenassist in screens for agents that increase NAD biosynthetic activity or agents that increase sirtuin activity in neurons.

[0076] Recombinant vectors that serve as carriers for a nucleic acid encoding a sirtuin molecule or an enzyme for biosynthesis of NAD are also within the scope of the present invention. Such recombinant vectors can comprise a promoter operatively linked to a sequence encoding a mammalian NMNAT1 protein or a mammalian sirtuin protein such as a SIRT1 protein. Such recombinant vectors can be any suitable vector such as, for example a lentivirus or an adeno-associated virus. Any suitable promoter can be also used such as, for example a ubiquitin promoter, a CMV promoter or a β-actin promoter.

[0077] The invention can be further understood by reference to the examples which follow.

Example 1

[0078] This example demonstrates that transected axons from neurons transfected with a vector expressing Wlds protein show a delayed degeneration compared to control neurons.

[0079] In wlds mice, Wallerian degeneration in response to axonal injury has been shown to be delayed (Gillingwater, et al., J Physiol, 534:627-639, 2001). Genetic analysis has shown that the wlds mutation comprises an 85 kb tandem triplication, which results in overexpression of a chimeric nuclear molecule (Wlds protein). This protein is composed of the N-terminal 70 AAs of Ufd (ubiquitin fusion degradation protein) 2a, a ubiquitin chain assembly factor, fused to the complete sequence of nicotinamide mononucleotide adenylyltransferase1 (NMNAT1), an enzyme in the NAD salvage pathway that generates NAD within the nucleus. The Wlds protein has NMNAT activity but lacks ubiquitin ligase function, suggesting that axonal protection is derived from either increased NMNAT1 activity or a 'dominant negative' inhibition of Ufd2a function.

[0080] To identify the mechanism of delayed axonal degeneration mediated by the Wlds protein, we employed an in-vitro Wallerian degeneration model. Primary DRG explant neurons were infected with lentivirus expressing the appropriate proteins, and axons were injured by either removal of the neuronal cell body (transection) or growth in vincristine (toxic).

[0081] Lentiviral expression constructs were kindly provided by D. Baltimore (Lois, et al., Science 295:868-72, 2002). We modified the FUGW vector to generate a general expression shuttle FUIV (ubiquitin promoter-gene of interest-IRES-enhanced YFP (Venus)) vector that enables enhanced YFP expression in cells that express the gene-of-interest. The following proteins, each with a hexahistidine tag at the C-terminus, were cloned into the FUIV vector: Wlds chimeric mutant protein; Ufd2a containing a point mutation (P1140A), which has previously been shown to inhibit wild-type Ufd2a function as a "dominant-negative" (Ufd2a (P1140)). The following genes were cloned into FUGW vector 1) The first 70 AAs of Ufd2a (the portion contained in Wlds protein) fused to the N-terminus of EGFP (Ufd2a(1-70)-EGFP) or EGFP with nuclear localization signal at the C-terminal (Ufd2a(1-70)-nucEGFP). 2) The NMNAT1 portion of Wlds protein fused to the C-terminus of EGFP (EGFP-NMNAT1).

[0082] The murine cDNA for Ufd2a/Ube4b (mKIAA0684) was provided by Kazusa DNA Research Institute. Murine cDNAs for NMNAT1 (accession number: BC038133) were

purchased from ATCC. PCR-mediated mutagenesis was used to generate point mutations in Ufd2a, NMNAT1 and Wlds.

[0083] We generated siRNA constructs in the FSP-si vector generated from the FUGW backbone by replacing the ubiquitin promoter and GFP cDNA with the human U6 promoter and Pol I termination signal followed by the SV40 promoter-puromycin-N-acetyl-transferase gene. Cloning of siRNA construct was performed as described previously, so that the siRNA is transcribed from the U6 promoter (Castanotto, et al., RNA, 8:1454-60, 2002). Sequences used for siRNA downregulation of protein expression were 1692-1710 of SIRT1, 1032~1050 of SIRT2, 538~556 of SIRT3, 1231~1249 of SIRT4, 37~55 of SIRT5, 1390~1408 of SIRT6, and 450~468 of SIRT7. The integrity of each lentiviral expression and siRNA construct was confirmed by DNA sequencing.

[0084] Mouse DRG explants from E12.5 embryos were cultured in the presence of 1 nM nerve growth factor. Non-neuronal cells were removed from the cultures by adding 5-fluorouracil to the culture medium. Transection of neurites was performed at 10-20 DIV using an 18-gauge needle to remove the neuronal cell bodies. Incubation with β-nicotinamide adenine dinucleotide (Sigma) or Sirtinol (Calbiochem) was performed using conditions indicated in the text or figures.

[0085] Lentiviral expression vectors were generated using HEK293T cells as described above. For confirmation of lentivirus-derived protein expression, HEK293T cells were infected with lentivirus and cells were lysed 3 days after infection. These lysates were analyzed by immunoblot to using anti-His tag monoclonal antibody (Qiagen) to detect expression of the respective hexahistidine-tagged proteins. Lentiviral infection of DRG neurons was performed by incubating ~106-107 pfu/ml virus with the DRG explant for 24 h beginning 3-7 days prior to axonal transection. The infected neurons were examined under an inverted fluorescent microscope to insure detectable lentivirus-mediated transgene expression in >95% of neurons.

[0086] Quantitative analysis of axonal degeneration was performed as previously described (Zhai, et al., Neuron 39:217-25, 2003). Briefly, the cultures were examined using phase contrast microscopy at the indicated times. Axons with a fragmented, non-refractile appearance were designated as "degenerated." At each time point, at least 200 singly distinguishable axons were blindly scored from several randomly taken images of each culture. Each condition was tested in triplicate explants in each experiment. Results were obtained from 2-4 independent experiments for each condition. Statistical analysis was performed by Student's T test. For calculations of neurite-covered area, digitally captured images from quadruplicate samples of two independent experiments were analyzed using analysis 3.1 software (Soft Imaging System, Lakewood, Colo.).

[0087] We found that transected axons from neurons expressing the Wlds protein degenerated with the delayed kinetics characteristic of neurons derived from wlds (Buckmaster, et al., Eur J Neurosci 7:1596-602, 1995) mice as shown in FIG. **1**A.

[0088] Next, we compared axonal degeneration after transection in neurons that overexpress Wlds protein with those that express the Ufd2a or NMNAT1 portions that make up the Wlds protein linked to EGFP. Results are shown in FIG. **18**.

[0089] We found that expression of EGFP-NMNAT1 delayed axonal degeneration comparable to Wlds protein itself, whereas the N-terminal 70 AA of Ufd2a (fused to EGFP), either targeted to the nucleus or cytoplasm, did not affect axonal degeneration. Quantification of these effects was performed by counting the percentage of remaining neurites at various times after removal of neuronal cell bodies. This analysis showed that EGFP-NMNAT1, like Wlds protein itself, resulted in a >10-fold increase in intact neurites 72 hr after injury. To further exclude direct involvement of the UPS in Wlds protein-mediated axonal protection, we examined the effect of Ufd2a inhibition using either a dominant-negative Ufd2a mutant or an Ufd2a siRNA construct. However, neither of these methods resulted in delayed axonal degradation in response to axotomy. Together, these experiments demonstrated that the NMNAT1 portion of the Wlds protein is responsible for the delayed axonal degeneration observed in wlds mice.

Example 2

[0090] This example shows that mutations in the full length NMNAT1 and in Wlds protein abolish the axonal protective effects of the proteins.

[0091] NMNAT1 is an enzyme in the nuclear NAD salvage pathway that catalyzes the conversion of nicotinamide mononucleotide (NMN) and nicotinate mononucleotide (NaMN) to NAD and nicotinate adenine mononucleotide (NaAD), respectively. The axonal protection observed in NMNAT1 overexpressing neurons could be mediated by its ability to synthesize NAD (i.e. its enzymatic activity), or perhaps, by other unknown functions of this protein. To address this question, we used the NMNAT1 crystal structure to identify several residues predicted to participate in substrate binding. A mutation in one of these residues (W170A) was engineered into full length NMNAT1 and Wlds protein. In vitro enzymatic assays confirmed that both of these mutant proteins were severely limited in their ability to synthesize NAD (FIG. **2**A). Each of these mutants and their respective wild type counterparts were introduced into neurons to assess their ability to protect axons from degradation. We found that neurons expressing these enzymatically inactive mutants had no axonal protective effects (FIG. **2**A), indicating that NAD/NaAD-production is responsible for the ability of NMNAT1 to prevent axonal degradation.

Example 3

[0092] This example illustrates that increased NMNAT activity in neurons injured with vincristine also show a delayed axonal degradation.

[0093] In addition to mechanical transection, axonal protection in wlds mice is also observed against other damaging agents such as ischemia and toxins (Coleman, et al., Trends Neurosci 25:532-37, 2002; Gillingwater, et al., J Cereb Blood Flow Metab 24:62-66, 2004). We sought to determine whether increased NMNAT activity would also delay axonal degradation in response to other types of axonal injury such as vincristine, a cancer chemotherapeutic reagent with well-characterized axonal toxicity. Neurons expressing either NMNAT1 or EGFP (control) were grown in 0.5 μM vincristine for up to 9 d. We found that axons of neurons expressing NMNAT1 maintained their original length and refractility, whereas axons emanating from neurons expressing EGFP gradually retracted and had mostly degenerated by day 9 (FIG. **28**). These results indicate that NMNAT activity by

itself can protect axons from a number of insults and mediate the protective effects observed in wlds mice.

Example 4

[0094] This example shows that exogenously administered NAD can protect injured neurons from axonal degeneration.

[0095] Previous experiments have shown that neuronal cells express membrane proteins that can bind and transport extracellular NAD into the cell (Bruzzone, et al., Faseb J 15: 110-12, 2001). This encouraged us to investigate whether exogenously administered NAD could prevent axonal degeneration. We added various concentrations of NAD to neuronal cultures prior to axonal transection and examined the extent of axonal degradation. We found that 0.1-1 mM NAD added 24 hr prior to axotomy significantly delayed axonal degeneration, although exogenously applied NAD was slightly less effective in protecting axons than lentivirus mediated NMNAT1 expression (FIG. **3**A). These results provide direct support for the idea that increased NAD supply can prevent axonal degradation.

Example 5

[0096] This example illustrates that NAD was required prior to the removal of the neuronal cell bodies to protect the injured neurons from axonal degeneration.

[0097] To gain insights into the mechanism of NAD-dependent axonal protection (NDAP), we examined whether NAD was required prior to the removal of the neuronal cell bodies, or whether direct exposure of the severed axons to high levels of NAD was sufficient to provide protection (FIG. **31**). Neuronal cultures were prepared and 1 mM NAD was added to the culture medium at the time of axonal transection or at various times (4 to 48 hr) prior to injury.

[0098] We found that administering NAD at the time of axonal transection or, for up to 8 hr prior to injury, had no protective effects on axons. However, significant axon sparing was observed when neurons were incubated with NAD for longer periods of time prior to injury, with the greatest effects occurring after at least 24 h of NAD pre-treatment, These results indicate that NAD dependent axonal protection is not mediated by a rapid post-translational modification within the axons themselves.

[0099] The requirement for extended exposure to NAD of the intact neurons to prevent axonal degradation in response to injury suggests that the protective process requires de novo transcriptional and/or translational events. Interestingly, both the Wlds protein and NMNAT1 are located within the nucleus (data not shown). Similarly, most enzymes that make up the NAD salvage pathway in yeast are also compartmentalized in the nucleus. We compared NAD levels in wild type and NMNAT1 expressing DRG neurons using sensitive microscale enzymatic assays (Szabo, et al., Proc Natl Acad Sci USA, 93:1753-58, 1996), however no changes in overall cellular NAD levels were found (data not shown). This is similar to observations in yeast, in which activation of this nuclear pathway did not change overall levels of NAD (Anderson, et al., J Biol Chem, 277:18881-90, 2002; Huh, et al., Nature, 425:686-91, 2003). Furthermore, levels of tissue NAD in the brains of wild type and wlds mice are similar despite the increased levels of NMNAT activity in wlds mice (Mack, et al., Nat Neurosci, 4:1199-206, 2001). These data suggest that an NAD-dependent enzymatic activity in the nucleus, as opposed to cytoplasmic NAD-dependent processes, is likely to mediate the axonal protection observed in response to increased NMNAT activity.

Example 6

[0100] This example shows that inhibition of Sir2 is involved in NAD-dependent axonal protection.

[0101] The Sir2 family of protein deacetylases and poly (ADP-ribose) polymerase (PARP) are the major WAD-dependent nuclear enzymatic activities. Sir2 is an NAD-dependent deacetylase of histones and other proteins, and its activation is central to promoting increased longevity in yeast and *C. elegans* (Bitterman, et al., Microbiol Mol Biol Rev, 67:376-99, 2003; Hekimi, et al., Science 299:1351-54, 2003). PARP is activated by DNA damage and is involved in DNA repair (S. D. Skaper, Ann NY Acad Sci, 993:217-28 and 287-88, 2003). These enzymes, in particular the Sir2 proteins, have generated great interest in recent years as they provide a potential link between caloric restriction and its effects on the ageing process. The importance of these NAD-dependent enzymes in regulating gene activity, prompted us to investigate their role in the self-destructive process of axonal degradation. We therefore tested whether inhibitors of Sir2 (Sirtinol) and PARP (3-aminobenzamide (3AB)) could affect NAD-dependent axonal protection (NDAP) (FIG. **4**A). Neurons were cultured in the presence of 1 mM NAD and either Sirtinol (100 μM) or 3AB (20 mM). Axonal transection was performed by removal of the neuronal cell bodies and the extent of axonal degradation was assessed 12 to 72 hr later. We found that Sirtinol effectively blocked NDAP, indicating that Sir2 proteins are likely effectors of this process. In contrast, 3AB had no effect on NDAP, indicating that PARP does not play a role in axonal protection. To further examine the role of Sir2 proteins in NDAP, we tested the effects of resveratrol (10~100 μM), a polyphenol compound that enhances Sir2 activity (Howitz, et al., Nature, 425:191-96, 2003). We found that neurons treated with resveratrol prior to axotomy showed a decrease in axonal degradation that was comparable to that obtained using NAD (FIG. **4**A), providing further support for the idea that Sir2 proteins are effectors of the axonal protection mediated by increased NMNAT activity.

Example 7

[0102] This example shows that SIRT1 is involved in NAD-dependent axonal protection.

[0103] In humans and rodents, seven molecules sharing Sir2 conserved domain (sirtuin (SIRT)1 through 7) have been identified, although some of these proteins do not appear to have histone/protein deacetylase activity (Buck, et al., J Leukoc Biol, S0741-5400, 2004). SIRT1 is located in the nucleus and is involved in chromatin remodeling and the regulation of transcription factors such as p53 (J. Smith, Trends Cell Biol, 12:404-406, 2002). The cellular location of other SIRT proteins is less clear, but some have been found in the cytoplasm and in mitochondria. To determine which SIRT protein(s) is involved in NAD-dependent axonal protection, we performed knockdown experiments using siRNA constructs to specifically target each member of the SIRT family. Neurons were infected with lentiviruses expressing specific SIRT siRNA constructs that effectively suppressed expression of their intended target (FIG. **4**B). The infected neurons were cultured in 1 mM NAD and axonal transection was performed by removing the cell bodies. We found that the SIRT1 siRNA

construct was just as effective at blocking the axonal protective effects of NAD as the Sirtinol inhibitor. In contrast, inhibition of the other SIRT proteins did not have significant effects on NDAP (FIG. **4**B). These results indicate that SIRT1 is the major effector of the increased NAD) supply that effectively prevents axonal self destruction. Although, SIRT1 may deacetylate proteins directly involved in axonal stability, its predominantly nuclear location, along with the requirement for NAD ~24 hr prior to injury for effective protection, suggest that SIRT1 regulates a genetic program that leads to axonal protection.

[0104] Axonal degeneration is an active, self-destructive phenomenon observed not only after injury and in response to chemotherapy, but also in association with aging, metabolic diseases such as diabetic neuropathy, and neurodegenerative diseases. Our results indicate that the molecular mechanism of axonal protection in the wlds mice is due to the increased supply of NAD resulting from enhanced activity of the NAD salvage pathway and consequent activation of the histone/protein deacetylase SIRT1.

EXAMPLES 8-11

[0105] The following Materials and Methods were used in Examples 8-11.

[0106] Construction of expression plasmids and mutagenesis. Coding regions of the NAD biosynthetic enzymes were PCR amplified from EST clones BC038133 for murine NMNAT1 and BC005737 for murine nicotinamide mononucleotide adenylyltransferase3 (NMNAT3), using Herculase (Stratagene). Human NAD) synthetase (QNS) hexahistidine-tagged cDNA was kindly provided by Dr. N. Hara (Shimane University, Shimane, Japan). Hexahistidine tag was added at the 3'-end of each cDNA. NMNAT1 cytosolic mutant (cytNMNAT1) was generated by PCR-mediated site-directed mutagenesis. Nuclear form of NMNAT3 (nucNMNAT3) was generated by adding a nuclear localization signal to the C-terminal end of NMNAT3. Each PCR amplified NAD synthetic enzyme fragment was cloned into FCIV lentiviral shuttle vector as previously described. The integrity of all the constructs was sequenced using Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems) and an Applied Biosystems 373 DNA sequencer.

[0107] NAD biosynthetic substrates. All substrates for NAD biosynthetic enzymes were purchased from Sigma (Na, Nam, NMN, NaMN, nicotininc acid adenine dinucleotide (NaAD), and NAD). NmR was synthesized from NMN. Conversion of NMN to NmR was confirmed by HPLC (Waters) using reverse phase column LC-18T (Supelco). NmR is eluted 260±10 seconds and NMN is eluted 150±10 seconds under 1 ml/min flow rate of buffer containing 50 mM K_2HPO_4 and 50 mM KH_2PO_4 (pH 7.0). Biological activity of NmR was accessed as previously described by using yeast strains kindly provided from Dr. Charles Brenner (Dartmouth Medical School, N.H., USA).

[0108] Real-time quantitative reverse transcription-PCR analysis. All the surgical procedures were performed according to National Institute of Health guidelines for care and use of laboratory animals at Washington University. For the expression analysis following nerve injury, the sciatic nerves of a C57BL/6 mouse was transected and L4 to L5. DRGs were collected at indicated time points and pooled to extract RNA, Rat DRG explants from E14.5 embryo were cultured for 14 days according to the method described and cultured with media containing 10 nM vincristin for indicated period and extracted RNA. Total RNAs from pooled tissue sources or DRG explant cultures were prepared. First-strand cDNA templates were prepared from 1 μg of each RNA using standard methods. Two independent cDNA syntheses were performed for each RNA sample. Quantitative reverse transcription (RT)-PCR was performed by monitoring in real-time the increase in fluorescence of the SYBR-GREEN dye on a TaqMan 7700 Sequence Detection System (Applied Biosystems).

[0109] Cell culture, in vitro axotomy, and quantification of axonal degeneration. Mouse DRG explants from E12.5 embryos were cultured in the DMEM containing 10% FCS and 1 nM nerve growth factor. Non-neuronal cells were removed from the cultures by adding 5-fluorouracil to the culture media. Transection of neurites was performed at 14-21 DIV using an 18-gauge needle to remove the neuronal cell bodies. Lentiviral expression vectors were generated. Lentiviral infection was performed 3-7 days prior to axonal transection for 24 hr. Quantitative analysis of neurite degeneration was performed.

[0110] Determination of protein expression and localization. For confirmation of protein expression, HEK293T cells were infected with a virus that expresses each of NAD biosynthetic enzymes. Cells were lysed 5 days after infection to be analyzed by immunoblot to detect expression of each protein with a hexa-histidine tag by anti-6×His tag monoclonal antibody (R&D Systems). Subcellular localization of each protein was analyzed using HEK293T cells transiently transfected with a viral shuttle vector for each NAD biosynthetic enzymes. Cells were fixed in 4% paraformaldehyde in PBS containing 0.1% tween-20 (PBS-T) and incubated with PBS-T containing 5% BSA for 1 hour, and then covered with 1:1000 diluted anti-6×His tag antibody (R&D Systems) in PBS-T containing 1% BSA and for 16 hours at 4° C. Cells were washed with PBS-T and incubated with Alexa Fluor 594-conjugated secondary antibody (Molecular Probes) in TBS-T for 1 hour and examined by fluorescence microscopy (Nikon).

[0111] NMNAT protein overexpression, affinity purification and enzymatic assay. HEK293T cells were transfected with an expression plasmid for each enzyme by using calcium phosphate precipitation. Three days later, cells were washed with PBS twice and then suspended in the buffer containing 50 mM Sodium Phosphate (pH 8.0), and 300 mM NaCl (buffer A). Cells were then homogenized by SONFIRE 450 (BRANSON) and supernatant was collected by centrifugation at 10,000 g for 10 min. His-select Nickel Affinity Gel (Sigma) was washed with buffer A and 0.1 ml of 50% gel suspension was added to 1 ml of supernatant and incubated for 10 min at 4° C., then beads binding hexa-histidine-tagged protein was extensively washed with the buffer A. Proteins were eluted by adding 100 μl of the solution containing 50 mM Sodium Phosphate (pH 8.0), 300 mM NaCl, and 250 mM imidazole. Relative NMNAT enzymatic activity was measured by using affinity purified proteins as described before and subtracted the value obtained from mock transfected cells and normalized by the amount of recombinant protein determined by densitometry.

[0112] Administration of NAD biosynthetic substrates and optic Nerve transection, Nam, NMN, NmR, or NAD was dissolved in PBS at the concentration of 100 mM or 1 M. Each of 5 μl solution was injected into left intravitreal component under the anesthesia at a rate of 0.5 μl ml per second. The left optic nerve was transected at 24 hours after intravitreal injec-

tion and optic nerve was recovered at indicated time. Optic nerve tissue was homogenized in 100 μl of a buffer containing 100 mM tris-HCl (pH 6.8), 1% SDS, and 1 mM DTT. Fifty μg of protein for each sample was analyzed by the Western blotting using anti-neurofilament antibody 2H3 (Developmental Studies Hybridoma Center) and peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). The degeneration rate was calculated from the ratio of the neurofilament immunoreactivity of transected vs. contralateral nerves.

Example 8

[0113] This example illustrates the NAD biosynthetic pathway and expression analysis of mammalian NAD biosynthetic enzymes.

[0114] NAD is synthesized via three major pathways in both prokaryotes and eukaryotes. In the de novo pathway, NAD is synthesized from tryptophan (FIG. **5**). In the salvage pathway, NAD) is generated from vitamins including nicotinic acid and nicotinamide. A third route from nicotinamide riboside called Preiss-Handler independent pathway has recently been discovered. The last enzymatic reaction of the de novo pathway involves the conversion of quinolinate to NaMN by QPRT (EC 2.4.2.19). At this point, the de novo pathway converges with the salvage pathway. NaPRT (EC 2.4.2.11) converts Na to NaMN, which is then converted to NaAD by NMNAT (EC 2.7.7.1). QNS1 (EC 6.3.5.1) converts NaAD to NAD. NmPRT (EC 2.4.2.12); also reported as visfatin) converts Nam to NMN. NMN is also converted to NAD by NMNAT. Nicotinamidase (PNC, EC 3.5.1.19), which converts Nam to Na in yeast and bacteria salvage pathway has not been identified in mammals. In the Preiss-Handler independent pathway, Nrk (EC 2.7.1.22) converts NmR to NMN and converge to salvage pathway. Most of these mammalian enzymes including QPRT, NmPRT, QNS1, Nrk1/2 and NMNAT1/2/3 have previously cloned and characterized. A mammalian homologue of NaPRT was also identified as an EST annotated as a mammalian homolog of a bacterial NaPRT.

[0115] To investigate the expression of mammalian NAD biosynthetic enzymes in the nervous system, we performed quantitative RT-PCR using RNA from mouse brain, retina, spinal code, and DRG at age of E14, P0, P7, P14 and P21. All enzymes are expressed ubiquitously in the nervous system throughout the development and in adulthood, with an exception of Nrk2 whose expression is very low in all examined tissues (data not shown). To identify inducibility of NAD-synthesizing enzymes in response to neuronal insults, we compared the RNA expression of each enzyme in DRGs at 1, 3, 7, and 14 days after sciatic nerve transection against non-injured DRG. As shown in FIG. **6**A, most of the enzymes were up-regulated 2 to 8-fold after injury. Among those, Nrk2 expression is exceptionally highly induced (more than 20-fold) at 14 days after axotomy. We also analyzed expression of NAD synthetic enzymes during the axonal degeneration caused by neurotoxin in cultured rat DRG neuron. DRG neurons were treated with 0.1 μM and 1 μM rotenone to cause axonal degeneration and collected RNA at 24 hours after the addition of rotenone. The expression of Nrk2 was increased more than 6 folds after rotenone treatment (FIG. **68**). These results suggest that, while all enzymatic activities in NAD synthesis pathway is ubiquitously present, Nrk2 may be responsible for supplying NAD synthesizing substrate after neuronal insults.

Example 9

[0116] This example illustrates that both nuclear and cytoplasmic Nmat enzymes save axons from degeneration.

[0117] To determine whether nuclear localization of NMNAT1 is essential to provide the axonal protection, we analyzed the effect of subcellular distribution of NMNAT enzyme in the in vitro Wallerian degeneration assay and compared the extent of axonal protection between overexpression of cytoplasmic and nuclear NMNAT, NMNAT1 has putative nuclear localization signal PGRKRKW in the 211-217 amino-acids of NMNAT1 protein. We generated a mutant NMNAT1 designated as cytNMNAT1 in which this nuclear localization signal was altered as PGAAAAW and examined subcellular distribution. As shown in FIG. **78**, the majority of cytNMNAT1 located in the cytosol as we expected.

[0118] Next we confirmed enzymatic activity of cytNMNAT1, NMNAT1 and its mutant cytNMNAT1 were purified from the cell lysate expressing either of proteins by using affinity gel. The enzymatic activity of affinity purified proteins was measured as described above and we found that cytNMNAT1 activity did not altered by its mutation (FIG. **7**C). After the overexpression of cytNMNAT1 in DRG neurons, we observed strong neurite protection as well as nuclear wild NMNAT1 (FIG. **7**A, E). We further confirmed this result by using NMNAT1 isoenzyme that lacks nuclear localization signal. Among two NMNAT isoenzymes. NMNAT3 is previously reported to locate outside nucleus and mitochondria, and have comparable enzymatic activity to NMNAT1. We added nuclear localization signal KPKKIKTED of human topoisomerase I to the C-terminal of NMNAT3 to generate nuclear NMNAT3. We expressed hexa-histidine tagged NMNAT3 or nucNMNAT3 in HEK293T cells and analyzed subcellular localization and its enzymatic activity. NMNAT3 was distributed outside the nucleus including bright punctuate staining as reported before and nucNMNAT3 mainly localized in the nucleus with some punctuate staining in the cytosol (FIG. **71**). The enzymatic activity of NMNAT3 and nucNMNAT3 were measured and both proteins have comparable enzymatic activity compared with NMNAT1 (FIG. **7**C). Then, in vitro Wallerian degeneration assay was performed after overexpression of these two NMNAT3 enzymes, and we found that overexpression of both NMNAT3 and nucNMNAT3 showed same extent of delay in neurite degeneration as well as NMNAT1 (FIG. **7**A, E). The lentivirus mediated expression of each enzyme was confirmed by Western blotting (FIG. **7**D). These experiments confirmed that NMNAT targeted to either the nucleus or cytosol protects neurite from degeneration.

Example 10

[0119] This example illustrates that exogenous application of substrates for NAD biosynthetic enzymes protects axon from degeneration.

[0120] We have previously shown that exogenously applied NAD in the culture medium shows axonal saving effect in vitro. Here we showed that expression of NmPRT also shows axonal protection suggesting that Nam is used as a substrate for NAD synthesis in neurons. To determine which substrate shown in FIG. **5** is used for NAD synthesis in neurons and to identify whether any of NAD precursors may be able to save axons similar to or possibly better than NAD, we applied Na, Nam, NmR, NaMN, NMN, or NaAD in the culture media and performed in vitro Wallerian degeneration assay. An applica-

tion of 1 mM NMN for 24 hours before neurite transection successfully saved neurites from degeneration. Quantitative analysis revealed that NMN treatment results in neurite protection to an extent similar to that achieved by exogenously applied NAD (FIG. **8**B). These results further suggested the possibility that increased supply of other NAD biosynthetic substrates have an ability to save neurites from degeneration. We then exogenously applied 1 mM of NAD biosynthetic substrates including Na, Nam, NaMN, NaAD, and NmR to the DRG neurons for 24 hours and performed neurite transection. As shown in FIGS. **8**A and B, NaMN or NmR treatment also saved neurites as well as NAD. NaAD showed slight protection but Na failed to save neurites, while Na and Nam had no effect. Quantitative analysis revealed that exogenous application of 1 mM NaMN, NMN, NmR, or NAD caused comparable increase in intact neurites at 48 hours after transection (FIG. **8**B). Because the protective effect of NaMN is equal to NMN, a step synthesize NAD from NaAD by QNS is active enough to save neurites under the increased supply of NaAD. Nevertheless, exogenous application of NaAD shows less increase in intact neurites at 48 hours compared with NAD (FIG. **8**B). This indicates insufficient incorporation into the cell or instability of NaAD in our assay condition. These experiments suggest that there are several different ways to save neurites including exogenous application of NMN, NaMN, and NmR. All of these treatments seem to cause increased supply of NAD and it is consistent to the previous experiments showing NAD application or NMNAT1 overexpression save neurites from degeneration.

Example 11

[0121] This example demonstrates that intraviteal application of NAD biosynthetic substrates delays the axonal degeneration of retinal ganglion cells.

[0122] Transection of optic nerve is an in vivo model which can be used to investigate mechanisms leading to Wallerian degeneration and following retinal ganglion cell (RGC) death observed in human diseases such as glaucoma. In the C57BL/Wlds mouse strain, optic nerve degeneration during Wallerian degeneration after axotomy is dramatically slowed. In addition, intravitreal injection is used for screening of compounds that protect ROC axon from degeneration in vivo and thus we can asses the axon protective effect of each NAD biosynthetic substrates in vivo by intraocular injection of compounds including NAD, NMN, NmR, and Nam. From in vitro Wallerian degeneration assay, 1 mM of NAD, NMN, and NmR in the culture media is enough to protect axon from degeneration. We initially injected 5 μl of 100 mM or 1 M NAD) solution into left intravitreal compartment. After 24 hours incubation, left optic nerve was transected and control (right) and axotomized (left) optic nerve were collected at 3, 4, and 5 days after transection. Neurofilament immunoreactivity from the axotomized optic nerve was measured and normalized against the value obtained from the right side of the optic nerve. We found that the immunoreactivity at 4 days after transection was 77±27% and 78±22% of non-axotomized optic nerve in 1 M and 100 mM NAD injected rats respectively, while control animal showed only 7±16% (FIG. **9**)

[0123] We then injected 5 μl of 100 mM NMN, NmR, and Nam into left intravitreal compartment and collected optic nerves at 4 days after left optic nerve transaction. The immunoreactivity obtained from NMN and NmR injected optic nerve was 60±25 and 72±19% of non-axotomized nerve. Nam injected animals did not show any difference from the control animals. These results are consistent with the in vitro study that showed NAD, NMN, and NmR have axon saving activity but Nam does not. Our in vivo study revealed that these small molecules that are involved in the NAD biosynthetic pathway are useful tools to save axon from degeneration.

Example 12-6

[0124] The following Materials and Methods were used in Examples 12-16.

Materials and Methods

[0125] Mice. Lkb1 foxed mice were the gift of Dr. Ronald A Depinho (Dana Farber cancer Institute, Boston, Mass.). SIRT1 heterozygous mice were provided by Frederick W Alt (Harvard University Medical School, Boston, Mass.).

[0126] Cell culture and Reagents. Neuro2 cells obtained from ATCC were grown in MEM with 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 2 mM L-glutamine with 10% FCS. For differentiation, cells were switched to serum-starvation medium (containing 0.2% FCS). For neurite outgrowth measurements, experiments were done in quadruplicate and 100 neurites per well were randomly measured using the Metamorph software. Dorsal root ganglia sensory and cortical neurons were established from E13.5 mouse embryos, maintained in neurobasal medium supplemented with B27 (and NGF for DRG neurons) and infected with lentiviruses as described (Araki et al., 2004, Science 305:1010-1013; Hawley et al., 2005. Cell Metab 2:21-23). Resveratrol was a gift from Sirtris Pharmaceuticals (Cambridge Mass.) and AICAR was obtained from Toronto Research (North York, Canada). Splitomycin and Compound C were purchased from Biomol (Plymouth Meeting, Pa.) and Calbiochem (San Diego, Calif.). Sirtinol and nicotinamide were purchased from Sigma (Saint Louis, Mo.)

[0127] Plasmids and viruses. The dominant negative (dnAMPK) and constitutively active (caAMPK) plasmids were gifts from Russell Jones (University of Pennsylvania, Philadelphia Pa.), and pCXN-Cre was a gift of Inder Verma (Salk Institute, San Diego, Calif.). All constructs were subcloned into the lentiviral shuttle vector FCIV and verified by nucleotide sequence analysis. Viruses were prepared as previously described (Araki et al., 2004, Science 305:1010-1013). Protein and mRNA analysis. Immunoprecipitations and western blot analysis were performed by standard methods using antibodies directed against total AMPK, phosphorylated AMPK, total ACC or phosphorylated ACC that were obtained from Cell Signaling Technology (Beverley, Mass.). The phospho-AMPK antibody detects endogenous AMPK α1 and α2 when phosphorylated at threonine 172. The LKB1 antibody was purchased from Upstate (Lake Placid, N.Y.). Quantitative RT-PCR analysis was performed using Sybr-Green methodology on a model 7700 instrument (Applied Biosystems) as previously described (Araki et al., 2004, Science 305:1010-1013). Primer sequences were those used in previous studies (Motoshima, et al., 2006. J Physiol 574:63-71).

Example 12

[0128] This example shows that resveratrol activates AMPK in neuronal cells.

[0129] To explore the role of polyphenols, in particular, of resveratrol, we tested whether resveratrol altered the activity of AMPK in neuronal cells. Neuro2a neuroblastoma cells were treated with resveratrol (10 μM) and AMPK activation was examined using phospho-AMPK specific antibodies. Resveratrol treatment resulted in a robust increase in AMPK Thr172 phosphorylation within 2 h that persisted for up to 72 h (FIG. **10**A). Interestingly, resveratrol activated AMPK to a similar extent as AICAR (5-aminoimidazole-4-carboxamide-β-D-ribofuranoside), a well characterized activator of AMPK that is converted to ZMP, an AMP mimetic (Culmsee, et al., 2001, J Mol Neurosci 17:45-48; Terai, et al., 2005, Mol Cell Biol 25:9554-9575). To confirm that AMPK activation by resveratrol results in typical AMPK-mediated downstream responses, we monitored phosphorylation of ACC (acetyl Co-A carboxylase), a primary target of activated AMPK. Using a phospho-ACC specific antibody, we found that resveratrol stimulation lead to robust phosphorylation of ACC both acutely and chronically to a similar degree as that observed with AICAR stimulation (FIG. **10**B). To examine a potential mechanism of resveratrol-mediated AMPK activation, we measured cellular ATP levels as a decreased ATP:AMP ratio increases the phosphorylation of AMPK. ATP levels were monitored using a luciferase-based assay in neuro2A cells treated with resveratrol or Oligomycin, an inhibitor of mitochondrial oxidative phosphorylation and therefore ATP production. While the ATP concentration dropped from 5.874 μM in DMSO-treated cells (control) to 0.976 μM in Oligomycin treated cells within 2 hr, the ATP concentration in resveratrol-treated Neuro2a cells was not altered from control either at 2 hr (6.33 μM) or 24 hr (6.42 μM). Taken together, these results demonstrate that resveratrol treatment results in potent activation of AMPK in Neuro2a cells.

Example 13

[0130] This example shows that AMPK activation by resveratrol stimulates neurite outgrowth in neuronal cells.

[0131] Neuro2a cells are a widely used as in vitro model of neuronal differentiation. These cells cease to proliferate and begin to differentiate, as evidenced by neurite outgrowth, in response to serum starvation, retinoic acid, or growth factors like neurotrophins and GDNF family ligands. As AMPK activation inhibits proliferation of a number of cell types, we first tested whether AMPK activation also inhibits Neuro2a cell proliferation. Neuro2a cells grown under serum starvation conditions (0.2% fetal calf serum) were treated with AICAR (1 mM) or resveratrol (10 μM). 24 h after plating the number of proliferating cells identified using Ki67 immunocytochemistry was decreased dramatically (12.8% and 11%) by AMPK activation using AICAR or resveratrol compared to 50% proliferating cells in DMSO controls (data not shown). Both AICAR and resveratol also induced differentiation of Neuro2a cells as evidenced by increased neurite outgrowth compared to serum starvation alone (FIG. **11**).

[0132] Since resveratrol activated AMPK and altered the differentiation of Neuro2a cells, we next asked whether AMPK activity was required for resveratrol-induced neurite outgrowth. To address this issue, we infected Neuro2a cells with lentivirus expressing either GFP alone (FUGW-control) or dominant negative AMPK (dnAMPK). After 3 days growth to allow robust lentiviral transgene expression, the medium was replaced with serum starvation medium. Resveratrol promoted robust neurite outgrowth in cells expressing GFP alone, whereas resveratrol-stimulated neurite outgrowth was severely diminished in Neuro2a cells expressing dnAMPK (FIG. **12**). Resveratrol-induced neurite outgrowth was reduced in the presence of the AMPK pharmacological inhibitor Compound C(CC 10 μM) further supporting the importance of AMPK activity for resveratrol-induced neurite outgrowth. We also infected Neuro2a cells with lentivirus expressing constitutively active AMPK (caAMPK) and found that constitutive AMPK activity significantly enhanced neurite outgrowth (FIG. **12**E). Notably, Compound C (FIG. **3**H) and dnAMPK (FIG. **3**F) by themselves did not cause any significant inhibition of neurite growth, indicating that AMPK inhibition specifically reversed resveratrol-stimulated neurite growth. Taken together these results indicate that AMPK activation is necessary and sufficient to inhibit Neuro2a proliferation and promote neuronal differentiation.

Example 14

[0133] This example shows that resveratrol induces mitochondrial biogenesis through AMPK activation.

[0134] We tested whether AMPK activation by resveratrol could promote mitochondrial biogenesis. We treated Neuro2a cells for 3d with either DMSO (control) or resveratrol (10 μM) in the presence or absence of the AMPK inhibitor Compound C (10 μM). We assessed mitochondrial biogenesis by monitoring mRNA levels of mitofusin 2 (MFN2), a mitochondrial protein and marker of mitochondrial mass and two key regulators of mitochondrial biogenesis, peroxisome proliferator activated receptor γ coactivator 1α (PGC cc) and mitochondrial transcription factor A (Tfam) (Kelly, et al., 2004, Genes Dev 18:357-368). Quantitative RT-PCR analysis revealed that resveratrol treatment increased Tfam mRNA ~18-fold while PGC-1α and MFN2 mRNA levels were increased 2-fold. The resveratrol-induced upregulation of these mitochondrial markers was severely diminished when cells were treated with resveratrol in the presence of Compound C (FIG. **12**J) or were expressing dominant negative AMPK (FIG. **12** L, M). These results suggest that one mechanism by which resveratrol exerts its protective effects is through promotion of mitochondrial biogenesis resulting from AMPK activation.

Example 15

[0135] This example demonstrates that resveratrol-stimulated AMPK activation is independent of SIRT1.

[0136] Since a number of biological effects of resveratrol and other polyphenols are dependent on SIRT1 function, we explored whether AMPK activation and neurite outgrowth by resveratrol are dependent on SIRT1. First, we confirmed that SIRT1 is expressed in Neuro2a cells by western blot analysis and immunocytochemistry (data not shown). Next, we stimulated Neuro2a cells with resveratrol (10 μM) for 2 h in the presence or absence of three inhibitors of SIRT1 (sirtinol (10 μM), splitomycin (10 μM) and nicotinamide (10 mM)$_j$. None of the SIRT1 inhibitors attenuated the robust activation of AMPK by resveratrol as judged by the increased phosphorylation of AMPK and its downstream target ACC (FIG. **13**A, B). Similarly, SIRT1 inhibitors had no effect on the ability of resveratrol to stimulate Neuro2a neurite outgrowth (FIG. **13**C). These results suggested that resveratrol effects on AMPK are independent of SIRT1 activity within the time period examined.

[0137] Two kinases, LKB1 and CaMKKβ, have been identified as upstream activators of AMPK. While no pharmacological inhibitors of LKB1 are presently available, we were able to use a selective CaMKKβ inhibitor STO 609 (2.5 μM) to test whether resveratrol activates AMPK in Neuro2a cells through CaMKKβ. We found that inhibition of CaMKKβ had no effect on resveratrol-mediated AMPK activation or neurite outgrowth (FIG. **13**). Together these results suggest that resveratrol-stimulated AMPK activation in Neuro2a cells is independent of rapid deacetylation by SIRT proteins or CaMKKβ function and predict the involvement of other upstream activators of AMPK.

Example 16

[0138] This example shows that LKB1, but not SIRT1 is required for resveratrol-stimulated AMPK activation in cortical and dorsal root ganglia sensory neurons.

[0139] Resveratrol activation of AMPK in Neuro2a cells along with the crucial role of AMPK in promoting neurite outgrowth in these cells encouraged us to examine this pathway in primary neurons. We treated E13.5 mouse dorsal root ganglia (DRG) sensory and cortical neuron cultures with resveratrol (10 μM) or AICAR (1 mM). Western blotting demonstrated that resveratrol stimulated phosphorylation of AMPK and ACC in neurons from both peripheral and central nervous systems (FIG. **14**C, **16**C).

[0140] The ineffectiveness of CaMKKβ inhibitors suggested that LKB1 is likely to be the major effector of AMPK activation in neurons. To directly test the role of LKB1 in the resveratrol-mediated activation of AMPK in primary neurons, we took advantage of genetic models of LKB1 deficiency in which LKB1 can be conditionally deleted using Cre recombinase. We cultured dorsal root ganglia (DRG) sensory neurons and cortical neurons from E 13.5 Lkb1 flox/flox mouse embryos. Neurons were infected with either FUGW lentivirus (GFP control) or lentivirus expressing Cre recombinase to excise the floxed LKB1 alleles (FIG. **14**A, B). Lkb1-positive (those infected with FUGW) and Lkb1-deficient (those infected with Cre) DRG and cortical neurons were treated with resveratrol (10 μM) for 2 h and AMPK phosphorylation was examined by western blot analysis. Loss of LKB1 significantly reduced resveratrol-stimulated phosphorylation of AMPK and its downstream target ACC in both DRG and cortical neurons (FIG. **14**C, FIG. **16**). As observed in Neuro2a cells, the CaMKKβ inhibitor STO 609 had no effect on resveratrol-induced AMPK phosphorylation in DRG neurons. However, STO 609 did inhibit AMPK and ACC phosphorylation by resveratrol in cortical neurons (FIG. **16**). These results indicate that the primary regulator of resveratrol-stimulated AMPK activation in DRG neurons is LKB1. However, in cortical neurons CaMKKβ also plays a role in AMPK activation by resveratrol, in accord with previous results indicating that it is a crucial regulator of AMPK in the brain.

[0141] To confirm the SIRT-independence of resveratrol-mediated AMPK activation that we observed in Neuro2a cells and in primary neurons, we performed experiments with pharmacological inhibitors as well as genetic experiments using neurons from SIRT1-deficient mice. Similar to our observations in Neuro2a cells, none of the SIRT inhibitors (Sirtinol, splitomycin and nicotinamide) inhibited resveratrol-stimulated AMPK phosphorylation in DRG or cortical neurons (FIG. **14**C, FIG. **16**). We also treated embryonic DRG and cortical neurons from SIRT1-deficient mice with resveratrol. Western blot analysis demonstrated that at this time point, resveratrol stimulated equivalent levels of AMPK and ACC phosphorylation in wild type and SIRT1-deficient DRG and cortical neurons (FIG. **14**E, FIG. **16**). Collectively, these results indicate that resveratrol activates AMPK through the LKB1 pathway.

Example 17

[0142] This example demonstrates that resveratrol acutely activates AMPK in vivo.

[0143] To extend our in vitro results, we tested whether treating mice with resveratrol activates AMPK in the brain. We injected intraperitoneally 2-month-old male mice with either resveratrol (20 mg/kg body weight) or DMSO (vehicle) (n=3 for each group). Western blot analysis revealed that a single intraperitoneal injection of resveratrol resulted in increased AMPK (2.5-fold) and ACC (2.1-fold) phosphorylation in the brain within 2 h (FIG. **15**). Taken together these results indicate that intraperitoneal administration of resveratrol acutely activates AMPK in the brain and, that this activation translates into the phosphorylation and presumed inhibition of ACC, an important downstream target.

[0144] All references cited in this specification are hereby incorporated by reference. Any discussion of references cited herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference or portion thereof constitutes relevant prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

<210> SEQ ID NO 1
<211> LENGTH: 747
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

```
Met Ala Asp Glu Ala Ala Leu Ala Leu Gln Pro Gly Gly Ser Pro Ser
1               5                   10                  15

Ala Ala Gly Ala Asp Arg Glu Ala Ala Ser Ser Pro Ala Gly Glu Pro
            20                  25                  30
```

-continued

```
Leu Arg Lys Arg Pro Arg Arg Asp Gly Pro Gly Leu Glu Arg Ser Pro
        35                  40                  45

Gly Glu Pro Gly Gly Ala Ala Pro Glu Arg Glu Val Pro Ala Ala Ala
    50                  55                  60

Arg Gly Cys Pro Gly Ala Ala Ala Ala Ala Leu Trp Arg Glu Ala Glu
65                  70                  75                  80

Ala Glu Ala Ala Ala Ala Gly Gly Glu Gln Glu Ala Gln Ala Thr Ala
                85                  90                  95

Ala Ala Gly Glu Gly Asp Asn Gly Pro Gly Leu Gln Gly Pro Ser Arg
            100                 105                 110

Glu Pro Pro Leu Ala Asp Asn Leu Tyr Asp Glu Asp Asp Asp Asp Glu
        115                 120                 125

Gly Glu Glu Glu Glu Glu Ala Ala Ala Ala Ala Ile Gly Tyr Arg Asp
    130                 135                 140

Asn Leu Leu Phe Gly Asp Glu Ile Ile Thr Asn Gly Phe His Ser Cys
145                 150                 155                 160

Glu Ser Asp Glu Glu Asp Arg Ala Ser His Ala Ser Ser Ser Asp Trp
                165                 170                 175

Thr Pro Arg Pro Arg Ile Gly Pro Tyr Thr Phe Val Gln Gln His Leu
            180                 185                 190

Met Ile Gly Thr Asp Pro Arg Thr Ile Leu Lys Asp Leu Leu Pro Glu
        195                 200                 205

Thr Ile Pro Pro Pro Glu Leu Asp Asp Met Thr Leu Trp Gln Ile Val
    210                 215                 220

Ile Asn Ile Leu Ser Glu Pro Pro Lys Arg Lys Lys Arg Lys Asp Ile
225                 230                 235                 240

Asn Thr Ile Glu Asp Ala Val Lys Leu Leu Gln Glu Cys Lys Lys Ile
                245                 250                 255

Ile Val Leu Thr Gly Ala Gly Val Ser Val Ser Cys Gly Ile Pro Asp
            260                 265                 270

Phe Arg Ser Arg Asp Gly Ile Tyr Ala Arg Leu Ala Val Asp Phe Pro
        275                 280                 285

Asp Leu Pro Asp Pro Gln Ala Met Phe Asp Ile Glu Tyr Phe Arg Lys
    290                 295                 300

Asp Pro Arg Pro Phe Phe Lys Phe Ala Lys Glu Ile Tyr Pro Gly Gln
305                 310                 315                 320

Phe Gln Pro Ser Leu Cys His Lys Phe Ile Ala Leu Ser Asp Lys Glu
                325                 330                 335

Gly Lys Leu Leu Arg Asn Tyr Thr Gln Asn Ile Asp Thr Leu Glu Gln
            340                 345                 350

Val Ala Gly Ile Gln Arg Ile Ile Gln Cys His Gly Ser Phe Ala Thr
        355                 360                 365

Ala Ser Cys Leu Ile Cys Lys Tyr Lys Val Asp Cys Glu Ala Val Arg
    370                 375                 380

Gly Asp Ile Phe Asn Gln Val Val Pro Arg Cys Pro Arg Cys Pro Ala
385                 390                 395                 400

Asp Glu Pro Leu Ala Ile Met Lys Pro Glu Ile Val Phe Phe Gly Glu
                405                 410                 415

Asn Leu Pro Glu Gln Phe His Arg Ala Met Lys Tyr Asp Lys Asp Glu
            420                 425                 430

Val Asp Leu Leu Ile Val Ile Gly Ser Ser Leu Lys Val Arg Pro Val
```

-continued

```
        435                 440                 445

Ala Leu Ile Pro Ser Ser Ile Pro His Glu Val Pro Gln Ile Leu Ile
    450                 455                 460

Asn Arg Glu Pro Leu Pro His Leu His Phe Asp Val Glu Leu Leu Gly
465                 470                 475                 480

Asp Cys Asp Val Ile Ile Asn Glu Leu Cys His Arg Leu Gly Gly Glu
                485                 490                 495

Tyr Ala Lys Leu Cys Cys Asn Pro Val Lys Leu Ser Glu Ile Thr Glu
            500                 505                 510

Lys Pro Pro Arg Thr Gln Lys Glu Leu Ala Tyr Leu Ser Glu Leu Pro
        515                 520                 525

Pro Thr Pro Leu His Val Ser Glu Asp Ser Ser Ser Pro Glu Arg Thr
    530                 535                 540

Ser Pro Pro Asp Ser Ser Val Ile Val Thr Leu Leu Asp Gln Ala Ala
545                 550                 555                 560

Lys Ser Asn Asp Asp Leu Asp Val Ser Glu Ser Lys Gly Cys Met Glu
                565                 570                 575

Glu Lys Pro Gln Glu Val Gln Thr Ser Arg Asn Val Glu Ser Ile Ala
            580                 585                 590

Glu Gln Met Glu Asn Pro Asp Leu Lys Asn Val Gly Ser Ser Thr Gly
        595                 600                 605

Glu Lys Asn Glu Arg Thr Ser Val Ala Gly Thr Val Arg Lys Cys Trp
    610                 615                 620

Pro Asn Arg Val Ala Lys Glu Gln Ile Ser Arg Arg Leu Asp Gly Asn
625                 630                 635                 640

Gln Tyr Leu Phe Leu Pro Pro Asn Arg Tyr Ile Phe His Gly Ala Glu
                645                 650                 655

Val Tyr Ser Asp Ser Glu Asp Asp Val Leu Ser Ser Ser Ser Cys Gly
            660                 665                 670

Ser Asn Ser Asp Ser Gly Thr Cys Gln Ser Pro Ser Leu Glu Glu Pro
        675                 680                 685

Met Glu Asp Glu Ser Glu Ile Glu Glu Phe Tyr Asn Gly Leu Glu Asp
    690                 695                 700

Glu Pro Asp Val Pro Glu Arg Ala Gly Gly Ala Gly Phe Gly Thr Asp
705                 710                 715                 720

Gly Asp Asp Gln Glu Ala Ile Asn Glu Ala Ile Ser Val Lys Gln Glu
                725                 730                 735

Val Thr Asp Met Asn Tyr Pro Ser Asn Lys Ser
            740                 745


<210> SEQ ID NO 2
<211> LENGTH: 352
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Asp Phe Leu Arg Asn Leu Phe Ser Gln Thr Leu Ser Leu Gly Ser
1               5                   10                  15

Gln Lys Glu Arg Leu Leu Asp Glu Leu Thr Leu Glu Gly Val Ala Arg
            20                  25                  30

Tyr Met Gln Ser Glu Arg Cys Arg Arg Val Ile Cys Leu Val Gly Ala
        35                  40                  45

Gly Ile Ser Thr Ser Ala Gly Ile Pro Asp Phe Arg Ser Pro Ser Thr
```

-continued

```
    50                  55                  60

Gly Leu Tyr Asp Asn Leu Glu Lys Tyr His Leu Pro Tyr Pro Glu Ala
65                  70                  75                  80

Ile Phe Glu Ile Ser Tyr Phe Lys Lys His Pro Glu Pro Phe Phe Ala
                85                  90                  95

Leu Ala Lys Glu Leu Tyr Pro Gly Gln Phe Lys Pro Thr Ile Cys His
            100                 105                 110

Tyr Phe Met Arg Leu Leu Lys Asp Lys Gly Leu Leu Leu Arg Cys Tyr
        115                 120                 125

Thr Gln Asn Ile Asp Thr Leu Glu Arg Ile Ala Gly Leu Glu Gln Glu
    130                 135                 140

Asp Leu Val Glu Ala His Gly Thr Phe Tyr Thr Ser His Cys Val Ser
145                 150                 155                 160

Ala Ser Cys Arg His Glu Tyr Pro Leu Ser Trp Met Lys Glu Lys Ile
                165                 170                 175

Phe Ser Glu Val Thr Pro Lys Cys Glu Asp Cys Gln Ser Leu Val Lys
            180                 185                 190

Pro Asp Ile Val Phe Phe Gly Glu Ser Leu Pro Ala Arg Phe Phe Ser
        195                 200                 205

Cys Met Gln Ser Asp Phe Leu Lys Val Asp Leu Leu Leu Val Met Gly
    210                 215                 220

Thr Ser Leu Gln Val Gln Pro Phe Ala Ser Leu Ile Ser Lys Ala Pro
225                 230                 235                 240

Leu Ser Thr Pro Arg Leu Leu Ile Asn Lys Glu Lys Ala Gly Gln Ser
                245                 250                 255

Asp Pro Phe Leu Gly Met Ile Met Gly Leu Gly Gly Gly Met Asp Phe
            260                 265                 270

Asp Ser Lys Lys Ala Tyr Arg Asp Val Ala Trp Leu Gly Glu Cys Asp
        275                 280                 285

Gln Gly Cys Leu Ala Leu Ala Glu Leu Leu Gly Trp Lys Lys Glu Leu
    290                 295                 300

Glu Asp Leu Val Arg Arg Glu His Ala Ser Ile Asp Ala Gln Ser Gly
305                 310                 315                 320

Ala Gly Val Pro Asn Pro Ser Thr Ser Ala Ser Pro Lys Lys Ser Pro
                325                 330                 335

Pro Pro Ala Lys Asp Glu Ala Arg Thr Thr Glu Arg Glu Lys Pro Gln
            340                 345                 350


<210> SEQ ID NO 3
<211> LENGTH: 399
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Ala Phe Trp Gly Trp Arg Ala Ala Ala Ala Leu Arg Leu Trp Gly
1               5                   10                  15

Arg Val Val Glu Arg Val Glu Ala Gly Gly Gly Val Gly Pro Phe Gln
            20                  25                  30

Ala Cys Gly Cys Arg Leu Val Leu Gly Gly Arg Asp Asp Val Ser Ala
        35                  40                  45

Gly Leu Arg Gly Ser His Gly Ala Arg Gly Glu Pro Leu Asp Pro Ala
    50                  55                  60

Arg Pro Leu Gln Arg Pro Pro Arg Pro Glu Val Pro Arg Ala Phe Arg
```

-continued

```
65                  70                  75                  80

Arg Gln Pro Arg Ala Ala Ala Pro Ser Phe Phe Phe Ser Ser Ile Lys
                85                  90                  95

Gly Gly Arg Arg Ser Ile Ser Phe Ser Val Gly Ala Ser Ser Val Val
            100                 105                 110

Gly Ser Gly Gly Ser Ser Asp Lys Gly Lys Leu Ser Leu Gln Asp Val
        115                 120                 125

Ala Glu Leu Ile Arg Ala Arg Ala Cys Gln Arg Val Val Val Met Val
    130                 135                 140

Gly Ala Gly Ile Ser Thr Pro Ser Gly Ile Pro Asp Phe Arg Ser Pro
145                 150                 155                 160

Gly Ser Gly Leu Tyr Ser Asn Leu Gln Gln Tyr Asp Leu Pro Tyr Pro
                165                 170                 175

Glu Ala Ile Phe Glu Leu Pro Phe Phe Phe His Asn Pro Lys Pro Phe
            180                 185                 190

Phe Thr Leu Ala Lys Glu Leu Tyr Pro Gly Asn Tyr Lys Pro Asn Val
        195                 200                 205

Thr His Tyr Phe Leu Arg Leu Leu His Asp Lys Gly Leu Leu Leu Arg
    210                 215                 220

Leu Tyr Thr Gln Asn Ile Asp Gly Leu Glu Arg Val Ser Gly Ile Pro
225                 230                 235                 240

Ala Ser Lys Leu Val Glu Ala His Gly Thr Phe Ala Ser Ala Thr Cys
                245                 250                 255

Thr Val Cys Gln Arg Pro Phe Pro Gly Glu Asp Ile Arg Ala Asp Val
            260                 265                 270

Met Ala Asp Arg Val Pro Arg Cys Pro Val Cys Thr Gly Val Val Lys
        275                 280                 285

Pro Asp Ile Val Phe Phe Gly Glu Pro Leu Pro Gln Arg Phe Leu Leu
    290                 295                 300

His Val Val Asp Phe Pro Met Ala Asp Leu Leu Leu Ile Leu Gly Thr
305                 310                 315                 320

Ser Leu Glu Val Glu Pro Phe Ala Ser Leu Thr Glu Ala Val Arg Ser
                325                 330                 335

Ser Val Pro Arg Leu Leu Ile Asn Arg Asp Leu Val Gly Pro Leu Ala
            340                 345                 350

Trp His Pro Arg Ser Arg Asp Val Ala Gln Leu Gly Asp Val Val His
        355                 360                 365

Gly Val Glu Ser Leu Val Glu Leu Leu Gly Trp Thr Glu Glu Met Arg
    370                 375                 380

Asp Leu Val Gln Arg Glu Thr Gly Lys Leu Asp Gly Pro Asp Lys
385                 390                 395


<210> SEQ ID NO 4
<211> LENGTH: 314
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Met Lys Met Ser Phe Ala Leu Thr Phe Arg Ser Ala Lys Gly Arg Trp
1               5                   10                  15

Ile Ala Asn Pro Ser Gln Pro Cys Ser Lys Ala Ser Ile Gly Leu Phe
            20                  25                  30

Val Pro Ala Ser Pro Pro Leu Asp Pro Glu Lys Val Lys Glu Leu Gln
```

-continued

```
        35                  40                  45

Arg Phe Ile Thr Leu Ser Lys Arg Leu Leu Val Met Thr Gly Ala Gly
    50                  55                  60

Ile Ser Thr Glu Ser Gly Ile Pro Asp Tyr Arg Ser Glu Lys Val Gly
65                  70                  75                  80

Leu Tyr Ala Arg Thr Asp Arg Arg Pro Ile Gln His Gly Asp Phe Val
                85                  90                  95

Arg Ser Ala Pro Ile Arg Gln Arg Tyr Trp Ala Arg Asn Phe Val Gly
            100                 105                 110

Trp Pro Gln Phe Ser Ser His Gln Pro Asn Pro Ala His Trp Ala Leu
        115                 120                 125

Ser Thr Trp Glu Lys Leu Gly Lys Leu Tyr Trp Leu Val Thr Gln Asn
    130                 135                 140

Val Asp Ala Leu His Thr Lys Ala Gly Ser Arg Arg Leu Thr Glu Leu
145                 150                 155                 160

His Gly Cys Met Asp Arg Val Leu Cys Leu Asp Cys Gly Glu Gln Thr
                165                 170                 175

Pro Arg Gly Val Leu Gln Glu Arg Phe Gln Val Leu Asn Pro Thr Trp
            180                 185                 190

Ser Ala Glu Ala His Gly Leu Ala Pro Asp Gly Asp Val Phe Leu Ser
        195                 200                 205

Glu Glu Gln Val Arg Ser Phe Gln Val Pro Thr Cys Val Gln Cys Gly
    210                 215                 220

Gly His Leu Lys Pro Asp Val Val Phe Phe Gly Asp Thr Val Asn Pro
225                 230                 235                 240

Asp Lys Val Asp Phe Val His Lys Arg Val Lys Glu Ala Asp Ser Leu
                245                 250                 255

Leu Val Val Gly Ser Ser Leu Gln Val Tyr Ser Gly Tyr Arg Phe Ile
            260                 265                 270

Leu Thr Ala Trp Glu Lys Lys Leu Pro Ile Ala Ile Leu Asn Ile Gly
        275                 280                 285

Pro Thr Arg Ser Asp Asp Leu Ala Cys Leu Lys Leu Asn Ser Arg Cys
    290                 295                 300

Gly Glu Leu Leu Pro Leu Ile Asp Pro Cys
305                 310


<210> SEQ ID NO 5
<211> LENGTH: 310
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Arg Pro Leu Gln Ile Val Pro Ser Arg Leu Ile Ser Gln Leu Tyr
1               5                   10                  15

Cys Gly Leu Lys Pro Pro Ala Ser Thr Arg Asn Gln Ile Cys Leu Lys
            20                  25                  30

Met Ala Arg Pro Ser Ser Ser Met Ala Asp Phe Arg Lys Phe Phe Ala
        35                  40                  45

Lys Ala Lys His Ile Val Ile Ile Ser Gly Ala Gly Val Ser Ala Glu
    50                  55                  60

Ser Gly Val Pro Thr Phe Arg Gly Ala Gly Gly Tyr Trp Arg Lys Trp
65                  70                  75                  80

Gln Ala Gln Asp Leu Ala Thr Pro Leu Ala Phe Ala His Asn Pro Ser
```

-continued

```
                85                  90                  95

Arg Val Trp Glu Phe Tyr His Tyr Arg Arg Glu Val Met Gly Ser Lys
            100                 105                 110

Glu Pro Asn Ala Gly His Arg Ala Ile Ala Glu Cys Glu Thr Arg Leu
        115                 120                 125

Gly Lys Gln Gly Arg Arg Val Val Val Ile Thr Gln Asn Ile Asp Glu
    130                 135                 140

Leu His Arg Lys Ala Gly Thr Lys Asn Leu Leu Glu Ile His Gly Ser
145                 150                 155                 160

Leu Phe Lys Thr Arg Cys Thr Ser Cys Gly Val Val Ala Glu Asn Tyr
                165                 170                 175

Lys Ser Pro Ile Cys Pro Ala Leu Ser Gly Lys Gly Ala Pro Glu Pro
            180                 185                 190

Gly Thr Gln Asp Ala Ser Ile Pro Val Glu Lys Leu Pro Arg Cys Glu
        195                 200                 205

Glu Ala Gly Cys Gly Gly Leu Leu Arg Pro His Val Val Trp Phe Gly
    210                 215                 220

Glu Asn Leu Asp Pro Ala Ile Leu Glu Glu Val Asp Arg Glu Leu Ala
225                 230                 235                 240

His Cys Asp Leu Cys Leu Val Val Gly Thr Ser Ser Val Val Tyr Pro
                245                 250                 255

Ala Ala Met Phe Ala Pro Gln Val Ala Ala Arg Gly Val Pro Val Ala
            260                 265                 270

Glu Phe Asn Thr Glu Thr Thr Pro Ala Thr Asn Arg Phe Arg Phe His
        275                 280                 285

Phe Gln Gly Pro Cys Gly Thr Thr Leu Pro Glu Ala Leu Ala Cys His
    290                 295                 300

Glu Asn Glu Thr Val Ser
305                 310


<210> SEQ ID NO 6
<211> LENGTH: 355
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Ser Val Asn Tyr Ala Ala Gly Leu Ser Pro Tyr Ala Asp Lys Gly
1               5                   10                  15

Lys Cys Gly Leu Pro Glu Ile Phe Asp Pro Pro Glu Glu Leu Glu Arg
            20                  25                  30

Lys Val Trp Glu Leu Ala Arg Leu Val Trp Gln Ser Ser Ser Val Val
        35                  40                  45

Phe His Thr Gly Ala Gly Ile Ser Thr Ala Ser Gly Ile Pro Asp Phe
    50                  55                  60

Arg Gly Pro His Gly Val Trp Thr Met Glu Glu Arg Gly Leu Ala Pro
65                  70                  75                  80

Lys Phe Asp Thr Thr Phe Glu Ser Ala Arg Pro Thr Gln Thr His Met
                85                  90                  95

Ala Leu Val Gln Leu Glu Arg Val Gly Leu Leu Arg Phe Leu Val Ser
            100                 105                 110

Gln Asn Val Asp Gly Leu His Val Arg Ser Gly Phe Pro Arg Asp Lys
        115                 120                 125

Leu Ala Glu Leu His Gly Asn Met Phe Val Glu Glu Cys Ala Lys Cys
```

-continued

```
    130                 135                 140

Lys Thr Gln Tyr Val Arg Asp Thr Val Val Gly Thr Met Gly Leu Lys
145                 150                 155                 160

Ala Thr Gly Arg Leu Cys Thr Val Ala Lys Ala Arg Gly Leu Arg Ala
                165                 170                 175

Cys Arg Gly Glu Leu Arg Asp Thr Ile Leu Asp Trp Glu Asp Ser Leu
            180                 185                 190

Pro Asp Arg Asp Leu Ala Leu Ala Asp Glu Ala Ser Arg Asn Ala Asp
        195                 200                 205

Leu Ser Ile Thr Leu Gly Thr Ser Leu Gln Ile Arg Pro Ser Gly Asn
    210                 215                 220

Leu Pro Leu Ala Thr Lys Arg Arg Gly Gly Arg Leu Val Ile Val Asn
225                 230                 235                 240

Leu Gln Pro Thr Lys His Asp Arg His Ala Asp Leu Arg Ile His Gly
                245                 250                 255

Tyr Val Asp Glu Val Met Thr Arg Leu Met Lys His Leu Gly Leu Glu
            260                 265                 270

Ile Pro Ala Trp Asp Gly Pro Arg Val Leu Glu Arg Ala Leu Pro Pro
        275                 280                 285

Leu Pro Arg Pro Pro Thr Pro Lys Leu Glu Pro Lys Glu Glu Ser Pro
    290                 295                 300

Thr Arg Ile Asn Gly Ser Ile Pro Ala Gly Pro Lys Gln Glu Pro Cys
305                 310                 315                 320

Ala Gln His Asn Gly Ser Glu Pro Ala Ser Pro Lys Arg Glu Arg Pro
                325                 330                 335

Thr Ser Pro Ala Pro His Arg Pro Pro Lys Arg Val Lys Ala Lys Ala
            340                 345                 350

Val Pro Ser
        355


<210> SEQ ID NO 7
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Met Ala Ala Gly Gly Leu Ser Arg Ser Glu Arg Lys Ala Ala Glu Arg
1               5                   10                  15

Val Arg Arg Leu Arg Glu Glu Gln Gln Arg Glu Arg Leu Arg Gln Val
            20                  25                  30

Ser Arg Ile Leu Arg Lys Ala Ala Ala Glu Arg Ser Ala Glu Glu Gly
        35                  40                  45

Arg Leu Leu Ala Glu Ser Ala Asp Leu Val Thr Glu Leu Gln Gly Arg
    50                  55                  60

Ser Arg Arg Arg Glu Gly Leu Lys Arg Arg Gln Glu Glu Val Cys Asp
65                  70                  75                  80

Asp Pro Glu Glu Leu Arg Gly Lys Val Arg Glu Leu Ala Ser Ala Val
                85                  90                  95

Arg Asn Ala Lys Tyr Leu Val Val Tyr Thr Gly Ala Gly Ile Ser Thr
            100                 105                 110

Ala Ala Ser Ile Pro Asp Tyr Arg Gly Pro Asn Gly Val Trp Thr Leu
        115                 120                 125

Leu Gln Lys Gly Arg Ser Val Ser Ala Ala Asp Leu Ser Glu Ala Glu
```

-continued

```
    130                 135                 140

Pro Thr Leu Thr His Met Ser Ile Thr Arg Leu His Glu Gln Lys Leu
145                 150                 155                 160

Val Gln His Val Val Ser Gln Asn Cys Asp Gly Leu His Leu Arg Ser
                165                 170                 175

Gly Leu Pro Arg Thr Ala Ile Ser Glu Leu His Gly Asn Met Tyr Ile
            180                 185                 190

Glu Val Cys Thr Ser Cys Val Pro Asn Arg Glu Tyr Val Arg Val Phe
        195                 200                 205

Asp Val Thr Glu Arg Thr Ala Leu His Arg His Gln Thr Gly Arg Thr
    210                 215                 220

Cys His Lys Cys Gly Thr Gln Leu Arg Asp Thr Ile Val His Phe Gly
225                 230                 235                 240

Glu Arg Gly Thr Leu Gly Gln Pro Leu Asn Trp Glu Ala Ala Thr Glu
                245                 250                 255

Ala Ala Ser Arg Ala Asp Thr Ile Leu Cys Leu Gly Ser Ser Leu Lys
            260                 265                 270

Val Leu Lys Lys Tyr Pro Arg Leu Trp Cys Met Thr Lys Pro Pro Ser
        275                 280                 285

Arg Arg Pro Lys Leu Tyr Ile Val Asn Leu Gln Trp Thr Pro Lys Asp
    290                 295                 300

Asp Trp Ala Ala Leu Lys Leu His Gly Lys Cys Asp Asp Val Met Arg
305                 310                 315                 320

Leu Leu Met Ala Glu Leu Gly Leu Glu Ile Pro Ala Tyr Ser Arg Trp
                325                 330                 335

Gln Asp Pro Ile Phe Ser Leu Ala Thr Pro Leu Arg Ala Gly Glu Glu
            340                 345                 350

Gly Ser His Ser Arg Lys Ser Leu Cys Arg Ser Arg Glu Glu Ala Pro
        355                 360                 365

Pro Gly Asp Arg Gly Ala Pro Leu Ser Ser Ala Pro Ile Leu Gly Gly
    370                 375                 380

Trp Phe Gly Arg Gly Cys Thr Lys Arg Thr Lys Arg Lys Lys Val Thr
385                 390                 395                 400
```

What is claimed is:

1. A method of promoting axonal growth in a mammal in need thereof, the method comprising administering to the mammal an agent in an amount effective for promoting axonal growth by increasing at least one of AMP activated kinase (AMPK) activity, LKB1 activity and CaMKKβ activity in at least one of diseased neurons, injured neurons and supporting cells.

2. A method according to claim **1**, wherein the agent is a stilbene, a chalcone, a flavone, an isoflavanone, a flavanone or a catechin.

3. A method according to claim **2**, wherein the stilbene is selected from the group consisting of resveratrol, piceatannol, deoxyrhaponfin, trans-stilbene and rhapontin.

4. A method according to claim **2**, wherein the chalcone is selected from the group consisting of burtein, isoliquiritigenin and 3,4,2',4',6'-pentahydroxychalcone.

5. A method according to claim **2**, wherein the flavone is selected from the group consisting of fisetin, 5,7,3',4',5'-pentahydroxyflavone, luteolin, 3,6,3',4'-tetrahydroxyflavone, quercetin, 7,3',4',5'-tetrahydroxyflavone, kaempferol, 6-hydroxyapigenin, apigenin, 3,6,2',4'-tetrahydroxyflavone, 7,4'-dihydroxyflavone, 7,8,3',4'-tetrahydroxyflavone, 3,6,2',3'-tetraliydroxyflavone, 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 5,7-dihydroxyflavone, morin, flavone and 5-hydroxyflavone.

6. A method according to claim **2**, wherein the flavanone is selected from the group consisting of naringenin, 3,5,7,3',4'-pentahydroxyflavanone and flavanone.

7. A method according to claim **2**, wherein the catechin is selected from the group consisting of (−)-epicatechin, (−)-catechin, (−)-gallocatechin, (+)-catechin and (+)-epicatechin.

8. A method according to claim **1**, wherein the agent is an isoflavone selected from the group consisting of daidzein and genistein.

9. The method of claim **1**, wherein the agent is selected from the group consisting of resveratrol, fisetin, butein, piceatannol and quercetin.

10. The method of claim **1**, wherein the agent is resveratrol.

11. A method according to claim **1**, wherein the mammal in need is a mammal having a neuropathy or axonopathy.

12. A method according to claim **11**, wherein the neuropathy or axonopathy is hereditary or congenital or is associated with a neurodegenerative disease, a motor neuron disease, neoplasia, an endocrine disorder, a metabolic disease, a nutritional deficiency, atherosclerosis, an autoimmune disease, convulsions and seizures, mechanical injury, chemical or drug-induced injury, thermal injury, radiation injury, nerve compression, optic neuropathy, retinal or optic nerve disorder, mitochondrial dysfunction, progressive dementia, a demyelinating disease, ischemia, stroke, an infectious disease or an inflammatory disease.

13. A method according to claim **11**, wherein the neuropathy or axonopathy is induced by a cytotoxic anticancer agent.

14. A method according to claim **12**, wherein the optic neuropathy is glaucoma, retinal ganglion degeneration, optic neuritis and/or degeneration, macular degeneration, ischemic optic neuropathy, traumatic injury to the optic nerve, hereditary optic neuropathy, metabolic optic neuropathy, optic neuropathy due to a toxic agent, optic neuropathy caused by adverse drug reactions or optic neuropathy caused by vitamin deficiency.

15. A method according to claim **12**, wherein the mitochondrial dysfunction is selected from the group consisting of a dysfunction resulting from oxidative damage, a dysfunction resulting from one or more mutations in one or more mitochondrial proteins, a dysfunction resulting from toxin exposure, a dysfunction resulting from aging and a combination thereof.

16. A method according to claim **15**, wherein a mitochondrial protein of the one or more mitochondrial proteins is encoded by the mitochondrial genome.

17. A method according to claim **15**, wherein a mitochondrial protein of the one or more mitochondrial proteins is encoded by the nuclear genome.

18. A method according to claim **1**, further comprising assessing the need of the mammal for increased activity of at least one of AMPK, LKB1 and CaMKKβ before, during or after treatment.

19. A method of screening an agent for axonal growth promoting activity, the method comprising:

providing a cell culture comprising a culture medium and mammalian neuronal cells under neurite growth-permissible conditions;

applying to the culture a candidate neurite outgrowth promoting agent which acts at least in part by increasing at least one of AMPK activity, LKB1 activity and CaMKKβ activity; and

detecting an increase in neurite outgrowth compared to a control culture.

20. A method according to claim **19**, wherein the neurite growth-permissible conditions comprise at least one of serum starvation, including retinoic acid in the culture medium and including a growth factor in the culture medium.

21. A method according to claim **20**, wherein the growth factor is a neurotrophin or a GDNF family ligand.

22. A method of promoting axonal growth in a mammal in need of treatment for an optic neuropathy, the method comprising administering to the mammal an agent in an amount effective for promoting axonal growth by increasing at least one of AMP activated kinase (AMPK) activity, LKB1 activity and CaMKKβ activity in at least one of diseased neurons, injured neurons and supporting cells.

23. A method according to claim **22**, wherein the supporting cells are glial cells.

24. A method according to claim **22**, wherein the administering to the mammal comprises intraocular administering.

25. A method according to claim **24**, wherein the intraocular administering comprises intraocular administering of a sustained release delivery system.

26. A method according to claim **24**, wherein the intraocular administering comprises intravitreal injection, administration by eyedrops or administration by trans-scleral delivery.

27. A method according to claim **22**, wherein the optic neuropathy is a glaucoma, a retinal ganglion degeneration, an optic neuritis and/or degeneration, a macular degeneration, an ischemic optic neuropathy, a traumatic injury to the optic nerve, a hereditary optic neuropathy, a metabolic optic neuropathy, a neuropathy due to a toxic agent, a neuropathy caused by adverse drug reaction, or a neuropathy caused by a vitamin deficiency.

28. A method according to claim **22**, wherein the mammal is a human.

29. A method according to claim **22**, wherein the agent is a stilbene, a chalcone, a flavone, an isoflavanone, a flavanone or a catechin.

30. A method according to claim **29**, wherein the stilbene is selected from the group consisting of resveratrol, piceatannol, deoxyrhaponfin, trans-stilbene and rhapontin.

31. A method according to claim **29**, wherein the chalcone is selected from the group consisting of burtein, isoliquiritigenin and 3,4,2',4',6'-pentahydroxychalcone.

32. A method according to claim **29**, wherein the flavone is selected from the group consisting of fisetin, 5,7,3',4',5'-pentahydroxyflavone, luteolin, 3,6,3',4'-tetrahydroxyflavone, quercetin, 7,3',4',5'-tetrahydroxyflavone, kaempferol, 6-hydroxyapigenin, apigenin, 3,6,2',4'-tetrahydroxyflavone, 7,4'-dihydroxyflavone, 7,8,3',4'-tetrahydroxyflavone, 3,6,2',3'-tetraliydroxyflavone, 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 5,7-dihydroxyflavone, morin, flavone and 5-hydroxyflavone.

33. A method according to claim **29**, wherein the flavanone is selected from the group consisting of naringenin, 3,5,7,3', 4'-pentahydroxyflavanone and flavanone.

34. A method according to claim **29**, wherein the catechin is selected from the group consisting of (−)-epicatechin, (−)-catechin, (−)-gallocatechin, (+)-catechin and (+)-epicatechin.

35. A method according to claim **22**, wherein the agent is an isoflavone selected from the group consisting of daidzein and genistein.

36. A method according to claim **22**, wherein the agent is selected from the group consisting of resveratrol, fisetin, butein, piceatannol and quercetin.

37. A method according to claim **22**, wherein the agent is resveratrol.

* * * * *